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Dracula ant phylogeny as inferred by nuclear 28S rDNA sequences and implications for ant systematics (Hymenoptera: Formicidae: Amblyoponinae)

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Abstract

Ants are one of the most ecologically and numerically dominant families of organisms in almost every terrestrial habitat throughout the world, though they include only about 1% of all described insect species. The development of eusociality is thought to have been a driving force in the striking diversification and dominance of this group, yet we know little about the evolution of the major lineages of ants and have been unable to clearly determine their primitive characteristics. Ants within the subfamily Amblyoponinae are specialized arthropod predators, possess many anatomically and behaviorally primitive characters and have been proposed as a possible basal lineage within the ants. We investigate the phylogenetic relationships among the members of the subfamily, using nuclear 28S rDNA sequence data. Outgroups for the analysis include members of the poneromorph and leptanillomorph (*Apomyrma*, *Leptanilla*) ant subfamilies, as well as three wasp families. Parsimony, maximum likelihood, and Bayesian analyses provide strong support for the monophyly of a clade containing the two genera *Apomyrma* + *Mystrium* (100% bpp; 97% ML bs; and 97% MP bs), and moderate support for the monophyly of the Amblyoponinae as long as *Apomyrma* (Apomyrminae) is included (87% bpp; 57% ML bs; and 76% MP bs). Analyses did not recover evidence of monophyly of the *Amblyopone* genus, while the monophyly of the other genera in the subfamily is supported. Based on these results we provide a morphological diagnosis of the Amblyoponinae that includes *Apomyrma*. Among the outgroup taxa, *Typhlomyrmex* grouped consistently with *Ectatomma*, supporting the recent placement of *Typhlomyrmex* in the Ectatomminae. The results of this present study place the included ant subfamilies into roughly two clades with the basal placement of *Leptanilla* unclear. One clade contains all the Amblyoponinae (including *Apomyrma*), Ponerinae, and Proceratiinae (Poneroid clade). The other clade contains members from subfamilies Cerapachyinae, Dolichoderinae, Ectatomminae, Formicinae, Myrmeciinae, and Myrmicinae (Formicoid clade).

Keywords: Formicidae; Poneromorph; Ectatomminae; Ponerinae; *Apomyrma*; rDNA; Maximum parsimony; Maximum likelihood; Bayesian inference

1. Introduction

Although there is no question about the close relationship of the ants (Formicidae) to the rest of the wasps (Hymenoptera: Aculeata), we know little about the evolutionary origin and diversification of ants subsequent to divergence of this lineage from their wasp ancestors. This lack of knowledge is exacerbated by lack of a comprehensive fossil history, and disagreements over reconstruction of ancestral morphological character states (Baroni Urbani et al., 1992; Grimaldi et al., 1997; Hashimoto, 1996; Ward, 1994; Wilson et al., 1967). Ants within the subfamily Amblyoponinae have often been

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thought to possess characters that reflect an early lineage in ant evolution (Brown, 1954; Ward, 1994; Wheeler, 1965; Wilson, 1971), but relationships within the subfamily are largely unresolved. This poor understanding of amblyoponine relationships in turn impedes our ability to understand behavioral, ecological, and social evolution (Bourke and Franks, 1995; Gotwald and Lévieux, 1972; Traniello, 1978, 1982; Wilson, 1971). Better resolution of relationships among the amblyoponine ants and clarification of their placement within the poneromorph subfamilies may shed some light on current controversies in ant evolution.

The family Formicidae, which contains all ant species, is currently divided into 21 extant subfamilies (Bolton, 2003). The Amblyoponinae subfamily of ants is a member of the poneromorph subfamilies. This group is comprised of 6 subfamilies: Amblyoponinae (9 genera), Ectatomminae (4 genera), Heteroponerinae (3 genera), Paraponerinae (1 genera), Ponerinae (25 genera), and Proceratiinae (3 genera). Bolton (2003) completed the last comprehensive treatment of this group during which he elevated the Amblyoponini tribe to subfamily status.

As a world-wide group of ants, the subfamily Amblyoponinae consists of nine extant genera: *Adetomyrma* Ward, *Amblyopone* Erichson, *Bannapone* Xu, *Concoctio* Brown, *Myopopone* Roger, *Mystrium* Roger, *Onychomyrmex* Emery, *Paraprionopelta* Kusnezov, and *Prionopelta* Mayr. *Adetomyrma*, a recently described genus (Ward, 1994), is endemic to Madagascar. *Amblyopone* is the most diverse genus within the amblyoponines constituting almost two-thirds of the known species and is found worldwide in tropical and temperate regions. *Bannapone* was recently described from one dealate female specimen collected in the Yunnan Province, China. Currently this is the only known specimen of this new genus. *Concoctio* is represented by only one species and is found only in Central Africa. *Myopopone* has a range from Sri Lanka east to the Philippines, and south into Australia and is comprised of only one species. *Mystrium* is found in Madagascar with six described and at least three undescribed species, *Mystrium silvestrii* is known from West and Central Africa, and *Mystrium camillae* is known from South-east Asia east to the Philippines and south into northern Australia. *Onychomyrmex* is limited to Australia with only three described species and at least four undescribed. The subfamily also contains the genus *Paraprionopelta*, which is known only from males collected at lights at Tucumán, Argentina. *Prionopelta* contains 13 known species and is found throughout the world in tropical and subtropical regions.

