

A Social Parasite Evolved Reproductive Isolation from Its Fungus-Growing Ant Host in Sympatry

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Summary

Inquiline social parasitic ant species exploit colonies of other ant species mainly by producing sexual offspring that are raised by the host. Ant social parasites and their hosts are often close relatives (Emery's rule), and two main hypotheses compete to explain the parasites' evolutionary origins: (1) the interspecific hypothesis proposes an allopatric speciation scenario for the parasite, whereas (2) the intraspecific hypothesis postulates that the parasite evolves directly from its host in sympatry [1–10]. Evidence in support of the intraspecific hypothesis has been accumulating for ants [3, 5, 7, 9–12], but sympatric speciation remains controversial as a general speciation mechanism for inquiline parasites. Here we use molecular phylogenetics to assess whether the socially parasitic fungus-growing ant *Mycocepurus castrator* speciated from its host *Mycocepurus goeldii* in sympatry. Based on differing patterns of relationship in mitochondrial and individual nuclear genes, we conclude that host and parasite occupy a temporal window in which lineage sorting has taken place in the mitochondrial genes but not yet in the nuclear alleles. We infer that the host originated first and that the parasite originated subsequently from a subset of the host species' populations, providing empirical support for the hypothesis that inquiline parasites can evolve reproductive isolation while living sympatrically with their hosts.

Results and Discussion

An enduring controversy in evolutionary biology is whether the origin of species always requires a period of geographic separation, or whether new species can arise in sympatry [13–16]. A firm demonstration of sympatric speciation requires documentation that the species pair in question is composed of sister species that (1) exist in sympatry, (2) are currently reproductively isolated, (3) exchanged genes freely prior to speciation, and (4) were not allopatric at the time of speciation [13, 17–20].

Ant social parasites and their hosts are often closely related (Emery's rule) [21], and two main hypotheses compete to

explain the parasites' evolutionary origins: the interspecific hypothesis proposes that host and parasite speciated in allopatry [2, 4, 6–8], whereas the intraspecific hypothesis postulates that the parasite evolved directly from its host in sympatry [1, 3, 5, 7, 9]. To distinguish between the two, it is useful to interpret Emery's rule in a phylogenetic framework. Strictly phrased, Emery's rule states that host and parasite are each other's closest relatives (i.e., sister taxa), whereas a looser interpretation postulates close relatedness (e.g., congeners) [7, 8]. Phylogenetic studies provide evidence for both scenarios (see Table S2 available online), but sympatric speciation continues to be controversial. A convincing phylogenetic study of sympatric speciation needs to demonstrate reproductive isolation between host and parasite as well as exhaustively explore all possible sister-group relationships. To assess the origin of the inquiline social parasite *Mycocepurus castrator*, we reconstructed the evolutionary history of the fungus-growing ant genus *Mycocepurus*.

Biogeography

Mycocepurus castrator is a workerless inquiline social parasite of the fungus-growing ant *Mycocepurus goeldii*. The parasite queens coexist and reproduce alongside the host queen (or queens) inside the same colony (Figure 1) [22]. The host is geographically widespread, occupying diverse biomes throughout South America (Figure 2) [22, 23]. The only known population of the parasite is limited to a single stand of *Eucalyptus* trees on the campus of São Paulo State University in Rio Claro, Brazil, which is nested inside *M. goeldii*'s extensive geographic distribution range (Figure 2). Despite comprehensive population studies and nest excavations of *M. goeldii* in the Amazon [24] and in the Brazilian Cerrado (C.R., unpublished data; [23]), we have only encountered *M. castrator* in Rio Claro, and free-living colonies are unknown [22]. Hence, extant populations of host and parasite are sympatric on a geographic scale as well as on a colony-level scale.

Phylogenetic Inference

We reconstructed the evolutionary history of the genus *Mycocepurus* from a complete sampling of all six known and five undescribed species. The host species was represented by samples from 17 geographically distant populations spanning *M. goeldii*'s extensive distribution range in South America (Figure 2), and the parasite species by six samples from the only known population (Table S1). The complete DNA sequence alignment consisted of 4,918 bp from three nuclear and two mitochondrial markers (Table S1).

The aim of our phylogenetic analyses was to distinguish between two mutually exclusive evolutionary hypotheses: (1) host/parasite reciprocal monophyly, in which the host and the parasite arose simultaneously from a most recent common ancestor (MRCA) and are thus true, reciprocally monophyletic sister species, and (2) host paraphyly, in which the host originated first and the parasite speciated subsequently, sharing a MRCA with a subset of extant host populations but not with all of them, rendering the host paraphyletic.

