International Journal of Evolutionary Biology

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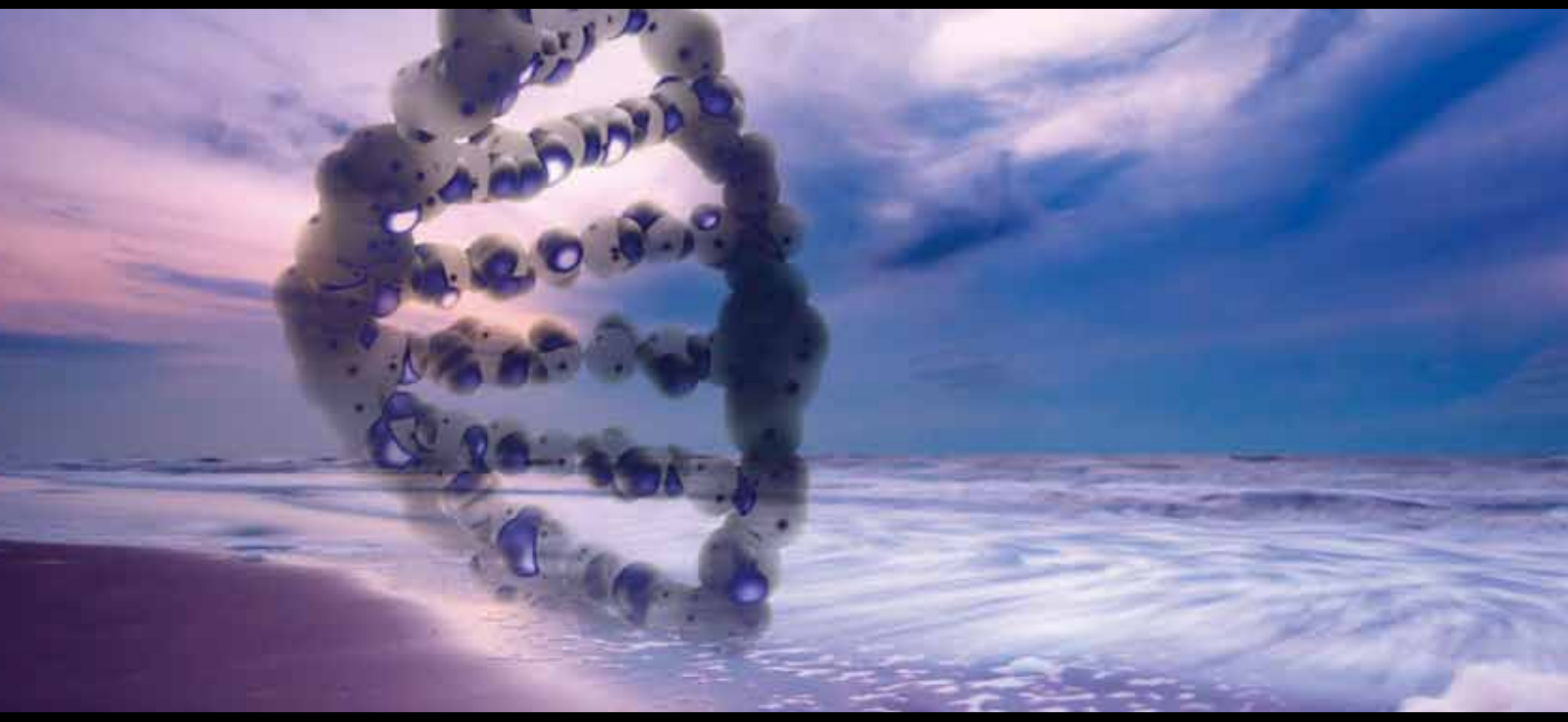
Guest Editors: Stephan Koblmüller, R. Craig Albertson, Martin J. Genner, Kristina M. Sefc, and Tetsumi Takahashi



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Editorial Board

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Editorial

Cichlid Evolution: Lessons in Diversification 2012

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This is the second special issue on cichlid evolution hosted by the International Journal of Evolutionary Biology. Once more, we are overwhelmed by the vivid responses to our call for contributions, and thank the authors for their great work. The thirteen papers in this issue, including two reviews, span geographically from Africa to South America and address a wide variety of evolutionary topics including speciation and hybridization, phenotype evolution, and reproductive behaviour. Papers are summarized below in the order in which they appear in this special issue.

Linking Diversification to the Physical Environment. The East African cichlid radiations represent the most spectacular vertebrate radiations known to date, and a multitude of studies have sought to elucidate the phylogenetic relationships among extant East African cichlid lineages. The East African rift valley is also extremely dynamic from a geological perspective, and numerous studies have aimed at clarifying the geologic, hydrologic, and climatic history of this region. However, while these fields have enormous potential to inform one another, a uniting synthesis combining evidence from studies on geology/hydrology/climatology and cichlid phylogeny/phylogeography has been lacking. In their review "*The impact of the geologic history and paleoclimate on the diversification of East African cichlids,*" P. D. Danley et al. provide an up-to-date synthesis of the geologic history and paleoclimate of the East African Great Lakes (and surrounding water bodies) and the evolution of the region's endemic cichlid species assemblages. They discuss how tectonic processes and climatic changes changed the hydrological systems in East Africa and affected the diversification of cichlid fishes. Thus, their review links data on cichlid diversity with the highly variable geological and paleoclimatic history of East Africa and serves as an important reference for the impact of geological, paleoecological, and paleoclimatic factors on biogeography.

Potential Roles for the Visual System in Promoting Adaptive Radiation. Animals differ dramatically in their ability to the perceive signals in their natural environment, and during the last ten years we have gained a much better understanding of how closely related species differ in both their signalling and receiving abilities during information transfer. In cichlids, the role of spatial environmental variation in driving natural selection on these sensory traits has become increasingly appreciated, and it has been proposed that such "sensory drive" may even have promoted speciation through sexual selection in the Lake Victoria cichlid radiation. In their thought-provoking review "*An evaluation of the role of sensory drive in the evolution of Lake Malawi cichlid fishes,*" A. R. Smith et al. consider whether this model of evolution could also help to explain the evolution of remarkable cichlid species richness in Lake Malawi. They explore three aspects of the hypothesis. First, they ask if the broad light spectrum of the relatively clear waters of Lake Malawi is capable of driving strong natural selection on visual systems, as it has been shown to be the case in Lake Victoria. Second, they consider the extent of variation in visual perception present both within and among populations of Lake Malawi cichlids, with a specific consideration of the relative roles of functional genetic differences and the extent of developmental plasticity in determining adult visual phenotypes. Finally, they

consider if links are present between male courtship colours and the ability of females to perceive those traits. On balance, the authors conclude that sensory drive is unlikely to be a major force in the evolution of Lake Malawi cichlids under present environmental conditions. However, it is also clear from the review that considerably more work is required to understand how cichlid sensory ecology is related to the process of adaptive radiation.

Determining the Effects of the Visual Environment on Coloration. In Lake Victoria visual environments are sufficiently diverse to provide divergent signalling conditions and create associations between sensory systems, signals, and environmental variation. In their study "*Species-specific relationships between water transparency and male coloration within and between two closely related Lake Victoria cichlid species,*" R. F. Castillo Cajas et al. demonstrate that the effects of visual environment on cichlid coloration can differ between closely related species, and, both within and across species, between body regions. While saturation and hue of the yellow/red body coloration of *Pundamilia nyererei* varied between populations in a way to enhance conspicuousness under local conditions, this was not apparent in the bluish body coloration of *P. pundamilia*. Different depth distributions or different sexual selection regimes in the two species may be responsible for species-specific responses to heterogenic signalling environments. In contrast, covariation of anal fin spot coloration with environmental conditions was congruent between species, and variation of the red coloration at the dorsal fin lappets of both species followed no consistent pattern. The study offers an illuminating glance on the complexity of the interactions among the multifarious influences and constraints acting on colour pattern evolution.

Divergence and Genetic Basis of Internal Bone Geometry. Beyond their rich colour variation, cichlids exhibit unparalleled levels of ecomorphological diversification, and this phenomenon has been extensively studied and characterized from the standpoint of external craniofacial bone morphology. In contrast, relatively little is known about whether biomechanically relevant shifts in internal bone architecture have accompanied this adaptive radiation. In "*More than meets the eye: functionally salient changes in internal bone architecture accompany divergence in cichlid feeding mode,*" R. C. Albertson and coauthors used μCT analysis to show clear differences in the internal anatomy and load-bearing function of craniofacial bone in species that occupy distinct foraging niches. Moreover, a mapping experiment was used to characterize the genetic architecture of bone biomechanics. These results shed new light on the evolutionary diversification of feeding architecture in cichlids and highlight the importance of internal skeletal geometry in studies of adaptive radiations.

The Feasibility of Hybridization in Divergent Species: A Genomic Perspective. Hybridization is increasingly recognized as a positive force in the evolutionary diversification of cichlids. In "*Analysis of the meiotic segregation in intergeneric hybrids of tilapias,*" E. Bezault et al. analyzed meiotic segregation in the hybrid genomes of two divergent tilapia species, *Oreochromis niloticus* and *Sarotherodon melanotheron*. The authors found that in reciprocal F2 and backcross progeny patterns of segregation were similar to those of both parental species. These results provide important insights into genome evolution and the roles for hybridization in promoting cichlid evolution.

Hybridization and Diversification in Lake Malawi Cichlids. The East African lacustrine cichlid radiations have been particularly shaped by introgressive hybridization and even hybrid speciation. Even the origin of whole adaptive radiations via ancient hybridization has been repeatedly postulated. In their study "*Extensive introgression among ancestral mtDNA lineages: Phylogenetic relationships of the Utaka within the Lake Malawi cichlid flock*," D. Anseeuw et al. provide evidence for past hybridisation among divergent Lake Malawi cichlid lineages and thus further highlight the importance of interspecific gene flow for shaping the evolutionary history of East African cichlid fishes. Specifically, highly divergent mtDNA lineages have been found in several species of the Utaka, an informal group of Lake Malawi cichlid species. Nuclear data, on the other hand, did not show comparable patterns of intraspecific divergence. The authors conclude that the observed discrepancy between mtDNA and nuclear DNA is best explained by introgression of divergent mtDNA into ancestral representatives of the Utaka.

Deepwater Communities Are Not Shaped by Introgression. Several molecular phylogenetic studies inferred hybridization among littoral cichlid species in Lake Tanganyika, a phenomenon typically attributed to recurrent climate-driven lake level fluctuations altering shoreline and habitat structure. In "*Evolutionary history of Lake Tanganyika's predatory deepwater cichlids,*" P. C. Kirchberger et al. show that in contrast to the predominantly littoral Lake Tanganyika cichlid lineages studied so far, the evolutionary history of the large predominantly piscivorous species of the tribe Bathybatini appear to have been not affected by introgressive hybridization and that inconsistencies between nuclear and mitochondrial phylogenetic trees are likely due to ancient incomplete lineage sorting. Their findings are consistent with the hypothesis that lineages evolving in the weakly structured deepwater habitat would develop stronger reproductive isolation than the often allopatric lineages in the highly fragmented littoral. However, whether this lack of hybridization among deepwater taxa is representative of a general pattern typical for cichlid lineages inhabiting a weakly structured habitat or applies to just particular lineages remains to be tested by analysing additional openwater and deepwater lineages.

Genetic Evidence in Support of Divergence between Sympatric Colour Morphs. A key issue in cichlid taxonomy is what characters we should use to delimit species. In African haplochromine cichlids, for example, male breeding colours appear to be effective indicators of species boundaries, at least among sympatric populations. In the monotypic Lake Tanganyika genus *Cyathopharynx* (tribe Ectodini) two

distinct colour morphs occur sympatrically around most of the lake; however they have been treated as a single species in the scientific literature. In "*Genetic and morphological evidence implies existence of two sympatric species in Cyathopharynx furcifer (Teleostei: Cichlidae) from Lake Tanganyika*," T. Takahashi and M. Hori convincingly demonstrate that these two distinct morphs actually represent two distinct species.

Genetic Evidence Fails to Support Divergence between Sympatric Colour Morphs. In a similar study, "*Deep phylogenetic divergence and lack of taxonomic concordance in species of Astronotus (Cichlidae),*" O. P. Colatreli and coauthors tested for genetic differences between sympatric and allopatric populations of *A. ocellatus* and *A. crassipinnis,* two Amazonian cichlids from the "oscar" genus that can be distinguished based on adult colour patterning. Their results showed no clear evidence of genetic differences among the sympatric colour forms. The analyses did however uncover the presence of five very substantially divergent allopatric lineages that may be new species. Further research would help to confirm this interpretation of the data and will require more genetic, morphological, and behavioural data. Oscars are among the most iconic of all cichlids, yet O. P. Colatreli and coauthors have shown a good deal of work is still required to catalogue the true diversity of their genus. This equally applies to many other genera of Neotropical and African cichlids.

Phylogeography of a Rapidly Dispersing Species. In "*Phylogeographic diversity of the lower Central American cichlid Andinoacara coeruleopunctatus (Cichlidae),*" S. S. McCafferty et al. used phylogeography to disentangle the relative contributions of historical and ecological processes in determining the biogeography of the lower Central American cichlid species *Andinoacara coeruleopunctatus*. Specifically, the authors used mtDNA sequence and RFLP data to test the hypothesis that, given its high dispersal capabilities, phylogeographic patterns in *A. coeruleopunctatus* will differ from those of other species. Significant phylogeographic structure was observed across lower Central American populations of *A. coeruleopunctatus*, and phylogeographic patterns were consistent with the rapid colonization and dispersal of this species following the rise of the Isthmus of Panama. This study provides insight into the processes that determine biogeography and underscores the value of studying species with distinct life histories and ecologies within this context.

Exploring the Life History and Reproductive Behaviour of a Shell-Brooding Cichlid. New details on the life cycle of a cichlid from Lake Tanganyika are provided by K. Ota et al. in their study, "*Alternative reproductive tactics in the shell-brooding Lake Tanganyika cichlid Neolamprologus brevis.*" Among their notable findings, the authors show that the distribution of this species across divergent habitats depends on their reproductive stage, and that they migrate to communal nests, that is, the shell patches assembled by another shell-brooding species, only temporarily for reproduction. Moreover, behaviours, body, and gonad sizes of males among shell patches provide evidence for a multimale polygynous mating system, and for the employment of sneaking as an alternative reproductive tactic in this lamprologine cichlid.

Coordination of Spawning in the Absence of Visual Cues. Another study in this issue focuses on the builder of these communal shell nests. The shell-brooding *Lamprologus callipterus* from Lake Tanganyika exhibits the most extreme sexual size dimorphism among cichlid fishes (males > females). Females spawn their eggs while inside gastropod shells, and males fertilize eggs from outside the shell. Although males of many cichlid species use visual stimulation to induce egg laying in their partners, pairs of this species cannot see each other during spawning. This obviously raises the question of how gamete release is synchronized between the sexes. In "*Spawning coordination of mates in a shell brooding cichlid,*" D. Schütz et al. inferred, based on field observations and laboratory experiments, that the male initiates the spawning sequence and that sperm release and egg laying are very well synchronised despite the limited communication possibilities during spawning. Females attempt to extend the egg laying period to increase the chance for parasitic males to participate in spawning in order to induce sperm competition. The authors also discuss the possibility that this exceptional spawning pattern reflects a conflict between the sexes.

Cross-Species Amplification of Microsatellites. The concluding paper of this issue is a technical note by E. Bezault et al., "*Microsatellites cross-species amplification across some African cichlids*," which tested the applicability of a large panel of microsatellite markers in a total of 15, mostly tilapiine, African cichlid species, and provided the community with a powerful marker arsenal for further molecular genetic studies.

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Research Article

Phylogeographic Diversity of the Lower Central American Cichlid *Andinoacara coeruleopunctatus* (Cichlidae)

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It is well appreciated that historical and ecological processes are important determinates of freshwater biogeographic assemblages. Phylogeography can potentially lend important insights into the relative contribution of historical processes in biogeography. However, the extent that phylogeography reflects historical patterns of drainage connection may depend in large part on the dispersal capability of the species. Here, we test the hypothesis that due to their relatively greater dispersal capabilities, the neotropical cichlid species *Andinoacara coeruleopunctatus* will display a phylogeographic pattern that differs from previously described biogeographic assemblages in this important region. Based on an analysis of 318 individuals using mtDNA ATPase 6/8 sequence and restriction fragment length polymorphism data, we found eight distinct clades that are closely associated with biogeographic patterns. The branching patterns among the clades and a Bayesian clock analysis suggest a relatively rapid colonization and diversification among drainages in the emergent Isthmus of Panama followed by the coalescing of some drainages due to historical connections. We also present evidence for extensive cross-cordillera sharing of clades in central Panama and the Canal region. Our results suggest that contemporary phylogeographic patterns and diversification in Lower Central American fishes reflect an interaction of historical drainage connections, dispersal, and demographic processes.

1. Introduction

Species distribution patterns are determined in large part by a combination of ecological (e.g., competition, predation, and demography) and historical (e.g., vicariance and dispersal opportunities) processes. Though in the past there was a general tendency to emphasize the role of ecology in structuring communities, historical processes have received increasing attention of late [1–5]. This is particularly true for the freshwater fishes where dynamic patterns of habitat loss (vicariance) and movement across freshwater connections (dispersal) are key determinants of species distributions [4, 6]. In addition, there is increasing evidence that contemporary patterns in species distributions and phylogeography tend to reflect historical rather than contemporary drainage connections in many freshwater species [7–10]. It is widely accepted that phylogeographic patterns have the potential to yield important insights into the mechanisms driving biogeographic structure. In particular, a close correspondence between intraspecific phylogeographic patterns and biogeographic provinces can be readily explained by historical processes. However, when discordance occurs, we must consider other factors to account for this disparity. Ecological differences in dispersal ability and demographics are two potentially important factors that can lead to differences between biogeographic and phylogeographic association [11].

The importance of the lower Central American region (LCA, defined here as northwestern Colombia north to Lake Nicaragua) in determining the distribution patterns for many species is well known [12–15]. The rising Isthmus of Panama acted as a corridor for many freshwater fishes, enabling the conquest of Mesoamerica by South American species from northwestern Colombia [4, 16–20]. Smith and Bermingham [4] were able to show that this region is divided

into a number of distinct biogeographic provinces based on species presence/absence, and proposed a model of historical patterns of vicariance and dispersal (through stream capture and anastomosis) to explain their results.

To date, detailed phylogeographic analyses and models of the colonization of LCA by freshwater fishes have focused on primary freshwater fishes (species that are relatively intolerant of seawater) in the Characiformes, Siluriformes, and Gymnotiformes [20–24]. In this paper, we provide a detailed phylogeographic description of a presumptive secondary freshwater fish (species with an elevated physiological tolerance for brackish or salt water), the cichlid *Andinoacara coeruleopunctatus*, in order to test expectations regarding the role of dispersal and diversification based on ecological differences. Our interest in *A. coeruleopunctatus* derives from the fact that despite being considered a secondary freshwater fish species by Myers [25], its distribution is relatively limited (Panama and southern Costa Rica) and occurs commonly in all but one (Bocas del Toro) of the biogeographic provinces defined in southern LCA. In addition, it shares a similar though not identical distribution pattern across LCA as many of the primary freshwater fishes previously used to construct models of colonization in this area [20]. Here, we specifically test the hypothesis that the phylogeographic pattern found in *A. coeruleopunctatus* differs from the biogeographic provinces defined by Smith and Bermingham [4] due to potentially greater opportunities for dispersal among drainages. In addition, we compare these patterns to those found in past studies based on primary freshwater species and put forward an explanation for differences found.

2. Materials and Methods

Samples of *Andinoacara* were collected by electroshocking or seining in various drainages from Costa Rica, the Republic of Panama, Columbia, Venezuela, Trinidad, and Peru. Figure 1 summarizes drainage locations sampled in this study. The geological history of this area is detailed in Bermingham and Martin [20], while the biogeographic structure is characterized in Smith and Bermingham's [4]. Drainage boundaries follow those of Smith and Bermingham and are specified in Table S1.1 (Supplementary Data available online at doi: 10.1155/2012/780169).

Preliminary sample identifications were made in the field. Individuals for DNA analysis were tagged and samples collected by excising gill tissue from the right side of the specimen. Gill tissue was preserved in an ambient temperature DMSO/EDTA buffer [26] or in 95% EtOH. The specimens were subsequently preserved in formalin, transferred to 70% ethanol, and deposited in the Neotropical Freshwater Fish Collection located at the Smithsonian Tropical Research Institute in the Republic of Panama [27]. Table S2 lists the STRI identification numbers, drainage locales, and Genbank accession numbers for all samples used in this study.

DNA sequence data was collected from the mitochondrial ATP synthase 6/8 (ATP6/8) using routine laboratory procedures [28]. The entire ATP6/8 region was amplified and sequenced using primers L8331/H9236/L8524 [28]. DNA sequence was determined using ABI 377 and 3100 automated sequencers following the manufacturers' recommendations.

For all phylogenetic analyses, *A. biseriatus* and *A. rivulatus* were used as outgroups [29, 30]. Redundant haplotypes were combined into single OTUs keeping track of geographic origin. A maximum likelihood (ML) approach was used to estimate phylogenetic relationships among mtDNA haplotypes using the program GARLI v2.0 [31]. The optimal evolutionary model(s) (those within 95% CI of the optimal) was inferred using jModelTest [32] and PhyML [33] following the author's recommendation. The resulting best models estimated in jModelTest based on the Bayesian Information Criteria (BIC) were used in ML analyses in GARLI with branch support estimated by bootstrapping ($n = 1000$) with the assistance of SumTrees found in the Python package DendroPy [34].

Additional estimates of clade support were determined by Bayesian inference using the program MrBayes 3.1 [35]. We ran four independent Bayesian analyses for 1,000,000 generations with 4 Markov chains sampling every 100 generations using the GTR+I+G model. The resulting log-likelihood scores were plotted against generation time to search for stationarity in the results. For all analyses, stability was reached within ca. 1,000 generations. The burn-in was set at 1000 generations and the remaining tree samples used to generate a 50% majority rule consensus tree to calculate the posterior probability of each clade.

In order to boost the confidence in the phylogeographic patterns observed, 318 individuals of *Andinoacara* distributed throughout the LCA region were studied using PCR restriction fragment length polymorphism genotyping (RFLP) similar to that of Reeves and Bermingham [24]. Five restriction enzymes (AluI, DdeI, HaeIII, HinfII, and RsaI) were used that in combination allowed us to uniquely identify samples to one of the primary clades found in the ML analysis. Individuals from each clade that were sequenced were included in the analysis as reference. This allowed us to estimate the frequency of the primary clades within the various drainage areas in Panama.

To test if the ATP6/8 region was evolving in a clock-like manner, we performed a likelihood ratio test using PAUP* v4.0d64 [36]. A GTR+I+R model was used with parameters estimated as above. Based on these results, a relaxed clock Bayesian estimation of the dates of diversification of the primary clades was performed using BEAST v1.6.2 [37]. The TRN+I+G model was used in four independent runs with a chain length of 10^7 generations, a burn-in of 10000 generations, and parameter sampling every 1000 iterations using the uncorrelated lognormal distribution option for rate variation across branches. A Yule tree prior was assumed and all other options were set to default. Lacking any well-defined calibration points (i.e., fossil representation) in the resulting phylogeny, we used a per lineage rate of divergence (divergence per lineage per 10^6 years) estimated from McCafferty et al. [38] of 0.0065 ± 0.002 based on parametric bootstrapping of *Abudefduf saxatilis*/*trossulus* (Pomacentridae) geminate species pair for the ATP6/8 gene region [39]. We feel, this estimate of divergence rate is appropriate for our purposes given that the estimate is from a homologous

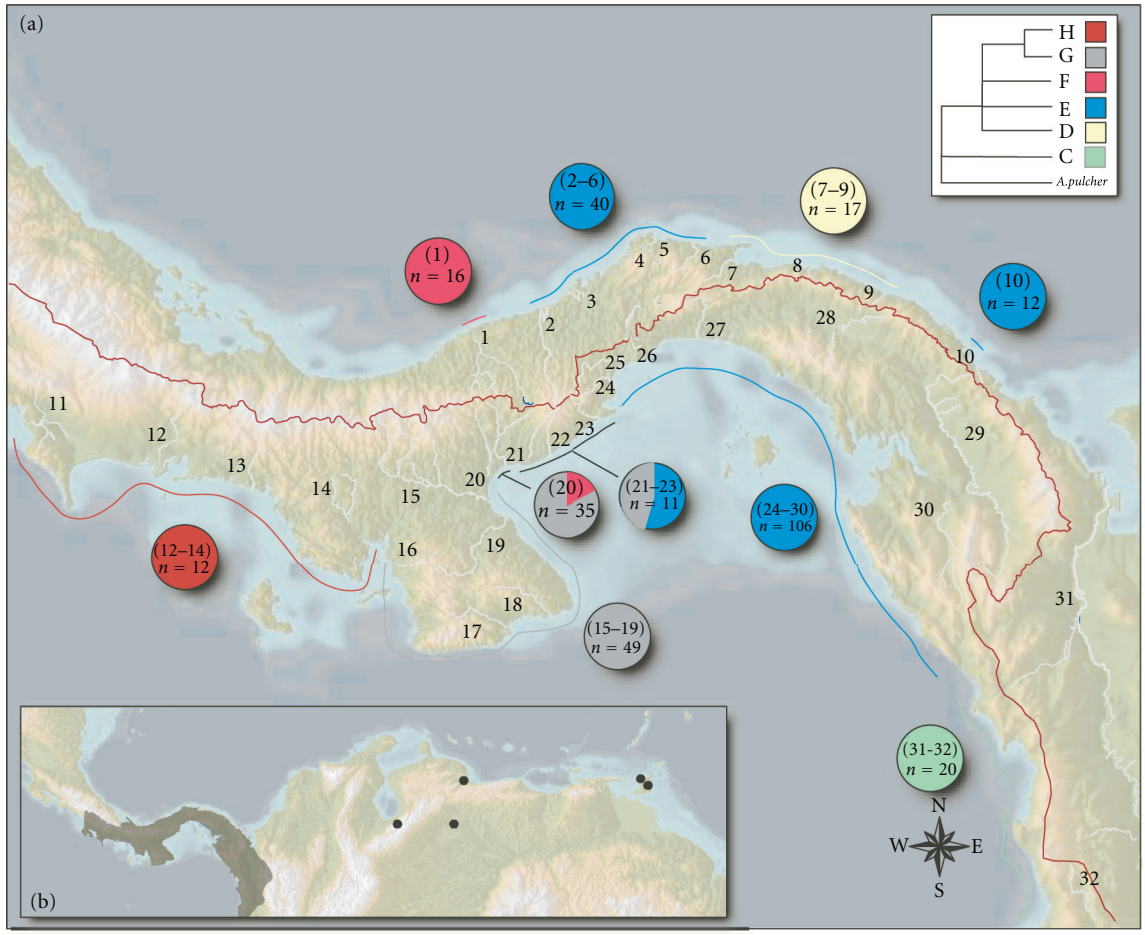

Figure 1: Map showing (a) sampling locations used in this study and (b) the distribution range of *A. coeruleopunctatus*. In addition, the frequency of ATP6/8 clades and the sample size used to estimate clade frequencies are included. Drainage areas and biogeographic regions follow those of Smith and Bermingham [4]: (1) Rio Cocle del Norte; (2) Rio Indio; (3) Rio Chagres; (4) Rio Cascajal; (5) Rio Pina Pina; (6) Rio Cuango; (7) Rio Mandinga; (8) Rio Azucar; (9) Rio Playon Chico; (10) Rio Acla; (11) Rio Coto; (12) Rio Chiriqui; (13) Rio San Felix; (14) Rio San Pablo; (15) Rio Santa Maria; (16) Rio Tebario; (17) Rio Tonosi; (18) Rio Oria; (19) Rio La Villa; (20) Rio Cocle del Sur; (21) Rio Anton; (22) Rio Farallon; (23) Rio Chame; (24) Rio Capoeira; (25) Rio Caimito; (26) Rio Grande; (27) Rio Pacora; (28) Rio Bayano; (29) Rio Tuira; (30) Rio Iglesia; (31) Rio Atrato, Colombia; (32) Rio Baudo, Colombia. The biogeographic areas are Chagres: 1–10; Chiriqui: 11–13; Santa Maria: 14–24; Tuira: 25–31. Values within pie charts are the drainage ID numbers used to estimate the frequency and the sample size.

mtDNA gene region, the value falls squarely in the middle of estimates from other fish species, and that the Cichlidae and Pomacentridae are relatively closely related. The independent runs were combined using LogCombiner v1.4.8, dates of divergence along with their 95% confidence intervals (HPD) were estimated using Tracer v1.4, and the resulting phylogeny and 95% HPD for the dates of divergences for the major clades visualized using FigTree.

3. Results

Complete ATP6/8 gene sequences were determined for 47 individuals of *A. coeruleopunctatus* collected throughout the species' range, plus an additional 8 individuals representing the closely related *A. pulcher* from 5 different geographic locations in Trinidad and Venezuela. The outgroup comprises *A. biseriatus* ($n = 2$) from the Atrato River, Colombia and *A. rivulatus* ($n = 8$) representing four drainages along the Pacific versant of Peru. The final dataset contained a total of 842 bp of sequence data for 65 individuals, of which 173 bp are parsimony informative. All sample identification codes and GenBank accession numbers can be found in Table S1.2 (Supplementary Data). Finally, we used RFLP analysis to genotype 318 individuals from 33 rivers to better estimate the distribution of mtDNA clades within drainages and biogeographic regions in Panama.

The resulting ML gene tree for ATP6/8 using the TRN+I+G model (based on the results of model selection using jModelTest; Supplement S1A) is shown in Figure 2. There are several noteworthy features of the topology shown in Figure 2 that can be summarized as follows: (1) the phylogeny identifies eight well-supported mtDNA clades within *A. coeruleopunctatus* and *A. pulcher*; (2) *A. pulcher* forms a monophyletic grouping with two mtDNA clades representing the Orinoco region of Venezuela (labeled clade A) and

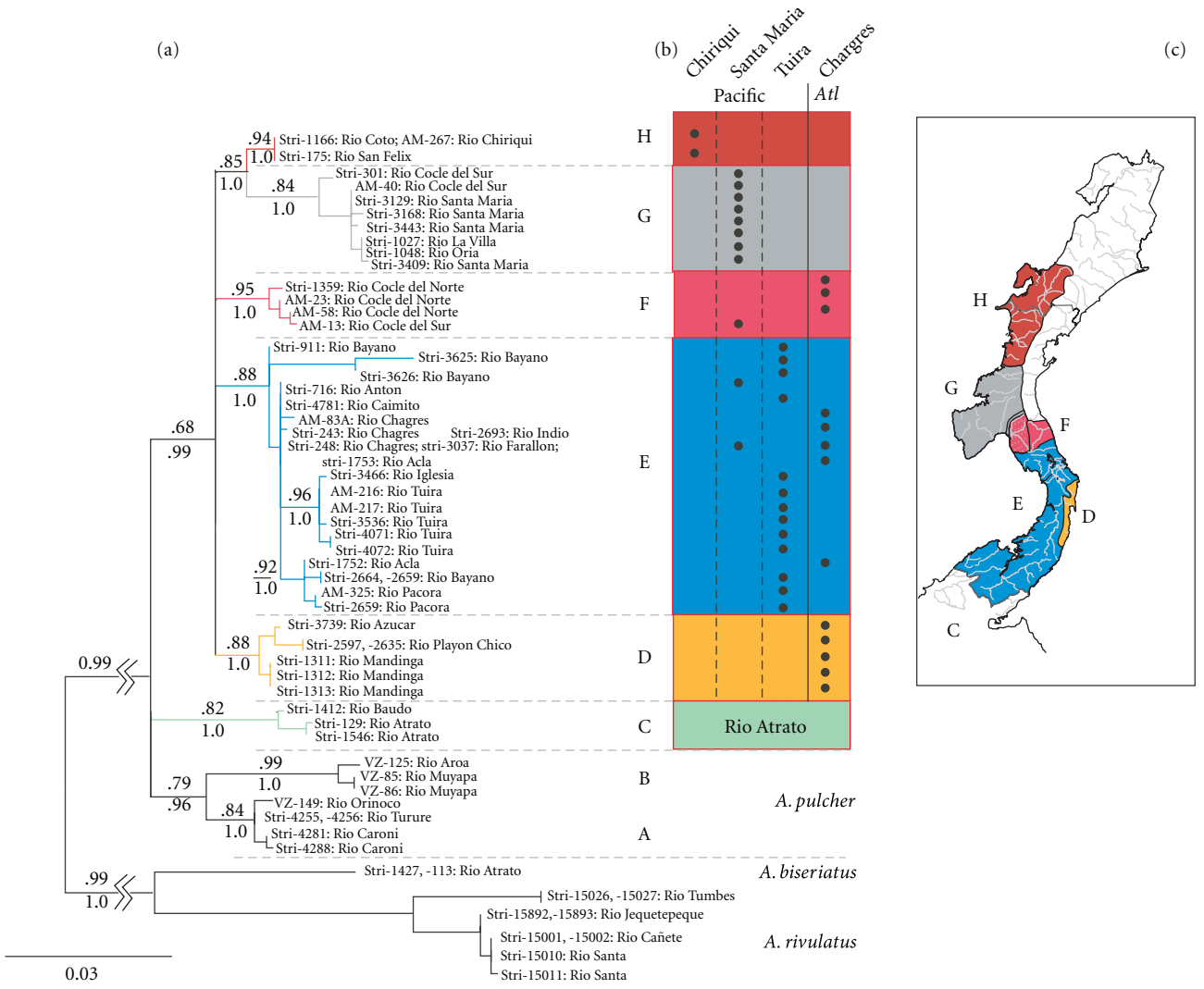

Figure 2: Phylogenetic relationship among mtDNA haplotypes. (a) ATP6/8 ML tree using the TRN+I+G model. Bootstrap support and clade confidence values (posterior probabilities from a Bayesian analysis) for major clades are above and below branches, respectively. (b) Origin of samples by rivers assigned to their biogeographic regions as described in Smith and Bermingham [4]. Branch colors reflect biogeographic regions or drainage areas as found in Figure 1. The inset map is a stylized representation of the range of the major clades along drainage and biogeographic boundaries.

Rio Maracaibo River/Trinidad (clade B); (3) the relationship between *A. pulcher* and *A. coeruleopunctatus* could not be resolved overall, with a three-way polytomy occurring among *A. pulcher*, a clade consisting of *A. coeruleopunctatus* from the Rio Atrato and Rio Baudo drainages in Colombia (clade C), and the remaining *A. coeruleopunctatus*; (4) *A. coeruleopunctatus* from Panama and Costa Rica form a monophyletic group consisting of five primary clades (labeled clades D through H) with a three way polytomy at its root.

3.1. Paraphyly of A. coeruleopunctatus with respect to A. pulcher. The polytomy describing the relationship among *A. pulcher*, Colombian *A. coeruleopunctatus*, and the remaining Panama and Costa Rica *A. coeruleopunctatus* appears to be robust based on the results of various phylogenetic analyses (Supplement S1B), and is unchanged by an additional 1048 bp of ND2 sequence (Supplement S1C). In addition, there is no evidence for saturation effects in either the ATP6/8 or combined data sets (Supplement S1F). Therefore, we consider the relationship unresolved; both *A. pulcher* and *A. coeruleopunctatus* from Colombia form reciprocal monophyletic clades that are approximately equally divergent from the remaining *A. coeruleopunctatus* from Panama and Costa Rica.

3.2. Phylogeographic Patterns and Cross-Cordillera Exchange. Following Figure 2, the next principal clade consists of four major clades (D, E, F, and [G,H]) that form an unresolved polytomy among the Panama *A. coeruleopunctatus* though each clade is well supported. Clade D is composed of samples derived from Rio Mandinga, Rio Azucar, and Rio Playon Chico in the Western San Blas region. In most of our

analyses, this group forms a poorly supported basal clade leading to the remaining Panama *A. coeruleopunctatus*, with an average bootstrap support of ca. 55% but with 100% support based on the Bayesian analysis. Due to the rather low support from most analyses for a basal D clade, we choose to show clade D as part of a four-way polytomy. Whether one considers this Western San Blas clade, a sister clade to all other Panama *A. coeruleopunctatus* or a member of an unresolved polytomy is not particularly important to the discussion below. The key point is that this region appears to form a unique, reciprocal monophyletic group closely related to all other Panama *A. coeruleopunctatus*.

The remaining three clades (E, F, and [G,H]) form an unresolved polytomy among the remaining Panama *A. coeruleopunctatus* in all analyses though each clade is well supported. Clade E is composed of samples derived from rivers and drainages in the Chagres, Tuira, and Santa Maria biogeographic regions of Smith and Bermingham [4] and includes samples from both sides of the continental divide including a disjunct population from Rio Acla in Eastern San Blas. Clade F represents haplotypes collected from the Rio Cocle del Norte (1 in Figure 1; Chagres biogeographic region) and Rio Cocle del Sur (20 in Figure 1; Santa Maria biogeographic region). This particular clade shows clear evidence of cross-cordillera sharing of mtDNA haplotypes. Finally, the last two clades (G and H) form a sister group, with samples derived from river drainages found in the Santa Maria (G) and Chiriqui (H) biogeographic regions forming separate well-supported clades. Mapping of these clade distributions can be found in Figure 2.

3.3. RFLP Analysis of Clade Frequencies. We sampled a total of 318 individuals from 33 different drainages for 5 restriction enzymes that in combination permit us to uniquely identify individual samples to one of the primary *A. coeruleopunctatus* clades. The resulting clade frequencies are summarized in Table 1 and graphically in Figure 1. Detailed haplotype frequencies can be found in Supplemental Table S1.3). If a particular biogeographic region contained a single clade (e.g., Tuira and Santa Maria in Table 1), then the data were pooled within biogeographic region. Otherwise, the clade frequencies were pooled by drainage area.

The RFLP data reinforce and expand on the phylogeographic patterns found for the ATP6/8 sequence data. One hundred and fifty-eight individuals representing 13 rivers in the Tuira and Chagres biogeographic regions, including the Rio Acla in eastern San Blas, all are haplotypes specific to the E clade. Furthermore, the 17 individuals sampled from three rivers in the western San Blas area within the Chagres all contained haplotypes specific to the D clade, and the 16 individuals collected from the Cocle del Norte river on the western edge of the Chagres biogeographic region all carried haplotypes specific to the F clade. The RFLP data also confirm that the Atrato in Colombia, the Santa Maria (49 individuals from 6 drainages), and Chiriqui (12 individuals from three drainages) biogeographic regions in Panama are fixed for mtDNA clades C, G, and H, respectively, with no evidence of interchange between the Santa Maria and Chiriqui regions.

RFLP analysis of 35 individuals also confirmed that Rio Cocle del Sur, a Pacific slope drainage, carried both clade F (17%), characteristic of Rio Cocle del Norte, and clade G (83%), characteristic of the Santa Maria. Again, these results are consistent with the phylogeny based on mtDNA sequence data and provide strong evidence for the movement of fish between the Cocle del Norte to the Cocle del Sur.

A. coeruleopunctatus populations collected from the Rio Anton, Rio Farallon, and Rio Chama drainages (21, 22, and 23 in Figure 1) at the western edge of the Tuira/Chagres biogeographic region contain haplotypes from both the E clade, characteristic of the Tuira and Chagres regions, and the G clade, characteristic of the Santa Maria region, in roughly equal frequencies when pooled (45% and 55%, resp.). This is a finding that would have been missed in the absence of the more complete geographic sample collected for the RFLP analysis.

3.4. Relaxed Clock Estimates of the Time of Divergence. The estimated dates of divergence based on relaxed clock Bayesian analysis for the major clades are summarized in Figure 3. The MCMC process appeared to perform well with stationarity achieved in all runs and ESS values well exceeding 200. Due to the unresolved relationship among the major clades in Figure 2, our analyses focused on the general time of divergence among these clades rather than the detailed timing of divergence within clades (see Supplement S1E for more information on these results). The estimated date of diversification of the Panama *A. coeruleopunctatus* mtDNA clades is around 3.4 Ma, with a 95% HPD from 1.5 to 5.5 Ma. We estimated the date of divergence among Panamanian A. *coeruleopunctatus*, Colombian *A. coeruleopunctatus*, and *A. pulcher* to have occurred around 5.9 Ma with an estimated HPD of 2.7 to 10.5 Ma.

4. Discussion

The phylogeographic pattern of *A. coeruleopunctatus* in the LCA region was described above based on mtDNA sequence and RFLP data. There are four aspects of our results that are most relevant to our discussion of the relationship to biogeographic patterns and previous work on primary freshwater fishes in this region. First, including *A. pulcher*, the eight unique clades (two in *A. pulcher*, one from *A. coeruleopunctatus* in Colombia, 5 in Panama *A. coeruleopunctatus*) detected are closely associated with the biogeographic regions described by Smith and Bermingham [4] and Abell et al. [40]; however, there are some important differences. Second, the branching order of four of the principle Panama clades was unresolved, suggesting a period of rapid diversification of colonizing populations followed by allopatric differentiation. Third, the timing of this rapid diversification based on a relaxed clock analysis corresponds to the final rise of the Isthmus of Panama in the late Pliocene. Fourth, there is strong evidence for historical cross-cordillera exchange in at least two regions of LCA: one centered in the Cocle del Sur/Cocle del Norte drainages, the other in the Chagres/Tuira region. Before we proceed to discuss the implications of these

Table 1: Frequency of clades among drainages and biogeographic regions based on RFLP analysis of the ATP6/8 gene region.

River	Biogeographic region or drainage	E	F	G	H	D	C	*n*
Rio Acla	Acla	12						12
Rio Anton	Anton	5		2				7
Rio Farallon	Anton	1		2				3
Rio Cham	Anton			1				1
	Anton total	6		5				11
Rio Atrato	Atrato						9	9
Rio Baudo	Atrato						5	5
Rio San Juan	Atrato						6	6
	Atrato total						20	20
Rio Cascajal	Chagres	4						4
Rio Chagres	Chagres	23						23
Rio Cuango	Chagres	6						6
Rio Pina Pina	Chagres	1						1
Rio Indio	Chagres	6						6
	Chagres total	40						40
Rio Chiriqui	Chiriqui				8			8
Rio Coto	Chiriqui				3			3
Rio San Felix	Chiriqui				1			1
	Chiriqui total				12			12
Rio Cocle del Norte	Cocle del Norte		16					16
Rio Cocle del Sur	Cocle del Sur		6	29				35
Rio Azucar	San Blas					8		8
Rio Mandinga	San Blas					6		6
Rio Playon Chico	San Blas					3		3
	W. San Blas total					17		17
Rio San Pablo	Santa Maria			10				10
Rio Tebario	Santa Maria			1				1
Rio La Villa	Santa Maria			6				6
Rio Oria	Santa Maria			3				3
Rio Santa Maria	Santa Maria			27				27
Rio Tonosi	Santa Maria			2				2
	Santa Maria total			49				49
Rio Bayano	Tuira	54						54
Rio Iglesia	Tuira	4						4
Rio Tuira	Tuira	30						30
Rio Caimito	Tuira	6						6
Rio Capira	Tuira	4						4
Rio Grande	Tuira	2						2
Rio Pacora	Tuira	6						6
	Tuira total	106						106

findings, it is worth considering the placement of *A. pulcher* with respect to *A. coeruleopunctatus*.

4.1. Paraphyly of A. coeruleopunctatus. Our results indicate that the relationship among *A. pulcher*, *A. coeruleopunctatus* from Colombia, and Panama *A. coeruleopunctatus* is best characterized as a three-way polytomy, implying that either *A. pulcher* may require reconsideration as a separate species, or *A. coeruleopunctatus* from the Atrato and Baudo River drainages be elevated to specific status, changing the distribution of *A. coeruleopunctatus* to strictly Panama and southern Costa Rica to make it a monophyletic group. Though we strongly emphasize that these results are based on a single mitochondrial gene and therefore should be viewed with caution, they are generally consistent with recent detailed studies on neotropical cichlids that included members of the genus *Andinoacara* [29, 30], though these studies

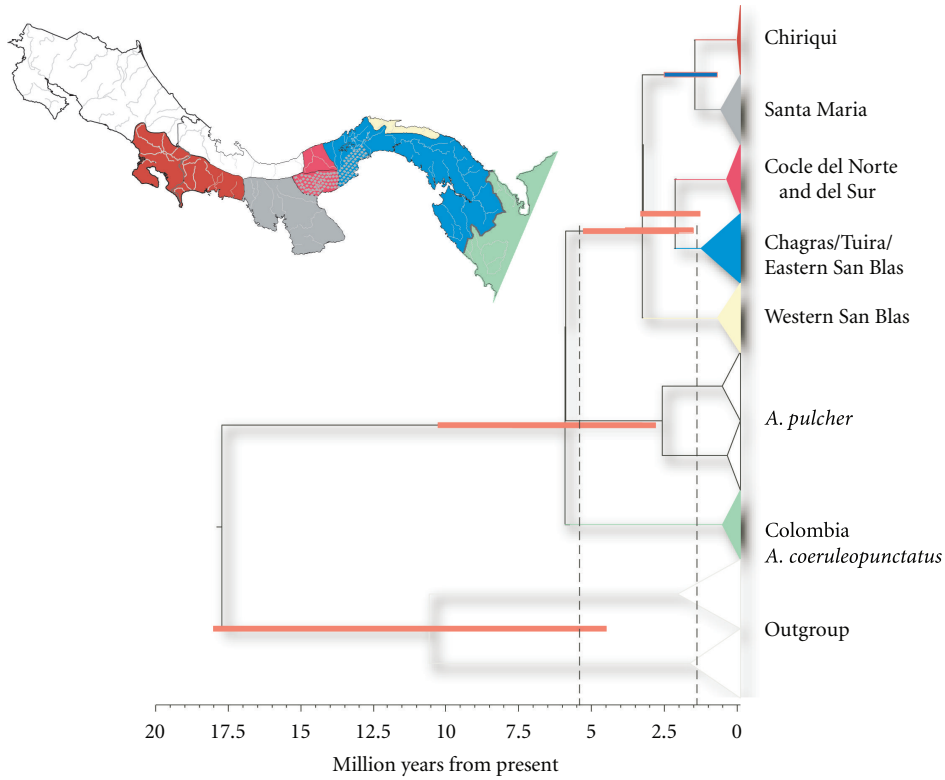

Figure 3: Estimated range of divergence of major clades based on a relaxed Bayesian analysis. Clades are colored as in Figure 1. The inset map is a stylized representation of the range of the major clades along drainage and biogeographic boundaries.

did not include *A. coeruleopunctatus* from Colombia. The unresolved branching order among these three clades dates to between 2.7 and 10.5 Ma with an estimated mean of 5.9 Ma, suggesting that *A. coeruleopunctatus* originated either in northern Colombia or in areas within an evolving LCA landscape itself prior to the estimated final rise of the Isthmus of Panama. Unfortunately the results of our Bayesian clock analysis do not exclude the possibility of a pre-Pliocene dispersal event, though our estimate suggests a much later origin than estimated for secondary freshwater fishes in these regions (ca. 18 to 15 Ma; [20, 23, 41–43]).

4.2. Phylogeographic Patterns within A. coeruleopunctatus and Biogeography. One of the key results from our study of *A. coeruleopunctatus* in LCA is that we find significant phylogeographic structure. To summarize, based on both DNA sequence and RFLP data we detected the presence of six unique clades that are consistent in many respects with the biogeographic provinces described by Smith and Bermingham [4]: clade C is from the Atrato region of Colombia, E combines the Chagres, Tuira, and portions of the Santa Maria regions, G is Santa Maria-specific, and H is from the Chiriqui region including Costa Rica. However, there are some key differences that may lend important insights into the forces driving phylogeographic and biogeographic structure in this region. First, our results divide Smith and Bermingham's Chargres region into three groups: (1) a reciprocal monophyletic Western San Blas clade (D), (2) a clade (F) that contains samples from Rio Cocle del Norte (Chargres) and Rio Cocle del Sur (Santa Maria), and (3) the remaining drainages from the Chargres region that all fall within a single clade (E) that includes samples from the Tuira region and two rivers from the Santa Maria (Rio Anton and Rio Farallon). It is interesting to point out that these two rivers are located on the border region between the Tuira and Santa Maria assemblages in Smith and Bermingham [4] and along with Rio Chama appear to represent a transition zone between the Tuira/Chagres and the Santa Maria biogeographic zones on the Pacific coast. The western extent of the Tuira/Chargres clade appears to be in Rio Anton, as there is no evidence for clade E haplotypes in Cocle del Sur. Smith and Bermingham [4] discuss the difficulties of assigning biogeographic boundaries in this area, further highlighting the similarity between the molecular and biogeographic patterns. In summary, our results coalesce the Tuira and Chargres biogeographic zones, excluding the rivers from the Atlantic versant found in Western San Blas and Cocle del Norte, and defines a fuzzy transition zone between the Tuira and Santa Maria assemblages centered around Rio Anton.

This clear association between phylogeography and biogeography is punctuated by the general unresolved branching order among the clades. A general lack of resolution of branching order among otherwise well supported clades is observed in most of the LCA freshwater fishes studied to date [20, 23, 24] and is not due to saturation effects (Supplement S1F). Whether this basal polytomy is due to rapid

diversification or simply reflects the limits of resolution of our data remains to be tested. Nonetheless, the rate of diversification of lineages in the newly emergent landscape must have been quite fast. Estimates of the timing of this diversification event based on a relaxed molecular clock are slightly older but nonetheless still consistent with the estimated date for the final rise of the Isthmus of Panama at 3.2 Ma [14] and are consistent with previous results reported for other freshwater fishes in this region. Though recent evidence suggests a more fully emergent LCA earlier then previously estimated [44], the final closure of the seaway connecting the Pacific and Caribbean remains as estimated in Coates and Obando [14], and appears to have been an effective barrier to the migration of many freshwater species. However, once a newly emergent bridge connected South America with the evolving LCA landscape, it was rapidly colonized by lineages of *A. coeruleopunctatus* that diversified rather quickly in the separate drainage areas.

Being characterized as a secondary freshwater fish species, we anticipated finding little if any phylogeographic structure among LCA drainages for *A. coeruleopunctatus*. Yet we find strong evidence for the formation of clades along biogeographic regions and drainage areas, a pattern characteristic of primary freshwater fishes in this region. This finding suggests that, regardless whether *A. coeruleopunctatus* is considered a primary or secondary freshwater species, similar forces may be acting to drive community composition and phylogeographic structure in freshwater fishes in this region. It also suggests that ecological differences among species may play a lesser role in determining phylogeographic patterns, a topic we will explore further below after discussing other important aspects of the phylogeography in *A. coeruleopunctatus*.

4.3. Cross-Cordillera Exchange. Another notable result from our analysis is a clear pattern of sharing of mtDNA clades across the central cordillera of LCA. We see this in two regions: the Chagres-Tuira area and further west around Cocle del Norte-Cocle del Sur. In both cases the degree of clade sharing is extensive and readily interpreted in light of the local physiography. In Western Panama, the central Cordillera rises steeply to over 2000 meters in most places as a consequence of late Pliocene and early Pleistocene subduction of a low-density Cocos Ridge [14]. The Cordillera gradually diminishes in relief to the east reaching a low of 200 meters near the headwaters of the Cocle del Sur in Central Panama and 100 meters in the region of the Panama Canal. The San Blas region (the Atlantic slope rivers of eastern Panama) and rivers of the eastern Pacific slope of Panama are separated by mountains rising only about 200 to 500 meters. The Chagres-Tuira region then encompasses one of lowest points of the central cordillera around the Panama Canal region, while the Cocle del Sur-Cocle del Norte are in the region of El Valle, an area of ancient volcanic activity. In both cases it is easy to infer processes of drainage rearrangements (stream capture) to account for cross-cordillera exchange.

Previous studies of other freshwater fishes in this region also show evidence for cross-cordillera exchange in these two regions. In a total of ten species studied to date, we find three with unequivocal evidence for cross-cordillera sharing of highly divergent haplotypes: *Bryconamericus emperador*, *Brycon argenteus,* and *Rhamdia guatemalensis* [23, 24]. In addition, three species provide limited evidence (due to small sample sizes or phylogenetic branching patterns) for cross-cordillera sharing of clades: *Hypopomus*, *Roeboides*, and *Rhamdia laticauda* [20, 23]. Four of the ten species show no evidence for cross-cordillera exchange: *Pimelodella* (both the A and B types), *Brycon striatulus*, *Bryconamericus scleroparius*, and *Cyphocharax magdalenae* [22, 24].

Compared with the above studies, our results for *A. coeruleopunctatus* are unique in that, based on large sample sizes from both the mtDNA sequence and RFLP analysis, we are able to make a strong statement concerning the extent of cross-cordillera exchange. We found that not only is cross-cordillera exchange extensive in central Panama and the Canal area, but also there is a key difference in the pattern of clade sharing between the two regions, with important implications. In the Chagres-Tuira area, there is evidence for extensive sharing of a diverse clade (E) by both Atlantic and Pacific drainages (except for western San Blas, which we discuss below), suggesting a long history of dispersal among drainages in this area.

This is in stark contrast to the situation in the central Panama. In the Cocle del Norte Atlantic drainage, there is evidence for a single clade (F) only, while the Cocle del Sur Pacific drainage contains two clades in very different frequencies, the F clade characteristic of Cocle del Norte (17%) and the G clade (83%) characteristic of the adjacent Pacific biogeographic area of Santa Maria. It is interesting to note that even though the Rio Anton drainage is immediately adjacent to the Cocle del Sur drainage, there is no evidence for sharing the Atlantic derived Cocle del Norte clade (F) in Rio Anton based on both sequence and RFLP data. One possible explanation for this result is that cross-cordillera exchange has occurred by one-way invasion of the Pacific Cocle del Sur by Atlantic Cocle del Norte lineages and that this migration event was relatively recent (occurring well after the final rise of the Isthmus perhaps due to tectonic events in the El Valle area) and limited in extent.

We attempted to test this hypothesis using a Baysian approach implemented in the program Migrate-n (Supplement S1D). However, the results from a series of analyses were contradictory and unconvincing. Maximum likelihood estimates of migration between Cocle del Norte (CdN) and Cocle del Sur (CdS) were consistent with the above hypothesis ($M_{\mathrm{CdN}^-} > M_{\mathrm{CdS}} = 85.030$; $M_{\mathrm{CdN}^-} > M_{\mathrm{CdS}} = 0$), though we have little faith in these estimates. Further Baysian analyses proved suspect, as the data appeared to be insufficient for the analysis to reach stationarity in the estimates of the parameters theta or M in most models (Supplement S1D). A test of three competing models of migration between the two regions (model 1 : CdN to CdS only; model 2 : CdS to CdN only; model 3: equal migration) using Bayes Factors suggested that model 1 (CdS to CdN only) had the highest probability, contrary to the ML estimates and our intuitive interpretation.

Our interpretation of these conflicting results is that our dataset is simply insufficient for this type of analysis and that the results from the Bayes factor approach should be viewed with caution. Nonetheless, determining the migration patterns in this region is important to understand the history or migration in this important region of Panama (see Supplement S1D).

In stark contrast to this extensive sharing of clades across the cordillera found in central Panama and the Chagres region, we find that the western San Blas region, represented by the Rios Azucar, Mandinga, and Playon Chico, maintains a reciprocal monophyletic clade (D) that roughly forms a polytomy with the other major clades characterizing *A. coeruleopunctatus* in the LCA. There is mixed evidence for this pattern in other neotropical freshwater fishes. *Pimelodella*, *Roeboides*, and *Bryconamericus emperador* evidence a reciprocal monophyletic group in the western San Blas area [20, 22, 24], while *Rhamdia guatemalensis*, *Hypopomus*, and *Brycon argenteus* join western San Blas with the Tuira or Chagres biogeographic region [20, 23, 24]. Smith and Bermingham [4] found no evidence for a separate western San Blas biogeographic province, subsuming it under the Chagres. Nonetheless, the evidence in *A. coeruleopunctatus* for a distinct, allopatric western San Blas clade is unequivocal.

4.4. Relationship to Historical Drainage Patterns. Previous studies on other LCA primary freshwater fishes studied to date suggest that the observed phylogeographic patterns are driven at least in part by historical patterns of river anastomosis (dispersal) and stream capture (vicariance and dispersal) driven by tectonic events and eustatic sea level changes. Though we lack a detailed paleodrainage model for Panama, Smith and Bermingham [4] were able to make good first-order approximations of historical drainage patterns and suggest a model of river anastomosis and stream capture that are consistent with the phylogeographic patterns found here with three important differences. First, cross-cordillera exchange via stream capture may well explain the presence of a distinct Atlantic Cocle del Norte clade that shares haplotypes with Pacific Cocle del Sur. Second, the coalescing of Chargres and Tuira biogeographic regions is also possibly explained by cross-cordillera exchange perhaps enhanced by the construction of the Panama Canal [45]. Finally, the presence of a reciprocal monophyletic WSB clade within the Chargres biogeographic region suggests long-term isolation of this region from surrounding drainages.

It is difficult to determine why the Tuira/Chargres clade was able to expand into many of the Atlantic drainages yet not into the Cocle del Norte or the Western San Blas region. There are no obvious geological features that might serve to isolate these two regions from the surrounding areas other than a limited continental shelf, yet these two regions retain deep monophyletic lineages dating to the rise of the isthmus. Why would we see the expansion of clades into some regions (e.g., into eastern San Blas and Atlantic drainages from Chagres) but not others that are geographically proximate (e.g., western San Blas) when no clear barrier to dispersal is obvious? The remainder of this paper will explore a possible explanation for the long-term retention of deep monophyletic lineages even when barriers to dispersal appear limited.

4.5. Retention of Long-Term Phylogeographic Patterns. To summarize, we have shown that the phylogeographic pattern found in *A. coeruleopunctatus* can be explained at least in part by historical drainage patterns driven by a combination of eustatic sea level changes and drainage rearrangements consistent with the biogeographic assemblages described in Smith and Bermingham [4] and consistent with the model first proposed by Bermingham and Martin [20]. However, there are aspects of the phylogeographic relationships that are not easily explained by drainage history alone. In particular, though there appear to have been extensive opportunities for dispersal among drainages following the rise of the isthmus, we see the retention of relatively deep monophyletic lineages in some drainage regions that appear to have no distinctive geological barriers to dispersal. This is somewhat of a paradox; how can deep monophyletic lineages be retained despite repeated and periodic opportunities for dispersal? The simplest explanation is of course that dispersal simply did not occur into these areas for some reason. We do not find this a particularly satisfying explanation. We suggest that there may be other mechanisms acting to retain (reciprocal) monophyly even in the presence of dispersal events.

Bermingham and Martin [20] argue that lineage turnover and ecological differences among species explain in large part the differences in phylogeographic patterns found among primary freshwater fishes in this region. Reeves and Bermingham [24] expanded on this and argued that demographic processes can also have a profound impact on the phylogeographic patterning in colonizing species. The basic thrust of their explanation is that once resident populations (the descendants of early colonizers) approach an environment's carrying capacity, those populations will tend to be more resistant to the influx of new immigrants, a process previously described as persistent founder effects [46] or priority effects.

Boileau et al. [46] first evoked priority effects to explain observed high levels of divergence among freshwater invertebrate populations in the presence of high rates of dispersal, demonstrating that high Fst values can be retained over long periods of time in populations at or near their carrying capacity even when dispersal rates were substantial; in effect, dispersal becomes effectively decoupled from gene flow. De Meester et al. [47] expanded on this concept, pointing out that adaptive divergence among populations can reinforce priority effects, a process they refer to as the monopolization hypothesis. Though priority effects are commonly put forward as an explanation for the long-term maintenance of phylogeographic variation in freshwater invertebrates (e.g., [47–50]; though see [5]), they are rarely evoked for vertebrates. There is clear evidence for the important role played by priority effects in driving community composition (e.g., [51–55]), yet very limited empirical evidence for its role

in maintaining phylogeographic structure [56]. Nonetheless, if a population is at or close to its carrying capacity, then priority effects can potentially play a role in determining long-term patterns of divergence. Waters [57] recently made a similar argument, pointing out the important role that competitive exclusion of secondary dispersers (similar to the monopolization hypothesis mentioned above) may play in maintaining phylogeographic structure within species even in the face of dispersal.

In the case of *A. coeruleopunctatus* and primary freshwater fishes in this region, we propose that contemporary phylogeographic patterns are determined by a combination of historical and contemporary drainage patterns and demographic effects. When population size is below carrying capacity, then drainages may be susceptible to invasion by migrant lineages that disperse from adjacent drainages during times of river anastomosis and stream capture. If the dispersal event is relatively recent and/or population sizes are relatively high but below carrying capacity, then the presence of multiple divergent lineages can occur, as seen, for example, in the Cocle del Sur drainage. Lineage turnover and local clade extinction can then occur over time, consistent with what we see in the Chagres-Tuira area and eastern San Blas. However, those drainages that retain populations close to the carrying capacity will tend to be resistant to invading lineages during bouts of dispersal. If a particular lineage has an adaptive advantage to invading lineages, then priority effects will be further reinforced (Monopolization hypothesis). This may explain the retention of the reciprocal monophyletic Western San Blas clade in Caribbean Panama and unique clades in the Cocle del Norte and Santa Maria/Chiriqui region.

5. Conclusion

In conclusion, we found significant phylogeographic structure in the LCA cichlid *A. coeruleopunctatus* that is concordant with the biogeographic regions described based on community composition data and consistent with phylogeographic patterns previously described for primary freshwater fishes in this region. However, our results lend a slightly different perspective to previous studies in that we find a coalescing of some biogeographic zones (Chagres/Tuira), evidence for phylogeographic structure within others (Chargres), and strong evidence for cross-cordillera dispersal. Though the branching order of the various phylogeographic clades could not be determined, the date for the diversification of the primary clades is consistent with the final rise of the Isthmus of Panama. We explain our results as due to rapid colonization and diversification in the emerging landscape, with the resulting contemporary phylogeographic pattern a result of both historical drainage connections and demographic processes (i.e., priority effects). This model highlights the dynamic between historical timing, rates of dispersal, and demographic processes with the end result being that priority effects and lineage turnover can potentially have a profound impact on the phylogeographic patterns found in some species. Understanding the extent to which we can begin to tease apart the complex interactions of historical and demographic processes will depend on multispecies phylogeographic studies coupled with careful estimates of rates of dispersal, historical trends in effective population sizes, and statistical testing of these models. Our results with *Andinoacara* demonstrate the added insights into the processes driving phylogeographic patterns that can be gained from studying species with varying life history strategies, ecologies, and phylogenetic histories.

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Research Article

Spawning Coordination of Mates in a Shell Brooding Cichlid

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Aim. External fertilisation requires synchronisation of gamete release between the two sexes. Adequate synchronisation is essential in aquatic media because sperm is very short-lived in water. In the cichlid *Lamprologus callipterus*, fertilisation of the eggs takes place inside an empty snail shell, where females stay inside the shell and males have to ejaculate into the shell opening. This spawning pattern makes the coordination of gamete release difficult. *Methods*. This study examined the synchronisation of males and females during egg laying. *Results*. The results showed that the male initiates each spawning sequence and that sperm release and egg laying are very well synchronised. 68% of all sperm releases occurred at exactly the same time when the female laid an egg, and 99% of ejaculations occurred within ±5 seconds from egg deposition. On average 95 eggs are laid one by one with intervals of several minutes between subsequent eggs, leading to a total spawning duration in excess of six hours. *Conclusions*. We discuss this exceptional spawning pattern and how it might reflect a conflict between the sexes, with males attempting to induce egg laying and females extending the egg laying period to raise the chance for parasitic males to participate in spawning.

