

Molecular phylogenetic analysis of ant subfamily relationship inferred from rDNA sequences

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The relationships among ant subfamilies were studied by phylogenetic analysis of rDNA sequences of 15 species from seven subfamilies. PCR primers were designed on the basis of the rDNA sequence of the Australian bulldog ant, *Myrmecia croslandi*, previously determined. Phylogenetic trees were constructed using sequences of a fragment of 18S rDNA (1.8 kb), a fragment of 28S rDNA (0.7 kb excluding variable regions) and a combination of the 18S and 28S rDNAs, by neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML). rDNA sequences corresponding to the same fragments from three non-ant hymenopteran species (a sawfly, a bee and a wasp) were employed as outgroups. These trees indicated that the ant subfamilies were clustered singly, and, among the seven subfamilies examined, Ponerinae and six other subfamilies are in a sister-groups relationship. The relationship among the six subfamilies, however, was not clarified. The phylogenetic trees constructed in the present study are not in contradiction to the tree from cladistic analysis of morphological data by Baroni Urbani et al. (1992) and the tree from morphological and molecular data (Ward and Brady, 2003), but are inconsistent with the traditional phylogeny. The present results thus raise a question as to the status of some traditionally employed “key” morphological characters. The present results also call for a reexamination of *Amblyopone* traditionally treated as a member of Ponerinae as belonging to a new subfamily.

Key words: Formicidae, Hymenoptera, phylogeny, ribosomal RNA gene

INTRODUCTION

The ants are known as one of the most flourishing eusocial insects. They are taxonomically classified in the family Formicidae in the order Hymenoptera, and about 10,000 ant species have been described from the whole world excepting the north and south poles. Formicidae includes 16 extant subfamilies, but their phylogenetic relationships are yet to be clarified (Bolton, 1994). Since the work by Brown (1954), various alternative phylogenetic trees have been proposed (e.g., Wilson, 1971; Taylor, 1978; Hölldobler and Wilson, 1990; Baroni Urbani et al., 1992).

The subfamily relationship of ants has traditionally been constructed by using a small number of morphological key characters (e.g., post-petiole and abdominal segment IV). Myrmicinae and Ponerinae, were traditionally treated as members of the same group with key charac-

ters of laterally fused abdominal segment IV and a sting. Baroni Urbani et al. (1992) used a large number of morphological characters and reported a cladogram that is different from the traditional subfamily relationship, placing Myrmicinae and Ponerinae in separate groups. The cladistic analysis, as in the traditional phylogenetic tree, resulted in a basal division of extant Formicidae into two groups, although different in their constitutions. However, it is not always better to increase the number of morphological characters for phylogenetic analysis. Since workers in social insects have degenerated morphologies, one finds much convergence that is difficult to distinguish from synapomorphy (Hölldobler and Wilson, 1990).

Molecular phylogenetic studies may provide better resolution of ant phylogeny than that based solely on morphology, and could reveal a precise relationship among subfamilies. rDNAs have been used extensively for phylogenetic analysis (Long and Dawid, 1980). Since a unit of rDNA contains both conserved and variable regions, the comparison of sequences of rDNAs provides informa-

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tion on the relationships of both distantly and closely related groups. Variable regions of 28S rDNA have often been used in molecular phylogenetic studies on closely related species (Pélandakis and Solignac, 1993; Gimeno et al., 1997). They are not suitable, however, for studies on the subfamily relationship of ants, because highly variable sequences generally can not be aligned between distantly related species. We previously obtained a complete sequence of an entire single unit of rDNA of a bulldog ant, *Myrmecia croslandi* (Ohnishi and Yamamoto, in press), that hopefully allowed us to design PCR primers to amplify conserved region of rDNA of any ant species and other hymenopteran insects.

In the present paper, we analyzed sequences of 18S and 28S rDNA fragments amplified from 15 species from seven subfamilies of ants, employing those of three species (a wasp, a bee and a sawfly) as outgroups. The PCR primers were designed so as to amplify the majority of 18S rDNA and a portion of 28S rDNA including variable regions. Based on the sequence data, we constructed phylogenetic trees for 15 ant species and three non-ant hymenopteran species, which do not contradict the trees obtained by Baroni Urbani et al. (1992) and by Ward and Brady (2003) but are inconsistent with the traditional one. The results thus raise a question as to the relevance of the traditionally employed "key" morphological characters.

