Review Article

Towards a Better Understanding of the Evolution of Specialized Parasites of Fungus-Growing Ant Crops

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Fungus-growing ants have interacted and partly coevolved with specialised microfungal parasites of the genus *Escovopsis* since the origin of ant fungiculture about 50 million years ago. Here, we review the recent progress in understanding the patterns of specificity of this ant-parasite association, covering both the colony/population level and comparisons between phylogenetic clades. We use a modified version of Tinbergen's four categories of evolutionary questions to structure our review in complementary approaches addressing both proximate questions of development and mechanism, and ultimate questions of (co)adaptation and evolutionary history. Using the same scheme, we identify future research questions that are likely to be particularly illuminating for understanding the ecology and evolution of *Escovopsis* parasitism of the cultivar maintained by fungus-growing ants.

1. Introduction

1.1. The Attine Fungus-Growing Ants. Fungus-growing ants (Hymenoptera: Formicidae: Attini) form a monophyletic tribe of primarily tropical ants that obligately depend on fungal cultivars (Agaricales: mostly Lepiotacea: Leucocoprineae). The ants provide the fungus with optimal growth conditions, and in return, the fungus serves as the main food source for the ants [1, 2]. The symbiosis between fungus-growing ants and their fungi originated about 50 million years ago [3–6] from a single ancestor that was most likely a generalist forager [3]. Subsequently, the Attini have diversified to encompass approximately 230 described species, distributed across 14 ant genera [4, 7]. Colonies of fungus-growing ants are typically founded by a single queen, who carries a piece of the fungus cultivar from her natal colony in the infrabuccal pocket [8] during her nuptial flight [9–11]. The Attini are divided into the "higher attine" and the phylogenetically basal "lower attine" genera based on their cultivars [5]. Lower attine cultivars are largely unmodified and resemble free-living Leucocoprini, whereas higher attine cultivars (including those of leaf-cutting ants) are highly derived [12].

The clonal propagation of cultivars through vertical transmission [2, 13] predicts ancient association and congruence between the ant and fungal cultivar phylogenies. High degrees of congruence have indeed been found at the deep phylogenetic levels in both higher [14, 15] and lower attines [12, 14]. However, the phylogenetic interaction specificity breaks down within, and occasionally between, ant genera and their cultivar strains, indicating that switches and/or reacquisitions of new garden cultivars have occurred (e.g., [12, 16–19]). While the higher-attine fungi no longer persist outside the symbiosis, lower attine fungi have free-living close relatives, which is likely to facilitate gene flow and reacquisitions of symbionts [12]. Cultivar switches can be induced in the laboratory, including the formation of chimeric gardens [20–22]. However, consistent with predictions from host-symbiont conflict theory [23], mature individual colonies appear to consistently maintain a single fungus clone, at least in leaf-cutting ants (*Atta* and *Acromyrmex*) where this has been best studied [24, 25].

Clonally propagated monoculture crops are expected to be particularly prone to infection with parasites and pathogens [26], because they represent an attractive resource that should be easy to exploit. This "Red Queen" logic

[27, 28] assumes that parasites and hosts are involved in evolutionary arms races, in which unpredictable genetic heterogeneity, due to sexual recombination, is the most powerful defence against parasites that have short generation times relative to their hosts [28–30]. Single asexual cultivar clones thus seem to represent a liability for the farming symbiosis [25] that needs to be overcome by active protection by the host ants (see below). Colony-level monoculture does not imply population-wide monoculture, as is often the case in modern human crops that are vulnerable to disease. With the possible exception of some species, there is likely to be considerable strain diversity across neighbouring colonies [16, 18] that should discourage the spread of infections between colonies.

1.2. Specialised Coevolved Parasites.

Microfungal parasites in the genus *Escovopsis* (anamorphic Hypocreales) have been known for more than a century to overgrow fungus gardens of laboratory colonies [1, 13, 31], but the formal status of *Escovopsis* as a disease was confirmed only just over a decade ago when Currie et al. [32] showed that *Escovopsis* fulfils Koch's four defining postulates [33] for causative disease agents. This included evidence that *Escovopsis* (i) is found in abundance in diseased but infrequently in apparently healthy colonies, (ii) can be isolated from diseased colonies, (iii) can cause disease when colonies are artificially infected, and (iv) can be reisolated from diseased experimental colonies [32]. It was also shown that *Escovopsis* has a directly negative impact on the ant cultivar [32, 34, 35] through the secretion of compounds that break down the cultivar mycelium [36]. As fungus-growing ants rely on healthy fungus gardens for growth and reproduction, this implies that *Escovopsis* is a potentially serious threat to ant fitness [35].

