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 sistergroups 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-

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: Myrmeciinae (*Myrmecia croslandi*), Nothomyrmeciinae (*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'-AATCATTCAATCGGTAGTAG-3' (18S-L1). The 28S rDNA primer set was 5'- ACTAAGCGGAGGAAAAGAAACTA -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* version4.0b10 (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.
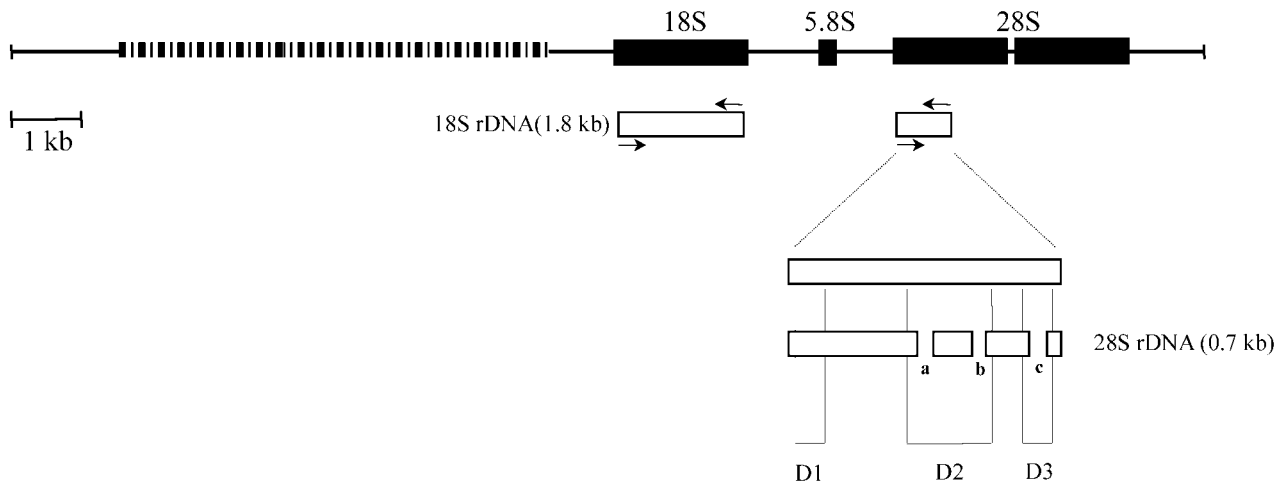
Fig. 1. A schematic presentation of a single unit of rDNA in *M. croslandi*. The rRNA coding regions are indicated by filled boxes. Spacer regions without repeats are indicated by thin lines and that with repeats by striped area (top). The PCR amplified fragments are indicated by open boxes. The PCR 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 sub-families (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.
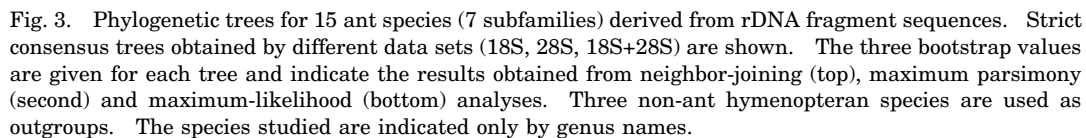
Phylogenetic relationship. Analysis of the 18S data set yielded a NJ tree with Kimura two-parameter distances, and a single MP tree (length 220, consistency index 0.736, retention index 0.681) and a ML tree (The selected model (TIMef + I + G) was a transitional model (TIM, Rodríguez et al., 1990) with 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 Myrmeciinae and Nothomyrmeciinae 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

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Aphaenogaster   434  CGATGTCCC- GCGGGG-CC- ---------T CGCGGCTCG- -CGCGCGGGC  469
Pristomyrmex    434  CGATGTCCC- GCGGAG-CC- ---------T CGCGGCTCG- -CGCGCGGAC  469
Tetramorium     434  CGATGTCCC- GCGGGG-CC- ---------T CGCGGCTCG- -CGCGCGGAC  469
Camponotus      434  CGATGTCCC- GCGGAGTCC- ---------T CGCGGCTCG- -CGCGCGGGC  470
Formica         434  CGATGTCCC- GCGGAG-CC- ---------T CGCGGCTCT- -CGCGCGGGC  469
Paratrechina    434  CGATGTCCC- GCGGAGTCC- ---------T CGCGGCTCG- -CGCGCGGGC  470
Ochetellus      435  CGATGTTCC- GCGGAGCCC- ---------T TGCGGATCCT -ACCGCGGAC  472
Myrmecia        434  CGATGTCCC- GCCGAGCAGC GTTCTTCGAG CGCGGTTCG- -AGCGCGGGC  480
Nothomyrmecia   434  CGATGTCTC- GCAGAGTCGC ---------T TGCGGCTCG- -AGCGCGGGC  471
Pseudomyrmex    434  CGATGACCCC GCGGCGTGTC CT-------G AGCGGCACGC GCGCGCGGGC  476
Pachycondyla    434  CGATGTCCGC GCGG------ ---------- -----TTTCT -CGCGCGGGC  461
Cryptopone      438  CGATGCATGC GCGG------ ---------- -----ATCAT CCGTGCGTGC  466
Diacamma        436  CGGTGCGCGC GCGG------ ---------- ------TTCT CCGCGTTCGC  463
Odontomachus    434  CGATGTCCT- GCGG------ ---------- -----TTTCT -CGCGCGGGC  460
Amblyopone      434  CGATGTCCC- GCGG------ ---------- ------GTTT ACTCGCGGGC  460
Vespa           437  CGATGTTGCC GAC------- ---------- --------TAC GGTTGGCGAC  462
Apis            433  CGATGCTCCG GATGAATCC- ---------T CGTGGTTCAT CGGCGAGGGC  472
Athalia         434  TGATGCTGGG ACCT------ ---------- --------CG CGTCCACGGC  459
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b

