Molecular Phylogenetics and Evolution 56 (2010) 294–304

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Phylogeny, divergence-time estimation, biogeography and social parasite–host relationships of the Holarctic ant genus *Myrmica* (Hymenoptera: Formicidae)

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ARTICLE INFO

Article history:
Received 29 October 2009
Revised 26 January 2010
Accepted 27 January 2010
Available online 14 February 2010

Keywords:
Emery's rule
Inquilines
Molecular phylogeny
Nearctic
Palearctic
Social parasitism
Temporary social parasites

ABSTRACT

We reconstructed a molecular phylogeny of the ant genus *Myrmica*, tested reciprocal monophyly of the Nearctic and Palearctic representatives, and inferred social parasite–host relationships for five workerless inquilines and four temporary parasites. We sequenced six gene fragments of 106 specimens (17 not identified to species), analysed the data with Bayesian phylogenetic inference and maximum likelihood, and estimated divergence times using penalized likelihood. Our well resolved phylogeny supported most morphologically defined species groups. The Nearctic and Palearctic species were not reciprocally monophyletic, which suggested repeated species interchange across the Beringian land bridge. Parasitism evolved several times in *Myrmica*. Three inquilines and one temporary parasite were closest relatives of their host, two inquiline species and one temporary parasite clustered basally to their host(s), and two temporary parasites more distantly. *Myrmica* probably diversified following drastic climatic cooling at the Eocene–Oligocene boundary ca. 34 Ma, the oldest species groups being rugosa and ritae in central and southeastern Asia. The oldest inquiline, *Myrmica karavajevi*, was estimated at 17 Ma, the youngest species *M. hirsuta* at 0.8 Ma, whereas the microgyne of *M. rubra* is an intraspecific inquiline.

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1. Introduction

1.1. The genus Myrmica

Ants play a prominent role in most terrestrial ecosystems. Many species reach locally high numbers, and thus profoundly influence ecological communities through predation, seed dispersal, soil turnover and a plethora of symbiotic relationships (Hölldobler and Wilson, 1990). In boreal and temperate regions, ant assemblages usually include one or several species of the genera *Formica*, *Lasius, Camponotus, Leptothorax* and *Myrmica* (Dlusskij, 1981; Francoeur, 1983; Savolainen et al., 1989; Gallé, 1991; Heinze, 1993; Punttila et al., 1996; Maes et al., 2003; Czechowski and Czechowska, 2006; Palladini et al., 2007). *Myrmica* are generalist zoophages and trophobionts, foraging mainly above ground (Arnoldi, 1968), and their omnipresence is seen in their relatively high species diversity and ecological versatility.

Myrmica belong to the largest ant subfamily, Myrmicinae, and are the closest relatives of *Manica* (Brady et al., 2006; Astruc et al., 2004). Currently about 200 species are known (Bolton et al., 2006; Elmes et al., 2008; Elmes and Radchenko, 2009; Radchenko and Elmes, 2009a,b,c; Radchenko et al., 2008a,b). The Palearctic distribution of the genus extends from Siberia to Vietnam, Southern China and Taiwan, and from Western Europe to the Far East (Elmes and Radchenko, 1998; Radchenko, 1995a; Radchenko and Elmes, 2001a). In North America, *Myrmica* range from the northern tree line in Canada to the highlands of Mexico (Weber, 1947). They appear in diverse habitats, including forests, bogs, meadows and human-altered landscapes. Most species are adapted to cool conditions, with the exception of a few xerophilous steppe forms (Arnoldi, 1968). In the southern parts of their distribution, *Myrmica* are confined to high altitudes.

Colonies of *Myrmica* may be found in the ground, frequently under stones and pieces of wood, under moss, in tussocks of grass, and in the litter (Czechowski et al., 2002). Nests are usually small (200–1500 individuals; Seifert, 2007), and contain a varying number of functional queens (Elmes and Petal, 1990). Intraspecific competition for nest sites may be strong (Czechowski, 1984), and some *Myrmica* may also take over nest sites of other ant species (Czechowski, 1985). Nevertheless, they are mostly submissive in interactions with other ants, which allows them to coexist with aggressive, territorial species (Vepsäläinen and Savolainen, 1990).

The taxonomy of *Myrmica* is well understood in the Palearctic (Seifert, 1988, 2003, 2005, 2007; Radchenko, 1995a,b,c,d; Radchenko and Elmes, 1998, 2001b, 2003, 2004; Radchenko et al., 2006, 2007). Illustrated guides with identification keys are avail-

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doi:10.1016/j.ympev.2010.01.029

able mainly for the species of Central Europe (Czechowski et al., 2002; Seifert, 2007), and a key for the Palearctic taxa is provided by Radchenko (1995e). The Palearctic species are classified into species groups, informal sets of species which resemble each other morphologically (Radchenko and Elmes, 2001b). This study aims to test the monophyly of the species groups with molecular-phylogenetic data.

In contrast to the situation in the Palearctic, the taxonomy of Nearctic species has not been studied since Weber (1947, 1948, 1950), who summarised the Palearctic *Myrmica* known at the time and revised the Nearctic forms. He simplified the taxonomy by relinquishing quadrinomens, but continued the tradition that regarded North American forms as variations of European species. Creighton (1950) tackled the entire Nearctic ant fauna, including *Myrmica*, but in less detail. More recently, Francoeur (1981, 2007) established three species groups and described five new species. Jansen et al. (2009), using the DNA barcoding method, established additional tentative species groups, but could not delineate species. Other publications are local ant lists and keys that usually review the available information (Gregg, 1963; Wheeler and Wheeler, 1986; Ward, 2005). Hence, the taxonomy of Nearctic *Myrmica* remains wanting and species identification problematic.

1.2. Social parasitism

The genus *Myrmica* includes a number of social parasites. The term parasite is usually associated with viruses, bacteria, fungi, mites and other small organisms living in or on their victim. Social parasites, however, do not target single individuals. Instead, they capitalise on the social accomplishments of other species (Buschinger, 1986). Exploiting the behaviour and organisation of the host society, they take advantage of nurture, protection and brood care. Social parasites are found in diverse insect taxa, including aphids (Miller and Crespi, 2003), beetles (Geiselhardt et al., 2007), butterflies (Pierce et al., 2002), hoverflies (Elmes et al., 1999), allodapine bees (Smith et al., 2007), wasps (Carpenter et al., 1993; Arévalo et al., 2004), and ants (Hölldobler and Wilson, 1990).