The amblyoponine ants are of much interest to the ant systematics community because the subfamily appears to retain many morphologically primitive characters (Brown, 1954; Ward, 1994; Wheeler, 1965; Wilson, 1971). The broad attachment of the second abdominal segment (petiole) to the third segment (gaster) seen in the Amblyoponinae is argued to be similar to extant vespid and tiphiid wasps (Brown, 1954; Hölldobler and Wilson, 1990; but see Hashimoto, 1996; Ward, 1994), and suggests that these ants may be a basal lineage within the Formicidae. Their behavior and ecology also suggests ancestral affinities (Bourke and Franks, 1995; Gotwald and Lévieux, 1972; Hölldobler and Wilson, 1990; Ito, 1993; Traniello, 1978). Furthermore, *Amblyopone* and *Mystrium* queens exhibit an unusual feeding behavior: non-destructive cannibalism (Fisher and Girman, 2000; Masuko, 1986; Wheeler and Wheeler, 1988). The queen cuts a hole in the integument of the larvae and feeds upon the exuding hemolymph. This behavior is considered non-destructive cannibalism because it does not seem to harm the larvae, which continue to grow and finally emerge as adults. The amblyoponines are sometimes referred to as "Dracula ants" due to this behavior.

Amblyoponine ants are hypothesized to be the sister group to either: all remaining extant ants (Sullender and Johnson, 1998), the remaining poneroid complex (Brown, 1954; Hölldobler and Wilson, 1990; Taylor, 1978), or the basal clade to the remaining members of the poneromorph subfamilies (Ward, 1994). It should be noted, however, that monophyly of the poneromorph group has also been questioned (Grimaldi et al., 1997; Hashimoto, 1991; Keller, 2000; Lévieux, 1972; Ward, 1994; Wheeler et al., 1999). Due to the possibility that this subfamily could be a very ancient lineage, a careful assessment of evidence for monophyly of this subfamily is essential.

The research presented here involves inference of the relationships and monophyly among the genera within the Amblyoponinae subfamily, investigates the relationship of the subfamily within the ants as a whole, and addresses the question of the hypothesized basal position of the amblyoponine ants within the Formicidae using the 28S ribosomal nuclear molecular marker. The rDNA 28S marker has been successful in recovering phylogenetic relationships among other groups of Hymenoptera (Belshaw and Quicke, 1997; Belshaw et al., 1998, 2001; Cameron and Mardulyn, 2001; Dowton and Austin, 1998, 2001; Lopez-Vaamond et al., 2001; Mardulyn and Whitfield, 1999). In order to most accurately reconstruct the relationships among this group of ants, several methods of phylogenetic analysis were performed: maximum parsimony, maximum likelihood, and Bayesian inference. In addition the confidence of these results was examined using bootstrap (Felsenstein, 1985; Hillis and Bull, 1993) and Bayesian analysis (Larget and Simon, 1999; Rannala and Yang, 1996).

This study is the first attempt to utilize molecular sequence data to investigate the relationships of the Amblyoponinae. The results of this study will be used to understand the relationships within this "primitive"

subfamily and will allow a better understanding of morphologically ancestral characters by addressing the position of basal ants and the characters they possess. The relationships resolved in this phylogeny based on molecular data may also lend insight into behavioral, ecological, and social evolution (Bourke and Franks, 1995; Gotwald and Lévieux, 1972; Traniello, 1978, 1982; Wilson, 1971).

2. Methods and materials

2.1. Taxon sampling

The analysis presented here includes a total of 51 specimens. Included are 30 specimens representing all genera of the Amblyoponinae subfamily (with the exception of *Bannapone* and *Paraprionopelta*). Nine specimens from three additional subfamilies within the poneromorph subfamilies are included (Ectatomminae, Ponerinae, and Proceratiinae). The poneromorph subfamilies Heteroponerinae and Paraponerinae (represented by three genera) were not included. Also, eight specimens from seven additional subfamilies (Apomyrminae, Cerapachyinae, Dolichoderinae, Formicinae, Leptanillinae, Myrmeciinae, and Myrmicinae) (Table 1) were included. Finally, three Hymenoptera outside of the Formicidae were included as outgroups (Vespidae, Bradynobaenidae, and Mutillidae), but only Mutillidae was defined as an outgroup in the analyses. Table 1 contains a full list of all specimens, their taxonomic status (Bolton, 2003), their country collection localities, and museum accession numbers. Sequence for *Myrmecia croslandi* was obtained from GenBank Accession No. AB052895 (Ohnishi et al., unpublished). All sequences have been deposited in GenBank under Accession Nos. AY325916–AY325965. The exact collection data for each specimen can be obtained from the author. Voucher specimens have been deposited at the California Academy of Sciences, San Francisco, CA.

