



Evolutionary history of inquiline social parasitism in *Plagiolepis* ants

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ABSTRACT

Social parasitism, i.e. the parasitic dependence of a social species on another free-living social species, is one of the most intriguing phenomena in social insects. It has evolved to various levels, the most extreme form being inquiline social parasites which have lost the worker caste, and produce only male and female sexual offspring that are reared by the host worker force. The inquiline syndrome has been reported in 4 species within the ant genus *Plagiolepis*, in Europe. Whether inquiline social parasitism evolved once or multiple times within the genus remains however unknown. To address this question, we generated data for 5 inquiline social parasites – 3 species previously described and 2 unidentified species – and their free-living hosts from Europe, and we inferred their phylogenetic relationships. We tested Emery's rule, which predicts that inquiline social parasites and their hosts are close relatives. Our results show that inquiline parasitism evolved independently at least 5 times in the genus. Furthermore, we found that all inquilines were associated with one of the descendants of their most related free-living species, suggesting sympatric speciation is the main process leading to the emergence of the parasitic species, consistent with the stricter version of Emery's rule.

1. Introduction

The hallmark of eusocial insect colonies (the ants, termites, and social bees and wasps) is reproductive division of labour between queens and males that are specialized in reproduction, and sterile workers performing all tasks required for colony development and growth. Division of labour is recognized as being at the root of social insects' modern ecological dominance (Wilson, 1971; Oster and Wilson, 1978). However, their social lifestyle based on altruistic behaviours also fostered the evolution of social parasitism, whereby a social species exploits parental care and targets host nest resources for the parasite's own fitness gain. The most extreme form of social parasitism is permanent inquilinism. Inquiline social parasites usually do not produce sterile

workers, but reproductives only. Queens of the parasite infiltrate a host colony and allocate all reproduction into male and female sexuals which are reared by host workers (Wilson, 1971; Hölldobler and Wilson, 1990; Nash and Boomsma, 2008; Buschinger, 2009).

Inquiline social parasites have evolved from free-living species repeatedly across multiple ant subfamilies (Hölldobler and Wilson, 1990; Buschinger, 2009). Suggested by the strong morphological similarities observed between parasites and their hosts, Emery's rule states that socially parasitic ants are evolutionarily closely related to their hosts (Emery, 1909). The emergence of a parasitic lifestyle may indeed be facilitated when the host is a related species, thanks to compatible communication systems, and/or similar pheromones for nestmate recognition and feeding habits (Buschinger, 2009). However, different

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degrees of relatedness between hosts and parasites must be considered (Bourke and Franks, 1991; Lenoir et al., 2001). In the 'strict' form of Emery's rule, hosts and parasites are the closest relatives, and parasites evolved from their host through sympatric speciation (Buschinger, 1990; Bourke and Franks, 1991). In the 'looser' form of the rule, the inquiline species and its host are somehow related, but not necessarily sister species. The two forms of Emery's rule are not necessarily incompatible; a 'strict' form could become a 'loose' form because of the emergence of new sister species. While some studies have indeed supported the strict form of Emery's rule for the evolution of inquiline social parasitism (Buschinger, 1990; Bourke and Franks, 1991; Savolainen and Vepsäläinen, 2003; Huang and Dornhaus, 2008; Rabeling et al., 2014), others have supported a looser version (Ward, 1996; Sumner et al., 2004; Parker and Rissing, 2007; Fischer et al., 2020).

An inquiline social parasitic lifestyle has been reported in 4 species of the ant genus *Plagiolepis* in Europe: in *P. xene* and *P. grassei*, both parasites of the free-living host *P. pygmaea* (Le Masne, 1956), as well as in *P. ampeloni* (Faber, 1969) and in *P. delaugerrei* (Casevitz-Weulersse, 2014), both found in nests of free-living species of the *P. pallescens* complex (previously *P. taurica* complex; Seifert, 2020). This complex consists of at least six Mediterranean species and a taxonomic revision using adequate data is under progress (Kirschner et al. in prep.). The *P. schmitzii* complex includes four species, such as *P. atlantis* (Seifert, 2020).

