Research article

Six origins of slavery in formicoxenine ants

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Summary. Slave-making (dulotic) ants have long fascinated biologists because of their intriguing behavior and highly specialized lifestyle. Dulosis evolved convergently several times within the two ant subfamilies Myrmicinae and Formicinae. Here, we demonstrate that it originated at least six times independently within the small myrmicine tribe Formicoxenini alone. Our phylogenetic trees, based on 1386 base pairs of the mitochondrial cytochrome oxidase gene, document different degrees of genetic divergence between different monophyla of slave-makers and their host species, which suggests that they evolved from non-parasitic Formicoxenini at different times. Two nearctic slave-makers, *Temnothorax duloticus* and a new species still to be formally described, appear to be of particularly recent origin. In contrast, the other parasitic monophyla clearly diverged much earlier from their non-parasitic ancestors and have a much longer evolutionary history.

Key words: Social parasitism, slave-making ants, phylogeny, dulosis, Formicoxenini.

Introduction

Slave-making in ants (dulosis) has fascinated scientists and the public ever since its first detailed description almost 200 years ago (Huber, 1810). For example, Charles Darwin dedicated several pages of his groundbreaking book 'On the origin of species' to the complex behavior of slave-making ants during slave raids (Darwin, 1859). However, despite much debate, the evolutionary origins of slave-making are still not well understood.

Slave-makers are socially parasitic ants, which permanently depend on the help of already established colonies of related ant species for all tasks in the nest (Buschinger, 1986; Hölldobler and Wilson, 1990; Stuart, 2002). Their colonies are predominantly initiated in summer by young, mated slave-maker queens, which usurp a host colony by killing or expelling the resident queen and, in some genera, also all adult workers. The first host workers to emerge from the conquered brood then care for the slave-maker queen and her offspring. In subsequent years, the slave-maker workers produced by the queen attack neighboring host nests and capture their pupae in order to replenish the stock of host workers.

Slavery is believed to have evolved independently at least nine times within two of the 21 known ant subfamilies, the Formicinae and the Myrmicinae (Buschinger, 1986; Hölldobler and Wilson, 1990; Stuart, 2002). The small myrmicine tribe Formicoxenini appears to be a particular hot spot in slave-maker evolution: five independent origins of slave-making ants (*Chalepoxenus* spp.; *Harpagoxenus* spp.; *Myrmoxenus* spp. – formerly *Epimyrma*, Schulz and Sanetra, 2002; *Protomognathus americanus*; *Temnothorax duloticus*, formerly *Leptothorax duloticus*, Bolton, 2003) have previously been identified (Buschinger, 1990; Baur et al., 1995). All these taxa occur throughout temperate and boreal habitats of Eastern North America, Eurasia, the Mediterranean and North Africa and parasitize various *Temnothorax* and *Leptothorax* species (Buschinger, 1986, 1990; Hölldobler and Wilson, 1990).

The different lineages of slave-making Formicoxenini exhibit strikingly different degrees of species richness. For example, whereas *Myrmoxenus* comprises eight or more morphologically similar species, suggesting an adaptive radiation, other monophyla, such as *Protomognathus americanus*, consist of only a single taxon. One reasonable hypothesis for this varying diversity is that different lineages evolved at different times in the past, with old lineages being richer in species than young lineages. Our attempt to quantify variation within extant slave-maker species and their host species revealed a sixth independent origin of slavery in a yet undescribed nearctic *Temnothorax* ant. This species shares its host

species *T. longispinosus* and *T. ambiguus* with *P. americanus* and *T. duloticus*, but, as we show here, is clearly distinct from the other nearctic social parasites.