Deep-level phylogenetic congruence has been found between the fungus-growing ants, their cultivars, and *Escovopsis* parasites, suggesting a long history of codivergence within the attine agricultural systems [37]. However, cophylogenies at lower levels appear to be punctuated with occasional host switching of the parasites [38], consistent with ongoing arms races [37], although null hypotheses of genetic drift in isolated parasite populations can usually not be dismissed.

Even within ant genera, there is some evidence for ant-cultivar-*Escovopsis* pairing specificity. Four morphologically and genetically distinct *Escovopsis* types parasitize the cultivars maintained by *Apterostigma*, a basal fungus-growing ant genus [39]. These have so far been categorised as "brown," "yellow," "pink," and "white," but are genetically distinct and likely different subspecies or species (cf. [39]). Even within these groupings, there is evidence for specificity: "pink" *Escovopsis* appears to infect only G3 cultivars and (rare) "white" *Escovopsis* only G2 cultivars, whereas "brown" and "yellow" *Escovopsis* commonly coinfect G2 cultivars (cf. Table 1 in [39]). Current evidence suggests that these pathogen lineages display patterns of phylogenetic congruence with their fungal host [39], maintained by chemotaxis and host resistance in nonnative (i.e., not naturally occurring) combinations [40]. A similar scenario

of association specificity is apparent for the lower attine genus *Cyphomyrmex*, where subclades of a single *Escovopsis* morphotype (pink) are phylogenetic congruent with corresponding clades of cultivar host genotypes [41]. In the higher attine ants, *Trachymyrmex* and *Sericomyrmex* are infected by specific *Escovopsis* parasites that are phylogenetically distinct from the two clades that parasitize *Atta* and *Acromyrmex* leaf-cutting ants [37, 38]. Within the leaf-cutting ant genera, *Escovopsis* infections are nonspecific [38], confirming the high degree of ant-cultivar specificity of all extant leaf-cutting ants to a single species of *Attamyces* symbiont [42].

1.3. Defence Strategies against Escovopsis.

Fungus-growing ants, especially the leaf-cutting ants, have elaborate prophylactic fungus grooming and weeding behaviours to keep their cultivar free from parasites [44, 45]. In *Acromyrmex*, minor workers are particularly efficient at restricting spore germination [45], and major workers appear to recruit minor workers to infected sites, thereby potentially increasing the efficiency of disease suppression [46]. If spores manage to escape the attention of minor workers and germinate, major workers appear to perform the task of removing infected garden pieces (weeding) [45]. Task specialization between castes thus appears to make hygienic policing more efficient in general, which has been proposed to be normally sufficient for eliminating generalist fungal parasites, but not for completely eradicating *Escovopsis* infections [44].

To control *Escovopsis* infections, fungus-growing ants may also use metapleural gland secretions, which contain an array of compounds with antibiotic properties [48, 69]. In a seminal study, Fernández-Marín et al. [47] described highly coordinated and challenge-specific foreleg movements along the metapleural gland opening (metapleural gland grooming), which allowed *Atta* and *Acromyrmex* ants to precisely target the application of antibiotic secretion to their gardens. In combination with metapleural gland grooming, fungus-growing ants utilize their infrabuccal pocket (located in the oral cavity) as a further filtering and sterilising device. After grooming, the ants collect *Escovopsis* spores in this pocket, where they are sterilised by an as-of-yet unknown mechanism (potentially metapleural gland compounds), after which the infrabuccal pellet is expelled on the colony refuse pile [47, 49, 70].