2.2. DNA isolation

Field collections were made in 95% EtOH and kept at −20 °C in the laboratory until the time of DNA extraction. Total genomic DNA was isolated by grinding either an entire ant specimen or part of a specimen in lysis buffer with a Teflon grinding implement, followed by purification using phenol/chloroform (Werman et al., 1990) or the DNeasyTissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocols. Phenol/chloroform extraction followed the procedure in Spicer (1995) with some modifications: lysis in 500 μL grinding buffer (0.5 M EDTA, 100 mM Tris, pH 8.0, 1% SDS, and 20 mg/mL proteinase K). Despite beliefs that the gaster contains compounds that inhibit PCR or contribute to amplification of gut bacteria (Feldhaar et al., 2003; Johnson et al., 2003), we found that including the gaster in the extraction homogenate did not affect amplification or sequencing.

2.3. Polymerase chain reaction amplification

For each specimen, a fragment approximately 1200 base pairs (bp) in length containing the divergent domains D1, D2, and D3 in the nuclear ribosomal gene 28S was amplified via PCR (Mullis et al., 1987; Saiki et al., 1988). Double-stranded DNA was amplified using the PCR Optimizer Kit (Invitrogen, Carlsbad, CA), with some modifications: 50 μL volume reactions of 21 μL ultra pure (HPLC quality) water, 10 μL 5× buffer (300 mM Tris–HCl, 75 mM (NH_4) SO_4, pH 8.5, and 2.5 mM $MgCl_2$), 5 μL of 10 mM dNTP, 5 μL of each 10 mM primer, 2 μL of 100% DMSO, and 0.25 (units) μL AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA). Polymerase chain reaction (PCR) amplification primers for this fragment (Table 2) are Mo6 (Schmitz and Moritz, 1994) and D3B (present study). All reactions were initially denatured at 94 °C for 2 min in a MJ Dyad Thermal Cycler (MJ Research, Waltham, MA), then subjected to 35 cycles of 30 s at 94 °C denaturation, 30 s at 56 °C for annealing, and 2 min at 72 °C extension. Amplified PCR products were cleaned using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) prior to sequencing.

2.4. Sequencing

All sequencing was done using dye terminator cycle sequencing following the protocol specified by the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Revision B, August 1995, Perkin–Elmer, Norwalk, CT). Primers used for amplification served as sequencing primers. Additional internal primers were designed for sequencing purposes (Table 2) to provide overlapping sequence coverage for the entire region. All samples were sequenced in both the forward and reverse directions by way of an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA) using a membrane comb (The Gel Company, San Francisco, CA) and following the manufacturer's protocols.

2.5. Sequence alignment

After rDNA 28S sequence was collected, it was analyzed and initially aligned using the computer programs Sequencing Analysis 3.4 (ABI Prism 1999) and Sequencher 3.1.1 (GeneCodes 1998), respectively. Then, conserved regions were identified and aligned, and gaps were assigned to minimize changes using ClustalX 1.9a169 (Thompson et al., 1997) then SeqPup 0.6 (Gilbert, 1996). This aligned data set was finally viewed

Table 1
List of all specimens, taxonomic status, country collection localities, and museum accession numbers