The analysis of the fully concatenated DNA sequence alignment indicated with high support that (1) host and parasite are

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Figure 1. A Queen of the Socially Parasitic *Mycocepurus castrator* Interacts with Its Host *M. goeldii*

The social parasite (center) is antennated by a worker (right) while standing on the host queen (left). Note the significant size difference between parasite and host queens. The scale bar represents 1 mm. Photograph courtesy of Scott E. Solomon.

sister species (maximum-likelihood bootstrap proportion [MLBP] = 96–100; Bayesian posterior probability [BPP] = 1; Bayesian estimation of species tree posterior probability [BESTPP] = 1; StarBEAST posterior probability [SBPP] = 1) and (2) the parasite is monophyletic (MLBP = 100; BPP, BESTPP, SBPP = 1). Interestingly, however, differing methods of analyses of the concatenated data varied in their support for the monophyly of the host species: ML analyses supported host monophyly only poorly (MLBP = 42 under an mtDNA nucleotide model; MLBP = 72 under an mtDNA codon model; see [Supplemental Experimental Procedures](#)), whereas Bayesian analyses strongly supported host monophyly (BPP, BESTPP, SBPP = 0.97–1). The low support for host monophyly under ML criteria contrasted markedly with the high support for the monophyly of all other *Mycocepurus* species in all analyses ([Figure 3](#)). This disagreement indicated a potential conflict between individual gene trees and the species tree inferred from analyses of the concatenated data, motivating additional analyses.

To explore this conflict, we separately analyzed each of the three nuclear genes, the mitochondrial genes, and a data set consisting of the concatenated nuclear genes; we also conducted phylogenetic constraint analyses of the same data sets ([Table 1](#); see [Supplemental Experimental Procedures](#)). Analyses of the individual and concatenated nuclear genes invariably supported the host paraphyly hypothesis, indicating that the host alleles are paraphyletic, with parasite alleles arising from within clades of host alleles ([Figure 3](#); [Table 1](#)). In contrast, mitochondrial trees were equivocal about reciprocal monophyly versus host paraphyly ([Figure 3](#); [Table 1](#)).

Following speciation, we would expect host and parasite to pass through an evolutionary interval beginning with reproductive isolation and ending with the completion of lineage sorting in mitochondrial and nuclear alleles. During this process, mitochondrial sequences will become reciprocally monophyletic more rapidly than nuclear alleles due to their relatively smaller effective population sizes [26], and mitochondrial sequences will lose information about population-level relationships relatively early, whereas at least some nuclear alleles will retain that information until complete lineage sorting has occurred [27–29]. In addition, individual nuclear alleles will have experienced different histories so that, until all alleles

have undergone lineage sorting, they will agree that the parasite is more closely related to some host populations than to others, but they will disagree about the particular populations involved [26–28]. Once that interval has ended and lineage sorting has occurred in all alleles, the only recoverable pattern will be that of host/parasite reciprocal monophyly.

Our results are best explained by a scenario in which we have fortuitously captured the host and parasite at a point in this interval in which lineage sorting has occurred in mitochondrial markers, producing a pattern of weakly supported reciprocal monophyly (two analyses) or weakly supported host paraphyly (one analysis) in mitochondrial-only analyses, whereas lineage sorting is incomplete in the nuclear markers, producing differing patterns of host paraphyly due to differing allelic histories ([Table 1](#)). Under this scenario, we would not expect the results of analyses of the concatenated data to reliably reflect species and population-level host/parasite relationships.

In addition to the above lineage-sorting scenario, discordance between nuclear and mitochondrial markers could be caused by hybridization and/or gene duplication [27, 30]. A recent hybridization event is unlikely, because some host and parasite individuals would be expected to share identical mitochondrial haplotypes (i.e., introgression) and divergent nuclear genotypes. Instead, the opposite pattern is observed in our study. Specifically, we found diverging mitochondrial alleles, and nuclear host alleles that were paraphyletic with respect to parasite alleles. Gene duplication is also unlikely as a source of phylogenetic discordance, because paralogs of some of the targeted genes were identified in previous studies [31, 32], and our primers were designed to amplify particular paralogs in *Mycocepurus* ants.

In summary, the combined results of our phylogenetic analyses support hypothesis 2, host paraphyly, in which the host originated first and the parasite speciated subsequently, sharing a MRCA with a subset of extant host populations but not with all of them.