In the ants, social parasitism may take three forms (Buschinger, 1986; Hölldobler and Wilson, 1990)—here, we follow Emery (1909) and do not classify guest ants as parasites. First, the queens of temporary parasitic species found a colony by usurping a hetero- or homospecific colony and replacing its queen. The orphaned host workers then raise the parasite brood, until they die out and the intruder's colony continues independently. Second, enslaving or dulotic species raid colonies of other species and transport the brood to their own nest. The abducted brood then assume the colony tasks in the parasite nest, whereas the parasite workers usually refrain from such activities. Third, inquilines are functionally workerless parasites that live inside the colony of another species. Their eggs usually develop exclusively into sexuals, whereas the host queen produces mostly workers. Thus inquilines are obligate parasites, fully dependent on their host. On the other hand, temporary parasites and dulotic species may, depending on species, be facultative or obligate parasites.

At least 230 of the 12,567 described ant species are known to be socially parasitic (Agosti and Johnson, 2009; Buschinger, 2009). This is almost certainly an underestimation, as many social parasites are rare and never abundant. Known socially parasitic species are concentrated in the two largest subfamilies, Formicinae (ca. 80 of ca. 3000 species) and Myrmicinae (ca. 110 of ca. 6150 species) (slightly outdated numbers of parasitic species from Hölldobler and Wilson (1990); total numbers of species from Agosti and Johnson (2009). In both subfamilies, the parasites occur in a limited number of genera. In Formicinae, they are mainly found in *Formica*, *Lasius, Plagiolepis* and *Polyergus*. In Myrmicinae, they chiefly belong to *Leptothorax*, *Monomorium*, *Myrmoxenus*, *Strongylognathus,* and *Myrmica* (Hölldobler and Wilson, 1990).

Myrmica has many inquilines and several temporary social parasites that infest other species of the genus (see Table 1 for an overview). Inquilines are characterised by a set of adaptations to a parasitic life style, termed the inquiline syndrome (Wilson, 1971). The altered characters may, among others, include reduced body size, loss of worker caste, broadened postpetiole, often with a ventral projection, reduced sculpture, lack of teeth on the mandibles, hairiness, and simple spurs on the middle and hind tibiae (instead of the pectinate spurs typical for *Myrmica*). Details on the morphology of each parasitic species may be found in a review of socially parasitic *Myrmica* (Radchenko and Elmes, 2003). For basic information on the parasites and their hosts, see Table 1.

Here we reconstruct the phylogeny of *Myrmica* using multi-locus DNA sequence data. First, we will test the reciprocal monophyly of the Nearctic and Palearctic *Myrmica* species. Second, we will test the species group concept based on morphological characters (Francoeur, 1981, 2007; Seifert, 1988, 2003; Radchenko, 1995a). Third, we will investigate the relationships between socially parasitic *Myrmica* and their hosts. Fourth, we will use divergence-time estimates to compare the ages of the social parasites and discuss possible biogeographical scenarios for the diversification of *Myrmica*.

2. Material and methods

2.1. Data collection and molecular protocols

We collected colony samples of *Myrmica* over its distributional range (see Supplementary Table A). We collected at least ten individuals per colony in 95% ethanol and kept them at 4 °C. We received additional samples from several colleagues (see Acknowledgements and Supplementary Table A). Voucher specimens of each sample are kept in the collection of R. Savolainen, University of Helsinki; for voucher identification codes, please see Supplementary Table A. When referring to species groups named by a species of that group, we use non-italicised names to distinguish them from species names in italics.

We crushed whole ants in liquid nitrogen and used Proteinase K (Fermentas, St. Leon-Roth, Germany) to digest cells at 60 °C overnight. We then extracted genomic DNA using the Nucleo Spin Tissue kit (Macherey–Nagel). We amplified the following gene fragments using specific primers for each gene (Table 2): mitochondrial *Cytochrome Oxidase* subunit I (*COI*) and *Cytochrome b* (*Cytb*), ribosomal 28S extension region 2 (28S), nuclear *Arginine Kinase* (*ArgK*) exons 1 and 2, the F1 copy of *Elongation Factor 1 alpha* (*EF-1α*), and *Long Wavelength Rhodopsine* (*LwRh*). Genbank accession numbers are given in Supplementary Table A. The 20 μl mixture for PCR amplification included the following final concentrations of reagents: 0.75× buffer, 0.09 mM dNTPs, 3.1 mM $MgCl_2$, 0.5 units Taq polymerase (Fermentas), 0.5 μM each primer and 0.8–1.5 μl of DNA. We amplified DNA through an initial step of 3 min at 94 °C, 30 cycles of 30 s at 94 °C, 45 s at 49–58 °C (depending on the primers used), and 2 min at 72 °C and a final step for 2–7 min at 72 °C (depending on the gene). We visualised each PCR reaction with electrophoresis on 1% agarose gels and subsequently purified the PCR products using ExoSAP-IT (USB Corporation). In the sequencing reaction, we used the BigDye Terminator v1.1 sequencing kit (Applied Biosystems). We purified the cycle sequencing reactions with the Montage SEQ_{96} sequencing reaction cleanup kit (Millipore) and sequenced both strands on a MegaBace 1000 DNA analysis system (GE Healthcare). We compiled and edited the sequences with Sequencher 4.5 (Gene Codes).

2.2. Phylogenetic analyses

We aligned the sequences using Mafft 6.06b (Katoh et al., 2005) with the following options: iterative refinement with local pair-

Table 1
Socially parasitic *Myrmica* species, their recorded or suggested host(s), known localities and occurrences, following Bolton (1988) and Radchenko and Elmes (2003). We supplemented the data with *semiparasitica*, new references on collecting information, and excluded all species considered dubious as parasites (suggested to be malformed individuals). An asterisk (*) indicates that we included the parasite in our study; footnotes give further remarks.