1. Introduction

In species with external fertilisation, males and females must synchronize gamete release. In fish, various forms of information that transfer between the sexes are involved to ensure fertilisation. Visual communication involves the use of colour signals [1–3] and behaviour [4, 5]. Chemical signals may be used to coordinate spawning, as fish possess a very powerful olfactory apparatus detecting odours in very low concentrations [6–8]. Some fish use also sound production during courtship and spawning [9–11].

In the highly polygynous cichlid, *Lamprologus callipterus* (Boulenger), fertilisation of eggs takes place inside an empty shell of the snail *Neothauma tanganyicense*. Large nest males collect these shells as spawning substrate and defend shell nests as their territories [12]. Females ready to spawn visit these nests, select a shell, spawn inside of them, and take care for the brood for 10 to 14 days [13]. Among all animals, this fish species shows the most extreme sexual size dimorphism (SSD) with males being larger than females. In our study population, nest males are on average 10 times heavier than the females they spawn with, but the magnitude of SSD varies greatly between populations, with up to 60 times heavier males than females in Muzimo, a Northern population in the Democratic Republic of Congo [14]. Results of an earlier study suggest that sexual selection mechanisms are probably not as important as natural selection mechanisms for the evolution and maintenance of the SSD in *L. callipterus* [15]. Rather, this extreme SSD appears to be mainly affected by ecological constraints, with opposing selection pressures on the two sexes: males need to pass a threshold size to be able to carry shells, and female size is constrained by the limited size of their breeding substrate, shell size, and by intrasexual competition for shells ([14, 15], see also [16]).

During spawning, males and females have highly restricted visual contact and hardly any direct bodily contact, because the female head sticks deep inside the shell and the male is much too large to enter the shell [13, 15]. On average 91 eggs are laid (range: 35–160, data from ref. [17]), and laying of a whole clutch lasts exceptionally long. In an

earlier laboratory experiment, spawning took 9.3 h (range: 5.5–12 h) and in the field 6.9 h ($n = 29$ spawnings at 10 nests, range: 2.16–10.28 h, [18]). Nest male spawning may be parasitized by two other male types performing alternative mating tactics, medium sized sneaker males and dwarf males, which can reside inside the shell during the course of spawning [12, 19, 20]. Parasitic males are always exposed to sperm competition, whereas nest males monopolize spawning without the participation of reproductive parasites in most spawnings [12, 21]. Preliminary results suggest that nest males' sperm lived longer than dwarf male sperm due to the longer sperm head size, but that dwarf male sperm swam straighter and faster than nest male sperm [22]. Little is known about the different spawning behaviours of the two sexes in this species.

Previous work on male-female timing and coordination of spawning behaviour has focused on species in which males and females have full visual and often also bodily contact [23, 24]. This is the first study addressing such male-female interactions where visual and bodily contact is highly limited. Using field and laboratory experiments we examined who is initiating a spawning bout and whether and how mating pairs of *L. callipterus* mutually synchronize gamete release under the limited availability of visual information.

2. Methods

2.1. Behaviours. In the field, the following behaviours of males and females were recorded without the observers entering their nests or disturbing the spawning process: male mouthing, female shifting, and male moving forward to put his genital papilla motionless over the shell entrance, which equals the duration of sperm release [18] (Table 1). In the laboratory, the behaviour and location of males and females were recorded simultaneously, including male head shake, male head in, female egg laying, and female moving out of the shell. Distinct behaviour events were separated from each other either by inactivity or other behaviours. Behaviours in the laboratory were recorded as frequencies (event) or durations (state) with "the Observer 3.0" (Noldus Information Technology).

2.2. Field Observations. Field data were collected in May 2002 at Kasakalawe, Lake Tanganyika, Zambia (8°46′S; 31°5′E) by Scuba diving at a depth of 12.8–15.9 m. The colony consisted of more than 100 nests, 97 of which were marked with numbered stones and observed in total 250 times. Spawning males were detected by hovering about one meter over the colony and observing each nest for 5 min. If spawning was detected, this nest was observed for 10 to 20 minutes (mean observation duration: 11.52 minutes), and the behaviours of all participants were recorded. Analyses were done at the female level to avoid pseudoreplication.

2.2.1. Mouthing Experiment. The male's opening and closing of the mouth when his head sticks into the shell entrance produce a water flow that can be detected by the female [25]. Therefore, we hypothesized that by mouthing a male might stimulate the female to lay an egg, and if she reacts, release sperm. To test this hypothesis, the mouthing was imitated experimentally. Twenty spawning and 165 guarding females were tested to check whether they reacted differently to this potential signal. Females of *L. callipterus* never hide in snail shells from predators [13] and no female, that is not ready to spawn, already spawning, or guarding, is accepted in the territory by the nest owner. Therefore, females staying inside snail shells are all guarding females and not individuals that simply hide in shells. We predicted that spawning females should react with shifting to prepare egg release, while guarding females should not show such behaviour. As spawning females, we used females that were currently laying eggs in shells of nest males, and spawning always restarted within 3 minutes after our short disturbance. As guarding females, we used females that currently took care for their broods in a nest male's shell, also without influencing their natural behaviour any further. Each of the groups was treated in two different ways in randomised succession: in the experimental treatment, male mouthing was simulated with our fingertips by creating a water current into the shell entrance. In the control treatment the fingertips were held still over the shell entrance. For each group of females, half of the fish (determined randomly) started with the experimental treatment and the other half with the control treatment, and female reaction to the manipulation was recorded. Shifting behaviour looks exactly the same in the situation when the water current is produced by a mouthing male versus when the water current is produced by moving fingers. We could not standardize the water current between traits completely. However, one person stimulated the mouthing behaviour in all trials (ZHB) and attempted to keep the water current as constant as possible between trials. A hierarchical log-linear analysis with backward elimination of terms was performed to test whether the group (guarding or spawning females), the treatment (control or experimental), or their interaction influenced the female's reaction (yes: female shifting; no: no reaction).

2.2.2. Undisturbed Observations. During 65 observed spawning events within 40 nests, the frequency of the nest male mouthing the shell with a spawning female inside was determined. Subsequently, it was noted whether the female showed shifting behaviour and whether the male released sperm. Mouthing was also observed at shells with non-spawning (guarding) females inside ($n = 140$), and the reaction to male mouthing behaviour was compared between spawning and guarding females. The occurrence of different sequences of different spawning behaviours was analysed with χ^2-tests.

2.3. Laboratory Experiment. For the laboratory experiment, shells were prepared with Plexiglas windows to allow the observation of egg laying without disturbing the animals. Spawning observations were performed in four 100-litre tanks, into each of which five empty *N. tanganyicense* shells were introduced that were fixed to a PVC plate with silicon glue. Three of the five shells were closed with a small stone

Table 1: Spawning behaviours and locations of males and females. (f): Behaviours of which frequencies were recorded, for all other behaviours frequency and duration were recorded.

	Behaviour/location	Description
Male behaviour	Mouthing	The male puts his head into the shell entrance and opens and closes his mouth repeatedly
	Head shake (f)	The male shakes his head quickly side wards in front of the shell entrance
	Head-in (f)	The male puts his head a few mm into the shell entrance but without actively opening and closing the mouth
	Sperm release (i.e. ejaculation)	The male puts his genital papilla over the shell entrance and stays motionless in this position for up to 4 seconds
Female behaviour	Shifting	The female partly moves out of the shell, flickers with the caudal fin and immediately moves back again to her original position. At all times, her tail remains visible and her head remains inside the shell, so no direct visual contact with the male or our fingers appears possible.
	Egg laying (f)	The females deposits an egg at the inner surface of her snail shell
	Moving out of shell	The female comes partly out of the shell
Male location	At the shell	The male is close to the shell (<4 cm)
	Away	The male is >4 cm away from the shell and cannot communicate with the female inside the shell
Female location	In the shell	The female is completely or partly inside the shell*
	Out of the shell	The female is completely out of the shell

*Data analyses were conducted starting when the female was inside the shell.

to prevent females from entering. The two other shells were positioned with their Plexiglas windows against the front screen of the tanks to enable video recording. Into each tank, a nest male (SL range: 89–123 mm) and five adult females (SL range: 41–50 mm) were introduced. In all four tanks, the two visible shells were continuously recorded on videotape for 13 hours a day (from 08:00 hours to 21:00 hours). These recordings were analysed for all periods during which a female was inside a shell and then started spawning.

2.3.1. Analysis of Laboratory Data. Nine spawning events could be used to analyse all behaviours involved in egg laying and sperm release. Since males released sperm already before the female started to lay eggs, the time the male released sperm for the first time and the time until the female laid her first egg ("prelaying period") were determined. The time for which males continued to release sperm after the female stopped laying was also determined ("postlaying period"). Male sperm release frequencies and durations between the pre-, post-, and egg-laying periods were compared. Descriptive statistics show means ± SD if data were normally distributed (Kolmogorov Smirnov-tests, $P > 0.1$) and medians with quartiles if data differed from a normal distribution ($P < 0.1$). Note that period durations and behaviour traits varied widely between females, and some periods were missing for some females due to impeded sight. Therefore, we appropriately used paired t-tests (per female) to test for male differences in sperm release characteristics between the pre- and laying periods, rather than ANOVA to test the differences among three periods at once. For the pre-and postlaying periods, $n = 6$ spawnings, because three females were already inside the shell when recording started (but had not started egg laying yet), and for three females, the recording ended before the male had ceased sperm release in the postlaying period. For the laying period, all 9 spawnings could be used for data analysis of sperm release frequency and duration, but only 6 cases for comparing period durations (pre- versus egg-laying versus postperiod).

To test whether and how male and female spawning patterns were synchronised, we analysed for each egg laid the behaviours shown in the period ranging from 15 sec before until 15 sec after it was laid (31 seconds, where $0 = -0.5$ to +0.5 sec around the egg being laid). Fourteen cases were observed where two eggs were laid and one case where three eggs were laid in one spawning bout, that is, within 15 seconds after the first egg was laid. Here, behaviours were analysed around the timing of the first of these multiple eggs. The proportion of time males and females showed different spawning behaviours within these 31 seconds around egg laying and the median time differences of each behaviour to the moment of egg laying were determined. The relationship between the number of eggs laid and the number of ejaculations was determined, as were the time intervals between two laid eggs and two subsequent sperm releases. Additionally, it was checked whether and how the intervals between two subsequent eggs, between two sperm releases, and the sperm release duration varied between pairs and within the egg laying period. All analyses were performed by SPSS and report two-tailed probabilities. χ^2-values are from chi-square cross-tabulation tests, unless otherwise specified.

3. Results

3.1. Field Observations, Mouthing Experiment. As expected, in the field spawning females reacted significantly more often to the experimental treatment (simulating mouthing

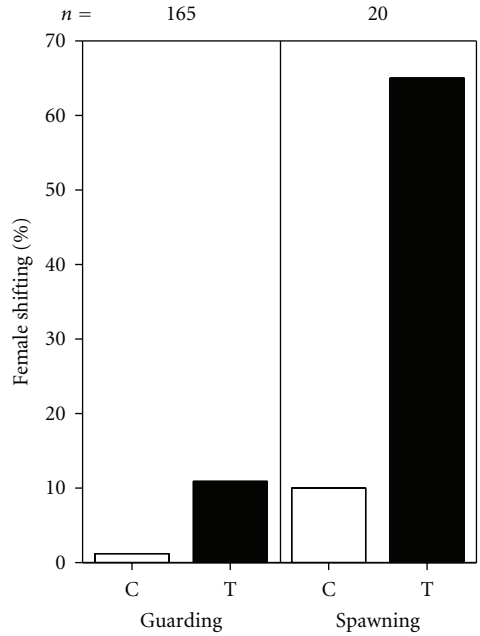

Figure 1: Percentage of cases in which guarding and spawning females reacted by shifting to the control, no finger movements (C), and experimental treatments, water current induced by finger movements (T).

behaviour) by shifting behaviour than guarding females (hierarchical loglinear analysis with backward elimination of nonsignificant terms: group × reaction: $\chi^2 = 27.8$, $P < 0.0001$, Figure 1). Additionally, experimentally tested females shifted significantly more often than females of the control treatment (treatment × reaction: $\chi^2 = 25.8$, $P < 0.0001$, Figure 1). Other interactions were all nonsignificant (group × treatment: $\chi^2 = 3.8$, $P = 0.059$, group × treatment × reaction: $\chi^2 = 0.198$, $P = 0.656$). Note that moving our fingers to the shell entrance (control) and subsequently induction of the water current (treatment) never induced the females in any of the 2 × 2 treatment arms to retreat into the shell to flee from this disturbance.

3.2. Field-Undisturbed Observations. In the field, out of seven possible behaviour sequences of male mouthing, female shifting, and sperm release, only three were actually shown (see Table 2 for frequencies of sequences). It never occurred that the female shifted and/or the male released sperm without male mouthing behaviour shown before. In 110 of the 796 observed mouthings, a rapid sequence of mouthing with no reaction was shown, followed quickly again by mouthing, female shifting, and sperm release. Altogether, female shifting occurred 473 times and sperm release occurred 476 times after 796 mouthing events (three times the male released sperm after mouthing without the female showing shifting behaviour in between). Therefore, female shifting and male sperm release showed almost the same frequency and always followed mouthing, but 40% of mouthings did not result in spawning. Mouthing was also performed at shells with guarding females inside ($n = 140$), but guarding females never responded by any behaviour. The reactions to male mouthing behaviour differed highly significantly between spawning and guarding females ($\chi^2 = 168.2$, df = 1, $P < 0.001$).

Table 2: Frequencies and proportions of all possible sequences of three essential behaviours of males and females during spawning in the field.

Male mouthing	Female shifting	Male sperm release	Frequency	% of total
Yes	Yes	Yes	473	59.4
Yes	Yes	No	0	0
Yes	No	Yes	3	0.4
Yes	No	No	320	40.2
No	Yes	Yes	0	0
No	Yes	No	0	0
No	No	Yes	0	0

3.3. Laboratory Experiment

3.3.1. Timing of Female Egg Laying and Male Ejaculation. During nine observed spawning events of an entire clutch, females laid the first egg 66.3 ± 41.0 min (mean ± SD, range: 31.7–110.1 min) after entering the shell for spawning (prelaying period, see Figure 2(a)). Egg laying lasted 279.6 ± 34.2 min ($n = 9$, range: 242.4–326.4 min, laying period), during which an egg was laid on average every 2.14 min (0.47 ± 0.37 eggs laid per min). Males started to release sperm long before the female laid her first egg, about four minutes after the female went into the shell (median = 3.98, quartiles: 1.29–9.60, range: 0.08–68.53 min). Males continued to ejaculate after the female laid her last egg on average for 35.7 ± 15.8 min ($n = 6$, range: 16.1–57.9 min; i.e., post laying period, Figure 2(a)). Ejaculation rates were significantly lower during the pre- and postlaying periods compared to the laying period (paired t-tests; prelaying versus laying period $t_7 = -2.8$, $P = 0.026$; laying versus postlaying period $t_5 = -4.4$, $P = 0.007$, Figure 2(b)). Sperm release lasted significantly shorter during the prelaying period than during the laying and postlaying periods (paired t-tests; prelaying versus laying period $t_7 = -4.5$, $P = 0.003$; prelaying versus postlaying period $t_4 = -4.6$, $P = 0.01$, Figure 2(c)), whereas there was no difference between the postlaying versus the laying period.

3.3.2. Synchronisation between Males and Females. Synchronisation between males and females was very high: males spent significantly different proportions of time at the shell within the 15 sec before and 15 sec after the deposition of an egg (Friedman test, $\chi^2 = 194.4$, df = 30, $P < 0.001$, Figure 3(a)), but when the female laid an egg, the nest male was almost always present at the shell. Out of 902 eggs laid, only 10 were laid with the males ejaculating more than 5 sec before or after the eggs were laid. Also the frequencies of male mouthing behaviour, male head shaking, and male head in varied systematically around egg laying (Friedman

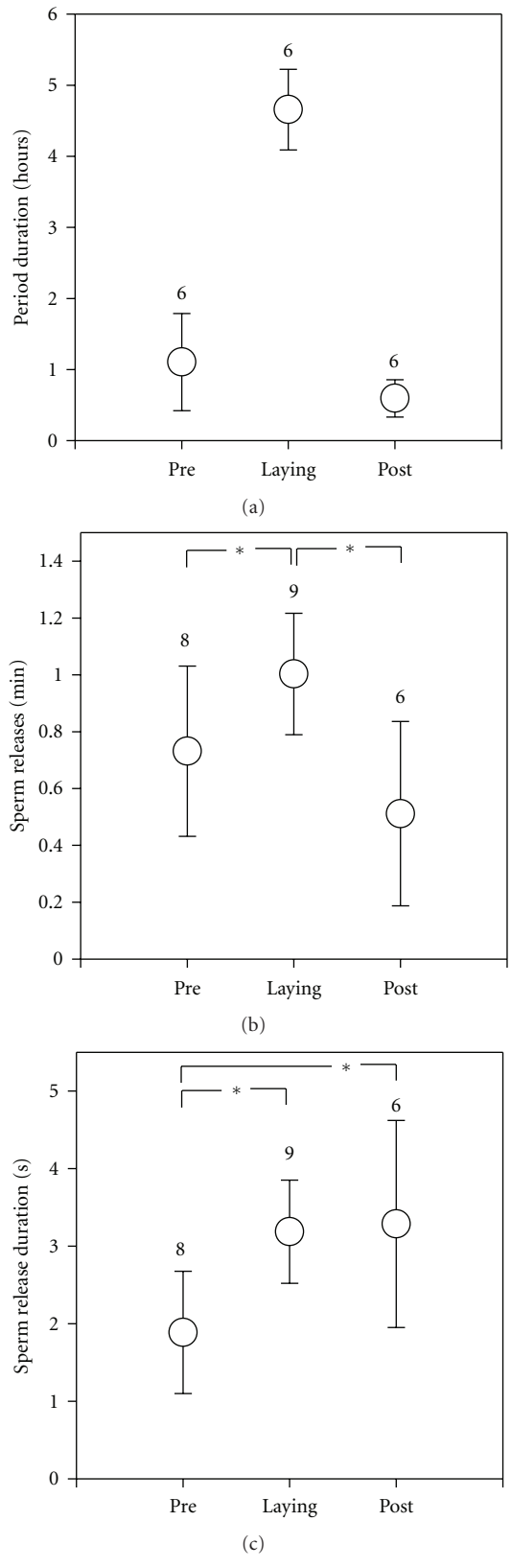

Figure 2: (a) Durations of the prelaying, laying, and postlaying periods in hours; (b) numbers of sperm releases per minute; (c) sperm release durations in seconds. Depicted are means and standard errors of the mean, numbers indicate sample sizes, *mark significant differences $P < 0.05$.

tests, $77.3 < \chi^2 < 189.4$, df = 30 in each test, $P < 0.001$ for each behaviour, Figures 3(b)–3(d)). Briefly before egg deposition females showed shifting behaviour (median = −1.6 sec, Friedman test, $\chi^2 = 202.9$, df = 30, $P < 0.001$, Figure 3(e)). 68.3% of all sperm releases occurred at exactly the same time when the female laid an egg, and in 98.9% of all cases, the male released sperm between 5 sec before and 5 sec after egg laying (Friedman test, $\chi^2 = 211.9$, df = 30, $P < 0.001$, Figure 3(g)). On average 2.26 (±0.7 SD) ejaculations occurred per laid egg. After egg deposition, the female often moved briefly partly out of the shell (Friedman test, $\chi^2 = 167.7$, df = 30, $P < 0.001$, Figure 3(h)). The interval between two subsequent egg depositions (mean ± SD: 128 ± 101.5 sec) was on average more than twice as long as the interval between two sperm releases (mean ± SD: 62.5 ± 58.2, Wilcoxon's paired test, $z = -2.666$, $P = 0.008$), but both intervals were highly variable.

3.3.3. Behavioural Changes during Laying of a Clutch. With increasing laying duration, females laid the eggs more quickly, but towards the end of laying the egg deposition rate slowed down (Table 3: egg interval, Figure 4(a)). Double eggs and triple eggs (see Figure 3 for definition) occurred evenly distributed over the spawning period (logit General Linear Model, effect of the time course of clutch production $P = 0.64$). The intervals between subsequent sperm releases also varied systematically over the laying period of a clutch (Table 3: ejaculation interval, Figure 4(b)). In seven pairs the sperm release intervals decreased during laying, but in two pairs they increased. The sperm release duration differed significantly between males and generally increased during the laying of a clutch, before slightly decreasing again at the end of laying (Table 3: sperm release duration, Figure 4(c)).

4. Discussion

Despite the limited communication possibilities during spawning, the synchronisation between sperm and egg release is very high. The male signals his readiness to spawn to the female by mouthing into the shell entrance. If the female is ready to spawn she responds to male mouthing by shifting (as confirmed in the mouthing experiment), after which she deposits an egg (median = 1.6 sec after shifting). Egg laying appears highly synchronised with male sperm release (median 0.2 sec after female shifting): in 98.9% of all cases, male sperm release occurred between 5 sec before and 5 sec after egg laying. Good synchronisation is important mainly for three reasons. First, sperm are very short lived in fresh water, so they should meet with the egg within about a minute [22]. Second, female *L. callipterus* usually lays one egg at a time, with long intervals between successive eggs, so each egg needs to be fertilized separately. Third, the prevalence of sperm competition in this species, where dwarf males can reside inside the shell during the course of spawning, which should raise the importance of spawning coordination with their female, at least from the perspective of nesting males [12, 19, 20].

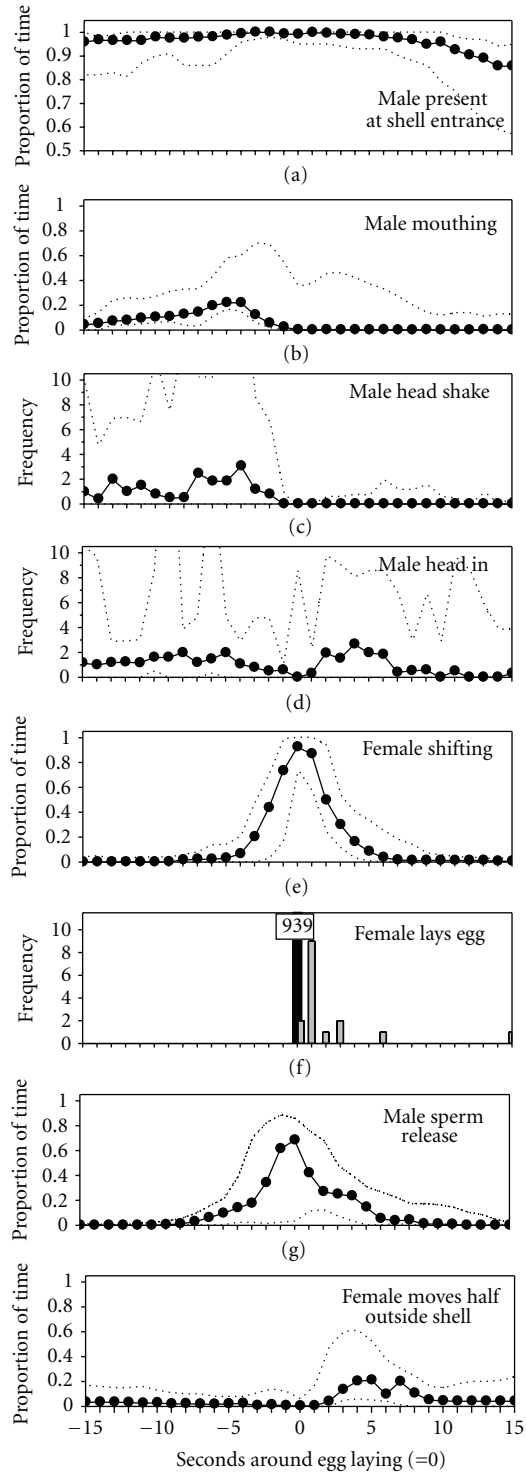

Figure 3: Proportions of time and behavioural frequencies during 31 one sec intervals around 939 eggs laid of (a) the male being present at the shell, (b) the male showing mouthing behaviour, (c) male "head shake" behaviour, (d) male "head-in" behaviour, (e) female shifting, (f) egg laying ($n = 939$: black bar); note that in 14 of 939 cases a second ($n = 14$ eggs) or a third egg ($n = 2$ eggs) was laid within 15 sec (grey bars, total $n = 955$ eggs). (g) Sperm release, (h) female moving half outside the shell. All panels show medians (black dots) and the total range (dotted lines) for $n = 939$ eggs.

Table 3: Changes of the intervals between subsequent egg depositions and sperm releases and sperm release durations in relation to the time course of laying a clutch standardised (time: 0 = first egg laid to 1 = last egg laid). GLM with covariate time (and time squared for egg interval and sperm release duration), random effect of observation number (Nr 1 to 9), and their interaction.

		Egg interval ($n = 946$)		Ejaculation interval ($n = 2052$)		Sperm release duration ($n = 2053$)	
	df	F	P	F	P	F	P
Intercept	1	39.5	<0.001	87.4	<0.001	108.4	<0.001
Time	1	14.0	<0.001	1.0	0.32	15.8	<0.001
Time^2	1	7.5	0.006			9.3	0.002
Nr	8	10.3	<0.001	8.5	<0.001	8.8	<0.001
Nr × time	8	5.5	<0.001	2.0	0.042	4.4	<0.001
Nr × time^2	8	3.8	<0.001			3.9	<0.001

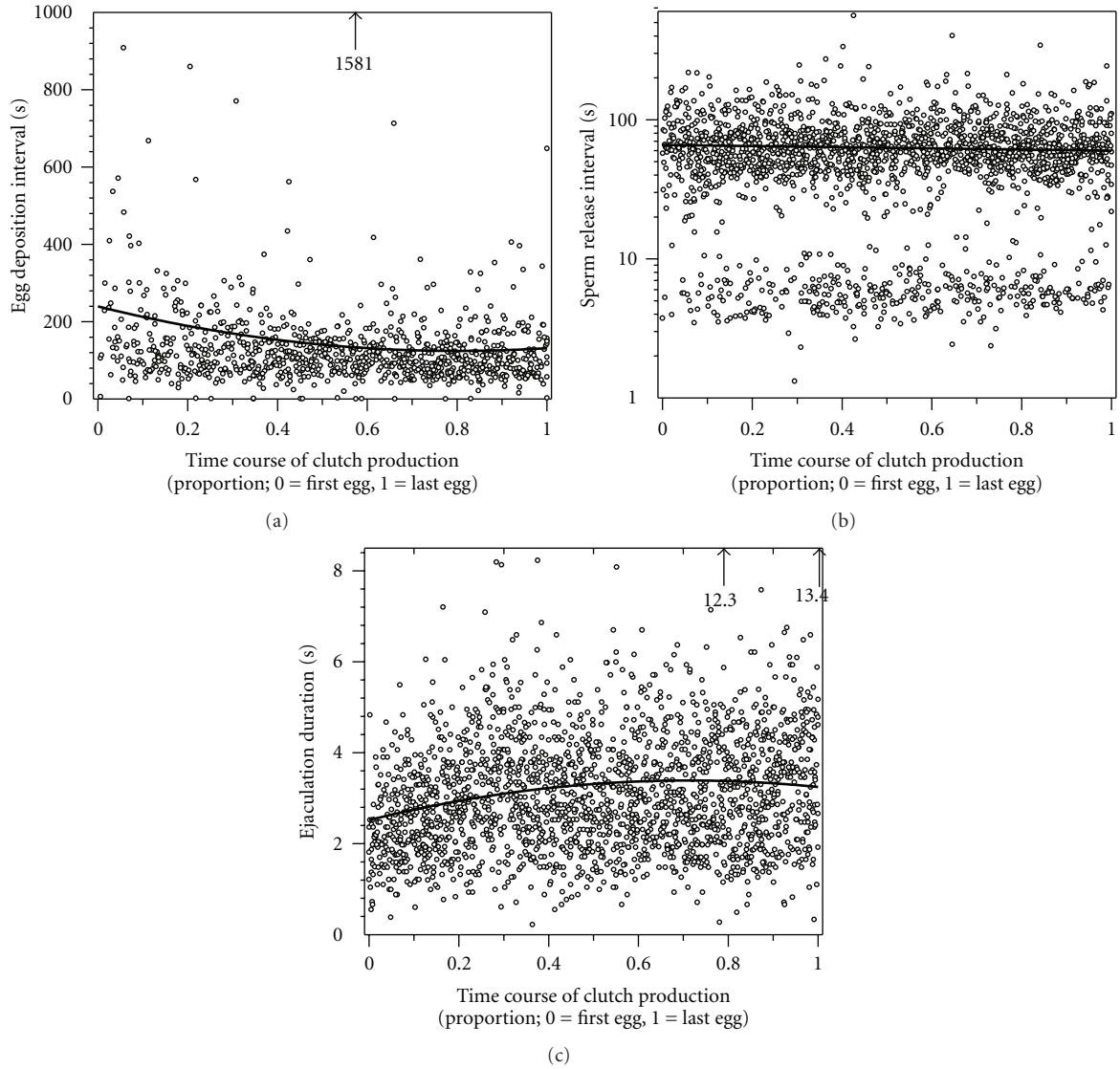

Figure 4: (a) Egg deposition interval, (b) sperm release interval (on a log scale), and (c) sperm release duration in the time course of producing a clutch (0 = first egg laid, 1 = last egg laid). Black lines show the fitted mean values for the 9 spawnings observed (see Table 3), and numbers point towards outliers.

These results show that males and females intensely communicate to synchronise their gamete release inside the snail shell. Apparently, this involves mainly behavioural cues like in the lekking cichlid *Lethrinops parvidens* (Trewavas; [26]) and the Atlantic cod *Gadus morhua* L. [27]. The field experiments suggested that "mouthing" is a crucial male behaviour to induce female egg laying. In the mouthing experiment, spawning females reacted significantly more often to the experimental treatment (simulating mouthing behaviour) by shifting behaviour than guarding females. One might argue that guarding females may detect the water current as signal of predators that try to enter shells. Then, they should not react to the created water current, which also possibly results in the different reactions between spawning and guarding females. However, predators are hardly ever able to perform behaviours similar to male mouthing behaviour above a shell in the field, because the nest male rigorously defends his territory against intruders. Moreover, guarding and spawning females react to a major disturbance (e.g., us touching the shell) by moving and hiding deep inside the shell and then freeze. This is completely different from shifting behaviour. We never observed females to retract into the shell due to the presence of our finger tips at the shell entrance in either treatment. Thus, we do not think that guarding females differently perceived the imitated mouthing behaviour and therefore reacted differently than spawning females.

In undisturbed observations in the field, out of seven possible sequences of important male and female behaviours at spawning, only three occurred, with the sequence "male mouthing—female shifting—sperm release" being most frequent (see Table 2). Female shifting and male sperm release occurred at almost the same frequency (about every 2 min), while mouthing was displayed almost twice as often. Apparently, males attempt to stimulate females by mouthing, and if they react, males respond by releasing sperm.

Males began to ejaculate already in the first five minutes after the female entered the shell for spawning, but females waited more than an hour before laying their first egg. This may suggest that male ejaculations are required for an extended period of time before females start laying, so this may be regarded as part of their courtship. Males might need to signal the availability, quantity, or quality of ejaculates to the female to induce egg deposition. However, given that males suffer from sperm shortage at late stage of spawning [18], prolonged ejaculation in the prelaying period will reduce fertilization success. Then, females may not necessarily prefer ejaculation in the prelaying periods, particularly if such ejaculates result in reduced fertilization success due to sperm shortage, and males may not release sperm in this stage. Males also continued to ejaculate for more than half an hour after the female had laid her last egg. This could be due either to limited information of the male that the female has stopped laying eggs, or it could reflect attempts of the male to induce the female to lay more eggs (see below).

During the egg laying phase in the laboratory experiment, males ejaculated on average more than twice as often as females deposited an egg. Both in the field and in the laboratory, females deposited one egg about every other minute. In the field, the interval between two ejaculations was almost the same as the egg-laying interval, but in the laboratory it was only about half as long. This discrepancy might be due to the higher sperm availability of males in the laboratory, allowing them to challenge their partner to spawn more often. In the field, males spawn with up to 4 females simultaneously (unpublished data), and sperm shortage seems to be a limitation for nest male reproduction [18]. Male timing of sperm release showed two interesting patterns (see Figure 4(b)). First, males often perform a second sperm release shortly after the first one (3–10 sec later, exemplified by the lower band of cases in Figure 4(b) around 3–10 sec). Second, males almost invariably spawned simultaneously with egg release and once again halfway between two eggs (40–140 sec later, exemplified by upper band of cases in Figure 4(b) around 40–140 sec). The second sperm releases were often performed without the female reacting to the males' mouthing behaviour.

The total spawning duration of a clutch was 6.3 hours in the lab (from the first until the last sperm release), during which time the females laid one egg approximately every 2 minutes within 4.55 hours (0.47 eggs/min). In other cichlid species, spawning lasts much shorter, for instance about 1 hour in the mouth-brooder *Tramitichromis intermedius* (Trewavas; [3]), in *Neolamprologus pulcher* (Trewavas & Poll; own observations), and in *Neolamprologus leleupi* (Poll; [28]), during which time the latter two species may lay more than 150 eggs. Longer spawning durations of 2 to 3 hours have been recorded in *Neolamprologus hecqui* (Boulenger; [28]) and *Cichla monoculus* (Spix & Agassiz; [29]), during which time these species lay usually less than 100 eggs. Therefore, it seems that the long spawning duration per clutch of more than 6 hours in *L. callipterus* is exceptional among cichlids and perhaps in fish in general. A possible function of this extended spawning period might be the induction of sperm competition by females (cf. [30]). There is increasing evidence that better sperm competitors sire higher quality offspring [31–33]. By extending the spawning duration females can increase the chances that other males participate in fertilising the eggs, which seems particularly appropriate in a species with two different male parasitic spawning tactics. Females may even prefer to spawn with many males, independently of sperm competition, because sperm shortage may cause reduced fertilization success of females if only nest males participate in the spawning events. However, the fact that egg deposition seems to be mainly induced by males does not support the hypotheses that the prolonged spawning duration is induced by females.

The complete spawning pattern in *L. callipterus* suggests that the male has an overall expectation of the actual egg-laying pattern, and that the exact timing and duration of sperm release are targeted to cumulatively fill the shell with sufficient sperm numbers to fertilize the majority of eggs, taking into account the expected longevity of his sperm. During laying a clutch, males may change strategies of ejaculates as time goes by, because sperm reserves will decrease with

increasing number of ejaculates. In *L. callipterus*, males first slightly increase sperm release duration but slightly decrease it during late stage of spawning males, which might also be due to males suffering from sperm shortage (see also [18]). This shows that it is important to know the time since egg laying started, to understand male strategy of ejaculation, and also may explain the variation of ejaculate interval among males.

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Review Article

The Impact of the Geologic History and Paleoclimate on the Diversification of East African Cichlids

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The cichlid fishes of the East African Great Lakes are the largest extant vertebrate radiation identified to date. These lakes and their surrounding waters support over 2,000 species of cichlid fish, many of which are descended from a single common ancestor within the past 10 Ma. The extraordinary East African cichlid diversity is intricately linked to the highly variable geologic and paleoclimatic history of this region. Greater than 10 Ma, the western arm of the East African rift system began to separate, thereby creating a series of rift basins that would come to contain several water bodies, including the extremely deep Lakes Tanganyika and Malawi. Uplifting associated with this rifting backponded many rivers and created the extremely large, but shallow Lake Victoria. Since their creation, the size, shape, and existence of these lakes have changed dramatically which has, in turn, significantly influenced the evolutionary history of the lakes' cichlids. This paper reviews the geologic history and paleoclimate of the East African Great Lakes and the impact of these forces on the region's endemic cichlid flocks.

1. Introduction

East Africa had a highly dynamic geological and ecological history. Over the past 35 million years (Ma), tectonic plates have shifted, rifts in the landscape have opened, rivers have reversed course, and lakes have formed and desiccated. It is within this environment that the world's largest extant vertebrate radiation has originated. Centered within the East African Great Lakes, over 2,000 species of cichlid fish have diversified to fill nearly every niche available to a freshwater fish. All of these fish are endemic to East Africa, many are single lake endemics, and several are microendemics found only at isolated areas within a given lake. Here, we examine the geologic and climatic history of East Africa and discuss how these forces have influenced this spectacular vertebrate radiation.

1.1. Geologic Setting and East African Climate. The East Africa rift system (EARS) is the roughly north-south alignment of rift basins in East Africa (Figure 1) that defines the boundary between the Somalian and African plates [2, 3]. The EARS is divided into two structural branches that are also oriented roughly north-south (Figure 1). Rifting in the eastern branch began ~30–35 Ma in the Afar and Ethiopian Plateau and propagated north-south until it impinged on the strong Precambrian Tanzanian cratonic block, which is in the center of the East Africa Plateau [4]. The extensional stress associated with the rifting or with widespread plume-related uplift was then transported westward across the craton to weaker mobile crust on the craton's western edge creating the western branch of the rift [4, 5]. The timing of the initiation of the western branch of the EARS is uncertain and has been suggested to have begun as early as ~25 Ma to as recently as ~12–10 Ma [4, 5]. After its onset, rifting then continued to propagate in the western branch of the EARS forming the rift basins that encompass Lakes Tanganyika and Malawi [2–7]. Extension and uplift associated with rifting created a reversal in rivers flowing westward across the East African Plateau and caused backponding into a topographic low in between the two branches of the rift, forming Lake Victoria [6–12].

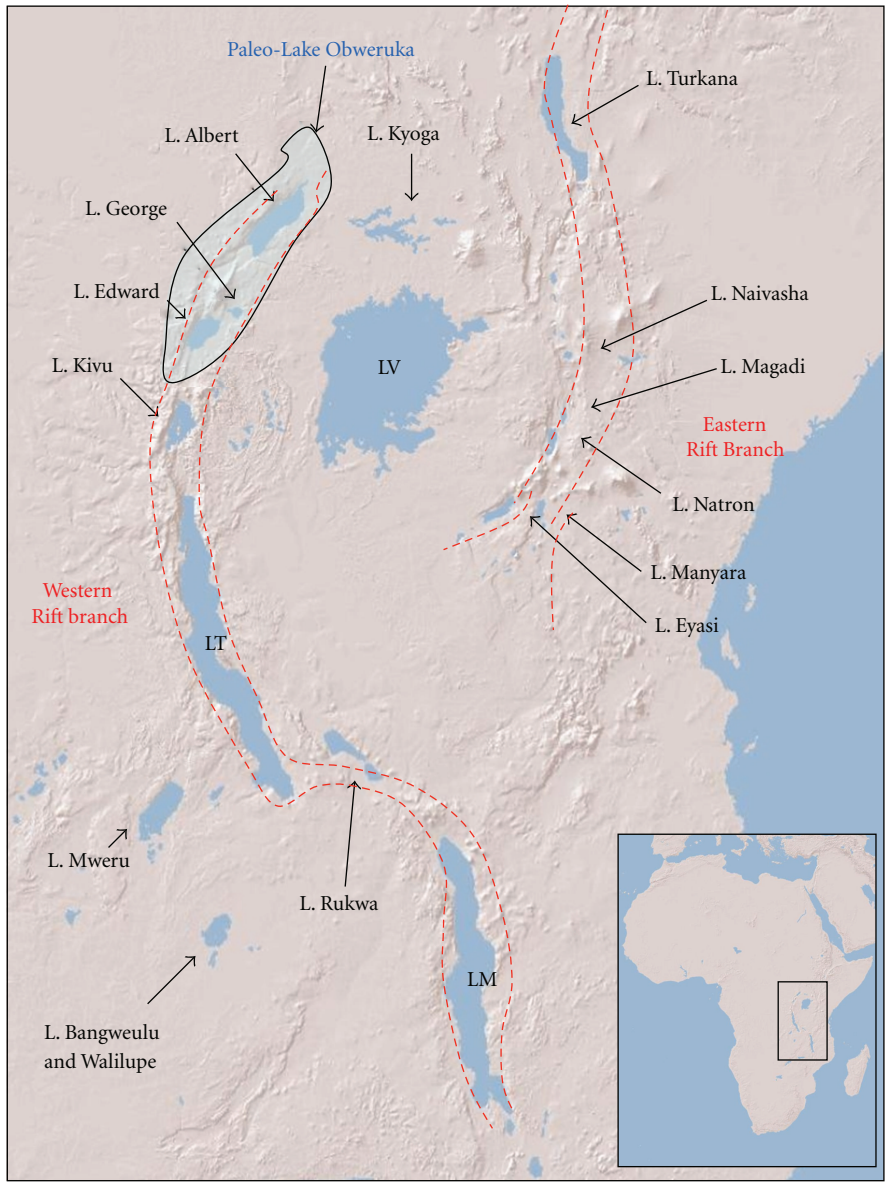

Figure 1: Geographic position of the study region and location of the East African rift. The position of paleo-Lake Obweruka is displayed in light blue [1]. The approximate locations of the two main branches of the East African rift system are displayed in red-dashed line (LV: Lake Victoria, LT: Lake Tanganyika, and LM: Lake Malawi).

The climate of the African Great Lakes (Lakes Tanganyika, Malawi, and Victoria) is driven primarily by annual changes in precipitation associated with the migration of the intertropical convergence zone (ITCZ) (Figure 2). The ITCZ is the zone of maximum insolation received by the Earth's surface and seasonally migrates between Tropics of Cancer and Capricorn in June and December, respectively. The warm air in the region of maximum heating rises, drawing the cooler trade winds equatorward, where they converge, effectively increasing convection and rainfall at the location of the ITCZ. The movement of the ITCZ across the African continent results in a wet-dry monsoonal climate for the African Great Lakes. The southernmost extent of the ITCZ is south of Lake Tanganyika; thus, it crosses the lake twice between September and May as it migrates southward to the Tropic of Capricorn and then back northward towards the Tropic of Cancer. As a result, the lake experiences a long wet season during which there is a lull in precipitation in January and February [31] when the core of the ITCZ is south of Lake Tanganyika and a pronounced, shorter dry season. The ITCZ crosses Lake Malawi once a year producing pronounced wet and dry seasons. The ITCZ crosses Lake Victoria twice due to the lake's position on the equator, resulting in two wet and two dry seasons. In each of the African Great Lakes, a significant portion of water loss is a result of evaporation; thus, the lakes are very sensitive to changes in precipitation.

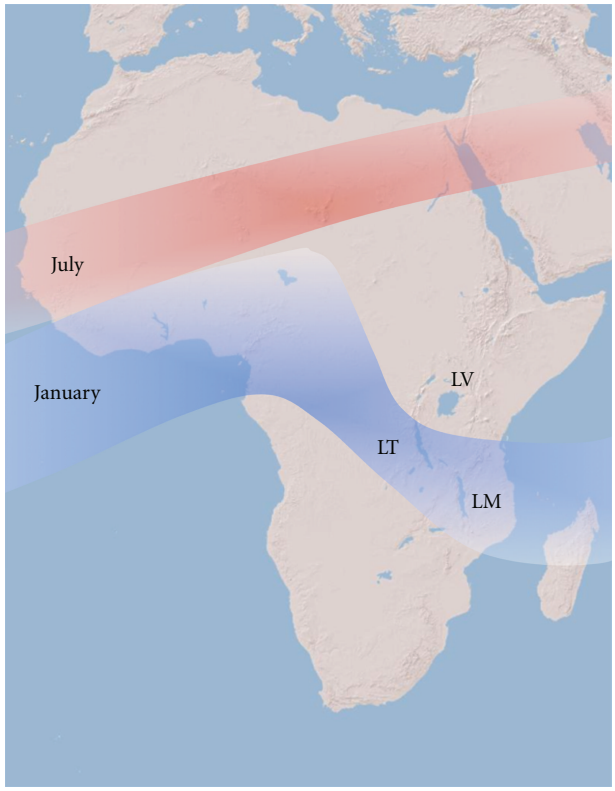

Figure 2: Seasonal position of the intertropical convergence zone (ITCZ). LV: Lake Victoria, LM: Lake Malawi, and LT: Lake Tanganyika.

Variations in the position and intensity of the ITCZ can affect the duration of the wet seasons in each lake, causing aridity and significant changes in lake level [14, 15, 32].

1.2. East African Paleoclimate (10 Ma–Present). The East African climate has been and continues to be dynamic [33]. Late Miocene (~8–10 Ma) climate in East Africa was humid and supported a variety of savanna and forest habitats, including rain forests [34]. Following this humid period, from ~7–5 Ma, the ice volume of the Antarctic ice sheet expanded and global temperatures fell [35–38]. This time period is also associated with aridification across East Africa [39, 40], as well as the uplift of the Himalayas and the resulting intensification of the Indian Monsoon [41], which may also have contributed to increased aridity. The early Pliocene (5–3 Ma) is characterized by warmer and wetter conditions globally and across Africa [42–46]. During this global warm, wet period, East Africa was also very humid [44], perhaps driving the expansion of Lake Tanganyika during the middle Pliocene [47]. Significant Northern Hemisphere Glaciation began and intensified between 3.2 and 2.6 Ma [48, 49] and beginning at ~2.0 Ma, Southern Hemisphere Glaciation expanded [50]. The interval beginning at ~2.8 Ma represents the onset of the glacial-interglacial cycles that characterizes the Pleistocene [51–53].

Simultaneous with this onset of bipolar glaciation and glacial-interglacial cyclity is a cyclic trend in aridity across Africa [53, 61–64]. In particular, the climate in East Africa during the last 500 thousand years (ka) has been extremely variable transitioning between wet and dry intervals that have caused significant fluctuations in the lake levels of the African Great Lakes (Figure 3) [10, 13–18, 37, 65–71]. Of particular importance for cichlid populations is an interval from 135 to 70 ka when there were at least two intervals of extreme aridity, called megadroughts, during which lake levels in Lakes Malawi and Tanganyika probably fell dramatically, and Lake Victoria was likely completely desiccated (Figure 3) [15, 17–19]. Following this megadrought interval, climate variability decreased considerably [19]. During the Last Glacial Maximum (LGM), ~20–15 ka, Africa again experienced an increase in aridity, which caused the complete desiccation of Lake Victoria, a significant drop in lake level of Lake Tanganyika (~250–300 m), and only a relatively minor drop in the lake levels of Lake Malawi [9, 12, 15, 17, 19, 20, 31, 64, 65, 70, 71]. The Holocene represents an interval of a moderately fluctuating climate during which there have been modest fluctuations in the lake levels of the African Great Lakes [21, 47, 72].

Table 1: Characteristics of the three great East African Lakes and their cichlid lineages.

	Lake Tanganyika	Lake Malawi	Lake Victoria
Maximum water depth (m) [54]	1470	700	79
Average water depth (m) [54]	580	264	40
Anoxic hypolimnion [54]	50–240	250	None
Surface area (km^2) [54]	32,600	29,500	68,800
Approximate formation of lake [7, 9, 10, 55–57]	9–12 Ma	>8.6 Ma	>0.4–1.6 Ma
Approximate number of species [58]	~250	~700	~700
Number of cichlids tribes [28, 59, 60]	12–16	2	2

References in the first column refer to the table's sources.

1.3. East African Cichlids. Few taxa have been as influenced by the environmental and geological history of this region than fishes in the family Cichlidae. Cichlids are believed to have originated 121–165 Ma [73] within the Gondwanal supercontinent. Their Gondwanan origin is reflected in the current distribution of cichlids [74]: cichlids can be found throughout Africa, the Neotropics, and Madagascar with several additional species occurring in the Middle East, India, and Sri Lanka. With an estimated 3,000 species, cichlids are the most species-rich teleost family, and the focus of this extraordinary species diversity is the East African Great Lakes (Table 1, Figure 4).

An estimated 2,000 species of cichlids occur in Lakes Victoria, Tanganyika, and Malawi, the majority of which are believed to have diverged within the past 10 Ma [75]. Many of these species are narrow endemics that are not found outside

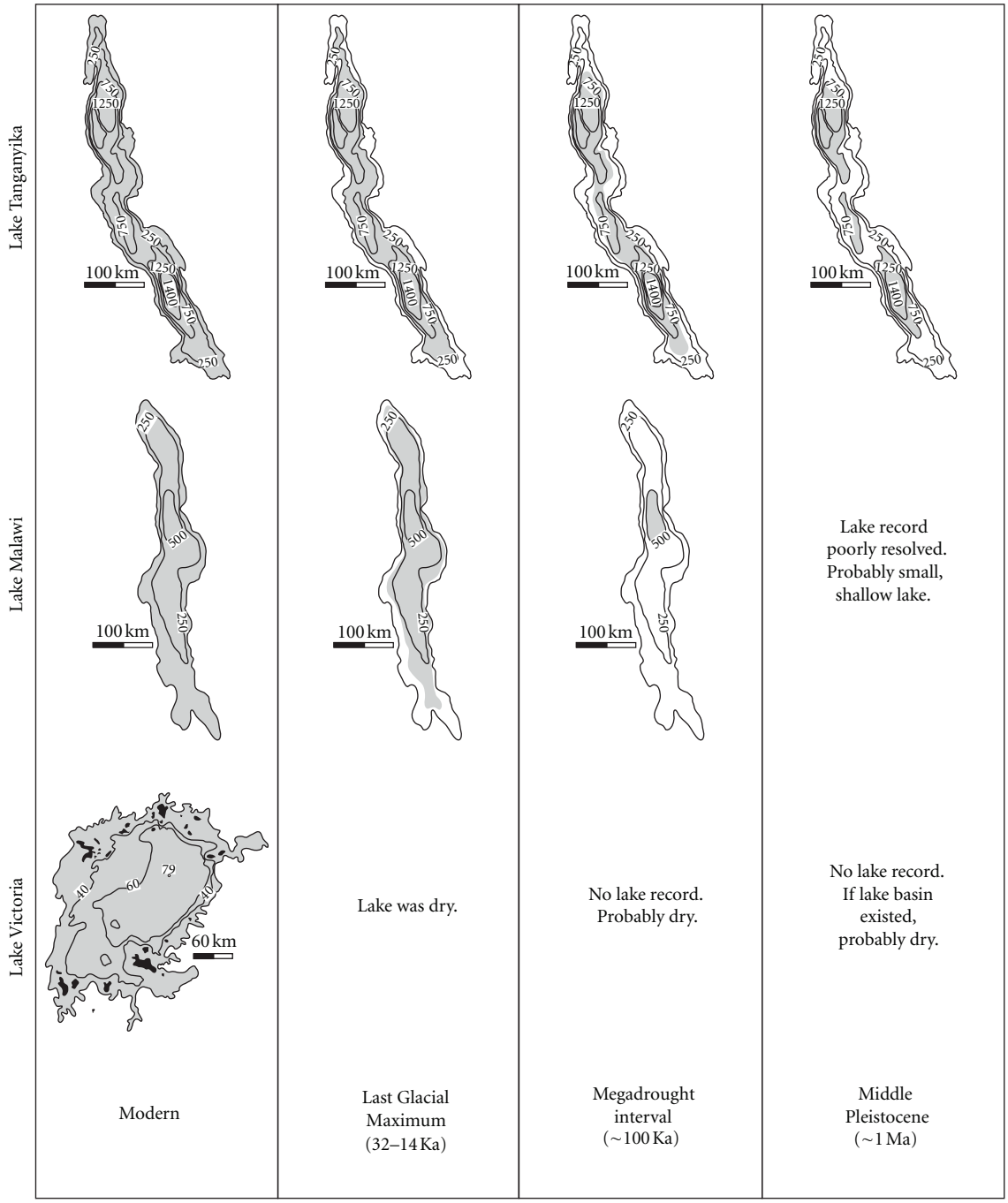

Figure 3: Bathymetric maps of Lakes Tanganyika, Malawi, and Victoria for modern, Last Glacial Maximum (15–32 ka), megadrought period (~100 ka), and the middle Pleistocene (~1 Ma). Reconstruction for ~1 Ma is based on data from Lake Tanganyika's subbasin [13], which has been extrapolated to the rest of the lake. Thus, this reconstruction is speculative and must be verified by additional data from the central and southern subbasins of Lake Tanganyika. Shaded areas show the maximum extent of lake during each interval. Lake Victoria's islands are shown in black. Lake levels based on [10, 13–22]. Bathymetric maps based on data from the World Lake Database (http://wldb.ilec.or.jp/).

the lake (or a location within the lake) in which they exist. This extraordinarily rapid, recent, and extensive species radiation has been shaped by the environmental and geological features that have affected the age, depth, and patterns of connectivity of the waters in which the cichlids diversified.

The aim of this paper is to synthesize the current understanding of the relationships between paleoclimate, geology, and the diversification of the East African cichlid species flock. Below, we explore these relationships in each of the lakes. In doing so, we hope to summarize the evolutionary

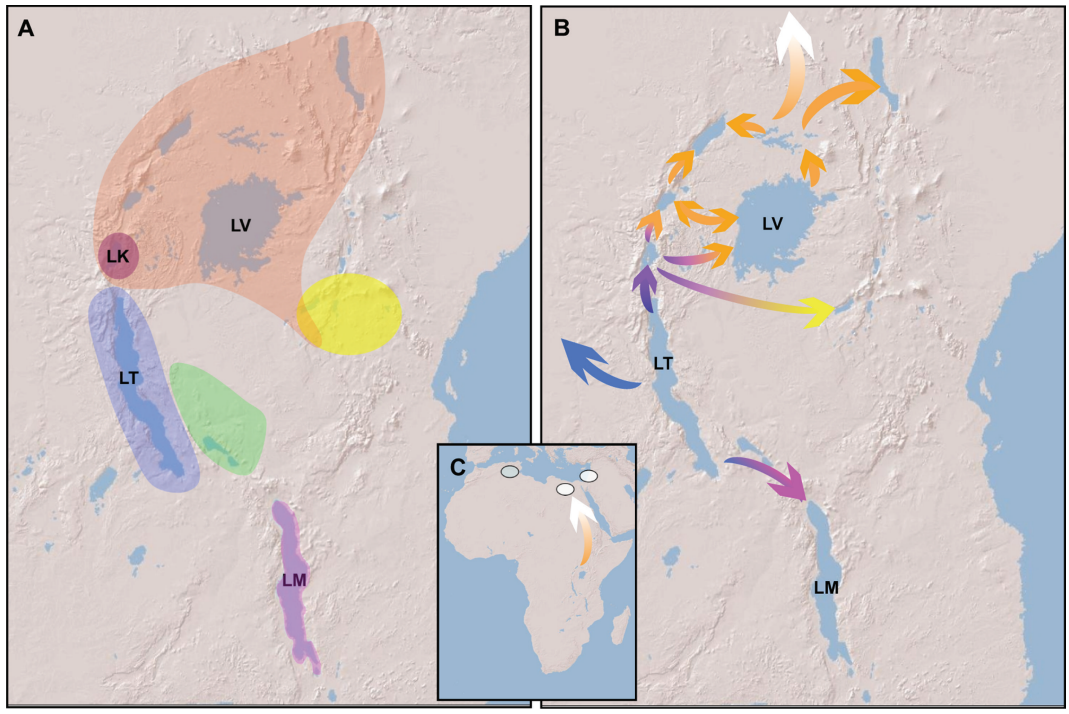

Figure 4: (A) The distribution of phylogenetic lineages. Colors indicate the distribution of genetic lineages: the distribution of the Lake Malawi lineage is displayed in purple, the Lake Tanganyika lineage is shaded in dark blue, the Malagarasi and Rukwa lineage are shown in green, the Lake Kivu lineage is colored purple, light red shading indicates the distribution of the Lake Victoria Superflock (LVSF), and the distribution of the South Kenyan—North Tanzanian lineage is displayed in yellow. (B) The possible colonization scenario for East African cichlids; the color of the arrows coincides with the colors of the lineages illustrated in part (A). (C) The distribution and possible colonization pathway for the North African and Israeli outposts of the LVSF. Phylogenetic data and colonization pathways are based on data from [23–30] and modified from a Figure 4(a) of [23].

diversification of East African cichlids within the context of the environmental and geological factors that have shaped their divergence.

2. Lake Tanganyika

2.1. Paleoclimate and Geologic History of Lake Tanganyika. Lake Tanganyika sits within the annual migration path of the ITCZ [76] and thus experiences both a rainy season (September–May) and a dry season (June–August), as well as changing wind direction and strength throughout the year [77]. Mean annual precipitation (MAP) in the region is ~1200 mm/yr [78] across most of the lake except on the eastern margins near the Mahale Mountains where orographic effects increase MAP to ~1800 mm/yr [76].

A number of paleoecological factors influence the cichlid diversity in Lake Tanganyika. Principle among these factors is the historic variability in lake level. Variation in lake level has been driven primarily by two major forces: tectonism and climate. Below, both mechanisms for lake level change will be summarized chronologically, and lake level lowstands will be identified. In addition, the historic connections between Lake Tanganyika and other water bodies in East Africa will be explored to (1) identify the likely origin of Lake Tanganyika's cichlids and (2) identify possible migratory pathways for Tanganyikan cichlids that colonized Lakes Victoria and Malawi.

2.1.1. Tectonic History of Lake Tanganyika. Lake Tanganyika formed as a series of half-grabens, which are down dropped blocks of land that are bordered by normal faults, within the western arm of the EARS. The geometry of rifting in Tanganyika is highly dependent upon the prerifting basement terrain and the remnants of a preexisting Permian-aged ancient rift [79, 80]. The series of half-grabens are ~160–180 km long by 30–60 km wide [79]. The half-grabens alternate east and west along the length of the lake and are deepest along the border faults and slope upwards towards the opposite shore via a series of faults and folds [79]. They are separated by bathymetric highs known as "high-relief" and "low-relief" accommodation zones forming intra- and interbasinal ridges that separate the lake into three subbasins [81].

The onset of rifting, the evolution of the lake basin, and the early history of Lake Tanganyika are dated relatively imprecisely because there are no direct dates from that time interval. Most of the lake basin's early record has been dated using the reflection seismic-radiocarbon method (RSRM). RSRM estimates ages using sediment thickness estimates

derived from reflection-seismic data combined with short-term sedimentation rates calculated from radiocarbon-dated cores. There is some inherent uncertainty in the reflection-seismic estimates of sediment thickness, and RSRM must make the sometimes tenuous assumption that sedimentation rates do not vary through time. Therefore, RSRM age estimates have large uncertainties. The RSRM ages discussed in this paper must be interpreted cautiously until corroborated by direct dating methods. The more recent history of Lake Tanganyika is very well constrained, and the dates for the last ~150 ka can be considered very reliable because they are derived from sediment core data.

Persistence of previous drainage patterns is a common early-stage feature in developing rifts [82]. Based on this analog, it has been suggested that prior to the Miocene until the onset of rifting, the area that would become Lake Tanganyika might have been the site of an ancient river system that drained primarily through a paleo-Congo river system [5, 83]. However, this potential river drainage pattern is uncertain. Rifting probably began ≥9–12 Ma in the central basin and extended northward, then southward [55, 79]. The creation of the Kivu-Ruzizi dome on the north end of modern Lake Tanganyika probably also occurred during this time [18]. The central subbasin infilled first, followed by the north basin, and finally the south basin as the rift opened [13, 55, 84].

Detailed seismic studies of the early history of Lake Tanganyika have been primarily focused on the north basin [13, 55, 84]. Each subbasin in Lake Tanganyika is structurally distinct, and the seismic record from the north basin cannot necessarily be extrapolated to the central and southern subbasins. However, significant tectonic changes and major sequence boundaries documented in the north basin may represent lakewide events. Much of the early tectonic and lake level history presented here is based on data from the north basin and thus must be cautiously interpreted until the events are also documented in the central and southern basins.

The timing of the earliest deposition in the central basin is difficult to resolve [20]. However, there is evidence that during early stages of rifting within the north basin, roughly 7.5 Ma, deposition began as a small, swampy lake formed and then expanded to fill the developing rift [13, 55, 84]. From the onset of rifting until about a million years ago, extension and faulting continued during which time the half-grabens found in each subbasin formed [13, 84]. The end of this initial rifting and depositional phase is marked by a nearly lakewide erosional surface [13, 84].

Following this early period of extremely active tectonism, a second period of geologic activity associated with modification of the existing half-grabens, uplift within the subbasins, renewed volcanic doming, and formation of syn-rift deposits occurred in the northern basin from about one million years ago until ~0.40 Ma [13, 47, 84]. The end of the second period of tectonism and deposition is marked by an erosional surface, which is thought to represent a lowstand event in the northern basin [13]. Subsequently, the basin has been largely inactive with only limited small-scale faulting occurring, allowing the formation of Lake Tanganyika's modern subbasins [13].

2.1.2. Lake Level History of Lake Tanganyika. Lake level has fluctuated dramatically throughout the history of Lake Tanganyika. During the first phase of deposition from approximately seven and a half million to one million years ago, there is evidence for several major unconformities in the northern basin [13, 84, 85]. The timing of these events is difficult to resolve; however, they are most likely related to both tectonic and climatic factors [13]. During the initial phase of tectonism, proto-Lake Tanganyika grew to fill the developing rift basin, and at approximately three and a half million years ago, there is evidence for a dramatic expansion in the size of proto-Lake Tanganyika in the north basin, either as a result of downwarping or due to a transition to a wetter climate across Africa [13]. Following this high stand at about three and a half million years ago, there is a pronounced aridification trend across Africa that is associated with Northern Hemisphere glaciation [39, 48, 52, 53, 63, 64, 86], which likely affected lake level.

The next phase of tectonism began at approximately one million years ago. During this time, lake level in the northern basin was 650–700 m below present lake level (bpll) [13]. The onset of this lowstand is unclear, but it was likely prolonged and may have begun considerably earlier than one million years ago [20, 85]. Based on the modern bathymetry of Lake Tanganyika, a reduction in lake level this large could have split the lake into three hydrologically and biologically isolated basins [83] (Figure 3). However, continued tectonism over the last one million years suggests that the bathymetry of the Lake Tanganyika could have significantly changed during that time. Further, no estimates of lake level in the central and southern subbasins have been made for this time interval. Until new data from the central and southern basin are obtained for this interval, this separation into three subbasins is somewhat speculative.

Following this lowstand, lake level fluctuated dramatically, and the lake significantly contracted in the northern basin several times at ~390–~360, ~290–~260, ~190–~160, ~120–~100, ~40, and 32–14 ka [13, 15, 20, 47, 87–89]. These lowstand events were related to either tectonic factors (~390–~360 and ~190–~160 ka) or major intervals of aridity (~290–~260, ~120–~100, ~40, and 32–14 ka). During the first three lowstand events at ~390–~360, ~290–~260, and ~190–~160 ka, the deepest areas in the lake may have either been separated or have only been connected by emergent, swampy areas [13]. Since 106 ka, Lake Tanganyika has remained a single connected water body, even during significant intervals of aridity [20]. All of these lowstand events, except for the most recent events (younger than ~150 ka), have been well documented mainly in the lake's northern basin, and the exact ages of the events are somewhat uncertain because they were made using RSRM. These RSRM ages for the lake level change events must be corroborated by direct dating methods before they can be considered reliable enough for calibration purposes. Further research in the central and southern basins is also needed to determine whether all of the lowstand events were lake wide, which would indicate a climatic, rather than a tectonic process.