Family/subfamily	Tribe	Genus	Species	Collection country	Museum Accession Nos.
Formicidae/Amblyoponinae	Amblyoponini	*Adetomyrma*	sp. *2a*	Madagascar	CASENT0500143
		Adetomyrma	sp.*2b*	Madagascar	CASENT0500384
		Adetomyrma	sp.	Madagascar	CASENT0500401
		Amblyopone	*oregonesis*	USA	CASENT0500002
		Amblyopone	*pallipes*	USA	CASENT0500058
		Amblyopone	*silvestrii*	Japan	CASENT0500525
		Amblyopone	*silvestrii*	Korea	CASENT0500443
		Amblyopone	sp.*1*	Madagascar	CASENT0500011
		Amblyopone	sp.*2*	Madagascar	CASENT0500013
		Amblyopone	sp.*6*	Tanzania	CASENT0500396
		Amblyopone	sp.*7*	Gabon	CASENT0500028
		Amblyopone	sp.*8*	Gabon	CASENT0500022
		Amblyopone	sp.*9*	Madagascar	CASENT0500003
		Amblyopone	*mutica*	Gabon	CASENT0500006
		Amblyopone	*elongata1*	Brazil	CASENT0500027
		Amblyopone	*elongata2*	Brazil	CASENT0500043
		Concoctio	*concenta1*	Gabon	CASENT0500145
		Concoctio	*concenta2*	Gabon	CASENT0500147
		Concoctio	*concenta3*	Gabon	CASENT0500149
		Myopopone	*castanea*	Soloman Is.	CASENT0501682
		Mystrium	*voeltzkowi*	Madagascar	CASENT0500430
		Mystrium	sp.*2*	Madagascar	CASENT0500431
		Mystrium	*rogeri*	Madagascar	CASENT0500097
		Mystrium	sp.*1a*	Madagascar	CASENT0500093
		Mystrium	sp.*1b*	Madagascar	CASENT0500070
		Onychomyrmex	*hedleyi*	Queensland	CASENT0501443
		Prionopelta	*Africa* sp.*1a*	Gabon	CASENT0500357
		Prionopelta	*Africa* sp.*1b*	Gabon	CASENT0500361
		Prionopelta	sp.*1*	Madagascar	CASENT0500351
		Prionopelta	sp.*2.*	Colombia	CASENT0500456
Formicidae/Apomyrminae	Apomyrmini	*Apomyrma*	*stygia*	Central African Republic	CASENT0501442
Formicidae/Cerapachyinae	Cerapachyini	*Cerapachys*	sp.*9*	Central African Republic	CASENT0517739
Formicidae/Dolichoderinae	Dolichoderini	*Linepithema*	*humile*	USA	CASENT0500524
Formicidae/Ectatomminae	Ectatommini	*Ectatomma*	*rudium*	Colombia	CASENT0500450
		Ectatomma	*tuberculatum*	Colombia	CASENT0500452
	Typhlomyrmecini	*Typhlomyrmex*	*rogenhoferi*	Peru	CASENT0501441
Formicidae/Formicinae	Camponotini	*Camponotus*	*vicinus*	USA	CASENT0500526
	Myrmoteratini	*Myrmoteras*	*iridiuom*	Malaya	CASENT0500268
Formicidae/Leptanillinae	Leptanillini	*Leptanilla*	*VB03*	South Africa	CASENT0501680
		Leptanilla	*VB04*	South Africa	CASENT0501681
Formicidae/Myrmeciinae	Myrmeciini	*Myrmecia*	*croslandi*	Australia	GenBank No. AB052895
Formicidae/Myrmicinae	Solenopsidini	*Solenopsis*	*invicta*	USA	CASENT0500523
Formicidae/Proceratiinae	Proceratiini	*Proceratium*	sp.*1*	Madagascar	CASENT0500379
		Discothyrea	sp.*1*	Madagascar	CASENT0500162
		Discothyrea	sp.*2*	Gabon	CASENT0500366
Formicidae/Ponerinae	Platythyreini	*Platythyrea*	*bicuspis1*	Madagascar	CASENT0500170
		Platythyrea	*bicspis2*	Madagascar	CASENT0501446
	Ponerini	*Hypoponera*	*sakalava*	Madagascar	CASENT0500383
Bradynobaenidae		*Chyphotes*	sp.	USA	CASENT0501449
Vespidae		*Polistes*	sp.	USA	CASENT8050694
Mutillidae		*Odontophotopsis*		USA	CASENT0501450

and further manually aligned using MacClade 4.03 (Maddison and Maddison, 2001).

2.6. Preliminary sequence analysis

Base composition bias was calculated (Irwin et al., 1991) for the entire fragment. A value of zero indicates no bias and a value of one indicates complete bias. An extreme overabundance of one nucleotide state can increase the tendency for those sites to become saturated (Irwin et al., 1991). In addition, a strongly skewed mutation bias can violate the assumption in maximum parsimony analysis that there is an equal probability of change at all sites (Perna and Kocher, 1995). The heterogeneity χ^2 test in PAUP*4.03b10 was used to test for bias among taxa.

Table 2
Primer sequences for amplification and sequencing of the nuclear large subunit (28S) rDNA divergent domains D1–D3

Primer	Sequence	Utility	*D. mel* Ref.	Primer citation
Mo6	5′-CCCCCTGAATTTAAGCATAT-3′	Amplification/sequencing	3318–3337	Schmitz and Moritz (1994)
D2B	5′-GTCGGGTTGCTTGAGAGTGC-3′	Sequencing	3549–3568	(Present study)
D2B-r	5′-GCACTCTCAAGCAACCCGAC-3′	Sequencing	3549–3568	(Present study)
D3A	5′-GACCCGTCTTGAAACACGGA-3′	Sequencing	4046–4065	(Present study)
D3A-r	5′-TCCGTGTTTCAAGACGGGTC-3′	Sequencing	4046–4065	(Present study)
D3B	5′-TCGGAAGGAACCAGCTACTA-3′	Amplification/sequencing	4395–4414	(Present study)

Drosophila melanogaster reference sites listed. Formicidae amplification of these sites is larger (~1200 bp) due to indels.