Parasite species	Host(s)	Locality	Occurrence
Inquilines			
colax	*striologaster*	Texas, USA	Known from types only
ereptrix[a]	*aimonissabaudiae*	Kashmir, India	Only one type queen known
hirsuta[*b]	*sabuleti*, *lonae*	Europe	Widespread
kabylica	*cagnianti*	Algeria	Known from types only
karavajevi[*c]	*scabrinodis, rugulosa, sabuleti, lonae, gallienii*	Europe	Most widespread Palearctic parasite
lampra	*alaskensis*	Quebec, Canada	Found in two localities
laurae[*d]	*spinosior*, *scabrinodis*	Central Italy	Collected several times
lemasnei[e]	*spinosior*	Pyrenees	Found in two localities
microgyne of *rubra*[*f]	*rubra*	Europe	Relatively common
myrmicoxena	*lobicornis*	Switzerland, Austria	Known from types only
quebecensis[*]	*alaskensis*	NE North America	Relatively common
Temporary parasites			
arnoldii[*g]	Not documented	S Siberia, Mongolia	Found only independently
bibikoffi[h]	*sabuleti, spinosior*	Switzerland, Germany,	Found in five localities Spain
luteola[*i]	Uncertain	Russian Far East, Korea, Japan	Mostly freeliving
semiparasitica[*j]	*punctiventris*	Quebec, Canada; Illinois, New York and Ohio, USA	Found in four localities
vandeli[*k]	*scabrinodis*	Europe	Relatively common

[a] Extreme parasite morphology (inquiline syndrome).
[b] Rarely produces workers.
[c] Several other host recordings are misidentifications, here we follow Radchenko and Elmes (2003) and Seifert (2007); recently *M. karavajevi* was also found in the Iberian peninsula (Espadaler et al., 2004).
[d] First reported host *M. sabuleti*, collected from the same locality as the host *M. spinosior* of our sample; see 5 – because of the revised taxonomy, the validity of the host status of *M. scabrinodis* and *M. sabuleti* needs re-evaluation.
[e] The "West-Mediterranean" form of *M. sabuleti* (Seifert, 1988) is now known as *M. spinosior* (Seifert 2005).
[f] Seifert (1993) raised this inquiline of *M. rubra* to species (*M. microrubra*), but Steiner et al. (2006) synonymised it with *M. rubra*.
[g] Morphology suggests parasitism; presumed host *M. lobicornis*.
[h] Found twice with *M. sabuleti* and once without host in the Alps; recently recovered from a *M. spinosior* nest in Catalonia (Spain) and males were captured in a swarm in Galicia (García et al., 2008).
[i] morphology suggests parasitism; presumed host *Manica yessensis*, though no *Manica* in the Russian Far East.
[j] Described by Francoeur (2007).
[k] Mostly found with host; possibly independent colony founding in optimal habitats.

Table 2
Oligonucleotide primers used in this study. For *ArgK* exon 1, initial amplification was done using the first two primers, reamplification with the last two.

Gene	Primer	5′–3′ sequence	Reference
COI	LCO	GGTCAAACAAATCATAAAGATATTGG	Folmer et al. (1994)
	HCO	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
	Ron	GGATCACCTGATATATAGCATTCCC	Simon et al. (1994)
	Jerry	CAACATTTATTTTGATTTTTTGG	Simon et al. (1994)
	Pat	TCCAATGCACTAATCTGCCATATTA	Simon et al. (1994)
Cytb	CB 1	TATGTACTACCATGAGGACAAATATC	Jermiin and Crozier (1994)
	CB 2	ATTACACCTCCTAATTTATTAGGAAT	Jermiin and Crozier (1994)
28S D2	28SF2	AGAGAGAGTTCAAGAGTACGTG	Belshaw and Quicke (1997)
	28S3DR	TTGGTCCGTGTTTCAAGACGGG	Belshaw and Quicke (1997)
ArgK exon 1	AK1F2	TGGTTGAYGCYGCYGTTYTGGA	P.S. Ward, unpublished
	AK461R	GTGCTRGAYACYTTCTCYTCCAT	P.S. Ward, unpublished
	AK4F2	GTTGAYGCYGCYGTTYTGGAYAA	P.S. Ward, unpublished
	AK392R	TCCAARGAGCGRCCGCATC	P.S. Ward, unpublished
ArgK exon 2	AK346EF	AGGGTGARTACATCGTRTCHACT	P.S. Ward, unpublished
	AK720ER	ACCTGYCCRAGRTCACCRCCCAT	P.S. Ward, unpublished
EF-1α	TRS4F	GCGCCKGCGGCTCTCACCACCGAGG	Brady et al. (2006)
	TRS9.1b	GGAAGGCCTCGACGCACATCGG	Brady et al. (2006)
	TRS10R	ACGGCSACKGTTTGWCKCATGTC	T.R. Schultz, unpublished
LwRh	LR143F	GACAAAGTKCCACCRGARATGCT	Ward and Downie (2005)
	LR182F	CACTGGTATCARTTCGCACCSAT	P.S. Ward, unpublished
	LR639R	YTTACCGRTTCCATCCRAACA	Ward and Downie (2005)
	LR672R	CCRCAMGCWGTCATGTTRCCTTC	P.S. Ward, unpublished

wise alignment information (L-INS-i), maximum 1000 iterations and default gap settings. To maximise homology and to ascertain the absence of stop codons in protein-coding fragments, we translated the nucleotides to amino acids and concatenated the gene alignments in MacClade 4 (Maddison and Maddison, 2000). The concatenated dataset included 106 ingroup taxa and the following six outgroups: *Crematogaster auberti*, *C. scutellaris*, *Manica invidia*, *Manica rubida* (Myrmicinae), *Formica fusca* and *Lasius alienus* (Formicinae). The aligned DNA sequence matrix has been deposited in TreeBase (Study accession number S2637).

For each gene fragment we chose the most fitting nuclear substitution model using the Akaike information criterion (AIC) in Modeltest 3.7 (Posada and Crandall, 1998). We analysed the combined data using Bayesian inference in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). We applied three partitionings: (1) no partition; overall general time reversible model with an estimated gamma shape parameter and an estimated proportion of invariable sites (GTR + G + I); (2) partitioned to gene fragment, each analysed with the model chosen by Modeltest; (3) partitioned to four categories of DNA: mitchondrial, ribosomal, nuclear protein-coding and nuclear intron, again each with the appropriate model. For partition scheme 2, Modeltest chose GTR + G + I for each fragment except for ArgK (SYM + G + I) and 28S (TVM + G + I). We applied SYM + G + I to ArgK by setting the nucleotide frequency prior to be equal (statefreqpr = fixed(equal)). Since TVM (a submodel of GTR with equal transition rates) is not available in MrBayes, we used GTR + G + I. For partitioning scheme 3, Modeltest chose GTR + G + I for each partition. Each analysis was run for ten million generations, sampling every 1000th generation. We unlinked the parameters for each partition except the topology and branch lengths. We assessed burn-in by plotting the tree likelihood for each sampling point using Tracer 1.4 (Rambaut and Drummond, 2007). When the likelihood values reached a plateau, we considered the Markov chains stationary. We thus discarded trees that were obtained before burn-in and calculated 50% majority rule consensus trees of the remaining trees. Hence, we removed 2000 (partitions 1 and 2) or 4000 (partition 3) trees from the samples and calculated a consensus of 18002 or 16002 trees. The obtained posterior probabilities (PP) reflect the relative representation of splits in the sampled trees, and can be interpreted directly as measures of support.