To date, only the species pairs *P. xene* – *P. pygmaea* and *P. grassei* – *P. pygmaea* have been the focus of detailed studies (Le Masne, 1956; Passera, 1969; Aron et al. 1999, 2004; Passera et al., 2001; Trontti et al., 2006; Mardulyn et al., 2014; Schifani, 2017). While the host species *P. pygmaea* is largely distributed across Europe and North Africa (Fig. 1), the geographic distribution of its two social parasites seems restricted to Europe. As is typically the case for inquiline ants, *P. xene* and *P. grassei* are rare and extremely difficult to find. Extensive sampling over the years has shown that the *P. xene* distribution ranges from Portugal to Turkey. This species has completely lost the worker caste and produces exclusively male and female sexual brood. In contrast, *P. grassei* has only

been found in France, Spain and Sicily, so far (Fig. 1). Queens of *P. grassei* still produce a handful of workers; however, workers hatch after the production of sexuals, suggesting they have little or no adaptive role. Both *P. xene* and *P. grassei* inflict a severe cost to *P. pygmaea* since queens of the parasites eat the host eggs, thereby significantly reducing productivity of host colonies (Passera 1972; Passera et al. 2001).

It remains unknown whether inquiline social parasitism in *Plagiolepis* arose from one or multiple independent evolutionary transitions. To address these questions, we inferred the phylogenetic relationships among several populations of *P. pygmaea* and their inquiline parasites *P. xene* and *P. grassei*, as well as of populations of two other European free-living *Plagiolepis* complexes (*P. pallescens* complex and *P. schmitzii* complex) and their associated inquiline parasites, based on the analysis of 1 mitochondrial and 4 nuclear genes. Based on this phylogenetic hypothesis, we tested whether inquiline social parasitism has emerged multiple times independently or whether it has evolved only once in the genus. Furthermore, for each independent emergence, we tested whether hosts and parasites were randomly distributed in the phylogeny or whether the Emery's rule (loose or strict form) applied.

2. Methods

2.1. Sampling

Our study includes 48 *Plagiolepis* samples corresponding to at least 3 free-living species: *P. pygmaea*, species of the *P. pallescens* complex, species of the *P. schmitzii* complex; and 5 inquiline social parasites: *P. xene*, *P. grassei*, *P. delaugerrei*, and 2 unidentified species *P. sp.* (Table S1). When available, hosts and parasites were collected from the same nest. No samples identified as *P. ampeloni* were included in our analysis due to the scarcity of this inquiline parasite. We used one *Lepisiota* sp. and one *Anoplolepis* sp. as outgroups to root our phylogeny (Ward et al., 2016; Schär et al., 2020).