2.7. Phylogenetic analysis

To infer relationships among the amblyoponine ants, several phylogenetic analyses were performed using PAUP*4.03b10 (Swofford, 2001) and MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001). A variety of model-based methods, in addition to maximum parsimony (MP), were employed to infer phylogenetic relationships. Parsimony has been shown to be inconsistent under certain situations (Felsenstein, 1978), particularly when dealing with certain types of molecular sequence data (Hasegawa and Fujiwara, 1993; Kuhner and Felsenstein, 1994; Huelsenbeck, 1995), so maximum likelihood approaches were also used. First, parsimony searches were performed using the random stepwise addition option of the heuristic search for 1000 replicates with tree bisection-reconnection (TBR) branch swapping, collapse of zero-length branches, and equal weighting of all characters. Two maximum parsimony analyses were conducted: one including all sites in the alignment (the ''complete'' data set), and the other ''reduced'' data set with 150 ambiguously aligned sites excluded. If searches produced more than one tree, a strict consensus was performed to summarize data analyses. To measure the robustness of branching patterns of the parsimony trees, bootstrap analyses (bs) (Felsenstein, 1985; Hillis and Bull, 1993) were executed by using the closest stepwise addition of the heuristic search for 500 replicates.

To evaluate the fit of the data, a maximum likelihood analysis was conducted using the complete data set with both PAUP*4.03b10 (Swofford, 2001) and MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001). To determine which model best fit the data, a series of nested [i.e., the null hypothesis (H_0) is a special case of the alternative hypothesis (H_1)] hypotheses were performed on various nucleotide substitution models, the likelihood ratio test (LRT). The data were also subjected to Modeltest 3.06 (Posada and Crandall, 1998) to corroborate the findings of the likelihood ratio test. After a best-fit model was found a heuristic search was begun using the initial parameter estimates obtained from a neighbor-joining (NJ) tree generated in PAUP*4.03b10 (Swofford, 2001). Once a better tree was found, the parameters were re-estimated and the search was repeated. This process was continued until a tree converged on the same maximum likelihood tree. To test the robustness of the final maximum likelihood (ML) tree, a bootstrap analysis was performed using the closest stepwise addition option of the heuristic search for 1000 replicates.

The maximum likelihood model was used to determine whether the sequence among taxa was evolving at a constant rate and fit a molecular clock (Felsenstein, 1993). We used a procedure proposed by Felsenstein (1993) to test the H_0 of a molecular clock. This test uses a LRT to determine if there are significant differences between the likelihood scores obtained from an analysis where the branch lengths are unconstrained as compared to an analysis where the branch lengths are constrained so terminal ends are contemporaneous. The likelihood test statistic was assumed to be approximately equal to a χ^2 distribution with $n-2$ degrees of freedom, where n equals the number of taxa sampled (Felsenstein, 1981).

Analyses were also performed with MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001), with model parameters being estimated during the run, and using the default value of four Markov chains. Multiple chains can assist in more easily navigating tree-space and help avoid entrapment in local topological optima. Incremental heating of each chain resulted because a ''temperature'' parameter of 0.2 was implemented. Higher temperature values result in greater differences in heating between chains, and hotter chains are less constrained by likelihood scores in moving through tree-space (Wilcox et al., 2002). The Markov chain Monte Carlo (MCMC) length was 20^5 generations and 10^5 generations (results not shown), and we sampled the chain every 100 generations after the initial burn-in period of 100,000 generations. Bayesian posterior probabilities (bpp) were estimated as the proportion of trees sampled after burn-in that contained each of the observed bipartitions (Larget and Simon, 1999).

3. Results

3.1. Simple sequence statistics

This study produced a final aligned 1353 bp fragment for each taxon for a region spanning the nuclear 28S

rDNA fragment of the divergent domains: D1–D3. The aligned fragment contained 476 sites that were variable (35%) and 314 sites that were parsimoniously informative (23%). Examinations of base composition in the entire data set resulted in the following: A, 0.21739; C, 0.27821; G, 0.32253; and T, 0.18187. This data set exhibited low to relatively low base composition bias (bias = 0.043). A χ^2 test for homogeneity of base frequency among taxa was non-significant when all characters were included resulting in a value of 65.883 with 150 degrees of freedom ($P = 1.00$). When uninformative sites were eliminated, the χ^2 value was 174.310 with 150 degrees of freedom, and once again the test for heterogeneity of base frequency among taxa remained non-significant ($P = 0.085$). The heterogeneity test suggests that none of the sequence was heterogeneous.