2.1.3. History of Connectivity. Uplift related to rifting processes has caused Lake Tanganyika to have a highly dynamic history of connections to many of the major lakes in East Africa. These variable connections between Lake Tanganyika and the other waters of East Africa have allowed the dispersal of many cichlid lineages, including the haplochromines that seeded the highly diverse species flocks in Lakes Malawi and Victoria [23].

In the Cretaceous and early Cenozoic, prior to the initiation of rifting in the eastern arm of the EARS, the main drainage direction was west to east across the African continent into the proto-Indian Ocean [90]. The uplift of the East African Plateau and the initiation of rifting in the eastern arm of the EAR reversed the drainages west of this propagating rift causing the rivers to flow from east to west [90]. This suggests that as the central basin of Lake Tanganyika formed, it was probably infilled by rivers draining from the east. The most likely source was the proto-Malagarasi River inflow and Lukuga River outflow river system, which may be the only modern river system that also existed prior to the formation of the Tanganyika Rift [47, 78]. Rifting propagated northward from the central basin, and by about seven and a half million years ago, the northern basin had begun to form and was being infilled by a proto-Rusizi River [13]. This represents an early connection between Lake Tanganyika and the Rusizi-Kivu Basin, which was probably also forming at this time [91]. Variable rifting-related uplift has probably led to periodic connections between the Rusizi-Kivu and Tanganyika Basins [91]. These periodic connections may have also allowed for a direction connection between Lakes Kivu and Tanganyika after the formation of Lake Kivu $\geq$ 2 Ma [91]. The pre-Pleistocene history of this Kivu-Tanganyika connection is poorly understood, however, as result of the general lack of seismic data from Lake Kivu. Today the Rusizi River, which flows from Lake Kivu, is one of Lake Tanganyika's main inlets. Most recently, this connection was likely open ~13–9.5 ka when volcanism in northern Lake Kivu blocked its northern outlet to the Nile [92–94]. This inflow from the Ruisizi River increased lake levels in Tanganyika, causing renewed outflow via the Lukuga River [88]. Following these openings, the Rusizi inlet and Lukuga outlet have closed and reopened multiple times during the Holocene [47, 88, 93, 95]. It is possible that the Tanganyikan cichlids used the connection to Lake Kivu via the proto-Rusizi River to colonize northern bodies of water such as Lake Victoria. Alternatively, the connection between Lake Victoria and Lake Tanganyika may have been through the Malagarasi River, which may have been connected to both Lakes Victoria and Tanganyika during its geologic history.

Lakes Malawi and Tanganyika have a more complex relationship. Today, the two are not connected; however, the Malawian haplochromine cichlids are clearly derived from the Tanganyikan haplochromines [23]. Thus, fish from Lake Tanganyika migrated to Malawi via an unknown riverine connection.

2.2. Evolution and Diversification of Tanganyikan Cichlids. Lake Tanganyika contains one of the most diverse fish faunas in the world. Though the exact number of fish species in Lake Tanganyika (or any of the three Great Lakes) is unknown, estimates suggest that Lake Tanganyika supports more than 365 species of fish, at least 115 of which are noncichlids [58, 96]. Depending on how one groups these fish, the cichlids of Lake Tanganyika span either 12 [97] or 16 different tribes [59]. Within these tribes is a remarkable amount of phenotypic diversity in body shape, trophic structures, and behavioral and parental care strategies, making the cichlids of Lake Tanganyika the most phenotypically variable species assemblage in the East African Great Lakes [98]. The unique geological features of the lake, along with the dynamic evolutionary history of its cichlids, serve as an ideal model to study origin of the cichlid diversity [99].

2.2.1. The Origin and Diversification of Lake Tanganyikan Cichlids. Prior to rifting and the formation of any Tanganyikan basin, it has been suggested that an ancient river system that drained west into the Congo River system existed in the location of modern Lake Tanganyika (see above) [83]. This ancient connection between these two bodies of water is further supported by the similarities of fish fauna found in these systems. Eighteen of 24 fish families documented in the Congo are found in tributaries and marshes around the lake. Twelve of the 24 families from the Congo River system occur in littoral and sublittoral zones of the lake. Seven Congolese families are found in the benthic and four in the pelagic zone of Lake Tanganyika [78]. However, recent immigration of these families into this Lake Tanganyika cannot be ruled out.

Among the cichlids, it is clear that the Tanganyikan radiation is nested within cichlids endemic to the Congo River system [100]. According to Schwarzer et al. [100, 101], the East African cichlid radiation is a sister group to a clade containing the substrate brooding genus *Steatocranus* that is common in the Congo River system. Together with the several species of tilapia ([100], clade "AII"), the genus *Steatocranus* and the cichlids of Lakes Tanganyika, Malawi, and Victoria form the Austrotilapiini. The Austrotilapiini are further nested within the larger Haplotilapiini, which itself is nested within a collection of taxa that are widespread across the Congo River system and West Africa. The findings of Schwarzer et al. [100] are largely consistent with those of Farias et al. [74], both of which provide clear support for the Congolese origin of the cichlids of Lake Tanganyika.

The relationship between Tanganyikan and Congolese cichlids, however, is far from simple [24] (Figure 4). Poll [97] originally identified 12 polyphyletic cichlid tribes in Lake Tanganyika and concluded that the lake had been colonized multiple independent times. Since then, a number of molecular phylogenetic studies, summarized by Koblmüller et al. [25], support the multiple invasion hypothesis, and the 12 tribes that originated during the primary lacustrine radiation have been identified: the Haplochromini (including the Tropheini and the hyperdiverse haplochromines of Lakes Victoria and Malawi), the "new tribe" consisting of *Ctenochromis* species, the Cyphotilapiini, the Benthochromini, the Limnochromini, the Perissodini, the Cyprichromini, the Ectodini, the Lamprologini, the Eretmodini, the Orthochromini, and the Bathybatini. After the initial

invasion of the lake, many of these lineages experienced a secondary radiation, which formed the modern cichlid diversity found in Lake Tanganyika [99]. Following these radiations, species from several of the radiating lineages recolonized the rivers surrounding Lake Tanganyika [24, 75]. The lineages that secondarily invaded the surrounding rivers include two species rich tribes, the Lamprologini and Haplochromini, and one lineage currently found exclusively in rivers, the Orthochromini. Two additional lineages, the Tilapiini and the Tylochromini, recently invaded the lake [102, 103].

The diversification of the Haplochromini demonstrates the complex patterns of dispersal between the cichlids of Lake Tanganyika and its surrounding rivers. The common ancestor to the haplochromines evolved within a larger, lacustrine cichlid diversification in Lake Tanganyika. These haplochromines then colonized the rivers in the surrounding catchment of Lake Tanganyika. These generalized riverine haplochromines then secondarily invaded the lacustrine habitats in Lakes Tanganyika, Malawi, and Victoria. In each lake, the haplochromines (the "modern" haplochromines) then experienced a remarkable radiation [23]. Thus Lake Tanganyika is not only a sink for ancient African cichlid lineages, but also a source of recent cichlid diversity in East Africa.

In contrast to a predominately intralacustrine radiation, Lake Tanganyika could have been colonized by a larger number of more diverse taxa [75]. When the molecular clock is calibrated to the breakup of Gondwana, molecular clock estimates of divergence times suggest that nearly all Tanganyikan lineages began to diverge prior to the estimated onset of deep lake conditions [75]. In this model, the divergence of Lake Tanganyika's cichlid fishes did not occur in Lake Tanganyika, but rather these lineages began to diversify in the surrounding rivers prior to the formation of the lake. Though novel, this much older estimate of the diversification of Lake Tanganyikan cichlids conflicts with long-held assumptions concerning the habitats suitable for cichlid radiations, the evolution of resource partitioning, and the biogeographic patterns of species distributions [25]. In addition, it is well known that estimating recent diversification events with ancient calibration points may produce unreliable age estimates with large variances [104].

2.2.2. Ages of the Lake Tanganyikan Cichlid Radiations. To resolve these alternative hypotheses, an accurate estimate of the divergence time for Lake Tanganyika's cichlids is needed. Unfortunately, different calibration methods yield highly incongruent estimates. By calibrating the molecular clock to recent geologic events (e.g., the formation of Lake Malawi and the inundation of the Lukaga valley), Salzburger et al. [23] suggested that Lake Tanganyika's cichlids evolved since the formation of the lake basin 6–12 Ma and that the species-rich haplochromines originated approximately 2.4 Ma. While this estimate is widely accepted within the cichlid community, this calibration method relies on assumptions that are still debated within the geologic literature. For example, this calibration method ignores the fact that the timing of Lake Malawi's formation is poorly constrained.

Genner et al. [75] generated age estimates using two calibration methods. One method relied on the cichlid fossil record, while the other relied on the breakup of Gondwana. The cichlid fossil record calibration suggests that Lake Tanganyika's cichlids began diversifying coincident with deep lake conditions (6–12 Ma), a finding that is consistent with previous estimates [23]. Genner et al. [75] favor instead the Gondwana calibration. This calibration suggests that the diversification of Lake Tanganyika's cichlids had begun prior to the creation of Lake Tanganyika's basin, possibly in a now extinct paleolake. However, there is no geologic evidence to support this paleolake hypothesis. Genner et al.'s [75] conclusions were supported by estimates produced by Schwarzer et al. [100] who used the fossil record of *Oreochromis lorenzoi* (5.98 Ma), the divergence of *Tylochromis,* and the remaining East African cichlids (53–89 Ma) as calibration points. However, as was noted by Koblmüller et al. [25], Genner et al.'s [75] Gondwana calibration lacks constraints on the more shallow bifurcations which may lead to the incorrect assignment of divergence times. Koblmüller et al. [105] produced models calibrated to a number of geologic points including the occurrence of deep water conditions in the Great Lakes and Genner et al.'s [75] Gondwana calibration. From this analysis, Koblmüller et al. [105] conclude that the most parsimonious age estimate for the divergence of Lake Tanganyika's cichlids is ~6 Ma with the most recent common ancestor of the haplochromines occurring 5.3–4.4 Ma [105].

It is worth noting, however, that these divergence times are highly dependent on the estimated timing of geologic events that have large uncertainties. Estimating the time since divergence in many cichlid lineages is further complicated by the age of the events used to calibrate the molecular clock. Many of cichlid diversification events occurred relatively recently, while the events used to calibrate the molecular clock are comparatively old (e.g., the breakup of supercontinents, the formation of lake basins) [25, 104]. Thus, the continued analysis of the geologic history of this dynamic region is needed to accurately quantify the divergence times of the East African cichlids [106]. It is clear that the divergence times of Lake Tanganyika's cichlid and the haplochromines have yet to be resolved.

Incomplete taxon sampling is another major limitation of the current dating estimates [105]. In most studies, one or several of the major lineages are not included in the phylogenetic reconstruction. Further, based on recent publications, not all of haplochromines lineages in the region have been identified [26]. Dating estimates are further limited by the lack of good calibration points. Estimates from molecular clocks become more reliable when multiple geological and fossil calibration points are used [105, 107, 108] in combination with reliable rates of sequence evolution [109], estimates of sequence saturation [110], and a posteriori evaluation of estimated divergence times [108, 110]. Few of these requirements have been satisfied in previous analyses, and the necessary data are just now becoming available [106]. Thus, caution is necessary when considering the dates provided here.

2.2.3. Impact of Lake Level Fluctuations. The dynamic geological history and variable paleoclimate of Lake Tanganyika have shaped the cichlid diversity in this lake. The effect of these factors can be seen in three major areas: the maintenance of ancient cichlid lineages, the isolation of populations, and the admixture of previously isolated populations.

East Africa has experienced multiple periods of extreme aridity during the Pleistocene. For cichlid lineages to have persisted through such events, water sources must have remained available. Owing to the great depth of the lake, even during periods of extreme aridity [13, 20], Lake Tanganyika would have been a refugium for ancient cichlid lineages, which is reflected in age estimates of the diversification of Tanganyikan cichlids [23, 75].

Though these periods of aridity apparently did not extirpate the seeding lineages in Tanganyika, the resulting low lake levels likely had a significant impact on the distribution of genetic variation within and among these lineages. For example, an analysis by Sturmbauer et al. [111] of mtDNA regions from several *Tropheus* populations identified 6 phylogeographically unique haplotype clusters in three regions of the lake: the northern basin, the central basin, and the southern basin. Remarkably, in the central and southern parts of the lake, individuals from one side of the lake had mitochondrial haplotype identical to those found at the opposite shoreline. It is possible that during one of the major regressive events, Lake Tanganyika was separated into three near-distinct lakes (Figure 3), and populations that are currently separated by deep water were connected through the shorelines of these three separate lakes. Alternatively, during times of lake level fall, water levels at topographic highs that separate the subbasins may have become shallow enough to allow species to migrate from one side of the lake to the other. Given that there are discrete haplotypes clusters for each of the three subbasins and that their mitochondrial haplotype are shared by individuals on opposite sides of the lake in each subbasin, the separation into three near-distinct lakes seems to be the more likely scenario.

While the cross-lake affinities of mitochondrial haplotypes reflect the impact of major regressive events on the distribution of genetic variation in Lake Tanganyika's cichlids, the interaction of historic hydrology and bathymetry can have a more subtle influence. Examining the genetic diversity in a collection of *Tropheus moorii* populations, Koblmüller et al. [112] detected the effect that changes in lake level have had on the genetic diversity over extremely limited geographical distances. In this study, two distinct collections of populations were identified. One collection was located on the steeply sloping shores of the eastern side of the Chituta Bay, while the other was located on the more gently sloping shores west of the bay. Within the eastern populations, three distinct populations which corresponded to geographic locations were identified. In contrast, the western populations show greater degree of admixture. The authors conclude that this pattern is consistent with the horizontal displacement of the western shore populations as the lake regressed, causing those populations to admix as the available habitat shrank. The eastern populations migrated vertically along the steeply sloping habitat, were not forced into secondary contact and retained their accumulated genetic differences. In both the eastern and western populations, the authors detect the signature of population expansion that coincided with the end of the Last Glacial Maximum. Population expansion was greater in the western populations likely as a consequence of this area having relatively greater area of available habitat with a rise in lake level. The authors conclude that rapid, dramatic, and relatively recent climatic changes in East Africa drive both population divergence and population admixture.

3. Lake Malawi

3.1. Paleoclimate and Geologic History of Lake Malawi. At 700 m deep, 580 km long, and 30–80 km wide, Lake Malawi is one of the largest lakes in the world. Rifting in the Malawi Rift began during the Late Miocene, probably no less than ~8.5 Ma, and propagated from north to south resulting in three drainage basins [6, 7, 56, 85, 91, 113–116]. The two northernmost drainage basins are deeper and steeper sided, while the southern basin is shallower with a muddy bottom [19, 116]. The age of the formation of Lake Malawi is very uncertain. Geologic evidence from deposits surrounding the lake suggests that a deep lake may have first existed between ~4.5 and 8 Ma [6, 7, 56, 57, 114, 115]; however, it is possible that a lake was present even earlier during the earliest phases of rifting, 8–12 Ma [57, 117]. Since formation, the lake has undergone dramatic fluctuations in lake level during its history [15, 17, 85, 118].

The climate of Lake Malawi is strongly influenced by the seasonal migration of the ITCZ producing a wet-dry monsoonal cycle with the wet season extending from December to April. Annual precipitation is seasonal and ranges from <800 mm/yr in the south to >2400 mm/yr in the north [119]. The lake is close to the southern extent of the modern ITCZ path (Figure 2), and thus changes in the position of the ITCZ and its intensity considerably affect dry season length and have been linked to periods of aridity and drops in lake level during the Pleistocene [15, 17, 32].

Lake Malawi is permanently stratified with a chemocline depth of ~250 m [120], and today the lake is hydrologically open. Several large drainage systems enter the lake across different structural settings in the three drainage basins [121], and the sole outlet is the Shire River. Although outflow to the Shire is continuous, more than 90% of water loss in the lake is due to evaporation [119, 122, 123]. Because precipitation is seasonal and evaporation is the main contributor to water loss, lake level seasonally fluctuates up to a few meters. Variability in lake level has caused disruption of the outflow during historical times [72] and frequently throughout the geologic history of the lake. Considerable reductions in lake level during the lake's geologic history have caused the outlet to be disrupted, and the lake has become closed and more saline [18, 22].

The geologic and paleoclimatic history of Lake Malawi, which has influenced the connectivity of Lake Malawi to the surrounding waters and generated highly variable lake levels, has played an important role in the evolution of its endemic

species. Below we review the geologic and paleoclimatic history of Lake Malawi and discuss their influences on the divergence of its haplochromine flock.

3.1.1. Tectonic History of Lake Malawi. The Malawi Rift is located at the southern end of the western arm of East Africa rift between 9°and 14°S, and almost two-thirds of the rift is filled by Lake Malawi. The rift zone is comprised of four alternating asymmetrical half-grabens and several smaller basins, resulting in three main structural and drainage basins [114, 116]. Each half-graben is bounded by a steep border fault with high rift mountains (>1500 m above lake level) along the lake shore on one side and a shallower flexural or shoaling margin on the opposite side [121]. The border faults link across "transfer zones" [85] which strongly influence drainage and deposition patterns in each of the basins [124, 125]. The long-lived half-graben basins and associated deep subsidence have resulted in the development of a long-lived lake basin [95, 116]. As a result, the lake is underlain by >4000 m of lacustrine sediment that thins from the far north to the south, indicating that rifting has propagated from north to south [2, 113–116]. The northern and central basins, which are up to ~700 m deep, are each ~150 km long and are characterized by very steep offshore slopes at the border faults [116, 125]. In these basins, sediment is primarily transported downslope within channels and canyons and out onto well-developed fan complexes [125, 126]. The offshore slopes in the shallower southern basin (maximum depth = 450 m) are less steep than the northern and central basins, and the basin is primarily covered by fine-grained, muddy sediments [125, 126].

The exact timing of the onset of rifting is unknown; however, the earliest sediments that are associated with Cenozoic rifting in the Rungwe volcanic province, which borders the Malawi Rift to the north, are associated with welded tuffs dated to 8.6 Ma [7, 56]. The sediments are not directly correlative with sediments in the subsiding Malawi Rift; thus, 8.6 Ma is a minimum age for the onset of rifting. Age models based on sedimentation rates suggest that rifting commenced between 8 and 12 Ma [117]. The earliest interval of rifting and subsidence in the Malawi Rift probably occurred contemporaneously with two pulses of volcanism in the Rungwe volcanic province between 8.6 and 1.7 Ma during which most faulting occurred parallel or subparallel to the bounding faults [7, 56, 57, 117]. This was then followed by an ~1 Ma interval of quiescence until the latest phase of rifting beginning at ~500–400 ka when there was a shift in rifting style to oblique rifting and strike-slip deformation [117, 127].

3.1.2. Lake Level History of Lake Malawi. The early history of Lake Malawi is somewhat difficult to resolve. Radiometric dates from lavas and tuffs surrounding Lake Malawi [7, 56] and sedimentological evidence suggest that a small, shallow lake may have periodically existed after the onset of rifting [57]. Lacustrine deposits, structural evidence, and an increase in the rate of subsidence of the lake floor between 4.5 and 1.6 Ma suggest that a deep lake formed by ~4.5 Ma, if not earlier [57, 128, 129].

Sedimentation in the Malawi Rift occurred contemporaneously with two of the early pulses of the Rungwe Volcanic province to the north of the lake between 8.6 and 1.7 Ma [7, 56, 57]. During this period of volcanism, rifting, and deposition, there is evidence for multiple depositional hiatuses that likely correlate with significant reductions in lake level [57]. The age of the early hiatuses is poorly constrained. Two of them may be contemporaneous with onshore unconformities that have been dated to 2.3 and 1.6 Ma [56]; however, it is uncertain to which offshore unconformity the lake events correlate. This onshore evidence indicates a pronounced unconformity from 1.6 to 1.0 Ma during which time Lake Malawi was probably significantly reduced in size and possibly even completely desiccated [56, 57, 127] (Figure 3). Preliminary evidence from drill core records also suggests that Lake Malawi was a significantly reduced, saline lake at ~1.2 Ma [22]. Following this lowstand, lake level rose towards deeper lake conditions [22]; however, the history of lake level change is poorly resolved from 1.2 to ~0.15 Ma.

Between ~150 and 60 ka, there were dramatic fluctuations in lake level [15, 17–19]. During this time period, there were two intervals of pronounced lake level drops up to 550 m bpll: one from 135 to 124 ka and the other from 117 to 85 ka [15, 17, 19]. These two megadrought events would have severely restricted Lake Malawi, and lake level may have been reduced to as little as 2% of modern lake levels [19]. Between the two megadroughts was an interval of relatively high lake levels where the lake was stratified and the bottom water was anoxic [18, 19]. Beginning at ~60 ka, the lake rose to much higher levels, and changes in lake level were much less dramatic than during the preceding 90 ka. There have been modest fluctuations in lake level (100 m or less) since 60 ka, including during the LGM [19]. However, in general lake conditions during the last 60 ka have been relatively stable and similar to those at present.

3.2. Evolution and Diversification of Malawian Cichlids. In many respects, the origin and diversification of Lake Malawi's cichlid fish is the least complex of the three Great Lake radiations. Lake Malawi contains both tilapiine and haplochromine cichlids. The tilapia are represented by two distantly related lineages [102]: *Tilapia rendalli,* a substrate spawning species common throughout the region, and an endemic species flock, the chambo, containing three species (*Oreochromis karongae, Oreochromis lidole,* and *Oreochromis squamipinnis*) [130]. Given the paucity of endemic tilapiine species, this section will focus on the more diverse haplochromine lineage.

3.2.1. Origin and Diversification of Lake Malawi's Haplochromine Cichlids. With over 700 endemic species [58] most, if not all of which appear to have descended from a single common ancestor [131], the haplochromine cichlids of Lake Malawi are the largest monophyletic species flock of cichlid fishes. This species flock is nested within the Lake Tanganyikan haplochromine group and is sister to the clade containing the haplochromine cichlids of the Lake Victoria superflock [23].

The age of Lake Malawi's species flock, like the ages of other East African cichlid flocks, is debated. Sturmbauer et al. [111] calibrated the age of Lake Malawi's cichlids to the geologic history of the lake [57] and estimated the divergence of Lake Malawi's cichlids at 0.93–1.64 Ma. In contrast, Genner et al. [75] using two calibration methods (see above) suggested that Lake Malawi's cichlid flock originated either 4.6 Ma (Gondwanan calibration) or 2.4 Ma (fossil calibration). Genner et al.'s [75] estimate suggests that Lake Malawi's cichlids began to diversify prior to deep water conditions in the lake and/or persisted through multiple megadroughts that either desiccated the lake or dramatically altered the water chemistry thereby making the lake uninhabitable. Genner et al.'s [75] estimates were not supported by the work of Koblmüller and colleagues [25]. In Koblmüller et al.'s [25] analysis, the estimated age of the Lake Malawi's cichlids ranged between 0.72 and 1.80 Ma for five of the seven calibration methods used. Though the estimated age of Lake Malawi's cichlid flock is not known, an abundance of data suggests that this flock originated ~1 Ma. If or how Lake Malawi's haplochromine cichlids persisted through the megadroughts of 135 ka and 117 ka is unknown.

Assuming an origination age of ~1 Ma for the Malawi cichlid flock, a riverine generalist similar to *Astatotilapia calliptera* or *Astatotilapia bloyeti* [105] migrated from Lake Tanganyika to Lake Malawi during that time [23] (Figure 4). The cichlids of Lake Malawi then diverged into two large clades plus several oligotypic lineages. The two large clades, each containing ~250–300 species, are reciprocally monophyletic and consist of the rock-dwelling cichlids, or mbuna, and the sand-dwelling cichlids [132]. Genner et al. [75] suggest that the mbuna emerged 0.486 Ma (Gondwanan) or 0.313 Ma (fossil), while the more diverse sand-dwelling cichlids emerged 1.447 Ma (Gondwanan) or 0.855 Ma (fossil). Given the large variances of these estimates and lack of multiple calibration points [25], the reliability of these divergence estimates is unknown. Future research utilizing a broad sampling of taxa and multiple calibration points is needed to accurately estimate the ages of these highly diverse clades.

3.2.2. Impact of Lake level Fluctuations. Regressive events probably played an important role in shaping the evolutionary history of Lake Malawi cichlids. For example, Genner and Turner [133] recently discovered that one of Lake Malawi's most diverse clades evolved in response to a major regression event. Between ~75 and 135 ka, lake level dropped as much as 580 m bpll [15, 17]. As a consequence, the shallow benthic habitats of southern Lake Malawi completely desiccated, thereby reducing the proportion of shallow rock and sand habitats relative to deep benthic and pelagic habitats. In addition, this lowstand facilitated the hybridization of two diverging lineages: the rock dwellers and the sand dwellers. As a consequence of this event, this new hybrid lineage rapidly adapted to the now plentiful deep benthic habitats and gave rise to as many as one-third of all the species in Lake Malawi's haplochromine radiation.

During the following transgressive period which brought the lake to its current level, the littoral areas north and south of the central basin were reinundated. The newly emerging habitats provided the opportunity for expansion and diversification of many species, which is reflected in both the patterns of genetic [134] and species diversity [135]. This period that reestablished littoral habitats and permitted the rapid expansion of populations is likely synchronized with similar phenomenon in other East African lakes [111, 136].

Within historical times, Lake Malawi has experienced meaningful but less dramatic regressive/transgressive events. For example, Owen et al. [72] found that much of southern Lake Malawi was exposed land as recently as 300 years ago. Given the large number of species endemic to this area [135, 137, 138], a regressive event of this magnitude would suggest an exceptionally rapid diversification of southern Lake Malawi endemics. These rapid and recent regressive/transgressive events are believed to have disrupted and permitted gene flow between mbuna populations and thereby contributed to the high cichlid diversity in Lake Malawi [139].

4. Lake Victoria

4.1. Paleoclimate and Geologic History of the Lake Victoria Region. Lake Victoria is the largest freshwater lake in the tropics by surface area (68,800 km^2) and the second largest in the world. The lake spans the equator in between the western and eastern branches of the EARS (Figure 1), giving it a rectangular-shaped coastline. The timing of the formation of Lake Victoria is uncertain. The lake has been suggested to have formed between 1.6 and ~0.40 Ma due to backponding associated with the damming of westward flowing rivers by the uplifting of the western arm of the EARS [7–12]. Unlike the other African Great Lakes, Lake Victoria is not within a rift basin, and as a result, it is relatively shallow with a maximum depth of less than 100 m.

Modern climate in the Lake Victoria region is primarily controlled by the ITCZ, which crosses Lake Victoria twice a year in March (long rains) and again in October (short rains) [140]. The mean annual precipitation of the Lake Victoria region is ~1600 mm/yr [141]. The lake is monomictic, and mixing by the trade winds occurs during the dry season between May and August [142]. However, in modern times, the lake's water column has become more stable, so that as much as 40% of the lake's bottom waters are anoxic [143].

Today, the lake is hydrologically open with its major inlets being the Kagera and Katonga Rivers in the west. The primary outlet is the Victoria Nile at the northern end of lake. As much as 90% of water loss is from evaporation and 80% of the input is from direct precipitation on the lake surface [141, 144]. Because evaporation is consistent, whereas precipitation in the Lake Victoria region is variable, changes in precipitation have profound impacts on lake level [145]. Due to its shallow depth (<100 m) and strong dependence on precipitation to maintain lake level, Lake Victoria has desiccated completely multiple times, probably in response to increased aridity [10, 14, 16, 21, 70].

The geologic and paleoclimatic history of Lake Victoria is considerably different than that of Lakes Tanganyika and Malawi. Principal among these differences is the depths of the lakes and the influence of arid intervals on the lake's

persistence. Lake Victoria is a relatively young, shallow lake that completely desiccated ~15 ka. Despite this event, the cichlids of Lake Victoria are species-rich and widely distributed outside the lake basin [27, 28]. Below we describe the geologic and paleoclimatic history of Lake Victoria and its surrounding waters and discuss how the geologic and paleoclimatic history has influenced the extensive radiation of the Lake Victoria cichlid superflock.

4.1.1. Evolution of Lake Victoria Basin. Prior to the onset of rifting in the Miocene, the Lake Victoria region drained from east to west. Rifting in the western branch of EARS during the late Miocene and the Pliocene probably created an NE-SW-oriented basin that began to capture some of the tributaries feeding the Congo River [12, 146]. Eventually rifting in the western EARS completely truncated this network during the Pleistocene, causing the rivers to flow eastward [7]. This eastward flow from the western branch of the EARS, coupled with the westward flow of rivers draining the western flank of the eastern EARS [147], formed Lake Victoria as the low-relief areas between the two arms of the EARS filled with water. The timing of this formation is poorly constrained, and the maximum estimate for the timing of formation is ~ 1.6 Ma [9–11], but because the lake formed by backponding between the two arms of the EARS, it is possible that Lake Victoria, or a "proto-Lake Victoria," formed earlier than 1.6 Ma.

After formation of the lake, rifting continued to tilt the basin eastward, moving the center of lake 50 km to the east and exposing mid- to late-Pleistocene lacustrine sediments west of the lake [11, 143]. These sediments are exposed in the Kagera River Valley 100 km to the west and 130 m above present lake level. Doornkamp and Temple [11] used these sediments to suggest that Lake Victoria was younger than 0.8 Ma. Mid-Pleistocene lacustrine sediments identified in Kenya near the Kavirondo Gulf have been used to suggest that Lake Victoria may be as old as 1.6 Ma [9]. However, it is important to note that these dates (0.8 Ma and 1.6 Ma) are very imprecise and poorly constrained. Additional work is needed to better constrain the age of these lacustrine sediments surrounding modern Lake Victoria. RSRM estimates for the 60 m thick package of sediment in Lake Victoria suggest at least 0.4 Ma of deposition [10]. However, there are multiple hiatuses in the succession, and their durations are not possible to estimate, indicating that 0.4 Ma is a minimum estimate for the formation of the lake [10].

The original outflow of Lake Victoria was probably to the west directly into Lake Albert [148]. Uplift associated with continued rifting of the EARS likely blocked this connection and established the modern outflow through Lake Kyoga by ~35–25 ka [148]. The first connection of Lake Victoria to the White Nile may have been as early as ~0.4 Ma [149]. The timing of the modern connection of Lake Victoria to the White Nile via the Victoria Nile is uncertain though it has probably occurred in the last 13 ka [8].

4.1.2. Paleoclimate and Paleoenvironment. Lake Victoria is extremely dependent on precipitation because as much as 80% of water input is from direct precipitation on the lake surface [118, 141, 144]. Seismic data indicates that lake level has fluctuated significantly during the Pleistocene and Holocene and that there were multiple intervals when the lake completely desiccated [10]. Coring of the uppermost sediments provides evidence for the most recent desiccation events. Near the base of the core is a 16-17 ka paleosol that represents drying at the end of the LGM [10, 14, 16]. This paleosol has shrink-swell features that identify it as a paleo-Vertisol [10]. In order to form a Vertisol, the soil must be completely desiccated for at least one month per year [150]. Seismic data indicate that the paleo-Vertisol can be traced continuously across the entire lake basin [10, 14, 16, 143]. The bathymetry of Lake Victoria does not allow for the formation of separate basins where smaller lakes could have persisted [10, 14, 16, 143], leaving no refugium for cichlids. Following this desiccation event during the LGM, the lake dried up again between 14 and 15 ka [14, 16, 21]. Thus, Lake Victoria was completely desiccated for at least two intervals from the LGM to ~14 ka, and it is highly unlikely that the lake could have supported any cichlid populations during these events. Following these desiccation events, the lake probably filled relatively quickly [21].

In Lake Albert, two paleosols have been identified between 18 ka and 12.5 ka, indicating that Lake Albert probably also desiccated at least twice since the LGM [151]. Other evidence from the Burundi Highlands and from the Congo River Basin also suggests that the late Pleistocene in equatorial East Africa was arid [152, 153]. This evidence for aridity coupled with the roughly contemporaneous paleosol horizons in Lakes Victoria and Albert suggests that many of the lakes in which the Lake Victoria superflock currently persists (e.g., Lakes Victoria, Albert, George, and Kyoga) were probably completely dry for at least some period of time during the LGM and the subsequent arid interval during the latest Pleistocene.

Across Africa, the early- to mid-Holocene was generally much wetter [86], and by ~12 to 13 ka lake levels in Lake Victoria and other surrounding lakes began to fill to their current level [21, 143, 154]. Throughout the Holocene, Lake Victoria experienced changes in lake levels, but no other complete desiccations [21]. During the last 4 ka, climate has become more seasonal, and precipitation has decreased, which has likely caused lake level to decrease such that Lake Nabugabo separated from Lake Victoria [21, 155–157].

4.2. Evolution and Diversification of the Lake Victoria Superflock. The evolutionary history of the cichlids of Lake Victoria cannot be fully understood without a broader discussion of the greater Lake Victoria species flock. While Lake Victoria supports at least 150 endemic species of cichlids, this diversification is only a fraction of cichlids belonging to the Lake Victoria superflock (LVSF) [27]. The superflock consists of over 600 species of haplochromine cichlids spread across nearby lakes such as Lakes Albert, Edward, George, Kyoga, Kivu, and the rivers of the region [27], in addition to more distant locations such as the more southern Lake Rukwa and its drainage [27], Lake Turkana [105], smaller North-Eastern Tanzanian lakes [26], and water bodies as far north as Egypt, Tunisia, and Israel [29, 30, 105].

Thus, the LVSF has a geographic distribution far larger than those of Lakes Tanganyika and Malawi [28, 158].

In order to understand the complex relationships of fish in this superflock, multiple phylogenetic, biogeographic, and population genetic studies have been performed. These have revealed a complex phylogeographic pattern, which reflects the influence of past geological and climatic events on the colonization of new habitats [26, 28, 105]. Below we examine these patterns with special attention given to identifying the geographic origin of the superflock, its age, and how its members persisted through recent periods of extreme aridity in East Africa.

4.2.1. Age and Origin of the Lake Victoria Superflock. Molecular phylogenetic evidence indicates that the LVSF predates the most recent complete desiccation event at ~14-15 ka. The LVSF appears to have emerged at about 200 ka [159] with major diversification of lineages between 98 and 132 ka [28]. Similar patterns in which genetic lineages predate the refilling of the Lake Victoria basin were found in cyprinid fish [160], catfish [161], and snails [162, 163]. However, all of these estimated ages rely on the estimated timing of geological events which themselves may be revised through future research (see above).

On the basis of these estimates of lineage divergence, several authors suggested that the lake never completely desiccated [164, 165]. Yet, the geological evidence is unambiguous. Lake Victoria and its surrounding waters were completely dry at least once, and possibly twice, between ~14 and 20 ka [10, 14, 16, 143, 151]. This conclusion is consistent with phylogenetic analysis of the superflock. Based on estimates of speciation rates for all cichlid lineages in the Lake Victoria region, Seehausen [166] did not reject the Pleistocene desiccation event and concluded that the lake was colonized from a source outside of the basin rather than persisting in small refugia within the basin itself. Hence, the major diversification of the modern cichlid superflock in the Lake Victoria region coincides with the Holocene refilling of the lake when large areas of habitat became available again. This conclusion is supported by the apparent severe bottleneck [136] and subsequent range expansion [111] that occurred in this lineage. Together these studies support the conclusion that the present cichlid diversity endemic to Lake Victoria must be the result of recent colonization followed by intralacustrine speciation.

The rapid diversification of the LVSF has been attributed by some [167] to the formation of a hybrid clade leading to morphological novelty. Such hybridization events at the base of highly diverse clades have been documented in Lakes Tanganyika [168] and Malawi [60]. A similar event may have influenced the origin of Lake Victoria's cichlids [169].

To find the source of the lineages that colonized the Lake Victoria region, multiple phylogenetic and population genetic studies have been performed. These studies identified several potential colonization sources, including the Kagera and Katonga Rivers [170] and the Congo [171]. Another possible source for the ancestral lineages could have been paleo-Lake Obweruka that formed 8 Ma but desiccated during the late Pliocene [1]. Paleo-Lake Obweruka matched Lake Tanganyika in size and depth and hence provided a large area of habitat [1]. Such paleo-lakes have been implicated in the diversification of related cichlid taxa. Joyce et al. [172], for example, showed that much of the riverine cichlid diversity in southern Africa can be traced back to paleo-Lake Makgadikgadi, where the group radiated before the lake desiccated and the species dispersed into the surrounding waters. However, geological estimates of the desiccation of paleo-Lake Obweruka indicate that it likely did not play a role in seeding the modern constituents of the LVSF because it disappeared before the formation of modern Lake Victoria. Yet another hypothesis suggests that the LVSF arose out of a lineage that persisted in refugia in Tanzania. The lakes in the Eastern Arc region of Tanzania did not desiccate during the Pleistocene and have been suggested to have served as refugia for the Lake Victoria species flock [25, 173]. This, however, seems unlikely. Hermann et al. [26] demonstrated that the cichlids found in that region represent an ancient lineage which is not closely related to the LVSF. There now seems to be general agreement that the Lake Victoria superflock arose more recently in the much smaller Lake Kivu [28, 136] (Figure 4).

4.2.2. The "Out of Kivu Hypothesis". Lake Kivu harbors 15 endemic haplochromine species in addition to three tilapiine species one of which is native (*Oreochromis niloticus*) while the remaining two (*Oreochromis macrochir, Tilapia rendalli*) were introduced [174]). Among the haplochromine species, two phylogenetically distinct lineages can be distinguished both genetically and phenotypically [175]. Interestingly, these two groups correspond to the two lineages of haplochromines found in Lake Victoria [175]. Furthermore, phylogenetic and population genetic evidence clearly indicates that the ancestors of the superflock are derived from Lake Kivu's haplochromines [28, 136]. Molecular clock estimates suggest that the split between the Lake Victoria and Kivu lineages occurred less than 41.5–30.5 ka. This estimate roughly coincides with the eruptions of Virunga volcanoes (25–11 ka), which disrupted the connection between Kivu and the northern lakes in the Lake Victoria region (Lakes Albert, Edward, George, and Kyoga) [28]. However, a causative relationship between the split of Lake Kivu's and Lake Victoria's cichlids and the eruptions of the Virunga volcanoes is speculative. The apparent similarities in the timing of these events depends on a geologic calibration point, the origin of lacustrine conditions in Lake Malawi [57], which is relatively poorly constrained.

Lake Kivu may be an important, though not ultimate, source of cichlid diversity. While at least two lineages from Lake Kivu have invaded the Lake Victoria region and diversified, it appears that a third lineage left Lake Kivu earlier and seeded a smaller radiation in North Eastern Tanzania [26]. The lineage might have been separated from the western cichlids during the formation of the Kenyan-Tanzanian rift system formation [26].

4.2.3. Relationships within the LVSF and between the Other Lakes. While most researchers agree on the postdesiccation colonization and diversification of Lake Victoria's endemic

cichlids, the number of invading lineages appears to be less clear. Nagl et al. [27] suggested that the lake has been invaded at least twice, which is consistent with the results of Verheyen et al. [28]. Additionally, this study found evidence that Lakes Albert, George, and Edward have been seeded at least four times. It appears, however, that only one of the four invading lineages radiated extensively, while the others are rare relicts. Although the number of colonizers remains unclear, some phylogenetic patterns can be found within the LVSF.

Nagl et al. [27] found seven haplogroups within the Lake Victoria superflock. Two lineages (II, IV) are found only at and around Lake Rukwa. A third lineage is restricted to Lake Manyara and Tanzanian rivers (VI). A fourth lineage is found in these same rivers (III), while a fifth lineage is restricted to the Malagarasi River east of Lake Tanganyika (I). A sixth lineage (VII) is found in the Malagarasi River, the Kazinga Channel, and Lake George. However, all species endemic to Lake Victoria and its surrounding lakes and rivers fell into a single haplogroup (V). Within this haplogroup V, four subgroups were distinguished: one is endemic to Lake Victoria (VD), one is found in the rivers close to Lake Rukwa (VA), and the other two lineages have a wider distribution within the Lake Victoria region and can be found in Lakes Victoria, Albert, Edward, and George and adjacent rivers (VB, VC).

While these relationships within the lake and the region are fairly complex, the phylogeography of the superflock becomes even more complicated when one considers members of the LVSF that occur in water bodies far from Lake Victoria. Members of the LVSF have been found as far south as Lake Rukwa [27], in the North Eastern Lake Turkana [25], and as far North as Egypt and Israel [25, 29, 30]. These distributional patterns are interesting from a biogeographical as well as from a paleogeographical standpoint since they inform on past connectivity and colonization events. For example, the presence of members of the superflock in Lake Rukwa has been explained by a series of river capture events that might have enabled the colonization of Lake Rukwa from the Lake Victoria region [25]. Lake Turkana was probably colonized fairly recently in the early Holocene, when Turkana spilled over into the Nilotic system and a connection between the Turkana and the Nile was established [176]. This is supported by a fairly young age of the Lake Turkana endemic species *H. rudolfianus* which groups with the LV species flock [25]. Northern African locations in turn were likely colonized ~11 ka via the Nile [25].

5. Biogeographic Implications

The cyclical periods of aridity/humidity and the resulting contraction, diversification, and expansion of species resemble the classic biogeographic theory formulated by Bush [177] to explain Amazonian diversity [177]. In Bush's [177] diversity-instability hypothesis, species become fragmented due to climatic changes associated with glacial/postglacial environmental conditions [177]. While fragmented, these species undergo allopatric speciation. Repeated bouts of climatic change through the Pleistocene would act as species pumps that increase the species diversity in the tropics.

It is clear that similar climatic cycles have influenced the diversification of East African cichlids [60, 111, 112, 134, 136, 166]. In East Africa, periods of humidity facilitated the dispersal and fragmentation of species [23, 134, 136]. Periods of aridity may either have led to further fragmentation due to basin geomorphology [111] or facilitated admixture as the lake levels dropped, and the available area to cichlids dwindled [60, 168]. Populations and species diverged during these repeated climatic cycles at both the regional [23] and within-lake scales [112]. Thus, climatically driven cycles of desiccation and inundation may have acted as species pumps within East African cichlids [98].

The diversification of East African cichlids also informs on the "cradle" versus "museum" dichotomy in biogeographic theory. In attempting to explain higher diversity found at lower latitudes, Stebbins [178] suggested that tropical regions may act as either "cradles," areas with high rates of diversification, or "museums," areas supporting diversity with low extinction rates. Given the extraordinary diversification of East African cichlids, the East African Great Lakes are clearly cradles of diversity [58]. However, the great depths of Lakes Tanganyika, Malawi, and Kivu allowed for the persistence of cichlid lineages through prolong periods of aridity [25, 28, 75, 100, 105, 136, 175]. In this way, East African lakes also conform to the "museum" hypothesis. As with a growing list of tropical species [179, 180], East African cichlids split the false dichotomy of "cradle" versus "museum" because their habitats act as both cradles and museums.

6. Conclusions

Fundamental to the extraordinary diversification of East African cichlids is the geologically, climatically, and ecologically dynamic environment in which they arose. Beginning at least 10–12 Ma, the western East African rift opened and created a lake basin in place of a swampy, meandering tributary to the Congo River. Seeded by Congolese cichlids, proto-Lake Tanganyika expanded and its cichlids diversified. Several of these diversifying lineages reinvaded the surrounding rivers and one lineage, the haplochromines, migrated south, perhaps via Lake Rukwa, to Lake Malawi, and north, possibly via Lake Kivu, to Lake Victoria. In each of these Great Lakes, the haplochromine cichlids formed remarkably large species flocks in an exceedingly short length of time. The evolutionary histories of the East African Great Lake cichlids were further influenced by fluctuating climatic conditions. During episodes of aridification in East Africa, the lakes were reduced in size and occasionally fully desiccated. The reduction of lake levels reshaped the lake habitats, dividing once connected populations and causing the admixture of previously isolated populations. Such processes facilitated the continued diversification of species and, at least in one instance, lead to the creation of a diverse monophyletic clade of hybrid origin. Lake Victoria most recently completely desiccated ~15 ka causing the extirpation of its endemic cichlids. As the lake infilled in the Holocene, it was then recolonized by cichlids that persisted through the arid interval in the extremely deep, but relatively small, Lake Kivu. The cichlids of Lake Kivu then went on to seed

the Lake Victoria superflock, which while centered in Lake Victoria is distributed throughout the water bodies of East Africa and reaches far north into Israel via the Nile River. The cichlids of East Africa have long been recognized as an evolutionary model system in which to study phenotypic divergence and speciation. It is clear that this system also provides researchers with an exemplary system to study the impact of geologic, paleoecological, and paleoclimatic factors on the biogeography of a lineage.

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Research Article

Species-Specific Relationships between Water Transparency and Male Coloration within and between Two Closely Related Lake Victoria Cichlid Species

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Environmental variation in signalling conditions affects animal communication traits, with possible consequences for sexual selection and reproductive isolation. Using spectrophotometry, we studied how male coloration within and between populations of two closely related Lake Victoria cichlid species (*Pundamilia pundamilia* and *P. nyererei*) covaries with water transparency. Focusing on coloration patches implicated in sexual selection, we predicted that in clear waters, with broad-spectrum light, (1) colours should become more saturated and (2) shift in hue away from the dominant ambient wavelengths, compared to more turbid waters. We found support for these predictions for the red and yellow coloration of *P. nyererei* but not the blue coloration of *P. pundamilia*. This may be explained by the species difference in depth distribution, which generates a steeper gradient in visual conditions for *P. nyererei* compared to *P. pundamilia*. Alternatively, the importance of male coloration in intraspecific sexual selection may differ between the species. We also found that anal fin spots, that is, the orange spots on male haplochromine anal fins that presumably mimic eggs, covaried with water transparency in a similar way for both species. This is in contrast to the other body regions studied and suggests that, while indeed functioning as signals, these spots may not play a role in species differentiation.

1. Introduction

Heterogeneous signaling conditions exert divergent selection on animal communication traits, leading to the divergence of sexual signals between environments [1–3]. For example, bird song characteristics may covary with the sound transmission properties of the vegetation (e.g., [4]) and fish coloration may covary with underwater light conditions (e.g., [5]). These adaptations could contribute to reproductive isolation between populations and possibly promote speciation [6–9]. In addition, signalling conditions may influence the opportunity for sexual selection, by compromising signal perception or by increasing the costs of mate searching [10–12].

The haplochromine cichlids of East Africa constitute a species-rich assemblage with extensive variation in male coloration. Several lines of evidence suggest that variation in underwater light conditions influences the evolution of

these colour patterns. In Lake Victoria, for example, male colours tend to become more distinctive in locations with relatively high water transparency [13, 14] and some colour morphs are completely absent in turbid waters [15].

Haplochromine coloration mediates both intraspecific sexual selection [16, 17] and interspecific behavioural isolation [18–20]. Thus, environment-dependent adaptation in male colours may contribute to reproductive isolation. Indeed, there is a relationship between species diversity and colour diversity along water transparency gradients in Lake Victoria, indicating that constraints on visual communication may explain variation in species richness [13].

Here, we focus on the species pair *P. pundamilia* and *P. nyererei*. These two closely related species are morphologically similar, and the cryptically coloured females of both species are difficult to distinguish. Males however differ markedly in coloration: male *P. pundamilia* are blue-grey while male *P. nyererei* are bright red and yellow (Figure 1). The species co-occur at various locations in Lake Victoria that differ in water transparency. In the present study, we investigate how this variation in signalling conditions may affect male coloration in both species. Previous work indicated that, within *P. nyererei*, populations inhabiting turbid waters exhibit less red coloration in males [13, 14] and weaker colour preferences in females [14] compared to clear-water populations.

We address the following predictions. First, we predict that colours are less saturated (i.e., less chromatic) in turbid waters. Since less-saturated colours can reflect a broader range of wavelengths, we expect these to be favoured (i.e., reflect more light and thus be more conspicuous) in turbid water. Second, we assume that colour conspicuousness is constrained by the ambient light intensity at the wavelengths of reflectance. As a result, colours outside the dominant wavelengths of the ambient spectrum will be favoured only in clear waters where their absolute intensities are high enough for receivers to detect. We therefore predict that, in clear water, reflectance should shift towards either shorter (blue) or longer (red) wavelengths, away from the dominant wavelengths (green) in the ambient light.

Finally, as a result of the above changes, we predict that colour differentiation between *P. pundamilia* and *P. nyererei* will be more pronounced in clear waters.

2. Methods

2.1. Study Species and Sampling Locations. *Pundamilia pundamilia* and *P. nyererei* are two closely related species of haplochromine rock-dwelling cichlids that co-occur throughout a gradient of light environments in Lake Victoria. Both species are morphologically very similar. Females of both species show a yellowish cryptic coloration and are difficult to tell apart. *P. pundamilia* males are blue-grey while males of *P. nyererei* are red dorsally with yellow flanks (Figure 1). Females of both species exert species-assortative colour preferences [18]. In *P. nyererei,* male yellow and red coloration is subject to directional sexual selection as well [14, 17]. Due to its shallow depth, Lake Victoria has relatively turbid waters. Light scattering and absorption are mostly due to nonphytoplankton particles, derived from soil erosion and resuspended sediment [21]. In the present study, we focus on five islands in the south of the lake (Figure 2; Table 1). Two of these islands (Makobe and Ruti) are located offshore and have relatively clear waters. Here, *P. pundamilia* inhabits the top shallower waters while *P. nyererei* dwells in deeper waters. In turbid waters (Kissenda and Python), both species inhabit the same shallow depth layers [22]. At even more turbid locations (i.e., Luanso island), the two species are replaced by a single panmictic population with variable coloration, referred to as *P.* sp. "*Luanso*" [22]. *Pundamilia* sp. breed year-round, with no marked seasonality in breeding activity. All data were collected during May-June 2010.

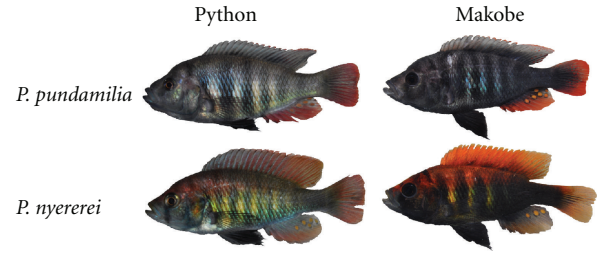

Figure 1: Examples illustrating the colour variation between species and populations.

2.2. Underwater Light Environments. At each island, water transparency was measured using a white Secchi disk (Table 1). We measured downwelling irradiance at each island using a BLK-C-100 spectrophotometer and an F-600-UV-VIS-SR optical fiber with CR2 cosine receptor (Stellar-Net, FL). Measurements were collected in 0.5 m depth increments down to 5 m depth and subsequent 1 m increments down to 12 m depth. At turbid locations, light intensities were too low to obtain reliable measures over this entire depth range (Luanso: measurements down to 4 m; Kissenda and Python: down to 7 m). During each measurement series, we took a minimum of two irradiance spectra at each depth and used the average for further analysis (for repeatability estimates see Supplementary Table S1 in Supplementary Material available online at doi:10.1155/2012/161306). We collected 2 independent measurement series for Luanso island, 3 series each for Kissenda and Ruti islands and 4 series each for Python and Makobe islands (Table 1).

To characterise variation in light environments between locations and depth ranges we calculated the orange ratio for each spectrum [24, 25]: the light intensity in the 550–700 nm range (yellow, orange, red) divided by the intensity in the 400–550 nm range (blue, green). This ratio reflects the spectral composition of the ambient light and tends to increase with depth and with increasing turbidity, as short wavelengths are selectively scattered and absorbed [22, 26]. We subsequently fitted island-specific exponential curves to obtain estimated orange ratios at each depth. Using the species-specific depth ranges (obtained from [22] and assuming equal distributions at Makobe and Ruti) we subsequently identified the range of orange ratios that each species experiences in its natural habitat.

Table 1: Study site characteristics and numbers of individuals collected.

	Luanso	Kissenda	Python	Makobe	Ruti
Maximum depth of the rock-sand interface (m)[1]	5-6	7-8	7-8	8–12	>13
Mean Secchi transparency (cm, mean ± se)[2]	54 ± 4 ($n = 9$)	78 ± 8 ($n = 8$)	106 ± 7 ($n = 11$)	222 ± 7 ($n = 88$)	250 ± 23 ($n = 7$)
Spectral width (and range, nm) of the light spectrum at 2 m depth and 0.002 W/m^2 light intensity	195 (497–692)	247 (477–724)	264 (455–719)	366 (362–728)	390 (343–733)
Sampling dates for irradiance spectrophotometry (2010)	29/5, 7/6	17/5, 1/6, 9/6	20/5, 26/5, 4/6, 5/6	22/5, 27/5, 3/6, 10/6	24/5, 31/5, 12/6
Sample size *P. pundamilia*	10^3	8	10	11	9
Sample size *P. nyererei*		6	16	19	17

[1] Data from [23] and *pers. obs*.
[2] Data collected between 2000 and 2010. Water transparency varies seasonally, but differences between sampling locations are highly consistent (for Secchi readings collected during 2000–2010 at our four sampling sites: anova controlling for sampling date: $F_{3,107} = 25.41$, $P \ll 0.0001$).
[3] *P. sp. "Luanso"* replaces both species at this locality.

2.3. Reflectance Spectrophotometry. Adult males of the three *Pundamilia* species were collected by gillnetting and angling (sample sizes are given in Table 1). Immediately after collection, reflectance spectra at different areas of the body (Figure 3) were taken using the above-mentioned spectrophotometer, an SL4-DT (Deuterium/Tungsten) light source and an R600-8-UV-VIS reflectance probe (StellarNet, FL). We focused on body parts that are potentially subject to (divergent) sexual selection. In *P. nyererei*, sexually selected coloration (red and yellow; [14, 17]) is mostly present on the flank, dorsum, and dorsal fin. In *P. pundamilia*, intraspecific sexual selection has not been explored and we therefore analysed the same body areas, that are grey-blue in this species. However, red coloration is present also in *P. pundamilia*, namely, on the edges ("lappets") of the unpaired fins. In order to capture potentially important variation in this trait, we included "dorsal fin lappets" as an additional body area for both species. Finally, for both species we also measured the spectra of the anal fin spots ("egg dummies") as these brightly coloured spots have been implicated in sexual communication [27–30]. For correlations between body areas, see Supplementary Tables S4 and S5.

About halfway through the field work, the light source stopped working and subsequent measurements had to be taken using the sun as a light source (see below for statistical incorporation of this variation).

Table 2: Coloration metrics.

Name/description	Formula	Reference
Chroma A measure of the "purity" or saturation of a colour; a function of how rapidly intensity changes with wavelength	$C = \sqrt{LM^2 + MS^2}$ $LM = B_R - B_G$ $MS = B_Y - B_B$ where: $B_B = \frac{\sum_{400}^{474} Q(\lambda, x)}{B}$ $B_G = \frac{\sum_{475}^{549} Q(\lambda, x)}{B}$ $B_Y = \frac{\sum_{550}^{624} Q(\lambda, x)}{B}$ $B_R = \frac{\sum_{625}^{700} Q(\lambda, x)}{B}$, $B = \sum_{400}^{700} Q(\lambda, x)$	[35]
λP_{50} Wavelength that divides the spectrum in two parts with equal spectral energy (i.e., the median of the cumulative distribution between 400–700 nm)	$\sum_{400}^{\lambda P_{50}} = \sum_{\lambda P_{50}}^{700}$	[33, 34]

2.4. Calculation of Colour Metrics. A minimum of two reflectance spectra were measured for each body region for each fish, and the mean of these was used for calculations (unless after visual inspection, one of the spectra was outside expected limits and was discarded, less than 10% of all spectra; repeatability estimates for included spectra are given in Supplementary Tables S2 and S3). We then extracted two colour metrics (see Table 2), excluding the UV part of the spectrum (300–400 nm) because UV-sensitive pigments have not been detected in Lake Victoria cichlids including *Pundamilia* species [31, 32]. (1) Chroma (or saturation): a measure of the purity of a colour, indicating how much of the reflectance is concentrated in a particular segment of the spectrum. It ranges from 0 (e.g., grey or white) to 1 (a pure colour). (2) Hue: related to the wavelength at the maximum absolute slope in the reflectance spectrum, and the property that in common language we understand as colour (e.g., red, blue, green, etc.). As a measure of hue, we calculated λP_{50}, the wavelength at which 50% of the total reflectance between 400–700 occurs [33, 34].

Brightness, that is, the total intensity of light reflected, is another potentially important component of coloration. However, due to the failure of the light source we did not

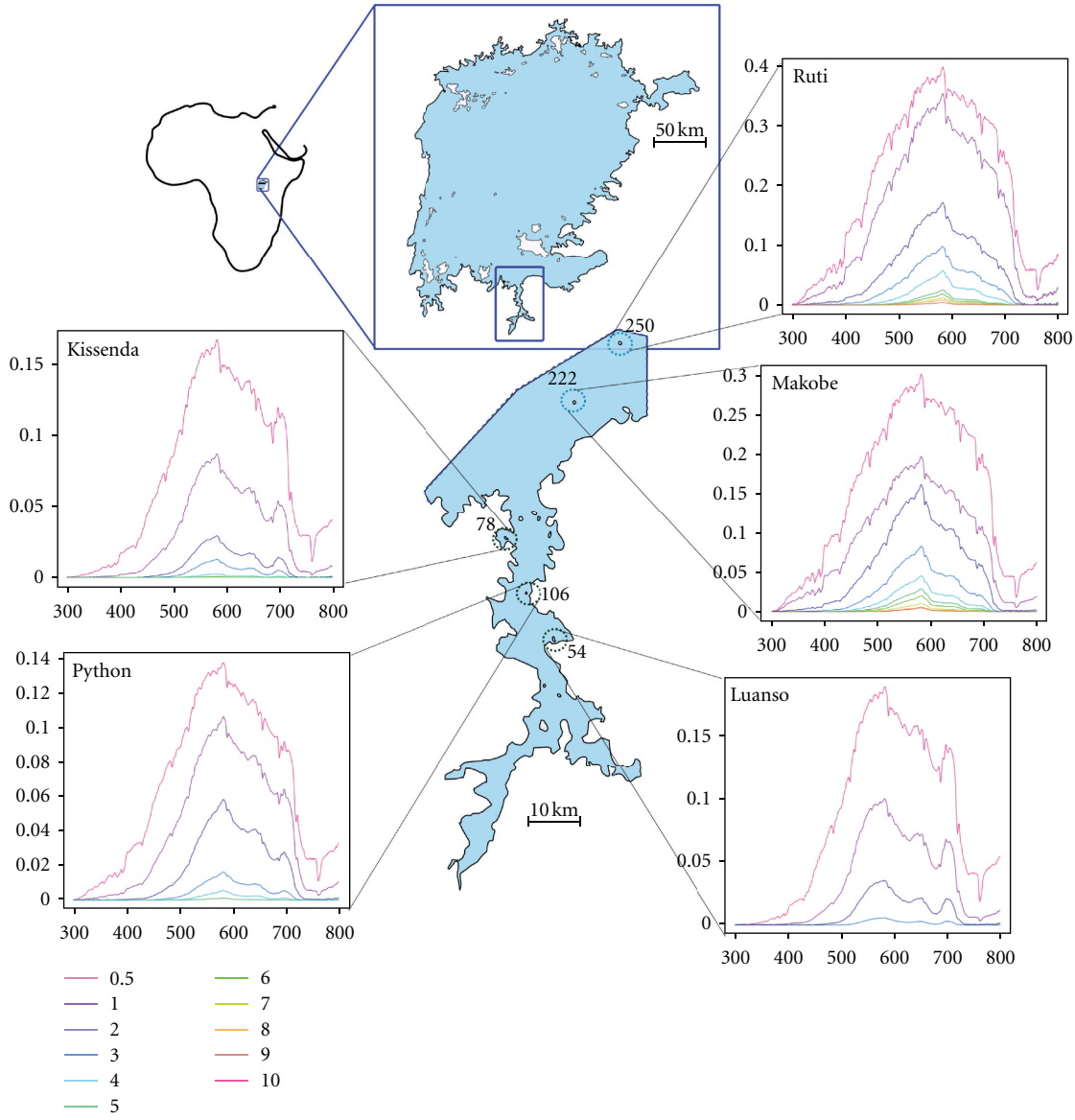

Figure 2: Sampling locations and their underwater light environments. In each panel, curves show underwater ambient light spectra at different depths (m). Numbers shown next to the islands are the mean Secchi disk measurements (cm).

obtain reliable brightness estimates (see below) and therefore excluded this property from the analyses.

2.5. Data Analysis. We built linear models allowing for random effects as well as differences in variances among the explanatory variables, using Linear Mixed Effect models (LME) [36]. We fitted models for each coloration property, each body area, and each species separately. We chose this approach (as opposed to collapsing metrics and body areas into, for example, Principal Components) because it allows evaluation of specific predictions and exposes potential differences between body areas. All analyses incorporated four populations of each species (Luanso was excluded from the analyses but included in the figures as a reference). Because water transparency was bimodal rather than continuous (i.e., the waters at Kissenda and Python islands were similarly turbid, and Makobe and Ruti similarly clear, Table 1; Figure 4), water clarity was modelled as a categorical variable (i.e., turbid versus clear). A factor for population was included as a random effect in all models. In addition to water clarity, the effect of using either the lamp or the sun as a light source was included as explanatory variable. To address colour differentiation between species, species identity was added as a third explanatory variable and the interaction with water clarity evaluated.

For model selection, we explored all possible variance structures (variance components were functions that included the actual Secchi depths (Table 1) and a factor for light source) and selected the most parsimonious model using restricted maximum likelihood ratio and Akaike's

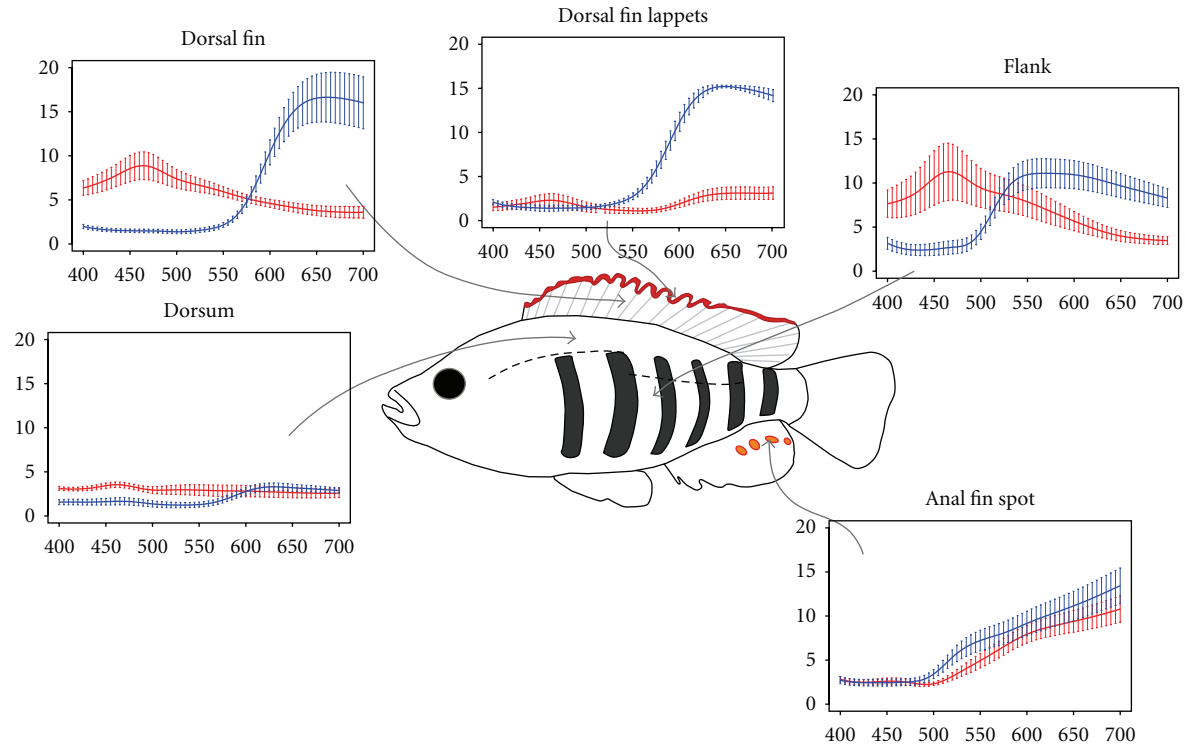

Figure 3: Body areas measured and reflectance spectra for each species (average with standard error; *P. pundamilia* in blue, *P. nyererei* in red; both from Makobe island).