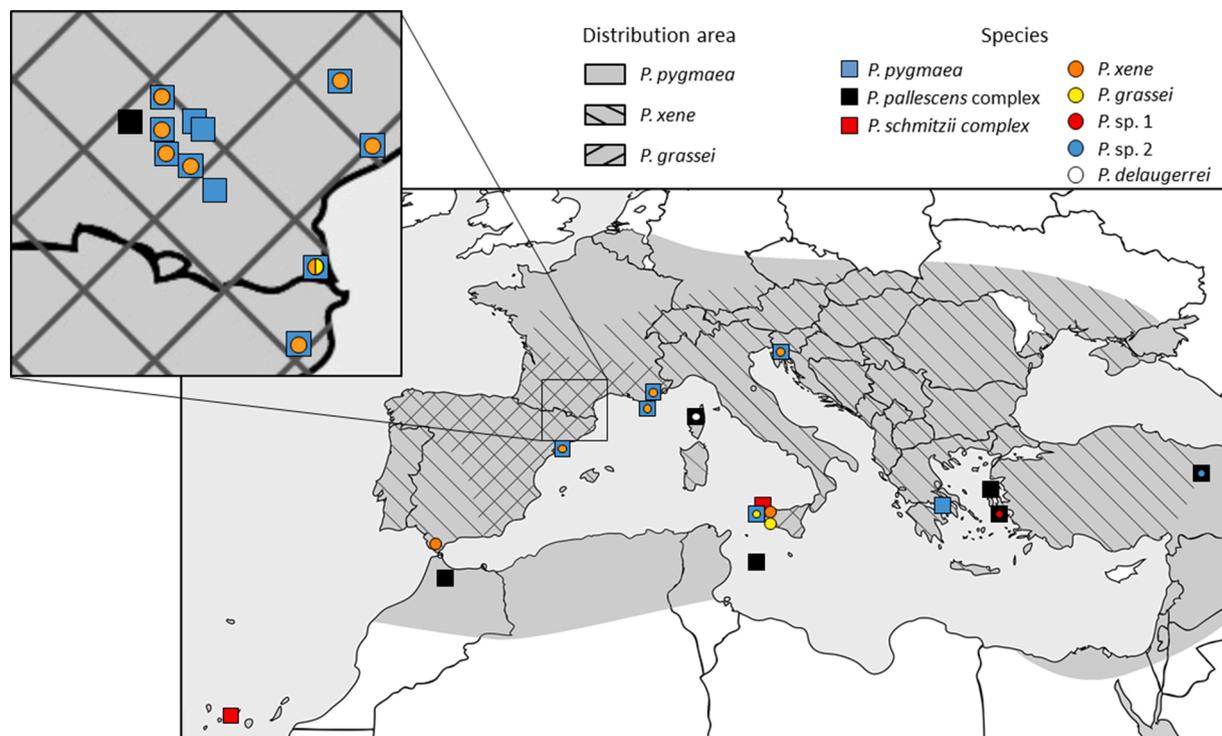


Fig. 1. Distribution area of the free-living host *Plagiolepis pygmaea* (dark grey) and its two inquiline social parasites *P. xene* and *P. grassei* (hatched lines). The sampling locations of the inquilines species (circles) and the free-living species (squares) are indicated. Parasites sampled within host nests are represented by circles within squares. Queens of both *P. xene* and *P. grassei* were found in the same host nest in Cerbère (France) (see inset).

2.2. DNA extraction, amplification and sequencing

DNA was extracted from all individuals using Chelex (Walsh et al., 1991). For each specimen, four nuclear genes (the *Internal Transcribe Spacer*, ITS; and three *Exon Primed Intron Crossing*, EPIC) and one mitochondrial gene (COI) were amplified via PCR using primers designed previously (Table S2; Folmer et al., 1994; Ji et al., 2003; Ströher et al., 2013; Kuhn et al., unpublished). PCR amplifications were conducted in a volume of 25 μ l, using 2 μ l of DNA template, 12.5 μ l of Apex™ Taq DNA Polymerase Master Mix, 0.1 μ l of each forward and reverse primers (100 μ M) and 10.3 μ l of PCR water. Cycling conditions are reported in Table S3.

PCR products were purified with Nucleofast® 96 plates. Sanger sequencing reactions were then conducted in a total volume of 11 μ l with 2.1 μ l of sequencing buffer, 0.2 μ l of forward or reverse primers (10 μ M), 0.6 μ l of Big Dye Terminator Mix, 5.1 μ l of sterilized water and 3 μ l of PCR products. Cycle conditions were as described in the manufacturer protocol. Electrophoresis of sequencing products was achieved on a 3730 DNA Analyzer (Applied Biosystems). All DNA fragments were sequenced in both directions. Not all sequences amplified successfully for some individuals (Table S1).

