Research Article

Evolutionary Relationship between Two Firefly Species, *Curtos costipennis* and *C. okinawanus* (Coleoptera, Lampyridae), in the Ryukyu Islands of Japan Revealed by the Mitochondrial and Nuclear DNA Sequences

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The phylogenetic relationship, biogeography, and evolutionary history of closely related two firefly species, *Curtos costipennis* and *C. okinawanus*, distributed in the Ryukyu Islands of Japan were examined based on nucleotide sequences of mitochondrial (2.2 kb long) and nuclear (1.1-1.2 kb long) DNAs. In these analyses, individuals were divided among three genetically distinct local groups, *C. costipennis* in the Amami region, *C. okinawanus* in the Okinawa region, and *C. costipennis* in the Sakishima region. Their mtDNA sequences suggested that ancestral *C. costipennis* population was first separated between the Central and Southern Ryukyu areas, and the northern half was then subdivided between *C. costipennis* in the Amami and *C. okinawanus* in the Okinawa. The application of the molecular evolutionary clocks of coleopteran insects indicated that their vicariance occurred 1.0–1.4 million years ago, suggesting the influence of submergence and subdivision of a paleopeninsula extending between the Ryukyu Islands and continental China through Taiwan in the early Pleistocene.

1. Introduction

The Ryukyu Islands form a chain of more than 200 islands extending for about 1,200 km between the Japanese mainland and Taiwan. The faunae of this area are divided among three portions, the Northern, Central, and Southern Ryukyu areas, and this pattern is considered to have been strongly influenced by changes of the land configuration of this area during the Neogene and Quaternary Periods that occurred due to the interaction between tectonic changes of the Ryukyu ridge and changes of the sea level [1–3]. Paleolands or land bridges connecting this area with Taiwan and continental China, super islands connecting neighboring islands, and geologically long-standing channels that emerged during these periods are considered to be major factors, as they acted as corridors and barriers to biological dispersal [3, 4].

The genus *Curtos* Motschulsky is a group of fireflies including 16 species mainly distributed in southeastern Asia [5, 6]. In Japan, only two species, *Curtos costipennis* (Gorham) and *C. okinawanus* Matsumura, are known to exist in the Ryukyu Islands. The former species, also extant on the Chinese continent and in Taiwan, is distributed widely in the Northern, Central, and Southern Ryukyu Islands excluding the Okinawa region in the southern half of the Central Ryukyu Islands, while the range of the latter species is restricted to the Okinawa region, interrupting the range of the former species. Based on morphological and behavioral observations of the two species, Ohba and Goto [7] suggested that *C. okinawanus* might have derived from *C. costipennis*. However, it is not clear where and how these species have evolved or how *C. okinawanus* came to occupy the Okinawa region in the middle of the range of *C. costipennis*. Otherwise, *C. costipennis* might have entered the Ryukyu Islands from

FIGURE 1: Map showing islands where *Curtos* fireflies were collected.

Taiwan or the Chinese continent, before and after the occurrence of *C. okinawanus* in the Okinawa region, while it is not clear whether or not many paleolands emerged during the evolutionary history of the two species. To answer these questions, we performed molecular phylogenetic analyses of the two species.

To do this, we collected specimens of the two species from 51 localities in the Ryukyu Islands, sequenced three sections of the mitochondrial and nuclear DNA of individual insects, and then constructed phylogenetic trees based on the sequences. Our objective was to examine (1) the phylogenetic relationship among local populations of the two species, (2) the geographic distribution patterns of genetically separated groups, and (3) the evolutionary history of the two firefly species. The results strongly suggested that *C. costipennis* is paraphyletic and that *C. okinawanus* have evolved from populations of *C. costipennis* that have been isolated in the Central Ryukyu Islands.