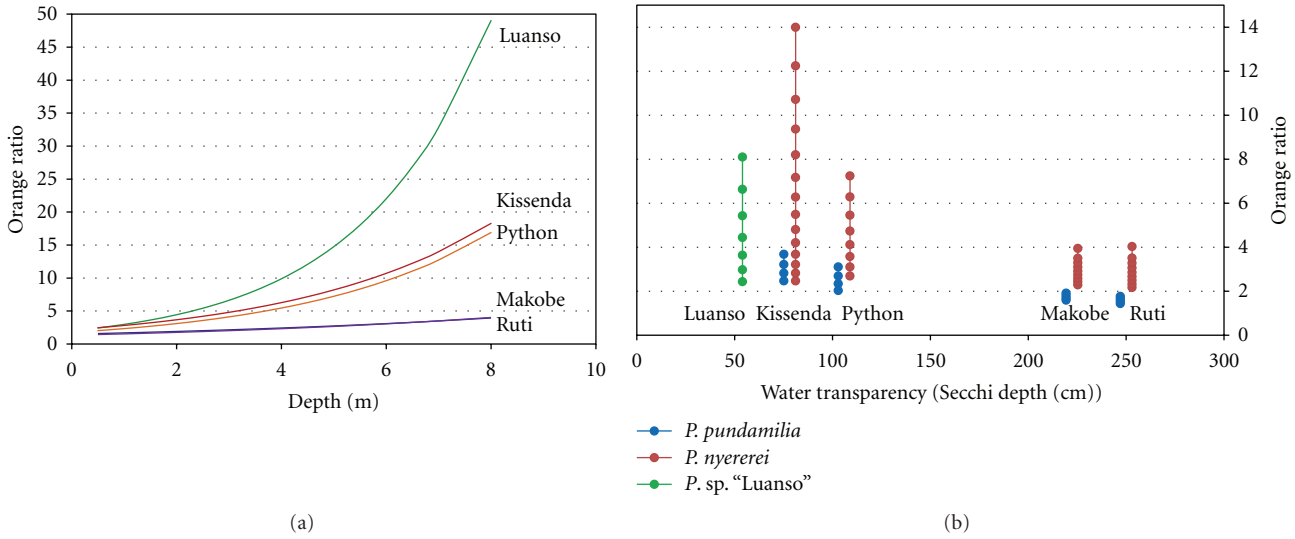

Figure 4: Variation in underwater light environments between sampling locations and species-specific depth ranges. For each location, plotted values derive from fitting an exponential function to all measured orange ratios at that location. (a) The increase in orange ratio with depth for the five sampling locations. Ruti and Makobe show virtually identical curves. (b) The orange ratios at the species- and island-specific depth ranges. Each symbol represents the orange ratio at a specific water depth (in 0.5 m increments) where the species occur.

information criterion, corrected for small sample size (AICc) [37]. After remaining with the best variance structure, we used maximum likelihood to reduce the complexity of the models and AICc to select the covariates that remain in the model. We then used ANOVA to test whether a model including the clarity covariate (or the interaction between species: clarity, when applicable) was significantly better than one that did not, and we report likelihood ratio and *P* values for this comparison.

All statistical analyses were conducted in R 2.12 [38], applying packages *nlme* and *MuMIn*. To adjust for multiple testing of the same prediction in multiple body areas, we used

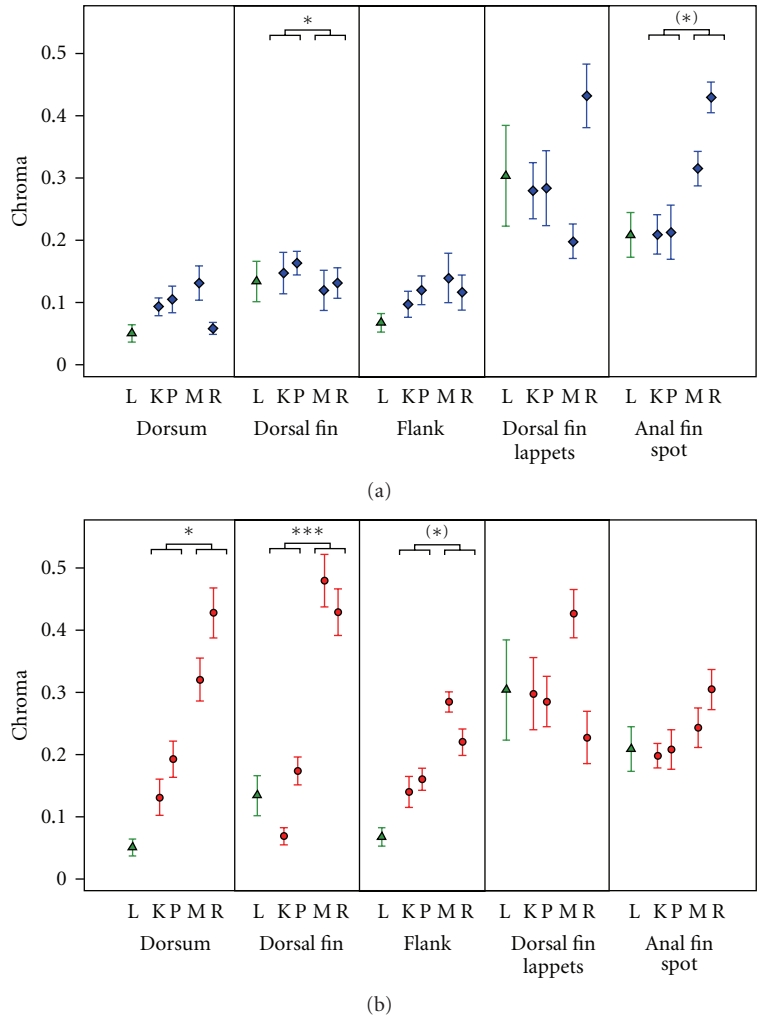

Figure 5: Chroma of different body parts at four sampling locations for (a) *P. pundamilia* (blue diamonds) and (b) *P. nyererei* (red circles). In both panels, *P.* sp. *"Luanso"* (green triangles) is included as a reference. Symbols indicate means with standard errors. Statistically significant differences between clear and turbid locations are indicated with asterisks (after correction for multiple testing; (*) $P < 0.10$; *$P < 0.05$; ***$P < 0.001$). L: Luanso, K: Kissenda, P: Python, M: Makobe, R: Ruti.

corrected P values (i.e., we multiplied the actual P values with the number of body areas, 5).

Our estimates of chroma and hue were not strongly influenced by the light source used (lamp or sun, see Supplementary Table S6) but there were major effects on brightness, showing significant interactions between water clarity and light source for all models. Therefore, we had to discard this metric.

3. Results

3.1. Light Environments. At all study sites, the proportion of longer wavelengths in the light spectrum (i.e., wavelengths >550 nm) increased towards deeper waters (Figure 4(a)). The increase was steepest at Luanso, intermediate at Kissenda and Python islands, and very gentle at Makobe and Ruti islands. Incorporating species-specific depth ranges at each location, we estimated the range of orange ratios that the two species experience in their natural habitats. Both species are exposed to higher orange ratios in the turbid waters of Kissenda and Python, compared to Makobe and Ruti (Figure 4(b)). *P. nyererei* in particular experiences a large difference in light environment between turbid and clear locations, although the decrease in orange ratio was not significantly different between the species (ANOVA, interaction effect between Secchi reading and species on orange ratio: $F_{2,4} = 4.49$, $P = 0.10$).

3.2. Chroma. In *P. pundamilia* (Figure 5(a)), we did not observe a significant increase in chroma in any of the measured body areas. There was a trend for anal fin spots ($L = 5.66$, $P = 0.087$), but a significant decrease in chroma for dorsal fin ($L = 6.81$, $P = 0.045$). There were no changes in the chroma of the dorsum, flank, or dorsal fin lappets. The changes in *P. nyererei* were more consistent (Figure 5(b)), with significantly increased chroma in clearwater populations for dorsum ($L = 9.16$, $P = 0.013$) and dorsal fin ($L = 12.53$, $P < 0.001$) and a trend in the same direction

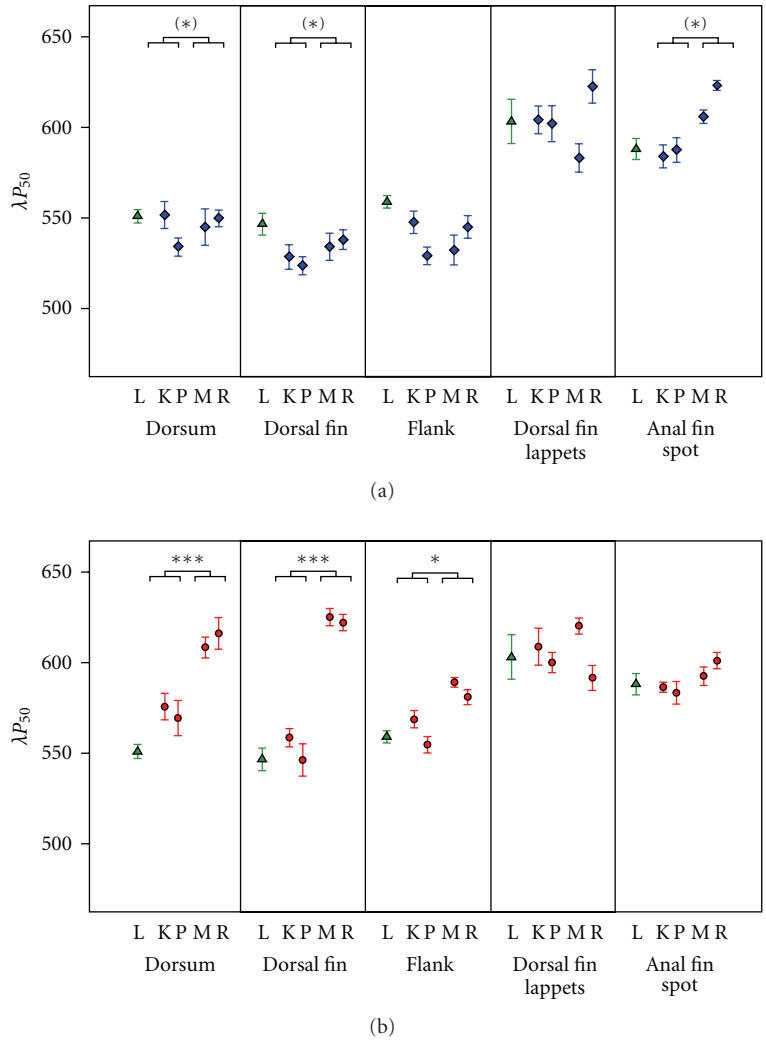

Figure 6: Hue (λP_{50}) of different body parts at four sampling locations for (a) *P. pundamilia* and (b) *P. nyererei*. Symbols and labels as in Figure 5.

for flank ($L = 5.99$, $P = 0.072$). No significant changes were observed in anal fin spots and dorsal fin lappets.

3.3. Changes in Hue. λP_{50} (the wavelength that halves the total reflectance) was expected to shift towards more extreme wavelengths in clear waters. For the blue coloration elements in *P. pundamilia*, results were inconsistent with this prediction (Figure 6(a)). We found small and nonsignificant changes towards longer rather than shorter wavelengths for dorsum ($L = 6.36$, $P = 0.059$) and dorsal fin ($L = 5.76$, $P = 0.082$). There was no significant change in the hue of flank coloration. The red dorsal fin lappets also did not increase in λP_{50}. Only the yellow anal fin spots tended to follow the prediction, but the increase towards longer wavelengths in clear water was not statistically significant ($L = 6.17$, $P = 0.065$).

In *P. nyererei* (Figure 6(b)), we observed a highly significant shift towards longer wavelength reflectance for the dorsum ($L = 11.51$, $P < 0.001$), dorsal fin ($L = 15.69$, $P < 0.001$) and flank ($L = 9.28$, $P = 0.012$). Anal fin spots and dorsal fin lappets did not show significant changes.

3.4. Colour Differentiation between Species. λP_{50} was also used to test for the extent of differentiation between the two species' coloration (Figure 7). We found increased differentiation in clear waters for dorsal fin ($L = 27.29$, $P < 0.001$), and flank ($L = 8.77$, $P = 0.016$) and a trend in the same direction for dorsum ($L = 5.52$, $P = 0.094$). In contrast, coloration of anal fin spots and dorsal fin lappets did not show increased differentiation with water clarity.

4. Discussion

We examined patterns of colour variation within and between two cichlid species that inhabit different signalling environments. We specifically tested whether fish coloration becomes more saturated and increasingly exploits wavelength ranges outside the dominant ambient light spectrum,

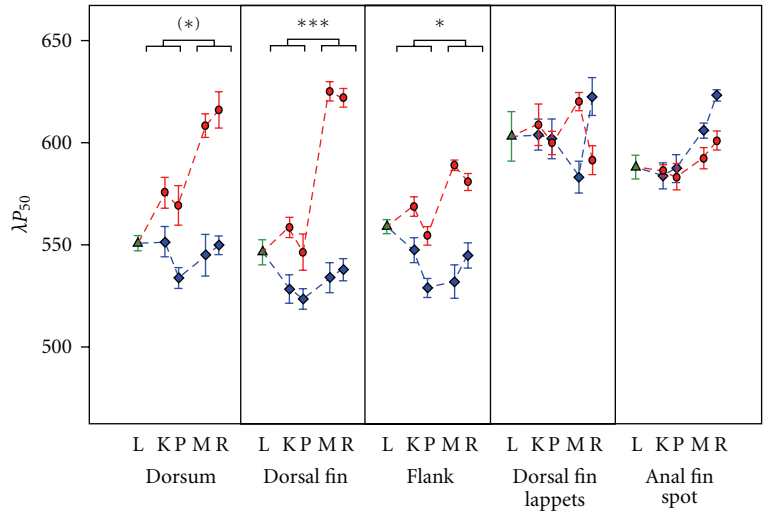

Figure 7: Differentiation in coloration hue (λP_{50}) of different body regions between *P. pundamilia* and *P. nyererei*. Symbols and labels as in Figure 5.

in populations inhabiting clearer waters. We found support for these predictions in *P. nyererei*, but inconsistent results for *P. pundamilia*. For those body areas that are differently coloured between the species, we observed increasing species differentiation in coloration towards clear waters.

For the red and yellow coloration elements in *P. nyererei*, we found that colours are more saturated and shifted towards longer wavelengths (i.e., redder) in clearer waters. For the blue coloration of *P. pundamilia* however, we did not observe any statistically significant shift towards greater chroma or shorter wavelengths (i.e., more blue). One reason for this incongruence may lie in the different depth distributions of the two species. The change in the environmental light spectrum from turbid to clear waters is more pronounced in the deeper waters where *P. nyererei* is most abundant (Figure 4), possibly generating stronger divergent selection between allopatric populations for this species. It is also possible that the importance of male coloration for intraspecific female choice differs between the species. Sexual selection on red and yellow colour elements is well established in *P. nyererei* [14, 17], but intraspecific sexual selection remains to be studied in *P. pundamilia*. Just like *P. nyererei*, *P. pundamilia* females use colour cues during interspecific mate choice [18]. However, they might use other characteristics, such as body size, behaviour or chemical cues in their choice among conspecific males. Recent work in these and other haplochromines indicates that chemical cues could play a role in mate choice in some species [39–41]. Methodological constraints may also contribute to the difference between species, as the blue-grey coloration of *P. pundamilia* may be more difficult to capture with spectrophotometry [42]. This is consistent with the observation that the yellow anal fin spots did tend to change in the predicted direction for both hue and chroma.

Although not statistically significant, we observed similar variation in anal fin spot coloration in both species. This is consistent with earlier suggestions of adaptation of these spots to environmental light: Goldschmidt [29] found that species inhabiting darker habitats had relatively large anal fin spots. Anal fin spots have been suggested to mimic eggs and contribute to fertilisation success (e.g., [43] but see [44]). This functional context raises the question whether the observed variation in spot coloration influences the resemblance to eggs. *Pundamilia* sp. eggs are orange, but no data exist regarding egg colour variation between species or populations. Anal fin spots have also been suggested to play a role in speciation (e.g., [30, 45–48]. Here, we do not find evidence for species-specific effects in spot coloration and a role in species recognition is thus unlikely.

We found no consistent changes in the coloration of the red dorsal fin lappets in either species. Interestingly, this trait is shared not only between our study species, that are very closely related, but also occurs in many other haplochromines [49]. This may indicate that there is little genetic variation in this trait, preventing adaptive divergence between populations and species.

We propose that the differences in coloration that we observed across the four studied populations are adaptations to different underwater light environments. Fish coloration can be phenotypically plastic [50, 51] and in haplochromines, colour expression varies with diet, territorial status [52, 53], and stress ([54]; pers. obs.). However, given the maintenance of colour differences in the laboratory, and significant genetic differentiation between populations [22], evolutionary adaptation is both feasible and likely. We hypothesise that the observed patterns are driven by selection for signal conspicuousness, which requires that signals have sufficient intensity as well as provide contrast against the sensory background [2].

Colour signals that rely on reflection of incident light (as opposed to luminescence or iridescence) will maximise signal intensity by reflecting most strongly in the wavelength range of the incident light (e.g., [55, 56]). However, maximising colour contrast requires reflectance of wavelengths

that are underrepresented in the background (e.g., [57]). When the illuminating and background spectra are similar, signal evolution will likely reflect a tradeoff between signal intensity and contrast. This situation occurs in many aquatic systems, where signals are viewed against the water column [58]. In some fish species, conspicuousness is achieved by reflectance of colours that contrast against the prevalent ambient light (e.g., [59, 60]). In other species, colour variation is positively correlated with the prevalence of the reflected wavelengths in the environmental light spectrum [5, 50, 61]. The patterns we observe in *Pundamilia* may reflect a compromise between these two strategies. The blue *P. pundamilia* are restricted to shallow waters where short wavelengths are still present, whereas the red and yellow *P. nyererei* inhabit deeper waters with red-shifted ambient light. At the same time, colour contrast against the background can be maintained by exploiting the shoulders rather than the peak of the ambient spectrum and by reflecting in a relatively narrow wavelength range. We hypothesise that this explains the shift in hue and chroma in the clearwater populations of *P. nyererei*, that experience a broader and more intense illumination spectrum than their counterparts in turbid waters. The failure of our light source precluded analysis of brightness variation in the present dataset. As a consequence, we are unable to test whether the conspicuousness of male coloration is optimised for local viewing conditions. Moreover, recent studies suggest that there is variation in visual systems between sympatric species and allopatric populations of *Pundamilia* [22, 31], and ongoing work is aimed at identifying the visual pigments and expression levels in the populations studied here. This information will subsequently be incorporated into quantitative visual models.

Different patterns of variation may also result from other factors than intraspecific perceptual processes. For example, colour production may be subject to physiological constraints [62, 63]. The red and yellow coloration in *Pundamilia* is carotenoid based [14] and the availability of dietary carotenoids may covary with underwater light intensity [64, 65]. Thus, redder coloration in clearer waters could be due to greater availability of carotenoids. Observations that colour variation between populations is maintained in the laboratory indicate a heritable component, but this does not rule out that carotenoid limitation selectively favours different levels of colour expression [66–68]. Testing this hypothesis requires evaluating whether haplochromines are carotenoid limited in their natural habitat. Second, sexually selected traits are often subject to increased predation (e.g., in fish: [69–72]). In Lake Victoria, however, piscivorous birds and fish tend to be more numerous in clearwater locations [13]; pers. obs), possibly because turbidity hampers visual predation [12, 73]. This would favour less chromatic and less contrasting colours in clearwater, which is not what we observe in *Pundamilia*. Finally, male colour evolution will likely reflect variation in female preferences among populations. Relaxed sexual selection on visual signals in turbid water has been documented in several fish species [74–76]. In addition to immediate effects of reduced signal perception, variation in water turbidity may lead to heritable changes in female preference behaviour. This seems to be the case in *P. nyererei*. Females from turbid waters are less selective with respect to male coloration, even when tested under broad-spectrum illumination in the laboratory [14]. The observed colour variation across populations might therefore be driven by heterogeneous sexual selection regimes, rather than selection for optimal local conspicuousness. To resolve this question, we need more detailed analyses of variation in female preference and choosiness to establish sexual selection strength for the different aspects of male coloration (hue, chroma), as well as quantitative estimates of visual conspicuousness in relation to these aspects. Such studies should also help to identify the mechanisms underlying preference variation. Beside sensory biases for conspicuous signals, haplochromine female preferences are likely influenced by selection for heritable benefits (e.g., parasite resistance [52]). Thus, if signal conspicuousness in turbid waters is maximised by lower carotenoid deposition, for example, carotenoid-dependent aspects of male coloration may become less informative and therefore less important in mate selection (e.g., [77–79]). We suggest that the interactions between sensory processes and signal content in shaping haplochromine colours constitute an important and rewarding avenue for further study.

Taken together, we found that different body regions and different species show different responses to environmental heterogeneity in visual conditions: divergence at the level of allopatric populations as well as sympatric species (flank, dorsum, dorsal fin), divergence between populations but not species (anal fin spots), or no consistent pattern of change (dorsal fin lappets). Importantly, our findings confirm earlier suggestions that divergent sexual selection is involved in haplochromine species divergence [13, 80], as we found significantly stronger species differentiation towards clear waters for the same body areas that were previously shown to be subject to intraspecific sexual selection in *P. nyererei* [17]. As such, our study implicates species- and habitat-specific selective pressures as well as potential genetic or functional constraints to adaptive divergence and thereby contributes to identifying the traits involved in the buildup of reproductive isolation.

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Research Article

Alternative Reproductive Tactics in the Shell-Brooding Lake Tanganyika Cichlid *Neolamprologus brevis*

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Alternative reproductive tactics (ARTs) are found in several Lake Tanganyika shell-brooding cichlids. Field studies were conducted in the Wonzye population to examine reproductive ecology and ARTs in the Lake Tanganyika shell-brooding cichlid *Neolamprologus brevis*. We discovered that this fish occurred in both rocky- and sandy-bottom habitats, but in rocky habitats, brood-caring females exclusively occurred in shell-patches that another cichlid species created. All *N. brevis* of both sexes in the patches were sexually mature, whereas immature males and females with unripe eggs were found frequently in sandy-bottom habitats. Males in sandy-bottom habitats were smaller, but fed more frequently and were in better somatic condition than males in the patches. Similar tendency was found in females. This indicates that *N. brevis* uses different habitats depending on the stage of its life history, with migration from sandy-bottom habitats to the shell-patches for reproduction. Males in the patches exhibited different behavior patterns: floating above the patches and lying in the patches. The former was larger, more aggressive, and invested less in gonads (relative to body size) than the latter. These results accord with those of other shell-brooding Lake Tanganyika cichlids with ARTs, and they therefore suggest the presence of ARTs in *N. brevis*.

1. Introduction

In species where males hold the resources required for breeding, reproductive behavior is often associated with "bourgeois" territorial tactics that involve defense of resources in an attempt to monopolize mating opportunities [1–4]. Bourgeois tactics are usually adopted by males who achieve competitive superiority (and also attractiveness) thorough behavioral (e.g., antagonistic behaviors) and morphological investments (e.g., large body size). These exclude less competitive males from reproduction. Such intrasexual competition for mating may lead to the evolution of alternative reproductive tactics (ARTs) [5, 6]. Competitively inferior, less attractive, and smaller subordinate males are unlikely to monopolize opportunities for mating, but they may evade monopolization by bourgeois males by using reproductively "parasitic" tactics that often involve "sneaky" behavior and the theft of reproductive efforts by bourgeois males [1, 3, 4, 6].

ARTs inherently give rise to sperm competition, that is, a competition among sperm from different males for the fertilization of ova [7]. Subordinate males exhibiting "sneaky" mating behavior (i.e., sneakers) face a high probability that their sperm will encounter sperm competition (sperm competition risk), as they obtain opportunities for fertilization by taking part in the mating of others. However, this is not necessarily true for bourgeois males, which engage in mating without any rivals (low sperm competition risk) unless

sneakers frequently intrude in their mating. The theoretical game model of sperm competition predicts that sneakers are forced to make a greater investment in ejaculate than are bourgeois males due to the increased risk of sperm competition [8]. Differential testes investment, as well as behavioral and morphological investments, are found in many species with ARTs (e.g., [9–15]; see also [1, 16–18], for review).

ARTs have been reported in a variety of taxa [4, 6] and particularly in fish [1, 3, 16]. ARTs are prevalent and diversified in the Lake Tanganyika cichlid tribe Lamprologini [19], although such ARTs can also be found in other Lake Tanganyika tribes ([20] and references therein). In the Lamprologini, ARTs are typically dichotomous, that is, bourgeois territorial and parasitic "sneaky" tactics (e.g., cooperative brooders, *Julidochromis ornatus* [21], *Julidochromis transcriptus* [22, 23], and *Neolamprologus pulcher* [24]; a shell brooder, *Lamprologus callipterus* [3, 25–27]; a rock-hole brooder, *Telmatochromis temporalis* [28]). In *T. temporalis*, piracy tactic (i.e., the takeover of spawning events from territorial males by the largest males) also appears but seasonally [29]. In the shell-brooder *Telmatochromis vittatus*, four reproductive tactics have been reported (sneaker, satellite, territorial, and piracy tactics, [30]), with piracy tactics being dependent on conditions [31]. Furthermore, mixed paternity is found in biparental breeders (*Neolamprologus meeli* [32] and *Variabilichromis moorii* [33]), suggesting the presence of parasitic tactics.

A Lamprologini cichlid, *Neolamprologus brevis* (a synonym of *Neolamprologus calliurus* [34]), is characterized as an obligate shell brooder that spawns and cares for broods inside empty gastropod shells [25]. The shell is also used as shelter when these fish encounter predators [35]. The Wonzye population, which is located in a southern region of the lake, occurs in a wide range of habitats in the littoral zone, from shallow rocky habitats to relatively deep offshore sandy-bottom habitats. However, ecological information for this fish is only available from a particular habitat, shell patches consisting of a number of gastropod shells (mean = 96 shells, [25]). It is not known how this fish uses other habitats. The patches are not spontaneous but are created by nesting males of *L. callipterus* [36]. *L. callipterus* is the only species that can transport shells. A female *L. callipterus* occupies a shell for breeding, which lasts for 10–14 days, during which period she spawns and subsequently provides brood care [36]. *L. callipterus* females that are ready to spawn or care for broods are not found in the patches [25], suggesting that they leave the patches immediately after the completion of a breeding event. *N. brevis* also breeds in the patches, and a number of females and several males are found at the same time, suggesting that this fish has a multimale polygynous mating system [25]. Two other species, *Neolamprologus fasciatus* and *T. vittatus*, also use shell patches for breeding [25]. *L. callipterus* is not aggressive toward *N. brevis* and tolerates their using the unoccupied shells for breeding. The shell patches are therefore communal nests.

In our pilot study, mature males of different size classes were found in the shell patches, with larger males floating several dozen centimeters and sometimes more than 1 m above the patches and smaller males residing in the shells. This resembles the bourgeois "nesting" males and parasitic "dwarf" males of *L. callipterus* [18, 26, 36, 37]. Therefore, we suspected that the male *N. brevis* may also exhibit ARTs. If parasitic males are present in this fish population, it is expected that there exist discontinuous differences in aggressiveness and relative testes investment (testes mass relative to body mass) between males of the different size classes [8, 15, 18, 38]. In this study, we examined habitat use and the presence of ARTs in *N. brevis* in the Wonzye population.

2. Methods

2.1. Sampling. Field studies using SCUBA diving techniques were conducted at Wonzye Point (8°43′S, 31°08′E) near Mpulungu, Zambia, from November to December 2002, and October to November 2007. Where this population is found, the ground is covered with rocks from the shoreline to about 9 m depth (rocky habitat) or with sand to about 7–11 m depth (sandy bottom habitat). In this population, *N. brevis* occurred in both habitats, each of which was divided into two subhabitats: (1) sandy bottoms that were almost covered with shells at 9–11 m depth (662.5 shells/m^2, [25]) (henceforth "shell bed" or SB); (2) sandy bottoms on which shells were widely distributed at 7–11 m depth (0.12 shells/m^2, [25]) (henceforth "separated shells on sandy bottom" or SS); (3) midwater aggregations in rocky habitats that often consisted of >100 individuals, at 4–9 m depth (henceforth "midwater aggregation" or MA); (4) shell patches of *L. callipterus* in rocky habitats, which often consisted of >100 shells, at 4–9-m depth (shell density = 496.8 shells/m^2, [25]) (henceforth "shell patch" or SP).

Habitat use was determined by differences in size structure, frequency of reproductively active individuals, and behavior among the habitats. To examine the size structure, we captured randomly selected *N. brevis* using gill nets in three habitats from November to December 2002 (N_{SB} = 60, N_{SS} = 63, N_{MA} = 34) and in one habitat from October to November 2007 (N_{SP} = 156, see below for details). We brought the collected fish back to the laboratory and measured their standard length (SL) to nearest 0.12 mm and body mass (BM) to nearest 0.002 g. All fish sampled from sandy bottom habitats and midwater aggregations and some of the fish sampled from the shell patches (N_{male} = 48, N_{female} = 31) were dissected immediately after sacrificing among crushed ice or by anesthetizing with eugenol. We then weighed their gonad mass (GM) to nearest 0.002 g and sexed individuals. Undissected females were released at the capture points. Male maturity was determined from developmental stages of gonads, because testes were either white and enlarged or transparent and threadlike. Males with white and enlarged testes were considered to be mature. Brood-caring females and females whose ovaries were filled with large, orange-colored (i.e., ripe) eggs were labeled as mature. Consequently, as the minimum size of mature females was 31.6 mm SL, we considered females ≥31.6 mm SL to be sexually mature, even if their ovaries were filled with unripe eggs. For the dissected fish (N_{SB} = 60, N_{SS} = 63, N_{MA} = 34, N_{SP} = 79), we calculated the gonad-free condition factor

(i.e., BM-GM $\times 10^5/SL^3$) and compared it among habitats and by sex. We also calculated the gonadosomatic index (GSI) of the dissected males as an estimate of testes investment and compared it among habitats (see below for details).

To examine the size structure and describe the reproductive ecology of *N. brevis*, we conducted an intensive field study in the shell patches in 2007. We determined the number of males and females in each shell patch, the maturity of males, and the breeding status of females from October to November 2007. We also examined whether there was a spawning cycle in *N. brevis,* which would be an important aspect of the reproductive ecology of this fish because *N. brevis* may have a periodic spawning cycle given that the reproductive cycle of the patch-owner species *L. callipterus* displays weak lunar-related periodicity [39]. Additionally, it was important to describe the mating system, as the different sampling days among habitats may have influenced reproductive parameters. In 17 randomly selected shell patches, we counted the numbers of *N. brevis* individuals that remained in shells in each patch once a week from 19 October to 12 November 2007, during which period a new and full moon occurred twice and once, respectively. Consequently, we obtained the counts for a continuous 6-week period in each shell patch. Because *N. brevis* resides in a shell in a head first position, the presence of an individual can be identified by observation of the caudal fin. We therefore visually checked whether *N. brevis* were present in shells by observing the entrance of shells. However, this visual counting could lead to an underestimation due to missing individuals that had retreated into the inner part of the shells or an overestimation due to the inclusion of males hiding in shells. To confirm the exact number of *N. brevis* and clarify the presence and absence of fish in a shell, all shells in the shell patches would need to be crushed. Because the application of this method is destructive, it was performed in only a portion of the shell patches (N = nine patches), minimizing the impact on the habitats. At the end of the observation (12 November 2007), we counted the number of shells in the nine shell patches by visual inspection and captured all shells and *N. brevis* individuals that were floating above the shell patches or that remained near the shells ($N = 145$). In the remaining eight nests, 11 floating *N. brevis* individuals were also captured. Hence, we sampled a total of 156 *N. brevis* in 2007. All fish and shells that were sampled were brought back to the laboratory. To determine the exact number of *N. brevis* individuals all collected shells were crushed using an iron hammer. The numbers revealed by visual counting and by actual counting (i.e., counting after crushing of shells) were strongly positively correlated (Poisson regression model, $\chi^2 = 52.42$, df = 1, $P < 0.001$, $N = 9$), although visual counting had identified only three-quarters of the actual counting (mean ± SD = 74.0 ± 26.7%, $N = 9$). This indicates that the number of *N. brevis* estimated in the field by visual counting is a good index of the exact number of *N. brevis*, although with some underestimation.

2.2. Behavioral Observations. To examine the differences in behavioral characteristics among the four habitats, behavioral observations were conducted at a variety of locations in each habitat in November and December 2002. A total of 123 individuals that were randomly selected ($N_{SS} = 22, N_{SB} = 19, N_{MA} = 23, N_{SP} = 59$) were observed for 10 minutes, and the time spent in the shells (i.e., hiding), pecking behavior (i.e., feeding), aggressive behavior (i.e., dashing and fin spreading to opponents), and the opponents (conspecifics or heterospecifics) were recorded. Observed fish were later captured and sexed based on the shape of genital papilla and body color under water. They were released at the original capture points immediately after the sex determination.

2.3. Statistical Analyses. To examine the difference in size structure within and among habitats, we compared the SL of both sexes and the proportion of individuals available for mating (i.e., gravid females or mature males). For females, we compared the SL among three habitats (i.e., shell beds, separated shells on a sandy bottom, and shell patches) because only one female was sampled from midwater aggregations. For males, we compared the SL among all four habitats and between mature and immature individuals in each habitat. Comparisons were performed by general linear models (GLMs) fitted to a Gaussian distribution. The proportions of gravid females and mature males were compared between sandy and rocky habitats using Fisher's exact probability test. This analysis included fish samples that were taken in different years, which may have resulted in a difference of the size structure among habitats. However, because the periodic observations showed that temporal variation in reproductive activity was very little during the season (see results) and the environments within the littoral zone of Lake Tanganyika are considered to be stable over time [40], the differences generated by such effects were assumed to be negligible.

The periodicity of spawning was examined by the repeated-measures procedure, that is, a generalized linear mixed model fitted to a Poisson distribution (the number of females in shells = the dependent variable, date = the fixed factor, patch identifier = the random factor).

Other phenotypic data (the condition factor and testes investment) were also compared among habitats. The condition factor was compared for each sex, whereas the GSI was compared only for males using GLMs. In these comparisons, we took into account the differences between males in the shell patches, which may employ different reproductive tactics. For this purpose, we divided them into two groups based on adherence to shells, resulting in a total of five categories. To compare testes investment among habitats, we first attempted to follow the methodology of Tomkins and Simmons [17] in which log gonad mass (GM) was compared among groups, with log soma mass (BM-GM) as a covariate. In this analysis, only mature males were included ($N = 128$ males). There was a significant interaction between habitat and log soma mass (GLM, habitat × log soma mass: $F_{4,88} = 3.570$, $P = 0.01$). We therefore estimated testes investment by GSI.

To examine differences in behavioral characteristics among habitats, behavior was also compared among three habitats for females and among four habitats for males.

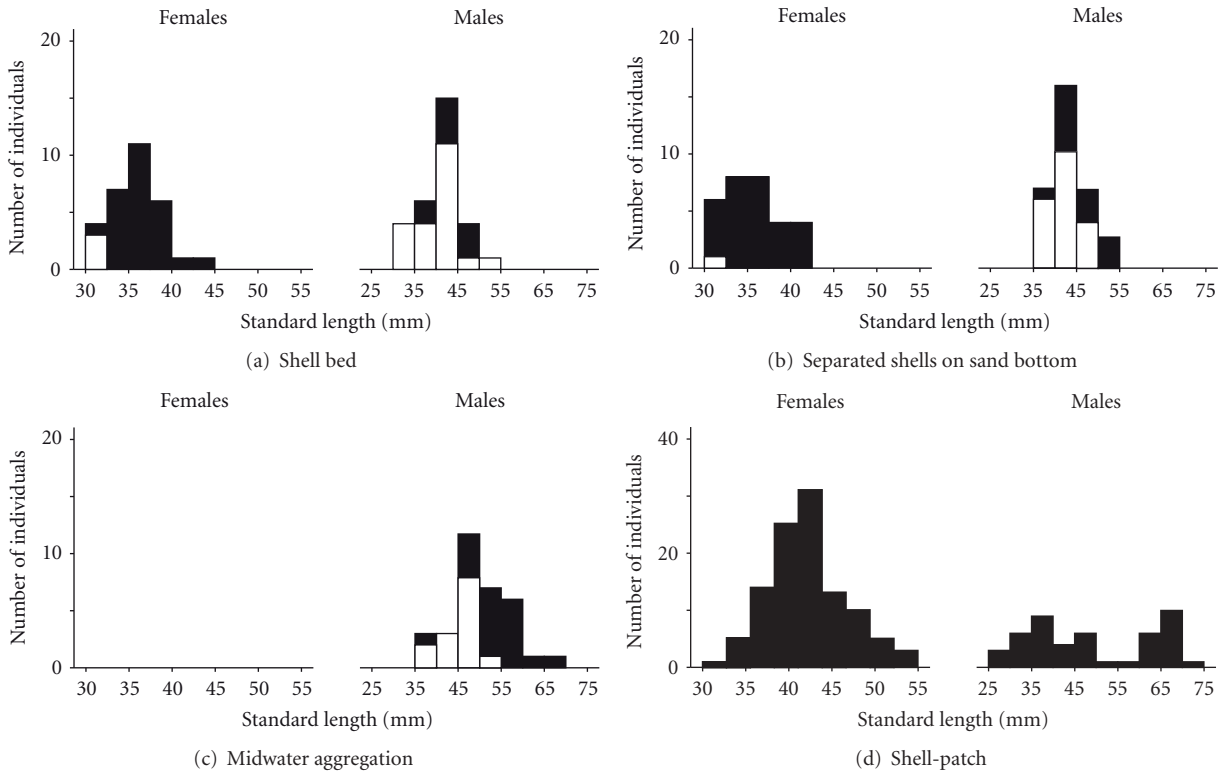

Figure 1: Body size histograms for *N. brevis* from four different habitats within the Wonzye population. Filled and blank bars indicate mature and immature individuals, respectively.

We also divided males in the shell patches into two groups based on adherence to shells as in the previous observations. Count data (the number of feeding events and attacks) were compared by use of a generalized linear model fitted to Poisson distribution models (Poisson GLMs). When the residual deviance divided by the degrees of freedom (deviance/df) of the model suggested overdispersion (deviance/df ≥ 2), we took account of this by fitting a generalized linear model to a negative binomial distribution (negative binomial GLM). We thereby succeeded in avoiding overdispersion (i.e., deviance/df < 2 for all models). Time spent in the shell was compared using the nonparametric Kruskal-Wallis test.

For all multiple comparisons (i.e., comparisons among habitats), we corrected the significant values using the Bonferroni method so as to avoid type I errors. All analyses were performed using R 2.13.0.

3. Results

3.1. Size Structure

3.1.1. Sandy-Bottom Habitats. Sixty *N. brevis* were captured in the shell beds including 30 females and 30 males. We found only one gravid female (34.5 mm SL). Of the remaining females, three were immature and 26 were mature females that had ovaries filled with unripe eggs. Sexually matured males were 30% ($N = 9$) of all males captured in the shell beds. There was no difference in SL between mature (mean ± SD = 42.9 ± 3.9 mm) and immature males (40.1 ± 4.4 mm, GLM, $F_{1,28} = 2.65$, $P = 0.12$, Figure 1(a)). Males were larger than females (mean ± SD = 35.7 ± 2.8 mm SL, $N = 30$, GLM, $F_{1,58} = 29.268$, $P < 0.001$, Figure 1(a)).

Of 63 *N. brevis* captured in the separated shells on a sandy-bottom habitat, 30 were females and 33 were males. Gravid females were 47% ($N = 14$) of all females, and the other females captured either had ovaries filled with unripe eggs ($N = 15$) or were immature ($N = 1$). Sexually mature males constituted 42% ($N = 14$) of all males captured. Mature males (mean ± SD = 45.5 ± 4.3 mm SL) were larger than immature ones (41.8 ± 3.0 mm, GLM, $F_{1,31} = 9.39$, $P = 0.007$, Figure 1(b)). Males were larger than females (mean ± SD = 35.5 ± 3.2 mm SL, $N = 30$, GLM, $F_{1,61} = 75.18$, $P < 0.001$, Figure 1(b)).

3.1.2. Rocky Habitats. Midwater aggregations mainly consisted of males (33 of 34 fish captured). Sexually mature males accounted for 58% ($N = 19$) of all males captured. The one female captured was mature and had an ovary containing unripe eggs (SL = 36.4 mm). Mature males (mean ± SD = 52.6 ± 6.3 mm SL) were larger than immature ones (45.6 ± 4.2 mm, GLM, $F_{1,31} = 12.95$, $P = 0.001$, Figure 1(c)).

Visual counting indicated that a number of *N. brevis* individuals occupied shells within the shell patches throughout the 6-week observation period (mean ± SD = 9.5 ± 1.1

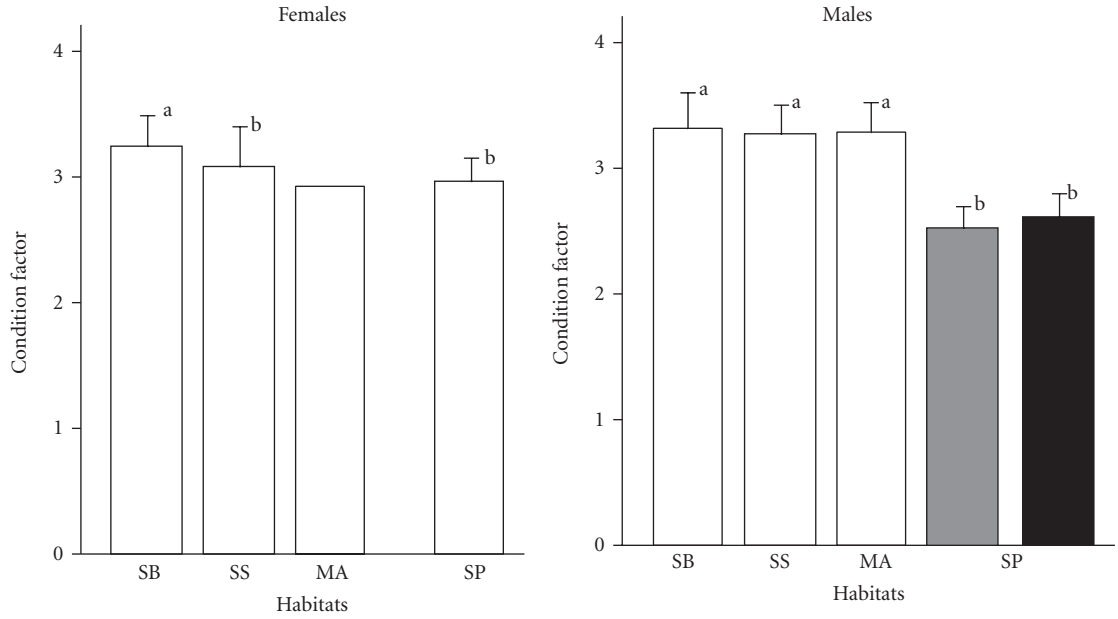

Figure 2: Comparisons of the condition factor among different habitats for each sex. SB, shell bed; SS, shells on a sandy bottom; MA, midwater aggregation in a rocky habitat; SP, shell patches in a rocky habitat. The single female in midwater aggregations was omitted from analysis. Filled and grey bars indicate males floating above nests and males in shells, respectively. Different letters beside the values indicate statistically significant differences as determined using a Bonferroni correction.

individuals/patch per week). There was no difference in the number of *N. brevis* individuals occupying shells over the 6-week observation period (Poisson mixed model, $\chi^2 = 10.87$, df = 5, $P > 0.05$), indicating that there was no spawning cycle corresponding to the lunar cycle.

Actual counting (i.e., counting after the crushing of shells) determined that a mean number of 16.1 *N. brevis* (SD = 11.3) occurred in a shell patch, with both males and females present (females: mean ± SD = 12.0 ± 9.0, males: 4.1 ± 3.2, $N = 9$ patches). Of the females, half were brood-caring females (mean number ± SD = 5.9 ± 6.5, $N = 9$ patches) and the other half were gravid females that had not yet spawned (6.1 ± 4.4, $N = 9$ patches). Of the males, 40% were floating above the patches (mean number ± SD = 1.6 ± 0.7, $N = 9$ patches), whereas 60% remained close to or in the shells (2.6 ± 2.7, $N = 9$ patches). All of the males in both locations were sexually mature.

Floating males (mean ± SD = 60.4 ± 8.3 mm SL, $N = 25$) were much larger than males residing in shells (35.2 ± 4.6 mm SL, $N = 23$, GLM, $F_{1,46} = 164.63$, $P < 0.001$), which resulted in a bimodality in the size distribution of males found in the shell patches (Figure 1(d)). Females (40.9 ± 4.0 mm SL, $N = 107$) were larger than the males in shells but were smaller than floating males (GLM, $F_{2,152} = 186.40$, $P < 0.001$, after Bonferroni correction). Of the males in shells 78% ($N = 23$) were found solely in shells, and the others were found with brood-caring females in shells. In the later cases, males remained at the shells where females had spawned ($N = 2$) were spawning ($N = 1$) or had not yet spawned ($N = 2$). In all cases, males were always found in a head-first position nearer to the entrance than females. The former "solo" males (mean ± SD = 34.3 ± 4.5 mm SL) were marginally smaller than the latter "partnered" males (38.6 ± 3.8 mm SL, GLM, $F_{1,21} = 3.65$, $P = 0.07$).

3.1.3. Comparisons among Habitats. Gravid females were more frequently found in rocky habitats (gravid : non-gravid = 107 : 1) than in sandy-bottom habitats (gravid : nongravid = 15 : 45, Fisher's exact probability test, $P < 0.001$). Likewise, the proportion of mature males was greater in rocky habitats (mature : immature = 67 : 14) than in sandy bottom habitats (mature : immature = 23 : 40, Fisher's exact probability test, $P < 0.001$). Females in the shell patches were larger than those in sandy-bottom habitats (GLM, $F_{2,164} = 40.01$, $P<0.001$, after a Bonferroni correction, Figure 1). Male size likewise differed among habitats (GLM, $F_{4,138} = 69.48$, $P < 0.001$, males floating above the shell patches > male_{MA} > male_{SB} = male_{SS} > males in a shell within the shell patches, after a Bonferroni correction, Figure 1).

Females in the shell beds were in better condition than those in other habitats (GLM, $F_{1,88} = 8.89$, $P < 0.001$, after a Bonferroni correction, Figure 2). Likewise, there was a variation in the condition factor for males among habitats: all males in the shell patches were in poorer condition compared with males in other habitats (GLM, $F_{4,138} = 75.06$, $P < 0.001$, after a Bonferroni correction, Figure 2).

There was also a great difference in testes investment (estimated by GSI) among habitats: males occupying shells within the shell patches had the greatest testes investment

Table 1: Differences in four behavior types for male *N. brevis* within and among habitats.

	Habitats				
	Sand-bottom habitats			Rocky habitats	
	Shell bed	Separated shell	Aggregation	Shell patch	
Females	($N = 7$)	($N = 3$)		($N = 14$)	
Time spent in shell (min)	0.28 ± 0.50^{a}	3.33 ± 5.77^{ab}		8.34 ± 2.82^{b}	
Number of feeding pecks	76.9 ± 35.6^{a}	73.3 ± 69.4^{a}		0.36 ± 1.33^{b}	
Number of intraspecific attacks	1.14 ± 1.57^{a}	0^{b}		0^{b}	
Number of heterospecific attacks	0.71 ± 0.76^{a}	1.33 ± 1.53^{ab}		0^{b}	
Males	($N = 12$)	($N = 19$)	($N = 23$)	($N = 22$)†	($N = 23$)‡
Time spent in shell (min)	0.09 ± 0.21^{a}	0^{a}	0^{a}	0.01 ± 0.04^{a}	4.78 ± 3.55^{b}
Number of feeding pecks	48.5 ± 25.6^{ab}	82.8 ± 100.1^{a}	57.2 ± 40.5^{a}	11.5 ± 24.7^{b}	1.3 ± 3.1^{c}
Number of intraspecific attacks	3.3 ± 4.5^{a}	2.5 ± 2.0^{a}	0^{b}	3.8 ± 3.9^{a}	0.09 ± 0.29^{b}
Number of heterospecific attacks	1.17 ± 1.64^{a}	1.58 ± 3.08^{a}	0.04 ± 0.21^{b}	0.77 ± 0.87^{a}	0.04 ± 0.21^{b}

Values are means ± SD.
Different letters beside the value indicate statistical significances determined using the Bonferroni correction.
†Males floating above shell patches.
‡Males found in shell of shell patches.

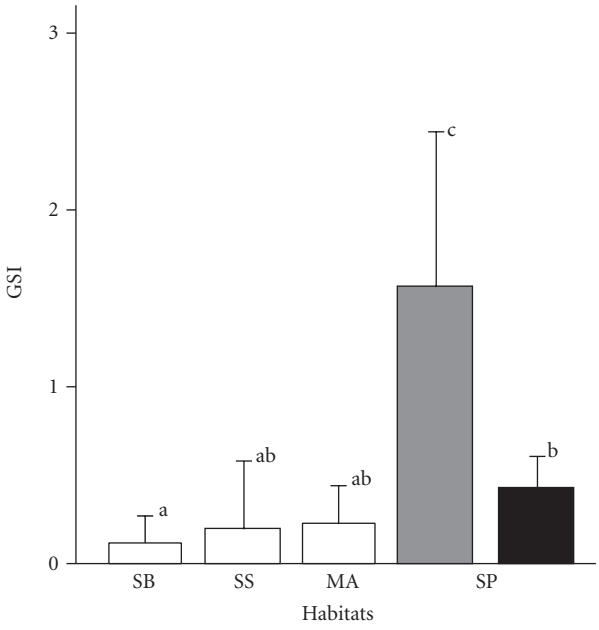

Figure 3: Comparisons of gonadosomatic index among the different habitats. SB: shell bed; SS, shells on a sandy bottom; MA: midwater aggregation in a rocky habitat; SP: shell patches in a rocky habitat. The single female in midwater aggregations was omitted from analysis. Filled and grey bars indicate males floating above nests and males in shells, respectively. Different letters beside the values indicate statistically significant differences determined using a Bonferroni correction.

among males, and males in SB had the smallest testes investment (GLM, $F_{4,139} = 49.49$, $P < 0.001$, Figure 3).

3.2. Behavioral Observations. Females in the shell patches spent more time in shells than did those in sandy-bottom habitats (Kruskal-Wallis, test, $\chi^2 = 15.16$, df = 2, $P = 0.001$, Table 1). Larger males in the shell patches seldom entered the shells, but this was not the case for the small males (Kruskal-Wallis, test, $\chi^2 = 82.58$, df = 4, $P < 0.001$, Table 1). *N. brevis* individuals usually floated a few meters above the lake bottom in midwater aggregations, whereas in sandy-bottom habitats, individuals foraged in relative close proximity to (several dozen centimeters to a few meters above) the bottom where shells used for shelter were distributed. The feeding frequency of females was lower in the shell patches than in sandy-bottom habitats (negative binomial GLM, $\chi^2 = 9.78$, df = 2, $P < 0.01$, Table 1). In accordance with this tendency of females, male feeding frequency was also lower in the shell patches than in the other habitats (negative binomial GLM, $\chi^2 = 66.51$, df = 4, $P < 0.001$, Table 1).

Aggressive interactions between *N. brevis* females occurred only in the shell beds (Poisson GLM, $\chi^2 = 19.71$, df = 2, $P < 0.001$, Table 1). Aggressive behavior toward other species was observed in sandy-bottom habitats but not in the shell patches (Poisson GLM, $\chi^2 = 16.59$, df = 2, $P < 0.001$, Table 1). Male attacks on conspecifics were much more frequently performed by males floating above the patches and males in sandy-bottom habitats than by the small males in the shell patches and midwater aggregations (negative binomial GLM, $\chi^2 = 74.22$, df = 4, $P < 0.001$, Table 1). A similar tendency was found in attacks on heterospecifics (Poisson GLM, $\chi^2 = 67.36$, df = 4, $P < 0.001$, Table 1).

4. Discussion

N. brevis obligately uses empty gastropod shells for breeding and seldom uses substrates other than shells for shelter. Therefore, the distribution of shells is expected to strongly influence the distribution of *N. brevis*. *N. brevis* was found in every habitat of the Wonzye population where shells were present. However, breeding females occurred only in the shell patches. This indicates that the breeding events of *N. brevis* exclusively take place in the shell patches for this

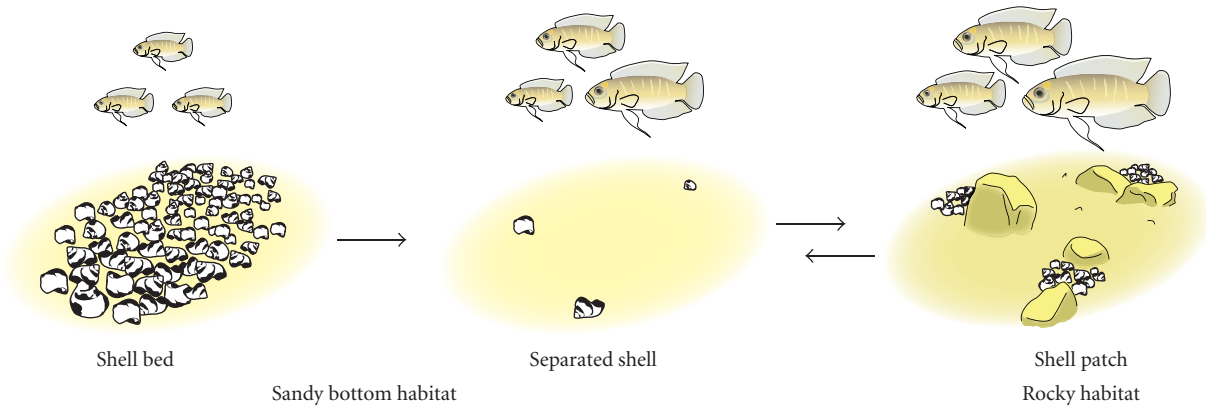

Figure 4: A schematic representation of the proposed scenario of life history in *N. brevis*. Arrows indicate the directions of fish movements.

population. In contrast, mature males and gravid females were also found in sandy-bottom habitats, although much less frequently than in rocky habitats. Both sexes, with the exception of the small males in the shell patches, were smaller in sandy-bottom habitats than in rocky habitats. This suggests that *N. brevis* spend their early life-history stages in sandy-bottom habitats and migrate to shell patches for reproduction, possibly through shells distributed on the sand bottom between the shell bed and rocky habitats (Figure 4).

Such movements among habitats are often found in fish (e.g., [41, 42]) and are favored if the benefits exceed the costs [43–45]. In *N. brevis*, the shell patches may be a favorable habitat for breeding compared with other habitats. Shell patches contained a number of conspecific and heterospecific females in the Wonzye population (this study and [25]), and these patches likely enable more effective avoidance of egg predation through dilution effects [46] than would breeding in sandy-bottom habitats. However, the shell patches may be unsuitable for later growth and survival compared with other habitats. The complex structures of rocks, for example, would disturb food delivery (plankton drifting in the water column) to the shell patches. Consequently for feeding, *N. brevis* would be required to float in the midwater column, where the fish would be exposed to predation risk by piscivorous (e.g., *Lepidiolamprologus* spp. and *Lamprologus* spp.) and scale-eating (*Perissodus* spp.) fish. In contrast, sandy-bottom habitats may provide reduced predation risk and high food delivery near shelters. In these habitats, *N. brevis* can feed in close proximity to shells because there are no rocks disturbing food delivery, and individuals could hide in the shells immediately in response to the perceived risk of predation. Indeed, we found that *N. brevis* in the shell patches infrequently fed on plankton and consequently had poorer somatic condition compared with *N. brevis* individuals in other habitats. This suggests that sandy bottom habitats and midwater aggregations may be favored for *N. brevis* to invest in somatic growth. We therefore hypothesize that *N. brevis* chooses favorable habitats according to the stage of the life history. However, there is another possible explanation for the movements among habitats. In Wonzye, movements from shell-patches might be inevitable. *N. brevis* females who had finished broodcaring would be obstacle, because they would no longer yield any profits for *L. callipterus* (such as avoidance of egg predation through dilution effect, see above). If so, *L. callipterus* would expel them so as to secure shells for their own breeding. Then, *N. brevis* individuals would be forced to migrate to near other habitats, that is, separated shells on sandy bottom. These hypotheses are not mutually exclusive, and it is therefore possible that both factors are responsible for movements among habitats.

Several males were found within the shell patches at the same time. These males were divided into two types based on morphological and behavioral phenotypes that were clearly differentiated. Males floating above the shell patches were larger in body size and exhibited relatively frequent aggressive behavior toward conspecifics. Males who usually stayed close to or in the shells were smaller and less aggressive. These discontinuous differences in phenotypes are found in the related shell-brooding cichlids with ARTs, *L. callipterus* [3, 26, 37], and *T. vittatus* [30]. In *L. callipterus*, there are two types of reproductively parasitic males: "sneaker" males dart toward the shell where spawning takes place and ejaculate from the entrance of the shell and "dwarf" males wriggle past spawning females and take up residence behind them so as to ejaculate in close proximity to females [37]. In *T. vittatus*, sneaker males get inside the shell for several seconds or longer and ejaculate, and they then leave the shell immediately [30]. In *N. brevis*, most sexually mature small males were found solely in shells where spawning had not yet taken place. Although these males may be simply hiding in the shells, it is possible that they may have a sit-and-wait tactic for "sneaking" in which they lie in wait for a female to spawn [37]. Because the sit-and-wait tactic enables *N. brevis* males to position themselves behind females, smaller bodies are required to succeed with this approach. Indeed, these males were marginally smaller than the males that remained in shells with females. We also found several males entering the shells after females, some of which had not yet mated. Additionally, small male *N. brevis* individuals were found behind females in shells (i.e., in the innermost parts of

the shells, [34]). This indicates the presence of wriggling "sneaking" by small male *N. brevis*. Together, these observations indicate that small mature male *N. brevis* may have a wide range of parasitic "sneaky" behavior types so as to steal fertilization opportunities.

If the small males really do employ parasitic tactics, their ejaculation should usually occur during mating by territorial males. In this situation, theory predicts that their testes investment is greater than territorial conspecifics [8]. This was the case in *N. brevis* as well as other Lake Tanganyika cichlids employing ARTs [18, 21, 22, 26, 28, 30, 37], but some controversy about this remains (see [47]). Testes investment among the floating males was the same as for males found in the midwater and sandy-bottom habitats and was much smaller than that of males within the shells of the shell patches. These findings are interpreted as demonstrating the presence of ARTs, although we did not directly observe the spawning behavior of *N. brevis*.

Several territorial males were found within some of the shell patches. Together with the finding of a number of females in the shell patches, this suggests that *N. brevis* exhibits multimale polygyny [48], in accordance with a previous study [25]. This mating system differed from that of other shell brooders found in the shell patches: both *L. callipterus* [37] and *T. vittatus* [30] are polygynous. The hierarchy among these species and the reason they share a shell patch remain to be investigated. Further studies that include paternity analysis are needed to clarify the mating system of *N. brevis*.

In conclusion, the results strongly suggest that the mating system of *N. brevis* is characterized by movements among habitats and multimale polygyny with ARTs. ARTs seem to be a common attribute in the Lake Tanganyika cichlid tribe Lamprologini, particularly in shell brooders. It is possible that the specific shape of the empty gastropod shells is at least partly responsible for this universality. Several studies have suggested that the presence of a space in a breeding substrate that large bourgeois males cannot enter is an important factor allowing "sneakers" to gain access to females [23, 28, 37]. As the entrance of the shells is very small (3.3 cm^2 on average in Wonzye), entering the shells requires specialized morphologies (e.g., small body size [27, 37]). However, territorial males are selected for large size because of the intense male-male competition and, particularly in case of *L. callipterus*, the requirements of shell transportation (e.g., [49–51]). This difference in optimal body size between tactics leads to biased access to the shells: "sneakers" can penetrate the spawning substrate to a depth that territorial males cannot reach. In accordance with this pattern, in *N. brevis*, smaller males entered shells, whereas large territorial males seldom entered. We therefore assume that the shape of a breeding substrate is one of the key factors leading to the prevalence of ARTs among the Lake Tanganyika shell-brooding cichlids.

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Research Article

Deep Phylogenetic Divergence and Lack of Taxonomic Concordance in Species of *Astronotus* (Cichlidae)

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The neotropical cichlid genus *Astronotus* currently comprises two valid species: *A. ocellatus* Agassiz, 1831 and *A. crassipinnis* Heckel, 1840. The diagnosis is based on color pattern and meristics counts. However, body color pattern is highly variable between regions and the meristic counts show a considerable overlap between populations differing in color patterning. They do not represent true synapomorphies that diagnose species. Purportedly the only truly diagnostic character is the presence or absence of one or more ocelli at the base of the dorsal fin, diagnosing *A. ocellatus* and *A. crassipinnis*, respectively. Using the 5′ portion of the mitochondrial COI gene and EPIC nuclear markers, the validity of the dorsal ocelli as diagnostic character was tested in individuals sampled from ten localities in the Amazon basin. Analyses rejected the hypothesis that dorsal ocelli are diagnostic at the species level. However, they revealed the existence of five hypothetical, largely allopatrically distributed morphologically cryptic species. The phylogeographic structure is not necessarily surprising, since species of the genus *Astronotus* have sedentary and territorial habits with low dispersal potential. The distribution of these hypothetical species is coincident with patterns observed in other Amazonian aquatic fauna, suggesting the role of common historical processes in generating current biodiversity patterns.

1. Introduction

The neotropical cichlid genus *Astronotus* currently comprises two valid species: *A. ocellatus* Agassiz, 1831 and *A. crassipinnis* Heckel, 1840 [1]. Kullander [1] reports a number of diagnostic characters, however, with the exception of the presence of ocelli at the base of the dorsal fin in *A. ocellatus* and their absence in *A. crassipinnis*, all other characters show considerable overlap in their statistical distributions. The two species are characterized by differences in the modal number of lateral line scales (35 to 40 in *A. crassipinnis* versus. 33 to 39 in *A. ocellatus*), and the number of rays and spines of the dorsal fin (modal XIII.20 in *A. ocellatus* versus. modal XII.21-22 in *A. crassipinnis*). There are also reported differences in color hue and patterning where *A. crassipinnis* is darker than *A. ocellatus*, the first light vertical bar is above the anal fin base in *A. ocellatus* versus more anteriorly in *A. crassipinnis*, and *A. crassipinnis* has two more or less well-separated dark vertical bars in the position of the first light bar in *A. ocellatus*. Although proposed as diagnostic characters, the position of the vertical bars and body color appears highly variable between localities and individuals (authors' obs.), and the meristic counts are not truly diagnostic (are not synapomorphies) since they represent modal values and overlap between species.