2.3. Phylogenetic analyses

All sequences were manually verified by examining their associated electropherograms and aligned with the MUSCLE algorithm in Aligner 8.0.2 (CodonCode Corporation, Dedham, MA, USA). The most appropriate model of nucleotide substitution was identified for each gene with the Akaike information criterion corrected for sample size (AICs), as implemented in Jmodeltest v2.1.7 (Darrriba et al., 2012): HKY + G for ITS, EPIC 1087 and EPIC 1281, HKY + I + G for COI and GTR + I for EPIC 1225. We also concatenated all gene sequences in a single alignment. We used PartitionFinder (Lanfear et al., 2012) on this concatenated data set to find the best-fit partitioning scheme and associated nucleotide substitution models. The best partitioning identified with PartitionFinder divided the data set in 5 partitions, each being assigned a separate substitution model (the COI gene was further subdivided into 3 codon positions): (1) COI, codon positions 1, 2 and 3 (GTR + G), (2) ITS (GTR + I + G), (3) EPIC_1087 (GTR + G), (4) EPIC_1225 (GTR + G), and (5) EPIC_1281 (GTR + G).

The phylogeny was then inferred using a Bayesian analysis with the software MrBayes v.3.2.6 (Ronquist et al., 2012) both for each individual gene separately and for the concatenated data set with the following parameters: nucmodel = 4, nruns = 2, nchains = 8 and samplefreq = 1000. Markov chain Monte Carlo included 10 million generations. Output trees from all analyses were visualized with FigTree v1.4.3 (available at <http://tree.bio.ed.ac.uk/software>). As an alternative to analyzing the concatenated data set with MrBayes, we also conducted a *BEAST (Heled and Drummond, 2010) analysis on the combined sequence data, in which the potential incongruence among gene trees is accommodated via a model of coalescence. The analysis was performed with a strict clock model and a Yule tree prior, and was run for 20 million steps while sampling trees and parameters every 5000 steps. For this analysis, species were redefined based on the phylogenetic tree inferred with MrBayes: when sequences defined initially as belonging to the same species (or species complex) were not recovered as a clade on this tree, they were split into two or more separate species (see Supplementary Information for detail). This was justified by our observations that we could not always rely on morphology alone to identify species correctly in the genus *Plagiolepis*, suggesting the existence of cryptic species. Also, since a requirement for a *BEAST analysis is the presence of at least two sequences per species and per locus in the data set, we artificially duplicated the few sequences for which a single sequence was available. A chronogram was also constructed based on the *BEAST phylogeny. Finally, using the inferred *BEAST phylogeny, we estimated the state (free-living or parasitic lifestyle) of each ancestral

node using an asymmetric two-rates model, based on a likelihood criterion, in Mesquite version 3.6. (Maddison and Maddison, 2018). For this analysis, we assumed that the transition from a free-living to a parasitic lifestyle is virtually non-reversible (Cruickshank and Paterson, 2006; Goldberg and Iqic, 2008), and therefore assigned transition rates of 0.9 for “free-living \rightarrow parasite” and of 0.1 for “parasite \rightarrow free-living”.

3. Results and discussion

3.1. Molecular phylogeny

Our phylogenetic analysis relies on a total of 214 sequences. The COI, ITS, EPIC 1087, EPIC 1225 and EPIC 1281 sequence alignments contained 744, 1180, 579, 618 and 519 nucleotides (including gaps), respectively. Of the 3 640 bp, 1 373 sites were variable. Overall, with the exception of the EPIC 1281 gene, the Bayesian phylogenetic trees inferred with MrBayes from each locus separately were mostly compatible with each other and with the phylogeny inferred from all loci combined (Figure S1 and Fig. 2). Nevertheless, a few conflicts did appear. For example, *P. grassei* did not appear as the closest taxa of *P. xene* and *P. pygmaea* in the phylogenetic trees inferred from the EPIC 1087 and ITS genes. In the latter tree, a species of the *P. pallelescens* complex (Samos) and its parasite *P. sp. 1* (Samos) are less phylogenetically related to each other than in the concatenated tree. Also, the phylogeny inferred from the EPIC 1281 gene is overall poorly resolved. Despite some divergences between the trees derived from each locus, our phylogenetic tree inferred from all loci combined is well supported: the majority of nodes that were retained in the final phylogenetic hypothesis (Fig. 2) have a posterior probability of 1, and all remaining nodes have probabilities > 0.90. The species tree inferred with *BEAST is less resolved (and clades are supported with lower posterior probability values) (Figure S2), but fully compatible with the tree of Fig. 2.

