



## Molecular phylogeny of the ant tribe Myrmicini (Hymenoptera: Formicidae)

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The interrelationships within ant subfamilies remain elusive, despite the recent establishment of the phylogeny of the major ant lineages. The tribe Myrmicini belongs to the subfamily Myrmicinae, and groups morphologically unspecialized genera. Previous research has struggled with defining Myrmicini, leading to considerable taxonomic instability. Earlier molecular phylogenetic studies have suggested the nonmonophyly of Myrmicini, but were based on limited taxon sampling. We investigated the composition of Myrmicini with phylogenetic analyses of an enlarged set of taxa, using DNA sequences of eight gene fragments taken from 37 representatives of six of the seven genera (*Eutetramorium*, *Huberia*, *Hylomyrma*, *Manica*, *Myrmica*, and *Pogonomyrmex*), and eight outgroups. Our results demonstrate the invalidity of Myrmicini as currently defined. We recovered sister-group relationships between the genera *Myrmica* and *Manica*, and between *Pogonomyrmex* and *Hylomyrma*. This study illustrates that to understand the phylogeny of over 6000 myrmicine species, comprehensive taxon sampling and DNA sequencing are an absolute requisite.

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### INTRODUCTION

Ants (Formicidae) numerically dominate terrestrial ecosystems from the arctic regions to the tropics. Formicidae includes about 12 000 described ant species grouped into 21 subfamilies (Bolton, 2003; Saux, Fisher & Spicer, 2004; Bolton *et al.*, 2006; Rabeling, Brown & Verhaag, 2008). The interrelationships among ants have long been elusive, as demonstrated by the thorough revisions in each general taxonomic work on Formicidae (Bolton, 1995, 2003; Bolton *et al.*, 2006). Moreover, cladistic analyses of morphological data have proven insufficient to resolve the phylogeny of ants (Baroni Urbani, Bolton & Ward, 1992). Only recently, Ouellette, Fisher & Girman

(2006) succeeded in defining the early branches of the ant tree of life, and Brady *et al.* (2006) and Moreau *et al.* (2006) made significant progress in resolving the interrelationships among the subfamilies. The recent discovery of the sister group to all extant ants, *Martialis heureka* (Rabeling *et al.*, 2008), further enhanced our understanding of early ant evolution.

In contrast to the emerging consensus on the overall relationships among ants, the classification within subfamilies remains controversial. The largest of the 21 subfamilies, Myrmicinae, comprises 149 extant genera (Agosti & Johnson, 2005), classified into 25 morphologically defined tribes (Bolton, 2003; Fernández, 2004). An analysis of the interrelationships of Myrmicinae is only available as part of the phylogenies of ants. Thus, the data in Brady *et al.* (2006) included 19 tribes representing 42 myrmicine genera, and in Moreau *et al.* (2006) 17 tribes covering 52 genera. Both studies agreed on the nonmonophyly of several tribes, including Dacetini, Stenammini, Solenopsidini, and Myrmicini.

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**Table 1.** Summary of the species numbers, distribution, basic ecology, and most important taxonomic work for the seven genera of the tribe Myrmicini, presented in decreasing order according to species number

Genus	Species	Distribution	Ecology	Taxonomic notes
<i>Myrmica</i>	~200	Holarctic, mountains of Southeast Asia	Common; cold adapted; nests in diverse habitats; omnivorous	Generic definition revised by Bolton (1988b); Palaearctic species well understood, divided into species groups (Radchenko, 1995a,b,c,d; Radchenko & Elmes, 2001, 2003); Seifert, 1988, 2003; Nearctic taxonomy outdated and problematic (Weber, 1947, 1948, 1950; Creighton, 1950); two new Nearctic species groups and four new species (Francoeur, 2007)
<i>Pogonomyrmex</i>	~70	USA, Mexico, South America	Mostly temperate; nests in arid regions; seed harvesters	<i>Ephebomyrmex</i> genus (Creighton, 1950; Wheeler & Wheeler, 1985; Hölldobler & Wilson, 1990); Former subgenera <i>Ephebomyrmex</i> , <i>Forelomyrmex</i> synonymized with <i>Pogonomyrmex</i> (Lattke, 1990); <i>Ephebomyrmex</i> paraphyletic (Parker & Rissing, 2002); monophyletic North American species complexes: <i>barbatus</i> , <i>occidentalis</i> and <i>californicus</i> (Taber, 1990; Parker & Rissing, 2002); keys, Chile (Snelling & Hunt, 1975), Argentina (Kusnezov, 1978), Mexico (MacKay <i>et al.</i> , 1985), Northern South America (Fernández & Palacio, 1997)
<i>Hylomyrma</i>	13	From Mexico to South Brazil, Paraguay, and Argentina	In leaf litter	Raised to genus (Brown, 1953); <i>Lundella</i> transferred from Tetramoriini and synonymized with <i>Hylomyrma</i> (Brown, 1953); revised to 12 species (Kempff, 1973)
<i>Manica</i>	6	Four in North America, one in Europe, and one in Japan	Cool habitats; mostly at higher altitudes	Synonymized with <i>Myrmica</i> (Roger, 1863); subgenus of <i>Myrmica</i> (Emery, 1921); raised to genus and placed in <i>Myrmicini</i> (Weber, 1947); sister group of <i>Myrmica</i> (Astruc <i>et al.</i> , 2004; Brady <i>et al.</i> , 2006)
<i>Eutetramorium</i>	2	Madagascar		Tetramoriini (Ashmead, 1905); Myrmecini (Emery, 1912, 1914); Tetramoriini (Emery, 1915)
<i>Huberia</i>	2	New Zealand	Forests	Stenammini (Ashmead, 1905); Solenopsidini (Emery, 1914, 1922; Forel, 1917; Wheeler, 1922); <i>Incertae cedis</i> in Myrmicinae (Ettershank, 1966); Myrmicini (Hölldobler & Wilson, 1990)
<i>Secostruma</i>	1	Malaysia	Reduced eyes and ventrally placed sting suggest hypogeic life	Tetramoriini (Bolton, 1988a); Lack of characteristic Tetramoriini sting results in transfer to Myrmicini (Bolton, 1994)

The morphological definition of the tribe Myrmicini has been problematic because of a lack of apomorphies. Since Emery (1921) revised the classification of ants, Myrmicini has consisted of *Myrmica*, *Pogonomyrmex*, and a changing array of other genera. Several additional genera have been transferred to the tribe and removed again later. The tribe is usually recognized by the presence of metatibial spurs (rarely absent) and a characteristic wing venation in winged individuals.

Currently, Myrmicini is formally defined by several morphological characters, many of which are not unique to Myrmicini, and includes the genera *Eutetramorium*, *Huberia*, *Hylomyrma*, *Myrmica*, *Manica*, *Pogonomyrmex*, and *Secostruma* (Bolton, 2003). Information on the distribution, ecology, and taxonomy of each Myrmicini genus is summarized in Table 1.

