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The evolution of social parasitism in *Acromyrmex* leaf-cutting ants: a test of Emery's rule

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Summary. Emery's rule predicts that social parasites and their hosts share common ancestry and are therefore likely to be close relatives. Within the leaf-cutting ant genus *Acromyrmex*, two taxa of social parasites have been found, which are thought to occupy opposite grades of permanent social parasitism, based on their contrasting morphologies: *Acromyrmex insinuator* differs little in morphology from its free-living congeneric host species and produces a worker caste, and is thus thought to represent an early grade of social parasitism. At the other extreme, *Pseudoatta* spp. exhibit a very specialised morphology and lack a worker caste, both of which are characteristics of an evolutionarily derived grade of social parasitism. Here we present a molecular phylogeny using partial sequences of cytochrome oxidase I and II of about half of the known *Acromyrmex* species including two social parasites, their hosts and all congeneric species occurring sympatrically. We show that the two inquiline parasites represent two separate origins of social parasitism in the genus *Acromyrmex*. The early-grade social parasite *A. insinuator* is highly likely to be the sister species of its host *Acromyrmex echinator*, but the derived social parasite *Pseudoatta* sp. is not the sister species of its extant host *Acromyrmex rugosus*.

Key words: Inquiline parasites, speciation, phylogeny, *Acromyrmex*.

Introduction

Emery's rule states that social parasites are close relatives of their hosts (Emery, 1909) and thus allows specific predictions about the ancestry of social parasites and inferences about their evolution and modes of speciation to be made. Emery's rule can be resolved at two levels and it is important to distinguish between them because they have different implications on the modes of evolution (Bourke and Franks, 1991). Social parasites may be their host's closest relative (the strict version of Emery's rule) and are likely to have evolved intraspecifically from their host's lineage by sympatric speciation (Bourke and Franks, 1991) or through a combination of allopatry and secondary sympatry (Lowe et al., 2002). Alternatively, social parasites may be close relatives of their host but not sister species (the loose version of Emery's rule). In this case the social parasite may have arisen in two different ways, either interspecifically from a non-parasitic species in a different lineage to their host (Bourke and Franks, 1991), or intraspecifically, from the same lineage as their host, after which the parasite may have switched hosts or undergone subsequent speciation (Buschinger, 1990). The problem with testing Emery's rule through phylogenetic analysis therefore is that it is difficult to distinguish between events that occurred at the origin of social parasitism and events that occurred afterwards. The highest probability of tracing events at the moment of speciation will be for species close to the speciation event, i.e. incipient parasites, which are unlikely to have switched from their original host or undergone secondary speciation. Such comparisons are particularly useful if they can be contrasted with closely related lineages of advanced social parasitism for which the strict version of Emery's rule is least likely to apply.

Of the few phylogenetic analyses conducted on hymenopteran social parasites and their hosts, most support at least the loose form of Emery's rule. A rare exception exists in which host and parasite belong to different subfamilies (Maschwitz et al., 2000). Social parasitism in bees (Danforth, 1999; Pedersen, 1996) and in wasps (Carpenter et al., 1993; Choudhary et al., 1994) tends to be concentrated in relatively few species-rich clades of monophyletic origin, which often exploit species of equally extensive sister clades. Many genera of ant social parasites are polyphyletic and often show highly convergent adaptations (e.g. Ward, 1989;

Agosti, 1994; Baur et al., 1996; Ward, 1996; Savolainen and Vepsäläinen, 2003). An exception is two *Pogonomyrmex* inquiline parasites which form a monophyletic group within the host clade (Parker and Rissing, 2002). Nonetheless, it has not been easy to assess if and when Emery's rule holds strictly amongst ant social parasites and their hosts because few host-parasite pairs have been subjected to molecular phylogenetic analysis (Ward, 1989). Furthermore, almost no studies have considered incipient parasites, because they are rare. The reported cases amongst the ants where Emery's rule does seem to hold strictly are based on morphological evidence (Elmes, 1978; Wilson, 1984; Schultz et al., 1998, but see Savolainen and Vepsäläinen, 2003).