MATERIALS AND METHODS

Ants, a wasp, a bee and a sawfly specimens and genomic DNA extraction. The following 15 ant species from seven subfamilies were examined: Myrmecinae (*Myrmecia croslandi*), Nothomyrmecinae (*Nothomyrmecia macrops*), Pseudomyrmecinae (*Pseudomyrmex kuenckeli*), Formicinae (*Camponotus obscuripes*, *Formica japonica*, *Paratrechina* sp.), Dolichoderinae (*Ochetellus glaber*), Myrmicinae (*Aphaenogaster famelica*, *Pristomyrmex pungens*, *Tetramorium caespitum*) and Ponerinae (*Amblyopone australis*, *Pachycondyla* (= *Brachyponera*) *chinensis*, *Cryptopone sauteri*, *Diacamma rugosum*, *Odontomachus rixosus*). Three non-ant hymenopteran species used as outgroups were: *Vespa mandarinia* (Vespidae), *Apis mellifera* (Apidae) and *Athalia rosae* (Tenthredinidae).

Genomic DNAs were extracted from frozen (−80°C) or 100% ethanol-stored specimens as described (Sambrook et al., 1989).

DNA amplification and sequencing. The primer set employed for amplification of a 18S rDNA fragment was 5'-AGTAGTCATATGCTTGTCTC-3' (18S-U1) and 5'-AAT-CATTCAATCGGTAGTAG-3' (18S-L1). The 28S rDNA primer set was 5'-ACTAAGCGGAGGAAAAGAACTA-3' (28S-U1) and 5'-ACTCCTTGGTCCGTGTTTCA-3' (28S-

L2). These primers were designed based on the sequence of *M. croslandi* (Ohnishi and Yamamoto, in press; see also database, accession numbers AB052895 and AB121787) (Fig. 1). PCR amplification was conducted with *Ex Taq* polymerase (Takara), according to the manufacturer's protocol. Amplification conditions were 30 cycles of 1 min at 96°C, 1 min at 58°C, and 2 min at 74°C following the initial denaturation at 96°C for 2 min.

After electrophoresis, an amplified fragment was purified by SUPREC-01 (Takara). DNA sequences of PCR-amplified fragments were determined by the DyeDeoxy Terminator Cycle Sequencing kit (Perkin Elmer) on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). The sequences were deposited in DDBJ/EMBL/GenBank (accession Nos. AB126778 ~ 126810, AB121786, AB064266 and AB064267).

Phylogenetic analyses. Gene sequences of the ants and outgroups were aligned with the Clustal W program version 1.74 (Thompson et al., 1994) with the default settings of a gap-opening penalty 10, and a gap-extension penalty 0.1 in pairwise and 0.05 in multiple alignments. The resulting alignment was modified by hand to correct a few obvious alignment errors. The highly-variable regions in D2 and D3 domains in 28S rDNA fragment (Fig. 2) were eliminated from analyses. After alignment, phylogenetic analyses were performed by neighbor-joining (NJ) (Saitou and Nei, 1987), maximum parsimony (MP) (Fitch, 1971) and maximum likelihood (ML) (Felsenstein, 1981) methods. The distance was computed with Kimura two-parameter distances method (Kimura, 1980). The NJ method with a bootstrap test (Felsenstein, 1985) was performed using the Clustal W program. The bootstrap values were based on 1,000 replicates. PAUP* version 4.0b10 (Swofford, 2002) was used for maximum parsimony (MP) and maximum likelihood (ML) methods of phylogenetic analyses. Strength of nodal support in the MP and ML analyses were estimated using the conventional nonparametric bootstrap (Felsenstein, 1985; 1000 replicates, heuristic search with random input orders, and tree-bisection-reconnection (TBR) branch-swapping), then generate bootstrap majority-rule consensus trees. We used MODELTEST version 3.06 (Posada and Crandell, 1998) to initially estimate maximum-likelihood values under 56 different substitution models, which were then subjected to hierarchical likelihood ratio tests to determine the most appropriate model to be used in ML analysis (Posada and Crandell, 2001). Combinability of different data sets may require a measure, the incongruence length differences (Farris et al., 1995), for the levels of heterogeneity among the data sets. Because of arguments against this procedure (Baker et al., 2001; Yoder et al., 2001; Dowton and Austin, 2002), however, we did not employ the test to evaluate combinability of data sets.