While the presence of ocelli on the dorsal fin is considered a diagnostic character of *A. ocellatus*, Kullander ([1]; see http://www2.nrm.se/ve/pisces/acara/astronot.shtml), only individuals from Peru were analyzed by Kullander [1] in his reanalysis of the genus. Moreover, Kullander [1] raises the possibility that ocelli are unique to specimens of western Amazonia, requiring a possible reinstatement or reclassification of species considered synonyms of *A. ocellatus*. The geographic distribution of *A. ocellatus* spans the whole Amazon basin and the Oyapock and Approuague drainages. It does not include the Bolivian basin which is a subbasin of the Amazon basin.

The quantity and size of ocelli further appear to be influenced by reproductive state. In a study by Queiroz and Barcelos [2] of *Astronotus ocellatus* (diagnosed as such by the presence of ocelli) from the Mamirauá Sustainable Development Reserve located in the western Amazon north of the city of Tefé, the authors demonstrated that the number of ocelli and their size are positively and linearly correlated with gonadal development in both males and females. These potential difficulties do not prevent, however, the common acceptance of ocelli as strictly diagnostic character of the two species (e.g., [3]).

Of the type series of *A. crassipinnis*, only two syntypes from the Guaporé River are known. Other type material reported from the Negro and Branco Rivers according to Kullander [1] likely represents *A. ocellatus* or some undescribed species. *Astronotus crassipinnis* is therefore restricted to the upper Paraguay River and the Bolivian Amazon including the Guaporé, Mamoré, and Madre de Dios rivers. However, pending designation of a lectotype from the Guaporé River, Kullander [1] considers the classification of Paraguayan and Bolivian Amazonian specimens as *A. crassipinnis* provisory. Kullander [1] also recognizes that *A. ocellatus* could be restricted to the western Amazon and that *Astronotus ocellatus* var. *zebra* Pellegrin, 1904 and *Astronotus orbiculatus* Haseman, 1911 both described from Santarem and currently considered junior synonyms of *A. ocellatus* could represent valid species or may be synonyms of *A. crassipinnis*. Kullander [1, 4] further mentions the existence of an *Astronotus* species from the Orinoco basin but does not recommend any kind of classification of these specimens.

Phenotypic variation of *A. ocellatus* at the scale of the Amazon basin would not be surprising given the extent of geographic distribution of the species and the biology of cichlids. Both species of the genus *Astronotus* inhabiting lentic environments are sedentary. Males have strong territorial behavior, and both sexes build nests and exhibit parental care. First gonadal maturation occurs between 15 and 24 months, and reproduction may occur more than once a year. Both species are also relatively large for fishes of the family Cichlidae (up to 35 cm SL and 1.5 kg). The geographic distribution of species of *Astronotus* as well as the species themselves may therefore carry signatures of climatic and geological events.

While phenotypic variation is evident in the species of *Astronotus*, it is not clear if the currently used sets of characters are fully diagnostic. An alternative approach to species diagnosis may be through the use of DNA barcoding [5]. DNA barcoding has rapidly expanded in the last years, and already the fish faunas of several countries have been barcoded (e.g., [6–9]). One of the objectives of the DNA barcoding initiative is to generate a curated database of reference material. The usefulness of this database depends on the quality of the reference specimens and the quality of the underlying taxonomic information. For example, recently diverged species may share DNA barcodes (COI haplotypes), or multiple species may be subsumed within the same morphospecies, and both cases will lower the quality of the database. Identifying these instances is the first step in generating a reliable biodiversity database.

Table 1: Number of *Astronotus* specimens sampled at each site. We have no information about the phenotype (*Astronotus ocellatus*/*Astronotus crassipinnis*, presence/absence of dorsal ocelli, resp.) for specimens identified as *Astronotus* sp., but, in each of the Careiro do Castanho and Araguari River localities, both species of *Astronotus* occurred, were sampled, and were included in the analyses.

Localities	Specimen identification			All
	A. crassipinnis	*A. ocellatus*	*Astronotus* sp.	
Tabatinga		4		4
Tefé/Mamirauá		4		4
Eirunepé		4		4
Guajará-Mirim	5			5
Borba	5	3		8
Barcelos		10		10
Sta Isabel do rio Negro		3		3
Careiro do Castanho			6	6
Oriximiná	3	2		5
Araguari river			8	8
Total	13	30	14	57

Many neotropical fish species have broad geographic distributions, often occurring allopatrically in the tributaries of the Amazon River, or are even shared between the Amazon and other South American basins (see [10]). While some species truly appear to be biological species with weak or nearly nonexistent population structuring across its distributional range (e.g., [11–14]), others probably comprise morphologically cryptic species complexes, recently diverged groups, or complexes of hybridizing groups (e.g., [15–18]).

The goal of this study was to assess population structuring and reassess the taxonomy of the genus *Astronotus* based on an analysis of molecular data and assess the utility of a traditionally used diagnostic character for the species *A. ocellatus* and *A. crassipinnis*.

2. Material and Methods

2.1. Sampling. Tissue samples (dorsal muscle or pectoral fins) were collected from specimens purchased directly from artisanal fishermen and from fishes sampled with 50 mm mesh gillnets. The tissues were deposited in the tissue collection of the Laboratory of Animal Genetics and Evolution, Federal University of Amazonas. Most individuals were photographed, and vouchers are being deposited at the ichthyological collection of the Instituto Nacional de Pesquisas da Amazonia (INPA).

We sampled 10 localities in the Amazon basin (Figure 1), and individuals were classified as *A. ocellatus* or *A. crassipinnis* based on the presence/absence of at least one ocellus or dark spot on the posterior part of the dorsal fin (Table 1). We do not have exact information about the state of ocelli for the Tabatinga and Mamirauá/Tefé specimens; however, based on field identification, fishes from Tabatinga and Mamirauá/Tefé were classified as *A. ocellatus*. Several studies [2, 19] also only report *A. ocellatus* from Mamirauá. Similarly although the presence/absence of ocelli was not recorded for

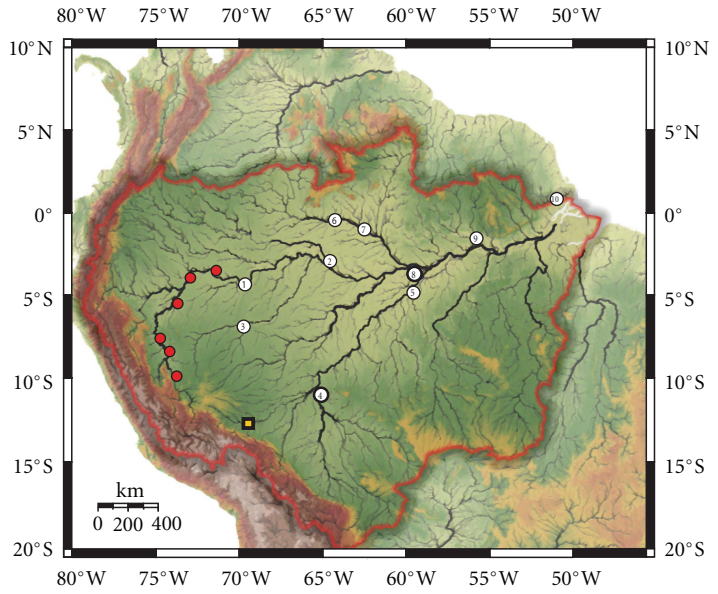

Figure 1: Sampling localities of species of *Astronotus* in the Brazilian Amazon. Base map was obtained from WWF (http://assets.panda.org/img/original/hydrosheds_amazon_large.jpg). Numbers correspond to sampling localities: (1) Tabatinga; (2) Mamirauá; (3) Juruá; (4) Guajará Mirim; (5) Borba; (6) Santa Isabel; (7) Barcelos; (8) Careiro Castanho; (9) Oriximiná; (10) Araguari. Red circles and yellow squares are localities of *A. ocellatus* and *A. crassipinnis*, respectively, studied by Kullander [1]. Reddish-brown line delimits the periphery of the Amazon basin.

Figure 2: Photograph of fishes of the genus *Astronotus* collected in the Araguari River and showing the presence and absence of dorsal ocelli in the same locality. In addition to the Araguari locality, both *A. ocellatus* and *A. crassipinnis* phenotypes were collected in Oriximiná, Careiro Castanho, and Borba. Photo by S. C. Willis.

individual specimens at the time of collection at the localities of Careiro do Castanho and the Araguari River, both *A. ocellatus* and *A. crassipinnis* phenotypes were observed and sampled (Figure 2).

2.2. Polymerase Chain Reaction (PCR) and Sequencing. We amplified and sequenced one mitochondrial and two nuclear gene regions. All PCR reactions were carried out in a final volume of 15 μL containing 7.0 μL of ddH_2O, 1.5 μL of $MgCl_2$ (25 mM), 1.5 μL of dNTPs (10 mM), 1.2 μL of 10x PCR buffer (100 mM Tris-HCl, 500 mM KCl), 1.2 μL of each primer (2 μM), 0.3 μL of Taq DNA Polymerase (1 U/μL), and 1 μL of DNA (concentration varied between 50 ng and 100 ng). We amplified the COI barcode region with the primers COIFishF.2 (5′-CGACTAATCATAAAGATATCGGCAC-3′) and COIFishR.1 (5′-TTCAGGGTGACCGAAGAATCAGAA-3′), and the EPIC region primers 18049E2 (18049E2f2—5′-GTGGTGGAGATGCAYGAYGTGAC-3′; 18049E2r2—5′-TAGTAAAGGTCYCCRTGGATGGTGAG-3′), and 14867E4 (14867E4f2—5′-TGTGATCAGGGGACAGAGRAAAGGTG-3′; 14867E4r2—5′-CAGTARATGAACTGBCCGGTGTGG-3′) obtained from the online supplement of Li and Riethoven [20]. PCR reaction consisted of 35 cycles of denaturation at 93°C for 5 seconds, primer annealing at 50°C; 50°C and 56°C, respectively, for 35 seconds, and primer extension at 72°C for 90 seconds, followed by a final extension at 72°C for 5 minutes. PCR products were purified using the polyethylene glycol/ethanol precipitation [21] and subjected to cycle sequencing reaction using both amplification primers following the manufacturer's recommended protocol for BigDye sequencing chemistry (Applied Biosystems). Subsequent to the cycle sequencing reaction, the products were precipitated with 100% ethanol/125 mM EDTA solution, resuspended in Hi-Di formamide, and resolved on an ABI 3130xl automatic sequencer (Applied Biosystems). Base calls were verified by viewing electropherograms in the program Bioedit [22], sequences were aligned in the program Clustal W [23], and alignment was verified by eye. Sequences of nuclear genes were separated into alleles prior to analyses. Sequences were deposited in Genbank (JQ965997-JQ966020).

2.3. DNA Barcode Analysis (COI mtDNA). Genetic distances between individuals were calculated using the JC69 model of molecular evolution [24], and individuals were clustered using the BIONJ algorithm [25]. The analyses

were implemented in the online version of the ABGD software [26] whose objective is to automatically and in an unbiased way delimit clades. Clade delimitation was done assuming a range of possible intraclade θs from 0.001 to 0.1. Once clades were identified, we also estimated average divergences between and within clades using the JC69 model of molecular evolution [24] in the program MEGA 5 [27]. Although the K2P model of molecular evolution [28] is the recommended [29] and has become the *defacto* model in DNA barcoding studies, it poorly fits the data at the species level divergence [30]. Collins et al. [30] recommend the use of uncorrected divergences or simplest models possible. Further, intraspecific divergences—employed in DNA barcoding threshold and barcoding gap methods, and pairwise divergences between sister taxa—employed in DNA barcoding gap methods, normally need no correction for multiple mutational hits and saturation due to their inherently shallow phylogenetic divergences.

We also performed an individual level Population Aggregation Analysis (PAA) [31] to identify clades. In the DNA barcoding literature, the use of molecular synapomorphies to delimit clades has been described by Rach et al. [32] under the acronym CAOS.

2.4. Phylogenetic Inference and Hypothesis Testing. Maximum likelihood topology for the mtDNA dataset was inferred in the program Treefinder [33], and the robustness of the tree topology was assessed using the nonparametric bootstrap with 1,000 replicates. The most appropriate model of molecular evolution for the mtDNA dataset was inferred as HKY85 [34] with a portion of the sites considered invariable in the program Treefinder [33]. Model selection criterion was the corrected Akaike Information Criterion [35]. Association of lineages and phenotypes was tested by comparing the constrained topology (phenotypes are monophyletic) with the most likely unconstrained topology. Significance was tested using the approximately unbiased test of Shimodaira [36]. A test of phylogenetic distribution of ocelli was performed using the CAPER package [37] in the statistical program R (http://www.cran.r-project.org/). A test of genetic structuring at nuclear loci, assuming the existence of groups identified in the ABGD [26] analysis of the COI barcode region, was performed in the software Arlequin 3.5.1 [38].

2.5. Phylogenetic Networks. Due to the low number of variable sites, phylogenetic relationships of nuclear haplotypes were inferred as a haplotype network using the PEGAS package [39] in the statistical program R (http://www.cran.r-project.org/).

3. Results

We sequence data for one mitochondrial and two nuclear DNA regions. We collected 664 bp of the mtDNA COI barcode region, representing 19 haplotypes separated by 31 mutations. No stop codons were observed in the COI barcode region. We also collected 397 bp of the nDNA 18049E2 EPIC regions, representing three haplotypes separated by three mutations. We further collected 248 bp of the nDNA 14867E4 EPIC region, resulting in two haplotypes separated by one mutation.

Table 2: Mean intra- and interspecific distances and their standard errors estimated between COI haplotypes using the Jukes Cantor model of molecular evolution [24]. Hypothetical species were inferred using the ABGD [26] algorithm.

Average divergence between groups (below diagonal), and associated standard errors (above diagonal)					
	East	West	Bolivia	Jurua	Negro
East		0.56%	0.36%	0.55%	0.36%
West	2.17%		0.55%	0.33%	0.57%
Bolivia	0.98%	2.20%		0.57%	0.42%
Jurua	2.08%	0.86%	2.42%		0.49%
Negro	0.97%	2.20%	1.31%	1.80%	
Average divergence within groups (left column), and standard errors (right column)					
East			0.03%		0.02%
West			0.06%		0.03%
Bolivia			0.10%		0.07%
Jurua			0.13%		0.12%
Negro			0.09%		0.05%

Using the ABGD software, we were able to infer five clades potentially representing species. Minimal divergence between these clades is 0.9% (Table 2). Individuals from all localities but Borba, a locality in the lower Madeira River, belong to just one clade. In the case of Borba, one individual is part of a clade that otherwise has a distribution in the Bolivian basin (upper Madeira River), while the remaining individuals are members of a clade found in the western Amazon basin. All five groups, with the exception of the Jurua group, are supported by at least one molecular synapomorphy (Table 3). For the sake of convenience, these clades will be referred to as East, Bolivia, Negro, West, and Jurua groups (Figure 3).

The 18049E2 nDNA gene was represented by three haplotypes (Figure 4), with the most common haplotype being present in all localities but Tabatinga-western-most locality of the West clade, the second most common haplotype not occurring in the Negro River and upper Madeira River, corresponding to the Negro and Bolivia groups, and the third haplotype being restricted to the upper Madeira River—Bolivia group. The 14867E4 nDNA gene was represented by only two haplotypes (Figure 5), one common haplotype not found in western localities corresponding to the West and Jurua groups and another restricted to the central Amazonian localities. Both nDNA gene regions show strong structuring, that is, alleles are not randomly distributed among the five groups identified in ABGD analysis. Analysis of molecular variance of the 18049E2 nDNA gene was significant ($F_{ST} = 0.4163$, $P < 0.001$) as was that of the 14867E4 nDNA gene ($F_{ST} = 0.8099$, $P < 0.001$).

Ocelli were not phylogenetically clustered (Figure 3). A constrained topology where individuals with and without ocelli were forced into reciprocal monophyly, that is an

Table 3: Matrix of molecular synapomorphies of the hypothetical species inferred using the ABGD [26] algorithm. Molecular synapomorphies are in bold. Column numbers indicate position within the sequenced COI fragment.

	89	98	131	143	152	209	215	227	236	248	260	305	443	447	464	539	578	590	596	662
East	G	C	G	G	T	T	C	A	A	T	T	C	T	C	A	A	**A**	T	C	T
Bolivia	G	C	G	A	T	T	C	A	A	T	C	C	T	C	A	**G**	G	T	**A**	**C**
Negro	**A**	C	G	G	T	T	C	G	A	C	C	T	T	C	A	A	G	T	C	T
West	G	T	**T**	A	C	C	A	A	G	T	C	T	C	**T**	G	A	G	A	C	T
Jurua	G	T	G	A	C	C	A	G	G	C	T	T	C	C	G	A	G	A	C	T

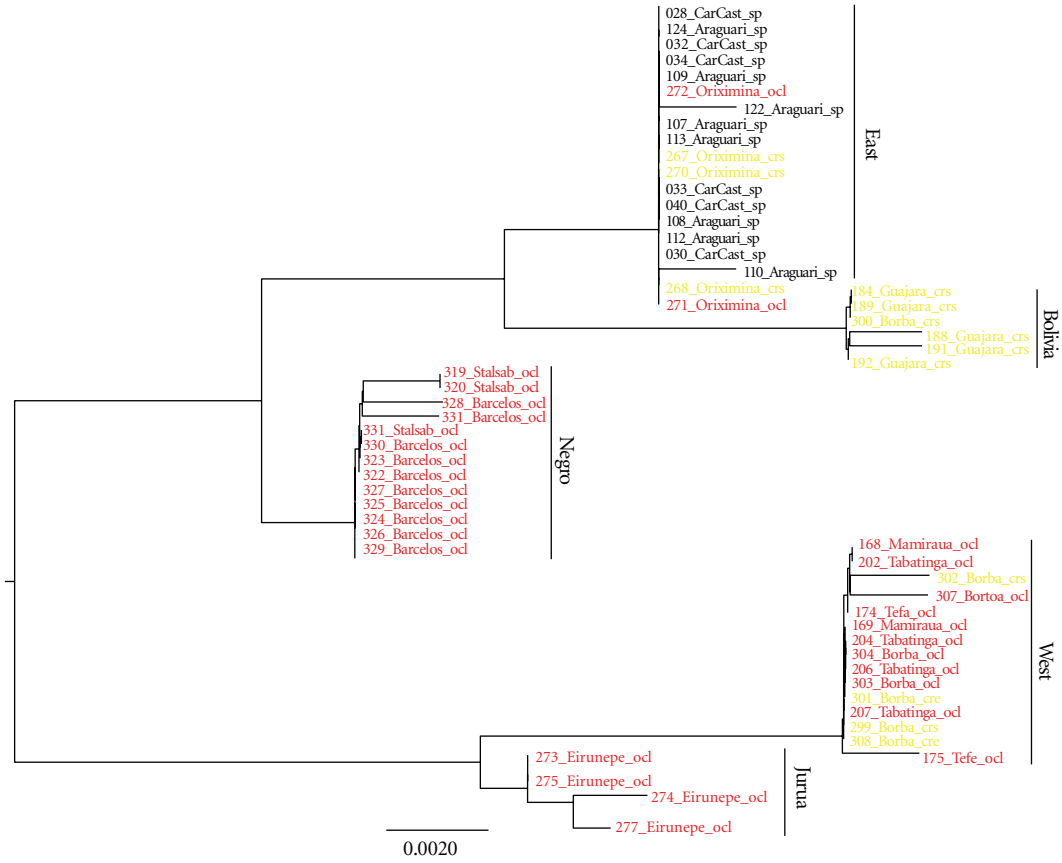

Figure 3: Maximum likelihood phylogenetic hypothesis ($-\ln = 1166.583$) of relationships of individuals of *Astronotus* sampled throughout Brazilian Amazônia based on the mtDNA COI barcode region. The topology is significantly different ($P = 0.003$) from constrained topology enforcing monophyly of *A. ocellatus* and *A. crassipinnis*. red—*A. ocellatus* (ocelli present); yellow—*A. crassipinnis* (ocelli absent); black—unknown.

explicit phylogenetic test of the usefulness of the presence/absence of ocelli as a diagnostic character, resulted in a significantly less likely topology ($P = 0.003$) and thus a rejection of the null hypothesis. However, analyses in CAPER indicated that ocelli were not distributed randomly across the ML topology (Fritz and Purvis' $D = 0.3862$, $P < 0.001$) but also were not clumped ($P = 0.021$).

4. Discussion

DNA barcode analyses revealed five, largely geographically restricted clades. Each clade with the exception of the Jurua group was supported by at least one molecular synapomorphy in the mtDNA dataset. While having less phylogenetic information, patterns of geographic distribution of nuclear DNA haplotype distribution did not contradict the mtDNA results and supported certain phylogeographic divisions observed in the mtDNA phylogeny. The Bolivia group had a private allele of the 18049E2 nDNA gene, while the second most common haplotype of this gene was absent in the Bolivia and Negro groups. Of the two 14867E4 nDNA alleles, the more common allele do not occur in the West and Jurua groups, while the rarer allele occurred infrequently in the group East.

The five groups predicted with Automatic Barcode Gap Discovery (ABGD) [26] and supported by the analyses of nuclear DNA loci can be taken as a first set of species hypotheses that need to be tested with other data. The algorithm is based on the statistical properties of the coalescent, and baring recent radiations, will identify evolutionary entities compatible with the coalescent. Other methods of

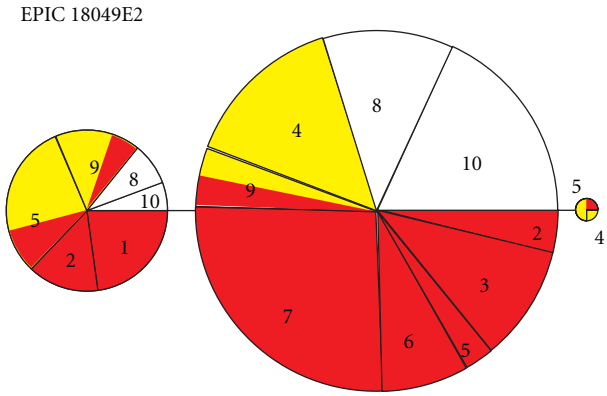

Figure 4: Haplotype network of the E0PIC 18049E2 nDNA region. Colors correspond to phenotypes: red—*A. ocellatus* (ocelli present); yellow—*A. crassipinnis* (ocelli absent); white—unknown. Numbers correspond to sampling localities: (1) Tabatinga; (2) Mamirauá; (3) Juruá; (4) Guajará Mirim; (5) Borba; (6) Santa Isabel; (7) Barcelos; (8) Careiro Castanho; (9) Oriximiná; (10) Araguari.

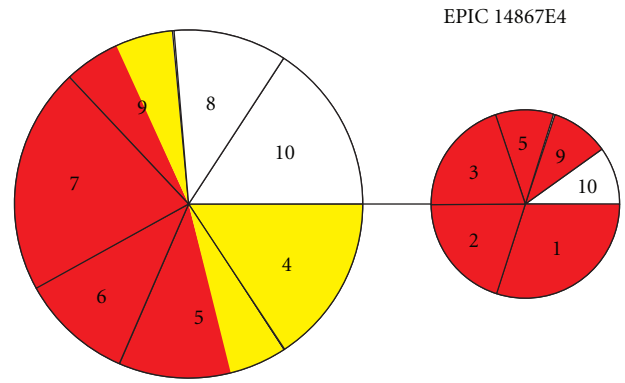

Figure 5: Haplotype network of the EPIC 14867E4 nDNA region. Colors correspond to phenotypes: red—*A. ocellatus* (ocelli present); yellow—*A. crassipinnis* (ocelli absent); white—unknown. Numbers correspond to sampling localities: (1) Tabatinga; (2) Mamirauá; (3) Juruá; (4) Guajará Mirim; (5) Borba; (6) Santa Isabel; (7) Barcelos; (8) Careiro Castanho; (9) Oriximiná; (10) Araguari.

identifying species from DNA barcode data are generally subjective or not generalizable across a broad range of organisms. The commonly used criterion of delimiting species such as the 3% interspecific divergence criterion, DNA barcodes differing by more than 3% belonging to different taxa [40], or the 10x rule, interspecific divergences that are 10x or larger than intraspecific divergences [41], fails to generalize for a number of taxonomic groups (e.g., [15, 42, 43]). Similarly, the interspecific and intraspecific divergences often overlap among closely related taxa (e.g., [15, 44–46]).

While it is clear that clades identified by ABGD [26] as potential species are geographically structured, the same cannot be said of the presence/absence of ocelli. Ocelli are not randomly distributed on the mtDNA phylogeny nor the nDNA haplotype networks; however, they also do not form monophyletic groups. Individuals of the Bolivia group do not have dorsal ocelli, while dorsal ocelli characterize all individuals of the Negro and Jurua groups. With the exception of individuals from the Borba locality, all other individuals pertaining to the group West are also characterized by the presence of ocelli. The group East is, on the other hand, characterized by a mix of individuals exhibiting both phenotypes (Figures 2 and 3). It should be noted that the Borba locality in the lower Madeira River is geographically intermediate between the Bolivia and the East groups. Thus, while some groups are monomorphic with respect to the presence/absence of ocelli, this character is not diagnostic and cannot be used to delimit species. Thus, currently, there are no morphological characters that can be used to diagnose and delimit species of *Astronotus*. On the other hand, ocelli are not randomly distributed throughout the phylogeny and do retain some phylogenetic information. In effect, specimens sampled from the vicinity of the main stream of the Amazon River (groups East and West) show both phenotypes, while specimens sampled from major affluents show either one or the other phenotype.

Broadly, however, the biodiversity patterns observed in the genus *Astronotus* are consistent with Kullander's [1] analysis. The group Bolivia is likely to be *Astronotus crassipinnis,* and one of its characteristics is lack of dorsal ocelli. What is currently considered *Astronotus ocellatus* harbors multiple species, a possibility also raised by Kullander, and while not diagnostic, specimens in the western Amazon basin have ocellated dorsal fin. Additional potential species currently subsumed under *A. ocellatus* include the groups from Jurua and Negro Rivers and from the central and eastern Amazon (group East).

The strong phylogeographic structure and the discovery of potentially new species of *Astronotus* are not necessarily surprising. *Astronotus* species are sedentary and territorial, have low power of dispersion, and therefore are likely to be influenced by climatic and geomorphological events. Perhaps the most interesting observation is that the division between the group East and West (not considering the Borba locality) parallels the division between the cichlid fishes *Symphysodon* sp. 2 (phenotype blue) and *Symphysodon tarzoo* (phenotype green) [16, 17, 47]. Also intriguing is that all but one specimen from the Borba locality in the lower Madeira River share haplotypes with the group West, which again parallels haplotype sharing between lower Madeira River and western Amazon observed by Ready et al. [47] in *Symphysodon*. The differentiation of the Bolivia group from all other *Astronotus* is potentially explained by the presence of the series of rapids on the Madeira River. These series of rapids are thought to delimit the geographic distributions of such diverse taxa as *Inia geoffrensis* and *I. boliviensis* [48, 49], *Cichla monoculus* and *C. pleiozona* [50], or they act as barriers, restricting gene flow in *Colossoma macropomum* [13] and *Podocnemis expansa* [51]. The physiochemical composition of the Negro River has also been suggested to act as a barrier between and within species [16, 17, 52, 53]. The patterns observed in *Astronotus* are likely to be general, implying that multiple additional species in broadly distributed Amazonian taxa are almost inevitably to be discovered.

Acknowledgments

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Review Article

An Evaluation of the Role of Sensory Drive in the Evolution of Lake Malawi Cichlid Fishes

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Although the cichlids of Lake Malawi are an important model system for the study of sensory evolution and sexual selection, the evolutionary processes linking these two phenomena remain unclear. Prior works have proposed that evolutionary divergence is driven by sensory drive, particularly as it applies to the visual system. While evidence suggests that sensory drive has played a role in the speciation of Lake Victoria cichlids, the findings from several lines of research on cichlids of Lake Malawi are not consistent with the primary tenets of this hypothesis. More specifically, three observations make the sensory drive model implausible in Malawi: (i) a lack of environmental constraint due to a broad and intense ambient light spectrum in species rich littoral habitats, (ii) pronounced variation in receiver sensory characteristics, and (iii) pronounced variability in male courtship signal characteristics. In the following work, we synthesize the results from recent studies to draw attention to the importance of sensory variation in cichlid evolution and speciation, and we suggest possible avenues of future research.

1. Introduction

The cichlid faunas of the east African rift lakes encompass some of the most species-rich extant vertebrate radiations [1]. Amongst the flocks from Lakes Malawi, Tanganyika, and Victoria, the radiation in Lake Malawi is the largest and most diverse in terms of species number [2]. Given the young age of the radiation (~1-2 mya, [3, 4]), Malawi cichlids are a valuable system for the study of both rapid speciation and niche evolution [5]. Albertson et al. [6] first laid out a model for species radiation in three stages: (i) habitat diversification, (ii) trophic diversification, and (iii) sexual selection. Sexual selection is likely a primary mechanism for speciation within genera, as is evidenced by clear differences in male breeding color displayed by ecologically similar congeners. Many studies have demonstrated that females can distinguish between conspecific and congeneric males based on nuptial coloration alone [7, 8], although cues from other sensory modalities are likely in play as well [9–11].

Despite the apparent importance of sexual selection in the radiation of Malawi cichlids, the evolutionary processes underlying diversification in female sensory sensitivities and mate preferences are as yet unknown. Given current knowledge regarding cichlid sensory systems [12–14], courtship signal structure [9, 15–17], and female behaviors [7, 18, 19], the experimental data is available to critically evaluate models of signal diversification and mate choice. In the following paper, we will synthesize contemporary cichlid research, particularly as it applies to cichlid visual systems in Lake Malawi. Furthermore, we will discuss some potential mechanisms underlying sexual selection in Lake Malawi.

2. Sensory Drive and the Stages of Communication

A linkage between sensory evolution and male courtship signals was eloquently laid out by Ryan and Rand [20] in their sensory exploitation model. This model was expanded upon by Endler [21], who incorporated environmental influences to develop an evolutionary model known as the sensory drive hypothesis. The sensory drive model of

evolution focuses on selection for the three primary steps of communication, which can be broadly defined as (i) passive or active emission of a signal by a signaler, (ii) transmission of the signal through the environmental channel, and (iii) perception of the signal by the receiver.

Sensory drive orders these three primary communication steps in a hierarchy to define a cascade of selection processes that link the environment, receiver sensory capabilities, and the properties of communicative signals. As a result, (i) the environmental transmission channel modulates signal intensity and fidelity, (ii) the sensory capabilities of the receiver should evolve such that greatest sensitivity is achieved at the region of highest environmental transmission, and (iii) signal properties should then evolve to match this sensory system (see Figure 1 in [21]). The model encompasses selective forces imposed by diverse ecological factors such as food detection, predation, and microhabitat choice for male displays. It is important to recognize that sensory drive emphasizes the role of the environmental transmission channel as a selective force constraining sensory and signal evolution [22].

3. Cichlids, Sensory Systems and Models of Sexual Selection

When considering sexual selection in Lake Malawi cichlids, it is useful to compare it with sexual selection in the sister flock in Lake Victoria. Much like Malawi, Lake Victoria harbors a young (<500,000 years; [3]) cichlid radiation. Anthropogenic eutrophication (primarily due to agricultural runoff) threatens species diversity in Victoria due to the breakdown of species-recognition barriers in murky waters [23]. This effect highlights the environmental constraint on sensory capacities and signals and the importance of visual communication in speciation processes. The dim, narrow-spectrum light environment in Lake Victoria strongly constrains both visual properties and male nuptial displays. It can also limit the depth range of haplochromines, increasing interspecific space use along the depth gradient [24]. Reduced visibility can lead to a breakdown of species recognition, leading to a loss of species diversity through hybridization (although it may promote speciation in certain instances; see [25]). This eutrophication process and the hydrology of Victoria as a whole have also imposed extreme selection on cichlid visual systems and mate selection mechanisms [26]. As a result, the cichlids of Lake Victoria are an important model system for sensory drive based on behavioral and LWS opsin sequence data [27].

Pronounced differences in the visual environments of Lakes Victoria and Malawi suggest that it is not necessarily appropriate to extrapolate findings from one lake to the other. Unlike Victoria, Malawi is a clear-water lake with high-intensity light in shallow waters and a broad transmission spectrum. While the global light spectrum found in Lake Malawi does shape the overall visual sensitivities used by cichlids in those lakes, the light spectrum typically changes gradually between habitats or with depth. Consequently, it is unlikely that the environmental transmission channel in the shallow littoral environments of Lake Malawi exerts quite the same constraining selective pressure on sensory systems and signals that it does in Lake Victoria.

4. Does the Ambient Light Environment in Lake Malawi Constrain Visual Communication?

The initial (and causative) step in the sensory drive model emphasizes the role of the environmental transmission channel in shaping sensory sensitivities. This raises the question, how often do environmental transmission channels impose significant selection on sensory systems? Satellite imagery of light transmission through the waters of Lakes Malawi and Victoria illustrates the fundamental differences in the visual environments of these two lakes (Figure 1). The turbid waters of Lake Victoria optimally transmit longer wavelengths (orange and red), while the typically clear waters of Lake Malawi optimally transmit intermediate wavelengths (blue and green). Although both habitats are subject to short-term seasonal perturbations, the fundamental difference in environmental transmission is stable throughout the year (Figure 1). Therefore, Malawi offers us the opportunity to investigate signal constraint in a light environment substantially different to that studied in Victoria.

The tendency of blue-green wavelengths to transmit well in Lake Malawi is significant because these wavelengths correlate well with the absorbance of several cichlid visual pigments. The cichlid fishes of Lake Malawi possess seven distinct cone opsin genes, which we group into six functional categories (SWS1—ultraviolet, SWS2B—violet, SWS2A—blue, RH2B—blue—green, RH2Aα and β—green, LWS—red; Figure 2(a)). Because of the spectral distribution of these pigment types, the blue-green wavelengths that are optimally transmitted in Lake Malawi closely match the area of predicted peak sensitivity for the Malawi cichlid visual system, particularly for the medium- and long-wavelength sensitive double cones (which express the RH2B, RH2A, and LWS opsin genes). This match is quite consistent and extends through the known depth distribution of many species ([14]; Figure 2(b)). However, the red-shifted light environments in Lake Victoria tend towards the long-wavelength end of the cichlid visual pigments (Figure 2(c)).

Smith et al. [14] used models of luminance sensitivity to predict differences in the total quantum catch of various visual systems in a given environment. These models highlighted the minimal impact of the spectral environment on cichlid vision in Lake Malawi, over a range of depths. Figure 3 shows how quantum catch of the six classes of cichlid visual pigments varies with depth in both Lakes Malawi and Victoria [12]. These are calculated using

$$Q_i = \int I(\lambda) T_w(\lambda, d) R(\lambda) d\lambda, \quad (1)$$

where $I(\lambda)$ is the solar light spectrum at the water surface as a function of wavelength λ, $T_w(\lambda, d)$ is the spectral transmission properties of the water at depth d, and $R(\lambda)$ is the absorption spectrum of the visual pigments calculated based on Govardovskii et al. 2000 [28]. We calculated these

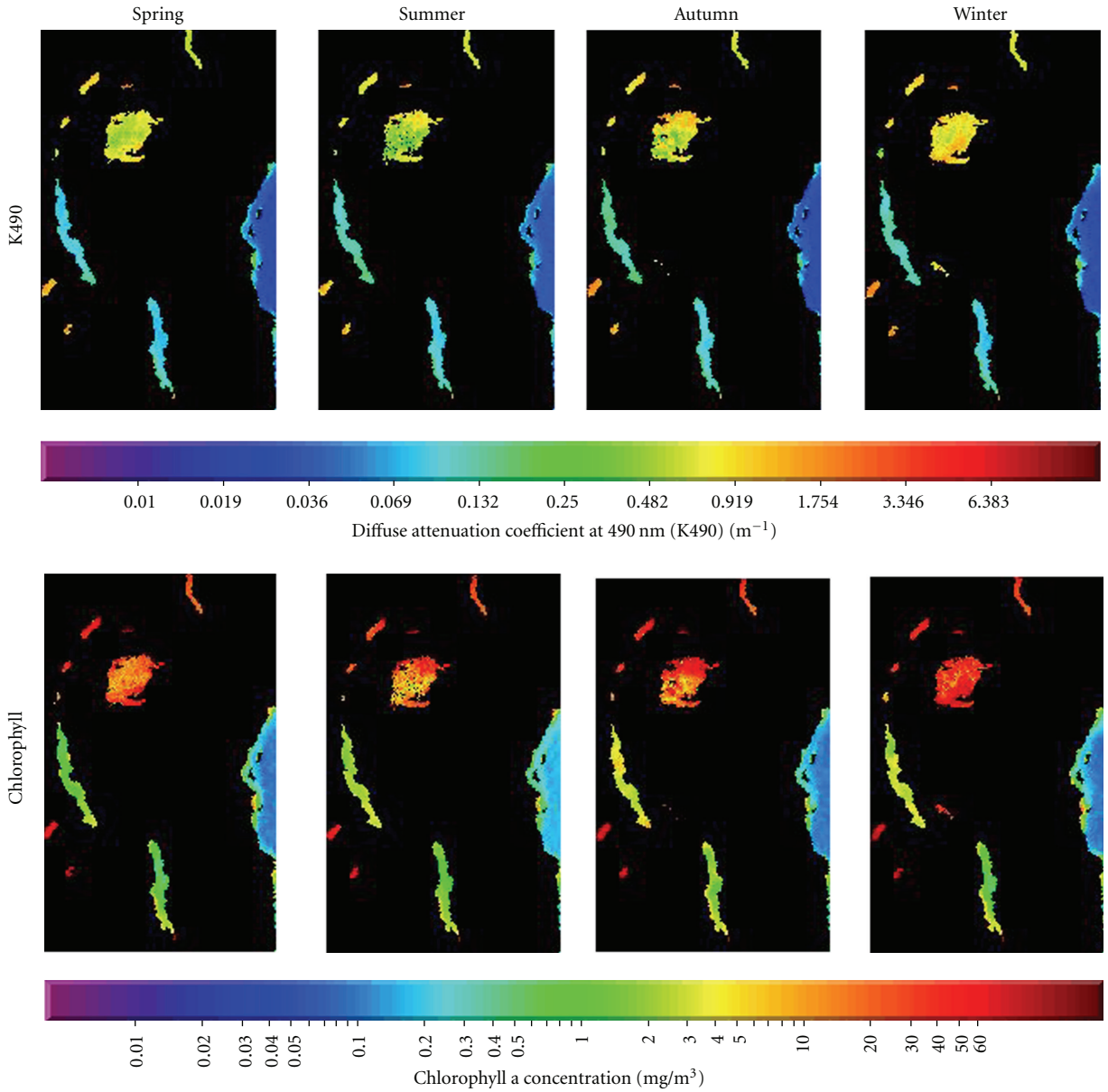

Figure 1: Seasonal satellite images for the three major African rift lakes, with Victoria at the top, Tanganyika in the middle, and Malawi at the bottom. The higher attenuation coefficients (K490, top row) and chlorophyll concentration (bottom row) estimates demonstrate that Lake Victoria is more turbid than the other lakes and, therefore, has a different fundamental ambient light environment. Lakes Tanganyika and Malawi are fairly similar and have much clearer waters than Victoria. Images constructed from averaged SEAWIFS data for the years 1998–2002. The large body of water on the right is a portion of the Indian Ocean that borders the African horn.

at depths up to 15 m, typical for the range where we collect fish. We then normalized the quantum catch calculations to reveal the relative tradeoffs between the different pigments with depth. In Malawi, the largest change occurs in the SWS1 pigment, with a decrease in quantum catchvaluesby a factor of 2 (Figure 3). The quantum catch of other visual pigments only varies by 5–15% over this 15 m depth range suggesting their quantum catch are all relatively good across this depth range. By contrast in Lake Victoria, the quantum catch for SWS1, SWS2B, and SWSA at 15 m decrease by 10^{-6}, 10^{-3}, and 0.03 relative to that at the surface. The relative quantum catch of LWS actually increases by 2.5 times over this depth range, supporting the idea that LWS is key to visual sensitivity in Lake Victoria. This highlights the fundamental differences in the light transmission properties of the two lakes as it applies to stimulating the cichlid cone visual pigments (Figure 4). It is also worth noting that absolute visual catch in Malawi can be 100 x greater than that in Victoria for similar depths due to greater availability of light (Figure 3), so that dim lighting is unlikely to have the same effects in Lake Malawi that it has in Lake Victoria.

In essence, the ambient light environments in Victoria and Malawi exhibit diametrically opposite effects on the cichlid visual system. In Lake Victoria, the environmental

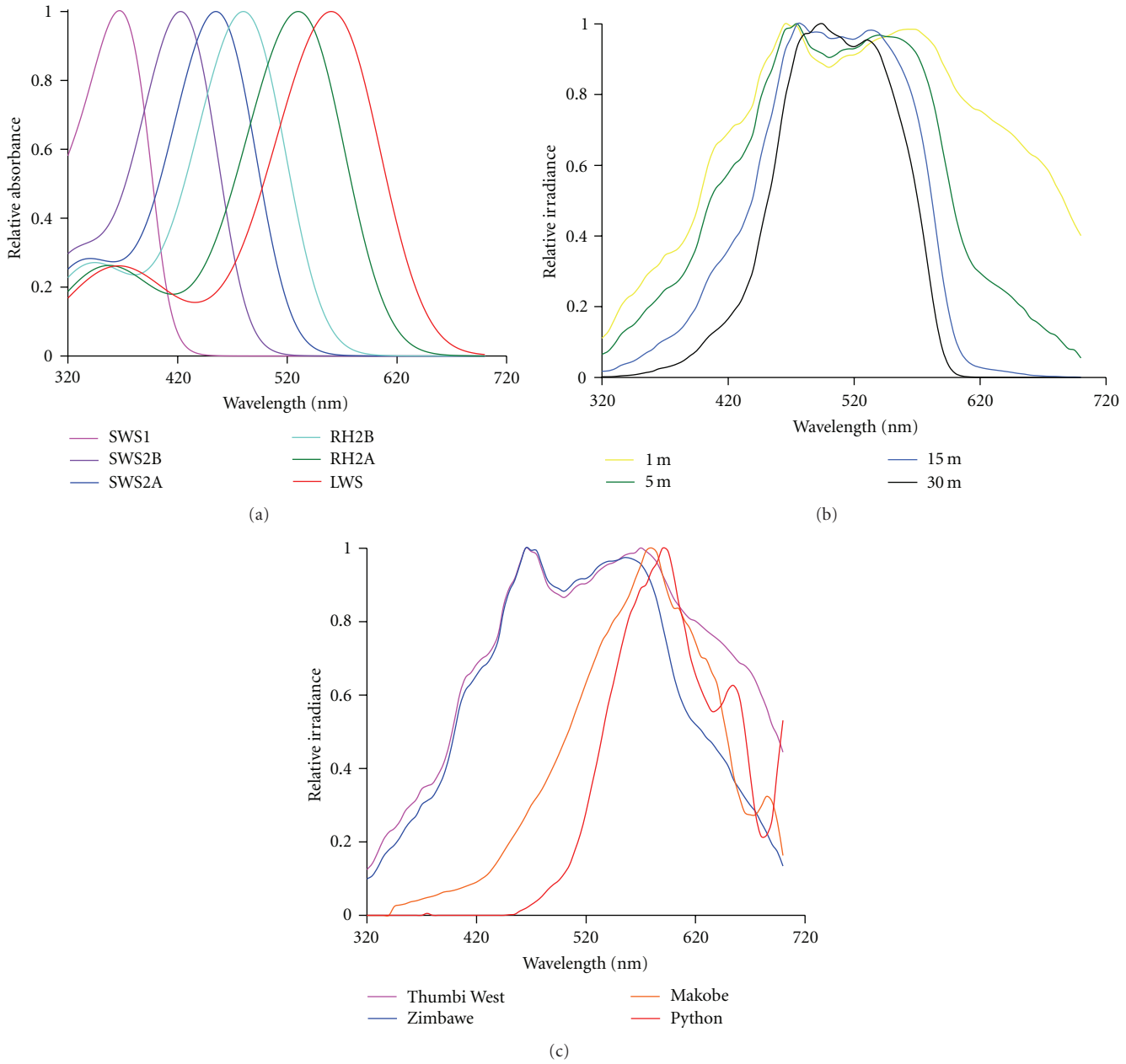

Figure 2: Comparison of cichlid cone opsin absorbance spectra in various environmental light environments. (a) Absorbance spectra for the six primary classes of cichlid cone opsins. (b) Relative irradiance measures of downwelling light at various depths at the Zimbawe Rock in Lake Malawi. (c) Comparison of the relative irradiance spectra for two Lake Malawi habitats (Thumbi West and Zimbawe) and two Lake Victoria habitats (Makobe and Python). Relative irradiance represents the proportion of total downwelling irradiance (photons/cm/s^2) normalized to the wavelength of maximum transmission.

transmission spectrum skews towards longer wavelengths, creating selective pressure favoring increased long-wavelength sensitivity with depth as we have previously observed [12, 29]. However, in Lake Malawi, the environment tends towards the center of the visual spectrum, which is an area where several of the cichlid visual pigments exhibit high quantum catch values. The relatively constant quantum catch of visual systems with differential opsin expression patterns suggests that changes in gene expression patterns do not confer improved luminance detection in littoral habitats [14]. Therefore, the broad signal transmission channel in Malawi does not likely impose significant selection pressure on the chromatic visual system that would select for or against changes in opsin expression patterns. Because we observe all possible opsin expression patterns in a variety of species cooccurring at the same shallow depth in the same habitat [12], we cannot explain this diversity simply from the light transmission properties in the clear waters of Lake Malawi. This result is inconsistent with the first tenet of sensory drive.

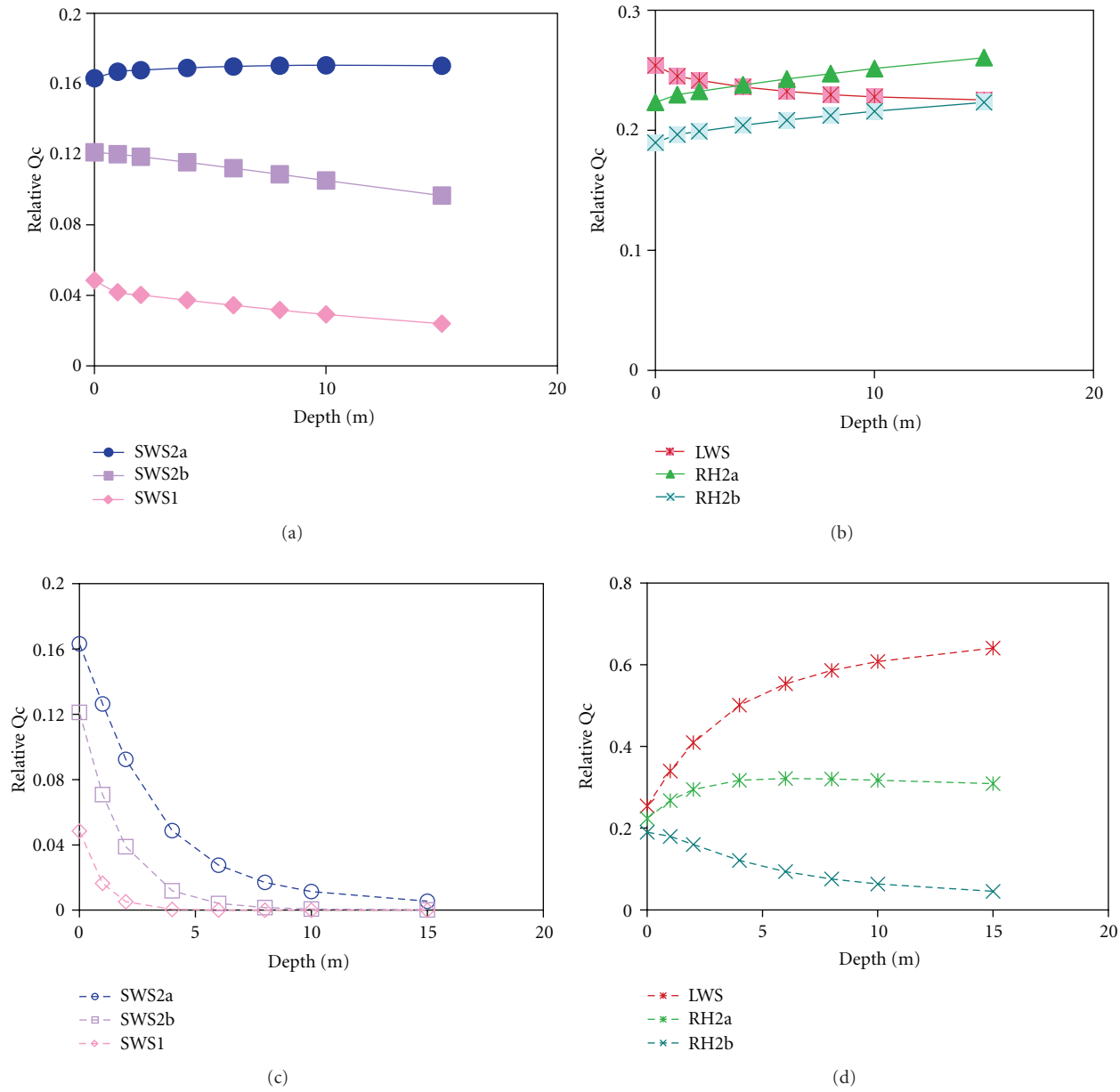

FIGURE 3: The relative quantum catch various opsin pigments for sample environments in Lakes Malawi and Victoria along depth gradients. The relative quantum catch of both short- (a) and long-wavelength (b) pigments is relatively constant in Lake Malawi. In Lake Victoria the short-wavelength pigments rapidly lose the ability to catch light with depth (c) while the relative quantum catch of the LWS pigment increases rapidly (d). Relative Qc represents the quantum catch (photons) of each cone pigment normalized to the total quantum catch for all pigments.

5. Receiver Systems: Variability in the Visual System

The second tenet of the sensory drive hypothesis centers on the characteristics of the sensory system of the receiver. More specifically, it predicts that the diversification in sensory systems results in different degrees of signal discrimination. Over time, this can lead to divergent responses to signals and subsequent speciation. To some extent, this appears to be true in Lake Malawi. Different species in the lake generally express subsets of the six opsin classes (grouping RH2Aα and β as RH2A), and these combinations have been termed "templates" [30]. These templates typically involve expression of three of the genes and occur as three primary types: (i) short—(SWS1, RH2B, RH2A), (ii) medium—(SWS2B, RH2B, RH2A), and (iii) long—(SWS2A, RH2A, LWS) sensitive gene sets [30].

However, significant variation within these primary templates has been found [14, 30]. This variability can manifest either as a quantitative variation in the relative expression of different opsin genes or as a qualitative shift from trichromatic to tetrachromatic opsin expression where additional

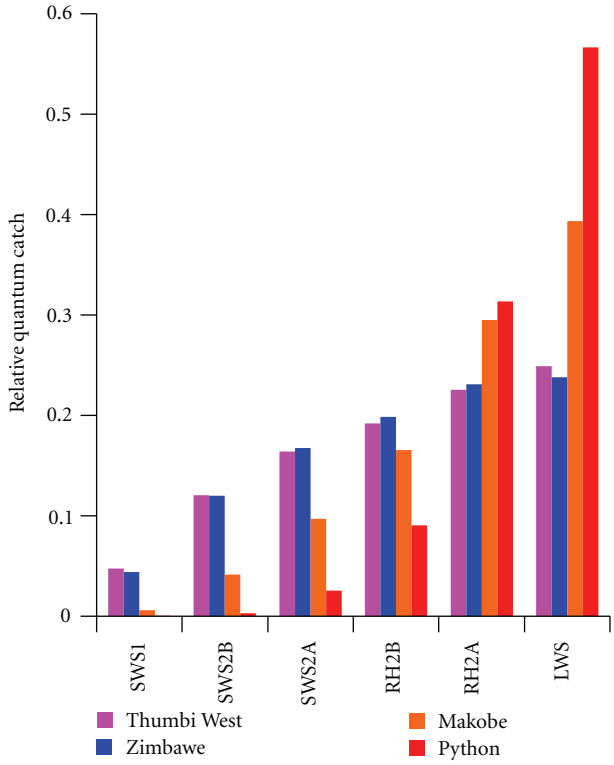

Figure 4: Absolute (a) and relative (b) cone opsin quantum catch comparisons distinct geographical locations in Lake Malawi (Thumbi West and Zimbawe) and Lake Victoria (Makobe and Python). Relative quantum catch represents the quantum catch (photons) of each cone pigment normalized to the total quantum catch for all pigments.

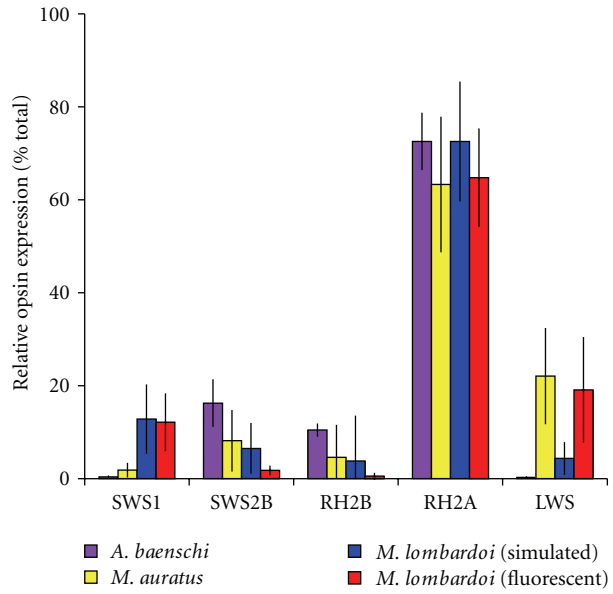

Figure 5: Gene expression patterns for fishes raised under laboratory conditions. *Aulonocara baenschi* and *Melanochromis auratus* both represent a primary medium-wavelength sensitive palette (SWS2B, RH2B, RH2A) in the wild. *A. baenschi* retains this template in the lab, while *M. auratus* develops substantial quantitative variation in LWS expression. *Metriaclima lombardoi* represents a short-wavelength sensitive palette (SWS1, RH2B, RH2A) in the wild but displays substantial variation in the expression of both the SWS2B and LWS genes in the laboratory. Furthermore, expression patterns for *M. lombardoi* in the lab are influenced by whether the fish are raised under fluorescent lighting or simulated sunlight. The SWS2A gene was omitted because it is not expressed in any of the groups depicted. Relative opsin expression represents the gene expression measure for each cone opsin gene normalized to the total measured opsin expression. Error bars indicate group standard deviations.

genes are expressed [14]. This intraspecific variation has been found in the wild, both along depth gradients (5 m versus 20 m) and across geographically distinct locales, and appears to be largely independent of the ambient light environment. As such, species-specific expression profiles appear to be far from being stable or predictable in a quantitative sense.

While much of the large-scale variation in opsin expression between the cichlid templates has been shown to be genetic [31], there is some evidence for subtler shifts in opsin expression due to environmentally induced plasticity. Inducible opsin expression plasticity was first observed by Hofmann et al. [30] when F1 progeny reared in the laboratory were found to have expression profiles that differed from their wild-caught parents, with the effect being most apparent in the expression of the SWS2B and LWS genes. The plasticity is manifested as quantitative variation in genes that would otherwise be expressed at low levels, rather than changes to the trichromatic expression template (illustrated in Figure 5). Hofmann et al. attributed the plasticity to the differences between the natural and laboratory lighting environments, but no attempts to rescue wild-type expression patterns in the lab were performed. Prior work in bluefin killifish (*Lucania goodei*) has demonstrated that variations in the ambient light environment can induce a plastic response in opsin gene expression [32], and the laboratory-manipulated spectra that induce plastic responses can be tied to specific (clear or tannin-stained) natural environments. A similar experiment performed by Smith et al. [33] with Malawi cichlids found that by manipulating the ambient light environment opsin expression plasticity could be induced through development in some species but not others. In plastic species, fish reared in simulated sunlight had expression profiles similar to wild-caught individuals, while those reared under standard fluorescent lights (which are substantially red-shifted compared to wild environments) were the same as the lab-bred individuals described by Hofmann et al. [30]. However, the difference in spectral content required to generate plasticity in the lab far exceeded the variation observed in natural light gradients in Lake Malawi (Figure 6). This suggests that although the potential for developmental expression plasticity exists in Malawi cichlids, the expression variation currently observed in shallow, clear-water habitats is probably not the result of environmental influences. Rather, it likely originates in genetic differences in the factors underlying opsin expression. In the event that the developmental environment becomes unstable (as might occur near river mouths during

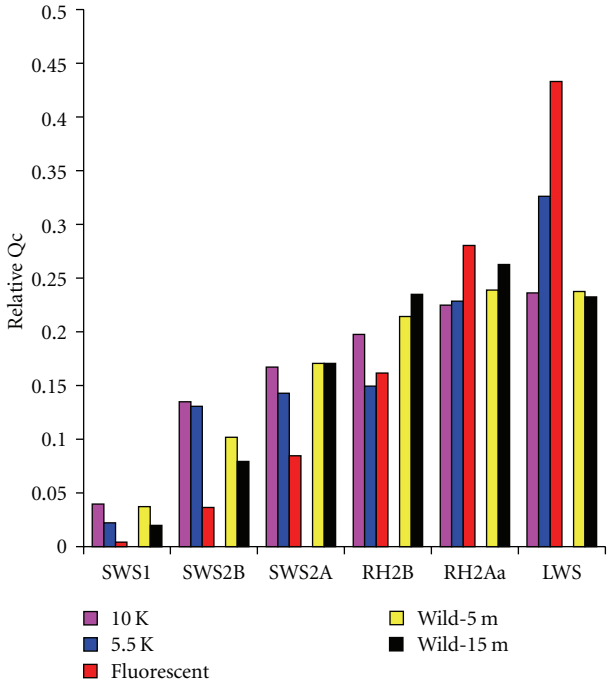

Figure 6: Relative quantum catch calculations for each of the primary cone opsin pigment classes for different light environments. The 10 K and 5.5 K bulbs were combined for a single simulated sunlight treatment, and pilot trials indicated no difference between the two in their effects on gene expression. However, the combination of these two had significant impact on gene expression when compared to fish raised under fluorescent lighting. Furthermore, the absolute difference in relative catch across treatments in the lab required to induce plasticity is much greater than that observed along the wild depth gradients where Smith et al. [14] collected samples for studies on wild expression plasticity. Relative Qc represents the quantum catch (photons) of each cone pigment normalized to the total quantum catch for all pigments.

floods or droughts), the environmental change could induce a rapid plastic change in the visual systems of developing fishes.

Opsin expression variation may simply represent genetic "noise" unless there are tangible consequences for neural processing and behavior. A strong link between opsin expression and complex behaviors such as mate choice was not found in bluefin killifish [34]. However, links between performance on an optomotor (OMR) task and both opsin gene sequence [35] and developmental light environment [33, 36] have been demonstrated in cichlid fishes. Smith et al. [33] determined that not only is OMR performance labile but also that this variation in the performance of a luminance-based task are linked to variation in LWS opsin gene expression. This highlights the potential for the generation of behavioral variation via shifts in the expression of cone opsin genes, regardless of whether or not these shifts are environmentally induced. It is important to note, however, that the OMR paradigm is not necessarily an ideal proxy for complex mate-choice behaviors, as it is a luminance-dependent mechanism that does not take into account visual contrast (discussed further in [33]).

6. Heterochrony and the Timing of Developmental Variation

In previous work, we have shown that opsin expression can vary through ontogeny and that these shifts appear to be an ancestral trait in African cichlids. By definition, ontogenetic shifts represent changes in developmental programs that are finalized prior to adulthood, that is, although variation will be observed across juveniles of different ages, adult animals with the same developmental program will be relatively homogenous. Data from the tilapia *Oreochromis niloticus* demonstrate that, for this species, the visual system progressively passes through the short-, medium-, and long-wavelength trichromatic opsin gene expression palettes from hatching to adulthood (~6 months; [37]). Heterochronic shifts in developmental timing result in (i) the retention of the neotenic short-wavelength template, (ii) direct expression of the long-wavelength palette, or (iii) accelerated development of the medium-wavelength palette [37]. For the sake of future discussion, we will define any period for opsin expression variation as the critical period and the achievement of the adult phenotype as crystallization per the literature on other phenotypically plastic traits (birdsong; reviewed by [38]).

The variation of gene expression through ontogeny in the lab suggests that the critical period required to achieve adult expression profiles in Malawi cichlids may vary between species. For example, Smith et al. [33] used two species in their study; one species exhibited environmentally induced plasticity (*Metriaclima lombardoi*) while the other did not (*Melanochromis auratus*). In the case of *M. auratus*, the final adult phenotype develops between 11 and 14 days after fertilization (dpf), with a shift from the expression of SWS1 to SWS2B. This switch in SWS gene expression occurred at the same developmental time point in both the broad- and narrow-spectrum laboratory light environments and appears to be ontogenetically fixed. For *M. lombardoi*, changes in gene expression were observed steadily through development over a period of four to six months, at which point the adult phenotype crystallized. The rate at which expression of the LWS pigment increased during the critical period differed between simulated sunlight and fluorescent lighting, resulting in adult phenotypes with differing levels of LWS expression [33]. If we consider developmental progressions in another known plastic species from the laboratory, we see a similar pattern in a different opsin gene. In *Melanochromis johanni* "black and white," SWS2B expression increases through time until crystallization, and the rate at which it increases determines the adult phenotype (Figure 7). This phenomenon is such that the ontogeny of gene expression in *M. lombardoi* and *M. johanni* "black and white" is similar, although the latter is a congener to a developmentally fixed species (*M. auratus*). This suggests that heterochrony can vary between closely related species and that ontogenetic

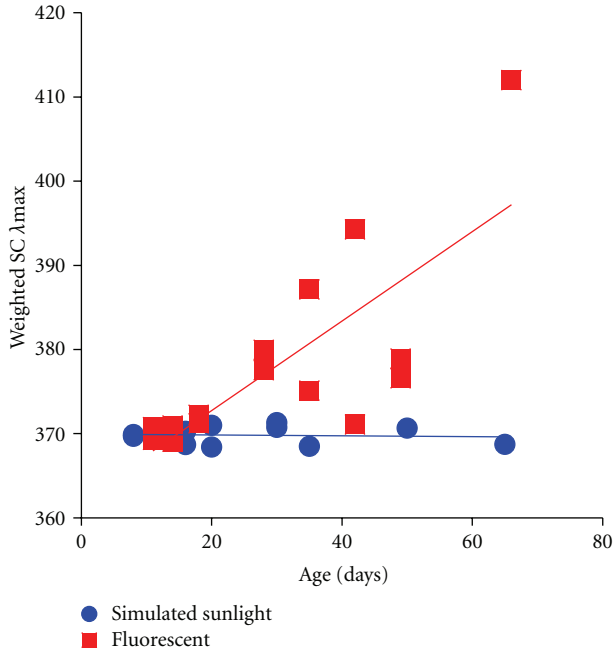

FIGURE 7: Change in the relative expression of the SWS gene group (SWS1, SWS2B, SWS2A) plotted using a weighted λ_{max} calculation through developmental time for *Melanochromis johanni* "black and white." For this species, an increase in the weighted λ_{max} indicates a shift from SWS1 to SWS2B expression. Individuals reared under simulated sunlight exhibit little plasticity through development, while individuals reared under fluorescent lighting express more SWS2B over time. Also, there was much greater quantitative variation in SWS1/SWS2B expression in fish raised under fluorescent lighting.

changes in gene expression can interact with environmental plasticity to produce distinct adult phenotypes.