The cuticle of major garden workers is often covered with a thick white growth of Actinobacteria [50, 51], which produce antimicrobial compounds that aid in the protection of the fungal cultivar from *Escovopsis* [49–51, 64, 71] and possibly other parasites [65]. These beneficial Actinobacteria are reared by the ants and housed in cuticular crypts, tubercles, or other modifications associated with subcuticular exocrine glands [52]. Most work on the Actinobacteria has focused on specifically associated lineages of *Pseudonocardia* [51, 55]. *Pseudonocardia* appears to be vertically transmitted by default [50], but phylogenetic evidence indicates that events of horizontal transfer and incorporation of free-living *Pseudonocardia* to the symbiosis have occurred [55–57]. Recent studies have further shown that other Actinobacteria

genera (mainly *Streptomyces*) are often also present [57–61], but their degree of specificity with the symbiosis is less clear. There is little doubt that cuticular Actinobacteria cultures serve active defence functions in the symbiosis, but clarifying the relative importance of predominantly vertically transmitted *Pseudonocardia* and horizontally transmitted other defensive microbes will need much further work.

Ant cultivars, the hosts of *Escovopsis* parasitism, are able to launch defences themselves by secreting chemical compounds that suppress *Escovopsis* growth. This has been tested in the *Apterostigma* and *Cyphomyrmex* [41, 43], where antifungal compounds secreted by the cultivar appeared to be more effective in suppressing the growth of *Escovopsis* strains that are unknown to infect them in nature, but less effective against their native *Escovopsis* strains [41, 43]. Such cultivar responses towards novel *Escovopsis* strains might result in limitations for *Escovopsis* host switching outside the agricultural system that they are adapted to. Overall, therefore, the defences of the ants, the Actinobacteria, and the cultivar appear to reinforce each other in suppressing *Escovopsis* infection and proliferation within attine ant fungus gardens (see e.g., Figure 10.1 in [22]).

1.4. Trade-Offs between Alternative Defence Functions. Over the course of millions of years of selection on the interaction between fungus-growing ants and *Escovopsis*, different ant genera have diversified in their specific utilization and combination of alternative defence mechanism to reduce the impact of *Escovopsis*. This has been best studied in species of the leaf-cutting ant genera *Atta* and *Acromyrmex*. *Escovopsis* infections appear to be more prevalent in *Acromyrmex* than *Atta* colonies [35], possibly due to differences in the efficiency of alternative defensive strategies. First, the chemical compounds in the metapleural glands differ between *Acromyrmex* than *Atta*, reviewed in [53], making it inevitable that compounds with different antimicrobial properties are produced (cf. [48]). Second, Actinobacteria are abundant in *Acromyrmex* and essentially absent in *Atta* [54]. Third, the rate of metapleural gland grooming differs in a contrasting manner, with *Atta* increasing grooming rates after *Escovopsis* infection and *Acromyrmex* maintaining a constantly low rate of metapleural gland grooming [54].

Differences in metapleural gland chemistry, grooming rate, and Actinobacteria coverage indicate that trade-offs between these alternative defensive strategies are likely, conceivably because these defences are known to be costly [72, 73]. Different defences may target the same parasite, but with different modes of action. For example, in *Acromyrmex*, metapleural gland secretions kill *Escovopsis* spores but show limited effect on hyphae [48], while Actinobacteria secretions suppress hyphal growth but do not kill spores [64]. A similar scenario has been proposed for two other genera of higher attine ants, *Trachymyrmex* and *Sericomyrmex*, as certain species from the former genus have abundant Actinobacteria cover and low frequencies of metapleural gland grooming, while *Sericomyrmex* has very few Actinobacteria and a higher frequency of metapleural gland grooming [72].

2. Using Tinbergen's Four Quadrats to Structure Attine-*Escovopsis* Research

Nikolaas Tinbergen was a Dutch ethologist and ornithologist who received a Nobel Prize in Physiology or Medicine in 1973 together with Karl von Frisch and Konrad Lorenz for their joint work on the organization and elicitation of individual and social behaviour in animals [74]. Tinbergen's four categories of evolutionary questions were originally developed to obtain an integrated explanation for animal behaviour, based on complementary understanding of proximate mechanisms (1) and ontogenetic developments (2), as well as ultimate selection forces resulting in adaptive evolution of individuals (3) and long-term evolutionary change of populations or higher-level clades (phylogenetic history) (4) [75]. Tinbergen's framework has since been used in many research programs throughout the life sciences [76–78] but has, to our knowledge, not been applied to host-parasite interactions. For the purpose of the present paper, we modify Tinbergen's framework to encompass a classification of questions that have been (Table 1), or could be (Figure 1), addressed to better understand the evolutionary ecology of attine ant-*Escovopsis* interactions.