The incipient inquiline parasite *Acromyrmex insinuator* was discovered recently (Schultz et al., 1998). It belongs to the leaf-cutting ants, Attini (Myrmicinae) and is found in Panama, Central America where it parasitizes a single host *Acromyrmex echinatior*. It is thought to be incipient because it closely resembles its host in morphology and produces a worker caste (Schultz et al., 1998). Morphological data suggest that *A. insinuator* is the sister species of its host, in agreement with the strict version of Emery's rule (Schultz et al., 1998; Bekkevold and Boomsma, 2000). Because few incipient social parasites with a worker caste are known, *A. insinuator* presents a rare opportunity to examine the true origin of inquiline behaviour (Sumner et al., 2003). Another two inquiline parasites of *Acromyrmex* leaf-cutting ants have been found in South America. Together they presently constitute their own genus, *Pseudoatta* (*Pseudoatta argentina,* host, *Acromyrmex lundi* (Santschi, 1926; Gallardo, 1929) and *Pseudoatta* sp., host, *Acromyrmex rugosus* (Delabie et al., 1993)). These two inquiline parasites represent an extreme grade of social parasitism where the worker caste has been lost and the morphology has become specialised and very different from that of their hosts.

Twenty six species of the genus *Acromyrmex* have been described from throughout the Neotropics (Hölldobler and Wilson, 1990 supplemented with recent literature). Two phylogenies based on larval morphology and molecular sequence data have so far been constructed for the attine ants. Both concentrate on higher level relationships, e.g. the relationship between higher and lower attines and include most of their genera (Schultz et al., 1998; Wetterer et al., 1998). They show that the Attini are a monophyletic group and that the higher attines form a derived clade in the paraphyletic lower attines. However, these phylogenies included only 2–4 species of *Acromyrmex*, none of which were the social parasites or their hosts. In this paper we examine the relationship of *A. insinuator* and *Pseudoatta* sp. with their respective hosts in more detail using partial sequences of the COI and COII genes.

Material and methods

We obtained mitochondrial DNA sequence data from 12 species of *Acromyrmex* leaf-cutting ants and combined these with published sequence data (see below) to reconstruct a partial phylogeny of the genus *Acromyrmex*. Our specific aim was to test the degree to which Emery's rule is supported and from this make inferences about likely modes of speciation of socially parasitic lineages within *Acromyrmex*. Our selection of species reflects this objective: we included the representatives of the two socially parasitic lineages, their extant host species and all other *Acromyrmex* species occurring sympatrically with these parasites. In addition, we included a random selection of other species from Central and South America that were made available to us (see table 1 for collection sites and species ranges). We combined our own sequence data with two *Acromyrmex* sequences (*A. octospinosus* and *A. volcanus*), three *Atta* sequences (*Atta sexdens sexdens*, *Atta sexdens rubropilosa* and *Atta cephalotes*) and two non-leafcutting attines (*Trachymyrmex saussurei* and *Sericomyrmex amabilis*) whose comparable sequences were available in Genbank (Wetterer et al., 1998). The latter two were used as outgroups.

Genomic DNA was extracted from the thorax of 1–4 individuals for each species using standard techniques. A mitochondrial DNA fragment was amplified using the polymerase chain reaction (PCR). The fragment included the 3′ part of COI, an intergenic spacer, the tRNA leucine locus and the 5′ end of COII. In total the fragment amplified was approximately 600 base pairs. These partial genes were amplified in each species using primers designed from existing leaf-cutting ant sequences (Wetterer et al., 1998). The primers used for each species are indicated in Table 1. Approximately 50 ng of template DNA were amplified in 50 µl volumes in 1 × reaction buffer, 0.2 mM dNTPs, 0.5 µM primers, with 1.25 U of Taq polymerase, using an initial denaturing step of 94 °C for 2 min, followed by 25 cycles of 94 °C for 30 s, 48–55 °C for 30 s (depending on species and primer pair), 72 °C for 30 s. A final elongation step of 72 °C for 10 min completed the amplification process. Products were run on 2% agarose gels stained with ethidium bromide. Single strong bands were cleaned up using the Qiagen kit and subsequently sequenced.