The taxonomic status of free-living species of the *P. pallelescens* complex and *P. schmitzii* complex is unclear – neither form a monophyletic group. This confusion likely stems from the difficulty in identifying species based on morphology, and the resulting poor taxonomy of *Plagiolepis* currently available (Seifert, 2018). It may reflect the existence of several cryptic species in the genus (Schulz and Sanetra, 2002; Kirschner et al. in prep., cf Seifert, 2018). The *pallelescens* complex and *schmitzii* complex consist of at least six and four species, respectively (Seifert, 2020; Kirschner et al. in prep.).

3.2. Multiple evolution of inquilinism

The estimated evolution of inquiline social parasitism on the tree inferred from all loci combined (Figs. 2 and S3) suggested that it evolved at least 5 times independently in the genus: in the species *Plagiolepis xene*, *P. grassei*, *P. delaugerrei*, as well as in two unidentified species: *P. sp. 1* from Samos (Greece) and *P. sp. 2* from Sivas (Turkey).

Samples from *P. xene* and from *P. grassei* form each a monophyletic group, with the clade *P. xene* more closely related to *P. pygmaea*, indicating independent evolution of inquilinism in these species. Our data support that *P. grassei* evolved from the free-living ancestor of *P. pygmaea* and that the social parasite *P. xene* later derived independently (Figure S4). This result contrasts with previous works based on morphological and behavioral studies that suggested a more recent evolution of the inquiline *P. grassei* (Passera, 1969). Size reduction of reproductives is part of the inquiline syndrome (Bourke and Franks, 1991). It has been suggested that reduced size evolved as a means to take advantage of the host's caste-determination system, so that parasite larvae develop into sexuals with less food than is required to produce host workers (Bourke and Franks, 1991). Miniaturization would also allow parasites to produce sexuals even when the host actively prevents production of its own sexuals (Aron et al., 1999; Aron et al., 2004). Queens of *P. grassei* are indeed smaller than queens of its host *P. pygmaea*, but still significantly larger than queens of *P. xene* (Passera,



Fig. 2. Phylogenetic relationships between inquiline social parasites and free-living host species in the ant genus *Plagiolepis* inferred in a Bayesian framework with MrBayes. Values along branches are posterior probabilities. Red branches indicate inquiline lifestyle. Inquiline parasites are shown in grey. Dashed lines link the parasite and their host. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1969). Furthermore, another inquiline syndrome is the lack of worker production. *P. grassei* still produces a handful of workers, whereas *P. xene* is completely workerless and produces exclusively sexual offspring. These observations led to the assumption that *P. grassei* is less adapted to the inquiline lifestyle compared to *P. xene* and, therefore, represents an intermediate stage to social parasitism (Passera, 1969).

Our phylogenetic analyses reveal another independent evolution of the inquiline parasitism in the species *P. delaugerrei*, which derives directly from a free-living species of the *P. pallescens* complex. This evolution seems recent (Figure S4) and localized to Corsica. As for the two unidentified social parasites *P. sp. 1* and *P. sp. 2*, they were also sampled in nests of a host of the *P. pallescens* complex. Both species