7. Evolution and the Cooption of Heterochronic Variation

With the substantial variation in visual systems and signals present in Lake Malawi, the question of which evolutionary factors are at play remains to be addressed. As previously discussed, the ambient light environment in Lake Malawi probably does not exert significant stabilizing or diversifying selective pressures on the various shallow water visual templates found in Lake Malawi. This would allow the genetic and neural capacity for visual variation to remain in natural populations as a neutral trait under stable environmental conditions. Intuitively, the second ecological factor that could select for specific visual characteristics is the habitat choice and dietary requirements of individual species. An association does exist between diet and SWS1 expression across a panel of distantly related Malawi cichlid species [12]. The resulting UV sensitivity has also been found to be important for foraging [39]. However, we found no link between ecology and expression of the other opsin genes, in particular the RH2 and LWS genes, suggesting that ecology does not explain diversity at the long-wavelength end of the spectrum. Further, functional ecological diversity within genera is fairly limited. Given that this is the phylogenetic level at which most diversification in visual signals has evolved, it is unlikely that ecology is the primary selective force on the visual system as well.

To the extent that visual systems are largely unconstrained by environmental and ecological selection pressures in Lake Malawi, the potential for "non-adaptive" evolutionary forces increases substantially. In particular, genetic drift could act to generate or limit diversity by randomly altering the nature of ontogenetic variation. Population sizes of Lake Malawi cichlids have been estimated to be as small as 3000 to 5000 individuals suggesting that drift could act with sufficient efficiency to fix nonadaptive alleles [40]. By turning the heterochronic critical period "on" or "off" or simply changing its duration, drift could drastically alter crystallized adult phenotypes without requiring changes to opsin sequences or the basic transcription machinery that governs opsin expression. If we compare this prediction with measurements of gene expression from wild populations, we hypothesize that the qualitative gain or loss of a critical period within a single species could generate two qualitatively different adult expression phenotypes, such as that observed for both *Mchenga eucinostomus* and *Metriaclima zebra* in the wild (see Figure 2 of [14]). Two populations of *M. eucinostomus* were found to vary in LWS expression, while populations of *M. zebra* were found to vary in SWS2b expression, perhaps as a result of changes to genetic factors underlying ontogenetic patterns of gene expression. Similarly, changes in the duration of a critical period could generate extensive quantitative variation based on the total time an individual's visual system can progress through a developmental shift before crystallization. This prediction would match the phenomenon observed in two populations of *Tropheops gracilior* that were sampled along a steep depth gradient (see Figure 2 of [14]) as well as variation in gene expression in *Melanochromis johanni* "black and white" in the laboratory (Figure 7). Here, gene expression is presented by converting it to weighted single cone λ_{max}, using

$$\lambda_{SC} = \frac{f_{SWS1}\lambda_{SWS1} + f_{SWS2b}\lambda_{SWS2b} + f_{SWS2a}\lambda_{SWS2a}}{f_{SWS1} + f_{SWS2b} + f_{SWS2a}}, \quad (2)$$

where λ_i is the peak sensitivities previously measured (λ_{SWS1} = 368 nm, λ_{SWS2b} = 423 nm, and λ_{SWS2a} = 455 nm [41]) and f_i are the gene expression fraction for the three single cone genes, SWS1, SWS2b, and SWS2a. Both variation in individual gene expression patterns with depth or differential environmental lighting in the lab could generate behavioral shifts such as those observed in the optomotor response, regardless of whether the differences in gene expression occurred in response to environmental conditions or if it was genetically programmed [33]. Perhaps most importantly, critical period changes could generate similar patterns of diversity by a random pattern of changes in the length of the critical period. This would explain the continual reevolution of phenotypic diversity that we observe in African cichlids [37] while accommodating the lack of major selection pressures as

posited by Smith et al. [14]. While these developmental changes account for the generation of substantial diversity in sensory systems and nuptial displays, the tendency of the sensory system to evolve free of environmental selection is inconsistent with the fundamental premise of the sensory drive hypothesis. Rather, it is more consistent with the sensory exploitation model of Ryan and Rand [20].

8. What Does Sensory Diversity Mean for Signalers?

The third and final tenet of the sensory drive hypothesis centers on the evolution of male courtship displays to match female sensory traits and potential coevolution of these two traits in concert. For example, in Lake Victoria males of different species have evolved either blue or red nuptial coloration based partially on the environment and the visual systems of females [26]. In this system, strong selection would cause females within a species/population to have very similar sensory capacities due to the need to retain sensitivity in a given environment. Therefore, male signals can more easily evolve to match those sensitivities closely. However, in some systems intrapopulation expression variation can be fairly extensive. For example, opsin gene expression in *Tropheops gracilior* collected from 20 m depth in Lake Malawi was significantly more variable than their conspecifics collected at 5 m [14]. In this example, female sensory capacities at 20 m would be a relatively unknown quantity to a courting male. This could create problems for males that attempt to mate with as many females as possible and has implications for signaling systems.

As many recent studies have highlighted, courtship in African cichlids is a multimodal affair. In particular, males often employ acoustic signals as part of their courtship dance [9, 11, 17]. These calls may be used to differentiate species based on call qualities such as frequency and duration [11, 18], but there is often extensive variation in call characteristics within species and even within successive signals from a focal individual [9]. This acoustic plasticity is further compounded by variation in the association of visual and acoustic signals [9], resulting in what van Staaden and Smith [42] posited is a complex communication system that may reflect contextual information, indicate male motivation, or even exploit a female's sensory system.

In principle, males could use complex communication to account for uncertainty in the preferences of multiple females. If this is indeed the case, we would hypothesize that males of species with variable sensory systems would exhibit more variable multimodal displays (concept illustrated in Figure 8). While no study has directly tested this to date, anecdotal evidence suggests that this is a question worth pursuing. Smith et al. [33] found that *Metriaclima lombardoi* had extensive variation in LWS expression and that this translated into substantial differences in behavioral measures of visual sensitivities. Smith and van Staaden [9] found that males of this species had highly variable acoustic and multimodal courtship displays. Similarly, *Melanochromis auratus* did not have variable opsin expression and little variation in their visual sensitivities on the OMR task [33]. Behavioral trials indicated that, while this species can vocalize, they rarely do so during courtship and therefore have fairly static unimodal displays [9]. Taken together, this suggests that the complexity of male signals in Lake Malawi may be a response to sensory variation. Although intriguing, this idea is admittedly speculative and will need to undergo extensive and rigorous testing.

9. Conclusions and Suggestions for Future Research

In order to summarize the ideas presented here, it is useful to revisit the original scheme for sensory drive so effectively laid out by Endler [21], layering in how facets of Lake Malawi cichlid biology relate to specific portions of his model (Figure 9). The sensory drive model is dominated by the qualities of the environment, and how these qualities influence sensory systems and communication. In the absence of strong environmental influences, many of the aspects of the model become less influential (as depicted by dashed arrows). If we remove these portions from the model, we can begin to see in which way the Lake Malawi system is unique (Figure 10). So where, in fact, does sensory variation fit in this model? In essence, data suggest that sensory variation, which can function in conjunction with or independent of the environment depending on the species and habitat, acts as a buffer between the "environmental channel" and "sensory characteristics" portion of the sensory drive model.

As an example, let us again consider the *Mchenga eucinostomus* collected at two different depths along a habitat gradient in Lake Malawi. These fish exhibited profound visual plasticity, with fish from 5 m depth being tetrachromats (SWS1-RH2B-RH2A-LWS) while fish from 20 m were trichromats (SWS1-RH2B-RH2A). Quantum catch models predict that this qualitative variation in LWS opsin expression would have no effect on luminance function for shallow littoral Lake Malawi habitats (i.e., the retina will not catch more light), although enhancing luminance detection is likely a driving selective force in Lake Victoria [14]. However, an increase in LWS function in Malawian taxa is known to have behavioral consequences in the OMR paradigm, with an increase in LWS expression increasing behavioral response/sensitivity [33]. This corresponds to a change in function of a particular neural pathway: the magnocellular visual pathway. Therefore, we can deduce that sensory variation in Lake Malawi cichlids is capable of generating variation in the behavior of wild fish such as that observed in Victorian cichlids [35]. However, this variation in Malawi can occur without the need for the same selective gradients that would be present for the same fish in Lake Victoria.

Although sensory variation in contemporary Malawi cichlid biology is clearly discernible, many avenues of research remain to determine exactly how important variation has been in cichlid evolution through time. Aside from further profiles of sensory variation in Lake Malawi, we envision two particularly important avenues of future

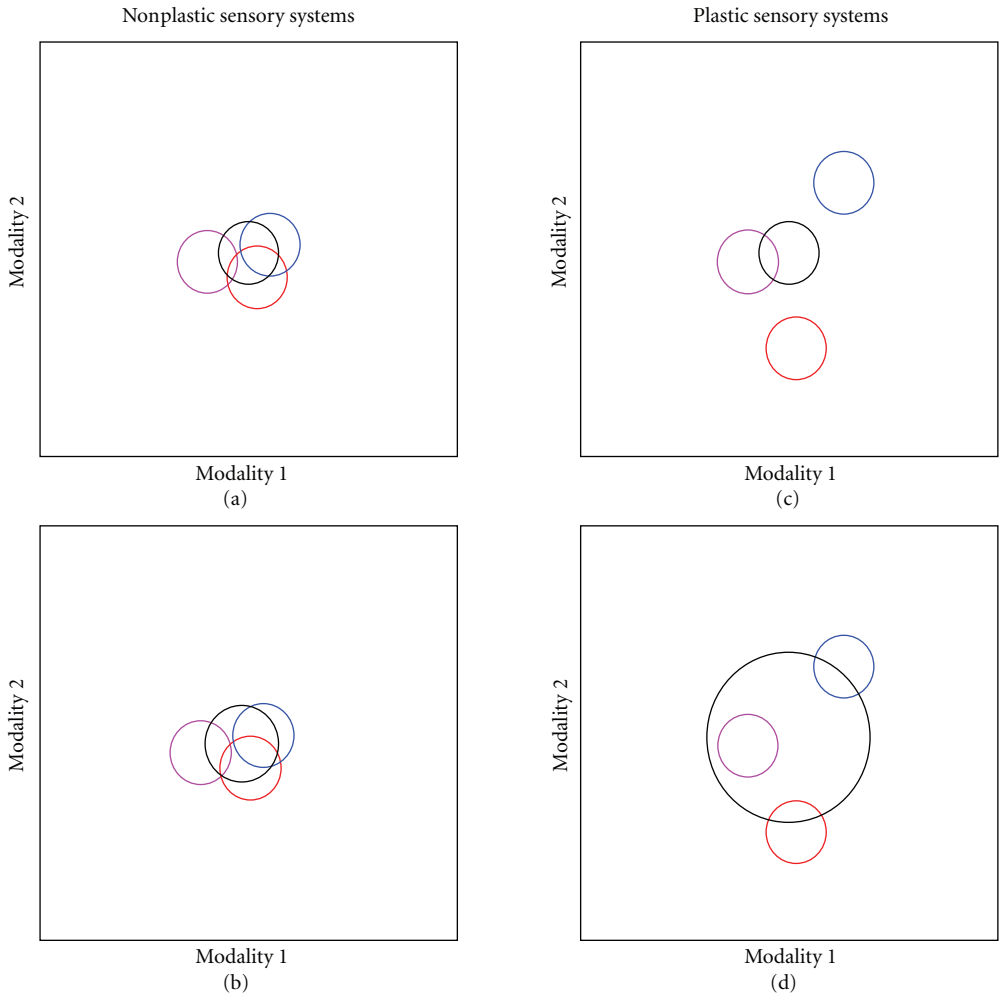

FIGURE 8: Illustrative cartoon of the effects of sensory plasticity on multimodal male courtship signals. In all panels, the male signal is indicated by the black circle and the female "choice zone" (i.e., the male signal that will elicit a positive response) for three individuals are indicated via colored circles. In the case of a nonplastic species (a), a static male signal will likely overlap the choice criteria for all females, and even a small expansion of male behavior through plasticity will result in effective stimulation of all females involved (b). However, in the case of a plastic species with highly distributed female preferences, a static male signal is unlikely to overlap many female choice zones (c). In order to overlap the choice zones for multiple females, a male must therefore employ a very plastic signal to increase his odds of eliciting a positive response from a given female (d).

research: (i) investigations of the role of variation in the cichlid tribes of Lake Tanganyika and (ii) studies of the molecular and cellular mechanisms involved in the stabilization of the developing retina. The former represents an important opportunity to test whether variation also functions in independent cichlid radiations. Since Tanganyikan cichlids represent multiple independent lineages that have evolved in a visual environment more similar to Lake Malawi than Lake Victoria, they may elucidate mechanisms of sensory evolution in relatively nonrestrictive environments. Indeed, work by Sugawara et al. [43] on the cichlid rod opsin suggests parallel evolutionary processes in Lakes Malawi and Tanganyika with respect to rod cellular sensitivities. The molecular and cellular mechanisms important in the developing retina are of considerable importance to the broader field of neuroscience, as mechanisms determining vertebrate neural plasticity are of great significance for both basic and clinical research.

In sum, we propose that sensory variation is quite diverse in the Lake Malawi cichlids. However, we cannot explain this diversity by simple models of sensory drive. Such variation could dramatically alter the nature and pace of sensory evolution and visual communication, though correlations between cichlid color and sensory variation have yet to be demonstrated in Lake Malawi. Given the potential for sensory variation to modify our understanding of both sensory evolution and cichlid speciation, contemporary research should consider the implications of these mechanisms when interpreting experimental results. Not only would a fresh view of cichlid communication biology further emphasize

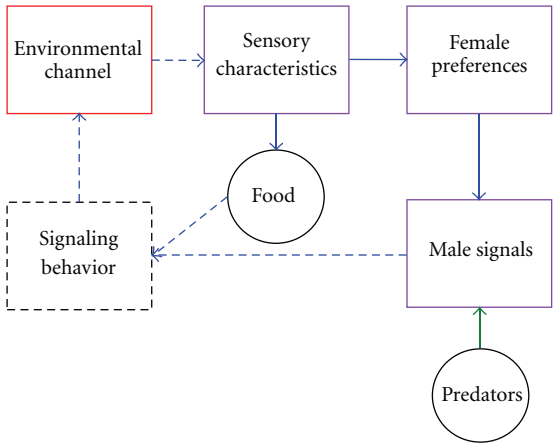

Figure 9: A modified version of the sensory drive schematic proposed by Endler [21]. Violet boxes denote the portions of the model that coincide with the original sensory bias hypothesis first proposed by Ryan and Rand [20]. The red box represents the primary driver of sensory stabilization or diversification (the environmental transmission channel). Solid arrows represent selective forces that are likely active in Lake Malawi, while dashed arrows represent factors whose selective influence on sensory evolution in Lake Malawi is marginalized due to environmental characteristics.

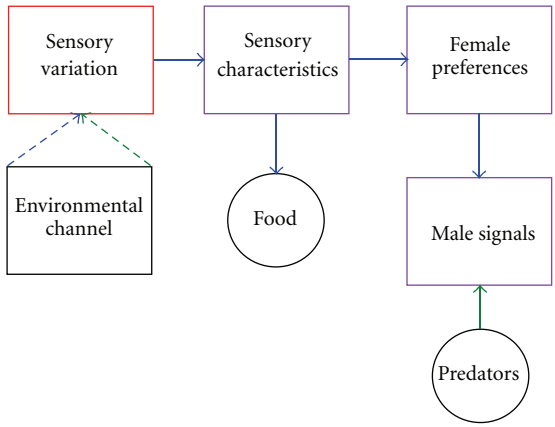

Figure 10: A further modification of the sensory drive framework that inserts sensory plasticity as a buffer between the environmental transmission channel and the sensory bias framework. Sensory plasticity is highlighted in red due to the potential for modulating variation in sensory sensitivities either independent of or in response to environmental effects.

the importance of these fishes as evolutionary models, it could also be an important model for questions in the neurosciences.

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Research Article

Analysis of the Meiotic Segregation in Intergeneric Hybrids of Tilapias

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Tilapia species exhibit a large ecological diversity and an important propensity to interspecific hybridisation. This has been shown in the wild and used in aquaculture. However, despite its important evolutionary implications, few studies have focused on the analysis of hybrid genomes and their meiotic segregation. Intergeneric hybrids between *Oreochromis niloticus* and *Sarotherodon melanotheron*, two species highly differentiated genetically, ecologically, and behaviourally, were produced experimentally. The meiotic segregation of these hybrids was analysed in reciprocal second generation hybrid (F2) and backcross families and compared to the meiosis of both parental species, using a panel of 30 microsatellite markers. Hybrid meioses showed segregation in accordance to Mendelian expectations, independent from sex and the direction of crosses. In addition, we observed a conservation of linkage associations between markers, which suggests a relatively similar genome structure between the two parental species and the apparent lack of postzygotic incompatibility, despite their important divergence. These results provide genomics insights into the relative ease of hybridisation within cichlid species when prezygotic barriers are disrupted. Overall our results support the hypothesis that hybridisation may have played an important role in the evolution and diversification of cichlids.

1. Introduction

Interspecific hybridisation has been suggested to be an important evolutionary force that generates biological diversity by the recombination of genetic material among divergent lineages [1–3]. Hybridisation has been shown to facilitate adaptation, the emergence of evolutionary novelty, and the isolation of new species [4–7]. The role of introgressive hybridisation has also been shown in case of human-mediated evolution and especially domestication [8]. For instance, interspecific hybridisation has been widely used for aquaculture purposes in a large variety of species [9].

Convincing evidence suggests that inter-specific hybridisation has played an important role during evolution and diversification of cichlid fish [10]. Some cichlid adaptive radiations may have been initiated through hybridisation between distantly related lineages, forming a "hybrid swarm," such as the radiations of Lakes Victoria [11], Malawi [12], and Makgadikgadi [13]. Hybridisation can also occur later during the process of radiation between divergent or already diverged species, forming "syngameon", as suggested for some Lake Tanganyika lineages [14–16]. The vast majority of the studies conducted so far on cichlid speciation and the potential influence of hybridisation have focused on the East African Great Lakes radiations, especially the Haplochromines. However, the Tilapiines *sensu lato* [17, 18] appear to be an extremely interesting group to study the evolutionary role of hybridisation. Several cases of introgressive hybridisation have been recorded in the wild, under natural conditions, either during the process of adaptive

radiation, as in Cameroonian crater lake [19], or during paleoenvironmental fluctuations [20]. Several cases have also been reported following anthropogenic perturbations, either between sympatric species after important habitat modifications such as dam building [21] or between allopatric species after introduction of species outside their natural distribution area [22, 23]. Furthermore, numerous interspecific hybrids have been produced for aquaculture [24], mainly among *Oreochromis* species [9, 25, 26]. The production of tilapia hybrids has two main purposes: the production of monosex populations by crossing between species with opposite sex determination system [27–31] and the improvement of phenotypic traits, such as body colour, growth, or tolerance to environmental conditions [24]. However, it is only for the latter that introgressive hybridisation is performed, with the production of breeding population of hybrid origin, aiming at being propagated and selected throughout successive reproductive events. This has been conducted mostly crossing pairs of closely related species (e.g., *Oreochromis niloticus* and *O. aureus*, [32]), but also using more complex crossing schemes involving multiple species (e.g., Red Florida stain, between *Oreochromis urolepis hornorum*, *O. mossambicus*, *O. niloticus,* and *O. aureus*) [33].

Theoretically, while the evolutionary potential of a hybrid lineage is ultimately dependent on its ability to successfully occupy a peak of the local adaptive landscape through the advantage conferred by its original combination of phenotypic traits, hybrid propagation initially requires the maintenance of a stable gene pool through generations. Most of theoretical and empirical studies are focussing on the adaptive role of hybridisation (see review by Stelkens and Seehausen [34]); however very few studies have investigated the transmission of hybrid genomes across generations.

Various hybrid meiotic mechanisms deviating from classical diploid Mendelian segregation and inheritance have been reported, especially in fish [35, 36]. Such mechanisms may be classified in four main categories: (1) hybridisation can induce variable ploidy levels by the production of nonreduced (diploid) gametes; this mechanism is likely to lead to the isolation of polyploidy lineages as, for example, in *Barbus* species [37]; (2) hybridisation can induce clonal gynogenetic reproduction by the suppression of syngamy (i.e., absence of fusion and elimination of male pronucleus) and the mitotic or meiotic restoration of diploidy, such as observed in *Poecilia formosa* [38]; (3) hybridogenesis can be achieved by selective meiosis, in which the genome of one of the parents (generally the paternal one) is preferentially eliminated from the gametes leading to the next generation, as described in hybrid females of *Poeciliopsis* [39]; (4) modification of recombination within the hybrid genomes, caused by the slight structural divergences between the associated genomes, might affect the homogeneity of the hybrid gene pool, leading, for example, to the maintenance of a mosaic hybrid genome and a fitness deficit [40–43].

To investigate hybrid meiotic segregation in cichlids, we previously produced an intergeneric hybrid between two highly divergent species using *in vitro* fertilisation: the Nile tilapia, *Oreochromis niloticus,* and the black-chinned tilapia, *Sarotherodon melanotheron* [44]. All hybrids were found to be viable and fertile for at least three generations [44]. The divergence between the two species is estimated between 6.4 Myrs and 21.4 Myrs using the age of radiation of *Oreochromis* and Oreochromines as an approximation [18]. Additionally, these two species show high levels of differentiation in morphology, ecology, behaviour, and physiology. *O. niloticus* is a maternal mouthbrooder, fast growing and freshwater stenotopic species, whereas *S. melanotheron* is a paternal mouthbrooder, slow growing, brackish water eurytopic species. Selecting two highly differentiated parental species for the experimental hybridisation potentially increases the possibility of generating original trait association in hybrids compared to the parental species (including transgressive characters) but also increases the likelihood for reproductive incompatibility or unusual meiotic mechanisms, compared to hybridisation between closely related or sister species. Thus, these inter-generic hybrids represent an original and well-adapted model to study the association and segregation of parental genes within cichlid hybrid genomes.

Microsatellite markers provide valuable tools for a wide range of genetic investigations, including species comparison using PCR primers developed in one species and cross-amplified in closely related taxa [45, 46]. Here we took advantage of the availability of a large number of markers cloned in *O. niloticus* [47] positioned onto the tilapia genetic maps [48–51] and their high rate of cross-species amplification among tilapias [52], to study the mechanism of hybrid meiotic segregation in experimental hybrids between *O. niloticus* and *S. melanotheron*. We tested (1) whether hybrid meiotic segregations follow diploid Mendelian inheritance and (2) whether this model of meiotic segregation allows the maintenance of a stable hybrid gene pool across generations.

2. Material and Methods

2.1. Biological Material. The meiosis of first generation hybrids (F1) between the 2 parental species *O. niloticus* (On) and *S. melanotheron* (Sm) was studied for both reciprocal hybrid crosses (♀ On × ♂ Sm called G1 and ♀ Sm × ♂ On called G′1) and both sexes. Analysis was conducted on backcross (BC) and second-generation hybrid (F2) families. While F2 crosses allow studying hybrid meiosis for both parents in each family, BC progeny allowing the study of only one hybrid meiosis per family can be more informative with respect to the parental origin of alleles, and further allow the analysis of a parental meiosis, which provide an internal pure-species control segregation.

Four backcross families performed on *O. niloticus* (due to its better known reproduction biology and greater ease to be manipulated) were analysed to study the meiotic segregation of both reciprocal hybrid types and both sexes (♀ G1, ♂ G1, ♀ G′1 & ♂ G′1), and the pure-species segregation of *O. niloticus* in both sexes (♀ On & ♂ On) (Table 1). In addition, two different hybrid F2 families (♀ G′1 × ♂ G′1 and ♀ G1 × ♂ G′1) were analysed to look at the segregations and allele associations within a full hybrid genome (Table 1). Finally, an independent pure *S. melanotheron* family (♀ Sm & ♂ Sm; P-Sm) was studied to provide information on

Table 1: Description of the experimental families analysed, including the type of cross, the genetic origin of breeders (i.e., pure *O. niloticus*) (On) or *S. melanotheron* (Sm), and reciprocal 1st generation hybrid G1 (♀ On × ♂ Sm) and G′1 (♀ Sm × ♂ On) and the number of studied individuals.

Families	Type of crossing	Breeders		No. Ind.
		Female	Male	
BC-A	Backcross	Hybrid Gl	*O. niloticus*	50
BC-B	Backcross	*O. niloticus*	Hybrid Gl	50
BC-C	Backcross	Hybrid G′l	*O. niloticus*	50
BC-D	Backcross	*O. niloticus*	Hybrid G′l	50
F2-A	Hybrid F2	Hybrid G′l	Hybrid G′l	50
F2-B	Hybrid F2	Hybrid Gl	Hybrid G′l	50
P-Sm	Pure Cross	*S. melanotheron*	*S. melanotheron*	50

the meiotic segregation of the other parental species (Table 1). For each family, 50 randomly sampled individuals were analysed.

2.2. Microsatellites Markers. Microsatellites markers were selected from published markers isolated in *O. niloticus* [47] and successfully amplified in both *O. niloticus* and *S. melanotheron* [52]. Markers were selected based on cross amplification efficiency, polymorphism among and within *O. niloticus* and *S. melanotheron*, and their position on the genetic map of tilapias [48–51]. The analysis of the meiotic segregation was conducted using a total of 30 microsatellite markers distributed across the tilapia genome, allowing to compare the segregation of both punctual genomic locations, represented by single independent loci, as well as larger genomic segments represented by two to four linked loci. Overall, the 30 selected markers represented 15 of the 24 linkage groups (LGs) defined in *O. niloticus* genetic map, 8 of each were represented by more than one marker. Two unmapped loci were also included in the analysis (Table 2).

2.3. Genotyping of Microsatellites. Genomic DNA was extracted from fin clips stored in ethanol using phenol-chloroform protocol [53]. Genotypes were obtained by PCR amplification using radioactively (P^{33}) labelled primers and 6% acrylamide gel electrophoresis [53, 54]. For each microsatellite marker, optimal amplification conditions, namely annealing temperature and $MgCl_2$ concentration for coamplification of alleles from heterospecific origin, were obtained from a cross-species amplification study in over 15 species of African cichlids, including both parental target species [52]. Specific PCR conditions for each locus are indicated in Table 2.

All parents used to produce the experimental progeny were genotyped first at all selected loci to identify informative markers for each family. Each family was genotyped for all informative loci in the entire set of optimised markers ($n = 30$), whereas the F2 hybrid progeny was only genotyped across the restricted set of independent markers ($n = 14$) (Table 2; details in supplementary Table S1 available online at doi:10.1155/2012/817562).

2.4. Statistical Analysis

2.4.1. Genetic Diversity. The test of amplification efficiency and the estimates of genetic diversity within and across the two-target parental species have been conducted over the set of individuals analysed during cross-species amplification study and the set of pure parents and grandparents of the seven experimental progenies included in the analysis of meiotic segregation [52]. For each locus, the number of observed alleles was recorded for each parental species, as well as the number of shared alleles between them. The presence of null alleles in the studied loci was estimated across all breeder individuals, based on the repetitive occurrence of a non-amplification result in parents, and/or the significant departure from Mendelian inheritance associated with a pattern characteristic of the segregation of at least one nonamplified allele (i.e., based on the overall pattern of alleles segregation in the entire progeny). For each species, the proportion of polymorphic loci ($P < 0.95$) and the mean allele number per loci were calculated. The number of shared alleles, between *O. niloticus* and *S. melanotheron*, as percent of total number of observed alleles, was calculated per locus and across loci. For the purpose of this study, the existence of shared alleles between parental species and/or their low allelic diversity can potentially lead to cases where marker segregation is not fully informative (e.g., when both parents are heterozygous for the same alleles). These cases were identified based on the genotyping of all parent individuals, prior to the progeny genotyping.

2.4.2. Analysis of Meiotic Segregation. The analysis of the meiotic segregation was conducted for each individual parent and family, as well as across all pure and hybrid segregations, considering either each locus independently or collectively. In order to accurately account for false positives due to multiple testing, a sequential Bonferroni correction was applied [55], considering the multiple tests performed either within a given progeny/breeder-segregation across independent loci or for a given locus or pair of loci across the independent tested segregations/families.

Accordance of the observed segregations to Mendelian expectations was tested using a Chi^2 test to detect possible

Table 2: Microsatellite loci analysed with indications of GenBank accession number, repeat structure, and linkage group according to *O. niloticus* [47, 48, 51], optimised PCR conditions established from cross-species amplification study of microsatellites across 15 African cichlid species (labeled primer "*", annealing temperature and Magnesium concentration (mM)—Bezault et al., [52]); the size range and diversity of alleles within each parental species as well as the number of shared alleles between them and the presence of null allele (N) have been estimated from the set of individuals used for cross-priming analysis and the parents of all families studied here; the set of independent loci analysed in all backcross and F2 hybrid families are indicated in **bold**.

Loci	GenBank accession	Structure	Linkage group	PCR conditions	Range (bp)	Allelic diversity		Shared alleles
						O. niloticus	S. *melanotheron*	
UNH-008	G31346	perfect	17	R* 56/1.2	196–236	3	2	0
UNH-102	G12255	perfect	16	R* 50/1.2	132–185	4	4	0
UNH-103	G12256	perfect	17	R* 48/1.2	171–260	3	4	0
UNH-106	G12259	compound	3	R* 50/1.2	115–189	4	2 + N	1
UNH-115	G12268	compound	3	F* 50/1.5	100–146	3	1	0
UNH-117	G12270	interrupted		R* 54/1.2	108–146	1	2	1
UNH-123	G12276	perfect	12	F* 48/1.2	142–232	6	2	0
UNH-124	G12277	perfect	4	F* 54/1.2	295–324	4	1	0
UNH-125	G12278	compound	16	R* 48/1.5	134–198	6	4	2
UNH-129	G12282	interrupted	8	R* 48/1.2	180–253	7	4	1
UNH-130	G12283	perfect	23	R* 50/1.2	174–242	6	1 + N	0
UNH-131	G12284	perfect	3	F* 48/2.0	283–303	4	2 + N	0
UNH-132	G12285	perfect	9	R* 52/1.2	100–134	2	1 + N	0
UNH-135	G12287	interrupted	3	R* 50/1.5	124–284	6	4	1
UNH-138	G12290	perfect	16	R* 48/1.5	144–250	7	2	0
UNH-142	G12294	interrupted		F* 48/1.2	142–192	3	2	0
UNH-146	G12298	interrupted	4	F* 60/1.0	111–149	3	3	1
UNH-149	G12301	perfect	5	R* 48/1.5	143–225	4	3	0
UNH-154	G12306	perfect	6	R* 50/1.2	98–176	8	5	2
UNH-159	G12311	perfect	2	R* 55/1.2	205–267	5 + N	3	1
UNH-162	G12314	perfect	4	R* 48/1.5	125–252	6	2	0
UNH-169	G12321	interrupted	5	R* 54/1.2	124–240	8	4 + N	1
UNH-173	G12325	perfect	13	F* 55/1.2	124–188	2	1 + N	0
UNH-174	G12326	perfect	20	F* 48/1.5	146–187	4	1	0
UNH-189	G12341	perfect	12	R* 52/1.2	135–208	3	3	1
UNH-190	G12342	compound	21	R* 60/1.0	133–202	2	1	0
UNH-197	G12348	interrupted	23	R* 50/1.2	154–228	6	5	0
UNH-207	G12358	interrupted	6	R* 60/1.2	90–198	3	4	1
UNH-211	G12362	perfect	19	R* 48/1.5	82–194	6	7	1
UNH-216	G12367	perfect	23	R* 52/1.2	126–212	2	4	0
				Average across loci		4.3	2.8	0.48
				Polymorphism *P* (0.95)		97%	77%	

distortion of segregation. Sequential Bonferroni correction of *P*-value for multiple tests was performed across loci separately within each cross. The balance of global meiotic transmission of both parental alleles was tested within each hybrid segregation using a Chi^2 test across all loci within each breeder segregation. Homogeneity of reciprocal backcrosses was tested by comparison of genotypic distributions between families for each locus using Chi^2 tests, with global tests combining these results across loci for each families comparison using Fisher's method [56]. Comparisons were carried out between progeny from the same hybrid type (G1 or G′1), or the same hybrid way (♀ or ♂), and overall cases. Pairwise linkage analysis was performed using LinkMFex [57] using an LOD score of 3 as threshold for significance. Linkage associations with a $2 \leq$ LOD Score < 3 were considered as suggestive. Recombination rates were compared using a Chi^2 test.

3. Results

3.1. Microsatellite Diversity. A high percentage of the markers were polymorphic in both species (Table 2), with a slightly lower diversity in *S. melanotheron* (77%) than in *O. niloticus* (97%). A similar pattern was observed for the mean allelic diversity per locus and species (2.8 and 4.3, resp.). Null alleles were detected for a total of 7 markers, with a higher frequency

Table 3: Test of balanced meiotic transmission of alleles from both parental species (On for *O. niloticus* and Sm for *S. melanotheron*) for each of the 8 hybrid F1 breeders (G1 (♀ On × ♂ Sm) and G′1 (♀ Sm × ♂ On)); Chi^2 values and associate *P*-values are given; significant (i.e., biased transmission) tests ($a = 0.05$) when applying sequential Bonferroni correction (n = independent tests) are indicated in **bold**.

Families	BC-A		BC-B		BC-C		BC-D		F2-A				F2-B			
Breeders	♀ G1		♂ G1		♀ G′1		♂ G′1		♀ G′1		♂ G′1		♀ G1		♂ G′1	
# loci	28		28		24		23		6		8		9		9	
Allele origin	On	Sm	On	Sm	On	Sm	On	Sm	On	Sm	On	Sm	On	Sm	On	Sm
n_{exp}	698	698	698	698	597	597	573	573	150	150	198	198	225	225	225	225
n_{obs}	682	713	710	686	583	611	506	639	137	162	195	201	203	247	251	199
Chi^2	0.689		0.413		0.657		15.449		2.09		0.091		4.302		6.009	
P	0.407		0.521		0.418		**8.47E − 05**		0.148		0.763		0.038		0.014	

in *S. melanotheron* ($n = 6$) than *O. niloticus* ($n = 1$). Markers showed a low percentage of shared alleles (7.7%) between the two parental species involved in the hybridisation, which allowed the accurate identification of the genetic origin of the alleles segregating in hybrid meiosis. Overall, the level of polymorphism present in both parental species population was sufficient to obtain a high frequency of informative segregation across the different types of progeny: 55% of the pure *O. niloticus* segregations appeared informative, 43% of *S. melanotheron* and 79.5% of hybrids segregations (90% in BC and 57% in F2—details in Supplementary Table S1).

3.2. Analysis of Meiotic Segregation. A total of 4 out of 232 segregations (1.7%) showed significant evidence of segregation distortion ($P < 0.05$ after correction for multiple testing). Three significant tests were observed in hybrid genomes (2 in ♂ G′1, UNH-008 & UNH-216, and 1 in ♀ G1, UNH-197) and a single significant test in pure species (♀ *S. melanotheron*, UNH-135) (details in Supplementary Table S1).

Out of the 8 hybrid parents analysed, equal transmission of alleles from both parental and species origin was observed in the vast majority of the cases ($n = 7$), representing at least one meiotic segregation of each hybrid cross (G1 and G′1) and sex (Table 3). The hypothesis of equal transmission of parental species alleles was rejected in a single hybrid male (BC-D family), which globally undertransmitted its paternal alleles of *O. niloticus* origin (Table 3). This was consistent with the results from the locus-level analysis for this individual, which revealed evidence of unequal allele transmission for 2 significant loci from 3 different LGs after Bonferroni correction, always in the direction of undertransmission of *O. niloticus* paternal alleles (details in Supplementary Table S1).

When we considered allelic distributions observed among backcross progeny, according to hybrid type and/or sex, only the comparison between hybrid males exhibited significant heterogeneity ($P < 0.01$) (Table 4). However, the comparison between the 2 types of hybrids, G1 versus G′1, and globally among the 4 different sex and hybrid types did not reveal any evidence of deviation from homogeneity.

Genetic linkage was detected in 16 cases (Table 5), of which 14 cases were expected from previous publications [48–51], but 2 being unexpected among these studies. The large majority of expected linkage associations (73%) were confirmed, including the 2 unexpected cases (i.e., UNH-008 & UNH-146, and UNH-154 & UNH-207, with LOD > 3 in 5 and 7 segregations, resp.). Three expected associations were not observed: UNH-008 & UNH-103 (LG 17) showed no cosegregation, whereas UNH-102 & UNH-138 (LG 16) and UNH-130 & UNH-197 (LG 23) showed a suggestive linkage (LOD > 2), but only in one case for each of these pairs. One unexpected linkage (UNH-008 & UNH-124) was significant in 6 parents. The other unexpected association (UNH-106 & UNH-207) was only found in one parent out of 3. Finally one unmapped marker, UNH-117, was assigned to the LG 5 with a significant linkage in 2 parents. The occurrence of these linkage associations was checked comparatively between the 2 pure and the hybrid genomes. All but one was significant in *S. melanotheron* parents, and every linkage significantly established in *O. niloticus* showed significant linkage in hybrid parents.

Table 4: Comparisons of allelic distributions observed among the 4 backcross progeny; the number of loci implicated, the Fisher's test value and the associated *P*-values are given; significant heterogeneity ($a = 0.05$) is indicated in **bold** (see Table 1 for detail about hybrid types).

Comparisons	# Loci	Fisher's test	*P*
Global	25	65.51	0.069
Hybrids G1	26	44.91	0.747
Hybrids G′1	20	54.46	0.063
Hybrids ♀	24	49.08	0.429
Hybrids ♂	22	68.8	**0.010**
♂ G1/♀ G′1	23	42.82	0.606
♀ G1/♂ G′1	21	55.93	0.074

Recombination rates were compared using a Chi^2 test between parents of opposite sex and/or genetic types in pure and hybrid individuals (see details in Supplementary Table S3). Out of 16 pairs of linked loci (i.e., for which a significant linkage has been detected in at least one parent), heterogeneity of recombination rate in at least one comparison was observed for 2 pairs of loci (i.e., UNH-125 & UNH-138 (LG16) and UNH-131 and UNH-135 (LG3)). These 2 cases where homogeneity of recombination rate was rejected were detected in comparisons involving pure-species parents.

Table 5: Comparison of linkage associations expected from tilapia genetic maps and observed within the 7 studied families; linkage group (LG) is taken from the previously published genetic maps (A: [48]; B: [50]; C: [49]; D: [51]); the recombination rate (Rec) is given with the associated LOD score; significant linkages (LOD > 3) are represented in **bold**, while suggestive linkages (LOD > 2) are underlined; see Table 1 for detail about hybrid types.

Families					BC-A				BC-B				BC-C				BC-D				P-Sm			
Locus A	LG	Locus B	LG	References	♀ G1		♂ O. nil.		♀ O. nil.		♂ G1		♀ G′1		♂ O. nil.		♀ O. nil.		♂ G′1		♀ S. mel.		♂ S. mel.	
					Rec	LOD	Rec	LOD	Rec	LOD	Rec	LOD	Rec	LOD	Rec	LOD	Rec	LOD	Rec	LOD	Rec	LOD	Rec	LOD
UNH-008	17	UNH-103	17	A, D	0.42	0.28	0.42	0.28	0.46	0.07			0.35	0.9					0.42	0.28				
UNH-008	17	UNH-124	4		**0.10**	**7.74**	**0.08**	**8.73**	**0.20**	**3.97**	**0.09**	**7.95**	**0.06**	**9.58**					**0.22**	**3.61**				
UNH-008	17	UNH-146	4	B versus A, D	**0.08**	**9.00**	**0.02**	**12.92**			**0.00**	**15.05**	**0.00**	**14.45**					**0.04**	**11.4**				
UNH-102	16	UNH-125	16	A, C	0.36	0.86			0.28	2.18	0.26	2.61	0.3	1.79	0.32	1.44			0.36	0.86	**0.24**	**3.08**	**0.22**	**3.61**
UNH-102	16	UNH-138	16	A, C	0.38	0.63			0.28	2.18	0.38	0.63	0.36	0.86	0.4	0.44			0.41	0.3			0.46	0.07
UNH-106	3	UNH-207	6								**0.24**	**3.08**					0.44	0.16	0.42	0.28				
UNH-115	3	UNH-131	3	A, D	**0.1**	**7.99**					**0.10**	**7.99**	**0.04**	**10.56**	**0.23**	**3.04**			**0.02**	**12.05**				
UNH-115	3	UNH-135	3	A, D	**0.02**	**12.92**					**0.04**	**11.4**	**0.02**	**12.92**	**0.02**	**12.92**			**0.00**	**15.05**				
UNH-117		UNH-149	5		**0.02**	**12.34**					**0.00**	**15.5**							NT					
UNH-123	12	UNH-189	12	A, C, D	**0.12**	**7.08**	**0.24**	**3.08**	**0.12**	**6.84**	**0.18**	**4.6**	**0.2**	**4.19**					**0.12**	**7.08**				
UNH-124	4	UNH-146	4	C, D	**0.06**	**9.85**	**0.10**	**7.74**			**0.09**	**7.95**	**0.06**	**9.85**	**0.10**	**7.74**			**0.18**	**4.82**				
UNH-125	16	UNH-138	16	A, B, C, D	**0.02**	**12.92**	**0.08**	**9.00**	**0.00**	**15.05**	**0.12**	**7.08**	**0.06**	**10.12**	**0.08**	**9.00**	**0.00**	**13.85**	**0.04**	**10.27**			**0.24**	**3.08**
UNH-130	23	UNH-197	23	A, C	0.46	0.07									0.28	2.18			0.48	0.02				
UNH-130	23	UNH-216	23	A, C	0.33	1.31	**0.02**	**12.63**											0.44	0.16				
UNH-131	3	UNH-135	3	A, D	**0.08**	**9.00**	**0.04**	**11.4**			**0.06**	**10.12**	**0.02**	**12.05**	**0.21**	**3.58**			**0.02**	**12.05**			**0.04**	**11.12**
UNH-154	6	UNH-207	6	A versus D	**0.02**	**12.92**			**0.02**	**12.92**	**0.02**	**12.92**	**0.04**	**11.4**			**0.02**	**12.92**			**0.00**	**14.75**	**0.04**	**11.12**
UNH-197	23	UNH-216	23	A, D	**0.23**	**3.42**					0.20	4.19							0.08	9.00	0.13	**6.60**	**0.08**	**8.47**

In both cases, evidence of heterogeneity was observed within species (in *O. niloticus* comparisons, between sexes and among males, resp.) as well as between species (i.e., between *O. niloticus* and *S. melanotheron*). No evidence of significant heterogeneity was observed among hybrid meioses.

4. Discussion

We have characterized meiotic segregation in experimental intergeneric hybrids between two highly differentiated Oreochromine cichlid species, *O. niloticus* and *S. melanotheron*, from which no natural hybrid has ever been reported, due to ecological and reproductive behaviour divergence, even if sympatric in some basins of West Africa [58–60]. Our analysis permitted to track the origin and transmission of alleles across 17 independently mapped anchors distributed over the tilapia genome, including 8 genomic segments represented by 2 to 4 linked loci to survey variation of recombination between hybrids, parental species, and/or sexes.

All hybrid parents showed systematic exclusion of the alleles from each of the two parental species (i.e., hybrid parents transmitted either the *O. niloticus* allele or the *S. melanotheron* allele but never both or none). The strict disjunction of both *O. niloticus* and *S. melanotheron* alleles at each locus confirms locus homology between these two species and indicates the diploid state of these hybrids, at both the sequence and genome organisation level. The balanced segregations of loci lead to Mendelian transmission of both parental species alleles to the next generation for both reciprocal ways of crosses (G1 & G′1). A stable hybrid gene pool was maintained through meiosis, although segregation distortions were observed in very few cases (1.7%). As we observed no preferential allele elimination, neither in terms of parental species nor cross-direction (maternal/paternal origin), the observed departures from expected Mendelian inheritance may be due to local meiosis irregularities (i.e., male G′1 hybrid from BC-D), rather than a general effect of hybridisation.

The vast majority of linkage associations between markers expected in *O. niloticus* based on existing genetic maps [48–51] were confirmed in this study. Moreover these associations appeared well conserved in *S. melanotheron* and hybrids. This is consistent with a high level of synteny conservation between tilapia species, as previously observed between the closely related *O. niloticus* and *O. aureus* [50] and further extends this observation to distantly related species among Oreochromines. Recombination rates appear very similar between sexes as well as among hybrids and pure species. We did not observe a contraction of the genetic map as may be expected in hybrids because of reduced recombination due to structural genomic differences between parental species [50]. Taken together our results allow us to reject the hypothesis of meiotic irregularities or specific meiotic processes (i.e., nonhomoploid biparental transmission) in the hybrid between *O. niloticus* and *S. melanotheron*.

Our study also allowed us to demonstrate Mendelian transmission during the F1 hybrid meiosis, independent of the sex and/or direction of crosses (G1 or G′1), leading to the maintenance of a stable and balanced hybrid gene pool through generations. These results, especially the strict disjunction of parental alleles at each locus and the conservation of linkage groups without genetic map contraction, suggest a close genomic structure between both parental species and their hybrids [50, 51]. The analysis of phenotypic traits segregating in hybrids is particularly interesting in this context, especially considering the numerous divergent traits between the two parental species, such as morphology, reproductive behaviour, and physiological adaptive capacity. Physiological traits, for example, growth and salinity tolerance, show intermediate phenotypes in F1 hybrids [44, 61]. In experimental conditions, the fertility of hybrids has been established up to the F2 and viability up to F3 (for both hybrid and backcross progeny—unpublished data). Fertility problems have been previously reported in interspecific fish hybrids (see review by Bartley et al. [9]), not only at the initial hybrid stage (i.e., sterility in F1 generally due to ploidy perturbations) but also in subsequent hybrid generations, as in the case of hybrid models where high fitness is observed in early hybrids (F1) followed by a strong fitness breakdown in subsequent generations (F2+). Such examples were observed in tilapia during a complex four-species crossing project between three *Oreochromis* and one *Sarotherodon* [49], where the viable and fertile two-way F1 hybrids led to four-way F2 hybrids unable to produce viable progeny [62]. Such rapid introgression of four genomes in only two generations might have exacerbated instability of the resulting gene pool leading to fertility problems. This phenomenon would have been avoided or strongly reduced in case of a two-way hybridisation and/or increasing the number of generations to mix the genomes, especially if it involved an introgressive (backcross) scheme and/or closely related parental species. In any case, the existence of a late fitness disruption in hybrids tends to suggest the absence of major genomic reorganisation between Oreochromines species, in favour of small genomic rearrangement(s), for example, microinversions and insertions, structural variations and/or divergent gene evolution. These elements can all be potentially responsible for the establishment of postzygotic isolation resulting from the increase of Dobzhansky-Muller genetic incompatibilities [63]. Over time, both premating and postmating incompatibilities are expected to accumulate between divergent lineages, with a relative rate depending on different parameters, including the geographical mode of speciation, the existence of sexual dimorphisms, and/or strong sexual selection, overall yielding to the "speciation clock" [64].

As cichlids are known to exhibit sexual selection, typically associated with male breeding colour polymorphism [65], theory predicts a faster loss of premating than postmating compatibility. In the species used in this study, pre-mating isolation is total, most notably due to the divergence of reproductive behaviour between the two parental species (i.e., *O. niloticus* is a maternal mouthbrooder whereas *S. melanotheron* is a paternal mouth-brooder, which explains the need for *in vitro* fertilisation for the production of F1 hybrids between these species). Post-mating isolation also appears to be relatively weak and probably only due to drift in the absence of reinforcement mechanisms between the two parental species. This process might be different depending

on the cichlid lineage considered, based on their rate of transition between reproductive systems. For instance, while Oreochromines exhibit maternal, paternal, and biparental mouth-brooding systems, the most species-rich lineage, Haplochromines, exhibits exclusively maternal mouth-brooding reproductive systems [66, 67]. The absence of pre-mating barrier based on reproductive behaviour among species would either be compensated by other component(s) of pre-mating isolation system, such as sexual selection mediated by male colour polymorphism, or represent an overall lower level of premating isolation, which would be prone to generate an increased rate of postzygotic accumulation (i.e., through reinforcement process).

Two recent studies have investigated the dynamics of hybridisation in Haplochromine cichlids [68]. While phenotypic novelty does increase with the genetic distance between parental hybridizing lineages [68], the accumulation of reproductive incompatibilities is also building up with divergence time [69]. This later study demonstrates that, along the axis of species divergence, pre-mating isolation is built up fast initially (i.e., due to mate choice and sexual selection) without increasing much later (e.g., due to the highly conserved courtship behaviour and the absence of change in reproductive system across the entire lineage), whereas post-mating incompatibilities only start accumulating at relatively later stages of divergence. Such a pattern may have led to "evolutionary viable" hybridisation between lineages diverging for a very long time (i.e., estimation of the hybrid unviability after 4.4–18.4 Myrs, depending on the molecular clock calibration used [69]). Considering the latest estimates of divergence time between the radiation of the entire Oreochromines tribe (12.8–21.4 Myrs) and the radiation of the genus *Oreochromis* (6.4–9.7 Myrs) [18], the experimental hybrids between *O. niloticus* and *S. melanotheron* stand at the later end of the continuum tested in Haplochromines. Our results then appear consistent with the findings drawn from hybridisation in Haplochromines, while adding an independent estimate from another major cichlid tribe. Additionally, the present study extends the previous results by demonstrating the maintenance of normal meiotic mechanisms in hybrids between highly divergent species, long after the completion of pre-mating isolation (i.e., once post-mating isolation would be expected to have already started accumulating).

The important role of interspecific hybridisation during the evolution of cichlids, especially the processes of adaptation and diversification, has been extensively discussed [10]. Hybridisation is thought to occur when populations are subjected to important environmental changes, such as anthropogenic perturbations [22, 23, 59, 70] and hydroclimatic changes [20], and when populations invade a new environment [10], enabling these populations to undergo rapid adaptive response and even radiation. Evidence suggests that many of the largest cichlid lake radiations may have been initiated and fuelled through hybridisation between distantly related lineages [11–13], which invaded the new empty environment and started interbreeding to form a hybrid swarm, predisposing the colonizing lineages to diversify. One of the crucial issues in the hypothesis of hybrid swarm origin of adaptive radiations is to identify the mechanism by which hybrid lineage(s) can be initiated and propagated. Demonstrating that hybridisation between highly distantly related Oreochromines species can lead to meiotic processes following diploid Mendelian segregation and the maintenance of a stable and recombining hybrid gene pool across generations appears to strongly support this hypothesis. Overall, this study provides functional insights into hybridisation in cichlids, when prezygotic barriers are disrupted, despite important divergence time between lineages, and therefore supports the idea that interspecific hybridisation has the potential to play an important role in the evolution and diversification of cichlids.

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Research Article

Microsatellites Cross-Species Amplification across Some African Cichlids

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The transfer of the genomic resources developed in the Nile tilapia, *Oreochromis niloticus*, to other Tilapiines *sensu lato* and African cichlid would provide new possibilities to study this amazing group from genetics, ecology, evolution, aquaculture, and conservation point of view. We tested the cross-species amplification of 32 *O. niloticus* microsatellite markers in a panel of 15 species from 5 different African cichlid tribes: Oreochromines (*Oreochromis, Sarotherodon*), Boreotilapiines (*Tilapia*), Chromidotilapines, Hemichromines, and Haplochromines. Amplification was successfully observed for 29 markers (91%), with a frequency of polymorphic (P_{95}) loci per species around 70%. The mean number of alleles per locus and species was 3.2 but varied from 3.7 within *Oreochromis* species to 1.6 within the nontilapia species. The high level of cross-species amplification and polymorphism of the microsatellite markers tested in this study provides powerful tools for a wide range of molecular genetic studies within tilapia species as well as for other African cichlids.

1. Introduction

African cichlid fish are of extreme interest for both evolutionary biology and applied genetics purposes, including amazing models for speciation, adaptation, behaviour and neurosciences [1–5] as well as groups of major importance for aquaculture and fisheries (strain selection and improvement, stock assessment, etc.) [6–10]. A wide range of structural and functional genomic resources have been developed for cichlids in the past 15 years, predominantly in the Nile tilapia, *Oreochromis niloticus* [11–14]. While genome sequencing projects are in progress for several African cichlids, the transfer of genomic resources from *O. niloticus* across the entire group of tilapias *sensu lato* as well as other African cichlid tribes would provide powerful tools to support a wide range of evolutionary biology studies, including comparative phylogenetics, genome mapping, evolution of gene family sequence and expression, candidate gene analyses for adaptation, and population genetics.

Microsatellite markers are one of the most interesting resources to transfer across lineages, as they can provide numerous locus-specific molecular markers and putatively homologous sequences across taxa. In addition to their high level of polymorphism, the evolutionary conservation of the flanking region of microsatellite loci allows large-scale heterospecific amplification [15, 16], as previously shown in various animal groups, particularly fish [17–19]. However, the rate of cross-species amplification varies widely among taxonomic groups and loci [18, 20]. In addition to their application in population genetics, conserved microsatellite markers are particularly useful for population, species or hybrid identification (especially at early developmental

stages) and candidate-marker analysis, comparative genetic mapping, and QTL analysis. Furthermore, compared to anonymous multilocus genomic markers (RFLP, AFLP, ISSR) and SNPs, microsatellites present the important advantages of (i) being highly reproducible and very easily transferable between laboratory (with limited equipment and computational requirement), (ii) providing a high polymorphism information contain (PIC) per locus, and (iii) being highly cost efficient when only a small number of loci are needed. For these reasons, microsatellites markers are likely to remain popular for a wide range of ecology and evolutionary studies (e.g., relatedness and parentage analysis, population diversity and demography assessment, noninvasive genetic analysis, and conservation).

Since the first publication of microsatellite markers cloned in *O. niloticus* [13], thousands have been published and more than 500 have been positioned onto the genetic map of *O. niloticus* and the closely related *O. aureus* [14, 21]. These microsatellites have been used to map traits of interest, such as sex determination factors [22, 23], and have also been found to influence the expression of genes associated to physiological adaptation [24].

Outside the tilapias, microsatellite markers have been developed in a few different Haplochromines species: *Copadichromis cyclicos* [25], *Tropheus moorii* [19], *Pseudotropheus zebra* [26], *Astatoreochromis alluaudi* [27], *Pundamilia pundamilia* [28], *Metriaclima zebra* [29], *Pseudocrenilabrus multicolor* [30], *Paralabidochromis chilotes* [31], and *Astatotilapia burtoni* [32]. However these studies reported a smaller number of markers than that in Nile tilapia. The use of microsatellite markers in Haplochromines has been almost strictly restricted to descriptive population genetics and parentage/relatedness analysis, which represent only a subset of the possibilities offered by having a large set of genome-anchored microsatellite markers, as available for *O. niloticus*.

Additionally, microsatellites developed outside tilapias were derived exclusively from the most species-rich group of African cichlids and there are very limited genomic resources in all the other "under-studied" African cichlid tribes [33–35].

Considering the central position occupied by the Tilapiines *sensu lato* in the African cichlid phylogeny [38], their large diversity within at least 3 monophyletic clades [39–41], and the important number of species involved in population transfers, hybridisation, and/or invasion [8, 42], we decided to investigate the cross-species amplification efficiency of Nile tilapia microsatellites among the different groups of the Tilapiines *sensu lato* as well as three other African cichlid tribes, to extend the use/availability of this resource across a wide range of African cichlid species, including "under-studies" groups. The panel of species investigated then spans a large section of the African cichlid radiation, with an estimated overall divergence time of 33.4–63.7 Myrs [41, 43].

2. Material and Methods

Tests of cross-species amplification were conducted in a panel of 15 African cichlid species, representing all three major genera of Tilapiines *sensu lato*: 7 *Oreochromis*, 2 *Sarotherodon*, both genera belonging to the Oreochromines, and 3 *Tilapia* (*Coptodon*), belonging to the Boreotilapiines; as well as representatives of 3 other African cichlid tribes, including the derived Haplochromines, and two more basal tribes, the Chromidotilapiines and the Hemichromines (see details in Table 1). Analyses were conducted using 3 to 9 individuals per species (Table 1). Genomic DNA was extracted from fin clips stored in ethanol using a standard phenol-chloroform protocol [44].

The panel of 32 microsatellites was selected from the markers isolated in *O. niloticus* [13]. Genotyping was obtained by PCR amplification with radioactive (P^{33}) labeled primers [44, 45]. Allele variants were separated on 6% acrylamide gel electrophoresis. For each marker, the annealing temperature and $MgCl_2$ concentration were adjusted to optimise the efficiency of PCR amplification based on *O. niloticus* and two others species: one closely related among *Oreochromis* (*O. mossambicus*) and one distantly related among the Oreochromines (*S. melanotheron*). Cross-species amplifications were carried out using these conditions in the 15 studied species (Table 2). For each microsatellite marker, the amplification success has been estimated qualitatively on a 4-level scale based on the quality of the electrophoresis pattern across the test individuals (i.e., "++" for strong and sharp amplification pattern, "+" for good quality pattern with some stutters, echo-alleles or low intensity, "−" for high variance of amplification quality across individuals, very high level of stutter, and/or high frequency of null alleles, and "−−" very poor quality pattern, nonspecific or lack of, amplification). For each locus by species combination ($n = 480$), we assessed the amplification success and counted the number of different alleles among individuals. The presence of putative null alleles (i.e., nonamplified alleles) was inferred when a few individuals consistently showed an absence of allele amplification while other individuals from the same species showed high-quality amplification pattern or in the complete absence of heterozygous individuals. Echo-alleles (i.e., supplementary allele coamplifying across individuals producing amplification pattern consistently representing 2 or 4 alleles per individuals, with the longest allele separated from the shortest "cosegregating" allele by an identical length across individuals/alleles) were also identified. Furthermore, the rate of amplification success, the frequency of polymorphic loci (P_{95}), and the mean number of allele per locus were calculated per species, genus, and tribe across all studied microsatellites markers.

3. Results and Discussion

Very high rates of microsatellite amplification and polymorphism were observed (both 97%), in the Nile tilapia, with a mean number of alleles per locus of 4.3. Across the 14 other test species, 29 loci gave good quality amplifications (91%-Tables 2 and 3), while 3 markers (9%) showed a high discrepancy of amplification efficiency and/or unclear amplification pattern (Table 2; *see details in supplementary material* which

Table 1: Species studied for cross-species amplification tests, with geographic origin, and number of samples analysed per species.

Lineages	Genus	Species	Geographic origin	*n*
Oreochromines				
	Oreochromis			
		O. (*Oreochromis*) *niloticus*	Bouake (Cote d'Ivoire)*	9
		O. (*Oreochromis*) *aureus*	Lake Manzala (Egypt)	5
		O. (*Oreochromis*) *mossambicus*	Mozambique	5
		O. (*Oreochromis*) *shiranus*	Lake Malawi	5
		O. (*Nyasalapia*) *macrochir*	Bouake (Cote d'Ivoire)**	5
		O. (*Nyasalapia*) *saka*	Lake Malawi	5
		O. (*Nyasalapia*) *squamipinnis*	Lake Malawi	5
	Sarotherodon			
		S. (*Sarotherodon*) *galilaeus*	Bamako (Niger)	3
		S. (*Sarotherodon*) *melanotheron*	Ébrié Lagoon (Ivory Cost)	5
Boreotilapiines				
	Tilapia			
		T. (*Coptodon*) *dageti*	Bamako (Niger)	5
		T. (*Coptodon*) *guineensis*	Ivory Cost/Senegal	4
		T. (*Coptodon*) *zillii*	Lake Manzala (Egypt)	5
Haplochromines				
	Haplochromis			
		Haplochromis sp. "rock kribensis"	Lake Victoria	3
Chromidotilapines				
	Chromidotilapia			
		Chromidotilapia guntheri	Bamako (Niger)	3
Hemichromines				
	Hemichromis			
		Hemichromis bimaculatus	Bandama (Ivory Cost)	5

Introduced stocks: *with mixed origin (Volta and Nile) [36]; **from wild population (RDC) [37].

is available online at doi:10.1155/2012/870935: Table S1). Excluding the Nile tilapia, the average intraspecific rate of successful amplification and polymorphism across the panel of 32 markers was more than 70% (Table 3).

The expected relationship between the success of cross-species amplifications and evolutionary distance from marker cloning species [15, 20] was observed, reflecting the phylogenetic relationships between the different groups of African cichlids [39–41] (Table 3; *see details in supplementary material:* Table S2). Within the Tilapiines *sensu lato*, species from both mouth-brooder genera (i.e., *Oreochromis* and *Sarotherodon*), constitutive of the monophyletic clade of the Oreochromines diverged 12.8–21.4 Myrs ago, showed very high and similar amplification (88% and 86%, resp.) and polymorphism (76% and 85%, resp.) rates, whereas species from the genus *Tilapia*, belonging to the Boreotilapiines with a divergence time from Oreochromines of 30.6–39.6 Myrs, showed lower rates of amplification (67%) and polymorphism (59%). The three other African cichlid tribes exhibited lower values for amplification and polymorphism rates: 38% and 50%, respectively, in the more derived lineage, Haplochromines, whereas a more heterogeneous pattern was found for the two more basal lineages, Chromidotilapiines (i.e., 47% and 20%, resp.) and Hemichromines (i.e., 19% and 50%, resp.). Allelic diversity varied with the same trends with a mean number of alleles per locus and species ranging from 3.7 and 3.3, respectively, for *Oreochromis* spp. and *Sarotherodon* spp. to 2.4 for *Tilapia* spp. and 1.6 in average (from 1.4 to 2.3) for the non-Tilapiines groups. The frequency of loci with putative null alleles also appeared to increase in the more distant species (*supplementary material:* Table S2). Rather than strictly reflecting reductions in polymorphism and/or the loss of the marker loci with increasing phylogenetic distance from the species in which the marker was cloned, these relationships are caused by mutations in the flanking regions complementary to the PCR primers. The conservation of microsatellites loci in the genomes has been shown to be potentially very long, and anyway much longer than the divergence time allowing successful cross-species amplification based on a given pair of primers, generally designed based on the only knowledge of the locus sequence in the species of cloning. The global success of cross-species amplification of a given microsatellite marker and/or the recovery of its different allelic variant (i.e., elimination of null allele) could then be enhanced in target species by either a specific optimisation

Table 2: Microsatellite loci tested for cross-species amplification with indications of repeat structure observed in *O. niloticus* (according to Lee and Kocher, [13]), allele size range of the amplified fragment across all tested species, PCR and electrophoresis conditions (labeled primer, annealing temperature/magnesium concentration (mM)/electrophoresis Volt-hour), and amplification quality obtained after PCR optimisation tests (from very good ++ to poor −−; see detail of the categories in main text); loci presenting a wide cross-species amplification efficiency are in bold.