Methods

Species of all presently known lineages of socially parasitic Formicoxenini and their dominant host species were collected in Europe and North America. In addition, we included the workerless inquiline *T. minutissimus* (Buschinger and Linksvayer, 2004). In Table 1, the

Table 1. Social parasites and their host species. *T. minutissimus* is not a slave-making ant, but a workerless inquiline parasite

Parasite species	Host species
Chalepoxenus muellerianus	*Temnothorax unifasciatus*
Myrmoxenus ravouxi	*Temnothorax nigriceps*
Harpagoxenus sublaevis	*Leptothorax acervorum*
Harpagoxenus canadensis	*Leptothorax canadensis*
Protomognathus americanus	*Temnothorax longispinosus*
	Temnothorax curvispinosus
	Temnothorax ambiguus
Temnothorax sp.	*Temnothorax longispinosus*
	Temnothorax ambiguus
Temnothorax duloticus	*Temnothorax longispinosus*
	Temnothorax curvispinosus
	Temnothorax ambiguus
Temnothorax minutissimus	*Temnothorax curvispinosus*

social parasites and their respective host species are listed. Genus names correspond to Bolton's new classification of Formicidae (Bolton, 2003). Details on collection sites are given in Table 2.

High molecular weight DNA was extracted from individual, frozen, female ants by grinding them in liquid nitrogen and subsequently following a cetyltrimethyl ammonium bromide protocol (Hamaguchi et al., 1993). We amplified a 1430 bp fragment of the subunits I and II of the mitochondrial gene cytochrome oxidase (CO I/II) using the primers C1-J-2195 and C2-N-3661 (Simon et al., 1994) and three primers designed by M. Brandt, B. Fischer-Blass, and A. Trindl: MIBI (5′-AGA TTT ATT CAC TGA TTC CC-3′), CW-3031rev (5′-TTT GCM CTW ATC TGC CMT ATT-3′) and CO1-516 for (5′-ATT TTT YTC TAT ATT TAT YGG A-3′). The 25 µl PCR reaction mixture contained 1 to 50 ng DNA, 2.5 µl 10 × polymerase buffer (without MgCl$_2$), 2.8 mM MgCl$_2$, 1.4 µM of each primer, 400 µM of each dNTP and 1 unit of *Taq* polymerase (MBI Fermentas). DNA was amplified with a Biometra T1 Thermocycler with the following temperature profile: 4 min at 94 °C, 41 cycles of 94 °C for 1.15 min, 50 °C for 1.15 min, 68 °C for 1.30–2.30 min, and a final extension at 72 °C for 5 min. PCR products were either purified from agarose gels after separation by electrophoresis for 45 min at 100 mA, using NucleoSpin® Extract columns (Macherey-Nagel), or directly using Montage™PCR Centrifugal Filter Devices (Millipore). The 20 µl cycle sequencing reaction mixture contained 20–100 ng DNA, 3 µl 5 × sequencing buffer, 0.5 µM primer and 2 µl ready mix (Big dye Terminator Cycle sequencing kit, Applied Biosystems). Both strands were sequenced on an ABI Prism 310 genetic analyzer.

The 27 sequences were compiled, edited and aligned in the program Bioedit 5.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html, Hall, 1999) and by eye. Two tree-building methods were used to reconstruct the phylogeny of the formicoxenine slave-makers and their host species, with an ant from the same tribe, *Cardiocondyla mauritanica*, serving as outgroup. Both phylogenetic trees are based on 1386 bp of the CO I/II sequence (789 bp CO I coding region, 54 bp intergenic region with a leucine tRNA motif, 543 bp CO II coding region). Eight to 13 bp in the non-coding region could not be aligned and were removed in frame. Three double peaks and four missing bases at the end of single sequences

Table 2. Overview of the sampled specimens, their collection sites and the GenBank accession numbers. In all instances, we sequenced the gene COI/COII, with the interjacent leucine tRNA. *Temnothorax* (*T.*) and *Leptothorax* (*L.*) host species were sampled from the same communities as their parasites. Some slave-maker species have a broader distribution than their host species and consequently co-occur with various hosts at different sites