Three hypotheses have been formulated on the composition of Myrmicini: one based on morphological

analysis (above) and two on molecular data. Molecular data suggested that Myrmicini as defined morphologically may not be monophyletic (Brady *et al.*, 2006; Moreau *et al.*, 2006). These studies included, respectively, four and three of the seven genera in Myrmicini, and sampled one or two species of each genus. In Brady *et al.* (2006), *Eutetramorium* did not form a monophyletic group with *Myrmica*, *Manica*, and *Pogonomyrmex*. In Moreau *et al.* (2006), *Pogonomyrmex*, *Eutetramorium*, and *Myrmica* clustered in separate clades.

To test the monophyly of Myrmicini, and to re-evaluate the conflicting ideas regarding the relationships among *Myrmica*, *Pogonomyrmex*, and *Eutetramorium*, we reconstructed the phylogenetic relationships among the members of Myrmicini using DNA sequence data, obtained from multiple genes. We included in our study all genera of Myrmicini, except *Secostruma* (which consists of one rare species from Malaysia), and represented each genus by multiple species. To investigate the position of Myrmicini within Myrmicinae, we complemented our data with published DNA sequences of other myrmicine genera (Brady *et al.*, 2006; Moreau *et al.*, 2006).

## MATERIAL AND METHODS

### ANT SAMPLES

We collected at least ten individuals from colonies of *Manica*, *Myrmica*, and *Pogonomyrmex* in Colorado, USA (1998), Japan and Taiwan (2002), and Quebec, Canada (2005) (Table 2). The ants were placed in absolute ethanol and stored at 4 °C. We also received additional specimens from colleagues. In total, 45 taxa were included, of which two *Eutetramorium*, three *Huberia* (two representatives of *Hu. brounii*), two *Hylomyrma*, two *Manica*, 19 *Myrmica*, and nine *Pogonomyrmex* (with two samples of *P. occidentalis*). Additionally, six myrmicine and two formicine outgroups were included. We took two representatives for each myrmicine outgroup genus to control for possible sample mix-ups and sequencing errors. In our study all Myrmicini genera were thus represented by several samples, except for *Secostruma*, for which one species, *S. lethifera* Bolton, has only been found rarely (Bolton, 1988a), and for which material was unavailable. A list of taxa with collection information and GenBank accession numbers is provided in Table 2. Vouchers were deposited in the collection of R. Savolainen, at the Department of Biological and Environmental Sciences, at the University of Helsinki.

### MOLECULAR METHODS

We crushed the ants in liquid nitrogen and added Proteinase K (Fermentas, St. Leon-Roth, Germany)

for overnight cell digestion at 60 °C. We extracted genomic DNA using the Nucleo Spin Tissue kit (Macherey–Nagel). We amplified eight gene fragments (using the primers in Table 3): mitochondrial *cytochrome oxidase subunit I (COI)*; nuclear ribosomal genes *18S* and *28S* (extension regions 1 and 2); and nuclear protein-coding genes *abdominal A (AbdA)*, *arginine kinase (ArgK)*, exon I, intron and exon II, the *F1* copy of *elongation factor-1 $\alpha$  (EF-1 $\alpha$ )*, and *long-wavelength rhodopsin (LwRh)*. GenBank accession numbers can be found in Table 2.

The 20- $\mu$ L mixture for PCR amplification included the following final concentrations of reagents: 0.75 $\times$  buffer, 0.09 mM deoxyribonucleotide triphosphates (dNTPs), 3.1 mM MgCl<sub>2</sub>, 0.5 U Taq polymerase (Fermentas), 0.5  $\mu$ M of each primer, and 0.8–1.5  $\mu$ L of DNA. We amplified DNA through 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. Subsequently, we 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<sub>96</sub> sequencing reaction clean-up 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).

### PHYLOGENETIC ANALYSES

We aligned the sequences for each gene fragment using Mafft 6.606b (Katoh *et al.*, 2005). We used the iterative refinement method with local pairwise alignment information (L-INS-i), and with the maximum number of iterations set at 1000. We checked and edited the alignments, and concatenated all gene fragments in MacClade 4 (Maddison & Maddison, 2000). The final alignment is available in TreeBase (study S2372; matrix M4504). The data included 37 ingroup taxa, and *Aphaenogaster rudis* Enzman, *Aphaenogaster senilis* Mayr, *Crematogaster auberti* Emery, *Crematogaster scutellaris* (Olivier), *Tetramorium caespitum* L., *Tetramorium impurum* (Foerster) (of Myrmicinae), *Lasius alienus* (Foerster), and *Formica fusca* L. (of Formicinae) as out-group taxa.

To increase our taxon sampling and infer the position of Myrmicini in the subfamily Myrmicinae, we supplemented our data with sequences of the subfamily Myrmicinae from two recent studies on ant phylogenetics (Brady *et al.*, 2006; Moreau *et al.*, 2006). We retrieved the two respective alignments (matrices M2958 and M2724) from Treebase, combined them, and excluded all taxa not belonging to Myrmicinae. Finally, we combined that data with our own sequences of Myrmicini. Because the data from the

**Table 2.** Overview of collection data for Myrmicini and outgroups used in this study, including sampling locality, geographical coordinates, voucher deposition and GenBank accession numbers