Table 1 summarizes how studies available so far can be grouped into Tinbergen four quadrats framework. This was relatively straightforward for the ultimate questions of adaptive evolution and phylogenetic history, but not always for the proximate ontogeny and mechanism categories, because available research tools have so far not allowed much understanding of the (epi)genetics behind developmental pathways and phenotypic plasticity. It is, therefore, also arguable that the questions addressed in our ontogeny and mechanism categories are rather ambiguous, in being both technologically challenging and relatively imprecise in their fit to a single Tinbergen quadrat. We nonetheless felt that making a first attempt to structure a research agenda was worthwhile and have chosen to group questions of *Escovopsis* specialization in the ontogeny quadrat and questions of cultivar utilization and defences by the ants and fungal symbionts in the mechanism quadrat. In the sections below, we utilise these groupings to formulate how new experimental work, combined with the increasing availability of genome sequences, may allow novel insights in *Escovopsis* parasitism.

3. Tinbergen's Ontogeny Quadrat

3.1. Escovopsis Recognition of Cultivars. In vitro assays have shown that *Escovopsis* can recognize native cultivar hosts through chemotaxis, followed by directed growth of the parasite towards the cultivar, the secretion of parasite enzymes breaking down cultivar cells, and absorption of cultivar cell contents [36]. In contrast, *Escovopsis* is not able to utilize nonnative cultivar strains and can even be inhibited by them [41, 43]. The mechanisms and genes underlying parasite differentiation between native and nonnative host cultivars remain unknown, that is, we neither know the identity or the evolution of the chemicals (what does *Escovopsis* recognize?) nor the genes coding for the chemicals produced and their

TABLE 1: Overview of available studies on *Escovopsis* virulence in gardens of fungus-growing ants, and our assortment of these studies into the four Tinbergen quadrats.

Quadrat	Study focus	References
Ontogeny	Pathology, impact, and prevalence	[32, 34, 35]
	Genetic and chemical basis of *Escovopsis* recognition of cultivars	[36, 38–41, 43]
Mechanism	Ant behavioural defences	[44–47]
	Chemical defences	[47, 48]
	Actinobacteria defences	[49–52]
	Cultivar defences	[40, 41, 43]
Phylogeny	Population-level specificity	[38–41, 43]
	Cross-phylogeny specificity	[38, 51]
Adaptation	Susceptibility/resistance to metapleural gland compounds	([48], reviewed in [53, 54])
	Degree of Actinobacteria specificity	[55–63]
	Susceptibility/resistance to Actinobacteria secretions	[50, 58, 60, 61, 64, 65]
	Host cultivar use	[38–41, 43]

		Objects of explanation	
		Development/Historical *Progression in current form*	**Single form** *What organisms need to function and why those functions arose*
Questions	Proximate *How organisms work by describing their developmental and functional traits*	Ontogeny (i) *Escovopsis* recognition of cultivars (ii) Genetic basis for *Escovopsis* recognition by the ants (iii) Trade-offs between alternative defences	Mechanism (i) *Escovopsis* transmission between colonies (ii) Colony-level virulence
	Evolutionary *How evolution has shaped organisms to acquire their extant forms*	Phylogeny (i) Origin and diversification of the association (ii) Phylo-geographic patterns, coevoluntionary interactions, and dispersal	Adaptation (i) Evolutionary potential of *Escovopsis* as a parasite (ii) Evolutionary consequences of host-parasite interactions

FIGURE 1: Tinbergen's four quadrat framework applied to evolutionary questions about *Escovopsis* parasitism of fungus-farming ant crops. Ontogeny refers to the description of development, from DNA to progressive phenotype, mechanism refers to the physiological and cellular processes that organisms have available, phylogeny refers to the idiosyncratic evolutionary history of a lineage, and adaptation refers to traits that acquired their extant function because of specific selective advantages, modified from [66–68].

evolutionary history. Ongoing genome sequencing of cultivars and *Escovopsis*, as well as efforts to isolate the chemicals involved, will thus allow considerable progress to be made.