3.2. Parsimony phylogenetic analyses of 28S rDNA

The maximum parsimony (MP) analysis of all characters resulted in 63 equally parsimonious trees ($L = 1282$). The strict consensus tree with bootstrap values is presented in Fig. 1. To examine the affect of the alignment on the analysis, 150 ambiguously aligned sites were removed, which created the ''reduced'' data set of 1203 positions. The parsimony analysis with ambiguously aligned sites removed resulted in 87 equally parsimonious trees ($L = 896$) (not shown). The consensus of this

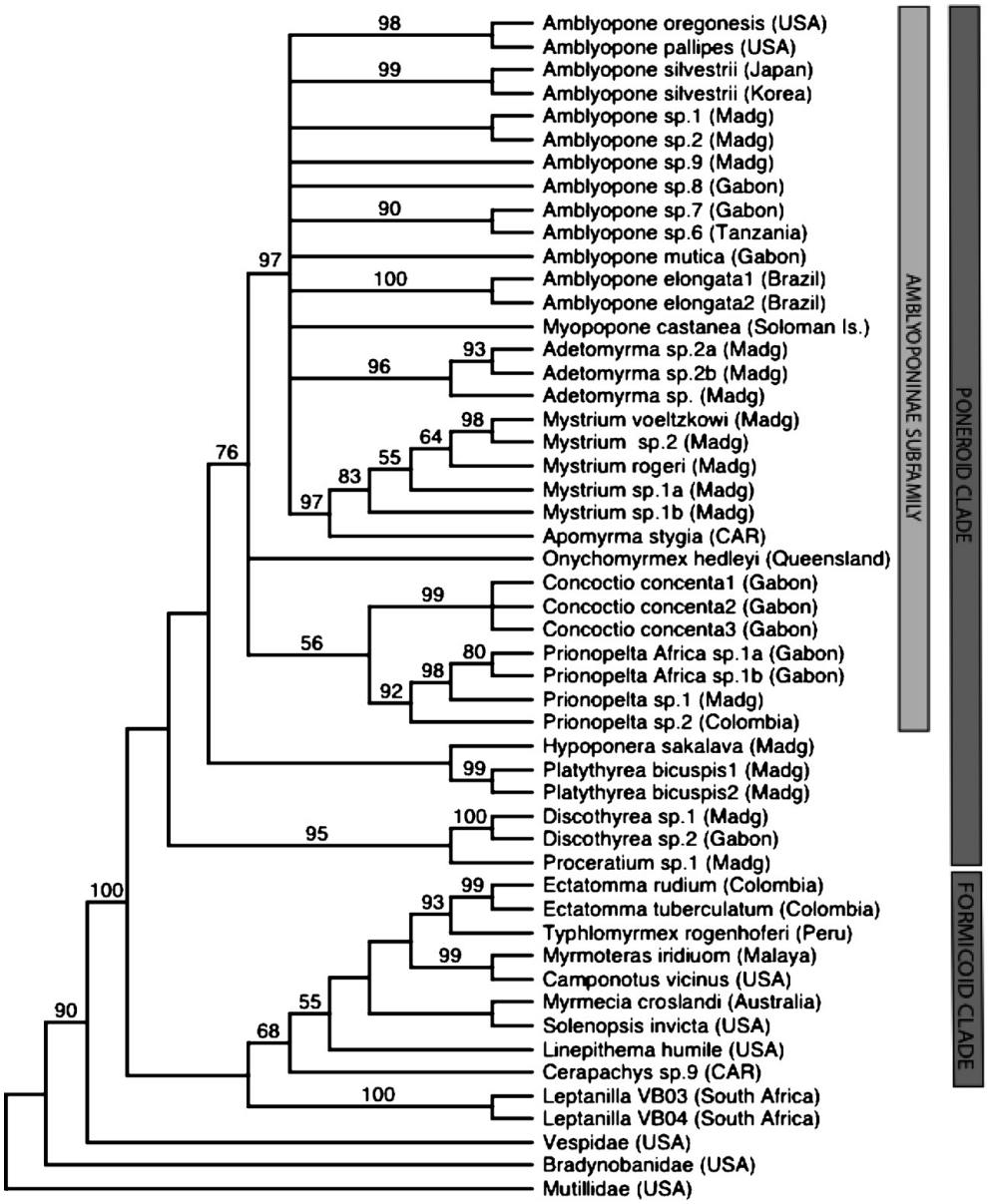

Fig. 1. Strict consensus tree calculated from the 63 equally parsimonious trees resulting from the analysis of the complete 28S data set. Values above the branches represent bootstrap percentages greater than 50%.

"reduced" data set tree is virtually the same as for the complete data set. The "reduced" data set consensus still supports the subfamily Amblyoponinae, including *Apomyrma*, but the *Concoctio* plus *Prionopelta* clade is no longer supported, although each genus remains a monophyletic clade. The variation in positioning of the latter two genera is not surprising since this grouping was not confirmed by bootstrap support in either analysis. Also in the "reduced" data set consensus topology the clade containing *Hypoponera* and *Platythyrea* is the sister clade to *Proceratium* plus *Discothyrea*. Where as the topology recovered in the complete data set the clade containing *Proceratium* plus *Discothyrea* is sister to all other Poneroid clade genera. Once again this difference lacked support in both analyses. Lastly, the positioning of *Leptanilla* as basal to the non-poneroid genera collapsed as a basal polytomy. The key differences present in the "reduced" data set consensus tree relative to the complete data set tree consists of a lack of resolution for certain taxa, but not an entirely different tree, suggesting that the gapped positions (ambiguously aligned sites) are not contributing a disproportionate effect on the topology. The major effect produced by removing the ambiguously aligned positions was to slightly lower the bootstrap support at some of the nodes, which is expected since fewer characters are present in the "reduced" data set. Since the inclusion of the ambiguously aligned characters does not appear to have any adverse affects on recovering the phylogeny, all subsequent analyses were conducted using all the characters.