All 19 sequences were aligned using the program Sequencher 3.11 (Gene Codes Corporation). Phylogenetic relationships were inferred from the aligned sequences using unweighted parsimony with PAUP* 4.0b8 and 10 (Swofford, 2001). Gaps were coded as missing data. The Branch and Bound option was used in PAUP to find the most parsimonious trees. Clade stability was assessed by 1000 bootstrap replications (Hillis and Bull, 1993), using heuristic searches to find the best trees in each replicate (a stepwise addition starting tree and 10 random addition sequences with TBR branch swapping). In addition to the parsimony analysis, three separate Bayesian analyses were performed using MrBayes 3.04b (Huelsenbeck and Ronquist, 2001, http://morphbank.ebc.uu.se/mrbayes/info.php). MrModeltest 1,1b* (Nylander, 2002) was used to select the best-fit model. The best-fit model was the general time reversible model of sequence evolution with gamma and a proportion of invariable sites (GTR + gamma + I; base frequencies estimated: A 0.3482, C 0.1932, G 0.0418, T 0.4168; rates: A–C 1.1676, A–G 5.7911, A–T 1.3983, C–G 0.0000, C–T 12.6436, G–T 1.0000; proportion of invariable sites: 0.3299; gamma distribution shape parameter: 0.8795). However, we also used the general time reversible model of sequence evolution with site-specific rates (GTR + SSR) since the used DNA sequence is protein coding. The analyses employed 4 chains, one cold and three incrementally heated chains, where the heat of the *i*th chain is $B = 1/[1 + (i-1)T]$ with $T = 0.2$. Starting trees for each chain were random and used the default starting values defined in MrBayes 3.04b. A single run consisted of 1,500,000 generations which were sampled in every 50th tree. Likelihood values reached a stable value after 10,000 generations. To ensure that we included only trees after the chain had reached a stable ('burnin') value, we set the burnin at 100,000 generations, which produced 28,000 sampled trees and corresponding posterior probability distributions based on these trees. A majority rule consensus tree of all the trees sampled in the analysis was created using PAUP* 4.0b10 (Swofford, 2001). Maximum likelihood analyses were performed using heuristic search options in PAUP* 4.0b10 (Swofford, 2001) using the general time reversible model and site specific rates, using the base frequencies and substitution rates estimated with MrModeltest. Starting trees were obtained by stepwise addition using 10 random addition sequences and TBR branch swapping.

Table 1. Species used in the phylogeny, with their ranges, place of collection and collector. Primer sequences are also given

Species	Subgenus	American range	Collecting site	Primer*
Acromyrmex echinatior	*Acromyrmex*	Central	Panama[1]	CO1andCOII
Acromyrmex insinuator	*Acromyrmex*	Central	Panama[1]	CO1andCOII
Acromyrmex octospinosus	*Acromyrmex*	Central and South	Panama[1]	CO1andCOII
Acromyrmex octospinosus	*Acromyrmex*	Central and South	Guadeloupe[6]	CO1andCOII
Acromyrmex octospinosus	*Acromyrmex*	Central and South	Genbank[5]	N/a
Acromyrmex volcanus	*Acromyrmex*	Central	Genbank[5]	N/a
Acromyrmex crassispinus	*Acromyrmex*	South	Brazil[3]	COIandCOII
Acromyrmex coronatus	*Acromyrmex*	Central and South	Panama[1]	COIandCOII
Acromyrmex heyeri	*Moellerius*	South	Uruguay[4]	COIandCOII(a)
Acromyrmex lundi	*Acromyrmex*	South	Buenos Aires[4]	COIandCOII(a)
Acromyrmex subterraneus subterraneus	*Acromyrmex*	South	Brazil[2,3]	COIandCOII
Pseudoatta sp.	*Acromyrmex*	South	Brazil[1]	COIandCOII(a)
Acromyrmex rugosus	*Acromyrmex*	South	Brazil[1]	COIandCOII(d)
Acromyrmex balzani	*Moellerius*	South	Brazil[1]	COIandCOII
Atta cephalotes	N/a	Central and south	Genbank[5]	N/a
Atta sexdens sexdens	N/a	Central and south	Genbank[5]	N/a
Atta sexdens rubropilosa	N/a	Central and south	Genbank[5]	N/a
Trachymyrmex saussurei	N/a	Central	Genbank[5]	N/a
Sericomyrmex amabilis	N/a	Central	Genbank[5]	N/a

** Primers:* CO1 = TATTTACTATTTGAGAAGC.; CO11 = TGATGTTC(T/A)AGGAGAAATC; COII(a) = GAAGGGATGGCAATAAAGA; COII(d) = ATTTTAATTGAAGGGATGGC).

Collectors: [1] the authors; [2] William O.H. Hughes; [3] Freddie-Jeanne Richard; [4] Flavio Roces, [5] Wetterer, Schultz, and Meier (1998); [6] Jenny Knapp. Voucher specimens have been placed in the Zoological Museum of the University of Copenhagen.