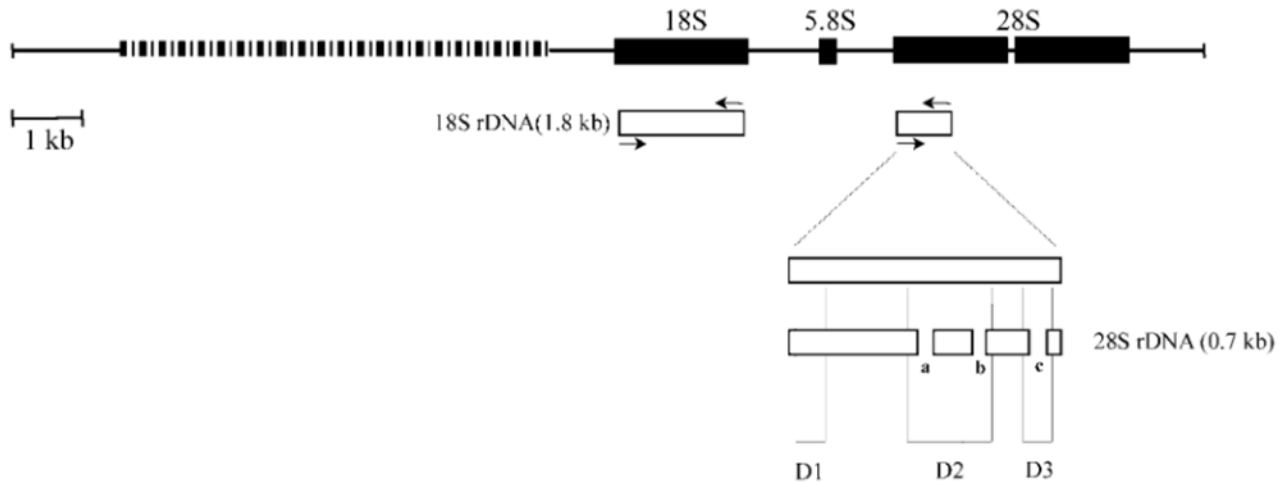


Fig. 1. A schematic presentation of a single unit of rDNA in *M. croelandi*. The rRNA coding regions are indicated by filled boxes. Spacer regions without repeats are indicated by thin lines and that with repeats by striped area (top). The PCR amplified fragments are indicated by open boxes. The PCR fragment of 28S rDNA (0.8 kb) is shown in detail, including the variable D1, D2 and D3 regions. The highly-variable subregions eliminated from analyses are shown by a, b and c. Arrows indicate the PCR primers (see Materials and Methods).

RESULTS

Structure of rDNA. Genomic DNAs were extracted from specimens of 15 ant species belonging to seven subfamilies (Family Formicidae) and three species belonging to different families of the Hymenoptera. The same regions of the rDNA, a large portion of 18S and a small portion of 28S, were PCR-amplified from each species using the same primer pairs. Single fragments were obtained in each and every species examined. Sequences were determined for the 18S rDNA fragments (1.8 kb) and the 28S rDNA fragments (0.8 kb).

The sequences of 1.8 kb fragments of 18S rDNA were very similar to each other among all the species studied. There were only limited numbers of base substitutions, some of which were subfamily-specific.

The PCR-amplified 0.8 kb fragments of 28S rDNA contained three (D1, D2 and D3) of the 12 variable regions previously identified (Hassouna et al., 1984) (Fig. 1). There was a stretch of conserved sequences in the middle of the D2 region when the sequences of the 18 species were aligned. Two highly variable subregions at D2 region and a subregion at D3 region (Fig. 2) were excluded from further phylogenetic analyses.

Phylogenetic relationship. Analysis of the 18S data set yielded a NJ tree with Kimura two-parameter distances, and a single MP tree (length 220, consistency index 0.736, retention index 0.681) and a ML tree (The selected model (TIMef + I + G) was a transitional model (TIM, Rodríguez et al., 1990) with γ -distributed rate heterogeneity (G) and a proportion of invariant sites (I), and which assumes equal base frequencies (ef)). Analysis of the 28S data set yielded a NJ tree with Kimura two-

parameter distances, and a single MP tree (length 456, consistency index 0.688, retention index 0.596) and a ML tree (The selected model (TrN + G) was Tamura-Nei model (TrN, Tamura and Nei, 1993) with γ -distributed rate heterogeneity). Kimura two-parameter distances of 18S and 28S were significantly correlated ($r = 0.915$, $p < 0.001$). The 18S rDNA evolved about one fifth as fast as the 28S rDNA. These two data sets were combined to yield an overall data set of about 2.5 kb. Analysis of the 18S+28S data set yielded a NJ tree with Kimura two-parameter distances, a single MP tree (length 702, consistency index 0.698, retention index 0.604) and a ML tree (The selected model (TrNef + I + G) was Tamura-Nei model with γ -distributed rate heterogeneity and a proportion of invariant sites, and which assumes equal base frequencies).