For the maximum likelihood analyses we used RaxML 7.0.4 (Stamatakis, 2006) compiled from source using gcc 4.2 in Linux. We applied a GTRCAT approximation to each gene partition and performed 1000 Rapid Bootstraps. We then conducted a complete ML search using the GTRCAT approximation. To obtain stable likelihood values, we evaluated the final tree topology with GTRGAMMA (a conventional GTR model). We also analysed each single gene using both Bayesian inference and maximum likelihood as described for the concatenated data. We analysed the data on a Macintosh G5, a Dell M6300, and a HP CP4000 BL ProLiant supercluster at the CSC-IT Center for Science, Ltd.

2.3. Divergence-time estimation

To obtain age estimates for our *Myrmica* phylogeny, we applied a relaxed molecular clock and performed a divergence-time estimation using penalized likelihood as implemented in r8s 1.71 (Sanderson, 2002, 2003). This method is a semi-parametric approach to divergence-time estimation, in which a parametric model (a different substitution rate on each branch) is combined with a non-parametric roughness penalty (which costs the model more when rates are changing too rapidly across branches). Using a smoothing parameter λ, the estimation may be averaged between the parametric and non-parametric component.

To infer absolute rates and ages, we calibrated our tree with two fossils, *Kyromyrma* and *Myrmica*. The former was found in New Jersey amber, dated at 92 million years (Ma). Because *Kyromyrma* likely possessed an acidopore, the apomorphy for Formicinae, it is a stem group formicine (Grimaldi and Agosti, 2000). We therefore used it for calibrating the root, i.e., the split between Formicinae and Myrmicinae. *Myrmica* fossils have been found in Baltic and Saxonian amber, dated at 44.1 Ma (Radchenko et al., 2007). Four of five fossil *Myrmica* groups resemble species of the ritae group (Radchenko et al., 2007), but they are considered basal to extant *Myrmica* (Radchenko, 1995a; Radchenko et al., 2007; Radchenko and Elmes, 2001b). We therefore conservatively consider them as stem group *Myrmica*, representing the minimum age for the genus. To assess the sensitivity of our analysis to these fossil calibrations, we performed two sets of analyses: (1) *Kyromyrma* and *Myrmica* fixed to 92 and 44.1 Ma, respectively; (2) *Kyromyrma* fixed to 92 Ma, *Myrmica* constrained to a minimum age of 44.1 Ma.

We analysed the majority rule consensus tree obtained from MrBayes using the truncated newton (TN) algorithm in r8s. To explore the possibility of multiple optima, we repeated all divergence-time analyses from multiple random starting points, with solutions randomly perturbed. To determine the best value of the smoothing parameter λ for our data, we performed a cross-validation analysis, during which each terminal in the tree is removed in turn, and the remaining parameters in the model are estimated without that branch. Subsequently, r8s predicts the estimated number of substitutions on the pruned branch and compares this to the original value. The user thus obtains an estimate of the accuracy of the method for each chosen level of smoothing.

Finally, to obtain confidence limits on our age estimates, we performed 100 bootstrap shuffles of our original dataset in Mesquite 2.6 (Maddison and Maddison, 2009) and used these to estimate branch lengths on the original Bayesian tree with the GTR + G + I model in Paup* (Swofford, 1998). For each bootstrap replication, we repeated the divergence-time estimation in r8s as explained above. Finally, we profiled the nodes of interest (crown ages of species groups and parasites) across the trees with bootstrapped branch lengths. This provided 95% confidence intervals for the previously obtained point estimates.

3. Results

3.1. Sequence characteristics

We successfully generated DNA sequences for 112 taxa, of which six were outgroups. For the majority of samples we amplified all gene fragments. However, one or two fragments were missing for the following taxa: *M. americana* (*Cytb*), *M. angulata* (exon 2 of *ArgK* and the 5′ part of *COI*), *M. arnoldii* (28S), *M.* sp. AC (Cytb), *M. excelsa* (*Cytb*), *M. forcipata* (28S), *M. hirsuta* (28S), *M. monticola* (*Cytb*), M295 (*Cytb*), *M. nearctica* (*Cytb*), *M. semiparasitica* (*Cytb*,

Table 3
Gene fragments, their length in base pairs (bp), variability, percentage of parsimony informative sites and GC content.

Gene fragment	Length	Variable sites (%)	Parsimony informative (%)	GC (%)
COI	1380	41.4	37.4	28.4
Cytb	390	50.0	44.1	24.5
28S	504	38.9	21.8	69.0
ArgK coding region	633	11.1	6.2	57.5
ArgK intron	175	30.3	12.6	23.6
Ef-1α	327	5.5	1.5	62.6
LwRh coding region	390	13.1	7.7	53.9
LwRh intron	87	24.1	13.8	54.1

EF-1α), *M. wesmaeli* (*EF-1α*), *Crematogaster auberti (Cytb)*, *Manica invidia* (*Cytb*) and *Manica rubida* (intron of *ArgK*). The concatenated data had 3886 base pairs, of which 1581 were variable. We did not detect any base composition bias (χ^2 test, $P > 0.9$, homogeneity not rejected). For sequence characteristics of each gene fragment, see Table 3.

3.2. Phylogenetics

The Bayesian and maximum likelihood analyses of the concatenated data yielded a mutually similar, well resolved and robust phylogenetic hypothesis (Fig. 1). Single gene topologies never resulted in fully resolved phylogenies (Supplementary Material B). Nevertheless, no conflict was detected among the gene trees, and none fundamentally conflicted with the concatenated analyses. In the Bayesian analyses of the concatenated data, the topology was similar for all three partitionings, but partitioning to gene fragments yielded the highest support values. The Nearctic and Palearctic species were not reciprocally monophyletic groups. Nevertheless, most clades united a set of either Nearctic or Palearctic species. We found only two exceptions: the Palearctic *M. arnoldii* clustered in the Nearctic incompleta species group, and *M. wheeleri,* an American species, with the Palearctic rugosa group. Maximally four dispersal events between Palearctic and Nearctic are implied in the tree (Fig. 1; see boxes in Fig. 2). The Nearctic species *M. wheeleri* is sister group to a clade of Palearctic species, and *M. arnoldii* is a Palearctic species nested within a Nearctic group. The Palearctic scabrinodis, schencki and lobicornis groups are interspersed between several Nearctic clades (punctinops-rugiventris, and the large Nearctic clade containing, a.o., *M. crassirugis* and *M. striolagaster*), which implies (at least) two switches from Nearctic to Palearctic (Fig. 2).