Results

The intergenic region and the tRNA gene (together comprising 150–358 base-pairs) were too variable for alignment and were removed from the analysis. The remaining partial COI and COII sequences were 111 and 151 base pairs long, respectively. One 3 base-pair insertion/deletion event had to be postulated which distinguished the *Atta* sequences from the *Acromyrmex* sequences. DNA sequences have been deposited under Genbank Accession Numbers AY265961-AY265972.

On average 76% of sites in the two genes were A and T. High AT richness is typical of hymenopteran mitochondrial DNA (e.g. 73.9% of COI in *Tetraponera rufoniger* (Jermiin et al., 1994) and 75% of COI in bumblebees (*Bombus* spp.) (Pedersen, 1996) is AT; 76% of COI and COII in dolichoderine ants (Chiotis et al., 2000) and 80% of COI and cytochrome b in carpenter bees (*Xylocopa)* (Leys et al., 2000) is AT). The mitochondrial CO (cytochrome oxidase) genes are relatively conserved because they evolve under functional constraints, and so most of the substitutions are expected to be in the third position of a codon. Our data set contained 136 variable positions, 92 of which were parsimony informative. Of the 136 variable positions, 25, 14 and 61% were at 1st, 2nd and 3rd positions, respectively, confirming this expectation. A single most parsimonious tree of length 375 was found in the parsimony analysis (c.i. = 51, r.i. = 0.59; Fig. 1). The maximum likelihood analysis was almost identical with the most parsimonious tree except for a minor difference in the position of *Acromyrmex subterraneus*. The three runs of Bayesian analysis resulted in similar topologies with posterior probabilities only differing slightly (maximum 3%). The results using the two models (GTR + gamma + I and GTR + SSR) were not positively different, but the GTR + SSR model gave a more resolved majority rule consensus tree. The Bayesian results were not positively different from the most parsimonious tree, so we present the most parsimonious tree with bootstrap values and Bayesian posterior probabilities (using the GTR + SSR model, Fig. 1). Some of the basal relationships are not well supported. We will therefore focus on the well-supported terminal branches, which are indicated by thick lines in Fig. 1.

In our analysis the genus *Acromyrmex* forms a paraphyletic group, with the genus *Atta* as a derived clade within the South American *Acromyrmex*. However, the support for the basal nodes producing this pattern is so low that we refrain from drawing conclusions at this level. The 12 species of *Acromyrmex* (including *Pseudoatta* sp.) cluster into two groups that correspond to their South and Central American distributions. The exception is *A. coronatus* which was sampled in Panama (Central America) but clustered with the South American group, perhaps reflecting the fact that its distribution extends throughout the Neotropics (Table 1; Kempf, 1972). Remarkably, *A. coronatus* is closely related to *Pseudoatta* sp. and comes out as the sister species in the phylogeny of Fig. 1 (bootstrap = 98, Bayesian probability = 100). The Central American group forms a well-supported clade (96% bootstrap and 100% Bayesian probability) and consists of species that were until recently considered to be different forms of *A. octospinosus sensu lato.* The pattern suggests that the Guadeloupe specimens of *A. octospinosus* represent a different species to the central American specimens of *A. octospinosus* and *A. echinatior*, which have recently been shown to represent good species on the basis of inde-