2. Materials and Methods

Adults of *C. costipennis* and *C. okinawanus* were collected from 51 localities (Table 1) in the Ryukyu Islands (Figure 1). Although several isles in the Tokara Islands of the Northern

Ryukyu Islands are also known to be habitats of the former species [5], we could not obtain samples from these islets. For an outgroup taxon in phylogenetic analyses, the firefly *Luciola kuroiwaie* Matsumura collected in Okinawa-jima Island was used. Specimens were stored in 99.9% ethanol. Template DNA was extracted from the insect body excluding the head and pronotum using a Wizard Genomic DNA Purification Kit (Promega Co., Madison, WI, USA) and dissolved in 100 μ L sterilized distilled water.

For template DNAs extracted from individual insects, three DNA fragments, the mtDNA fragment containing the tRNA^{leu} gene and portions of the cytochrome oxidase subunit I and II genes (CO), the fragment containing the 16S ribosomal RNA gene (rDNA), and the fragment containing internal transcribed spacer 2 (ITS2) of the nuclear rDNA, were amplified using the primer sets FFMT2210F/FFMT3578R, AAMT12948F/FFMT13911R, and ITS2F/ITS2R, respectively (Table 2). The primers FFMT2210F, FFMT3578R, and FFMT13911R were designed based on sequences obtained in the preliminary experiments using several firefly species. Numerals in the name of primers for COI-COII and 16S rDNA indicate nucleotide position in the total mtDNA sequence of *Drosophila yakuba* [8] corresponding to the 5' end of the primer. The primers ITS2F

TABLE 1: Materials used in this study.

Species	Locality	Date	Code
<i>Curtos costipennis</i>	Amami region		
	Amami-oshima Island		
	Uragami, naze, Amami city, Kagoshima Pref.	2010.VI.20	Amami A
	Chinase, Naze, Amami city, Kagoshima Pref.	2010.VI.20	Amami B
	Yamatohama, Yamato town, Kagoshima Pref.	2010.VI.21	Amami C
	Arangachi, Uken Vil., Kagoshima Pref.	2010.VI.22	Amami D
	Koniya, Setouchi town, Kagoshima Pref.	2010.VI.23	Amami E
	Wase, Sumiyo, Amami city, Kagoshima Pref.	2010.VI.24	Amami F
	Tokunoshima Island		
	Todoroki, Tokunoshima town, Kagoshima Pref.	2010.VI.25	Tokuno A
	Mt. Gusuku-yama, Tokunishima town, Kagoshima Pref.	2010.VI.25	Tokuno B
	Agon, Isen town, Kagoshima Pref.	2010.VI.26	Tokuno C
	Akirigami, Setaki, Amagi town, Kagoshima Pref.	2010.VI.26	Tokuno D
	Sakishima region		
	Miyako-jima Island		
	Higashinakasonezoe, Miyakojima city, Okinawa Pref.	2009.V.27	Miyako A
	Nobaru, Ueno, Miyakojima city, Okinawa Pref.	2009.VI.25	Miyako B
	Yonaha, Shimoji, Miyakojima city, Okinawa Pref.	2010.IX.30	Miyako C
	Yoshino, Gusukube, Miyakojima city, Okinawa Pref.	2010.X.16	Miyako D
	Minafuku, Gusukube, Miyakojima city, Okinawa Pref.	2010.X.16	Miyako E
	Irabu-jima Island		
	Kuninaka, Irabu, Miyakojima city, Okinawa Pref.	2010.X.9	Irabu A
	Shimojishima, Irabu, Miyakojima city, Okinawa Pref.	2010.X.9	Irabu B
	Ishigaki-jima Island		
	Mt. Maesedake, Ishigaki city, Okinawa Pref.	2009.V.20	Ishigaki A
	Kuura, Ishigaki city, Okinawa Pref.	2009.V.25	Ishigaki B
	Ibaruma, Ishigaki city, Okinawa Pref.	2009.V.27	Ishigaki C
	Inoda, Ishigaki city, Okinawa Pref.	2009.V.29	Ishigaki D
	Fukai, Ishigaki city, Okinawa Pref.	2009.VI.1	Ishigaki E
	Omoto, Ishigaki city, Okinawa Pref.	2009.VI.8	Ishigaki F
	Ozato, Ishigaki city, Okinawa Pref.	2009.VI.10	Ishigaki G
	Yasura, Ishigaki city, Okinawa Pref.	2009.VI.18	Ishigaki H
	Iriomote-jima Island		
	Ootomi, Taketomi town, Okinawa Pref.	2009.V.22	Iriomote A
	Uehara A, Taketomi town, Okinawa Pref.	2009.V.23	Iriomote B
	Funauki, Taketomi town, Okinawa Pref.	2009.VI.19	Iriomote C
	Utara, Taketomi town, Okinawa Pref.	2009.VI.20	Iriomote D
	Aira, Taketomi town, Okinawa Pref.	2009.VI.21	Iriomote E
	Kanokawa, Taketomi town, Okinawa Pref.	2010.IV.18	Iriomote F
	Uehara B, Taketomi town, Okinawa Pref.	2010.V.22	Iriomote G
<i>Curtos okinawanus</i>	Okinawa region		
	Okinoerabu-jima Island		
	Point A, China town, Kagoshima Pref.	2009.VII.3	Erabu A
	Point B, China town, Kagoshima Pref.	2009.VII.3	Erabu B
	Point C, Wadomari town, Kagoshima Pref.	2009.VII.4	Erabu C
	Point D, Wadomari town, Kagoshima Pref.	2009.VII.4	Erabu D
	Point E, Wadomari town, Kagoshima Pref.	2009.VII.4	Erabu E