Number	Species	Locality	Coordinates	Voucher	Genbank accession numbers							
					18S	28SD1	28SD2	AbdA	ArgK	EF-1 $\alpha$	LurH	COI
507	<i>Eutetrarium mocoquersi</i>	Andampibe, Cap Masc-ola, Madagascar	15°41'37"S, 50°10'53.40"E	leg. G. Alpert; R. Savolainen 92/2001	FJ824195	FJ824240	FJ824285	FJ824335	FJ824385	FJ824507	FJ824462	FJ824417
506	<i>Eutetrarium monticelli</i>	Morondara Kivindy Forest, Madagascar	20°04'28.50"S, 44°40'34"E	leg. G. Alpert; R. Savolainen 89/2001	FJ824196	FJ824241	FJ824286	FJ824333	FJ824386	FJ824508	FJ824463	FJ824418
M14	<i>Hylomyrma balzani</i>	Napo, Ecuador	1°17'10"S, 77°53'26"W	leg. J.M. Vieira, sent D. Donoso; G. Jansen M14	FJ824198	FJ824243	FJ824288	FJ824330	FJ824387	FJ824510	FJ824465	FJ824420
501	<i>Hylomyrma dentiloba</i>	Costa Rica	n.a.	leg. T. McGlynn 97681; R. Savolainen 2/2001	FJ824199	FJ824244	FJ824289	FJ824337	FJ824388	FJ824511	FJ824466	FJ824421
370	<i>Huberia brounii</i>	Lake Waikamoana, New Zealand	38°45'30"S, 177°30"E	leg D. Ward; G. Jansen 370	FJ824200	FJ824245	FJ824290	FJ824332	FJ824389	FJ824512	FJ824468	FJ824422
371	<i>Huberia brounii</i>	Bay of Plenty, Paenga-roa, New Zealand	n.a.	leg. RAB Leschen; sent D. Ward; G. Jansen 371	FJ824201	FJ824246	FJ824291	FJ824339	FJ824390	FJ824513	FJ824469	FJ824423
500	<i>Huberia striata</i>	The Sign of the Bellbird Nature Reserve, Port Hills, New Zealand	n.a.	leg. L. Booth; R. Savolainen 1/2001	FJ824202	FJ824247	FJ824292	X	FJ824391	FJ824514	FJ824467	FJ824424
M9	<i>Myrmica americana</i>	Medford, Suffolk Co., NY, USA	n.a.	leg. R. Savolainen 82/00	FJ824204	FJ824251	FJ824294	FJ824334	FJ824392	FJ824516	FJ824471	FJ824428
M8	<i>Myrmica angulata</i>	Cong Troi, Sa Pa, Lao Cai, Vietnam	n.a.	leg. K. Eguchi; R. Savolainen 196/2002	FJ824205	FJ824252	FJ824295	FJ824336	FJ824393	FJ824517	FJ824472	FJ824429
M6	<i>Myrmica arisana</i>	Yuanfeng, Nantou Co., Taiwan	n.a.	leg. R. Savolainen 148/2002	FJ824206	FJ824253	FJ824296	FJ824335	FJ824394	FJ824518	FJ824473	FJ824430
28	<i>Myrmica brevispinosa</i>	Saguenay-Lac S. Jean, Quebec, Canada	n.a.	leg. G. Jansen 28/2005	FJ824207	FJ824254	FJ824297	FJ824335	FJ824395	FJ824519	FJ824474	FJ824431
M13	<i>Myrmica excelsa</i>	Wadotage Pass, Mat-sumoto, Japan	n.a.	leg. R. Savolainen 2712	FJ824208	FJ824255	FJ824298	FJ824335	FJ824396	FJ824520	FJ824475	FJ824432
159	<i>Myrmica formosae</i>	Fancy Lake, Chiayi Co., Taiwan	n.a.	leg. R. Savolainen 159/2002	FJ824209	FJ824256	FJ824299	FJ824336	FJ824397	FJ824521	FJ824476	FJ824433
51	<i>Myrmica incompleta</i>	Chaudière-Appalaches, Quebec, Canada	n.a.	leg. G. Jansen 51/2005	FJ824210	FJ824257	FJ824300	FJ824334	FJ824398	FJ824522	FJ824477	FJ824434
M2	<i>Myrmica jessensis</i>	Takenegahara, Hokkaido, Japan	n.a.	leg. R. Savolainen 123/2002	FJ824211	FJ824258	FJ824301	FJ824334	FJ824399	FJ824523	FJ824478	FJ824435
M5	<i>Myrmica kotokui</i>	Shirataki-mura district, Hokkaido, Japan	n.a.	leg. R. Savolainen 125/2002	FJ824212	FJ824259	FJ824302	FJ824334	FJ824400	FJ824524	FJ824479	FJ824436
178	<i>Myrmica lobicornis</i>	Koverhar, Hanko, Finland	n.a.	leg. R. Savolainen 98/2001	FJ824213	FJ824260	FJ824303	FJ824333	FJ824401	FJ824525	FJ824480	FJ824437
508	<i>Myrmica monticola</i>	Grand Junction Co., Colorado, USA	n.a.	leg. R. Savolainen 67/1998	FJ824214	FJ824261	FJ824304	FJ824332	FJ824402	FJ824526	FJ824481	FJ824438
R510	<i>Myrmica nearctica</i>	Curecanati Natl. Rec. Area, Colorado, USA	n.a.	leg. R. Savolainen 92/1998	FJ824215	FJ824262	FJ824305	FJ824335	FJ824403	FJ824527	FJ824482	FJ824439
175	<i>Myrmica rubra</i>	Tvärminne, Hanko Finland	n.a.	leg. R. Savolainen 201/1998	FJ824216	FJ824263	FJ824306	FJ824337	FJ824404	FJ824528	FJ824483	FJ824440
109	<i>Myrmica ruginodis</i>	Tvärminne, Hanko, Finland	n.a.	leg. R. Savolainen 268/1996	FJ824217	FJ824264	FJ824325	FJ824339	FJ824405	FJ824529	FJ824484	FJ824441
218	<i>Myrmica rugulosa</i>	Kallvik, Helsinki, Finland	n.a.	leg. R. Savolainen 420/1999	FJ824218	FJ824249	FJ824307	FJ824335	FJ824406	FJ824530	FJ824485	FJ824426
M12	<i>Myrmica sabuleti</i>	Koverhar, Hanko, Finland	n.a.	leg. R. Savolainen 393/1999	FJ824219	FJ824265	FJ824308	FJ824338	FJ824407	FJ824531	FJ824486	FJ824442
209	<i>Myrmica scabrinodis</i>	Kaurastensuo, Lammi, Finland	n.a.	leg. R. Savolainen 391/1999	FJ824220	FJ824266	FJ824309	FJ824334	FJ824408	FJ824532	FJ824487	FJ824443