3.3. Maximum likelihood phylogenetic analyses of 28S rDNA

The best fit maximum likelihood (ML) model determined using the LRT, as well as, Modeltest 3.06 (Posada and Crandall, 1998) suggested that the best model for these data was the GTR + Γ + I. The maximum likelihood search in PAUP*4.03b10 (Swofford, 2001) using this model resulted in one maximum likelihood tree with a $-\ln L = 8136.34467$ (Fig. 2). The parameter values as estimated from this tree were: A $\Longleftrightarrow$ C: 1.28023, A $\Longleftrightarrow$ G: 2.40775, A $\Longleftrightarrow$ T: 2.25937, C $\Longleftrightarrow$ G: 1.35169, C $\Longleftrightarrow$ T: 5.97692, G $\Longleftrightarrow$ T: 1.0 for the GTR model, estimated base composition was A = 0.216632, C = 0.268598, G = 0.312668, T = 0.202102, $\alpha = 0.265147$ for the Γ distribution, and I = 0.238876 for the proportion of invariable sites. Maximum likelihood was also used to test for a clocklike evolution. The molecular clock tree produced with the same parameter estimates above gave a likelihood score of $-\ln L = 8208.61041$, which indicates that the molecular clock should be rejected ($\chi^2 = 144.54$, df = 49, $P = 0.0001$).

The maximum likelihood analysis of all characters in MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001) using the GTR + Γ + I (PINVAR) model of sequence evolution resulted in a tree topology (Fig. 3) with a likelihood score of $-\ln L = 8141.24226$, which allows for direct comparison of support values for the clades among all analysis techniques by allowing the authors to present support values recovered by maximum parsimony, maximum likelihood, and Bayesian inference on one topology. The parameter values as estimated from this tree were: A $\Longleftrightarrow$ C: 1.19429, A $\Longleftrightarrow$ G: 2.41538, A $\Longleftrightarrow$ T: 2.41132, C $\Longleftrightarrow$ G: 1.34209, C $\Longleftrightarrow$ T: 6.04970, G $\Longleftrightarrow$ T: 1.0 for the GTR model, estimated base composition was A = 0.215994, C = 0.270821, G = 0.313773, T = 0.199412, $\alpha = 0.249280$ for the Γ distribution, and I (PINVAR) = 0.223471 for the proportion of invariable sites.

3.4. Phylogenetic relationships within the ants

All parsimony and maximum likelihood tree topologies show moderate support for a monophyletic Amblyoponinae subfamily, as long as *Apomyrma* is included in the subfamily. No analysis recovered evidence of monophyly of the genus *Amblyopone*. In the Bayesian analysis *Myopopone* is nested within *Amblyopone* (73% bpp) but this relationship is not supported in the other analyses. The clades for the other amblyoponine genera (*Adetomyrma*, *Concoctio*, *Mystrium*, and *Prionopelta*) were consistently recovered as monophyletic groups under all analyses (Fig. 3). The clade containing members of *Adetomyrma*, *Amblyopone*, *Myopopone*, *Mystrium* and *Apomyrma* maintains high support (>90%) across all analyses, although many of the generic relations remain unclear. When this clade is included with the rest of the amblyoponines, Bayesian support value evidence for the monophyly of the subfamily decreases (87% bpp) (Fig. 3).

Although there is little parsimony (Fig. 1) or maximum likelihood (Figs. 2 and 3) bootstrap evidence for clades outside the Amblyoponinae, two results are noteworthy. First, among the outgroup taxa, *Typhlomyrmex* grouped consistently with *Ectatomma*, supporting the recent placement of *Typhlomyrmex* in the Ectatomminae. Second, the poneromorph subfamily group appears to be polyphyletic. This hypothesis is supported across all analyses (Fig. 3). One clade contains all the Amblyoponinae (including *Apomyrma*), Ponerinae, and Proceratiinae, which we term as the Poneroid clade. The other clade contains members from subfamilies Cerapachyinae, Dolichoderinae, Ectatomminae, Formicinae, Myrmeciinae, and Myrmicinae termed the Formicoid clade. The relationship of *Leptanilla* to other ants is unclear. *Leptanilla* is found basal to either the formicoid clade (Fig. 1), both clades (Fig. 2) or as a basal group lacking resolution as to their relationship to the other ants (Fig. 3).