The strict consensus topology from NJ, MP and ML trees based on the 18S, 28S and 18S+28S sequences data sets, respectively, are presented in Fig. 3. Those employed trees of MP and ML analyses were the bootstrap majority-rule consensus tree. Trees showed that the six subfamilies of ants (excluding Ponerinae) were in single cluster with bootstrap values of 91%, 93% and 100% for NJ, 89%, 96% and 100% for MP, 94%, 96% and 92% for ML, respectively. All five species belonging to Ponerinae were not included within the branches of the cluster of the 6 subfamilies of ants. These results suggest that Ponerinae is a sister group of the other 6 subfamilies. Figure 3 also shows that *Amblyopone* was not clustered with other Ponerinae species. The relationship among the 6 subfamilies could not be clarified, but Myrmecinae and Nothomyrmecinae appeared to be closely related on the basis of 28S and 18S + 28S data sets, although 18S data set did not support this relation-

a	<i>Aphaenogaster</i>	434	CGATGTCCC-	GCGGGG-CC-	-----T	CGCGGCTCG-	-CGCGCGGGC	469		
	<i>Pristomyrmex</i>	434	CGATGTCCC-	GCGGAG-CC-	-----T	CGCGGCTCG-	-CGCGCGGAC	469		
	<i>Tetramorium</i>	434	CGATGTCCC-	GCGGGG-CC-	-----T	CGCGGCTCG-	-CGCGCGGAC	469		
	<i>Camponotus</i>	434	CGATGTCCC-	GCGGAGTCC-	-----T	CGCGGCTCG-	-CGCGCGGGC	470		
	<i>Formica</i>	434	CGATGTCCC-	GCGGAG-CC-	-----T	CGCGGCTCT-	-CGCGCGGGC	469		
	<i>Paratrechina</i>	434	CGATGTCCC-	GCGGAGTCC-	-----T	CGCGGCTCG-	-CGCGCGGGC	470		
	<i>Ochetellus</i>	435	CGATGTTCC-	GCGGAGCCC-	-----T	TGCGGATCCT	-ACCGCGGAC	472		
	<i>Myrmecia</i>	434	CGATGTCCC-	GCCGAGCAGC	GTTCTTCGAG	CGCGGTTTCG-	-AGCGCGGGC	480		
	<i>Nothomyrmecia</i>	434	CGATGTCTC-	GCAGAGTCGC	-----T	TGCGGCTCG-	-AGCGCGGGC	471		
	<i>Pseudomyrmex</i>	434	CGATGACCCC	GCGGCGTGTCT	CT-----G	AGCGGCACGC	GCGCGCGGGC	476		
	<i>Pachycondyla</i>	434	CGATGTCCGC	GCGG-----	-----	-----TTTCT	-CGCGCGGGC	461		
	<i>Cryptopone</i>	438	CGATGCATGC	GCGG-----	-----	-----ATCAT	CGGTGCGTGC	466		
	<i>Diacamma</i>	436	CGGTGCGCGC	GCGG-----	-----	-----TTCT	CGCGGTTTCG	463		
	<i>Odontomachus</i>	434	CGATGTCTCT-	GCGG-----	-----	-----TTTCT	-CGCGCGGGC	460		
	<i>Amblyopone</i>	434	CGATGTCCC-	GCGG-----	-----	-----GTTT	ACTCGCGGGC	460		
	<i>Vespa</i>	437	CGATGTTGCC	GAC-----	-----	-----TAC	GGTTGGCGAC	462		
	<i>Apis</i>	433	CGATGCTCCG	GATGAATCC-	-----T	CGTGGTTCAT	CGCGGAGGGC	472		
	<i>Athalia</i>	434	TGATGCTGGG	ACCT-----	-----	-----CG	CGTCCACGGC	459		
b	<i>Aphaenogaster</i>	653	CCGCACC---	-----TC	ACTAAACGAA	GTGCGT	677			
	<i>Pristomyrmex</i>	653	CCGCACAACC	TCTC-----	--TCTTACGA	GAGGGGGAGA	GTGCGT	690		
	<i>Tetramorium</i>	654	CCGCAGCTCT	CACC-----	---ACCTGGT	GGTGACGAGT	GTGCGT	690		