Table 2. Continued

Number	Species	Locality	Coordinates	Voucher	Genbank accession numbers							
					J8S	28SD1	28SD2	AbdA	ArgK	EF-1 $\alpha$	L $\alpha$ Rh	COI
M4	<i>Myrmica serica</i>	Tsuifeng, Nantou Co., Taiwan	n.a.	leg. R. Savolainen 145/2002	FJ824221	FJ824267	FJ824310	FJ824355	FJ824409	FJ824533	FJ824488	FJ824444
R110	<i>Myrmica sulcinodis</i>	Täktom, Hanko, Finland	n.a.	leg. R. Savolainen 273/1996	FJ824222	FJ824250	FJ824311	FJ824342	FJ824410	FJ824534	FJ824489	FJ824427
190	<i>Manica inivida</i>	Crested Butte, Colorado, USA	n.a.	leg. R. Savolainen 166/1998	FJ824223	FJ824268	FJ824312	FJ824366	FJ824411	FJ824535	FJ824490	FJ824445
189	<i>Manica rubida</i>	Pieniny Mountains, Poland	n.a.	leg. W. Czechowski; R. Savolainen 312/1998	FJ824224	FJ824269	FJ824313	FJ824339	FJ824412	FJ824536	FJ824491	FJ824446
504	<i>Pogonomyrmex badius</i>	Appalachicola Natl Forest, Liberty Co., Florida, USA	30°15.85'N, 84°57.00'W	leg. S.P. Cover; R. Savolainen 39/2000	FJ824225	FJ824271	FJ824314	FJ824363	FJ824374	FJ824537	FJ824492	FJ824448
512	<i>Pogonomyrmex desertorum</i>	Portal, Arizona, USA	n.a.	leg. C.P. Strehli; R. Savolainen 4/2001	FJ824226	FJ824272	FJ824315	FJ824341	FJ824375	FJ824538	FJ824493	FJ824449
515	<i>Pogonomyrmex huachuacanus</i>	Portal, Arizona, USA	31°55'56.1"N, 109°12'26.1"W	leg. C.P. Strehli & J. Gadau; R. Savolainen 8/2001	FJ824227	FJ824273	FJ824316	FJ824347	FJ824376	FJ824539	FJ824494	FJ824450
516	<i>Pogonomyrmex imberbiculus</i>	Superior, Arizona, USA	33°19'09.2"N, 111°06'46.6"W	leg. C.P. Strehli; R. Savolainen 9/2001	FJ824228	FJ824274	FJ824317	FJ824336	FJ824377	FJ824540	FJ824495	FJ824451
513	<i>Pogonomyrmex maritropa</i>	Globe, Arizona, USA	n.a.	leg. C.P. Strehli; R. Savolainen 5/2001	FJ824229	FJ824270	FJ824318	FJ824353	FJ824378	FJ824541	FJ824496	FJ824447
503	<i>Pogonomyrmex occidentalis</i>	Fruita, Colorado, USA	n.a.	leg. R. Savolainen 52/1998	FJ824230	FJ824275	FJ824319	FJ824340	FJ824379	FJ824542	FJ824497	FJ824452
M10	<i>Pogonomyrmex occidentalis</i>	Arizona, USA	35°17'58"N, 112°52'04.8"W	leg. J. Gadau; R. Savolainen 7/2001	FJ824231	FJ824276	FJ824320	FJ824367	FJ824380	FJ824543	FJ824498	FJ824453
517	<i>Pogonomyrmex pima</i>	Salt River Rec. Area, Tucson, AZ, USA	n.a.	leg. J. Gadau; R. Savolainen 10/2001	FJ824232	FJ824277	FJ824321	FJ824352	FJ824381	FJ824544	FJ824499	FJ824454
514	<i>Pogonomyrmex rugosus</i>	Tempe, Arizona, USA	32°56'14.90"N, 111°42'18.1"W	leg. C.P. Strehli; R. Savolainen 6/2001	FJ824233	FJ824278	FJ824322	FJ824338	FJ824382	FJ824545	FJ824500	FJ824455
Out-groups												
R518	<i>Aphaenogaster rudis</i>	Medford, Suffolk Co., NY, USA	n.a.	leg. R. Savolainen 83/2000	FJ824191	FJ824236	FJ824281	X	FJ824372	FJ824503	FJ824458	FJ824413
R505	<i>Aphaenogaster senilis</i>	San Cugat, Catalonia, Spain	n.a.	leg. R. Savolainen 277/2000	FJ824192	FJ824237	FJ824282	FJ824329	FJ824373	FJ824504	FJ824459	FJ824414
R616	<i>Crematogaster auberti</i>	Sant Llorenç del Munt i' Obac, Catalonia, Spain	n.a.	leg. R. Savolainen 265/2000	FJ824193	FJ824238	FJ824283	X	FJ824383	FJ824505	FJ824460	FJ824415
R617	<i>Crematogaster scutellaris</i>	San Cugat, Catalonia, Spain	n.a.	leg. R. Savolainen 281/2000	FJ824194	FJ824239	FJ824284	FJ824326	FJ824384	FJ824506	FJ824461	FJ824416
R129	<i>Formica fusca</i>	Tvärminne, Hanko, Finland	n.a.	leg. R. Savolainen 262/1996	FJ824197	FJ824242	FJ824287	FJ824331	FJ824368	FJ824509	FJ824464	FJ824419
R62	<i>Lasius alienus</i>	Zscheplitz, Freyburg, Sachsen-Anhalt, Germany	n.a.	leg. R. Savolainen 360/1999	FJ824203	FJ824248	FJ824293	FJ824327	FJ824369	FJ824515	FJ824470	FJ824425
R192	<i>Tetramorium caespitum</i>	Tvärminne, Hanko, Finland	n.a.	leg. W. Czechowski; R. Savolainen 296/1998	FJ824234	FJ824280	FJ824323	X	FJ824370	FJ824546	FJ824501	FJ824457
R191	<i>Tetramorium impurum</i>	Pieniny Mountains, Poland	n.a.	leg. W. Czechowski; R. Savolainen 343/1998	FJ824235	FJ824279	FJ824324	FJ824328	FJ824371	FJ824547	FJ824502	FJ824456

Unavailable geographical coordinates are indicated with 'n.a.'. Missing sequences are denoted with an 'X'.

**Table 3.** Oligonucleotide primers used in this study (JS data)

Gene	Primer	5'–3' sequence	Reference
<i>18S</i>	18SA	AGCAGCCGCGGTAATACCAG	B. Sullender, unpublished (through T.R. Schultz)
	18SB	ATGCTTTCGCTTCTGGTCCGT	B. Sullender, unpublished (through T.R. Schultz)
<i>28SD1</i>	28SA	CCCCCTGAATTTAAGCATAT	Schmitz & Moritz, 1994
	28SC	CGGTTTCACGTACTCTTGAA	Schmitz & Moritz, 1994
<i>28SD2</i>	28SF2	AGAGAGAGTTCAAGAGTACGTG	Belshaw & Quicke, 1997
	28S3DR	TTGGTCCGTGTTTCAAGACGGG	Belshaw & Quicke, 1997
<i>AbdA</i>	AA1181F	ACCGGCGATATGAGTACGAAATT	De Menten <i>et al.</i> , 2003, modified by Ward & Downie, 2005
	AA1881R	GTTTGTGGCAGGATGTCAAAGG	De Menten <i>et al.</i> , 2003, modified by Ward & Downie, 2005
	AA1182F	CCGGCGATATGAGTACGAAATTC	De Menten <i>et al.</i> , 2003, modified by Ward & Downie, 2005
	AA1824R	TAGAAYTGTGCCGCCGCTGCCAT	De Menten <i>et al.</i> , 2003, modified by Ward & Downie, 2005
<i>ArgK E1</i>	AK1F2	TGGTTGAYGCGYCYGTTYTGGA	P.S. Ward, unpublished
	AK461R	GTGCTRGAYACYTTCTCYTCCAT	P.S. Ward, unpublished
	AK4F2	GTTGAYGCGYCYGTTYTGAYAA	P.S. Ward, unpublished
	AK392R	TCCAARGAGCGRCCGCATC	P.S. Ward, unpublished
<i>ArgK E2</i>	AK346EF	AGGGTGARTACATCGTRTCHACT	P.S. Ward, unpublished
	AK720ER	ACCTGYCCRAGRTCACCRCCCAT	P.S. Ward, unpublished
<i>COI</i>	LCO	GGTCAAACAATCATAAAGATATTGG	Folmer <i>et al.</i> , 1994
	HCO	TAAACTTCAGGGTGACCAAAAAATCA	Folmer <i>et al.</i> , 1994
	Ron	GGATCACCTGATATATAGCATTCCC	Simon <i>et al.</i> , 1994
	Jerry	CAACATTTATTTTGTATTTTTTGG	Simon <i>et al.</i> , 1994
	Pat	TCCAATGCACTAATCTGCCATATTA	Simon <i>et al.</i> , 1994
<i>EF-1<math>\alpha</math></i>	TRS4F	GCGCCKGCGGCTCTCACCACCGAGG	Brady <i>et al.</i> , 2006
	TRS9.1b	GGAAGGCCTCGACGCACATCGG	Brady <i>et al.</i> , 2006
	TRS10R	ACGGCSACKGTTTGWCKCATGTC	T.R. Schultz, unpublished
<i>LwRh</i>	LR143F	GACAAAGTKCCACCRGARATGCT	Ward & Downie, 2005
	LR182F	CACTGGTATCARTTCGCACCSAT	P.S. Ward, unpublished
	LR639R	YTTACCGRTTCCATCCRAACA	Ward & Downie, 2005
	LR672R	CCRCAMGCWGTGTCATGTTTCCTTC	P.S. Ward, unpublished

For *ArgK* exon 1, the initial amplification was performed using the first two primers, and the reamplification was performed with the last two.

above two studies had a different set of taxa and gene fragments than our study, we could only include those sequences that overlapped with ours. The final alignment thus included sequences stemming from *28SD1*, *28SD2*, *AbdA*, and *LwRh*. We deleted ambiguous regions, because of alignment uncertainty in introns and in regions with secondary structure (*28SD2*) outweighed the phylogenetic signal, resulting in a final alignment of 1508 sites for 119 taxa. We will refer in the methods and analyses to our data as JS (for Jansen and Savolainen), to separate it from the combined data (ALL).