Species	Locality	Accession Number
Chalepoxenus muellerianus	Savoillan, Provence, France (F)	AY909573
Myrmoxenus ravouxi	Waldenhausen, Bavaria, Germany (D)	AY909575
Harpagoxenus sublaevis	Abensberg, Bavaria, Germany (D)	AY754680
Harpagoxenus canadensis	Tadoussac, Quebec, Canada (CN)	AY909574
Protomognathus americanus	Huyck Preserve, New York, USA	AY754775
Protomognathus americanus	Harpersfield, Ohio, USA	AY754785
T. sp.	Huyck Preserve, New York, USA	AY909557
T. sp.	Huyck Preserve, New York, USA	AY909558
T. sp.	Huyck Preserve, New York, USA	AY909559
T. duloticus	Columbus, Ohio, USA	AY909560
T. duloticus	Columbus, Ohio, USA	AY909561
T. duloticus	Columbus, Ohio, USA	AY909562
T. duloticus	Columbus, Ohio, USA	AY909563
T. minutissimus	Bloomington, Indiana, USA	AY909564
T. minutissimus	Watoga State Park, West Virginia, USA	AY909566
T. minutissimus	Columbus, Ohio, USA	AY909565
T. unifasciatus	Savoillan, Provence, France (F)	AY909570
T. nigriceps	Waldenhausen, Bavaria, Germany (D)	AY909567
L. acervorum	Abensberg, Bavaria, Germany (D)	AY909571
L. canadensis	Tadoussac, Quebec, Canada (CN)	AY909572
T. longispinosus	Huyck Preserve, New York, USA	AY754805
T. longispinosus	Harpersfield, Ohio, USA	AY754798
T. longispinosus	Watoga State Park, West Virginia, USA	AY754797
T. ambiguus	Huyck Preserve, New York, USA	AY909568
T. curvispinosus	Harpersfield, Ohio, USA	AY754754
T. curvispinosus	Watoga State Park, West Virginia, USA	AY909569
Cardiocondyla mauritanica	La Gomera, Spain (E)	AY909576

were substituted by "N"s. The sequences used in the final analysis were without gaps, frameshifts, unexpected stop codons, insertions, deletions, or rearrangements. We also did not find any evidence of pseudogenes or gene duplications. The AT content of the selected region was 71.4%. The sequences comprised 551 variable sites, of which 457 were parsimony-informative (DnaSP 4.00.5, Rozas et al., 2004). We found 501 transitions and 288 transversions. Sequences are deposited in the Gen-Bank database under accession numbers shown in Table 2.

Maximum parsimony analysis was conducted using the program PAUP 4.0 (Swofford, 2000) at default settings in a heuristic search to find the most parsimonious tree. Deviating from these settings we used a random addition sequence with ten replications and the specification that the ingroup was monophyletic. We found a single tree and support for individual branches were assessed by 2000 bootstrap replicates. A Bayesian tree was generated using MRBAYES 3.0b3 (Huelsenbeck, 2000) with the general time reversible model with invariable sites and γ-distribution (GTR + I + G), calculated by Mr.Modeltest as included in the MRBAYES program. We used this model in the in MRBAYES implemented Monte Carlo algorithm with four Markov chains over two million generations, generating a tree each 500 generations. We excluded all trees generated within the first 10,000 generations before the chains converged to a stable value.

For scanning electron microscopy, specimens were fixed, washed in distilled water, dehydrated through a graded ethanol series, dried, coated with gold-palladium and examined in a Zeiss DSM950 scanning electron microscope.

Results

Assuming that slave-making is a derived state, both phylogenetic trees shown in Figs. 1A and 1B clearly show six independent origins of slavery in the Formicoxenini. The inquiline *T. minutissimus* forms a lineage separate from the other studied social parasites. Our research therefore supports previous claims that slave-making evolved convergently in *Chalepoxenus*, *Harpagoxenus*, *Myrmoxenus*, *P. americanus* and *T. duloticus* and beyond that reveals a sixth slave-making lineage, an as yet undescribed, nearctic *Temnothorax* parasitizing *T. longispinosus* and *T. ambiguus.*

Palearctic *H. sublaevis* and nearctic *H. canadensis* are presumably sister taxa, although branch lengths representing 127 mutations (9.2% sequence divergence) suggest an early separation and a long independent history of these two species, and *H. sublaevis* is grouped with *Leptothorax* in the Bayesian consensus tree. *Harpagoxenus* forms a robust monophyletic group with their host species *L. acervorum* and *L. canadensis* (bootstrap support value 100, posterior probability 1.00).