Loci	GenBank access No.	Structure	Range (bp)	PCR and electrophoresis conditions	Amplification efficiency
UNH-008	G31346	Perfect	196–236	R* 56/1.2/6000	++
UNH-102	G12255	Perfect	132–185	R* 50/1.2/4500	++
UNH-103	G12256	Perfect	171–260	R* 48/1.2/6000	+
UNH-106	G12259	Compound	115–189	R* 50/1.2/3500	+
UNH-115	G12268	Compound	100–146	F* 50/1.5/3500	++
UNH-117	G12270	Interrupted	108–146	R* 5411.2/4500	++
UNH-120	G12273	Compound	—	R* 48/2/—	− −
UNH-123	G12276	Perfect	142–232	F* 48/1.2/4500	++
UNH-124	G12277	Perfect	295–324	F* 54/1.2/7500	++
UNH-125	G12278	Compound	134–198	R* 48/1.5/4500	+
UNH-129	G12282	Interrupted	180–253	R* 48/1.2/4500	+
UNH-130	G12283	Perfect	174–242	R* 50/1.2/4500	+
UNH-131	G12284	Perfect	283–303	F* 48/2/6000	−
UNH-132	G12285	Perfect	100–134	R* 52/1.2/3500	+
UNH-135	G12287	Interrupted	124–284	R* 50/1.5/4500	+
UNH-138	G12290	Perfect	144–250	R* 48/1.5/4500	+
UNH-142	G12294	Interrupted	142–192	F* 48/1.2/4500	++
UNH-146	G12298	Interrupted	111–149	F* 60/1/3500	++
UNH-149	G12301	Perfect	143–225	R* 48/1.5/4500	+
UNH-154	G12306	Perfect	98–176	R* 50/1.2/3500	++
UNH-159	G12311	Perfect	205–267	R* 55/1.2/6000	++
UNH-162	G12314	Perfect	125–252	R* 48/1.5/6000	++
UNH-169	G12321	Interrupted	124–240	R* 54/1.2/3500	++
UNH-173	G12325	Perfect	124–188	F* 55/1.2/4500	+
UNH-174	G12326	Perfect	146–187	F* 48/1.5/4500	++
UNH-189	G12341	Perfect	135–208	R* 52/1.2/4500	+
UNH-190	G12342	Compound	133–202	R* 60/1/4500	+
UNH-193	G12386	Perfect	—	R* 48/2/3500	− −
UNH-197	G12348	Interrupted	154–228	R* 50/1.2/4500	+
UNH-207	G12358	Interrupted	90–198	R* 60/1.2/3500	++
UNH-211	G12362	Perfect	82–194	R* 48/1.5/3500	++
UNH-216	G12367	Perfect	126–212	R* 52/1.2/3500	++

of the amplification conditions or the modification of the sequence of the primers. This is especially appropriate when target species are distantly related to the cloning species of the markers and initial cross-species tests reveal low level of polymorphism with potentially high frequency of null allele (which would heavily bias any allele frequency-based estimates).

To represent the multi-locus pattern of genetic diversity across the 15 study species, we performed a population-based correspondence analysis using the software Genetix [46]. This multivariate analysis conducted on the genotype matrix allows to represent the clustering pattern among the different species groups, as well as among individuals within each of them in a factorial space (F1, F2, F3). This analysis allowed to clearly resolve the different species, except for *O. saka* and *O. squamipinnis* which are highly overlapping in the factorial space (Figure 1). Three separate groups of species were defined: the Oreochromines species, with all *Oreochromis* and *Sarotherodon* species, the Tilapia species, and all non-Tilapiines species. This clustering pattern reflects the phylogenetic relationships between the two tribes of Tilapiines *sensu lato*, that is, Oreochromines and Boreotilapiines. However the clustering of the three other tribes, which represent the most distant taxa from the source species, reveals the influence of the overall reduced polymorphism in highly distant taxa. This points out the limits of microsatellite size polymorphisms to estimate genetic divergence and/or phylogenetic relationship between too distantly related taxa, due to allele size homoplasy and/or increase of null allele frequency [19].

Table 3: Results of cross-species amplification performed over the 32 tested microsatellite loci on the 15 African cichlid species studied, including amplification rate, polymorphism rate, and mean number of alleles per locus, estimated per genus and tribe.

Groups	*N* species	Amplification rate	Polymorphism (P_{95})	Mean allele number per locus		% shared alleles per
				Per group	Per species	
O. niloticus		97%	97%	—	4.3	—
Oreochromis spp.*	6	88%	76%	17.8	3.7	37%
Sarotherodon spp.	*2*	86%	85%	6.4	3.3	9.2%
Tilapia spp.	*3*	67%	59%	6	2.4	19.7%
Tilapiines*	11	82%	74%	24.3	3.7	20.5%
non-Tilapiines	3	34%	36%	3.4	1.6	2.3%
Haplochromines	1	38%	50%	—	1.6	—
Chromidotilapines	1	47%	20%	—	1.4	—
Hemichromines	1	19%	50%	—	2.3	—
Total*		72%	70%	25.7	3.2	5.3%

* Excluding *O. niloticus.*

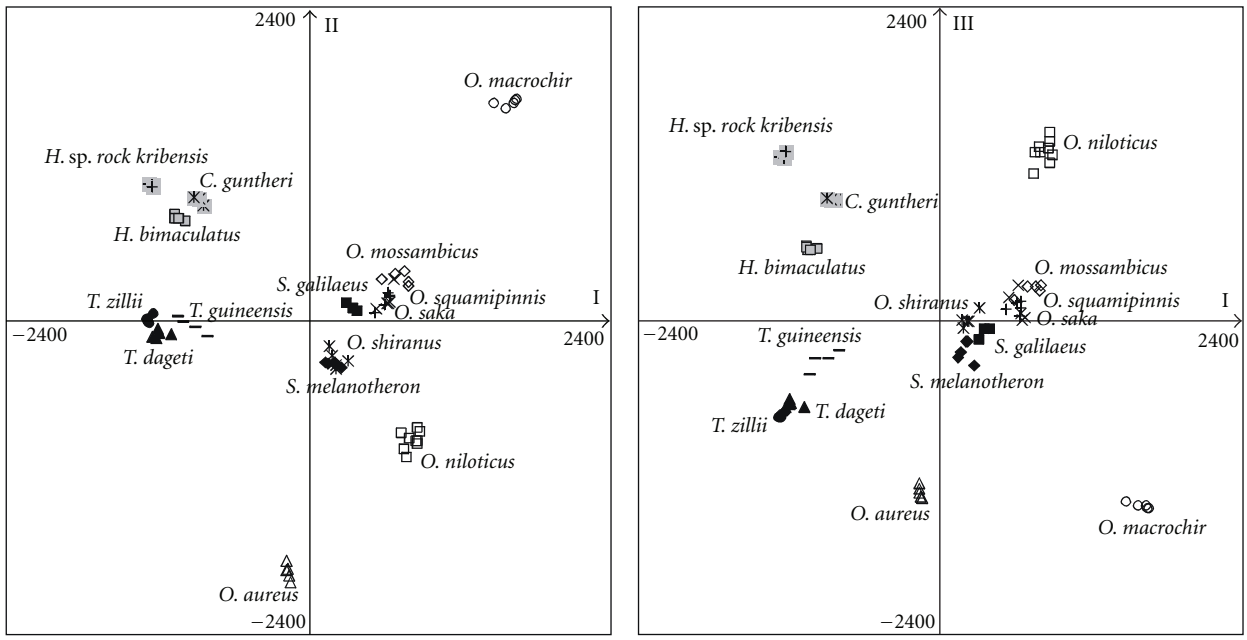

Figure 1: Clustering of the 15 study species based on multilocus diversity: correspondence analysis based on the individual genotypes over the 29 microsatellites loci successfully amplified and performed on the barycentre of the species: (a) factorial plane F1-F2 and (b) factorial planes F1–F3.

4. Conclusion

This study provides a quantitative estimate of the transferability of *O. niloticus*-derived microsatellites markers across 5 divergent African cichlid tribes, from the highly studied Haplochromines group to less studied tribes as Oreochromines, Boreotilapiines, Chromidotilapiines, and Hemichromines. The high rate of cross-species amplification and polymorphism highlights the usefulness of microsatellites markers for comparative genetic studies within Oreochromines and other African cichlids tribes, including stock/species identification, comparative genome mapping, candidate genes, or hybridisation surveys. Despite the fast growing opportunities to produce large-scale genomic data in nonmodel organisms, we believe that highly polymorphic, locus-specific markers such as microsatellites will continue to be useful for a wide range of genetic analyses in African cichlids.

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Research Article

Genetic and Morphological Evidence Implies Existence of Two Sympatric Species in *Cyathopharynx furcifer* (Teleostei: Cichlidae) from Lake Tanganyika

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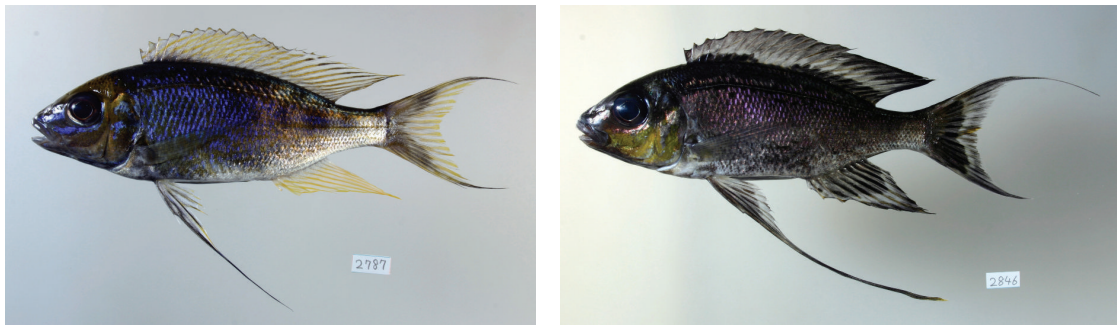
Although the cichlid fishes from Lake Tanganyika are treated as a textbook example of adaptive radiation, many taxonomic problems remain unresolved. *Cyathopharynx furcifer*, which belongs to the currently monospecific genus *Cyathopharynx*, contains two colour morphs at the southern end of the lake: one has a yellow anal fin, and the other has a black anal fin. Some books for hobbyists of ornamental fish treat these morphs as different species, but taxonomic studies have neither mentioned the existence nor addressed the status of these colour morphs. In the present paper, we analysed these two colour morphs using mitochondrial, microsatellite, morphometric, and meristic data sets. Both molecular and morphological data allowed clear discrimination between these morphs, suggesting the existence of two distinct sympatric species. Three taxonomic species have been described in this genus, and only *C. furcifer* is currently considered valid. Observations of type specimens of these three nominal species will be needed to determine the scientific names of these colour morphs.

1. Introduction

Lake Tanganyika is one of the ancient lakes of the East African Rift Valley. This lake harbours about 250 cichlid species, and 98% of these species are endemic to the lake [1]. These fish exhibit high morphological, behavioural, ecological, and genetical diversification, and are treated as a textbook example of adaptive radiation (e.g., [2–7]).

Cyathopharynx Regan is one of the genera belonging to the endemic tribe Ectodini from Lake Tanganyika [8, 9]. This genus is morphologically well defined, namely, fish of this genus have small scales on the sides of the body (48–64 scales in longitudinal line), a lower pharyngeal bone with a rounded posterior margin, and in males, long pelvic fins. These morphological features are also found in some other genera of Ectodini [8], but only *Cyathopharynx* has all of these features combined. A phylogenetic study based on mitochondrial DNA does not contradict the monophyly of *Cyathopharynx* and shows that this genus nest within a monophyletic group including *Ophthalmotilapia* Pellegrin and *Cardiopharynx* Poll [10]. Three species have been described in *Cyathopharynx*: *C. furcifer* (Boulenger) (originally described as *Paratilapia furcifer* in 1898 [11]), *C. foae* (Vaillant) (originally described as *Ectodus foae* in 1899 [12]), and *C. grandoculis* (Boulenger) (originally described as *Tilapia grandoculis* in 1899 [13]). The latter two nominal names are currently considered as junior synonyms of *C. furcifer*, and only *C. furcifer* is considered valid in this genus [8, 14].

Cyathopharynx furcifer is a common species in rocky shorelines of the lake and exhibits sexual dimorphism: males have a colourful, iridescent body, and elongated pelvic fins, whereas females are not colourful and their pelvic fins are moderate in length. This fish is a maternal mouth-brooder. Mature males build mating craters on the sandy lake bottom or on the flat surface of a large stone, to which they attract females. Females deposit eggs in the crater, and pick them up into their mouths before leaving the crater [15–17]. The function of the craters is not well known, but the size and neatness of craters may provide conspecifics with



(a) (b)

Figure 1: Two colour morphs of *Cyathopharynx furcifer*. (a) YA, male, 127.8 mm SL. (b) BA, male, 114.4 mm SL.

information about the owner's size, capability, and condition [17].

At Kasenga at the southern end of the lake, two colour morphs exist in males of *C. furcifer* (Figure 1). One morph has a bluish body, orange forehead, and a yellow anal fin (hereafter YA, which means yellow-anal-fin morph), while the other morph has a blackish body, orange cheeks, and a black anal fin (hereafter BA, which means black-anal-fin morph). No males with intermediate or mixed colour patterns between the morphs have been found. Some books for hobbyists of ornamental fish treat YA as *C. furcifer* because the body colouration of this morph accords with that of the type specimens of *C. furcifer*, and BA as *C. foae* (or *C. foai*) without any distinct reason [18]. However, taxonomic studies have neither mentioned the existence nor addressed the status of these sympatric colour morphs. In the present study, molecular and morphological analyses were conducted to test whether these sympatric morphs are different species.

2. Methods

2.1. Fish Samples. Fish were collected at Kasenga near Mpulungu, Zambia, at the southern end of Lake Tanganyika, with a screen net in November and December 2006. The right pectoral fins of the fish were fixed in 100% ethanol for DNA extraction. The bodies of the fish were fixed in 10% formalin and preserved in 50% isopropyl alcohol for morphological examination. The sex of the fish was determined from the shape of the genital papilla. Only large males with fully expressed body colour were used for molecular and morphological analyses in order to avoid misidentification of morphs ($N = 32$, 100.7–137.3 mm standard length (SL) in YA, $N = 32$, 121.5–138.8 mm SL in BA).

2.2. DNA Extraction and Amplification. Total DNA was extracted using an AquaPure Genomic DNA Kit (Bio-Rad). Polymerase chain reaction (PCR) was conducted using a PC 818 Program Temp Control System (Astec) for the amplification of the mitochondrial DNA (mtDNA) and the microsatellite loci using the following programme: one cycle of 94°C for 2 min; 30 cycles of 94°C for 15 s, annealing temperature specific to each primer set for 15 s, 72°C for 30 s; one cycle of 72°C for 7 min.

A partial mtDNA sequence, including a portion of cyt *b* (1125 bp), was amplified with the primers H15915 [19] and L14724 [20] (annealing temperature 53°C). The PCR fragments of the mtDNA were purified using the ExoSAPIT enzyme mix (USB), directly sequenced with BigDye sequencing chemistry (Applied Biosystems), and analysed on an ABI 3130xl sequencer (Applied Biosystems). Sequences are available in the DNA Data Bank of Japan (DDBJ Accession no. AB691241–AB691304).

Five microsatellite loci were used for genotyping: GM264 [21], Pzeb4 [22], Ttem8 and Ttem9′ [23], and UNH2050 [24] (annealing temperature 55°C). Forward primers were labelled with florescent dye NED (GM264), HEX (Pzeb4, UNH2050), or 6-FAM (Ttem8, Ttem9′). The microsatellite loci were analysed on an ABI 3130xl Sequencer using internal size marker Genescan 400 HD (Applied Biosystems).

2.3. Analyses of Molecular Data. For the mtDNA sequences, a haplotype network was constructed from the maximum-likelihood (ML) and maximum parsimonious (MP) trees, which were translated into maximum parsimony branch lengths in PAUP* version 4.0b10 [25]. The ML tree was generated based on the HKY model selected by hierarchical likelihood ratio tests implemented in ModelTest 3.5 [26].

Departure from Hardy-Weinberg (HW) equilibrium for every microsatellite locus and linkage disequilibrium (LD) for all pairs of loci were tested within each of the two morphs using Arlequin version 3.11 [27] (100 000 steps in the Markov chain, 1000 dememorization steps in the HW test; 10 000 permutations in the LD test). Critical significance levels were corrected following the sequential Bonferroni procedure [28]. A Bayesian model-based clustering algorithm was implemented in Structure 2.3.3 [29] to test the assignment of K ancestors with admixture and independent allele frequency models (100 000 iterations were run after an initial burn-in period of 50 000 iterations). K was set from 1 to 5, and 10 independent runs were performed for each K. The value of $K = 2$ was chosen, which showed the highest ΔK [30].

Genetic differentiation between the morphs was assessed by analyses of molecular variance (AMOVA) for

both mtDNA and microsatellite data as implemented in GENALEX version 6.41 [31]. Genetic significance tests between morphs were conducted using 9999 permutations.

2.4. Morphological Data. Methods for measuring 13 morphometric characters (SL, body depth, length and width of head, snout length, eye length, interorbital width, lower jaw length, length and depth of caudal peduncle, dorsal fin base length, anal fin base length, and pelvic fin length) and counting 9 meristic characters (numbers of spines and soft rays in dorsal fin, number of anal fin soft rays, number of pectoral fin soft rays, number of scales in longitudinal line, numbers of scales on upper and lower lateral lines, number of gill rakers on lower limb of the most rostral gill-arch, and number of outer teeth on premaxillae) correspond with those of Snoeks [32], except for pelvic fin length, which was measured from the base to the tip of the longest ray. Measurements were taken to the nearest 0.1 mm using dividers or digital callipers under a binocular microscope. The last two soft rays of dorsal and anal fins were counted as two soft rays, although those are sometimes counted as one soft ray in noncichlid fishes (i.e., [33]).

2.5. Analyses of Morphological Data. The 13 morphometric characters were $\log_{10}$ transformed. Twelve morphometric characters except for SL were analysed by the multivariate analysis of covariance (MANCOVA) with SL as covariate. The nine meristic characters were analysed by the multivariate analysis of variance (MANOVA, note that body size was not considered in this analysis because the meristic characters were not significantly correlated with SL : $F_{9,53} = 0.601$, $P = 0.791$). When the significant differences were found in these analyses, the analyses of covariance (ANCOVAs) with $\log_{10}$ transformed SL as covariate for the 12 $\log_{10}$ transformed morphometric characters and the analyses of variance (ANOVAs) for the 9 meristic characters were carried out in order to suggest which character was different between morphs. Critical significance levels were corrected following the sequential Bonferroni procedure [28].

The linear discriminant analyses (LDAs) were carried out in order to visualize the degrees of morphological differences between morphs. In the LDA based on the morphometric characters, each measured value was standardized with SL using the following formula:

$$Y'_{ij} = \log\left(Y_{ij}\right) - a_j \log(L_i), \quad (1)$$

where Y'_{ij} and Y_{ij} are the standardized and raw values of character j of individual i, respectively, a_j is the pooled regression coefficient of character j for the two morphs, and L_i is the SL of individual i. The LDA for the meristic characters was conducted based on the raw data.

3. Results

3.1. Analyses of mtDNA Sequences. A total of 27 mtDNA haplotypes was obtained in the 64 individuals. Proportion of the variance of genetic diversity between the two morphs was significantly larger than zero (AMOVA: degree of freedom = 1, proportion of variance between the morphs = 0.089, $P < 0.001$). The ML tree separated the 64 individuals into two clusters (Figure 2). One cluster consists of 31 out of the 32 individuals of YA (clade I), and the remaining 1 individual of YA and the 32 individuals of BA formed the other cluster (clade II). The separation of these two clusters was supported by a 94% bootstrap probability. One MP tree was obtained (CI = 0.976, RC = 0.966), which accorded with the ML tree in topology.

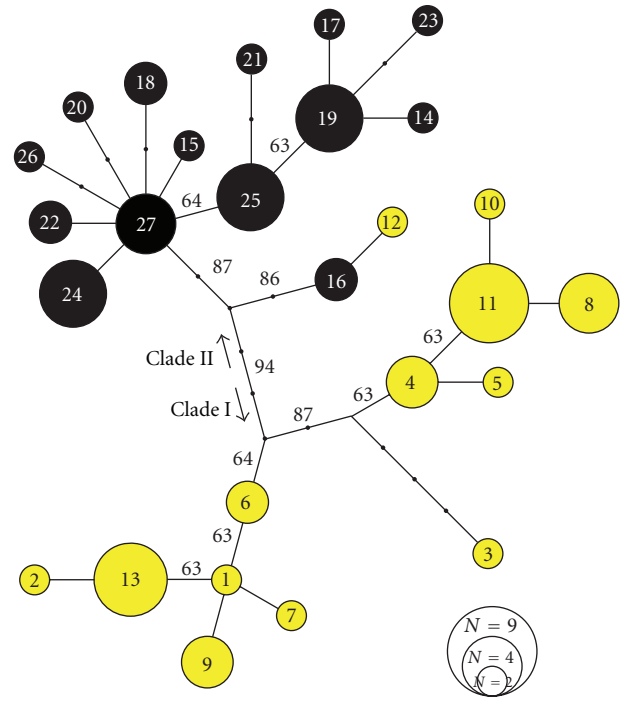


Figure 2: Unrooted haplotype network based on mtDNA sequences. Haplotypes are numbered from 1 to 27 and coloured according to morph (yellow circles: YA, black circles: BA). The size of circles reflects the number of specimens sharing the same haplotype (see explanation in the lower right corner). Only bootstrap values >50% are shown.

3.2. Analyses of Microsatellite Allele Frequencies. Based on the microsatellite data, no LD was found in any of the possible pairs among the five markers in the two morphs (likelihood ratio tests: $P > 0.05$ in 20 tests after sequential Bonferroni correction). Allele frequencies showed no significant departures from HW equilibrium (Table 1). Proportion of the variance of genetic diversity between the two morphs was significantly larger than zero (AMOVA: degree of freedom = 1, proportion of variance between the morphs = 0.190, $P < 0.001$). A Bayesian population assignment test to the two groups indicated that the 32 individuals of YA and 1 individual of BA were clustered together, and the remaining 31 individuals of BA formed the other cluster (Figure 3). The BA individual that was clustered in YA group

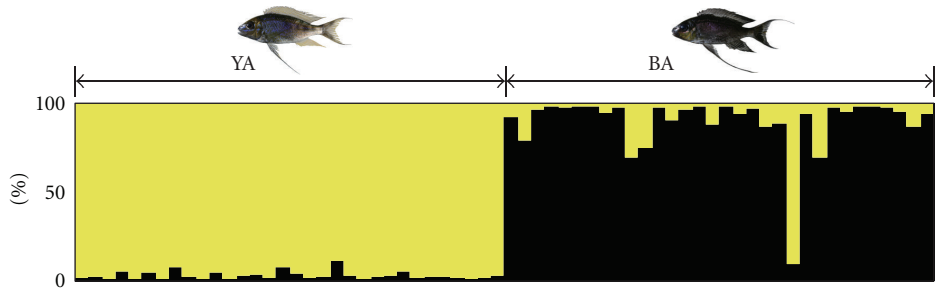


Figure 3: Results of the population assignment test based on five microsatellite loci.

Table 1: Details of microsatellite loci of the 72 large adults that are genotyped in the present study. (H_o: observed heterozygosity, H_e: expected heterozygosity, $^{NS}P > 0.05$ in a test of departure from Hardy-Weinberg equilibrium after a sequential Bonferroni correction).

	N	No. of alleles	H_o	H_e
YA				
GM264	32	7	0.750^{NS}	0.743
Pzeb4	32	11	0.750^{NS}	0.789
Ttem8	32	7	0.656^{NS}	0.600
Ttem9′	32	12	0.875^{NS}	0.820
UNH2050	32	8	0.469^{NS}	0.605
BA				
GM264	32	21	1.000^{NS}	0.930
Pzeb4	32	14	0.750^{NS}	0.879
Ttem8	32	16	0.750^{NS}	0.887
Ttem9′	32	14	0.906^{NS}	0.853
UNH2050	32	11	0.781^{NS}	0.743

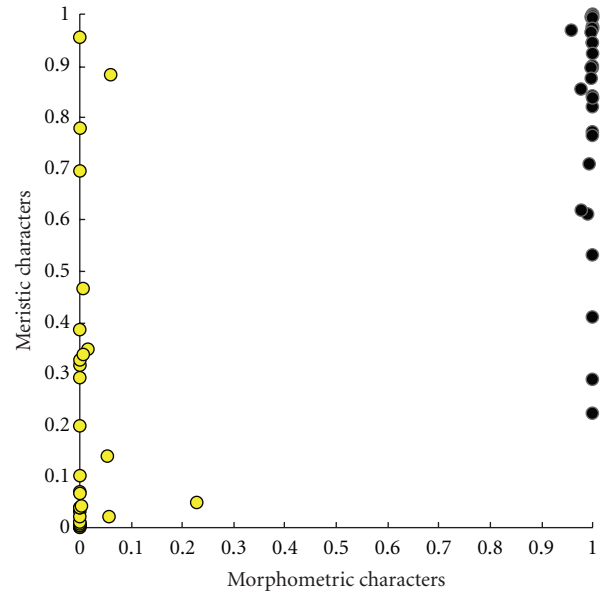


Figure 4: Plot of probabilities that an individual is BA estimated by the linear discriminant analyses of morphometric and meristic characters. Yellow circles indicate YA males, and black circles indicate BA males.

in the microsatellite data was included in the clade II of the mitochondrial tree (haplotype no. 19, Figure 2).

3.3. Analyses of Morphological Characters. The MANCOVA for morphometric characters and the MANOVA for meristic characters revealed significant morphological differences between colour morphs (Tables 2 and 3). The ANCOVAs for morphometric characters and the ANOVAs for meristic characters revealed that YA had significantly smaller head, smaller eyes, shorter pelvic fins, and smaller number of gill rakers than BA did, although the ranges of these characters largely overlapped between morphs (e.g., 14–16 gill rakers in YA, whereas15–18 gill rakers in BA). In the LDAs (Figure 4), the morphometric characters more clearly discriminated the morphs (error rate was 0.0%) than the meristic characters did (error rate was 10.9%).

4. Discussion

The present genetic analyses based on mtDNA sequences and microsatellites revealed that the gene flow is restricted between two colour morphs of *C. furcifer*. At Kasenga, males of these morphs build nests side by side on the lake bottom, and spatial and temporal barriers that would cause reproductive isolation between morphs are not found. Assortative mating by mate choice seems most likely to cause reproductive isolation between the morphs. These morphs were also different in morphological characters, supporting the idea that these morphs are distinct sympatric species. Some females have a yellowish anal fin and some other females have a blackish anal fin. These females may correspond to YA and BA, respectively. However, the colours of the anal fins of females are paler than those of large males, and it is difficult to determine the colours of the anal fins in some females. Molecular and morphological analyses will be useful to determine the morphs of females and small males, as the present data showed clear discrimination between the morphs in large males. In this study, one large male of YA and one large male of BA exhibited discrepancies in clustering between their mitochondrial and microsatellite data. This may have been caused by insufficient molecular data, by incomplete lineage sorting, or by hybridization

Table 2: Differences in $\log_{10}$ transformed morphometric characters between adult males of YA and BA ($^{**}P \leq 0.01$, $^{*}P \leq 0.05$, $^{NS}P > 0.05$ after a sequential Bonferroni correction).

	Morphs	$\log_{10}$ SL	Morph × $\log_{10}$ SL
MANCOVA	$F_{12,49} = 7.79^{**}$	$F_{12,49} = 77.5^{**}$	$F_{12,49} = 1.46^{NS}$
ANCOVAs			
Body depth	$F_{1,60} = 6.32^{NS}$	$F_{1,60} = 97.2^{**}$	$F_{1,60} = 0.0203^{NS}$
Head length	$F_{1,60} = 12.0^{**}$	$F_{1,60} = 146^{**}$	$F_{1,60} = 2.76^{NS}$
Head width	$F_{1,60} = 1.18^{NS}$	$F_{1,60} = 71.9^{**}$	$F_{1,60} = 0.266^{NS}$
Snout length	$F_{1,60} = 1.99^{NS}$	$F_{1,60} = 134^{**}$	$F_{1,60} = 1.96^{NS}$
Eye length	$F_{1,60} = 48.7^{**}$	$F_{1,60} = 21.1^{**}$	$F_{1,60} = 2.25^{NS}$
Interorbital width	$F_{1,60} = 0.0484^{NS}$	$F_{1,60} = 51.1^{**}$	$F_{1,60} = 0.454^{NS}$
Lower jaw length	$F_{1,60} = 5.77^{NS}$	$F_{1,60} = 32.5^{**}$	$F_{1,60} = 1.59^{NS}$
Caudal peduncle length	$F_{1,60} = 6.11^{NS}$	$F_{1,60} = 50.6^{**}$	$F_{1,60} = 2.24^{NS}$
Caudal peduncle depth	$F_{1,60} = 1.10^{NS}$	$F_{1,60} = 110^{**}$	$F_{1,60} = 1.04^{NS}$
Dorsal fin base length	$F_{1,60} = 8.21^{NS}$	$F_{1,60} = 460^{**}$	$F_{1,60} = 1.37^{NS}$
Anal fin base length	$F_{1,60} = 2.90^{NS}$	$F_{1,60} = 90.0^{**}$	$F_{1,60} = 0.0411^{NS}$
Pelvic fin length	$F_{1,60} = 12.8^{**}$	$F_{1,60} = 17.4^{**}$	$F_{1,60} = 4.08^{NS}$

Table 3: Differences in meristic characters between adult males of YA and BA ($^{**}P \leq 0.01$, $^{*}P \leq 0.05$, $^{NS}P > 0.05$ after a sequential Bonferroni correction).

MANOVA	$F_{9,54} = 7.70^{**}$
ANOVAs	
Dorsal fin spines	$F_{1,62} = 0.984^{NS}$
Dorsal fin soft rays	$F_{1,62} = 8.12^{*}$
Anal fin soft rays	$F_{1,62} = 2.00^{NS}$
Pectoral fin rays	$F_{1,62} = 0.463^{NS}$
Scales in longitudinal line	$F_{1,62} = 4.67^{NS}$
Scales on upper lateral line	$F_{1,62} = 8.27^{*}$
Scales on lower lateral line	$F_{1,62} = 1.16^{NS}$
Gill rakers	$F_{1,62} = 33.5^{**}$
Outer teeth on premaxillae	$F_{1,62} = 10.0^{*}$

between the morphs. In cichlid fish from Lake Tanganyika, incomplete lineage sorting is reported among tribes [34], and hybridization is reported between populations, between species, and between genera as a means by which rapid diversification can be achieved [35–42].

Boulenger published a description of *Cyathopharynx furcifer* on December 1898 [43]. This is the first full description of this species, but not the original description. Boulenger published a synopsis of this full description on June 1898 [11]. This short synopsis is the original description of this species because it was published earlier than the full description [14], although only a few morphological features are described. According to the full description, two syntypes of this species from Kinyamkolo, close to the present sampling locality, Kasenga, have elongated pelvic fins, bluish dorsal part and white ventral part of the body, some yellow marbling on the postocular part of the head, and some yellow streaks on the dorsal and anal fins [43]. These features accord with those of large males of YA (Figure 1), as some books for hobbyists of ornamental fish pointed out [18].

Although taxonomic studies currently treat *Cyathopharynx foae* and *C. grandoculis* as junior synonyms of *C. furcifer* [8, 14], some books for hobbyists of ornamental fish treat *C. foae* as a valid species that corresponds to BA, and *C. grandoculis* as a junior synonym of *C. foae* [18]. The taxonomic status of these two nominal species (*C. foae* and *C. grandoculis*) has not been tested with taking sexual and developmental variations into account (e.g., [44]). The holotypes of these two nominal species appear to be small males or females, as indicated by the small body size in *C. foae* (64 mm SL [12]) and short pelvic fins in *C. grandoculis* [13]. Morphological analyses, and if possible, molecular analyses, of type specimens of the three nominal species, and comparisons of these type specimens with nontype specimens of various body sizes, localities, and sexes will be needed to determine which nominal species corresponds to YA or BA, or possibly even to a yet undescribed species.

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Research Article

Evolutionary History of Lake Tanganyika's Predatory Deepwater Cichlids

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Hybridization among littoral cichlid species in Lake Tanganyika was inferred in several molecular phylogenetic studies. The phenomenon is generally attributed to the lake level-induced shoreline and habitat changes. These allow for allopatric divergence of geographically fragmented populations alternating with locally restricted secondary contact and introgression between incompletely isolated taxa. In contrast, the deepwater habitat is characterized by weak geographic structure and a high potential for gene flow, which may explain the lower species richness of deepwater than littoral lineages. For the same reason, divergent deepwater lineages should have evolved strong intrinsic reproductive isolation already in the incipient stages of diversification, and, consequently, hybridization among established lineages should have been less frequent than in littoral lineages. We test this hypothesis in the endemic Lake Tanganyika deepwater cichlid tribe Bathybatini by comparing phylogenetic trees of *Hemibates* and *Bathybates* species obtained with nuclear multilocus AFLP data with a phylogeny based on mitochondrial sequences. Consistent with our hypothesis, largely congruent tree topologies and negative tests for introgression provided no evidence for introgressive hybridization between the deepwater taxa. Together, the nuclear and mitochondrial data established a well-supported phylogeny and suggested ecological segregation during speciation.

1. Introduction

Cichlid fishes have undergone spectacular radiations in different parts of the world. In particular, the species flocks of the East African Great Lakes are well-known examples for rapid evolution and speciation [1–5]. Each of the three Great Lakes—Tanganyika, Malawi, and Victoria—is inhabited by hundreds of mostly endemic cichlid species [6, 7]. Notably, most of the diversity is found in the littoral habitat, whereas reduced species richness in the deep benthal and pelagial seems to be a common phenomenon in all East African Great Lakes [7–10]. At least three factors may have contributed to this pattern: (i) reduced niche diversity in the pelagic and in deepwater benthic zones, (ii) a narrow ambient light spectrum consisting only of short-wavelength blue light and hence less promotive of diversification mechanisms contingent on color perception than the shallow clear-water habitats [11–14], and (iii) the absence of strong barriers to gene flow. Indeed, deepwater cichlid species often have lake-wide distributions with very low, if any, population genetic structure over large geographic distances [10, 15, 16] (see also the Lake Tanganyika clupeid *Limnothrissa miodon* [17] and the centropomid *Lates stappersii* [18]). On the other hand, high levels of genetic differentiation, sometimes accompanied by phenotypic divergence on small geographic scales, are characteristic for the species-rich guild of stenotopic rock-dwelling cichlid species [19–31]. However, allopatric diversification in the fragmented littoral zone was not necessarily accompanied by the evolution of pre- or postzygotic isolation, so that secondary contact imposed by lake level fluctuations has often led to hybridization or introgression between previously allopatric taxa [32–35]. Even today, substrate breeders of the tribe Lamprologini can be found in mixed-species pairs [36], and interspecific fertilizations occur in communally nesting, shell-breeding lamprologines [37]. Indeed, phylogenetic analyses of predominantly littoral cichlid lineages revealed that interspecific hybridization has played, and still plays, an important role

in the evolution of these fish [32, 34, 36–41]. Thus, in the majority of recent molecular studies on species relationships within littoral tribes, especially when comparing mitochondrial and nuclear phylogenies, the explanation for the tree topologies involved the claim of introgression and hybridization between established lineages in addition to incomplete lineage sorting (reviewed in [42]).

We hypothesize that this is not the case in tribes composed of deepwater species. Lacking the geographic structure introduced by littoral habitat heterogeneity, deepwater species may still be spatially separated by distance, by segregation of breeding grounds, by variable hydrological conditions [16, 43, 44], or by large-scale fragmentation of the lake basin during major droughts [45, 46]. Generally, however, the potential barriers to gene flow for deepwater species are less insurmountable than those met by stenotopic littoral cichlids. Specifically in Lake Tanganyika, the evolution of stenotopy regarding depth, bottom type, or light intensity may have been prohibited by the seasonal upwelling of anoxic waters [47]. We postulate that given the high potential for gene flow, diversification of lineages will either be curtailed (as suggested by the relative species paucity) or be attended by strong reproductive isolation right from the start. This would imply that introgression following cladogenesis occurred at much lower rates, if at all, in the deepwater species than in littoral cichlids. We test this hypothesis in the deepwater cichlid tribe Bathybatini by comparing phylogenetic trees based on mitochondrial sequence data with trees obtained with nuclear multilocus AFLP data, an approach which has previously revealed hybridization in littoral cichlids and other contexts [34, 36, 37, 39, 40, 48–54]. Moreover, in several studies, phylogenetic inference on species relationships has benefited from the use of multilocus genetic data, and we expect that the AFLP data collected in the present study will also contribute to the resolution of intergeneric relationships within the Bathybatini, which are still debated due to conflicting or ambiguous molecular and morphological evidence [46, 55–59].

1.1. The Study Species. The tribe Bathybatini (sensu Takahashi [59]) comprises 17 species in three genera: (1) the genus *Bathybates* comprises six large (30–40 cm), piscivorous species preying mainly on pelagic freshwater clupeids (*B. fasciatus* and *B. leo*), benthic cichlids (*B. graueri*, *B. vittatus*, and *B. ferox*), or undefined prey (the rare, elusive *B. horni*), in addition to the small (20 cm) *B. minor*, which is a specialized clupeid hunter. In accordance with their trophic niches, Coulter [43] distinguished three morphotypes among the *Bathybates* species, the fast-swimming fusiform predators *B. fasciatus*, *B. leo*, and *B. horni*, the generalized shape of the benthic feeders *B. graueri*, *B. vittatus*, and *B. ferox*, and the small clupeid-mimicking *B. minor*, which mingles with its prey and accompanies the diurnal clupeid migrations. Based on trawl net and gill net catches, *B. minor* were classified as pelagic, *B. fasciatus* and *B. leo* as chiefly bathypelagic and the remaining four species (*B. graueri*, *B. vittatus*, *B. ferox*, and *B. horni*) as chiefly benthic [43]. Except for *B. minor*, which was never found below 70 m, *Bathybates* species descend to depths of 150–200 m. (2) The member of the monotypic genus *Hemibates*, *H. stenosoma*, is an abundant benthic species on the muddy bottom of southern Lake Tanganyika feeding on fish and shrimps mainly at depths between 100 and 200 m [43]. (3) The small-bodied (<15 cm) species of the genus *Trematocara* (formerly assigned to the genera *Trematocara* and *Telotrematocara*, [55]) comprise nine benthic and bathypelagic species feeding on a variety on invertebrate prey, fish larvae, and phytoplankton. They are found at maximum depths of 75 to 200 m [43]. Following the upward movement of zooplankton, many *Trematocara* species undertake nightly migrations along slopes into the littoral.

All members of the Bathybatini are maternal mouthbrooders. Some species release their fry in shallow areas, but overall, data on bathybatine breeding behaviour is anecdotal or lacking [43, 60]. The species are sexually dimorphic, with males of *Bathybates* and *Hemibates* exhibiting species-specific patterns of dark stripes, bars and dots on a silver background and egg-spots on the anal fins, and males of the silvery *Trematocara* with dark dorsal fin markings. Females of all species show a uniformly silver/brown coloration. All Bathybatini have large eyes, which promote not only the detection of prey and predators but possibly also mate-recognition in the dark depths. In line with the latter, the monochromatic patterning of males may be viewed as adaptation to the short-wavelength dominated visual environment [61], in contrast to the colourful patterns of the mouthbrooding cichlids in the shallow, light-flooded littoral.

A recent phylogenetic study based on three mitochondrial genes supported the monophyly of *Bathybates* as well as of the species therein and indicated a polytomy of three equidistant lineages representing *Bathybates*, *Hemibates*, and *Trematocara* [46]. Within *Bathybates*, *B. minor* appeared ancestral to a radiation of the six large species, which showed a basal split of *B. graueri* and low statistical support for the branching order of the remaining species (Figure 1). The short internal branches among the large *Bathybates* species supported a rapid radiation at approximately 2.3–2.7 MYA, coinciding with the rapid diversification of other Lake Tanganyika cichlids [28, 45, 62]. Competition and resource partitioning as well as potential geographic isolation during an extreme low-stand of the lake were proposed as promoters of *Bathybates* speciation [46].

2. Material and Methods

2.1. Sample Collection and DNA Extraction. This study is based on a total of 38 specimens, representing all seven *Bathybates* species, *Hemibates stenosoma* as well as *Trematocara unimaculata* and *T. macrostoma* (Table 1). All specimens were obtained between 1999 and 2011 from local fishermen at Lake Tanganyika and identified to species by S. Koblmüller. Unfortunately, it was not possible to obtain a comprehensive taxon sampling for the genus *Trematocara* as, because of their small size and hence low market value, these fish (except for the largest species *T. unimaculatum*) are not caught by local fishermen.

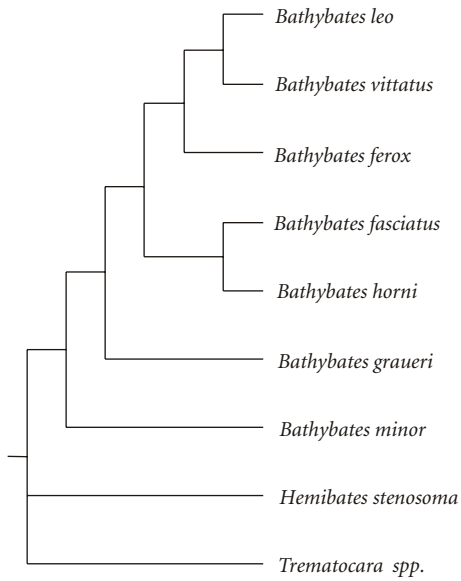

Figure 1: Schematic depiction of the the phylogenetic relationships within the Bathybatini as inferred from mtDNA data [46].

Fin clips or white muscle tissue were preserved in ethanol and DNA was isolated using a proteinase K digestion/high salt precipitation method [63]. DNA concentrations were measured using a NanoPhotometer (IMPLEN).

2.2. AFLP Data Collection. Amplified fragment length polymorphism (AFLP) genotyping followed the protocol described in [34]. The ten primer combinations used for selective amplification were *Eco*RI-ACA/*Mse*I-CAA, *Eco*RI-ACA/*Mse*I-CAG, *Eco*RI-ACA/*Mse*I-CAC, *Eco*RI-ACA/*Mse*I-CAT, *Eco*RI-ACT/*Mse*I-CAT, *Eco*RI-ACT/*Mse*I-CAA, *Eco*RI-ACT/*Mse*I-CAG, *Eco*RI-ACT/*Mse*I-CAC, *Eco*RI-ACC/*Mse*I-CAA, and *Eco*RI-ACC/*Mse*I-CAC. Selective amplification products were visualized using an ABI 3130xl automated sequencer (Applied Biosystems) along with an internal size standard (Genescan-500 ROX; Applied Biosystems). Polymorphic positions were initially identified using GeneMapper 3.7 software (Applied Biosystems) in a range of 50–500 bp. In order to adjust misaligned bins and avoid size homoplasy, bin positions were set manually. Bins containing ambiguous low intensity peaks in a large proportion of the samples and entire profiles with short read-lengths or very low peak heights were deleted. These preprocessed, unnormalized peak-height data were analyzed with AFLPScore 1.4a [64], which optimizes thresholds for locus retention and phenotype calling based on estimated error rates. Phenotype calling thresholds were set as absolute or relative depending on the number of retained loci and the achieved error rates obtained with each option. 20 replicate samples were included to calculate the mismatch error rate for all unique loci.

2.3. Phylogenetic Inference. A neighbour joining (NJ) tree based on Nei and Li's distances [65] was constructed in PAUP 4.0b5 [66]. Bootstrap values from 1000 pseudoreplicates were used as standard measure of confidence in the inferred tree topology.

Although accurate models for Bayesian tree construction using AFLP datasets do exist [67]), the high demands for processing power make them unfeasible to use [67, 68]. Thus, Bayesian phylogenetic inference (BI) was conducted in MrBayes 3.1.2 [69], employing the restriction site model

Table 1: List of samples with sample ID and sampling locality.

Sample ID	Species	Sampling locality
12879	*Bathybates fasciatus*	Mpulungu market
12885	*Bathybates fasciatus*	Mpulungu market
12889	*Bathybates fasciatus*	Kalambo Lodge
12890	*Bathybates fasciatus*	Kalambo Lodge
12917	*Bathybates fasciatus*	Tanganyika Lodge
12913	*Bathybates ferox*	Lufubu estuary
12877	*Bathybates graueri*	Mpulungu market
12878	*Bathybates graueri*	Mpulungu market
12883	*Bathybates graueri*	Mpulungu market
12893	*Bathybates graueri*	Mpulungu market
12897	*Bathybates graueri*	Mpulungu market
12901	*Bathybates graueri*	Mpulungu market
12902	*Bathybates graueri*	Mpulungu market
12911	*Bathybates graueri*	Mpulungu market
12912	*Bathybates graueri*	Mpulungu market
12919	*Bathybates graueri*	North of Sumbu
13101	*Bathybates horni*	Mpulungu market
12907	*Bathybates leo*	Mpulungu market
12921	*Bathybates leo*	Mpulungu market
12923	*Bathybates leo*	Mpulungu market
12925	*Bathybates leo*	Mpulungu market
13100	*Bathybates leo*	Mpulungu market
12909	*Bathybates minor*	Lufubu estuary
12910	*Bathybates minor*	Kalambo
12933	*Bathybates minor*	Sumbu
12882	*Bathybates vittatus*	Mpulungu market
12924	*Bathybates vittatus*	Mpulungu market
12926	*Bathybates vittatus*	Mpulungu market
12929	*Hemibates stenosoma*	Mpulungu market
12930	*Hemibates stenosoma*	Mpulungu market
12931	*Hemibates stenosoma*	Mpulungu market
12932	*Hemibates stenosoma*	Mpulungu market
12880	*Trematocara unimaculata*	Mpulungu market
12881	*Trematocara unimaculata*	Mpulungu market
12935	*Trematocara macrostoma*	Mpulungu market
12936	*Trematocara macrostoma*	Mpulungu market

Coordinates of sampling sites (if known): Kalambo, S 8°37′ E 31°12′; Kalambo Lodge, S 8°37′ E 31°37′; Lufubu estuary, S 8°32′ E 30°44′; Sumbu, S 8°31′ E 30°29′; Tanganyika Lodge, S 8°47′ E 31°05′.
Note that fish obtained at the fishmarket in Mpulungu might have been caught anywhere in southern Lake Tanganyika.

with the "noabsencesites" coding bias correction [68, 70]. The Dirichlet prior for the state frequencies was set to (2.44, 1.00) matching the actual 0/1 frequencies in the dataset. Posterior probabilities were obtained from Metropolis-coupled Markov chain Monte Carlo simulations (2 independent runs; 10 chains with 8,000,000 generations each; chain temperature: 0.2; sample frequency: 1,000; burn-in: 4,000,000 generations). Chain stationarity and run parameter convergence were checked in Tracer 1.5 [71].

To test for homoplasy excess introduced by hybridization, we conducted a tree-based method as outlined by Seehausen [74] by removing single species from the dataset and observing the change in bootstrap values in the NJ tree (see also [34, 40]). In theory, the inclusion of a hybrid taxon in a multilocus phylogeny introduces homoplasy with clades that contain its parental taxa. Hybrid taxa should be intermediate to the parental taxa since they carry a mosaic of parental characteristics. Thus, decreasing the amount of homoplasy in the dataset by removing the hybrid taxon should increase the bootstrap support for the clades that include the parental taxa or their descendants, whereas removing nonhybrid taxa should have no effect on the statistical support of other nodes (Figure 2).

2.4. Evaluating Alternative Tree Topologies. Testing for consistency between mtDNA- and AFLP-based tree topologies employed two different strategies. In a first test we evaluated whether our AFLP-NJ-topology can be explained by the mtDNA data of Koblmüller et al. [46]. Using the mitochondrial sequences, we inferred the log likelihood of the AFLP-NJ-topology data by constraining maximum likelihood (ML) tree search to a topology identical to the species tree suggested by the NJ analyses of our AFLP data, applying the substitution model used in this previous study (HKY+I+G). To test for significant differences between the unconstrained [46] and constrained mtDNA topology we performed an ML-based Shimodaira-Hasegawa (SH) test [75] (full optimization, 1,000 bootstrap replicates) in PAUP. In a second test we evaluated by means of a Bayes factors approach [76] whether the mtDNA tree [46] can be explained by our AFLP data, and whether the AFLP-NJ and BI trees differ significantly from each other. We performed BI searches constraining the topology to that of the mtDNA-topology [46] and the interspecific relationships implied by the AFLP-NJ-tree in MrBayes 3.1.2 employing the same settings as above. Bayes factor comparison—using the harmonic means of the likelihood throughout different runs [77, 78]—among the three alternative phylogenatic hypotheses was performed in Tracer 1.5. Values of $2 \times \ln \mathrm{BF}$ (two times the difference between the harmonic means of the two models) >10 are considered strong evidence for support of one model over another [76].

3. Results

The final AFLP dataset consisted of 659 unique loci with a mismatch error rate of roughly 3%, which falls within the acceptable limit for mismatch error rates as defined by [64]. Both the NJ and BI analysis yielded largely congruent and well-supported topologies with only minor differences between them (Figures 3(a) and 3(b)). Whereas all species were monophyletic in the NJ tree, *Bathybates graueri* was not resolved as a monophylum in the BI tree, but as paraphylum including the well-supported clade of the other large *Bathybates* species (Figure 3(b)). Despite this minor topological difference, Bayes factor comparison strongly supports the BI tree over the NJ tree ($2 \times \ln \mathrm{BF} = 26.828$; Figure 3(c)). We note, however, that the two-state model implemented in MrBayes does not fully cover the complex genetic process of AFLP evolution and thus provides accurate phylogenetic inference less likely than distance methods [67, 79, 80]. Hence, the observed differences between AFLP and NJ tree topologies might be attributed to this problem. Both the NJ and BI analyses support the monophyly of all three genera with the genus *Trematocara* representing the most ancestral branch (Figures 3(a) and 3(b)). Within the genus *Bathybates*, the small and morphologically most distinct member of the genus, *B. minor*, was sister taxon to the remaining large *Bathybates* species. Branch lengths among the large *Bathybates* species are rather short and some received rather low statistical support, indicating a period of rapid cladogenesis. Nevertheless, both NJ and BI analyses revealed a largely consistent phylogenetic pattern within the large *Bathybates* species. Both SH-test and Bayes factor comparison revealed significant differences between mtDNA and AFLP phylogenies (SH-test: $\ln L$ of -9505.385 versus -9585.142 for mtDNA versus AFLP-NJ-topology-constraint, $P < 0.001$; Bayes factors: $2 \times \ln \mathrm{BF}$ of 88.576 between mtDNA and AFLP-BI-topology and 61.748 between mtDNA and AFLP-NJ-topology, Figure 3(c)). The homoplasy excess test provided no evidence for introgressive hybridization (data not shown).

4. Discussion

In the original classification of Lake Tanganyika cichlid tribes by Poll [55], *Bathybates* and *Hemibates* were included in a tribe Bathybatini as sister group to the Trematocarini (equivalent to the genus *Trematocara*), a hypothesis supported both by lepidological [58] as well as allozyme data [57]. In contrast, based on morphological characteristics, Stiassny [56] and Takahashi [59] proposed a sister group relationship of *Bathybates* and *Trematocara*. Currently, all three genera, *Bathybates*, *Hemibates*, and *Trematocara*, are united in the tribe Bathybatini [59]. A previous mtDNA phylogenetic study remained equivocal with regard to the two competing morphological classifications and suggested that *Bathybates*, *Hemibates,* and *Trematocara* diverged rapidly from their common ancestor [46]. In the present AFLP phylogeny, the length differences between the most ancestral branches favour Poll's original classification with *Trematocara* as sister group to *Hemibates* + *Bathybates* [55, 57, 58]. Consistent with the mitochondrial phylogeny [46], the AFLP data confirm the split between *Bathybates minor* and the larger members of the genus *Bathybates* with *B. graueri* as their most basal representative and identifies a period of rapid cladogenesis at the onset of the diversification of the large *Bathybates* species. However, mtDNA and AFLP phylogenies

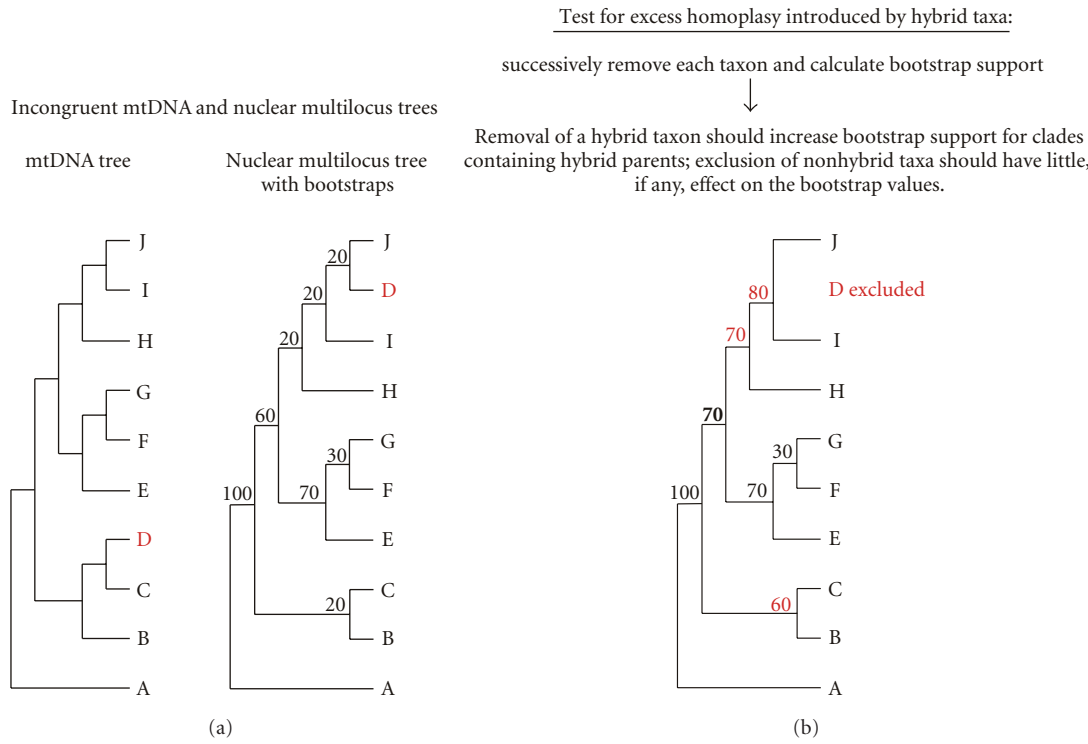

Figure 2: Incongruency between mtDNA and nuclear multilocus trees (e.g., AFLPs) and a test for hybridization in a multilocus phylogeny. (a) Incongruency between mtDNA and nuclear multilocus trees with regard to the placement of taxon D can result from ancient incomplete lineage sorting [72, 73] or the hybrid origin of taxon D. (b) As hybrid taxa combine nuclear alleles from both parental taxa, they introduce homoplasy into a multilocus phylogenetic tree and hence reduce bootstrap support of the nodes containing their parents [74]. Removal of the hybrid taxon from the phylogeny increases the bootstrap support of the parental clades (bold values in (b)). Conversely, removal of nonhybrid taxa should not or only slightly affect the bootstrap support of other nodes. To distinguish between informative (red values in (b)) and uninformative changes in bootstrap values, one taxon at a time is removed from the data and the resulting distribution of bootstrap values for each node is recorded. If removal of a certain taxon produces an outlier in these distributions, the removed taxon is considered a hybrid (or strongly introgressed taxon) and the clades for which support was raised are considered to contain the parental taxa (see, e.g., [34, 40, 48]). In the present example, taxon D is a hybrid between taxa C and J.

differ significantly with respect to the branching pattern among the remaining large *Bathybates* species. Introgressive hybridization (including the possibility of complete mtDNA replacement [41]) and ancient incomplete lineage sorting are two alternative sources of topological disagreement between nuclear and mitochondrial trees [40, 41, 81–83], resulting in similar phylogenetic patterns that are difficult to resolve by strict hypothesis testing [84]. Circumstantial inference can be based on the fact that lineage sorting is expected to lag behind rapid cladogenetic events, such that the rapid radiation of the large *Bathybates* species predisposes this clade to mitonuclear phylogenetic incompatibilities without implying postcladogenetic introgression [72, 73]. Likewise, monophyly of species in both mitochondrial and nuclear trees (excepting the paraphyly of *B. graueri* in Bayesian AFLP tree) and negative tests for homoplasy excess in the AFLP data do not indicate the presence of hybrid taxa in the genus *Bathybates*. These findings support our hypothesis that deep-water species are less prone to introgressive hybridization and hybrid speciation than littoral species, but reservations arise on the one hand from the possibility that events of introgression were not detected in our samples and data and given the power of our analyses, and on the other hand from the small number of species in the phylogeny. Principally, rates of interspecific introgression may not differ between littoral and deepwater cichlids, but will nonetheless lead to higher incidences of introgression in the species-rich groups than in a less speciose clade. If this was the case, the lack of a signal of introgression in *Bathybates* and *Hemibates* would be fully explained by the low diversification rate of the lineage and hence limited opportunity for interspecific hybridization, without implying the evolution of complete reproductive isolation early on during diversification.

The branching order of *Hemibates* and the basal *Bathybates* species, which is reconstructed congruently by mtDNA and AFLP markers, suggests repeated transitions between benthic and bathypelagic feeding mode (ecological data from [43]). The basal *H. stenosoma* represents a benthic generalist feeding on shrimps and various species of fish. The next split led to the specialized pelagic clupeid hunter *B. minor*, which mimics its prey in size and coloration and stages surprise attacks from within the sardine shoals. Then,

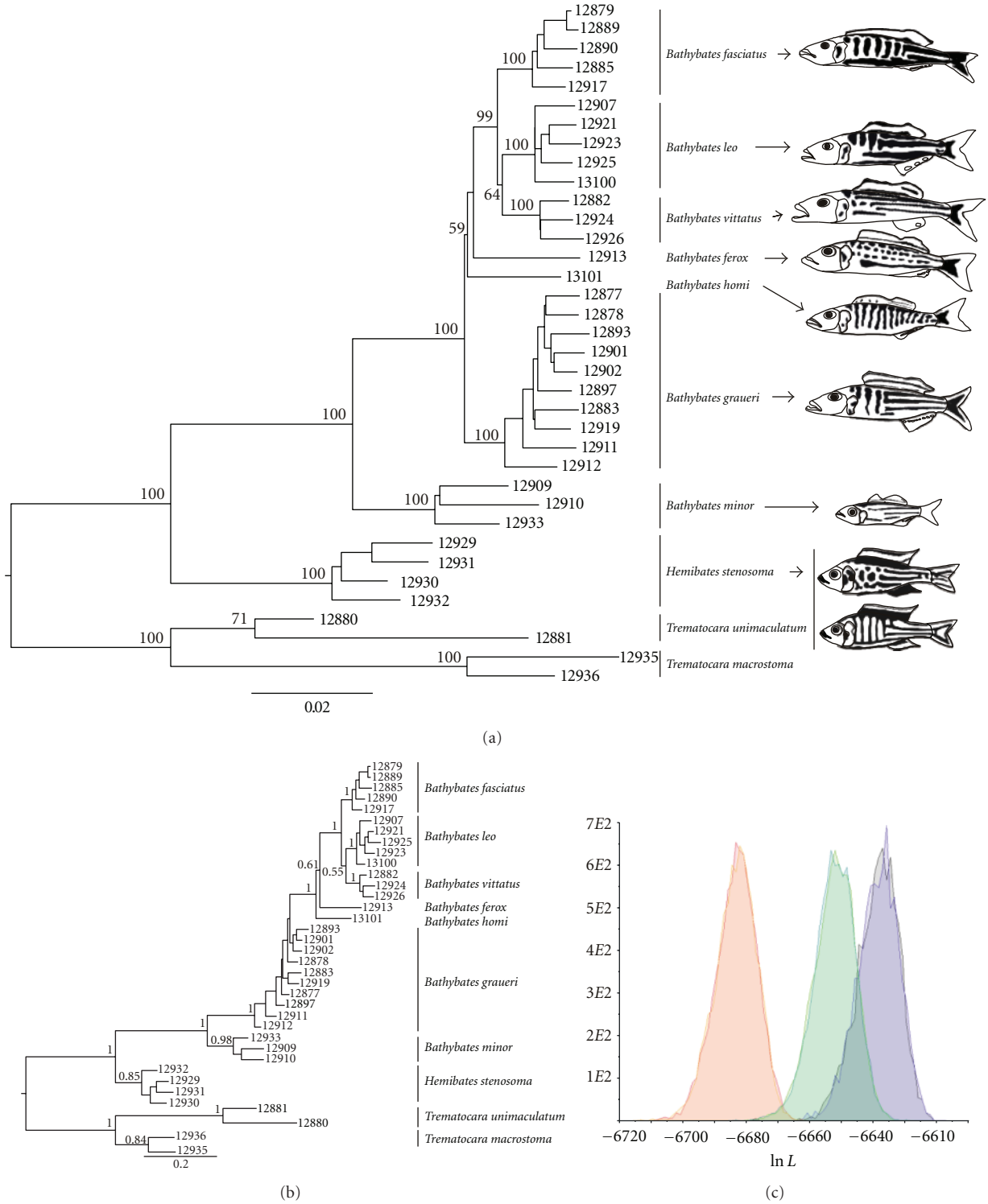

Figure 3: Phylogenetic relationships of the Bathybatini based on 659 polymorphic AFLP loci. (a) NJ tree (employing Nei and Li distances [65]) and (b) BI tree are shown. Only bootstrap values >50 and posterior probabilities >0.5 are shown. Branches are labelled with the samples' lab ID numbers. (c) The marginal density of posterior distribution of likelihood (Ln L) for each of the two MrBayes runs of the unconstrained AFLP data (violet, grey), the AFLP-NJ-topology-constraint (blue, green), and the mtDNA-topology-constraint (orange, red). Fish were drawn after photographs in [60] to demonstrate the interspecific differences in male nuptial patterns.

B. graueri took a step back to the benthic habitat, specialized on cichlid prey and evolved a large body size. The chronology of the following radiation of the large bathypelagic clupeid hunters and benthic cichlid hunters remains unresolved, but involved at least one transition from benthic to bathypelagic habitat preferences. Depth preferences may vary between these species [43], such that speciation may have involved both niche and spatial segregation. The apparent ecological differentiation among lineages may have reduced the fitness of hybrids [85–87] and may have promoted the evolution of a mate recognition system, perhaps based on the species-specific melanic patterns of male *Hemibates* and *Bathybates*. The efficacy of monochromatic black, silvery, and white body and fin patterns in mediating assortative mating in the dark, short-wavelength dominated environment has recently been demonstrated for deepwater cichlid species of Lake Malawi. These sympatric and morphologically similar species differ primarily in male nuptial patterns and their reproductive isolation is corroborated by genetic differentiation estimates [61]. However, there is increasing evidence that color pattern is not the only cue for mate recognition in cichlids fish and it is possible and likely that auditory [88] and olfactory cues [89] play a role in mediating assortative mating in deepwater species, too.