For JS, we analysed the genes separately and concatenated. For ALL, we only analysed the concatenated data. For each gene region of JS, we chose the best-fit nuclear substitution model (Table 4) according to the Akaike information criterion in Modeltest 3.7

(Posada & Crandall, 1998). We then analysed the data using maximum likelihood (ML) and Bayesian phylogenetic inference (BI).

We used RaxML 7.0.4 (Stamatakis, 2006a) for the ML analyses. We inferred the gene topologies using the GTRCAT approximation with 1000 rapid bootstraps. The GTRCAT approximation is a faster and more efficient way to accommodate rate heterogeneity in phylogenetic analyses than the conventional  $\Gamma$  model (Stamatakis, 2006b). For each site in an alignment the  $\Gamma$  model calculates the probability of evolving at any rate in a given distribution (the precise shape of the distribution is itself controlled by the empirically determined  $\alpha$  shape parameter). In contrast, the CAT-approximation maps each individual rate into one of a fixed number of rate categories (by default 50 in RaxML), and calculates only one prob-

**Table 4.** Gene fragments, their lengths in base pairs (bp), number of variable sites, and mean GC content per gene fragment in percentages (%)

Gene fragment	Length (bp)	Variable sites (%)	Mean GC (%)	Model
<i>COI</i>	1377	49.3	29.4	GTR + $\Gamma$ + I
<i>18S</i>	373	1.1	49.9	GTR
<i>28SD1</i>	349	7.2	55.9	GTR + I
<i>28SD2</i>	543	39.9	68.7	GTR + $\Gamma$ + I
<i>ArgK</i> (coding)	636	31.8	54.7	GTR + $\Gamma$ + I
<i>ArgK</i> intron	312	59.2	20.8	GTR + I
<i>AbdA</i>	540	21.1	62.9	HKY + $\Gamma$ + I
<i>LwRh</i> (coding)	405	33.1	51.6	GTR + $\Gamma$
<i>LwRh</i> intron	102	71.6	47.8	K81 + $\Gamma$
<i>EF-1<math>\alpha</math></i>	336	17.6	60.6	GTR + $\Gamma$
Concatenated JS	4973	35.0	48.1	GTR + $\Gamma$ + I
Concatenated ALL	1580	40.6	59.8	GTR + $\Gamma$ + I

Characteristics for the genes are listed separately, and are concatenated for JS data and concatenated for ALL data. The formicine outgroups *Formica fusca* and *Lasius alienus* were excluded from these statistics. The last column lists the best-fit substitution models as inferred using the Akaike information criterion in Modeltest 3.7.

ability per site. This results in dramatic performance increases, and often gives better likelihood values than ML analyses using a normal  $\Gamma$  model (Stamatakis, 2006b). Compared with GTRGAMMA, GTRCAT therefore allows for a more thorough analysis within any given period of time. We then conducted a complete ML search using the GTRCAT approximation, but switched to GTRGAMMA (a conventional GTR +  $\Gamma$  model) to evaluate the final tree topology (this yields stable likelihood values). For the concatenated JS data, we applied three partitioning strategies: (1) unpartitioned data with GTR +  $\Gamma$  + I; (2) partitioned data to gene regions using GTRCAT for each region (for *LwRh* and *ArgK* we gave a separate partition to the intron and to the coding region, making ten partitions in total); (3) partitioned data to gene regions, as in strategy 2, and codon position, combining for each protein coding gene (*AbdA*, *ArgK*, *COI*, *EF-1 $\alpha$* , and *LwRh*) the first two positions in one partition, and the third position in another (in total 15 partitions). We applied a GTRCAT approximation to each partition, and performed 1000 rapid bootstraps, as well as a full ML search, as described above. We similarly analysed the ALL data, using partitioning strategy 2.

We used Bayesian phylogenetic inference in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) for the two concatenated datasets (ALL and JS). For the BI analyses of JS we used the three partitioning strategies defined above, applying the models of Table 4 to each partition. For ALL, we used partition strategy 2. All partition parameters were unlinked, and the standard flat Dirichlet priors were used. However, we kept the topology linked across parti-

tions, with a uniform prior. For branch lengths we used an unconstrained Dirichlet prior (branch lengths were unlinked across partitions). We ran all BI analyses for 10 million generations. For most partitioned analyses we used the Murska supercluster of the CSC – IT Center for Science Ltd. For the remaining BI analyses, we used a Mac 5, and for the RaxML analyses, we used a Dell Precision M6300 running Ubuntu Linux (program compiled from source using gcc 4.2). We assessed burn-in by plotting likelihoods vs. generations in Tracer 1.4 (Rambaut & Drummond, 2007). We discarded all data obtained before likelihood values reached a plateau. Burn-in values varied between one million and two million generations. The burn-in numbers and statistics of consensus tree calculations are shown in Table 5.

## RESULTS

### SEQUENCE CHARACTERISTICS

We successfully sequenced all seven gene fragments for most of the 45 taxa in our study. Four sequences were missing for *AbdA* (*A. rudis*, *C. auberti*, *Huberia striata* Forel, *T. caespitum*), five were missing for exon 2 of *ArgK* [*Eutetramorium mocquerysi* Emery, *Hylomyrma dentiloba* (Santchi), *Huberia brounii* Forel, *Myrmica angulata* Radchenko, Zhou & Elmes, and *Pogonomyrmex huachucanus* Wheeler], and three *COI* sequences were incomplete [*E. mocquerysi*, *Hylomyrma balzani* (Emery), and *M. angulata*]. The concatenated JS data comprised 4973 aligned positions; the ALL data comprised 1580 aligned positions. The *18S* and *28SD1* fragments were almost invari-

**Table 5.** Data sets (JS and ALL), partition strategies 1–3, and number of partitions in each in parentheses, burn-in and consensus tree numbers in the Bayesian analyses

Data	Partitioning strategy	Burn-in ( $\times 1000$ generations)	Trees in consensus
JS	1 GTR + $\Gamma$ + I (1)	1000	18 002
	2 Genes (10)	1000	18 002
	3 Genes + codons (15)	1500	17 002
ALL	Genes (4)	2000	16 002

Each Bayesian run had ten million generations.

able (< 10% variable sites), whereas *COI* and the introns of *ArgK* and *LwRh* were rather variable (> 50%). Table 4 summarizes the length, variability, and GC content of each fragment of the concatenated JS and ALL data.