Two evolutionary explanations for the maintenance of *Escovopsis*-cultivar utilization patterns seem possible. The nonadaptive explanation would hold that *Escovopsis* strains (or species) would be subject to consistent genetic drift in isolated populations, so that they would lose adaptations to allopatric hosts by chance. The alternative adaptive explanation would hold that populations are mostly panmictic, so that genes coding for innovative pathogen traits and defensive recognition and resistance traits of cultivars would tend to coevolve. If so, *Escovopsis* would track cultivar evolution in continuous, but variable, arms races reminiscent of a geographic mosaic of coevolution [79]. If the latter is the case, expectations are that positive selection on specific gene complexes (e.g., recognition or resistance genes) will likely

have left signatures of enhanced dN/dS ratios compared to housekeeping and neutral genes, while nonsignificant dN/dS ratios would make the nonadaptive null hypothesis more likely. In general, it seems unlikely that *Escovopsis* populations are highly structured (see also below), but solid empirical evidence on this is lacking.

3.2. Genetic Basis for Escovopsis Recognition by the Ants. Ants are able to discriminate between *Escovopsis* and other fungi and behave accordingly [44, 45]. Natural selection in the ant host is expected to select for genes involved in the recognition and removal of *Escovopsis* from the fungus garden, as this is predicted to provide a selective advantage. Further, *Escovopsis* has the potential to be much more virulent than any general fungal weeds of attine ant colonies, at least in the higher attine system where virulence has been studied, implying stronger selection on *Escovopsis* recognition pathways in the

ants compared to pathways mediating the recognition of weed fungi. The genetic basis of *Escovopsis* recognition has not been explored, but genomic tools will make this possible in the years to come [80, 81]. For example, two leaf-cutting ant genomes are now published [82, 83] and a sequenced *Escovopsis* genome will soon follow (anonymous reviewer, personal communication), providing the tools necessary for such new approaches to studying behavioural recognition mechanisms. Recognition of, and concomitant behavioural responses to, *Escovopsis* infection are faster and last longer than the response to general fungal pathogens [44, 47], leading to the prediction of higher levels of recognition gene expression in the presence of *Escovopsis*. However, it is conceivable that the mechanism of recognition of *Escovopsis* and other fungi by the ants does not differ but that responses do, so that it is rather genes underlying behavioural removal responses that are differentially expressed.

3.3. Trade-Offs between Alternative Defences. Defences against *Escovopsis* include behavioural removal (including self- and allo-grooming), glandular secretions, cultivar defensive compounds against nonnative *Escovopsis*, and compounds with antibiotic properties derived from Actinobacteria. These defences all involve interactions on the ant cuticle and are expected to require coordinated interactions to avoid negative interference. In *Acromyrmex octospinosus*, the metapleural gland secretions do not appear to harm the Actinobacteria, so that both defences can be freely expressed [54]. Further, complementarity is expected to maximize cost-benefit ratios of defences as well as to avoid redundancies. It is conceivable that differences in Actinobacteria cover between closely related ant species, such as *A. octospinosus* and *A. echinatior* [72], reflect more recent adjustments (trade-offs) in the relative importance of defences between the species.

Explorations of defence trade-offs have only been done in some higher attines, leaving questions of this kind unexplored in most of the fourteen extant fungus-growing ant genera. We propose that utilizing the phylogenetic framework of structural modifications over the course of the association between fungus-growing ants and Actinobacteria [52] would offer a good basis for future work to understand the dynamics of defence components across the attine tribe. The relative usage of metapleural gland grooming and the chemistry of glandular and bacterial secretions in *Acromyrmex/Trachymyrmex* versus *Atta/Sericomyrmex* exemplify how such comparative approaches can be insightful [54]. However, considering the vast diversity of cultivar usage, Actinobacteria communities, substrate choice, and ant life-history traits, it is conceivable that defence strategies and trade-offs in unstudied attine ants might be different from those found in the higher attines.