TABLE 1: Continued.

Species	Locality	Date	Code
	Okinawa-jima Island		
	Benoki, Kunigami vil., Okinawa Pref.	2009.VI.5	Okinawa A
	Arume, Higashi vil., Okinawa Pref.	2009.VI.9	Okinawa B
	Kawakami, Nago city, Okinawa Pref.	2009.VI.9	Okinawa C
	Gokayama, Nakijin vil., Okinawa Pref.	2009.VI.14	Okinawa D
	Kin, Kin town, Okinawa Pref.	2009.VI.22	Okinawa E
	Maebaru, Ginoza vil., Okinawa Pref.	2009.VI.23	Okinawa F
	Gushiken, Motobu town, Okinawa Pref.	2010.V.19	Okinawa G
	Hija, Yomitan vil., Okinawa Pref.	2009.VI.4	Okinawa H
	Makabe, Itoman city, Okinawa Pref.	2009.VI.8	Okinawa I
	Ishikawa-Agariyama, Uruma city, Okinawa Pref.	2009.VI.15	Okinawa J
	Kochinda, Yaese town, Okinawa Pref.	2009.VI.30	Okinawa K
	Nakadomari, Onna vil., Okinawa Pref.	2010.V.11	Okinawa L
	Zakimi, Yomitan vil., Okinawa Pref.	2010.V.18	Okinawa M
	Noborikawa, Okinawa city, Okinawa Pref.	2010.V.20	Okinawa N
<i>Luciola kuroiwa</i> (outgroup)			
	Okinawa-jima Island		
	Arime, Higashi vil., Okinawa Pref.	2009.VI.9	<i>L. kuroiwa</i> A
	Kawakami, Nago city, Okinawa Pref.	2009.VI.9	<i>L. kuroiwa</i> B
	Yoza, Itoman city, Okinawa Pref.	2009.VI.13	<i>L. kuroiwa</i> C

TABLE 2: PCR primers used in this study shown in 5'–3' direction.