#### PHYLOGENETIC RESULTS

The Bayesian and ML analyses of the concatenated JS data showed that Myrmicini is polyphyletic (Fig. 1). The six genera of Myrmicini included in this study formed three separate, well-supported clades. We found sister-group relationships between *Myrmica* and *Manica* (posterior probability, PP = 100%; bootstrap support, BS = 100%), *Pogonomyrmex* and *Hylomyrma* (PP = 100; BS = 100), and *Huberia* and *Eutetramorium* (PP = 100; BS = 95). The last clade clustered with *Crematogaster* of the tribe Crematogastrini (PP = 100; BS = 92), and these clustered with the clade *Aphaenogaster* of the tribe Pheidolini and *Tetramorium* of the tribe Tetramoriini (PP = 100; BS = 100).

In the Bayesian analyses, the topology was insensitive to parametrizations, although convergence was difficult to obtain for partitioning strategy 3 (the data were divided into genes and codons, see Table 5). Nevertheless, the standard deviation of split frequencies was lower than 0.01 in all other analyses, indicating convergence was reached. ML bootstrap percentages were generally lower than BI probabilities, but the ML topology was identical to the one obtained with BI.

The analyses further found all genera to be monophyletic, with 100% posterior probability and bootstrap support. Within *Myrmica* we found support for the monophyly of several morphologically defined species groups: *ritae* (PP = 100; BS = 100), *rubra* (PP = 100; BS = 100), *scabrinodis* (PP = 100; BS = 100), and *lobicornis* (PP = 100; BS = 100). The Nearctic species groups were not monophyletic, as *Myrmica incompleta* Provancher did not cluster with the *Myrmica nearctica* Weber–*Myrmica americana* Weber clade.

Within *Pogonomyrmex*, we found support for the *californicus* (PP = 100; BS = 85) and *barbatus* (PP = 100; BS = 100) complexes. Because the analysis only included two representatives of *Pogonomyrmex occidentalis* (Cresson), we could not evaluate the monophyly of the *occidentalis* complex. The three representatives of *Ephebomyrmex* (*Pogonomyrmex imberciculus* Wheeler, *Pogonomyrmex huachucanus* Wheeler, and *Pogonomyrmex pima* Wheeler) were paraphyletic.

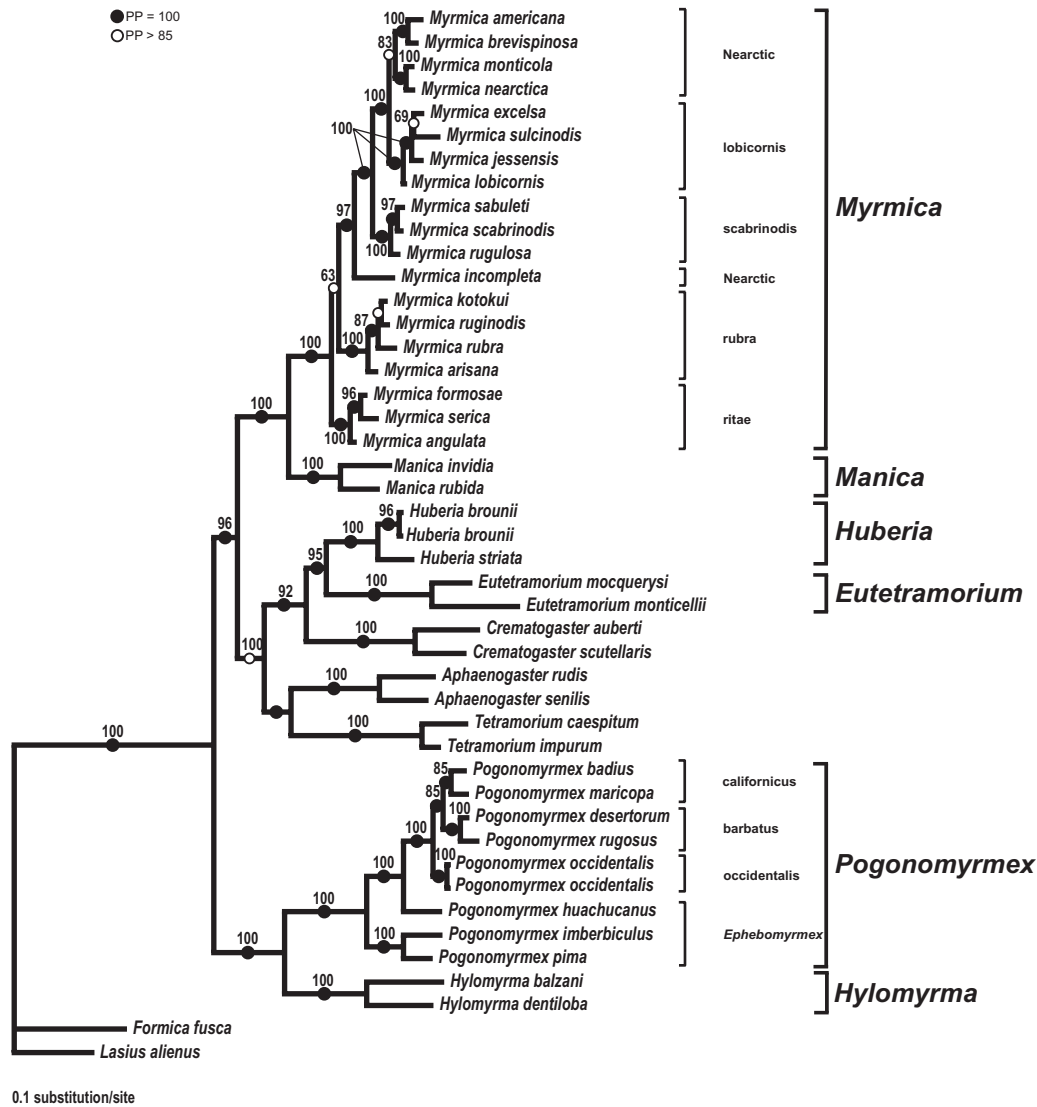
In the separate analyses of the genes (trees not shown), both BI and ML analyses failed to recover any structure for *18S*. All other gene trees (not shown) supported the monophyly of *Pogonomyrmex* (PP and BS = 91–100) and *Huberia* (PP and BS = 66–100). Most analyses also supported the monophyly of *Myrmica* (PP and BS = 64–96, except for *ArgK* and *EF-1 $\alpha$* ), *Eutetramorium* (PP and BS = 98–100, except for *28SD1* and *LwRh*), *Hylomyrma* (PP and BS = 78–100, except for *EF-1 $\alpha$*  and *28SD1*), and *Manica* (PP and BS = 66–100, except for *COI*). The sister-group relationships between *Hylomyrma* and *Pogonomyrmex* (PP and BS = 70–100, not present in *COI* and *EF-1 $\alpha$* ), and between *Myrmica* and *Manica* (PP and BS = 66–100, unresolved in *COI*), were found in most gene trees. Other, more basal relationships among genera were unresolved in the gene trees.