	<i>Camponotus</i>	654	CCGCACTTCT	ACA-----	-----	-----CG	GTGCGT	674		
	<i>Formica</i>	654	CCGCACTGTT	TA-----	-----	-----CG	GTGCGT	673		
	<i>Paratrechina</i>	654	CCGCACTCTT	TA-----	-----	-----CG	GTGCGT	673		
	<i>Ochetellus</i>	656	CCGCACTCTT	CTCAC-----	-----	-----GT	GTGCGT	678		
	<i>Myrmecia</i>	664	CCGCATACGC	GCGCGCGCAC	ATTGTTGCGC	GTTGCGCGTT	GTGCGT	709		
	<i>Nothomyrmecia</i>	655	CCGCACTC--	-----	-----	TCTATGTGTT	GTGCGT	678		
	<i>Pseudomyrmex</i>	661	CCGCAC----	-----	-----	-----CAACG	GTGCGT	677		
	<i>Pachycondyla</i>	644	CCGCAGCGCA	AAAG-----	-----	-----TCT	CTGCGT	666		
	<i>Cryptopone</i>	648	CCGCACAGAA	ACG-----	-----	-----T	GTGCGT	667		
	<i>Diacamma</i>	646	CCGCACAG--	-----	-----	---TCTATCT	CTGCGT	666		
	<i>Odontomachus</i>	643	CCGCAGTTTA	-----	-----	-----T	CTGCGT	659		
	<i>Amblyopone</i>	643	CCGCATAACT	TT-----	-----	CGAAACCCAT	TTGCGT	670		
	<i>Vespa</i>	645	CCGCAATTAT	CGA-----	-----	-----A	CTGCGT	664		
	<i>Apis</i>	654	CCGCACCAA	TCCATTTTTT	CGAA-----	-----TGTTG	GTGCGT	688		
	<i>Athalia</i>	635	CCGCATTTCG-	-----	-----	-----A	CTGCGT	650		
c	<i>Aphaenogaster</i>	778	GGCCTACG	AACGTCCCAC	GGGGTTTACC	CCAC-----	-----G	GGGACCGATA	828	
	<i>Pristomyrmex</i>	791	GGCCTGCG	AACGTCCCAG	GGCGGGCCCTC	GCGGGTTCGTC	GC-----	G	GTGACCGATA	842
	<i>Tetramorium</i>	791	GGCCTACG	AACGTTCCCC	CGCGTACATT	TATTGTGCAC	GGT-----	G	GGGACCGATA	833
	<i>Camponotus</i>	777	GGCCTGCG	AACGTCTCTC	TCACG-----	-----	-----G	GGGACCGATA	810	
	<i>Formica</i>	776	GGCCTGTC	GAACGTCCCT	CTCCCTCCCT	TGTGGGTCGC	GAG-----	G	GGGATCTATA	827
	<i>Paratrechina</i>	776	GGCCTGCG	AACGTCTTCC	GCGGTACGCG	G-----	-----G	GAGACCGATA	815	
	<i>Ochetellus</i>	778	GGCCTGCA	AACAA-----	-----	-----	-----	TATA	794	
	<i>Myrmecia</i>	812	GGCCTTTG	AGCGCGCGCG	CGCGCTCTCC	CCTTTGCGGG	GACGAGCTCG	CGCGCGGATA	869	
	<i>Nothomyrmecia</i>	778	GGCCTTCG	AACGTGTCT	CCCCGGGAGAG	CT-----	-----	CCGATA	813	
	<i>Pseudomyrmex</i>	787	GGCCTTGC	GAACGTCCCG	CGCCGCT---	-CTTTCGGGA	GCGCGCGCGC	GAGACCGATA	839	
	<i>Pachycondyla</i>	765	GGCCTCGC	GGGGCGAGGC	GCGTCCGCGC	GCCTCCG---	-----	CCCCAATA	807	
	<i>Cryptopone</i>	767	GGCCTCCA	TGCGCGGGCG	AGCGGGGCAA	CTCGTTTCGCT	CGCGCG---	-----	CATA	814
	<i>Diacamma</i>	765	GGCCTCGC	GGGGCGAGAT	CTCTCTCGCT	-----	-----	CCCGATA	799	
	<i>Odontomachus</i>	758	GGCCTTAC	GGGGCGACGT	GGGGGGTGTT	AGCCATTCCA	CGTCCGCCCT	-----	AATA	809
	<i>Amblyopone</i>	768	GGCCTTAA	CCCGGGGGGG	CGGTTCTCGC	CGCTCCCTC-	-----	-----	GATA	808
	<i>Vespa</i>	759	GGCAATAC	ACAAT-----	-----	-----	-----	-----	AATA	775
	<i>Apis</i>	754	GGCCNAGA	CT-----	-----	-----	-----	---	CGAATTA	832
	<i>Athalia</i>	748	GGCTCATT	-----	-----	-----	---	CGAATTT	TATATTGITA	772