4. Tinbergen's Mechanism Quadrat

4.1. Escovopsis Transmission between Colonies. The success of parasitism is tightly linked to the transmission frequency between host colonies [84]. The most common transmission for fungal spores is passive dispersal through the air, but this is unlikely to be the case for *Escovopsis* because it sporulates inside colonies and has wet spores [35]. The mechanism of *Escovopsis* transmission, therefore, continues to be enigmatic, with untested hypotheses of commensal garden arthropods vectoring spores between colonies, or foraging ants picking up spores via encounters outside the nest as reasonable leads [41, 84]. Both mechanisms could be further facilitated by attine colonies nesting in each others close proximity. Culture-based attempts to isolate *Escovopsis* from potential vectors are, therefore, needed for a better understanding of transmission modes. Expectations are that *Escovopsis* is more likely to be transmitted between colonies by commensal arthropods. This is so, because foragers presumably rarely, if ever, enter other colonies, and are therefore unlikely to pick up *Escovopsis* spores from nonnative infected colonies, and because workers are efficient at recognizing and removing *Escovopsis* spores from their cuticle (e.g., [85, 86]). In contrast, commensal arthropods moving between colonies are not expected to have evolved such avoidance behaviours towards *Escovopsis*.

4.2. Colony-Level Virulence. The within-nest dynamics of *Escovopsis* infections remain a frontier awaiting exploration. *Escovopsis* can coexist with other nonmutualistic filamentous fungi within colonies without colonies displaying signs of infection [62, 87–89]. However, it is not known if infection sets out shortly after *Escovopsis* introduction, or if *Escovopsis* spores remain dormant in the colonies until an outbreak of mycelial growth is triggered by external factors. To begin to understand these dynamics, two essential questions need to be addressed. Firstly, we need a better understanding of the level of metabolically active spores and hyphae of *Escovopsis* in normally functioning and apparently healthy colonies. This could be obtained through quantitative PCR approaches, so that amounts of *Escovopsis* biomass and levels of metabolic activity, measured as gene expression, can be estimated. Ideally, this should be explored over time to also determine temporal variation. Only when we have a better idea of such dynamics, we can begin to explore the role of the ants in mediating these threats. Secondly, if spore-dormancy is the rule, work should address what factors trigger within-colony outbreaks. One approach that could potentially address this is long-term field surveys of natural colonies to better understand the interplay between ecological fluctuations, (e.g., temperature, rainfall, and food availability), intrinsic factors (e.g., loss of queen, imbalance of worker to garden ratio, and emergence of reproductives), and infection dynamics.

5. Tinbergen's Phylogeny Quadrat

5.1. Origin and Diversification of the Association. The apparent presence of *Escovopsis* throughout the fungus-growing ants suggests that an ancestral *Escovopsis* was present as a parasite in the first ant cultivars that were domesticated ca. 50 million years ago (cf. [37, 90]). However, an alternative scenario is that *Escovopsis* parasitism originated shortly after the early attine ants had become irreversibly committed to farming. The latter would indicate that *Escovopsis* parasitism

was not merely a passive carry-over process, but that the highly peculiar garden phenotype of domesticated fungi created a novel niche to parasites like *Escovopsis*. Finding that *Escovopsis* parasitism would also occur in free-living relatives of lower attine garden symbionts would make an origin predating ant fungiculture more likely, but several lines of indirect evidence suggest that the "new garden niche" model is more likely to apply. First, Actinobacteria cultures on the cuticle of attine ants arose also shortly after these ants became farmers [52], and it would be hard to imagine that the origin of this costly biocontrol habit was not somehow related to *Escovopsis* infections. Second, the impact of *Escovopsis* on fungus-growing ant cultivars is likely to be particularly high because colonies keep a high density of cultivar mycelium without sufficient own defences. Third, it is striking that the only clade of attine ants that secondary developed a radically different and much less conspicuous garden phenotype, the yeast-rearing *Cyphomyrmex*, have secondary lost *Escovopsis* as a parasite [4].

To date, there are two described species of *Escovopsis*, with *E. weberi* from a Brazilian *Atta* species thought to be the monotypic species of the genus [91]. Later, a morphologically distinct *E. aspergilloides* was isolated from *Trachymyrmex ruthae* in Trinidad [92]. Both large scale (cf. [37, 38]) and lower-level lineage diversity [39–41] are considerable, suggesting that there are more *Escovopsis* species associated with fungus-growing ants. Molecular species delineation based on conserved genes such as EF-1α and 18S rRNA is unlikely to distinguish lineages that diverged recently, so that more sensitive marker studies are needed. Recent multilocus sequence analyses (MLSAs) have provided the opportunity to estimate divergence dates for crucial nodes in phylogenetic trees [4, 19] and would thus also offer novel insights when applied to an *Escovopsis* phylogeny [37]. Approaches of this kind will ultimately allow conclusions about the origin of *Escovopsis* parasitism (before or after attine ants became farmers) and the rates of *Escovopsis* evolution in different host clades.