Similarly, the nearctic parasites of *Temnothorax* and their hosts form a rather well supported monophylum (bootstrap support value 86, posterior probability 0.99). However, the four social parasites show very different divergence patterns. The long branch length and high intraspecific variability of *P. americanus* suggests a long, independent history, with its closest living relative being yet unknown. The two other nearctic slave-makers clearly cluster with one of their respective host species each and apparently have split from it or a related species much more recently. *T. duloticus* is close to *T. curvispinosus*, and both form a well-supported monophyletic group with the inquiline *T. minutissimus* (bootstrap support value 96, posterior probability 0.97).

The presently undescribed slave-maker, *Temnothorax* sp., is very close to *T. longispinosus* (bootstrap support value 92, posterior probability 1.00). It differs from *T. duloticus* in its larger and more pointed ventral postpetiolar sternite, its shorter and more robust epinotal spines and its more triangular mandibles (Fig. 2). The new species will be described in detail by R.J. Stuart and S.P. Cover.

Discussion

Our analysis revealed six independent evolutionary origins of slavery within the Formicoxenini, which is one more than previously thought. The very robust branching pattern of our phylogenetic tree suggests three convergent origins within the North American *Temnothorax* alone. The slave-makers *T. duloticus* and *T.* sp. cluster with their host species, which suggests a close phylogenetic relationship and a recent evolution from non-parasitic *Temnothorax* ancestors. This is in conflict with earlier suggestions that *T. duloticus* is a close relative of *Leptothorax muscorum* (Wesson, 1937; Wilson, 1975) but supports conclusions from a previous allozyme study (Heinze, 1991) and justifies the recent transfer of this species into the genus *Temnothorax* (Buschinger, 1990; Bolton, 2003).

Phylogenetic relationships between social parasites and their host(s) have been long debated. According to Emery (1909), social parasites are typically closely related to their hosts, with both species evolving from a common ancestor. In the strict form of Emery's rule, each parasite is the sister species of its host, whereas, in its looser version, the non-parasitic clade most closely related to the parasite contains all the parasite's host species and parasites can radiate to less related host species (Buschinger, 1990). Too few of the hundreds of *Temnothorax* species have been investigated to determine whether the evolution of socially parasitic Formicoxenini matches Emery's rule, and it is also not our aim to do so. Nevertheless, our data support previous reports on a more or less close relationship between parasites and hosts (Buschinger, 1990; Heinze, 1991, 1995; Baur et al., 1995). For example, *Harpagoxenus* forms a well-supported monophylum with its *Leptothorax* hosts. Though *H. canadensis* constitutes the outgroup to *H. sublaevis* and *Leptothorax* in the Bayesian analysis, the two *Harpagoxenus* species clearly form a monophylum in the maximum parsimony analysis and in an earlier tree based on enzyme electromorphs (Heinze, 1995). Monophyly is also suggested by morphology and karyotype (Buschinger and Alloway, 1978, 1979; Fischer, 1987; Buschinger, 1990).