Most morphologically defined species groups were monophyletic and received moderate to high support (89–100% for both posterior probability, PP, and bootstrap support, BS) (Fig. 1). The deepest split separated the luteola, ritae, rubra and rugosa groups from the other *Myrmica* species, but the likelihood analyses could not resolve the interrelationships among them.

Three species joined a different species group than expected from morphology (Fig. 1). *Myrmica divergens* clustered with lobicornis; *M. wittmeri* and *M. dshungarica* formed part of the rugosa group. Notably, support within the rugosa clade was partially low ($PP < 70$, $BS < 60$). Also, the interrelationships among several Nearctic groups (brevispinosa, fracticornis, group B and americana) were unresolved.

The inquiline species *M. hirsuta*, *M. quebecensis* and the microgynous inquiline morph of *rubra* were all closest relatives of their respective hosts (Fig. 2). *Myrmica laurae* was closely related but not closest to its *M. spinosior* host. *Myrmica karavajevi* joined the clade of those scabrinodis-group species that included all its hosts represented in our data (*M. rugulosa*, *M. scabrinodis*, *M. sabuleti*). The temporary parasites followed a similar but looser pattern of parasite–host relationships than the inquilines. *Myrmica semiparasitica* was the closest relative of its host *M. punctiventris*, *M. vandeli* was closely related but not sister species to *M. scabrinodis, M. luteola* joined the ritae group (not its host *Manica*), and *M. arnoldii* clustered in the incompleta, not lobicornis, group.

3.3. Divergence-time estimates

The crown age of *Myrmica* was estimated at 34.80 ± 3.67 Ma (±95% confidence interval; both fossils fixed) or 37.67 ± 11.80 Ma (*Kyromyrma* fixed, *Myrmica* constrained) (Fig. 2 and Table 4). We further inferred a Miocene crown group age for most species groups (ca. 8–25 Ma; Table 4). The rugosa, ritae and scabrinodis groups were the oldest clades (Upper Oligocene–Early Miocene). The most recently diverged species group was group B, followed by americana, group A, and schencki. Of the inquilines, *M. hirsuta* diverged from its host only recently (Pleistocene), but *M. laurae*, *M. quebecensis* and *M. karavajevi* were much older (Miocene). The branch length for the split between *M. rubra* and its microgynous inquiline was always inferred as zero with no time estimate of divergence. The temporary parasites were almost all estimated around 10–15 Ma old (Upper Miocene), although the crown age for *M. luteola* was much older (Upper Oligocene).

4. Discussion

We inferred a phylogenetic hypothesis for the genus *Myrmica,* and tested morphological and evolutionary hypotheses. The data included several undescribed and 70 of the 200 described *Myrmica* species, including eight socially parasitic species and one inquiline considered an intraspecific parasite. We analysed the most comprehensive sequence data so far collected for *Myrmica.* This yielded a mostly well resolved and supported phylogeny. We will use the phylogeny to discuss the systematics and biogeography of *Myrmica*, and evolutionary relationships of the social parasites to their hosts.

4.1. Myrmica systematics

The Palearctic *Myrmica* species have been divided into morphologically defined species groups (see Introduction). Such morphological groupings are controversial (Bolton, 2003). For example, Wilson (2003) proposed 19 species groups within the genus *Pheidole* based on morphological grounds. However, only one of the species groups was supported by a molecular phylogeny of the same genus (Moreau, 2008). In our molecular phylogeny, in contrast, morphological species groups all corresponded to well-supported monophyletic clades. The only exceptions were *M. wittmeri* (morphologically included in the smythiesii group, not indicated in Fig. 1) and *M. dshungarica* (morphologically placed in the rubra group), both of which nested within the rugosa group in our phylogeny. All species belonging to the smythiesii group and *M. dshungarica* have evenly curved antennal scapes in workers, a feature typical for the rugosa group. Nevertheless, the species of the smythiesii group are traditionally placed in a separate morphological group because the males have longer scapes than those of the rugosa group (Radchenko and Elmes, 2001b). The male scapes of *M. dshungarica* are even longer, and therefore it has been considered a member of the rubra group. Clearly, the morphological demarcation of the smythiesii and rugosa groups, and the taxonomic position of M. *dshungarica* are not phylogenetically supported, and may need to be revised.

To explore the poorly known diversity of North American *Myrmica* species, Jansen et al. (2009) applied the DNA barcoding approach (Hebert et al., 2003; Meier et al., 2006; Rach et al., 2008). The information comprised in the short *COI* fragment proved insufficient to distinguish species reliably, but successfully delineated tentative species groups. A common objective of barcoding is molecular species demarcation rather than phylogenetic reconstruction (Blaxter, 2004; Janzen et al., 2005). Nevertheless, it may serve well as an explorative tool when the fauna is taxonomically poorly known (Jansen et al., 2009). Therefore, our multi-gene phylogeny, which included several samples of all Nearctic clades identified by barcoding (Jansen et al., 2009), formed a test and extension of the barcoding results. We found support for most tentative Nearctic species groups. The americana group and group B were reciprocally monophyletic, but closely related. Their subdivision into two groups is thus mainly based on morphological grounds (A. Francoeur, pers. commun.; Jansen et al., 2009). But