The ML and BI analyses of the ALL data only supported the monophyly of some genera, including each genus of Myrmicini and some small clades (Fig. 2). Only the sister-group relationships between *Myrmica* and *Manica* (PP = 99; BS = 99), and between *Pogonomyrmex* and *Hylomyrma* (PP = 99; BS = 99), received high support; other interrelationships among the members of Myrmicini and among the other myrmicine genera were unresolved. The *Huberia* + *Eutetramorium* clade received lower support in ALL compared with the JS analyses.

#### DISCUSSION

The morphological definition of Myrmicini currently places seven genera in the tribe (Bolton, 2003).



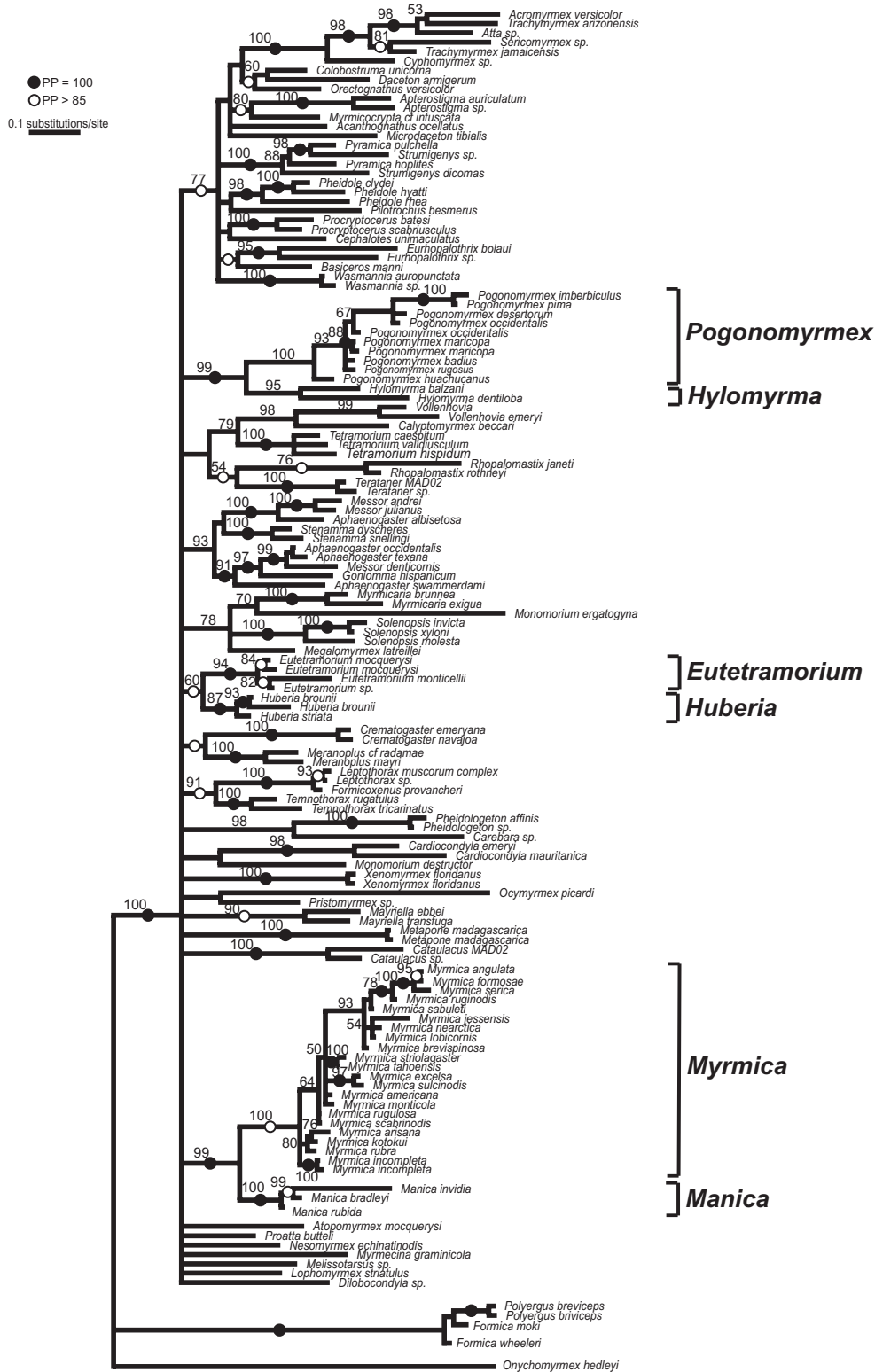


**Figure 1.** Majority-rule consensus tree (18 002 Bayesian trees, with a burn-in of one million generations) of 37 Myrmicini in-group taxa, and six members of Myrmicinae and two Formicinae as out-group taxa (JS data matrix, see Material and methods for details). This topology was obtained with all partition strategies (1–3, see Material and methods for details). Nodal support values were similar across analyses; the values shown were obtained from partitioning data according to gene regions (strategy 2). On branches, solid circles (●) indicate that the posterior probability = 100%; open circles (○) indicate that the posterior probability > 85%. Numbers above branches represent maximum-likelihood bootstrap support (1000 replicates). On the right, genera of Myrmicini and species groups of *Myrmica* and *Pogonomyrmex* are listed; Nearctic species groups are not known.

Recent molecular studies suggested, however, that the tribe may not be monophyletic (Brady *et al.*, 2006; Moreau *et al.*, 2006), although they differed on the generic composition of the tribe. Contrary to these molecular studies, we had representatives of all genera of Myrmicini, except for *Secostruma*. We obtained comprehensive DNA sequence data for almost all taxa, which yielded a robust and well-supported phylogenetic hypothesis. Our results clearly demonstrated that the tribe Myrmicini as cur-

rently defined is not monophyletic. Rather, we found that Myrmicini is a compound of three unrelated lineages. Unfortunately, the analysis of the Myrmicinae yielded a basal polytomy, and therefore we could not assess the placement of these three clades within the subfamily.

*Pogonomyrmex*, *Hylomyrma*, *Myrmica*, and *Manica* have always been placed in the tribe Myrmicini, based on their presumably unspecialized morphology (Wheeler, 1922). They resemble each other in having



**Figure 2.** Majority-rule consensus tree (16 002 Bayesian trees, with a burn-in of two million generations) of 111 Myrmicinae in-group taxa and *Polyergus*, *Formica*, and *Lasius* (Formicinae) as out-group taxa (ALL data matrix, see Material and methods for details). On branches, full circles (●) indicate that the posterior probability = 100%; open circles (○) indicate that the posterior probability > 85%. Numbers above branches represent maximum-likelihood bootstrap support (1000 replicates). The genera of Myrmicini are indicated on the right.

a complex metasternum, a nodiform petiole, and the absence of a promesonotal suture (Bolton, 2003). Brady *et al.* (2006) also recovered a close relationship between *Pogonomyrmex*, *Myrmica*, and *Manica*. However, that clade received low bootstrap support (61% in the parsimony analysis; 55% in the ML analysis), and was not present in their ML optimizations of alternative rootings. Moreau *et al.* (2006) presented a tree where *Pogonomyrmex* was unrelated to *Myrmica*. Our results suggest that *Myrmica* and *Manica* are unrelated to *Pogonomyrmex* and *Hylomyrma*. However, the inclusion of more myrmicine and formicine outgroups is needed to resolve the relationships of these two clades, because alternative rooting could make them monophyletic.