5. Summary and Conclusions

In concert with previous mitochondrial data, the present study provides an informative phylogeny of the species in the deepwater genera *Hemibates* and *Bathybates*. As far as the branching pattern can be resolved, it suggests ecological segregation during speciation. The rapid radiation within *Bathybates* mirrors a burst of speciation observed in several other cichlid tribes of Lake Tanganyika and reveals a congruent cladogenetic pattern across vastly different habitats, which suggests some kind of synchronization by environmental factors [27, 45, 46, 62]. Consistent with the hypothesis that lineages evolving in the weakly structured deepwater habitat would develop stronger reproductive isolation than the allopatric lineages of the fragmented littoral, our data provided no evidence for the presence of hybrid taxa in the deepwater dwelling genus *Bathybates.* In further support of the hypothesis, introgressive hybridization is also not indicated by the mitochondrial and AFLP phylogenies of the Lake Tanganyika cichlid genus *Xenotilapia* [90], which includes several deepwater-dwelling species comprising the prey of the benthic *Bathybates* and *Hemibates*. An increased sample size to evaluate this pattern will be attained by the analyses of additional open-water and deepwater species, for example, the tribe Limnochromini and the genus *Trematocara*.

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Research Article

More than Meets the Eye: Functionally Salient Changes in Internal Bone Architecture Accompany Divergence in Cichlid Feeding Mode

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African cichlids have undergone extensive and repeated adaptive radiations in foraging habitat. While the *external* morphology of the cichlid craniofacial skeleton has been studied extensively, biomechanically relevant changes to *internal* bone architecture have been largely overlooked. Here we explore two fundamental questions: (1) Do changes in the internal architecture of bone accompany shifts in foraging mode? (2) What is the genetic basis for this trait? We focus on the maxilla, which is an integral part of the feeding apparatus and an element that should be subjected to significant bending forces during biting. Analyses of μCT scans revealed clear differences between the maxilla of two species that employ alternative foraging strategies (i.e., biting versus suction feeding). Hybrids between the two species exhibit maxillary geometries that closely resemble those of the suction feeding species, consistent with a dominant mode of inheritance. This was supported by the results of a genetic mapping experiment, where suction feeding alleles were dominant to biting alleles at two loci that affect bone architecture. Overall, these data suggest that the internal structure of the cichlid maxilla has a tractable genetic basis and that discrete shifts in this trait have accompanied the evolution of alternate feeding modes.

1. Introduction

Adaptive radiations involve the concomitant evolution of ecological and phenotypic diversity within a rapidly multiplying lineage [1], and many of the most notable adaptive radiations are characterized by divergence in functional morphology. Hawaiian silverswords, for example, have evolved a suite of morphological traits associated with adaptations to an extreme range of environmental moisture (mesic to xeric) [2, 3]; *Anolis* lizards have diversified in regard to traits involved in clinging and climbing abilities [4–8]; both Galápagos finches and African cichlids are renowned for their extensive (and in the case of cichlids, repeated) adaptive radiations in trophic morphology that parallel, and presumably contribute to, microhabitat divergence in foraging niches [9–16]. Not surprisingly, considerable attention has been given to characterizing the phenotypic diversity associated with these extraordinary radiations [3, 4, 9, 14, 17–19].

In the case of the multiple adaptive radiations of East-African cichlids, extensive analyses of their anatomical diversity have only recently been undertaken [14, 20, 21]. Among the notable findings from this body of work is that patterns of diversification within each of the three large lakes in the region (Victoria, Tanganyika, and Malawi) are statistically similar to one another [14, 21]. In particular, previous work from our group has found that trophic variation among cichlid radiations is characterized by divergence along a conserved ecomorphological axis [14]. One end of this axis is defined by species that forage in the water column and possess elongated jaws, while the opposite end

is characterized by species that feed on benthic prey items using significantly shorter jaws. Thus, the primary axis of craniofacial variation defined by East-African rift-lake cichlids distinguishes benthic from pelagic ecotypes. The concordance between morphology and foraging mode observed in this study makes sense within the more general context of teleost functional morphology. Fish with short jaws have the potential, all other factors being equal, to produce bites that are proportionally more powerful due to an increased mechanical advantage employed by the jaw adductor muscles during biting, which is advantageous for herbivores that scrape tough, filamentous plant material from the substrate and for benthic predators that generate larger bite forces in order to crush, detach, or uncover their prey [22–25]. Longer jaws, on the other hand, facilitate the capture of more elusive prey by increasing bite speed and promoting greater jaw protrusion [23, 26–31].

While the functional implications of variation in external craniofacial geometry have been extensively studied in fishes [24, 28–30, 32–34] and many other vertebrates [18, 35–39], the examination of internal bone architecture has been less prominent with respect to adaptive radiations in fishes (but see [40, 41]). This paucity of data likely reflects the effort and expense associated with obtaining descriptions of these phenotypes. Specifically, while μCT scanning is becoming increasingly accessible to more research labs and is therefore being applied to the study of a steadily increasing number of taxa, the collection and processing of this type of data remains time-consuming, computationally intensive, and expensive in comparison to imaging external bone shape (which may only require light photography). Since adaptive radiations, by definition, result in species-rich and/or highly diverse lineages, the scanning of large numbers of skeletons is simply not feasible for most labs. Here we mitigate these limitations by focusing on one of the more stalwart, and functionally relevant, bones in the face (the maxilla) and by taking advantage of our current knowledge of cichlid adaptive radiations. In particular, we focus our analyses on species that define opposite ends of the functional continuum that characterizes the primary axis of craniofacial variation among Lake Malawi cichlid species [14]. In this way we can identify and describe trends that are associated with the primary axis of diversification of this lineage as well as generate a predictive framework for more global patterns of functional divergence among cichlids and other fish species. We find that discrete changes in the internal architecture of the maxilla have accompanied shifts in foraging mode within this group. These anatomical changes are biomechanically relevant and predict that biting species possess bone that is more resistant to force transmission compared to pelagic suction feeders. Finally, we show that variation in this trait has a relatively simple genetic basis, which suggests that it can respond quickly to natural selection. We submit that a more comprehensive understanding of the genetic architecture and phenotypic variation of this functionally important trait should be a priority of future research in this and other adaptive radiations defined by divergence in feeding morphology.

2. Methods

2.1. Focal Species. Two closely related Lake Malawi cichlid species that employ alternate modes of feeding (biting versus suction) were analyzed for this study. *Labeotropheus fuelleborni* (LF) is a member of the rock-dwelling clade of Malawi cichlids that is specialized to scrape tough, filamentous algae from the substrate [42, 43], and it has one of the most extreme craniofacial architectures of any lake-dwelling cichlid species within this region [14]. It possesses a short, stout head, steeply rounded craniofacial profile and wide jaws that are configured to employ high mechanical advantage during biting. *Maylandia zebra* (MZ; the genus name *Metriaclima*, which the authors have used elsewhere, is a junior synonym of *Maylandia*; [44]) is a closely related, but more generalized rock-dwelling species that collects plankton from the water column and brushes loose algae and detritus from rocky substrates. To accommodate this alternate mode of feeding, MZ has a relatively long head, shallow skull profile, and elongated jaws that are configured to produce faster but weaker bites (i.e., lower MAs) relative to LF [42, 43].

We have shown previously that the forces generated during biting will be transmitted from the lower jaw, through the maxillae, and to the anterior portions of the neurocranium and palatine ([34], Figure 1). Bending force load should also be high in the maxilla, since it acts as a lever that pivots around the pterygoid process of the palatine (Figure 1), and which is moved by the A1 division of the *adductor mandibulae* muscle during biting and by its connection to the lower jaw during mouth opening [32]. The shape of the maxillae is conspicuously different in LF and MZ (Figures 1 and 2(a)), with LF possessing an element that is much wider and more conspicuously bent along the medial-lateral axis compared to MZ, where the maxilla is thin and straight. See Albertson and Kocher [42] for a more comprehensive discussion of the craniofacial anatomy of these two species and Otten [45] and Cooper et al. [34] for a description of the functional anatomy of the cichlid oral jaws.

2.2. Microcomputed Tomography and Quantification of Internal Bone Architecture. Maxillae were scanned at 12-micron resolution with a microcomputed tomography (μCT) scanner (μCT 40, SCANCO Medical, Wayne, PA). Maxillae were oriented for scanning such that cross-sectional images were perpendicular to the long axis of the articular head of the bone (Figure 1(c)). These cross-sections were taken through the thinnest portion of the caudal (i.e., "neck") region of the articular head of the maxilla. This region is roughly semicircular in cross-section (Figure 2), and is caudal to the maxilla's premaxillary and palatinad wings and rostral to its dorsal wing (anatomy after [46]). It lies between the articulation of the palatinad wing of the maxilla with the maxillad process of the palatine (the fulcrum for maxillary rotation) and the two regions where closing and opening forces are applied to the maxilla: the insertion of the A1 division of the *adductor mandibulae* muscle on the medial surface of the dorsal wing (closing) and the ligamentous attachments between the shank of the maxilla and the lower jaw (opening). The maxillary shank is connected to the

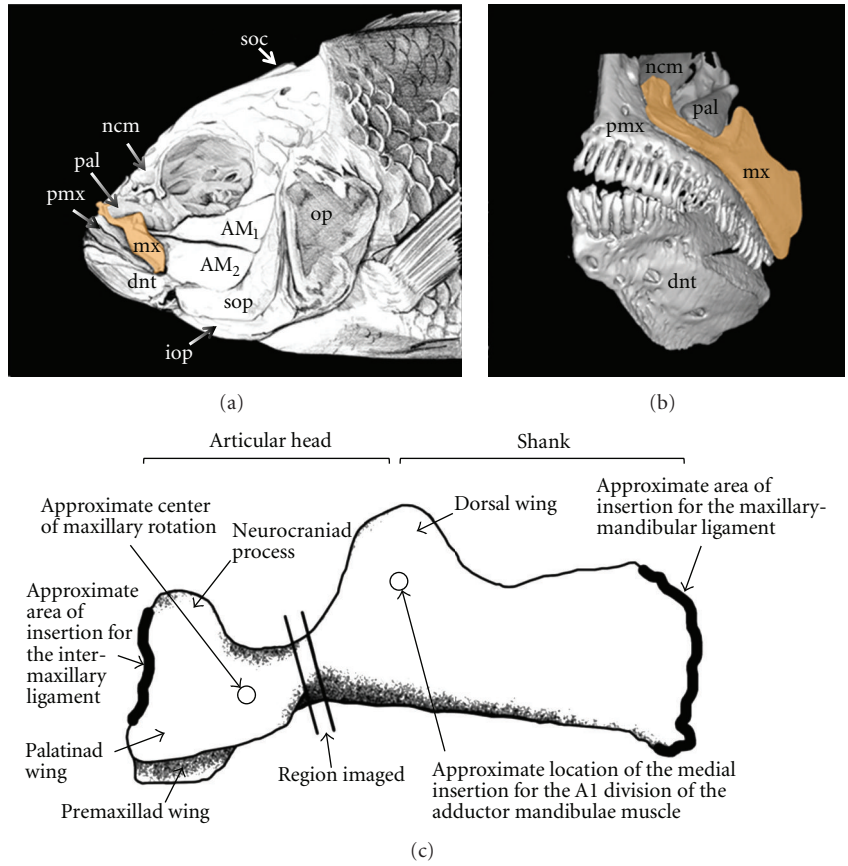

FIGURE 1: (a) Illustration of cichlid craniofacial anatomy in the lateral view. (b) Micro-CT scan of the oral jaws and associated elements. (c) Anatomy of a cichlid maxilla (left side, lateral view) showing the region imaged using μCT scanning. In panels (a) and (b) the maxilla (mx) is highlighted orange. Drawing by Kristen Ann Tietjen. AM_1: first division of the *adductor mandibulae* muscle; AM_2: second division of the *adductor mandibulae*; dnt: dentary; iop: interopercle; ncm: neurocranium; op: opercle; pal: pterygoid process of the palatine; pmx: premaxilla; soc: supraoccipital crest of the neurocranium; sop: subopercle.

lower jaw by connective tissue that attaches to both the primordial process of the articular and the coronoid process of the dentary in the fishes we examined (the maxillary connection to the dentary is sometimes less extensive in other fish species). The cross-sectional areas imaged lie almost immediately between the maxillary fulcrum and the point where biting (i.e., closing) forces are directly applied to this bone (Figure 1(c)), and an ability to resist bending should therefore be a particularly important aspect of the functional morphology of this region. Image sets were exported to ImageJ (http://rsbweb.nih.gov/), and a lower threshold was defined as 400 mg/cc hydroxyapatite equivalent to isolate bone. The BoneJ plug-in to ImageJ was used to quantify bone cross-sectional area (CSA, mm^2) and principal area moment of inertia (I_{max}, mm^4) of a single 2D slice within the articular neck of the maxilla (bracketed region, Figure 1(c)). CSA is a measure of the quantity of bone while I_{max} is a measure of the ability of bone to resist bending loads. In all, 7 LF, 7 MZ, 7 F_1, and 49 F_2 were scanned and analyzed in this study.

2.3. Pedigree and Linkage Analysis. Details concerning the mapping population, construction of the linkage map, and quantitative trait locus (QTL) analysis have been described elsewhere [13, 34, 47, 48]. In brief, we used a pedigree derived from crossing a single LF male to a single MZ female to generate an F_2 mapping population ($n = 173$) and a linkage map that assigned 165 markers (both microsatellites and SNPs) to 25 linkage groups using JoinMap 3.0 [49]. A linkage analysis was performed using MapQTL 4.0 [50] with I_{max} as the mapping variable. Because of the time and expense required to μCT scan cichlid maxillae, we chose 49 F_2 animals with a wide range of external maxillary thicknesses for our QTL analysis.

It is important to note that, while our experimental design (i.e., bulk segregants) captured much of the variance in maxillary width among our F_2, the relatively small number of F_2 used in this experiment makes the results susceptible to the Beavis effect, in which the number of QTL tends to be underestimated and QTL effects tend to be overestimated [51]. These specific variables should therefore be interpreted with caution as they likely represent a simplified view of the genetic architecture of these traits. However, both modeling and empirical data indicate that the accuracy of QTL localization is less affected by small sample sizes [52, 53]. Nevertheless, we consider this analysis to be largely a proof of concept and the results to be preliminary.

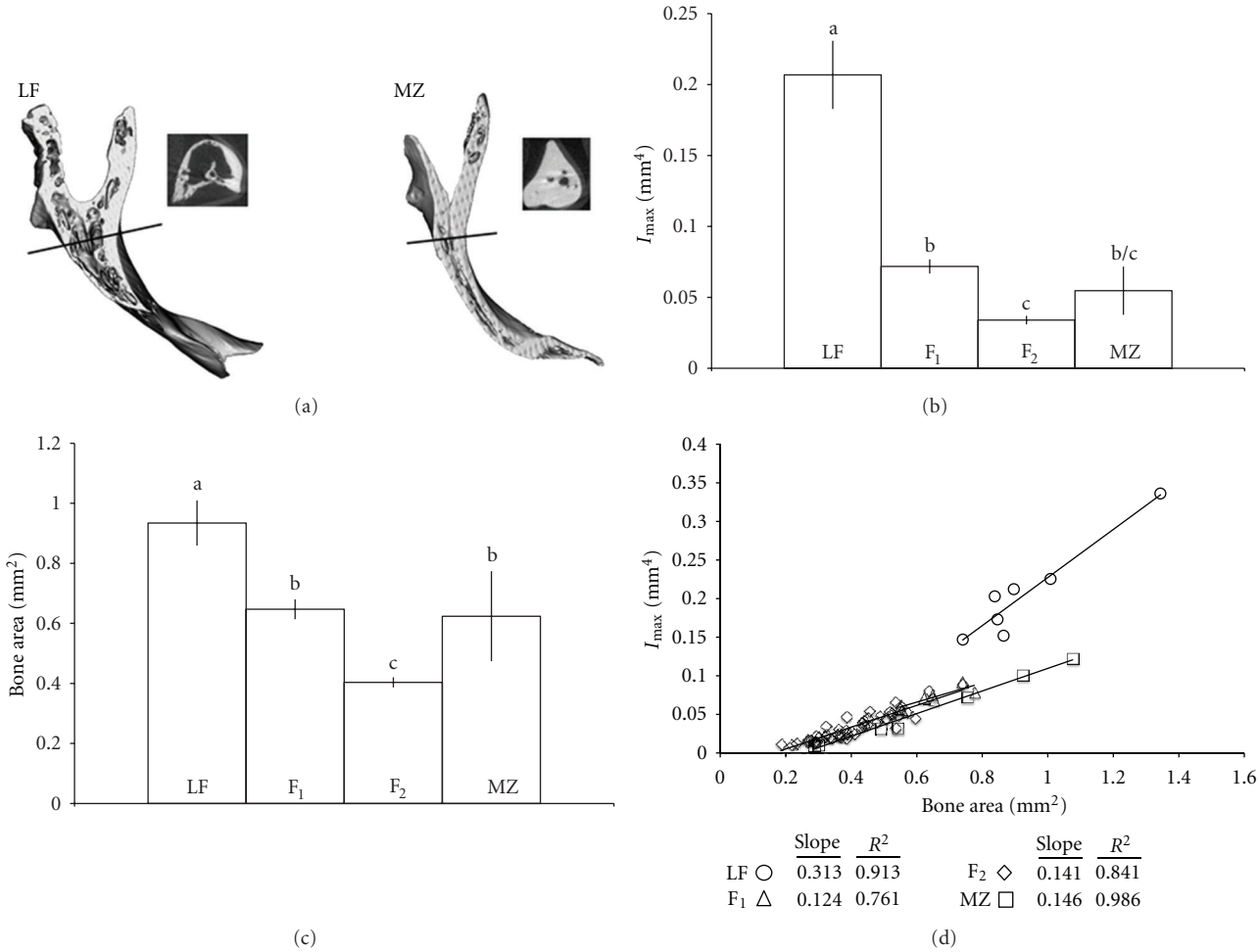

Figure 2: (a) LF and MZ exhibit clear differences in internal bone architecture. Both frontal and transverse sections are shown. Frontal sections were taken approximately halfway through the bone. Lines through the elements show the level at which transverse sections were taken. Differences in bone architecture were quantified as bone bending stiffness (i.e., I_{max} mm^4, (b)) and bone cross-sectional area (mm^2, (c)). For both measures, the F_1 and F_2 hybrid generations are intermediate, with a statistical bias toward MZ values. For both (b) and (c), the "a, b, and c" indicate statistical groupings according to a two-tail t-test, and bars indicate standard errors. (d) Linear regression of bone area on bending stiffness. The relationship between bone bending stiffness and area is approximately the same for MZ and both hybrid generations, but different for LF, which are more efficient in terms of generating greater bending stiffness via the distribution of bone.

3. Results

3.1. Distinct Internal Bone Architectures Are Associated with Divergent Feeding Modes. Micro-CT scanning revealed clear qualitative differences in internal bone morphology between LF and MZ (Figure 2(a)). We found that the maxilla in LF is hollow, with an internal bone architecture that closely resembles that of trabecular bone in mammals. The maxilla of MZ, on the other hand, is comparatively thin and solid. We reported previously that skull bone hydroxyapatite (HA) densities are roughly similar between these two species (LF: 708 ± 100 mg/cc HA; MZ: 757 ± 130 mg/cc HA; [34]), which suggests that any difference in biomechanical performance should be due to geometry rather than substance. To this end, we quantified differences in bone area moment of inertia (i.e., estimated bending stiffness, I_{max} mm^4, Figure 2(b)) and bone cross-sectional area (CSA, mm^2, Figure 2(c)). We found that the maxilla in LF contains significantly more bone than MZ and is also significantly more resistant to bending forces. Moreover, the relationship between bending stiffness and CSA suggests that the internal architecture of the maxilla in LF is more structurally stalwart per unit of bone compared to MZ. This assertion is supported by a steeper slope describing the relationship between bone stiffness and area in LF compared to that in MZ (Figure 2(d)). For both measures, the F_1 and F_2 hybrid generations were statistically biased toward MZ, suggesting a role for dominance in the inheritance of these traits. Moreover, the relationship between bending stiffness and CSA was approximately the same for the MZ, F_1 and F_2 populations.

3.2. Genetic Architecture of a Biomechanical Trait. Two significant QTL were detected for bone bending stiffness (Table 1). The first (I_{max} 1) localized to a narrow region on linkage group 7. The second QTL (I_{max} 2) localized to the

Table 1: Two distinct QTL on two linkage groups (LGs) were detected for bone bending stiffness. Both loci show evidence for dominance of the MZ allele, which is consistent with the mean values for each population reported in Figure 2. The LF/LF genotype increases mean stiffness at both loci, although the mean phenotypic values of each genotypic class were lower than what would be expected based on parental averages. This is likely due to our low F_2 sample size, which has also likely inflated the percent variance explained (PVE) by each QTL.

Trait	Parental means (SE)		95% range* peak					Mean phenotype/F_2 genotype			
	MZ	LF	QTL	LG	cM	cM	LOD	MZ/MZ	MZ/LF	LF/LF	PVE
Stiffness [I_{max} (mm^4)]	0.055 (0.017)	0.207 (0.024)	I_{max} 1	7	51–57	54	3.80	0.0326	0.0361	0.0606	23.4
			I_{max} 2	11	49–50	50	3.10	0.0298	0.0378	0.0630	38.5

* Significance ($\alpha = 0.05$) at the genomewide level.

distal end of linkage group 11. Both loci showed evidence for dominance of the MZ allele, which is consistent with the mean values for each population reported in Figure 2. The LF/LF genotype increased mean bending stiffness at both loci, although the mean phenotypic values of all genotypic classes were lower than what would be expected based on parental averages. This is likely due, at least in part, to our low F_2 sample size, which has also likely acted to inflate the percent variance explained (PVE) by each QTL. We cannot, however, rule out the possibly that other factors are leading to a downward bias in our F_2 values of stiffness, including environmental effects, or allometry. While we made every attempt to maintain constant rearing conditions across populations in terms of tank densities, substrate type, and diet, the F_2 were raised a couple of years after the parental and F_1 populations making it possible that there were unaccounted for differences in environment. Allometry could also be biasing our F_2 values. The average size of our F_2 population was smaller than that for either parental species or the F_1 (average standard length of 8.0 cm (F_2) versus 9.2 cm (LF), 9.3 cm (MZ), and 8.8 cm (F_1)). However, when using residuals from a regression of stiffness on size, the QTL results did not change. Clearly, this observation warrants further investigation.

We chose F_2 individuals for this analysis that exhibited a wide range of maxillary widths, with the intention of maximizing variance and thus the power to detect QTL. However, once these elements were scanned and stiffness was estimated, we found that width was only a weak predictor of bone stiffness ($R^2 = 0.087$, $P = 0.121$). In other words, these traits are segregating largely independent of one another, which suggests that they are under separate genetic control and that external skeletal anatomy cannot predict internal bone architecture. This assertion is supported by the observation that neither of the bending stiffness QTL fell within intervals that were previously implicated in maxillary shape [13]. In fact, QTL I_{max} 2 localized to a region that is distinct from all other cichlid craniofacial QTL identified to date [13, 34, 48, 54, 55]. QTL I_{max} 1, on the other hand, did localize to a region that overlaps with a QTL for jaw width [13] and exhibits a nearly identical LOD distribution with a QTL for the length of the retroarticular (RA) process of the lower jaw [48]. Similar to QTL I_{max} 1, LF alleles at this locus act to increase the trait value for RA length and MZ alleles appear to be dominant.

4. Discussion

4.1. Divergence in Bone Strength and Weight among Vertebrates. Bone strength and stiffness are critical for optimizing the function of skeletal elements associated with feeding and locomotion, and natural selection will favor animals that perform these functions with greater efficiency [56–58]. While both bone density and shape contribute to stiffness and strength, dense bone is heavier than less dense bone. Vertebrate bone therefore tends to be designed such that strength and stiffness are maximized and weight is minimized [58, 59]. This trade-off is especially important in flighted vertebrates, where skeletons must be lightweight to minimize the metabolic cost of flight but strong enough to withstand the torsion and shearing forces associated with powered flight. As a result, birds and bats have evolved bones that are hollow but more dense compared to those of terrestrial vertebrates [60–62].

There is also a dynamic relationship between bone strength and weight among aquatic vertebrates. Specifically, across a spectrum of vertebrate classes the modulation of bone density appears to be a mechanism for buoyancy control [63–65]. This trend is beautifully illustrated by the evolutionary history of whales, which is marked by discrete shifts in habitat from terrestrial, to semiaquatic, and finally to fully aquatic life histories. The mechanical constraints associated with locomotion in each of these habitats are very different, and as a result these evolutionary transitions were accompanied by dramatic changes in bone architecture. For example, the shift from terrestrial to semiaquatic habitats in ancient whales (i.e., archaeocetes) was accompanied by a dramatic increase in bone density. Like other large semiaquatic mammals, this adaptation was for increased mass, which is associated with benthic foraging [63]. Modern whales, on the other hand, are fully aquatic and possess a number of adaptations for life in the open water, including a largely osteoporotic skeleton [63]. While functional parameters including bone stiffness have not been examined in modern cetaceans, it is notable that osteoporotic bone in cetaceans is not observed in elements associated with feeding or locomotion (i.e., skull and vertebrae), where functional demands remain high [64]. Thus, a balance has been struck between increased buoyancy and efficient foraging and locomotion in the skeletons of modern whales.

The evolutionary history of Antarctic notothenioid fishes represents another striking example of how bone development has been modified to affect buoyancy. Antarctic notothenioids represent one of the best described adaptive radiations among marine fishes [66], and the hallmark of their evolution is the development of secondary pelagicism via alteration of buoyancy [67]. This lineage is thought to have evolved from a robustly mineralized bottom-dwelling perciform species beginning 40–60 mya when the waters of the Antarctic continental shelf were still temperate [67]. The grounding of the ice sheet on the continental shelf and changing trophic conditions led to the local extinction of the diverse late Eocene fish fauna, thus freeing pelagic niches into which the notothenioids radiated [68]. About 50% of notothenioid species now either live or forage in the pelagic habitat [69]. In many instances, secondary pelagicism has been achieved through pedomorphism, including the complete or partial retention of the notochord, delayed ossification of the skeleton, and replacement of bone by connective tissue [65, 70–72]. Similar to cetaceans, osteoporotic bone in pelagic notothenioids is most pronounced in areas of the skeleton that are not intimately associated with foraging (e.g., oral jaws) or locomotion (e.g., pectoral fins) [70].

While the examples above represent changes in bone structure at the macroevolutionary level, it is reasonable to assume that similar trends underlie microevolutionary divergence. As mentioned above, cichlids have diverged along a benthic-pelagic ecomorphological axis, and extensive modifications to the skeletal system have accompanied this divergence [14]. LF and MZ are closely related species that lie on opposing ends of this continuum, and while bone density does not appear to be different between these two species [34], LF has a more extensively mineralized skeleton (i.e., more bone in more places) [42], which is commensurate with other adaptations for a benthic mode of feeding. These findings suggest that levels and patterns of bone deposition are more evolvable in this group than are the material properties of bone (although a more rigorous survey of HA density in a greater number of elements and across more taxa is needed). We also show here that internal bone architecture appears to be surprisingly malleable among cichlids, as strikingly different cross-sectional bone shapes exist between species that employ alternate modes of feeding. This sets up clear predictions that can be tested in a larger number of species. For example, if species were arrayed along a benthic-pelagic ecomorphological axis, one might expect that this would establish a continuum of internal bone architectures. Alternatively, since LF represents a highly derived species, it is also possible that the internal bone architecture described here (i.e., high stiffness) is unique to this species. Clearly, this would be a fruitful area of future research.

4.2. Roles for the Environment versus Genetics in Determining Internal Bone Architecture. Bone geometry influences stiffness such that bone with a solid cross-section is less rigid whereas hollow bone with the same cross-sectional area is more rigid. Natural selection should therefore favor one configuration over the other depending on the task to be performed (e.g., biting versus sucking). Alternatively, given the varying functional demands imposed on the vertebrate skeletal system over ontogeny, or from season to season, selection might favor a plastic skeletal system that can respond to different mechanical stimuli. Distinguishing between these alternatives represents an important, but muddled area of research. In other words, the degree to which internal bone architecture is genetically preprogrammed or mechanically regulated remains unclear.

On one hand, both computational modeling and empirical studies offer strong support for the assertion that internal bone architecture responds to mechanical stimuli [59, 73, 74]. Alternatively, disparate vertebrate taxa have modified internal bone geometry due to novel functional demands (e.g., powered flight in birds and bats) [35, 61, 62], which suggests a genetic component for this trait. Unfortunately, compared to the relatively large body of literature dedicated to the study of the genetics of bone material properties (focused mainly on mouse mutants, reviewed by [75]), less is known about the genetic basis of bone geometry. Moreover, mutations that lead to aberrant bone architectures usually also affect material properties. "Wolff's Law" suggests that bone adapts to mechanical stimuli to maintain a narrow range of strain (reviewed by [76]). It is therefore thought that for many/most mouse mutants where both bone material and geometry are affected, deficient material properties are the primary defect and altered geometry represents a secondary response to compensate for abnormal bone strains [76].

Cichlids offer a genetic system where internal bone architecture varies independently from material properties, thus mitigating the confounding issues associated with Wolff's Law. Whereas HA density appears relatively conserved between the species examined here, internal geometry differs dramatically. This could be due to a fundamental constraint associated with changing material properties in fishes (e.g., higher-density bone is more brittle) or because altering bone architecture is a more efficient way to affect stiffness. For example, bending stiffness of a round bone is equal to EI, where the elastic modulus (E) is proportional to HA density of bone and area moment of inertia (I) is proportional to radius^4. Doubling HA would lead to a doubling of stiffness, whereas doubling the radius would lead to a 16-fold increase in stiffness. Changing bone architecture is therefore a more efficient way to change bone function due to increased demands on bending load. Either way, the decoupling of these two properties of bone, as well as the ability to perform genetic mapping studies, offers an excellent opportunity to examine the genetic basis of internal bone geometry. Moreover, the ability to rear cichlids on a range of diets (e.g., hard versus soft), thereby altering the mechanical environment in which the jaws develop, would enable an assessment of the degree to which this trait responds to the environment. Thus, cichlids represent an ideal system in which to characterize the genetic and environmental factors that influence this functionally salient trait.

4.3. Conclusions. We demonstrate that cichlids with divergent feeding morphologies and behaviors exhibit different

internal bone architectures that translate to different estimates of load-bearing function. We show further that this functional trait has a tractable genetic basis. Since bone geometry has a profound effect on skeletal performance and since performance determines resource use in nature, examining the genetic basis for this trait has the potential to yield important new insights into the mechanisms that have contributed to several notable adaptive radiations (e.g., those that involve divergence in feeding systems or locomotion). Moreover, continued work in the cichlid system may also contribute to an understanding of many unresolved issues in the biomedical literature, especially those focused on decoupling the genetic from epigenetic influences on internal bone geometry. In conclusion, external bone shape and size, while important in determining skeletal function, do not tell the entire story [76]. Future research should therefore be aimed at elucidating a better understanding of (1) the material properties and (2) internal geometry of skeletal elements associated with feeding and locomotion in this and other adaptive radiations.

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Research Article

Extensive Introgression among Ancestral mtDNA Lineages: Phylogenetic Relationships of the Utaka within the Lake Malawi Cichlid Flock

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We present a comprehensive phylogenetic analysis of the Utaka, an informal taxonomic group of cichlid species from Lake Malawi. We analyse both nuclear and mtDNA data from five Utaka species representing two (*Copadichromis* and *Mchenga*) of the three genera within Utaka. Within three of the five analysed species we find two very divergent mtDNA lineages. These lineages are widespread and occur sympatrically in conspecific individuals in different areas throughout the lake. In a broader taxonomic context including representatives of the main groups within the Lake Malawi cichlid fauna, we find that one of these lineages clusters within the non-Mbuna mtDNA clade, while the other forms a separate clade stemming from the base of the Malawian cichlid radiation. This second mtDNA lineage was only found in Utaka individuals, mostly within *Copadichromis* sp. "virginalis kajose" specimens. The nuclear genes analysed, on the other hand, did not show traces of divergence within each species. We suggest that the discrepancy between the mtDNA and the nuclear DNA signatures is best explained by a past hybridisation event by which the mtDNA of another species introgressed into the ancestral *Copadichromis* sp. "virginalis kajose" gene pool.

1. Introduction

The Lake Malawi cichlid fauna comprises over 800 species [1] offering a spectacular example of adaptive radiation with virtually all niches in the lake being filled by members of this family [2, 3]. With a few exceptions, all Lake Malawi cichlids form a monophyletic group as supported by mitochondrial [4–6] and nuclear ([7–9] but see [10]) markers as well as allozymes [11, 12].

The phylogenetic reconstruction of Lake Malawi cichlid fauna has recovered six main mitochondrial DNA (mtDNA) lineages [5, 6, 10, 13]. Two of these lineages correspond to the *Rhamphochromis* and *Diplotaxodon* genera. A third lineage contains the nonendemic riverine *Astatotilapia calliptera*. The remaining cichlid fauna has been traditionally divided into two groups: one containing predominantly the rock-dwelling species commonly called Mbuna and the second containing the remaining Lake Malawi cichlids. However, phylogenetic reconstructions have shown that both groups are artificial [5, 10, 13, 14]. Several *Lethrinops, Aulonocara,* and *Alticorpus* species (ecologically and morphologically typically assigned to the non-Mbuna) cluster within the Mbuna clade. Furthermore, the non-Mbuna genus *Copadichromis* has been shown to have representatives belonging to both the non-Mbuna clade, as well as to a separate lineage. The genus *Copadichromis*, together with the genus *Nyassachromis* and the newly erected genus *Mchenga,* constitute the Utaka, a species assemblage of midwater-feeding zooplanktivorous cichlid species. The phylogenetic position of this group remains unclear with Moran et al. [5] and Turner et al. [13] not recovering mtDNA monophyly within this assemblage: *M. eucinostomus* and *C. borleyi* were

placed within the non-Mbuna clade, while *C. mloto* (reidentified as *Copadichromis* sp. "virginalis kajose", J. Snoeks pers. obs.) and some other individuals of the *Copadichromis virginalis* complex seemed to represent a different, well-diverged lineage.

However only few Utaka specimens have been included in phylogenetic analyses so far. Therefore, currently available mtDNA phylogenies are inconclusive as to whether the Utaka are genetically associated with the non-Mbuna clade, whether they constitute an originally separate ancestral lineage, or whether only one or a few species or specimens cluster in a separate lineage. If specimens of a species cluster in genetically distant lineages, this may be a result of the retention of ancestral polymorphism, the existence of a cryptic species, or traces of a past hybridisation/introgression event. Support for these alternative hypotheses may be gained by using a multilocus approach (e.g., [10, 14–16]). We therefore combined mtDNA gene sequences with data from nuclear microsatellite loci. If the nuclear genetic signature is concordant with the mtDNA in subdividing a species into genetically separated units, this may point towards a cryptic species. On the other hand, if a mtDNA split within a species is not supported by the nuclear genetic data, this may be an indication of introgression of genetic material from another species, or of shared ancestral polymorphism.

Whereas the resolution of the specific interrelationships within the major clades remains problematic, the six main mtDNA clades of the Malawi cichlid flock are clearly delineated [5, 6, 13]. Shared polymorphism within taxa might result from incomplete lineage sorting, taxonomic inaccuracies, and/or hybridisation. While the other possibilities cannot be completely ruled out, there is a growing number of studies acknowledging the important role of hybridisation in the evolutionary history of adaptive radiations (e.g., [17–19]). In this study we present the most comprehensive mtDNA phylogeny of the Utaka assemblage so far. We aim at elucidating the phylogenetic position of the Utaka within the Malawian cichlid radiation and shed light on the causes for its taxonomic and molecular assignment inconsistency.

2. Material and Methods

2.1. Taxonomic Sampling. We examined individuals of five Utaka species (*Copadichromis* sp. "virginalis kajose", *C. quadrimaculatus, M. eucinostomus, C. chrysonotus,* and *C. borleyi*) from twelve localities throughout Lake Malawi and one locality in Lake Malombe (Figure 1). Pelvic fin clips were preserved in 100% ethanol and stored at room temperature. Voucher specimens were fixed in 10% formalin and are curated at the Royal Museum for Central Africa in Tervuren, Belgium. We included additional *Copadichromis* species that were sampled during the SADC/GEF project [1] and previously published mtDNA control region (complete D-loop) sequences of Lake Malawi cichlids, which we obtained from GenBank.

2.2. DNA Extraction, mtDNA Amplification, and Sequencing. Whole genomic DNA was extracted from ethanol-preserved fin clips using proteinase K digestion and salt precipitation, according to Aljanabi and Martinez [20]. DNA extracts were resuspended in 100 μL of autoclaved Milli Q water. The first fragment of the mtDNA control region was sequenced for 412 Utaka specimens (179 *Copadichromis* sp. "virginalis kajose", Genbank Accession EF211832-EF211945 and EF647210-EF647271; 55 *C. quadrimaculatus*, Genbank Accession EF647341-EF647438 and EF647578-EF647579; 67 *M. eucinostomus*, Genbank Accession EF647356-EF647390, EF647439-EF647460, EF647498-EF647505 and EF647581-EF647582; 70 *C. chrysonotus*, Genbank Accession EF647273-EF647340, and EF647571-EF647572; 41 *C. borleyi*, Genbank Accession EF647470-EF647497, EF647520-EF647531, and EF647548), using published primers by Meyer et al. [4]. We additionally sequenced the second fragment of the control region using the primers by Salzburger et al. [21] and Lee et al. [22] for 14 individuals, selected on the basis of the results of the phylogenetic reconstruction for the first fragment of the control region. Polymerase chain reactions (PCRs) were carried out in 25 μL buffered reaction mixtures, containing 5 μL template DNA, 5 μL of each primer (2 μM), 200 μM of each dNTP, 2.5 μL of 10x buffer (1 mM MgCl2), and 0.65 units of Red Taq Polymerase (Sigma Aldrich). PCRs were performed under the following conditions: 94°C for 120 s, followed by 35 cycles of 94°C for 60 s, 52°C for 60 s, 72°C for 120 s, followed by 72°C for 10 min. PCR products were purified following the TMQiaquick PCR purification Kit protocol and sequenced on an ABI 3130 automatic sequencer (Applied Biosystems) using standard protocols.

2.3. Microsatellite Variation. A total of 179 *C.* sp. "virginalis kajose", 230 *C. chrysonotus*, 252 *C. quadrimaculatus,* and 344 *M. eucinostomus* individuals were screened for genetic variation at nine microsatellite markers: Pzeb1, Pzeb3, Pzeb4, Pzeb5 [23], UNH002 [24], TmoM5, TmoM11, TmoM27 [25], and UME003 [26]. PCRs were performed under the following conditions: 94°C for 120 s, followed by 5 cycles of 94°C for 45 s; 55°C for 45 s; 72°C for 45 s, followed by 30 cycles of 90°C for 30 s; 55°C for 30 s; 72°C for 30 s, followed by 72°C for 10 min. 10 μL reaction mixes included 1 μL template DNA, 0.5 μM of each primer, 200 μM of each dNTP, 0.26 units Taq polymerase (Sigma Aldrich, Germany), 1 μL 10× reaction buffer (Sigma Aldrich). PCR amplification products were run on 6% denaturing polyacrylamide gels using an ALF Express DNA Sequencer (Amersham Pharmacia Biotech). Fragment sizes were scored with ALFWin Fragment Analyser v1.0 (Amersham Pharmacia Biotech), using M13mp8 DNA standards as external references, following van Oppen et al. [23].

2.4. Phylogenetic Reconstructions. For the reconstruction of the phylogenetic relationships of the Utaka, two datasets were analysed. Both were aligned using CLUSTALW [27] and visually checked afterwards using the program SEAVIEW [28]. The first dataset contained 412 *Copadichromis* spp. and *Mchenga* sp. sequences of the first fragment of the control region (328 bp). The program COLLAPSE v1.2 [29] was used to reduce this dataset to one individual sequence

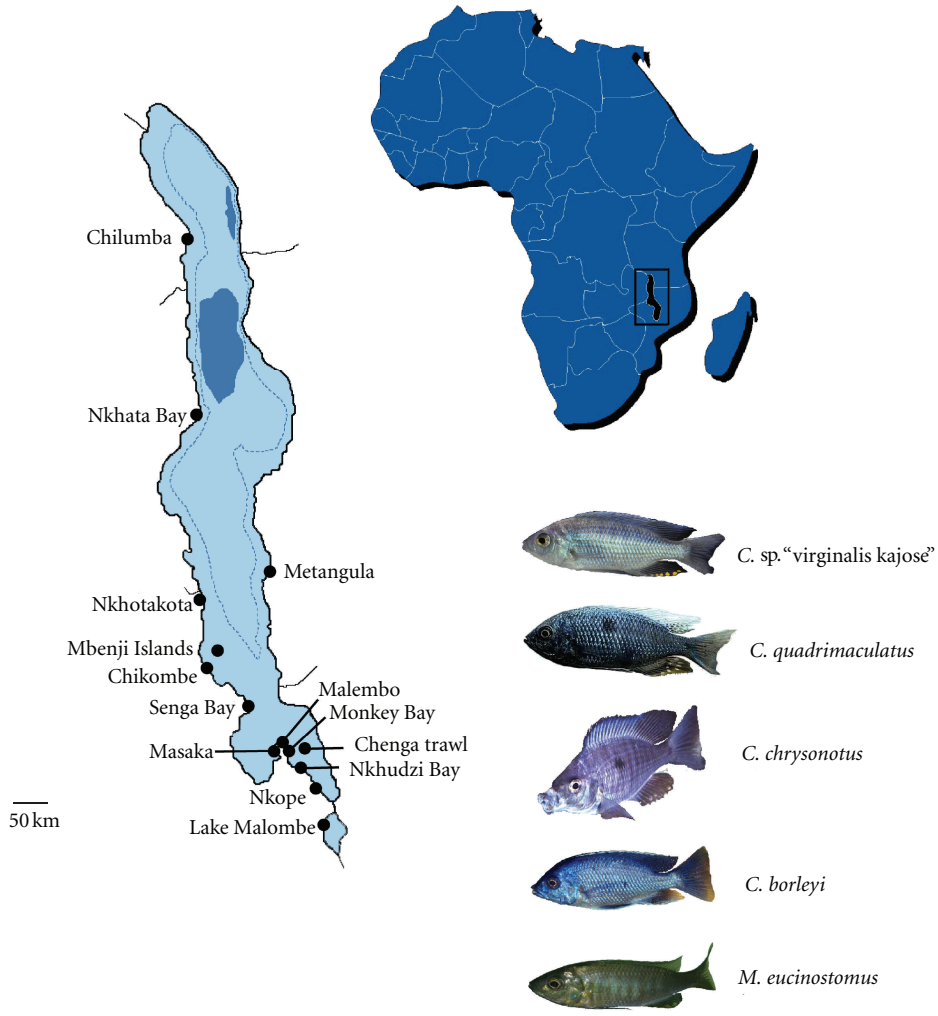


Figure 1: Map of Lake Malawi showing the localities sampled. A detailed listing on the origin of each specimen is presented in the Supplementary Table of the Supplementary Material available online at doi:10.1155/2012/865603.

per haplotype for further analyses. GTR+G+I model was the best-fitted model of sequence evolution inferred by MODELTEST v3.7 [30] according to the Akaike Information Criterion (AIC). A maximum likelihood (ML) heuristic search was performed with PHYML [31] starting from a neighbour-joining (NJ) tree. Parameters of the tree and of the substitution model were optimised sequentially until no increase in likelihood was found. The program TCS [32] was used to generate a haplotype network using statistical parsimony. Based on the results of the short control region phylogenetic reconstruction, we performed a second, more computationally intensive phylogenetic analysis with a smaller dataset containing 47 representatives of the different main lineages in the Lake Malawi cichlid flock (both new sequences and sequences extracted from GenBank) to test the interrelationships between these main lineages. Sites that could not be unambiguously aligned were removed prior to analysis. The final dataset, 837 bp long, was first run through MODELTEST, which selected the TrN+I+G model (AIC criterion).

Phylogenetic inferences were carried out using maximum-parsimony (MP, 100 replicates starting from random stepwise addition trees; TBR branch swapping) with different transition-transversion weights (1 : 1, 2 : 1 and 3 : 1) in PAUP* v4.0 [27]. ML reconstructions (100 replicates starting from random stepwise addition trees; TBR branch swapping) were run in PAUP*. Sequential searches were performed by reestimating the substitution model parameters upon the best tree found and then running a new search with these parameters. This was done until no change in the likelihood of the tree or in the estimated parameters was found. Support for the internal branches in the ML tree was assessed by analysing 100 bootstrapped replicates in the program PHYML. For Bayesian inference (BI) analyses, the GTR+G+I model was used since the TrN+G+I is not implemented in MRBAYES v.3.1 [33]. Markov Chain Monte Carlo samplings were run for 25 million generations. Two runs with four chains for each run were sampled every thousandth generation until the average standard deviation of split frequencies between runs reached ~0.003. Inspection

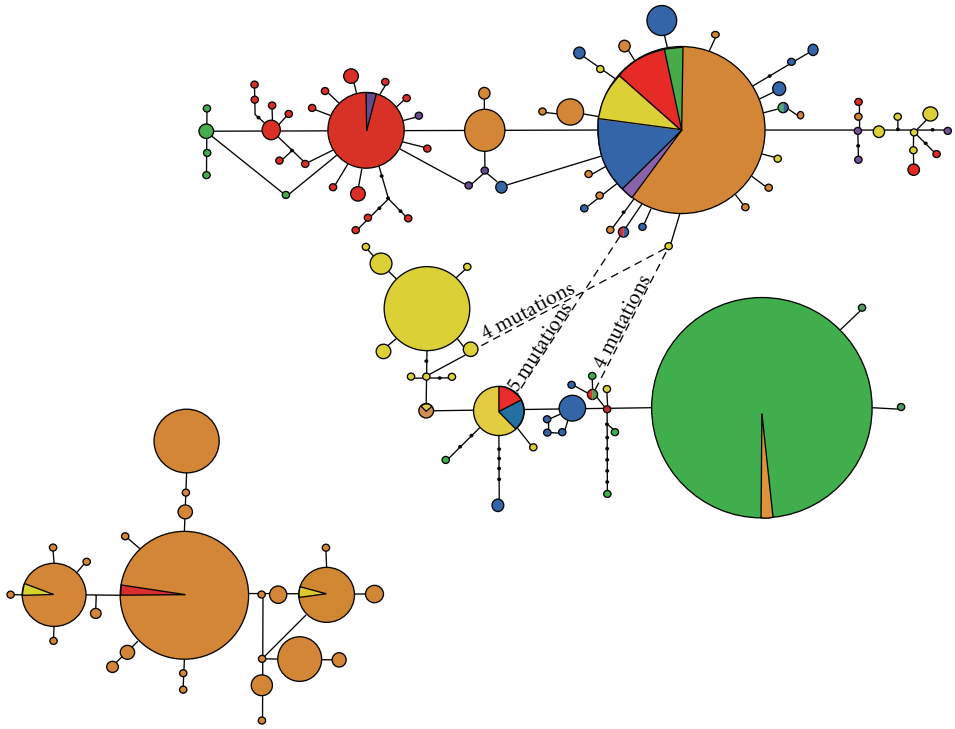


Figure 2: Haplotype networks of the Utaka obtained in this study. The upper network contains the majority of the specimens analysed and is separated from the lower network (named virginalis clade in text) by more than seven mutations. Each circle represents a haplotype and is coloured according to the respective species: blue: *C. borleyi*; orange: *C.* sp. "virginalis kajose"; red: *C. quadrimaculatus*; green: *C. chrysonotus* yellow: *M. eucinostomus*; purple: *C. mloto* and *C.* sp. "meta". Size of the circles is proportional to the frequency of each haplotype as indicated in the scaled circles. Small black circles in branches represent missing haplotypes. Dashed lines represent alternative connections with the number of missing haplotypes written above the lines.

of plot of likelihood versus generation revealed that the runs had reached stability and so did the analysis of the Potential Scale Reduction Factors.

Using the Shimodaira-Hasegawa test [34], as implemented in PAUP*, we tested the relative fit of two alternative tree topologies: the forced monophyly of all Utaka specimens was compared to the best, unconstrained tree. Significance of the difference in log-likelihood between the two trees was assessed by means of the Resample Estimated Log-Likelihood test (RELL).

2.5. Microsatellite Data Analysis. Linkage disequilibrium between loci was tested using exact tests as implemented by GENEPOP 3.3 [35]. We estimated the number of populations present in our microsatellite dataset using the program STRUCTURE [36, 37]. We calculated the posterior probability for different numbers of putative populations (K from 1 to 18 populations) using a model-based assignment. Burn-in was set at 100,000 steps followed by 300,000 MCMC iterations at each K. Simulations were run five times for each K to check for convergence of the MCMC. We performed clustering both under the admixture model without prior population information and with correlated allele frequencies between populations. To determine the most likely number of clusters, the rate of change in the log probability of data and in the statistic ΔK [38] between successive K values was estimated using StructureHarvester [39].

3. Results

3.1. Phylogenetic Reconstructions. The purpose of our phylogenetic analyses was twofold. First, we assessed the phylogenetic relationships among as many specimens as available from the five Utaka species that we collected throughout the lake. For this extensive dataset, we sequenced the short (328 bp) but most variable part of the mtDNA control region. By this analysis we aimed to detect specific or geographical patterns among the Utaka species studied. Second, we attempted to resolve the phylogenetic position of the Utaka species within the Lake Malawi cichlid flock. For this purpose we sequenced the complete mitochondrial control region for representative specimens ($n = 14$) of the previous dataset and included published sequences from species representing the main lineages in the Malawian cichlid flock. A total of 115 haplotypes were found amongst the 412 Utaka short mtDNA control region sequences (Figure 2). The ML tree presented two divergent clades within the Utaka: a large clade containing circa 70% of all sequences, and a smaller group. The latter almost exclusively contained *C.* sp. "virginalis kajose" individuals (125 *C.* sp. "virginalis kajose" individuals out of 179 sequenced clustered within this clade),

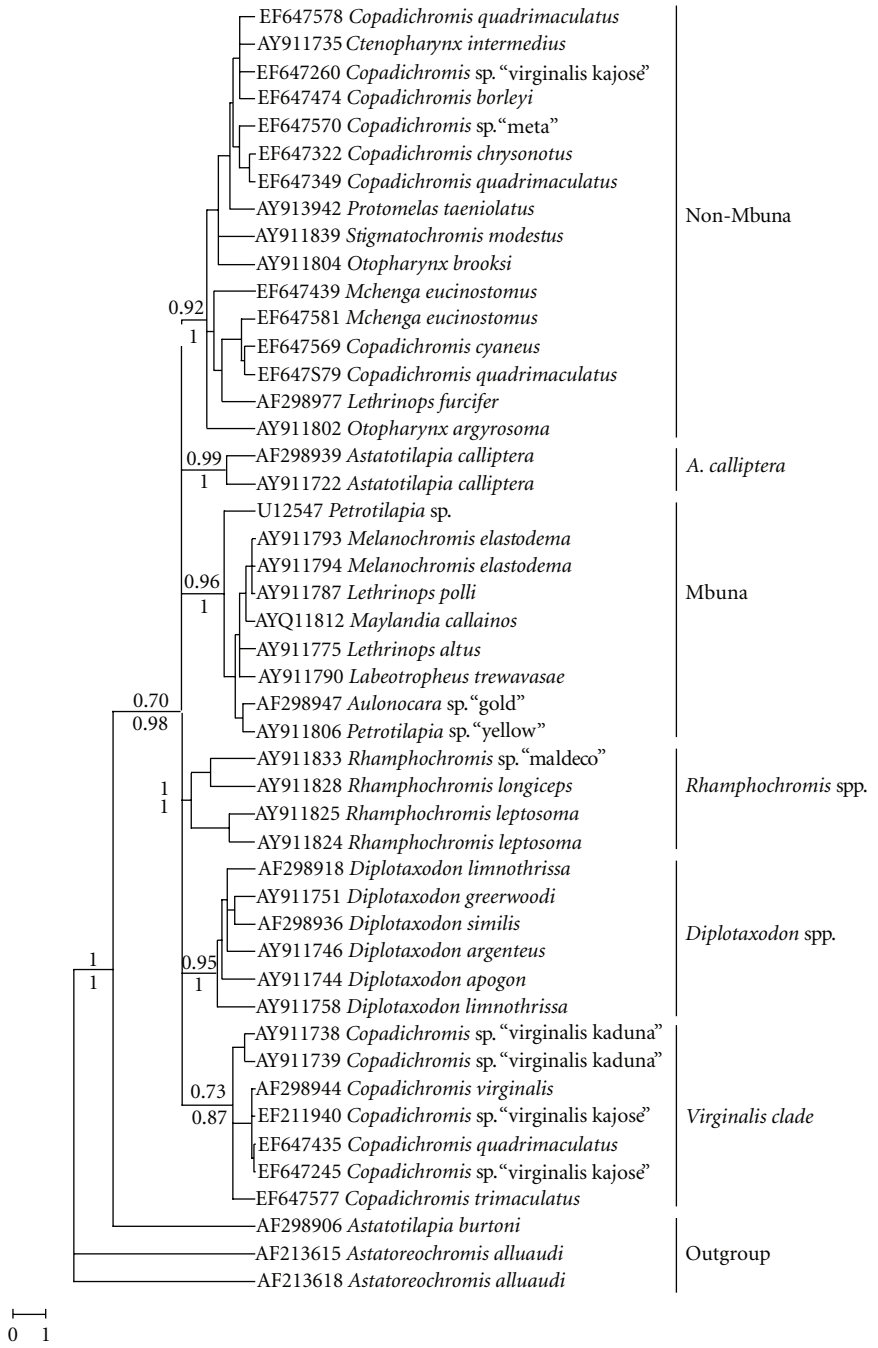
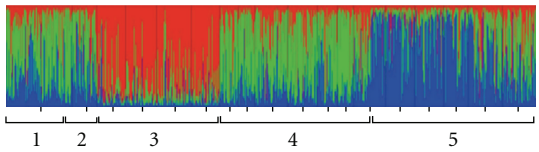


Figure 3: Maximum-likelihood reconstruction (PhyML) of the Lake Malawi cichlid flock using the complete mtDNA control region. Numbers next to the branches show bootstrap percentage support (upper) and bayesian posterior probabilities (below) of the main clades. Clade names follow denomination given in text. *Astatoreochromis alluaudi* and *Astatotilapia burtoni* represent the outgroups. Scale bar indicates substitutions per nucleotide site.

together with two (out of 67) *M. eucinostomus* and one (out of 55) *C. quadrimaculatus* individuals. Both mtDNA clades were present lake-wide in nearly all localities sampled, and within each lineage distinct geographic structuring was absent.

The complete mtDNA control region phylogenetic reconstructions using MP (with the different weighting schemes), ML, and BI consistently recovered the 6 main clades among the Malawian cichlids (Figure 3): (i) a lineage containing most non-Mbuna and Utaka species (non-Mbuna clade hereafter); (ii) a clade containing only *Copadichromis* individuals (virginalis clade hereafter); (iii) a Mbuna clade containing all Mbuna species plus some deep-water *Lethrinops* species and an *Aulonocara* specimen; (iv) a *Diplotaxodon* clade; (v) a *Rhamphochromis* clade; (vi) a clade containing *A. calliptera*. For these clades, bootstrap values



1 = *C.* sp."virginalis kajose" (non-Mbuna clade)
2 = *C.* sp."virginalis kajose" (virginalis clade)
3 = *C. chrysonotus*
4 = *C. quadrimaculatus*
5 = *M. eucinostomus*

Figure 4: Bar plot result of the STRUCTURE assignment test for $K = 3$ under the nonadmixture model. The two mtDNA groups within *C.* sp. "virginalis kajose" (non-Mbuna and virginalis clade) cannot be distinguished from each other based on the nuclear markers, whereas *C. chrysonotus* and *M. eucinostomus* clearly differentiate although they share the same mtDNA haplotype lineage.

and posterior probabilities displayed high node support values, except for the virginalis clade, which had a bootstrap support of 73 and a posterior probability of 0.87 (Figure 3). The phylogenetic relationships between the clades remained, however, unresolved: their branching order was variable, depending on the reconstruction methods used and was even resolved as a polytomy in the Bayesian analysis. The Shimodaira-Hasegawa test indicated a significant difference in likelihood score between the two topologies examined ($P = 0.03$), giving preference to the unconstrained topology (where Utaka are paraphyletic) over the best tree obeying to the monophyly of all Utaka specimens analysed.

3.2. Microsatellites. Linkage disequilibrium tests across loci and populations revealed no significant allelic associations. The model-based clustering approach implemented in the program STRUCTURE yielded estimated Ln probabilities for $1 \leq K \leq 18$ ranging from –37678 to –34160 with the highest posterior probability and ΔK (=20.605) for $K = 3$. In the most likely scenario, STRUCTURE assigned *M. eucinostomus* and *C. chrysonotus* to two different groups, while *C. quadrimaculatus* and *C.* sp. "virginalis kajose" formed a third cluster (Figure 4).

4. Discussion

The phylogenetic inferences of the Utaka assemblage performed herein showed that it contains two genetically distant and geographically widespread mtDNA lineages. The two lineages have been observed before [5, 10, 13, 14] but this is the first study to reveal the paraphyly not only of the genus *Copadichromis* in individuals from throughout Lake Malawi, but also of three (of the five analysed) Utaka species (based on the short mtDNA sequences). In a wider taxonomic context involving the other Malawian cichlid lineages, the most abundant of the two lineages in the Utaka clustered within the non-Mbuna mtDNA clade, while the other formed a separate clade containing exclusively Utaka specimens, mostly *C.* sp. "virginalis kajose" individuals. The paraphyly of the Utaka does not represent an artefact in our analyses, as corroborated by the long and well-supported branches that connect the non-Mbuna and the virginalis clades, as well as by the significant result of the Shimodaira-Hasegawa test.

One possible explanation is that the Utaka share ancestral polymorphic alleles and/or represent a truly paraphyletic group containing multiple lineages that have undergone convergent evolution. Importantly, the occurrence of two divergent mtDNA lineages within the Utaka is related neither to taxonomic clustering, nor to geographical structuring. If the two haplogroups observed within the Utaka indeed correspond to two ancestral lineages that are genetically isolated for such a long time that their mtDNA genotypes have become so deeply diverged, we would expect this to be also reflected in the nuclear genome of the species. However, we did not find any subdivision of nuclear gene pools that corresponds to the deep mtDNA divergence, neither across the Utaka species, nor within *C.* sp. "virginalis kajose" which yields the majority of the individuals in the divergent virginalis clade as well as a large number of individuals in the non-Mbuna clade. It thus seems unlikely that the presence of a cryptic species is the cause of the mtDNA divergence within *C.* sp. "virginalis kajose." Recently published phylogenetic reconstructions using AFLP loci [10, 14] also showed a discordance between the nuclear and mitochondrial placement of *Copadichromis virginalis* within the Malawi cichlid radiation, supporting our finding that the observed paraphyly of the Utaka and of *C.* sp. "virginalis kajose" is unlikely to be the result of incomplete ancestral lineage sorting or true paraphyly.

Alternatively, a disparate pattern of divergence between mitochondrial and nuclear DNA among conspecific individuals may be the result of a past hybridisation and introgression event, a process which has been documented in Malawian cichlids before (e.g., [40–42]) and for which evidence is accumulating (e.g., [10, 14, 16, 43]). Under this hypothesis we advance two possibilities regarding the original position of the Utaka within the Malawi cichlid phylogeny. A first scenario assumes that all Utaka species formerly constituted a separate ancestral clade within the Malawi cichlid flock, corresponding with the current virginalis clade. Subsequent unidirectional introgression of mtDNA from non-Mbuna into the Utaka could then explain the observed clustering of Utaka specimens within the non-Mbuna lineage. This scenario would involve that either all, or the ancestors of the current Utaka species, would have been extensively hybridised with a non-Mbuna species, resulting in the almost complete replacement of the original mtDNA of the Utaka. A second scenario assumes that all Utaka species initially belonged to the non-Mbuna lineage and a species from a distant mtDNA lineage hybridised with *Copadichromis* species. The mtDNA detected in the virginalis clade may then represent the introgressed mtDNA.

Interestingly and despite our extensive taxonomic sampling, the maternal species involved in the putative hybridisation event remains unidentified as the virginalis clade only contained representatives of the Utaka assemblage. It would seem that the species with which *Copadichromis* spp.

hybridised either has thus far not been subjected to molecular studies or may no longer be present in the lake. Empirical evidence for or against the above scenarios can be gained by examining mtDNA of supplementary Utaka species to validate whether the majority of the taxa cluster is within the non-Mbuna clade or within the virginalis clade. The more Utaka species cluster within the non-Mbuna clade, the less probable becomes the first scenario. Regardless of which of the two mtDNA lineages is the original or the introgressing one, and irrespective of the maternal species involved in the hybridisation event, our results show that the two mtDNA lineages have persisted within the gene pool of *Copadichromis* sp. "virginalis kajose" for a rather long period, as suggested by the diversity displayed by either of these two lineages (Figure 2). It thus suggests that either the population size of this species has remained very high since the hybridisation event (such that genetic drift would represent a lesser issue) or that some other mechanism is maintaining the two lineages within the same species (e.g., balancing or frequency-dependent selection).

Interspecific gene flow is increasingly recognized as an important factor in shaping speciation (e.g., [17–19, 44, 45]). Progressively more examples for hybridization are known from African cichlid fish: among Lake Tanganyika's cichlids evidence is found for ancient introgression (e.g., [15, 46–48]) and a complete replacement [49] of mtDNA in multiple tribes of the cichlid assemblage. From Lake Malawi, evidence for deep introgression leaving a long-term signal in its haplochromine radiation [10, 14, 43], as well as evidence for more recent natural hybridisation [16, 50, 51] among Malawi cichlids, has been provided. In the Lake Victoria cichlid flock recent or ongoing hybridisation [52–54] presumably affects large parts of the species' genomes by homogenization [54, 55], hampering the reconstruction of its young evolutionary history [54, 56, 57], yet potentially seeding the process of speciation [58] but see [55]. In Cameroonian crater lakes the hybridisation of two ancient lineages resulted in the formation of a new and ecologically highly distinct species [59]. Also for *Steatocranus* cichlids from the Congo basin it was recently shown that ancient as well as recent introgression of genes and hybridisation produced a genomic network that potentially promoted divergence and speciation [60]. Our results chime well with previous studies reporting hybridisation in the early stages of a cichlid radiation. Our findings reconcile with the recently reported evidence for ancient introgression between Mbuna and deep-benthic cichlids at the base of the Malawi radiation [14]. Remarkably, in our study we could not separate *Copadichromis* sp. "virginalis kajose" and *C. quadrimaculatus*, two phenotypically distinct taxa, by our microsatellite markers. However, it has already been reported that the performance of a clustering method may become poor for *Fst*'s below 0.05 ([61], J. Pritchard, *pers. comm.*). The estimates of population differentiation were low in both species ($\theta = 0.006$ in *C.* sp. "virginalis kajose" and $\theta = 0.007$ in *C. quadrimaculatus*, reported in [62]), and slightly higher among the two species ($\theta = 0.01$). Whether this observation might yield a demonstration of the relative ease of hybridisation among phenotypically well-differentiated taxa [14, 43] or be the result of an insufficient resolution of the markers used, deserves further research.

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