Gene	Name ⁽¹⁾	Sequence
COI-COII	FFMT2210F	TAC CAG GAT TTG GTA TAA TTT CTC AT
	FFMT3578R	GGA TAG TTC ATG AGT GGA TTA CAT C
	FFMT3140R	ATT GTT CTA TTA AAG GTG AAA TTC T
16S rDNA	AAMT12948F	ATC CAA CAT CGA GGT CGC AAA CT ⁽²⁾
	FFMT13911R	GTA GTT TTG TAC CTT GTG TAT CAG GGT
ITS2	ITS2F	TGT GAA CTG CAG GAC ACA TG
	ITS2R	CCT GTT CGC TCG CAG CTA CT
	FFITS2F	GGT GAG CTC GTC CCC GCA TCG
	FFITS2R	GTG TAA TAT CAT TTG ATA TCG

⁽¹⁾ Numerals in the name of primers for COI-COII and 16S rDNA indicate nucleotide position in the total mtDNA sequence of *Drosophila yakuba* [8] corresponding to the 5' end of the primer.

⁽²⁾ AAMT12948F was designed in our previous study [9].

and ITS2R were designed based on the aligned homologous sequences of several insects downloaded from the DDBJ nucleotide sequence database. Amplified nuclear ITS2 was cloned using a TOPO TA cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. One or two colonies were picked from individual insect and directly used for PCR and nucleotide sequencing. In addition to the primers used for PCR, FFMT3140R, FFITS2F, and FFITS2R designed based on sequences obtained in this study were used for sequencing. PCR, labeling, and sequencing were performed as described in Muraji et al. [9]. Sequences of representative individuals were submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases (accession numbers: AB671246-AB671262, AB672623-AB672630).

Sequences were aligned as described in Muraji et al. [9] and used to compute basic statistical data and to generate phylogenetic trees based on the neighbor-joining method using MEGA ver. 4.1 software [10]. Maximum parsimony analyses were performed with PAUP* ver. 4.0b10 [11], using a heuristic search procedure with TBR swapping and 100 max. tree options.

3. Results

Nucleotide sequences of mtDNA (CO: 1,300–1,304 bp long, rDNA: 889–895 bp long) were determined for 57 individuals. As in the mtDNA of many other insects, these sequences were biased toward A and T (A + T%: 74.3 in CO and 84.4 in rDNA). The frequency of nucleotide substitutions

FIGURE 2: Neighbor-joining tree based on the Jukes-Cantor distances calculated using the 2,206 bp long combined data set including both mitochondrial CO and rDNA sequences. Sequences of different individuals collected from the same locality are indicated by a numeral in the taxon name. Bootstrap confidence levels calculated based on 1000 replications are shown near the nodes.

was higher in CO (polymorphic sites: 25.8%, parsimony informative sites: 23.7%) than in rDNA (polymorphic sites: 15.7%, parsimony informative sites: 14.4%).

In the phylogenetic analyses using the neighbor-joining method, the CO, rDNA, and combined (CO + rDNA) data sets produced the same topology in terms of the relationships among the haplotypic groups irrespective of the method used to calculate the genetic distances. The same topology was also recognized in all of the equally parsimonious trees generated using CO (length: 512; CI: 0.801; RI: 0.972; RC: 0.778), rDNA (length: 184; CI: 0.886; RI: 0.982; RC: 0.870), and combined data sets (length: 697; CI: 0.882; RI: 0.974; RC: 0.801). At the basal node of these trees, individuals were divided into two major lineages, group A + B and group C (Figure 2), and the former group was then subdivided into groups A and B.

Groups A, B, and C were specific to the Amami (islands of Amami-oshima and Tokunoshima), Okinawa (Okinoerabu-jima and Okinawa-jima), and Sakishima regions (Miyako-jima, Ishigaki-jima, and Iriomote-jima), respectively. The ranges of groups A and C coincided with that of *C. costipennis* and that of B coincided with that of *C. okinawanus*. At the terminal nodes of the phylogenetic trees, 2, 3, and 3 minor haplotypic groups were also detected in groups A, B, and C, respectively. All of these groups were strongly supported by the bootstrap analyses (93–100%).