A close relationship between parasite and host is also suggested for *T. duloticus* and the new dulotic *Temnothorax* species, among whose hosts are what seem to be their closest free-living relatives. The comparatively short branch lengths suggest a recent evolution of both species and also *T. minutissimus* from the clade to which their host species belongs. In contrast, *P. americanus* appears to be phylogenetically old and only distantly related to its present hosts. Similarly, the palearctic, species-rich genera *Myrmoxenus* and *Chalepox-*
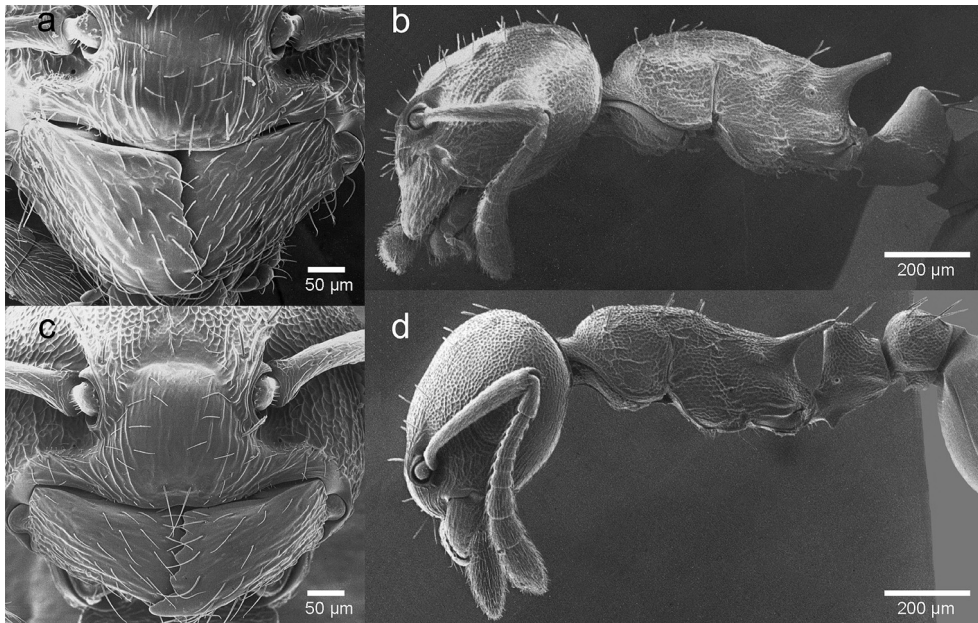
Figure 1. A. Bootstrap 55% majority-rule consensus tree for the ant taxa based on 1386 base pairs of the mitochondrial gene cytochrome oxidase I and II as calculated by PAUP 4.0 by heuristic search. Support for the branches are based on 2000 bootstrap replicates. Parasitic taxa are shown in bold. Parasites and their respective host(s) are marked with the same symbol. 1B. Majority rule consensus tree of 3980 Bayesian trees. Numbers above branches represent the posterior probability that the clade is correct given the model of evolution

Figure 2. Scanning electron microscope pictures showing morphological differences between the mandibles and the front part of a worker from *Temnothorax* sp. (a, b) and *T. duloticus* (c, d)

enus are all well separated from their *Temnothorax* hosts and their long branch lengths suggest a long independent evolutionary history (Buschinger, 1990; see also Baur et al., 1995).

Though *T.* sp. and *T. duloticus* are morphologically very similar, they differ in their geographic range and slave-raiding behavior. *T.* sp. was first discovered by R.J. Stuart in Southern Ontario and has subsequently been collected in Michigan, Vermont, and New York (Alloway, 1997; Herbers and Foitzik, 2002; R.J. Stuart, unpubl.), while *T. duloticus* occurs in Illinois, Ohio, and Michigan. The more northeastern distribution of *T.* sp. is also reflected in its usage of *T. ambiguus* as a second, and in some places even the main host. While *T. duloticus* conducts processions during slave raids (Alloway, 1979; Buschinger, 1986), scouts of *T.* sp. recruit nestmates by tandem running (R.J. Stuart, unpubl.). Colonies of *T.* sp. appear to be highly polydomous, because most nests are queenless and many contain less than six slave-maker workers (R.J. Stuart, unpubl.).