display the traits of the inquiline syndrome: (i) they were found in a host nest, (ii) they were not aggressed by the host individuals, (iii) queens are much smaller compared to host queens and comparable to host worker size, and (iv) they seem to produce no workers (H. C. Wagner and K. Kiran, unpublished data). These social parasites do not form a monophyletic group; they evolved from different ancestral free-living hosts of the *P. pallescens* complex. In Greece, the only inquiline parasite identified to date is *P. xene* (Borowiec and Salata, 2012; Bračko et al., 2016). Clearly, *P. sp. 1* does not belong to the *P. xene* clade and gynes differ morphologically from *P. ampeloni*; it could therefore correspond to a hitherto undescribed *Plagiolepis* inquiline social parasite. As for the inquiline *P. sp. 2* sampled in Sivas, it could be *P. ampeloni*, an inquiline

parasite of *P. pallescens* that was previously found in Turkey (Faber, 1969; Heinze and Kauffmann, 1993). This needs to be clarified through future morphological and genetic studies.

3.3. Emery's rule

Consistent with the strict Emery's rule, our results show that all inquiline parasite species surveyed in this study are associated with one of their closest host relatives, rejecting the random distribution and the loose form (Fig. 2). Sympatric speciation is therefore the most parsimonious explanation for the evolution of inquilinism in these species. A firm confirmation of sympatric speciation requires, however, demonstration that the host and parasite derived from sister species that (i) exchanged genes freely prior to speciation, (ii) were not allopatric at the time of speciation, (iii) exist in sympatry and (iv) are currently reproductively isolated (Rabeling et al. 2014). Emergence of parasitism is obviously more recent in some cases (*P. delaugerrei*, *P. sp. 2*) than others (e.g., *P. sp. 1*, *P. grassei*; Figure S4). The emergence of *P. sp. 1* for example likely occurred in the ancestral lineage leading to the clade of the *P. pallescens* complex from Greece. The repeated evolution of the inquiline syndrome in the ant genus *Plagiolepis* may have been promoted by polygyny (i.e., the occurrence of multiple reproductive queens in a colony), a characteristic of the hosts *P. pygmaea* and *P. pallescens* (Passera, 1969; Salata and Borowiec, 2018). Polygyny is indeed thought to be a prerequisite for the evolution of inquiline social parasitism in social Hymenoptera (Bourke and Franks, 1991; Buschinger, 2009; Boomsma and Nash 2015). In multiple-queen colonies, inquiline parasitism could evolve from mutant 'selfish' queens specializing in the production of sexual offspring at the expense of other queens producing the non-reproductive workforce. Selfish queens could then become reproductively isolated through temporal or spatial differences in mating behavior possibly due to their reduced size (Buschinger, 1990). Inquiline parasites may then insinuate themselves into new colonies and reproduce by thwarting the recognition mechanisms of host workers (Lenoir et al., 2001; Guillem et al., 2014). Such an evolutionary pathway has been documented in the red ant *Myrmica rubra*, where both large queens (macrogyne) and small queens (microgyne) can co-exist in a single nest. Macrogyne produce new macrogyne and workers, whereas microgyne produce mostly microgyne but few or no workers (Pearson, 1981). On average, microgyne give birth to nearly forty times more new reproductive queens than macrogyne. In line with an advanced sympatric speciation of the inquiline parasitism, there is high genetic divergence between the two queen morphs (Leppänen et al., 2015). A sympatric speciation in progress was also reported in *Mycocetopus* fungus-growing ants, where the inquiline *M. castrator* and its host are sister-species without gene-flow but have exchanged genes in the past and have speciated without being allopatric (Rabeling et al., 2014).

3.4. Conclusion

Our study shows multiple and independent evolution of inquiline social parasitism in the ant genus *Plagiolepis* in Europe. Furthermore, all phylogenetic relationships inferred between the studied parasites and their host are compatible with an emergence of the parasitic lifestyle through sympatric speciation, which shows that a close phylogenetic relationship to its host greatly facilitates the evolution of inquiline social parasitism in ants and, possibly, other eusocial insects.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ympev.2020.107016>.

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