The nucleotide sequence of ITS2 was determined for 50 clones obtained from 29 individuals. The length and sequence of the fragments were variable among and within the populations of the Amami (1,150–1,171 bp long), Okinawa (1,166–1,213 bp long), and Sakishima regions

FIGURE 3: Neighbor-joining tree based on the Jukes-Cantor distances calculated using the 856 bp long data set of the nuclear rDNA ITS2 sequence. Different sequences obtained from the same individuals were distinguished by lower-case letters, a and b, included in the taxon name. Bootstrap confidence levels calculated based on 1000 replications are shown near the nodes. This tree was unrooted.

(1,089–1160 bp long), and, due to insertion/ deletion mutations, the sequences could not be aligned in several sections. Thus, a 856 bp long data set generated excluding 14 (315–329 bp in total length), 15 (322–369 bp), and 13 (247–314 bp) sections from the Amami, Okinawa, and Sakishima populations, respectively, was used for phylogenetic analyses. In these analyses, the topology of the neighbor-joining trees was highly consistent among the different methods used to calculate the genetic distances (Figure 3). The topology also agreed with that of the equally parsimonious 100 trees (length: 269; CI: 0.903; RI: 0.990; RC: 0.894) obtained by the maximum parsimony method. In these trees, three groups, corresponding to groups A, B, and C in Figure 2, diverged simultaneously at the basal node. Subgroups at the lower level were recognized only in the populations that originated

in the Sakishima region. All these groupings were supported strongly by the bootstrap analyses (99–100%).

4. Discussion

In this study, we found that Japanese *Curtos* fireflies were divided among three genetically separated local populations, corresponding to *C. costipennis* in the Amami region, *C. okinawanus* in the Okinawa region, and *C. costipennis* in the Sakishima region (Figures 2 and 3). Although the nucleotide sequences of *C. okinawanus* formed a monophyletic group (group B), those of *C. costipennis* were separated between two distinct groups, A and C, and the monophyly of this species was not recognized in analyses using both mtDNA

FIGURE 4: Phylogeography of *Curtos* fireflies in the Central and Southern Ryukyu Islands.

and nuclear DNA sequences (Figures 2 and 3). In addition, the mtDNA haplotypes of *C. okinawanus* (group B) were positioned between those of *C. costipennis* (groups A and C) (Figure 2). These results indicate that the currently recognized *C. costipennis* species is paraphyletic. Otherwise, *C. okinawanus* must be a subspecies or a geographic strain included within the single species *C. costipennis*.

Although the two species closely resemble each other in both morphology and behavior [7], they are apparently distinct in terms of elytral coloration (*C. costipennis*: dark yellow except for elytral apex, *C. okinawanus*: entirely brownish black), and this characteristic is recognized as diagnostically important [6]. However, the present study revealed that such a characteristic did not reflect evolutionary relationships correctly. On the other hand, several researchers recognized variations in body size, the shape of the male genital organ, and the number of punctures on the elytra among local populations of *C. costipennis* [5, 6]. Reevaluation of these characteristics is needed to confirm the taxonomic status of the two species.

Among the three major groups detected in this study, a closer relationship was recognized between *C. costipennis* and *C. okinawanus* distributed in neighboring regions (the Amami and Okinawa regions in the Central Ryukyu Islands)

than between the populations of *C. costipennis* separated between remote areas (the Amami and Sakishima regions) (Figure 4). This phenomenon seems to be consistent with the view of Ohba and Goto [7] that *C. okinawanus* might have derived from *C. costipennis* in the Central Ryukyu Islands. As discussed below, their evolutionary history can be well understood by considering the influence of the Pleistocene paleogeography of this area.