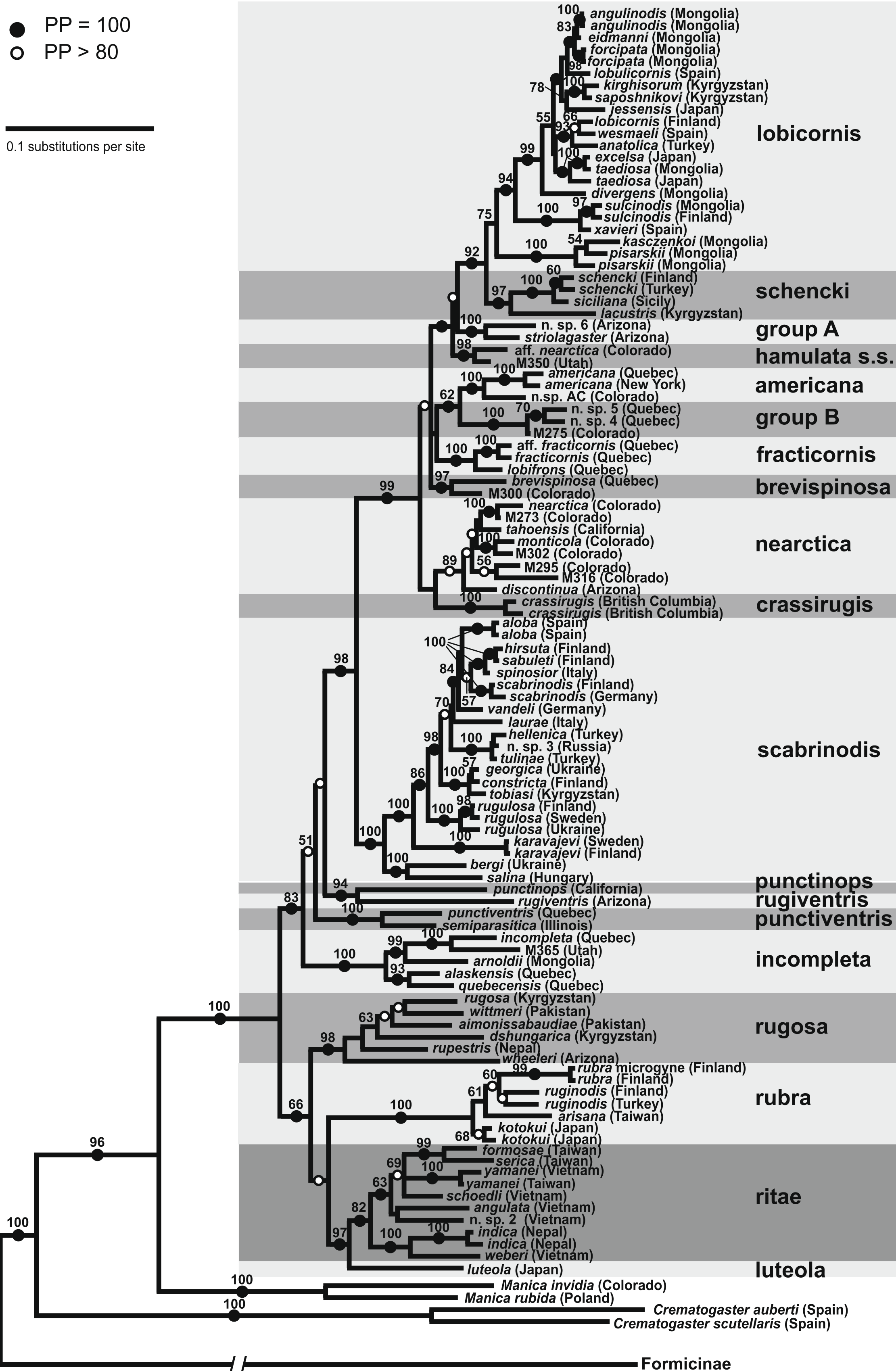

Fig. 1. Bayesian 50% majority rule consensus tree of 106 *Myrmica* ingroup samples and six outgroup taxa (*Manica invidia*, *M. rubida*, *Crematogaster scutellaris*, *C. auberti*, *Formica fusca* and *Lasius alienus*). Shown here is the topology with data partitioned to gene fragments (partition 2). Filled circles indicate posterior probability = 100%; open circles, PP > 80%. Numbers give maximum likelihood bootstrap support values. Names in bold (at right) refer to species groups which were monophyletic in our data. Origin of sample is given in parentheses.

Fig. 2. Chronogram of the *Myrmica* phylogeny obtained from r8s under penalized likelihood. The two fixed nodes are indicated with a star (*Kyromyrma* fixed at 92 Ma, *Myrmica* at 44.1 Ma). The scale under the chronogram gives age in millions of years and major geological eons. Nearctic species are surrounded by boxes. Social parasites are connected to their hosts with arrows (only those host species of which we have parasite–host samples; for additionally documented host species, see Table 1).

Table 4
Summary of crown age estimates and their 95% confidence intervals of the genus *Myrmica*, its species groups and social parasites obtained using penalized likelihood in r8s. Shown are values with *Kyromyrma* fixed at 92 Ma and *Myrmica* at 44.1 Ma (fixed), and *Kyromyrma* fixed at 92 Ma and *Myrmica* constrained at 44.1 Ma (constrained). Social parasites are given in italics, temporary parasites indicated with T. Species groups and parasites are arranged from oldest to youngest.

Node	Fixed	Constrained
Crown age of genus	34.80 ± 3.67	37.67 ± 11.80
M. luteola T	26.56 ± 4.43	28.84 ± 10.05
rugosa	26.24 ± 3.99	28.52 ± 9.90
ritae	22.78 ± 3.87	24.76 ± 8.97
scabrinodis	21.46 ± 4.00	23.49 ± 9.23
incompleta	17.33 ± 3.21	18.93 ± 6.99
M. karavajevi	16.93 ± 3.39	18.56 ± 7.35
punctiventris	14.77 ± 3.51	16.19 ± 7.05
M. semiparasitica T	14.77 ± 3.51	16.19 ± 7.05
M. arnoldii T	14.13 ± 2.49	15.40 ± 5.49
rubra	10.88 ± 2.12	11.79 ± 4.34
hamulata	10.84 ± 3.00	12.06 ± 5.68
M. quebecensis	10.40 ± 2.90	11.42 ± 4.79
brevispinosa	10.35 ± 2.58	11.44 ± 4.96
M. laurae	9.81 ± 2.62	10.80 ± 4.94
lobicornis	9.79 ± 2.03	10.70 ± 4.21
M. vandeli T	9.22 ± 2.53	10.21 ± 4.90
schencki	8.54 ± 1.86	9.32 ± 3.73
americana	8.75 ± 2.37	9.62 ± 4.14
group A	8.32 ± 2.21	9.18 ± 4.20
nearctica	6.03 ± 1.97	6.73 ± 3.55
group B	3.47 ± 1.11	3.79 ± 1.58
M. hirsuta	0.78 ± 0.54	0.85 ± 0.60
M. rubra microgyne	0	0

contrary to the barcoding results, the hamulata clade now broke into two unrelated clades (hamulata s. str. and nearctica). This illustrates that barcoding cannot unequivocally be used to estimate biodiversity, because *COI* data are insufficient and often incongruent with other sources of data (Meier et al., 2006; Rubinoff et al., 2006). Our results emphasise that any interpretation based on the limited information provided by barcoding needs careful evaluation. When more information becomes available, taxonomical interpretations derived from barcoding often require reassessment.