Fig. 2. Alignment of three variable regions in the 28S rDNA sequences. **a**, **b** and **c** correspond to the highly variable subregions shown in Fig. 1. Each species is indicated only by genus name. Alignment was done using Clustal W and then manually adjusted. Dashes indicate gaps created to make better alignment with conserved sequences. Sequences of variable regions are surrounded by the frame. Numbers are nucleotide positions as appeared in the sequences reported (accession numbers are shown in Materials and Methods).

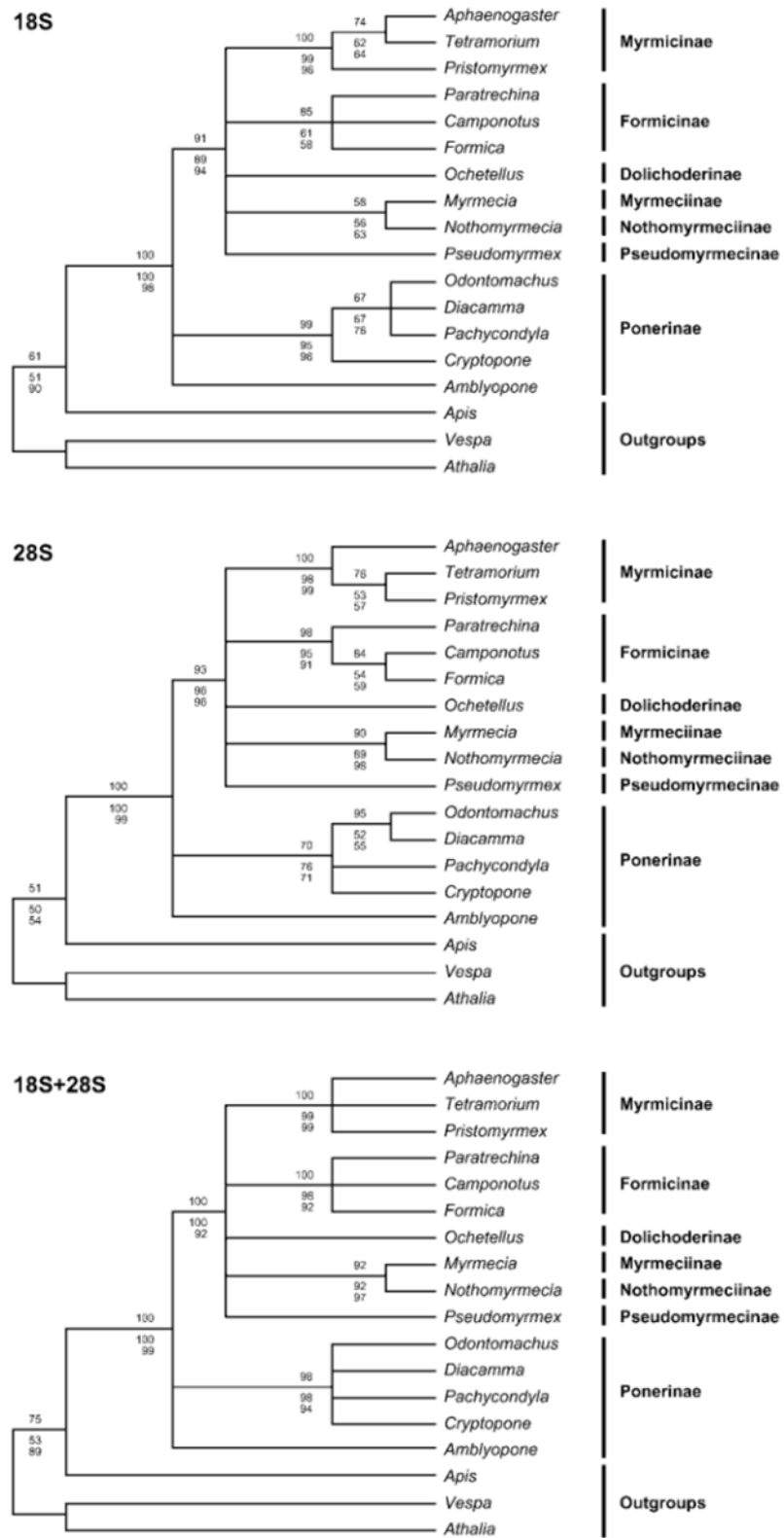


Fig. 3. Phylogenetic trees for 15 ant species (7 subfamilies) derived from rDNA fragment sequences. Strict consensus trees obtained by different data sets (18S, 28S, 18S+28S) are shown. The three bootstrap values are given for each tree and indicate the results obtained from neighbor-joining (top), maximum parsimony (second) and maximum-likelihood (bottom) analyses. Three non-ant hymenopteran species are used as outgroups. The species studied are indicated only by genus names.

ship strongly (bootstrap values: 58%, 90% and 92% for NJ, 56%, 89% and 92% for MP and 63%, 98% and 97% for ML).

DISCUSSION

The higher phylogeny of ants (Family Formicidae) has been studied for 50 years since Brown's (1954) pioneering work subdividing the family into two groups of subfamilies (reviewed by Hölldobler and Wilson, 1990). New methodologies have more recently been applied, such as cladistic analysis of morphological data (Baroni Urbani et al., 1994) and molecular phylogenetic analyses (Dowton and Austin, 1994; Gimeno et al., 1997; Sullender, 1998).