5.2. Phylo-Geographic Patterns, Coevolutionary Interactions, and Dispersal. Coevolutionary theory predicts that genotypic and phenotypic variation across the geographic range of a host-parasite association can lead to parasite adaptations to locally available host genotypes, while becoming maladapted to nonnative genotypes [93]. A prerequisite for such coevolutionary interactions is that host populations are genetically structured, so that gene flow between populations remains limited [93]. In fungus-growing ants, only a single study has attempted to explore such coevolutionary dynamics (in the ant species *Apterostigma dentigerum* [43]). This showed the presence of six distinct host genotype clusters across Central America, while structuring was essentially absent in the parasite, indicating that *Escovopsis* genotypes are not tightly tracking those of the host [43].

We would expect that other fungus-growing ant-cultivar-*Escovopsis* interactions will mirror the findings in *Apterostigma*, since cultivars are vertically transmitted by default while *Escovopsis* is horizontally transmitted. Therefore, the population structure in *Escovopsis* could be explainable if their sticky spores would use vectors for long distance dispersal that are not available to dispersing ants. It would be tempting to speculate that other arthropods living in attine nests might have this vector function, but examples of such long distance flyers vectoring spores are presently lacking. Alternatively, wind dispersal of small leaf fragments with *Escovopsis* spores would also seem a realistic mechanism for parasite populations to become less viscous than host populations. Future studies addressing relative dispersal efficiencies of partners in the attine ant symbiosis would seem most informative if they could span geographic areas that would be large enough to include natural barriers that would differentially affect *Escovopsis* spores and dispersing ant queens transmitting fungus-garden symbionts.

6. Tinbergen's Adaptation Quadrat

6.1. Evolutionary Potential of Escovopsis as a Parasite. As already mentioned, *Escovopsis* has probably persisted as a parasite of fungus-growing ant gardens since the origin of ant fungiculture 50 million years ago [4, 37]. If that is so, "Red Queen" like arms races with the ant and fungal hosts may have at least periodically occurred, so that genetic diversity of the parasite is likely to be substantial [26–29]. However, the sexual "teleomorph" of *Escovopsis* has never been observed so that *Escovopsis* may not have sexual reproduction, similar to many other Ascomycetes [94]. Lack of sex would not necessarily preclude the integration and exchange of genetic material between different anamorphous mycelia within nests, provided that coinfections occur with some frequency. This is because asexual Ascomycetes can undergo genetic exchange between strains after hyphal merging (anastomosis) and parasexual heterokaryosis (the exchange of cell nuclei) [95]. If such exchanges lead to mitotic crossovers, then there is potential for recombination between genetically different strains [95]. It will be very interesting to investigate whether the *Escovopsis* genome still shows signs of such genetic recombination.

The presence of coinfections within individual nests is a prerequisite for such genetic exchanges. Both *Atta* and *Acromyrmex* leaf-cutting ants appear to frequently harbour genetically distinct *Escovopsis* strains, including ones appearing in two separate phylogenetic clades [63]. Similarly, in the paleoattine genus *Apterostigma*, fungus gardens are infected by four distinct *Escovopsis* morphotypes "brown," "yellow," "pink," and "white" [39]. This implies the potential for exchange of genetic material between coinfecting strains within colonies. By explicitly addressing this question, we could gain insight both in the dynamics of coinfections (e.g., facilitation, inhibition, the role of the order of infection precedence) within colonies and in the putative species status of different *Escovopsis* morphotypes.