Divergence Dating

A molecular divergence dating analysis of the *Mycocepurus* nuclear gene alignment corroborated a secondary origin of the parasite, assigning a crown-group age to *M. castrator* in the late Pleistocene (37,000 years; confidence interval [CI] = 4,000–90,000) but assigning a crown-group age to the host clade, when constrained to be monophyletic, in the early Pleistocene (2.04 million years [My]; CI = 1.22–3.11). The stem-group age of the host/parasite clade was assigned to the mid-Pliocene at 3.31 My (CI = 2.02–4.92).

Given that the discovery of additional, genetically divergent parasite populations would probably increase the inferred age of origin, and considering the wide confidence intervals surrounding our estimates, we consider the divergence estimates as approximations accompanied by a high degree of uncertainty. In principle, however, a recent age for the origin of *M. castrator* is not unexpected, because haplodiploid species are known to diverge more rapidly than diploid species [33, 34]. Ant social parasites in particular have been shown to diverge faster than their eusocial relatives [35], and a comprehensive study of *Myrmica* also inferred a recent divergence date of 0.8 My for one parasite [12]. Nonetheless, considering the high degree of morphological and behavioral specialization that characterizes inquiline parasites (compare queens in [Figures 1](#) and [2](#)) [2], understanding the genetic mechanisms and selective pressures responsible for the convergent evolution of inquiline social parasites is of utmost biological interest.

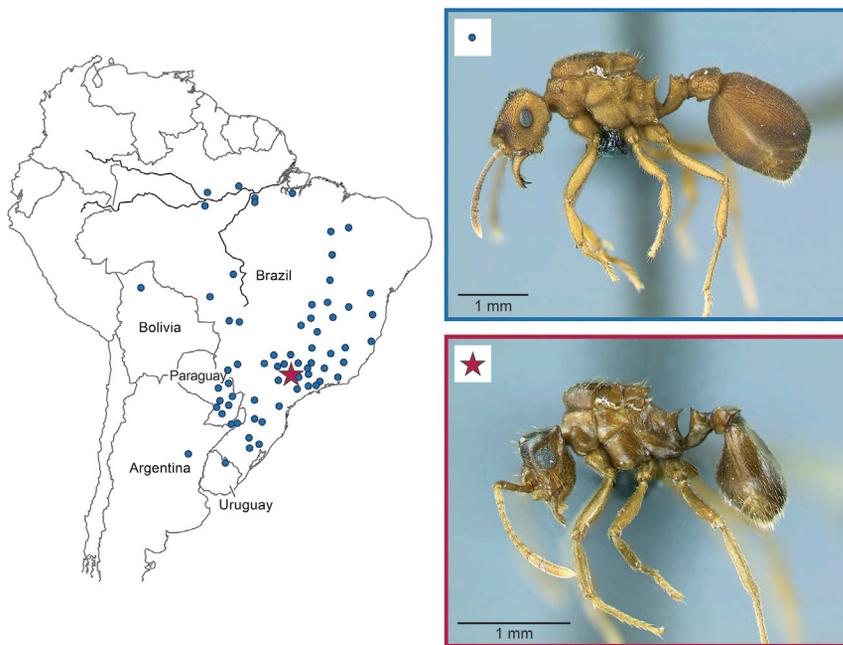


Figure 2. Sympatric Geographic Distribution of the Socially Parasitic *Mycocepurus castrator* and Its Host *M. goeldii* in South America

Left: blue dots depict geographic distribution records of the host species; the red star marks the parasite's type locality. Right: lateral views of the host *M. goeldii* queen (blue frame) and the parasitic *M. castrator* queen (red frame). The scale bars in both photographs represent 1 mm, indicating that the parasite is considerably smaller.

Morphology, Ecology, and Mating Behavior

The presence of multiple reproductively active queens per colony, i.e. polygyny, has repeatedly been identified as a first step for the evolution of “cheating” behavior in eusocial insect colonies, because polygyny provides the opportunity for a behavioral polymorphism in which some queens focus on selfish production of reproductives while others continue to altruistically produce sterile workers [6, 7, 9–11, 36–38]. In addition, polygyny facilitates the entry of additional queens into a colony due to increased tolerance of workers toward unrelated nestmates [6, 9, 10, 36].

Dissecting queens of *M. goeldii* and *M. castrator* showed that colonies of both species are at least facultatively polygynous [22, 39]. *M. goeldii* was facultatively polygynous in the Brazilian Amazon, whereas the single queenright colony we encountered in Rio Claro was monogynous [22, 24]. The only *M. castrator* colony that contained dealate reproductively active queens was polygynous with three reproductive queens [22, 39]. Polygyny in two sexually reproducing *Mycocepurus* species is remarkable, because lower Attini are usually monogynous [40, 41]. Facultative polygyny in *M. goeldii* may have been the vantage point for the evolution of social parasitism in this species.