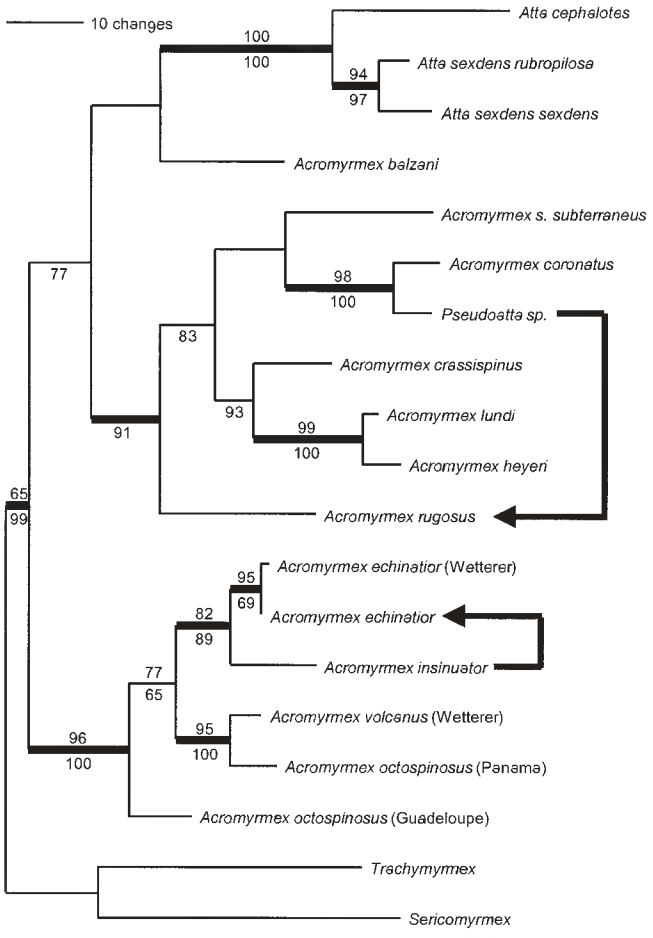

Figure 1. Phylogeny of 14 *Acromyrmex* and three *Atta* leaf-cutting ants reconstructed from partial sequences of COI and COII mitochondrial DNA, using two non-leaf-cutting attines (*Trachymyrmex saussurei* and *Sericomyrmex amabilis*) as outgroups. The figure represents the single most parsimonious tree (c.i. = 0.51, r.i. = 0.59), with non-parametric bootstrap support values (>50%, above branches) and Bayesian posterior probabilities (>50%, using GTR + SSR, below branches) given for each node. Arrows point from the social parasites to their hosts. The majority rule consensus trees sampled in Bayesian analyses (three separate analyses using different starting trees resulted in identical majority rule consensus trees) were not positively different from the most parsimonious tree. Well-supported branches (supported by either >90% support in one of the analyses and/or >80% in both of the analyses) are indicated with thick lines

pendent genetic markers (Schultz et al., 1998). The incipient social parasite *A. insinuator* is the sister species of its host *A. echinatior* as predicted by the strict form of Emery's rule. The host of *Pseudoatta* sp. *(A. rugosus)* is basal to the South American *Acromyrmex* clade and not closely related to its parasite.

Discussion

We have reconstructed a phylogeny of a representative sample of the leaf-cutting ant genus *Acromyrmex* with the express purpose of finding the relationship between two inquiline social parasites and their hosts. Further study of trees based on nuclear genes, would be informative since mitochondrial gene trees sometimes conflict with nuclear gene trees (e.g. Shaw, 2002). However, our results are also supported by morphological study (Schultz et al., 1998). We first draw attention to some general points about the systematics of the *Acromyrmex* genus and then discuss the evolution of social parasitism in *Acromyrmex* and suggest possible mechanisms by which the social parasites may have speciated.

Our phylogeny indicates that *Pseudoatta* sp. belongs to the genus *Acromyrmex,* as hypothesized by Hölldobler and Wilson (1990). The Central American *Acromyrmex* clade is made up entirely of the *A. octospinosus sensu lato* group (Santschi, 1925; Goncalves, 1961). Our study confirms that the systematics and species status of the *octospinosus* group is complex and still insufficiently resolved with current information (see Brown, 1957). For example, we found that *A. octospinosus* sequenced by Wetterer et al. (1998) ('*A. echinatior* Wetterer' in our study) was in fact the same species as the Panamanian *A. echinatior* used in our study. This is probably because the subspecies *A. octospinosus echinatior* was only recently elevated to species level, *A. echinatior* (Schultz et al., 1998). *A. volcanus* was also previously classified as a subspecies in the *octospinosus* group, *A. octospinosus volcanus* (Kempf, 1972), but was elevated to full species status by Wetterer (1993). Furthermore, *A. octospinosus* from Guadeloupe does not form a monophyletic group with *A. octospinosus* Panama, but is instead basal to the rest of the Central American clade, with high bootstrap support (96) and high Bayesian probability (100). This suggests that the species range of the 'typical form' of *A. octospinosus* may not be as wide as previously suggested (Weber, 1972), but that the group in fact consists of several different species. Clearly, more detailed taxonomic study of this group is required.