4.2. Biogeographical hypotheses

During the last 65 Ma, geological events and changing climatic conditions have profoundly influenced the distribution of plants and animals (Sanmartín et al., 2001; Zachos et al., 2001). The divergence-time estimates of *Myrmica* could thus illustrate responses of a now ecologically dominant group to the changing world. Our time estimates only allow a rough relative timing of events that may have been important in the evolution and biogeography of *Myrmica*. Below we present an overview of major historical episodes falling within the time frame we have inferred, and the possible effects they could have had on the diversification of *Myrmica*.

The extant *Myrmica* species in central and southeastern Asia (the Himalayas, southern China, Burma, Thailand, Vietnam, Taiwan) exhibit the most diverse morphologies of any biogeographic *Myrmica* assemblage and also contain the highest number of species, many of which are endemic (Radchenko, 1995a; Radchenko and Elmes, 2001b; Radchenko et al., 2007; Radchenko and Elmes, pers. commun.). Moreover, the ranges of the species groups evaluated oldest in the divergence-time estimation (rugosa and ritae) overlap in this region, but nowhere else. Although a centre of origin may never be found with certainty, currently available data suggest that central and southeastern Asia played an important role in the early diversification of *Myrmica*. Unfortunately, biogeographic dispersal-vicariance analysis (DIVA; Ronquist, 1996) could not infer a centre of origin for *Myrmica* (data not shown).

The Terminal Eocene Event (TEE) at the boundary of the Eocene and Oligocene (34 Ma) was one of the most dramatic climatic events of the Tertiary. Global temperatures plummeted rapidly (Zachos et al., 2001), causing abrupt turnovers in the European and Mongolian mammal fauna (Hartenberger, 1998; Meng and McKenna, 1998). The same events also stimulated the radiation of cold-adapted groups such as *Bombus* bumblebees (Hines, 2008). As comes to *Myrmica,* fossils were already present in the Eocene deposits (44.1 Ma), but their scarcity compared to other ants suggests that the genus was far from common then (Radchenko et al., 2007). Radchenko and Elmes (2001b) suggested that early Eocene *Myrmica* were rare until species adapted to cooler conditions had evolved. Our divergence-time estimates indeed place the crown age of the genus around the Eocene—Oligocene boundary. The TEE could thus have increased the availability of cool habitats in which modern *Myrmica* lineages could radiate and disperse.

The subsequent uplift of the Tibetan plateau was initiated by the collision of India and Eurasia (35–50 Ma ago), but was most dramatic at 21 Ma (Harrison et al., 1992). This period of tectonic activity was characterised by stochastic changes in temperature (Hines, 2008). Repeated phases of cooler and warmer conditions likely caused expansions and contractions of suitable habitats. Waves of geographic expansion of *Myrmica* during cold periods may have alternated with regional contraction and isolation at high elevations during warmer periods. Such dynamics may explain the current presence of phylogenetically old, endemic species in the Himalayas, Vietnam and Taiwan (Elmes and Radchenko, 1998; Radchenko and Elmes, 1998, 2001b).

The remarkable similarity between Asian and eastern North American flora and fauna is one of the most documented biogeographic patterns (Boufford and Spongberg, 1983; Tiffney, 1985a; Wolfe, 1975). It is typically explained as the remains of a continuous habitat that stretched across the Holarctic during the Tertiary (Tiffney, 1985a, b). From the Mid Cretaceous (ca. 100 Ma) throughout the Tertiary, northeastern Eurasia was almost permanently connected to the Nearctic via Beringia (McKenna, 1983b; Tiffney, 1985a,b). Additionally, the Atlantic Thulean and De Geer routes connected Europe and eastern North America in the late Cretaceous and Early Tertiary (McKenna, 1983a; Tiffney, 1985b).

The dispersal events (maximally four) between the Palearctic and Nearctic suggested by our phylogeny took place 10–30 Ma ago. The Atlantic land bridges probably broke down before they could serve as dispersal routes for *Myrmica* (Thulean bridge ca. 50 Ma, De Geer route ca. 39 Ma). Intercontinental dispersals therefore almost certainly involved the Beringian bridge, albeit probably not all during the time period. After the TEE, the Northern Hemisphere was covered with mixed mesophytic forest (Wolfe, 1987) that gradually became dominated by conifers as temperatures continued to decrease. By the Mid Miocene (ca. 14 Ma), a taiga-type vegetation was present in eastern Asia, Beringia and northern North America (Sanmartín et al., 2001). The Beringian land bridge therefore offered ample opportunities for dispersal of *Myrmica*.

Radchenko et al. (2007) considered it likely that stem group *Myrmica* be discovered in regions where relict fauna and flora may have persisted, such as southeast Asian or southern North American mountains. Such developments may yield insights that would substantially improve our understanding of the early evolution of *Myrmica* and amend our divergence-time estimates and biogeographic suggestions.

4.3. Social parasite–host relationships

Emery (1909) proposed that social parasites are closely related to their respective slaves or hosts. This generalisation became known as Emery's rule (Le Masne, 1956). The original, loose form of the rule almost always holds, because most social parasites be-

long to the same genus as their host, or a closely related genus, as *Polyergus*, which enslave *Formica* species (Emery, 1909). This is understandable, because similarity between parasite and host facilitates their coexistence (Buschinger, 1986). Rare exceptions to the loose form of the rule are inquilines of the formicine genus *Polyrhachis*, which parasitise ponerine hosts (Maschwitz et al., 2000, 2003).

The strict form of Emery's rule (SFER) is an evolutionary-biologically attractive hypothesis. By testing the hypothesis that social parasites are the closest relatives of their respective hosts, it helps to clarify whether social parasitism evolved once or several times within a genus. Moreover, the validity of SFER has been used as evidence in favour of sympatric speciation (Buschinger, 1990; Bourke and Franks, 1991; Savolainen and Vepsäläinen, 2003; Vepsäläinen et al., 2009).