Among the combined mtDNA sequences obtained in this study, mean substitution rates of 6.8% (6.1–7.4%) and 5.7% (5.4–6.0%) were estimated between groups A + B and C, and between A and B, respectively. From these values, divergence times of 1.2–1.4 and 1.0–1.1 million years were estimated by applying an mtDNA evolutionary clock, 5–5.7% per million years, calibrated for several coleopteran insect groups [12–14]. For these periods, the hypothesized paleogeography of the Ryukyu Islands [1, 15] postulated a large paleopeninsula extending from the Chinese continent to the Northern Ryukyu Islands through Taiwan (1.2 and 1.7 million years ago). Recent studies of the paleomarine environment indicated that this peninsula began to break around 1.6 million years ago [16]. Subsequently, the peninsula submerged to form a chain of small islands, which has remained for more than one million years. Although the land

configuration in the late Pleistocene is rather controversial, the seabed topography suggests the emergence of several large paleo-islands connecting neighboring islands [2, 4].

If such a hypothesis is accepted, the common ancestor of major groups is considered to have dispersed through the Ryukyu Islands along the large paleopeninsula, and their separation is considered to have occurred during the course of the gradual subsidence and subdivision of the paleopeninsula. The divergence times estimated above suggest that the channel between the Central and Southern Ryukyu areas (Kerama Gap in Figure 4) and that between the Amami and Okinawa regions opened sequentially around 1.3 and 1.0 million years ago, respectively. After a long period of isolation among small islands, the local populations must have been connected again by superislands that emerged in the late Pleistocene. Because Miyako-jima Island is known to have submerged beneath the sea in the mid-Pleistocene [17], the occurrence of fireflies on this island suggests a land connection in the late Pleistocene. The integrity of the nucleotide sequences within the respective regions (Figures 2 and 3) suggest that the super islands in this period were also separated among the Amami, Okinawa, and Sakishima regions. Minor haplotypes at the terminal nodes of the phylogenetic trees are considered to be due to the subdivision of the super islands caused by the sea level rise thereafter. The coexistence of two different minor haplotypes on the islands of Ishigaki-jima, Iriomote-jima, and Okinawa-jima (Figure 4) suggests that super islands emerged several times in this period.

The fauna of the Ryukyu Islands is known to be separated among the Northern, Central, and Southern Ryukyu areas by geologically long-standing channels at the Tokara and Kerama Gaps (Figure 4). These channels are considered to have long acted as barriers to biological dispersal [3], and their role was reflected in the relictual distribution and endemism of many terrestrial animals including mammals, birds, reptiles, amphibians, and insects [3, 18, 19] in the Central Ryukyu Islands. Although the faunal borderline is ambiguous between the Amami and Okinawa regions within the Central Ryukyu area, differences in species, subspecies, and mtDNA sequences have also been seen in many terrestrial animals among the regions [3, 19–22]. As in the fireflies examined in this study, *Neolucanus* beetles [22] showed sequential population vicariances in which the mtDNA haplotype was first separated between the areas north and south of the Kerama Gap, and the northern half was then further subdivided between the Amami and Okinawa regions, suggesting that the channel between the Amami and Okinawa regions (>500 m depth) opened after the opening of the Kerama Gap (>1,000 m depth) and had slightly less importance in determining faunal differences.

We believe that the speciation of *C. okinawanus* must be understood in this context. Considering the paleogeography of this area, the repetitive entry of fireflies into the Central Ryukyu Islands from neighboring areas is unlikely. Our conclusion is that the species derived in the Okinawa region from a population of *C. costipennis* isolated in the Central Ryukyu area. The fact that the two species can engage in interspecific copulation under laboratory conditions [23]

suggests that the speciation was based on the geographic segregation between the Amami and Okinawa regions.

In this study, we examined the evolutionary scenario of *Curtos* fireflies distributed in the Ryukyu Islands. However, because this study did not treat specimens that originated from the Northern Ryukyu Islands and Taiwan, we could not evaluate the function of two geologically long-standing channels at the Tokara Gap and the Yonaguni Depression (Figure 4). To complete the evolutionary scenario of this species group, further studies using these populations as well as populations from the Chinese continent are needed.

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