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Aphaenogaster   653  CCGCACC--- ---------- --------TC ACTAAACGAA GTGCGT  677
Pristomyrmex    653  CCGCACAACC TCTC------ --TCTTACGA GAGGGGGAGA GTGCGT  690
Tetramorium     654  CCGCAGCTCT CACC------ ---ACCTGGT GGTGACGAGT GTGCGT  690
Camponotus      654  CCGCACTTCT ACA------- ---------- -------CG GTGCGT  674
Formica         654  CCGCACTGTT TA-------- ---------- -------CG GTGCGT  673
Paratrechina    654  CCGCACTCTT TA-------- ---------- -------CG GTGCGT  673
Ochetellus      656  CCGCACTCTT CTCAC----- ---------- -------GT GTGCGT  678
Myrmecia        664  CCGCATACGC GCGCGCGCAC ATTGTTGCGC GTTGCGCGTT GTGCGT  709
Nothomyrmecia   655  CCGCACTC-- ---------- ---------- TCTATGTGTT GTGCGT  678
Pseudomyrmex    661  CCGCAC---- ---------- ---------- -----CAACG GTGCGT  677
Pachycondyla    644  CCGCAGCGCA AAAG------ ---------- -------TCT CTGCGT  666
Cryptopone      648  CCGCACAGAA ACG------- ---------- --------T GTGCGT  667
Diacamma        646  CCGCACAG-- ---------- ---------- ---TCTATCT CTGCGT  666
Odontomachus    643  CCGCAGTTTA ---------- ---------- --------T CTGCGT  659
Amblyopone      643  CCGCAAAACT TT-------- ---------- CGAAACCCAT TTGCGT  670
Vespa           645  CCGCAATTAT CGA------- ---------- --------A CTGCGT  664
Apis            654  CCGCAACCAA TCCATTTTTT CGAA------ -----TGTGT GTGCGT  688
Athalia         635  CCGCATTCG- ---------- ---------- --------A CTGCGT  650
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c

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Aphaenogaster   778  GGCCTACG AACGTCCCAC GGGGTTTACC CCAC------ ---------G GGGACCGATA  828
Pristomyrmex    791  GGCCTGCG AACGTCCCGC GGCGGCCCTC GCGGGTCGTC GC------G GTGACCGATA  842
Tetramorium     791  GGCCTACG AACGTTCCCC CGCGTACATT TATTGTGCAC GGT------G GGGACCGATA  833
Camponotus      777  GGCCTGCG AACGTCTCTC TCACG----- ---------- ---------G GGGACCGATA  810
Formica         776  GGCCTGTC GAACGTCCCT CTCCCTCCCT TGTGGGTCGC GAG------G GGGATCTATA  827
Paratrechina    776  GGCCTGCG AACGTCTTCC GCGGTCACGC G--------- ---------G GAGACCGATA  815
Ochetellus      778  GGCCTGCA AACAA----- ---------- ---------- ---------- ------TATA  794
Myrmecia        812  GGCCTTTG AGCGCGCGCG CGCGCTCTCC CCTTTGCGGG GACGAGCTCG CGCGCGGATA  869
Nothomyrmecia   778  GGCCTTCG AACGTGCTCT CCCCGGAGAG CT-------- ---------- ----CCGATA  813
Pseudomyrmex    787  GGCCTTGC GAACGTCCCG CGCCGCT--- -CTTTCGGGA GCGCGCGCGC GAGACCGATA  839
Pachycondyla    765  GGCCTCGC GGGGCGAGGC GCGTCCGCGC GCCTCCG--- ---------- ---CCCCAATA  807
Cryptopone      767  GGCCTCCA TGCGCGGGCG AGCGGGGCAA CTCGTTCGCT CGCGCG---- ------CATA  814
Diacamma        765  GGCCTCGC GGGGCGAGAT CTCTCTCGCT ---------- ---------- -CCCGATA  799
Odontomachus    758  GGCCTTAC GGGGCGACGT GGGGGGTGTT AGCCATTCCA CGTCCGCCCT -----AATA  809
Amblyopone      768  GGCCTTAA CCCGGGGGGG CGGTTCTCGC CGCTCCCTC- ---------- ------GATA  808
Vespa           759  GGCAATAC ACAAT----- ---------- ---------- ---------- ------AATA  775
Apis            754  GGCCAAGA CT-------- ---------- ---------- ---------- ---CGAATTA  832
Athalia         748  GGCTCATT ---------- ---------- ---------- ---CGAATTT TATATTGTTA  772
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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, Nothomyrmeciinae and Myrmeciinae 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 Myrmeciinae and Nothomyrmeciinae are sister-groups. In a recent study, Myrmeciinae and Nothomyrmeciinae 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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