Caution is needed, however, when interpreting evidence for SFER. It may be falsely supported in three cases. First, inadequate taxon sampling may have excluded the sister species of a host. Second, lineages intermediate between parasite and host may have gone extinct. Third, host switching or tracking, possibly combined with host extinction, may have confounded the original host-parasite relationships. We aimed to minimise the risk of false interpretations by incorporating the hosts and parasites in a large phylogeny of *Myrmica*. To avoid taxon sampling errors, we included non-parasitised species that morphologically resemble hosts and parasites. This approach is likely to cover intermediate lineages, because parasites often retain morphological similarities to their hosts (see Bolton, 1988; and morphological descriptions in Radchenko and Elmes, 2003). Further, to identify cases where host extinction, host switching and host tracking may have been important, we performed a divergence-time estimation. Ancient social parasites are more likely than recently diverged parasites to have experienced such events, and are thus less likely to obey SFER (Smith et al., 2007). Finally, finding multiple examples of SFER within one genus increases the credibility of the results.

Savolainen and Vepsäläinen (2003), studying three inquiline–host pairs of *Myrmica*, found support for SFER, thus showing that inquilinism has evolved independently multiple times in the genus. Our present study improved taxon and gene sampling, and included more parasites. Our results confirm that social parasitism has evolved several times within *Myrmica*.

Two inquilines were sister species of their hosts (*M. hirsuta*, *M. quebecensis*). *Myrmica hirsuta* diverged recently from its host lineage (ca. 0.8 Ma), and therefore strongly supports SFER. The support for SFER is weaker for *M. quebecensis,* which is older (ca. 10 Ma), and belongs to a small Nearctic species group. Because close relatives of *M. alaskensis* (the host species) do not exist or are in any case unknown, taxon sampling could not be improved. The microgyne of *M. rubra* necessarily clustered with its conspecific host, and exemplifies the potential for speciation through intraspecific parasitism (Savolainen and Vepsäläinen, 2003; Vepsäläinen et al., 2009). Current data thus strongly suggest that SFER applies to at least three inquilines of *Myrmica.* Two other inquilines, *M. laurae* and *M. karavajevi*, obeyed the loose form of Emery's rule; they both belonged to clades containing all of their respective hosts. Both are relatively ancient parasites (ca. 10 and 17 Ma, respectively), leaving ample time for processes that may obscure SFER. The observation that old inquiline species use more than one host (Table 1; Radchenko and Elmes, 2003) has, indeed, been interpreted, e.g., in *M. karavajevi*, such that they may actually be species complexes (Bolton, 1988). Old inquilines may with time have adapted to new host species, but may also have speciated. To detect possible complexes of inquiline sister species would, however, necessitate a more representative survey of parasite–host pairs than done in this study.

The inferred crown age of ca. 17 Ma for *M. karavajevi* makes it the oldest *Myrmica* inquiline. In view of its specialised morphology, a long socially parasitic life seems plausible. Its multiple host use and the phylogenetic relationship to its hosts may thus be explained in several ways (Savolainen and Vepsäläinen, 2003). Ancestral *M. karavajevi* may have colonised new host species, possibly after extinction of the original host. Alternatively, *M. karavajevi* may have diverged from its host so long ago that the host has meanwhile speciated. The parasite may then have tracked all or part of the sibling species. Finally, *M. karavajevi* may consist of several cryptic host-specific species (Bolton, 1988), though in our phylogeny the *M. karavajevi* parasitising *M. scabrinodis* and *M. rugulosa* differed only slightly. None of the above scenarios conflicts fundamentally with SFER, although it would now be impossible to infer from which host *M. karavajevi* diverged originally.

Temporary parasites are less likely than inquilines to obey Emery's rule. They only depend on the host in the earliest stages of the colony cycle, and may develop their dependency as an adaptation to unfavourable conditions. Indeed, several temporary parasites, including *M. bibikoffi*, *M. vandeli* and *M. luteola,* are facultatively parasitic (Radchenko and Elmes, 2003). Moreover, the parasitic life styles of temporary parasites are often inferred from their morphology. Only *M. bibikoffi*, *M. semiparasitica* and *M. vandeli* have been unequivocally found in the nests of their respective hosts (Radchenko and Elmes, 2003; Francoeur, 2007). In our analysis we found one temporary parasite obeying SFER (*M. semiparasitica*), one obeying the loose form (*M. vandeli*) and two clustering distantly from their respective host (*M. arnoldii*, *M. luteola*).

Despite partially supporting evidence in *Myrmica*, SFER has not received much support in other taxa. Several studies report that in polistine paper wasps, social parasites form a monophyletic group (Carpenter et al., 1993; Choudhary et al., 1994; Arévalo et al., 2004). In yellowjackets, *Lasioglossum* bees and bumblebees, SFER was rejected (Williams, 1994; Pedersen, 1996; Danforth, 1999; Carpenter and Perera, 2006). Even in several ant groups, social parasites are unrelated to their hosts (Ward, 1996; Parker and Rissing, 2002; Janda et al., 2004; Maruyama et al., 2008). Thus empirical support for SFER is limited to a few cases, including allodapine bees (Smith et al., 2007), attine leafcutting ants (Sumner et al., 2004) and some *Myrmica* (Savolainen and Vepsäläinen, 2003; Vepsäläinen et al., 2009; this study).

The scarcity of support for the strict form of Emery's rule implies that evolution of social parasitism has followed different routes. This observation opens the door for a stimulating research field, where detailed studies on the biology, population genetics and ecology of social parasites may shed light on how social parasites originate.

Acknowledgements

We thank the following colleagues for samples: A. Azuma, G. Bračko, S.P. Cover, S. Csősz, W. Czechowski, K. Eguchi, G.W. Elmes, X. Espadaler, R. Higgins, J.T. Longino, D. Lubertazzi, B. Markó, A.G. Radchenko, L. Rericha, R. Schultz, P.S. Ward and S. Yamane. Phil S. Ward kindly provided information on oligonucleotide primers. We thank A. Radchenko and A. Francoeur for help with determining the samples and for discussions on *Myrmica* taxonomy. We also thank Graham W. Elmes, Alexander G. Radchenko and two anonymous referees for helpful comments on the manuscript. This research was funded by the Academy of Finland (Project No. 210523 to R. S.), the Finnish Cultural Foundation and Ella and Georg Ehrnrooth Foundation (to G.J.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.01.029.

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