In the present study, we examined the subfamily relationship of Formicidae by molecular phylogenetic approaches using rDNA sequences. For this purpose, we amplified two regions of rDNA, a portion of 18S rDNA (1.8 kb) and a portion of 28S rDNA (0.8 kb) (Fig. 1). A comparison of the bootstrap consensus trees obtained from analyses of the two data sets, 18S and 28S (Fig. 3), does not reveal any strong disagreement, and suggests that Ponerinae is a sister group of the other six subfamilies. Although there are a few clades that appear in only one of the two consensus trees, there are no instances in which such clades conflict with groups appearing in the other tree. Thus, any disagreement between the two data sets involves groups that have no strong support of bootstrap values. Consequently a combined treatment of the data (18S + 28S, Fig. 3) appears to be of merit. The results further strengthen the notion that Ponerinae and other six subfamilies form a sister group relationship (Fig. 3). The sequences employed for analyses, in fact, contained many substitutions which were common only among six subfamilies and were not shared by Ponerinae and outgroups. Such substitutions should have occurred after the ancestor of six subfamilies diverged from the ancestor of Ponerinae.

The D domains are regions with variable sequences in an otherwise conserved sequence of 28S rDNA (Hassouna et al., 1984). The present study included examination of the D1, D2 and D3 domains. There was a conserved subregion in the middle of D2 common to all the hymenopteran species examined here (Fig. 1). The sequences of the variable subregion of D2 and of D3 (Fig. 2) were generally too diversified to align across all the families/subfamilies of hymenopteran species examined, and thus were excluded from the analysis. The eliminated highly-variable regions within the 0.8 kb fragments thus are not useful for construction of the trees of species belonging to different subfamilies. After the initial alignment by the program Clustal W, we made adjustment in the three variable regions by eye (Fig. 2).

The sequence of one of the variable subregions (Fig. 2a), was shared by Myrmicinae and Formicinae, subfamilies

that have traditionally been treated as belonging to separate groups but in the present study are shown to be closely related. This suggests the possibility that the highly variable regions can be used for phylogenetic analyses of closely related taxa. Support for this suggestion comes from the fact that the sequences of highly variable regions in *Vespa mandarinia* (Fig. 2 a, b, c) were identical to those in *Vespa crabro* (Schmitz and Moritz, 1998). Our notions here are in general agreement with those of previous reports (Schmitz and Moritz, 1998, and the references therein).

The present results, taken together, demonstrate that Myrmicinae, Formicinae, Dolichoderinae, Pseudomyrmecinae, Nothomyrmecinae and Myrmecinae belong to the same clade, to which Ponerinae forms a sister group (Fig. 3). Since Brown's (1954) phylogenetic studies, Myrmicinae had been thought to be related to Ponerinae and to belong to the same poneroid complex. The present results do not support this notion. In fact, the present phylogenetic trees (Fig. 3) basically agree with the tree by Baroni Urbani et al. (1992) based on morphological data and the tree obtained through a combination of morphological and molecular data by Ward and Brady (2003), but do not support the traditional phylogenetic tree (Hölldobler and Wilson, 1990). The present results and those of Baroni Urbani et al. (1992) and Ward and Brady (2003) thus raise a serious question as to the status of such morphological characters as post petiole and fused abdominal plates of segment IV which have been treated as key characters in the traditional phylogeny.

Our analysis suggests that *Amblyopone* should be included in another new subfamily (Fig. 3). The *Amblyopone* has been thought to be the most primitive member of the poneroid complex (Brown, 1954). A number of researchers also have questioned the monophyly of the Ponerinae (Sullender, 1998; Wilson, 1971). This issue needs to be investigated further so as to settle the question of fundamental division in the ants. The consensus phylogenetic trees derived from NJ, MP and ML methods based on the combined 18S and 28S rDNA sequences (Fig. 3) demonstrate the sister group relationship between Ponerinae, (*Amblyopone*, possibly as belonging to a separate subfamily), and the six other subfamilies, but fail to clarify the relationship among the latter subfamilies. The trees, however, suggest that Myrmecinae and Nothomyrmecinae are sister-groups. In a recent study, Myrmecinae and Nothomyrmecinae are in fact considered to be a single subfamily (Ward and Brady, 2003). Some groups classified as members of the Ponerinae, and that were not used in the present study (e.g. tribe Ectatommini), may be placed in the non-Ponerinae cluster (Sullender, 1998; Ward and Brady, 2003). In order to resolve these problems, analysis of more sequences, including both conserved and variable regions, will be necessary.