Miniaturization of the sexual offspring of parasitically behaving queens has been proposed as a mechanism promoting assortative mating once cheating behavior has evolved, because size reduction would shift the mating time and potentially the mating locality, resulting in reproductive isolation [7, 9, 10, 42]. A comparison of the mesosoma lengths of *M. castrator* and *M. goeldii* reproductives showed that the parasites are significantly smaller than the hosts (one-tailed Wilcoxon rank-sum test: $n = 76$, $W = 1300$, $p < 0.001$; Figures 1 and 2); however, *M. castrator* reproductives are similar in size to the host workers [22]. Miniaturization is frequently observed in socially parasitic queens that are not constrained to large body sizes to store energy reserves, because they depend on the host workers for colony foundation [7, 10, 42, 43].

To infer whether host and parasite reproductives were still capable of interbreeding, we also studied the morphology of

sclerotized male genitalia. Host and parasite male genitalia differ distinctly in that parasite males lack the characteristic teeth on the ventral border of the aedeagus. The aedeagal teeth are functionally important during copulation because they lock the aedeagus into the female genitalia to stabilize the copula [44], a requirement for successful mating of leafcutter ants in midair and on the ground [44]. Under laboratory conditions, host and parasite reproductives did not copulate,

even though parasite reproductives did mate readily in laboratory nests [22].

Relevant to the hypothesis that miniaturization shifts mating locality and influences assortative mating of host and parasite, we previously (2003–2013) observed the mating behaviors of *M. castrator* and of geographically distant *M. goeldii* populations in Brazil. *M. goeldii* performed seasonally timed mass nuptial flights, in which alates copulated when perched on lower vegetation [22, 39, 45]. In contrast, *M. castrator* reproductives mated inside the nest (i.e., adelphogamy) [22]. Observations of one parasitized laboratory colony showed that *M. castrator* males and females mated readily while inside the nest, a behavior that was not observed in the host [22]. In addition, observations of a second parasitized colony in the field revealed that *M. castrator* alates did not perform nuptial flights but instead left the maternal colony on foot [22]. The parasite's switch to adelphogamy is directly relevant to speciation and is common in inquiline parasites [5–7, 9]. If host and parasite adhered strictly to the separation of mating locality during the early stages of speciation, instantaneous reproductive isolation would be expected as a consequence.

In addition to the fact that they choose different mating localities, host and parasite reproductives of extant populations are unlikely to interbreed inside the same colony because in the presence of the social parasite, only worker brood was found in the parasitized *M. goeldii* colonies. Host sexual offspring were absent in the parasitized colonies, even though they were present in neighboring colonies, indicating that *M. castrator* queens suppressed the production of host sexual offspring [22].

To summarize, morphological and behavioral studies indicate that *M. castrator* and *M. goeldii* are fully reproductively isolated and that assortative mating is correlated with differences in body size and genital morphology, as well as with different preferences for mating locality. A temporal separation of mating episodes, as predicted by the miniaturization hypotheses, was not observed but cannot be ruled out as a factor during the early phases of the speciation process.

Table 1. Bayes Factor Comparisons of Bayesian and Maximum-Likelihood Constraint Phylogenetic Analyses

	Host Monophyly Enforced (RM)	Host Paraphyly Enforced (HP)	Bayes Factor Comparison 2ln(BF)	Interpretation of Bayes Factors
Maximum-Likelihood Results				
Nuclear and mitochondrial (nucleotide model) data combined	-19403.745	-19406.839	6.186	strong RM
Nuclear and mitochondrial (codon model) data combined	-19014.401	-19021.087	13.372	very strong RM
Mitochondrial data only (nucleotide model)	-12927.699	-12926.485	-2.428†	positive HP†
Mitochondrial data only (codon model)	-12546.105	-12547.728	3.246	positive RM
Nuclear data only, three genes combined	-6263.690	-6263.288	-0.803†	weak HP†
<i>EF1-alpha</i> F1-copy only	-2983.950	-2982.625	-2.651†	positive HP†
<i>LW Rh</i> only	-1014.251	-1009.503	-9.496†	strong HP†
<i>Wg</i> only	-2120.847	-2108.804	-24.086†	very strong HP†
Bayesian Results				
Nuclear and mitochondrial (nucleotide model) data combined	-19555.469	-19560.655	10.372	very strong RM
Nuclear and mitochondrial (codon model) data combined	NA	NA	NA	NA
Mitochondrial data only (nucleotide model)	-13103.081	-13102.644	-0.874†	weak HP†
Mitochondrial data only (codon model)	NA	NA	NA	NA
Nuclear data only, three genes combined	-6257.568	-6244.711	-25.714†	very strong HP†
<i>EF1-alpha</i> F1-copy only	-3109.357	-3108.931	-0.852†	weak HP†
<i>LW Rh</i> only	-1115.048	-1109.641	-10.814†	very strong HP†
<i>Wg</i> only	-2172.073	-2163.457	-17.232†	very strong HP†