The genus *Acromyrmex* has been divided into two subgenera, *Acromyrmex* and *Moellerius,* on the basis of two morphological traits (Emery, 1905). *Moellerius* is distinguished from *Acromyrmex* by its short, stout mandibles and the absence of lateral spines above the eyes of workers. *A. balzani* and *A. heyeri* were placed in the subgenus *Moellerius,* whilst the remaining species included in our phylogeny were placed in the *Acromyrmex* subgenus. The position of *A. balzani* is poorly supported in our study, but there is good support for *A. heyeri* and *A. lundi* being closely related (see Fig. 1). To verify this puzzling result, new samples of *A. heyeri* and *A. lundi* from the same set of laboratory colonies were sequenced and found to produce identical results. The validity of the division of *Acromyrmex* into the two subgenera *Acromyrmex* and *Moellerius,* may therefore be questionable, but also this issue cannot be settled until more sequencing data are available.

We found that social parasitism has a polyphyletic origin in the *Acromyrmex* leaf-cutting ants, i.e. *Pseudoatta* sp. and *A. insinuator* are not sister groups. Inquiline parasitism has thus arisen at least twice independently, once in each of the two regional groups. This agrees with the evidence to date that social parasitism in specific ant genera can easily have

multiple origins (e.g. Baur et al., 1996; Savolainen and Vepsäläinen, 2003). Our results support Emery's rule in the loose sense in that clades of parasites and their hosts are related. Thus, similarities in their morphology, behaviour and life-cycle are likely to have arisen from common ancestry rather than convergent evolution (Ward, 1989). However, we found that the incipient parasite (*A. insinuator)* and its host were sister species (confirming Schultz et al., 1998), but that the derived parasite *(Pseudoatta* sp.) and its host were not. We discuss the likely modes of evolution for both parasites below.

For *A. insinuator* to have evolved allopatrically from its host and to be sister species requires either that the non-parasitic ancestral species from which the extant parasite evolved has gone extinct (Bourke and Franks, 1991) or that there has been phyletic speciation (i.e. without lineage splitting), else its closest relative would not be its host. Given the likely recent origin of *A. insinuator* and the fact that all currently known *Acromyrmex* species from Central America were included in our study, sympatric speciation is likely to be the more parsimonious explanation for the origin of *A. insinuator* (Buschinger, 1990). However, caution is required as further cryptic species may easily be found in the future (Schultz et al., 1998). A major problem in the sympatric speciation model for social parasitism is in explaining how an incipient parasite becomes reproductively isolated from its host. Buschinger (1990) suggested that social parasites evolve intraspecifically from their host through some 'deficient' mutant, e.g. producing a queen-biased caste ratio. Reproductive isolation of these 'preparasites' or cheaters is then feasible for example through temporal or spatial differences in mating behaviour. Polygyny (multiple queens in a colony) may therefore favour the evolution of dependent nest foundation and parasitism, indeed most inquiline hosts are polygynous (Buschinger, 1990). This scenario for the evolution of *A. insinuator* is consistent with the data, since its host *A. echinatior* is facultatively polygynous (Bekkevold et al., 1999), a trait that is uncommon in *Acromyrmex*. The sympatric non-host *A. octospinosus* from Panama is obligately monogynous (Boomsma et al., 1999). Colonies of this species are invaded by newly mated *A. insinuator* gynes in approximately the same frequency as proper (*A. echinatior*) host colonies, but successful reproduction of *A. insinuator* with *A. octospinosus* as host has so far not been observed in the field (J. J. Boomsma, unpublished data). The queen-biased caste ratio scenario may also apply, because *A. insinuator* has retained its caste of small workers, but has lost its caste of large workers, which is most similar in body size with queens (Schultz et al., 1998, Sumner et al., 2002, 2003).

In contrast to *A. insinuator,* our study indicates that the derived inquiline, *Pseudoatta* sp., is only distantly related to its extant host, and that host and parasite do not form sister groups. One explanation for why host-parasite pairs are not sister species is that they evolved allopatrically and that one non-parasitic species then became the parasite of another non-parasitic species after sympatry was restored (Bourke and Franks, 1991). An alternative explanation, suggested by Buschinger (1990), is that the novel socially parasitic lineage evolved intraspecifically (i.e. sympatrically, in a similar way as suggested for *A. insinuator)* and subsequently speciated when new host species were successfully colonised. A scenario similar to this might occur in the future if a mutant *A. insinuator* manages to reproduce in sympatric *A. octospinosus* colonies (see also Buschinger, 1986).

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