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REFERENCES

- Baker, R. H., Wilkinson, G. S., and DeSalle, R. (2001) Phylogenetic utility of different types of molecular data used to infer evolutionary relationships among stalk-eyed flies (Diptera). *Syst. Biol.* **50**, 87–105.
- Baroni Urbani, C., Bolton, B., and Ward, P. S. (1992) The internal phylogeny of ants (Hymenoptera: Formicidae). *Syst. Entomol.* **17**, 301–329.
- Bolton, B. (1994) *Identification Guide to the Ant Genera of the World*. Harvard University Press: Cambridge, MA, USA.
- Brown, W. L. J. (1954) Remarks on the internal phylogeny and subfamily classification of the family Formicidae. *Insectes Sociaux* **1**, 21–31.
- Dowton, M., and Austin, A. D. (1994) Molecular phylogeny of the insect order Hymenoptera: Apocritan relationships. *Proc. Natl. Acad. Sci. USA* **91**, 9911–9915.
- Dowton, M., and Austin, A. D., (2002) Increased congruence does not necessarily indicate increased phylogenetic accuracy - the behavior of the incongruence length difference test in mixed-model analyses. *Syst. Biol.* **51**, 19–31.
- Farris, J. S., Källersjö, M., Kluge, A. G., and Bult, C. (1995) Testing significance of incongruence. *Cladistics* **10**, 315–319.
- Felsenstein, J. (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. *J. Mol. Evol.* **17**, 368–376.
- Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using bootstrap. *Evolution* **39**, 783–791.
- Fitch, W. M. (1971) Toward defining the course of evolution: minimum change for a specified tree topology. *Syst. Zool.* **20**, 406–416.
- Gimeno C., Belshaw, R., and Quicke D. L. (1997) Phylogenetic relationships of the Alysiinae/Opiinae (Hymenoptera: Bracoonidae) and the utility of cytochrome b, 16S and 28S D2 rRNA. *Insect Mol. Biol.* **6**, 273–284.
- Hassouna, N., Michot, B., and Bachellerie, J. P. (1984) The complete nucleotide sequence of mouse 28S rRNA gene. Implications for the process of size increase of the large subunit rRNA in higher eukaryotes. *Nucleic Acids Res.* **12**, 3563–3583.
- Hölldobler, B., and Wilson, E. O. (1990) *The Ants*. The Belknap Press of Harvard University Press, Cambridge, Mass.
- Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**, 111–120.
- Long, E. O., and Dawid, I. B. (1980) Repeated genes in eukaryotes. *Annu. Rev. Biochem.* **49**, 727–726.
- Ohnishi, H., and Yamamoto, M.-T. The structure of a single unit of ribosomal RNA gene (rDNA) including intergenic subrepeats in the Australian bulldog ant *Myrmecia croslandi* (Hymenoptera: Formicidae). *Zool. Sci.* in press.
- Pélandakis, M., and Solignac, M. (1993) Molecular phylogeny of *Drosophila* based on ribosomal RNA sequences. *J. Mol. Evol.* **37**, 525–543.
- Posada, D., and Crandell, K. A. (1998) Modeltest: testing the model of DNA substitution. *Bioinform.* **14**, 817–818.
- Posada, D., and Crandell, K. A. (2001) Selecting the best-fit model of nucleotide substitution. *Syst. Biol.* **50**, 580–601.
- Rodríguez, F., Oliver, J. L., Marin, A., and Medina, J. R. (1990) The general stochastic model of nucleotide substitution. *J. Theor. Biol.* **142**, 485–501.
- Saitou, N., and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual, 2nd ed.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schmitz, J., and Moritz, R. F. A. (1998) Molecular phylogeny of Vespidae (Hymenoptera) and the evolution of sociality in wasps. *Mol. Phylogenet. Evol.* **9**, 183–191.
- Sullender, B.W. (1998) A molecular phylogeny for the Formicidae. http://www.ruf.rice.edu/~bws/molecular_phylogeny.html
- Swofford, D. L. (2002) *PAUP*. Phylogenetic analysis using parsimony (*and other methods), version 4.0*. Sinauer associates: Sunderland, MA, USA.
- Tamura, K., and Nei, M. (1993) Estimation of the number of nucleotide substitutions in control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **10**, 512–526.
- Taylor, R. W. (1978) *Nothomyrmecia macrops*: a living-fossil ant rediscovered. *Science* **201**, 979–985.
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680.
- Ward, P. S., and Brady, S. G. (2003) Phylogeny and biogeography of the ant subfamily Myrmecinae (Hymenoptera: Formicidae). *Invertebrate Systematics* **17**, 361–386.
- Wilson, E. O. (1971) *The Insect Societies*. Harvard University Press: Cambridge, MA, USA.
- Yoder, A. D., Irwin, J. A., and Payseur, B. A. (2001) Failure of the ILD to determine data combinability for slow loris phylogeny. *Syst. Entomol.* **50**, 408–424.