Our analyses were designed to distinguish between two alternative phylogenetic hypotheses: (1) host-parasite reciprocal monophyly (RM) and (2) host paraphyly with respect to the parasite (HP). Topologies were minimally constrained to enforce monophyly of the host and nonmonophyly of the host, respectively. Bayes factor comparisons were calculated such that positive Bayes factors favor hypothesis 1 (RM) and negative Bayes factors favor hypothesis 2 (HP; values indicated †). Bayes factors were calculated as the ratio of marginal likelihoods (i.e., the differences in $-\ln L$) to produce the test statistic $2\ln(BF)$. Bayes factor interpretations follow Nylander et al. [25], and interpretations should be regarded not as rejecting or accepting a given hypothesis using a cutoff value but instead as providing a guideline for the evaluation of competing hypotheses. Bayesian codon-model analyses were computationally intractable.

that strong selective pressures can lead to the rapid evolution of reproductive isolation between host and parasite, supporting West-Eberhard's "alternative adaptation hypothesis," which proposes that interspecific diversity can arise from polymorphic traits already present within a species [47], such as a shift from eusocial to parasitic behavior [48].

The evolutionary biology of the inquiline parasite *M. castrator* is consistent with the sympatric speciation scenario for social parasite evolution and includes the following components: (1) facultative polygyny of the host species, providing the opportunity for cheating behavior to evolve; (2) selection favoring the disproportionate production of sexual offspring by a subset of cheater queens; (3) miniaturization of the parasite, allowing it to escape host recognition; and (4) a shift in mating locality, promoting assortative mating among cheating and altruistic lineages, resulting in reproductive isolation. The alternative scenario, allopatric speciation, would require that the host and future parasite evolved reproductive isolation in allopatry and then secondarily became sympatric, after which one of the two sister species evolved a parasitic behavior where it exclusively parasitized only its closest relative. Even if this alternative scenario were deemed likely on other grounds, it is inconsistent with our observation that the parasite renders the host paraphyletic. Indeed, arguments that have been made to reconcile a host-parasite sister-group relationship with allopatric speciation postulate (1) the extinction of the parasite's free-living ancestor [2, 6] or (2) the anagenetic evolution of social parasitism in one of the sister species without cladogenesis [4], both of which are incompatible with host paraphyly.

Ant inquiline parasites evolved at least 80 times [2, 6, 10, 49], providing a series of natural experiments and allowing for independent tests of sympatric speciation as a general mechanism for the origins of social parasite species. The exciting task ahead is to document the behavioral, ecological, and genetic conditions under which reproductive isolation has arisen in

convergently evolved inquilines, which will in some species prove challenging in the face of secondary host shifts, secondary speciation events, and extinctions.

Experimental Procedures

We analyzed a DNA sequence alignment (five markers, 4,918 bp) for a comprehensive sampling of *Mycocrepurus* taxa ($n = 55$) (Table S1) using partitioned Bayesian inference (BI) and maximum likelihood (ML) analyses, as well as multispecies coalescent analyses and phylogenetic constraint analyses under BI and ML criteria. Marginal likelihoods obtained from constraint analyses were compared using Bayes factors (Table 1). A Bayesian relaxed-clock uncorrelated lognormal approach was used to estimate the divergence times of host and parasite populations. Field and laboratory observations were conducted in Brazil between 2003 and 2013 at UNESP in Rio Claro, IBGE in Brasília, and EMBRAPA in Manaus. Detailed methods and statistical analyses are provided in the Supplemental Experimental Procedures.

Accession Numbers

Sequences reported herein (see Table S1) have been deposited at NCBI GenBank with the accession numbers KJ443357–KJ443649.

Supplemental Information

Supplemental Information includes one figure, Supplemental Experimental Procedures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2014.07.048>.

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