6.2. Evolutionary Consequences of Host-Parasite Interactions. A common question in the evolutionary study of host-pathogen interactions is whether coevolutionary arms races are almost continuous or relatively rare. This is partly because of the difficulty of testing such dynamics when exploring

biological systems in real time. Fungus-growing ants have evolved extensive complementary defences to deal with *Escovopsis*, but the parasite nevertheless prevails at relatively high population-level frequencies, ranging from 27–75%, depending on the ant genus and geographic location (e.g., [32, 88]). This finding suggests that *Escovopsis* continues to exert selection pressure on the ant hosts, potentially leading to concomitant changes in ant defences. All this is suggestive of, but not decisive evidence for, antagonistic coevolution (cf. [96]).

The efficiency of behavioural defences (grooming/weeding) in attine ants is known to have an impact on the virulence of *Escovopsis* [44, 45]. Under a coevolutionary scenario, expectations are that *Escovopsis* has exerted selection pressures on the ants to optimize their behavioural response towards native parasite strains. Such a scenario would predict that infections with (avirulent) nonnative strains of the parasite would not elicit the same efficient response from the ants. Similarly, if metapleural gland grooming behaviour and chemistry have been shaped by coevolutionary interactions with *Escovopsis*, then we would expect that the grooming rate and the chemical secretion cocktail would be adapted to inhibit native parasite strains more than nonnative strains.

The coevolutionary patterns arising from interactions between *Escovopsis* and the Actinobacteria are inevitably different from those between *Escovopsis* and direct defences by the ants. Two, perhaps nonmutually exclusive, scenarios derived from Red-Queen dynamics in relation to Actinobacteria defence have been proposed. The first scenario suggests that Actinobacteria in the genus *Pseudonocardia* evolve in response to antibiotic resistance evolving in *Escovopsis*. Evidence supporting the potential for this to be the case comes mainly from observations of variation in the propensities of different *Pseudonocardia*-derived antibiotics, including the presence of *Escovopsis* strains that are resistant [55, 64]. Phenotypic variation is a prerequisite for such dynamics to be maintained, as this is what natural selection can act on. However, no studies have as yet shown that changes in *Pseudonocardia* genes for antibiotic production do indeed change in response to *Escovopsis* susceptibility. A second possible scenario is that attine ants frequently acquire strains of bacteria from the environment that have novel antibiotic properties against *Escovopsis*, be it either *Pseudonocardia* [55] or other Actinobacteria [57, 61, 65]. Evidence for such acquisitions comes from survey data showing that free-living *Pseudonocardia* are phylogenetically interspersed with ant-associated clades [55], and that additional Actinobacteria with antibiotic properties (mainly *Streptomyces*) can be obtained from the ant cuticles or gardens of colonies. We expect that characterizations of the antibiotic profiles produced by the major clades of *Pseudonocardia* that associate with fungus-growing ants will clarify the role that these alternative acquisition mechanisms have played in maintaining a successful *Pseudonocardia*-defence against *Escovopsis*. Further studies will also benefit from a more explicit emphasis on exploring how and to what extent such horizontal acquisitions of novel Actinobacteria occur, and whether they have a selective advantage for ant colony fitness.

7. Conclusions

Since the discovery of *Escovopsis* parasitism of fungus-growing ants less than 15 years ago, we have obtained a broad understanding of prevalence, impact, role, and coevolution of the parasite with the attine ant-fungus symbiosis. Nevertheless, many fundamental questions remain unanswered, including the origin of the host-parasite association, its presence and potential role outside attine ant nests, parasite transmission between colonies, and within-colony disease dynamics. We know that *Escovopsis* is attracted to specific ant cultivars in some cases, but the generality of this phenomenon and the underlying recognition mechanisms are unknown. Several defences against *Escovopsis* are known, including prophylactic behaviours, metapleural gland grooming and compounds, and Actinobacteria symbionts, which all contribute to reducing the impact of *Escovopsis*. However, we know little about the context-specific efficiency of these alternative and complementary defences, and only in some cases do we have a crude understanding of the potential trade-offs involved. More detailed phylogenetic studies of the association specificity of ants, fungal cultivars, *Escovopsis*, and Actinobacteria are needed to improve our interpretations of reciprocal interactions observed. Although the Tinbergen framework did not allow us to do full justice to the complexity of this host-parasite interaction, we feel that it does provide a useful structuring device for the research agenda that will be required to make further progress in understanding this unique genus of crop-pests of fungus-growing ants.

Acknowledgments

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