*Myrmica* and *Manica* are biologically similar, and share a number of morphological characters. *Manica* differs from *Myrmica*, however, in the absence of propodeal spines (although a few *Myrmica* also lack spines), a deeper mesoepinotal suture, mandibles with smaller and more numerous teeth, and males with worker-like mandibles and distinct genitalia (Weber, 1947; Creighton, 1950). Our results agree with Astruc *et al.* (2004) and Brady *et al.* (2006), who previously recovered *Myrmica* and *Manica* as sister groups.

The sister-group relationship between *Pogonomyrmex* and *Hylomyrma* in our tree agreed with previous studies (Bolton, 2003, and references therein). *Hylomyrma* closely resembles *Pogonomyrmex*, especially the species of the former subgenus *Ephebomyrmex*, in also having short frontal carinae, a lack of transverse sutures on the dorsum of the thorax, armed propodea, pedunculate petioles, and pectinate spurs (Kempf, 1973). But, *Hylomyrma* differs from *Pogonomyrmex* in mandible shape, structure of clypeus, metasternal lobes, and petiole shape (Kempf, 1960).

For *Pogonomyrmex*, the species of the californicus, barbatus, and occidentalis species complexes included in this study all formed monophyletic groups. The analyses also supported the basal position of *Ephebomyrmex*. Taber (1990) found a sister-group relationship between the californicus and occidentalis complexes. In contrast, we discovered a sister-group relationship between the californicus and barbatus complexes, although only with medium support. Lattke (1990) synonymized *Ephebomyrmex* and *Forelomyrmex* with *Pogonomyrmex*, although others considered *Ephebomyrmex* to be a subgenus of *Pogonomyrmex* (Kempf, 1972; Snelling, 1982; MacKay *et al.*, 1985; Taber, 1998) or even a genus (Creighton, 1950; Wheeler & Wheeler, 1985; Hölldobler & Wilson, 1990). In their molecular analysis of social parasites in *Pogonomyrmex*, Parker & Rissing (2002) found *Ephebomyrmex* to be paraphyletic. In our tree,

*Ephebomyrmex* was paraphyletic but basal within *Pogonomyrmex*.

Finally, *Huberia* and *Eutetramorium* have been difficult to classify. Both were only recently placed in Myrmicini, although they have a small metasternal process instead of the large, conspicuous one found in the other genera of Myrmicini (Bolton, 2003). *Huberia* may resemble *Myrmica* in general habitus, but differs from all other Myrmicini genera (*sensu* Bolton, 2003) in having an 11-jointed antenna in females, and a forewing with a single small cubital cell (Forel, 1893). *Eutetramorium* was inferred to be unrelated to *Myrmica*, *Pogonomyrmex*, and *Manica* (Brady *et al.*, 2006; Moreau *et al.*, 2006, *Manica* absent in latter). Instead, it clustered with *Metapone*, the only member of the tribe Metaponini (Brady *et al.*, 2006), or *Colobostruma*, a member of Dacetini (Moreau *et al.*, 2006). Neither study aimed to resolve the relationships within Myrmicinae, and therefore taxon sampling was too limited to obtain good statistical support for most clades. Thus, the positions of *Eutetramorium* and *Huberia* relative to the other Myrmicinae remain disputable.

As illustrated above, the morphological diagnosis of Myrmicini is difficult and contradicts molecular data. The commonly used morphological characters to recognize the genera of Myrmicini are metatibial spurs on the middle and hind tibia, and the presence of two closed cubital cells in the forewing (Forel, 1893). However, these characters are not apomorphic (Bolton, 2003). For instance, tibial spurs show a sequence of degradation from long and pectinate to absent in *Myrmica* (Bolton, 1988b). The lack of apomorphies for Myrmicini and the presumably unspecialized morphology of its genera may thus be interpreted as a result of convergence rather than close relatedness. Bolton (1976) also noted such morphological similarities between *Myrmica* and *Tetramorium*: morphological resemblances thus form an unreliable proxy for the interrelationships among myrmicine genera.

The monophyly of the tribe Myrmicini may also be questioned from a biogeographical point of view. Divergence time estimations placed the crown age of ants at 111–137 Mya (Brady *et al.*, 2006) or 140–168 Mya (Moreau *et al.*, 2006). Modern Myrmicinae, including *Myrmica* and *Pogonomyrmex*, are much younger, and have only been found from Eocene deposits (*Myrmica* in Baltic and Saxonian amber of 44.1 Mya, Radchenko, Dlussky & Elmes, 2007; *Pogonomyrmex* in Florissant shales of 34.0 Mya, Carpenter, 1930). However, Madagascar and New Zealand were separated from other land masses tens of millions of years before the rise of ants, since the breakup of Pangaea 180 Mya (Raven & Axelrod, 1974). Genera endemic to remote areas such as New

Zealand (*Huberia*) and Madagascar (*Eutetramorium*) are thus unlikely to form a monophyletic clade with the genera found in the Holarctic (*Manica* and *Myrmica*). The current composition of the tribe therefore would require a complicated but not impossible biogeographical scenario to explain its distribution. However, our data offers an easier explanation: they are unrelated.

Our analysis only included a few representatives of the subfamily Myrmicinae, but inferred the interrelationships among the genera of Myrmicini with good support. However, we could not ascertain the position of the genera within Myrmicinae. With 6149 described species (Agosti & Johnson, 2005), Myrmicinae represents nearly 50% of the total known ant diversity, and is thus by far the largest, most diverse, and successful of the ant subfamilies. The higher relationships among myrmicines are unascertained, except for a few small, specialized tribes such as Attini, Melissotarsini, and Myrmicariini (they did not undergo many taxonomic changes; see Bolton *et al.* 2006). Most are grouped based on inclusive characters, and therefore their monophyly is only a suggestion (Bolton, 2003). Thus, it is not surprising that molecular data have questioned the monophyly of several tribes, including Dacetini, Pheidolini, Solenopsidini, and Stenammini (Brady *et al.*, 2006; Moreau, 2008). Shattuck (1992) even abolished the use of tribes in the subfamily Dolichoderinae.

We found that the tribe Myrmicini is not monophyletic. The composition of Myrmicini and its relationships to other tribes in the subfamily Myrmicinae can only be addressed with a comprehensive molecular phylogeny of the entire subfamily. The insertion of our data into large published datasets increased taxon sampling, but reduced sequence length, yielding no resolution. This illustrates that many independent molecular markers and dense taxon sampling are required before robust conclusions can be drawn about the classification and evolution of this hyperdiverse subfamily.

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