The small tribe Formicoxenini, with less than 4% of the presently known ant species, contains 60% of all independent origins of slavery. In addition, a number of inquilines (Buschinger, 1982, 1990; Heinze, 1989a; Heinze and Alloway, 1991), as well as the guest ant genus *Formicoxenus* (Francoeur et al., 1985) all have arisen in this group. Why are the Formicoxenini such a peculiar hot spot in the evolution of social parasitism? Three different behavioral traits have been suggested as starting points in the evolution of slave-making: predation on other ant species (Darwin, 1859), territoriality (Alloway, 1980; Stuart and Alloway, 1982, 1983), and polygyny, usually combined with polydomy (Buschinger, 1970, 1990; for recent reviews see D'Ettorre and Heinze, 2001; Stuart, 2002). The evolution of slavery from specialized predation on ant larvae for food is not likely in the Formicoxenini as they are food generalists and do not prey on other ants. Territorial disputes lead to raiding and intraspecific slavery

in *Myrmecocystus* (Hölldobler, 1976; Kronauer et al., 2003), and similar results have been obtained in laboratory studies with *Temnothorax* (Alloway, 1980; Alloway et al., 1991; Stuart and Alloway, 1982). However, formicoxenine ants typically do not engage in fights with neighboring colonies in the field (Dobrzañski 1965, 1966; Heinze et al., 1996), and molecular analyses have not revealed the genetic heterogeneity that should result from frequent intraspecific slavery (Foitzik and Herbers, 2001; Foitzik et al., 2004). Nevertheless, the fact that some sort of slave raiding can be elicited documents that the repertoire for this behavior is present also in non-parasitic species.

Finally, polygyny and polydomy were suggested as starting points for the evolution of slave-making (Wasmann, 1909; Buschinger, 1970, 1990; Elmes, 1973, 1978; Bourke and Franks, 1991). Parasitic colony founding might have originated from the return of newly mated young queens into their maternal colonies, and slave-raiding might have evolved from brood transport. Though the transport of brood between neighboring nests of a polydomous colony resembles brood transport during slave-raids (Buschinger, 1986; 1990), it completely lacks the scouting and fighting behavior exhibited by slave-maker workers. And although the host species of *Harpagoxenus*, *T. duloticus*, *T.* sp. and *P. americanus* are all facultatively polygynous, most *Temnothorax* hosts of *Chalepoxenus* and *Myrmoxenus* are monogynous and monodomous (see also D'Ettorre and Heinze, 2001). However, it must be recognized that the latter often live in ephemeral nests and might be frequently forced to move and transport brood.

Other aspects of the behavioral ecology and population biology of the Formicoxenini might therefore have contributed to the evolution of slavery in this group. Slave-makers can only be successful when the nests of their hosts are relatively dense and cannot easily be defended (Alloway

et al., 1982; D'Ettorre and Heinze, 2001; Stuart, 2002). This certainly applies to formicoxenine ants more than to many other ant taxa. *Leptothorax* and *Temnothorax* are among the most common ants in boreal and temperate forests, locally reaching densities of 10 nests/m² and more, and their colonies typically contain only a few dozen individuals and nest in often fragile, preformed cavities in wood and nuts or under stones. The large effective population sizes and the ubiquity of nesting sites have probably facilitated the high rate of diversification and speciation in Formicoxenini. There are several hundred *Temnothorax* species (Schulz and Verhaagh, 1999; MacKay, 2000; Bolton, 2003), and the diversity of *Leptothorax* is presumably also considerably higher than previously thought, with numerous nearctic taxa still unnamed (Heinze, 1989b) and several Eurasian taxa only recently recognized as valid species (Heinze et al., 1993; Radchenko and Heinze, 1997). The occurrence of large numbers of closely related sympatric species with asymmetries in their fighting abilities and in their discrimination abilities between nestmates and non-nestmates might similarly have facilitated the repeated evolution of slavery (Alloway, 1997; Stuart, 1988, 1993, 2002).

Detailed investigations of the behavior and ecology of non-parasitic formicoxenine species and slave-makers at an early stage in their parasitic evolution, such as *T. duloticus* and *T.* sp., might shed light on the evolutionary origin and development of slave-making in this fascinating group of ants.

Acknowledgements

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