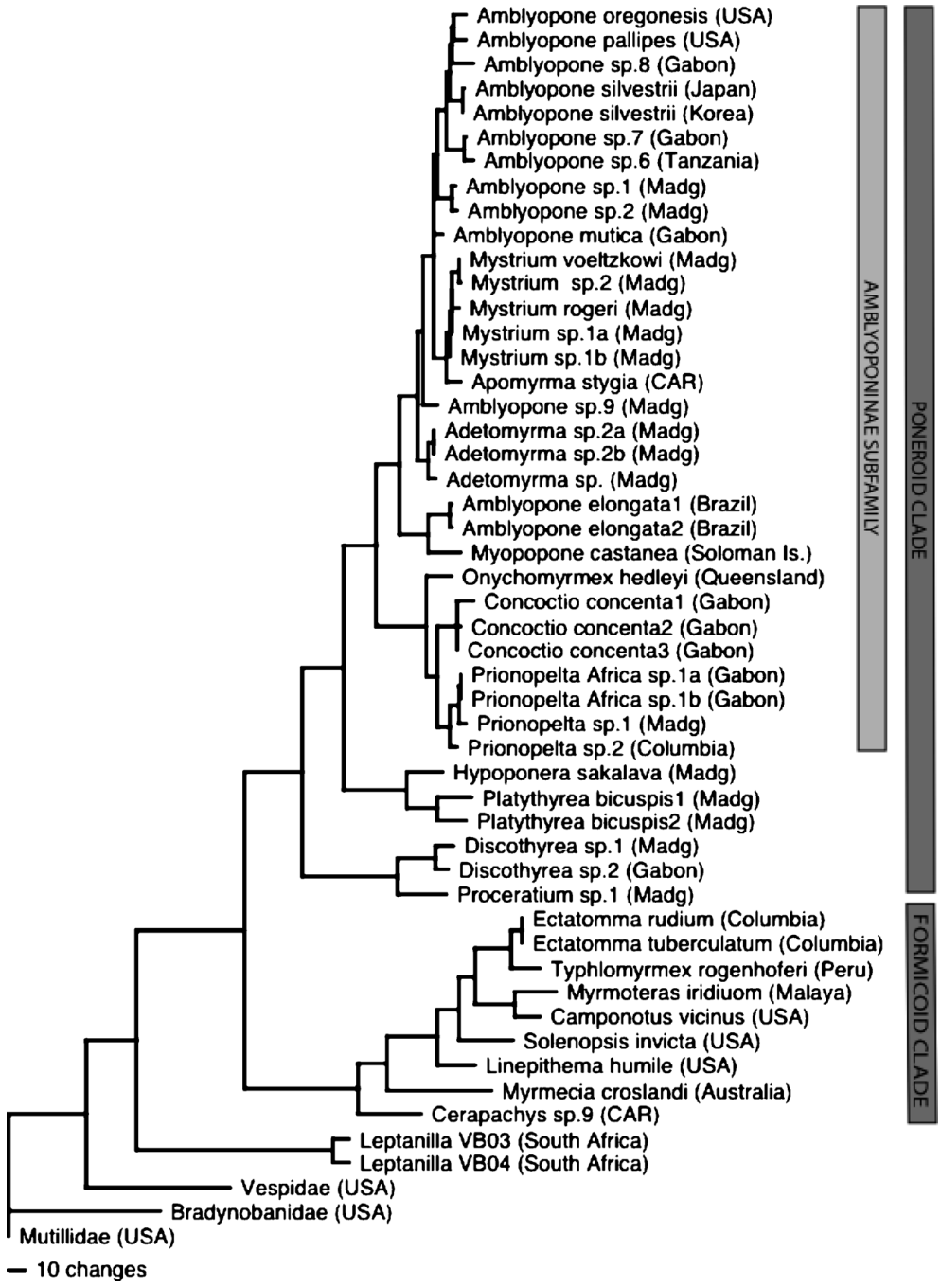

Fig. 2. Single tree inferred under maximum likelihood search shown with branch lengths proportional to estimated divergence with a GTR + Γ + I model of sequence evolution from the analysis of the complete 28S data set.

The ants (Formicidae) are consistently recovered as a monophyletic group. Within the outgroups analyzed the wasp family Vespidae is the sister group to the Formicidae, with support for this relationship consistent across all analyses (95% bpp; 72% ML bs; and 90% MP bs). Using morphological evidence, Brothers (1999) also suggested this relationship while investigating the Hymenoptera.

4. Discussion

Phylogenetic relationships of the Amblyoponinae ants are investigated. Analyses were performed using several different methods: maximum parsimony bootstrap analyses (Felsenstein, 1985; Hillis and Bull, 1993), maximum likelihood non-parametric bootstrapping (Felsenstein, 1985) and Bayesian analysis (Larget

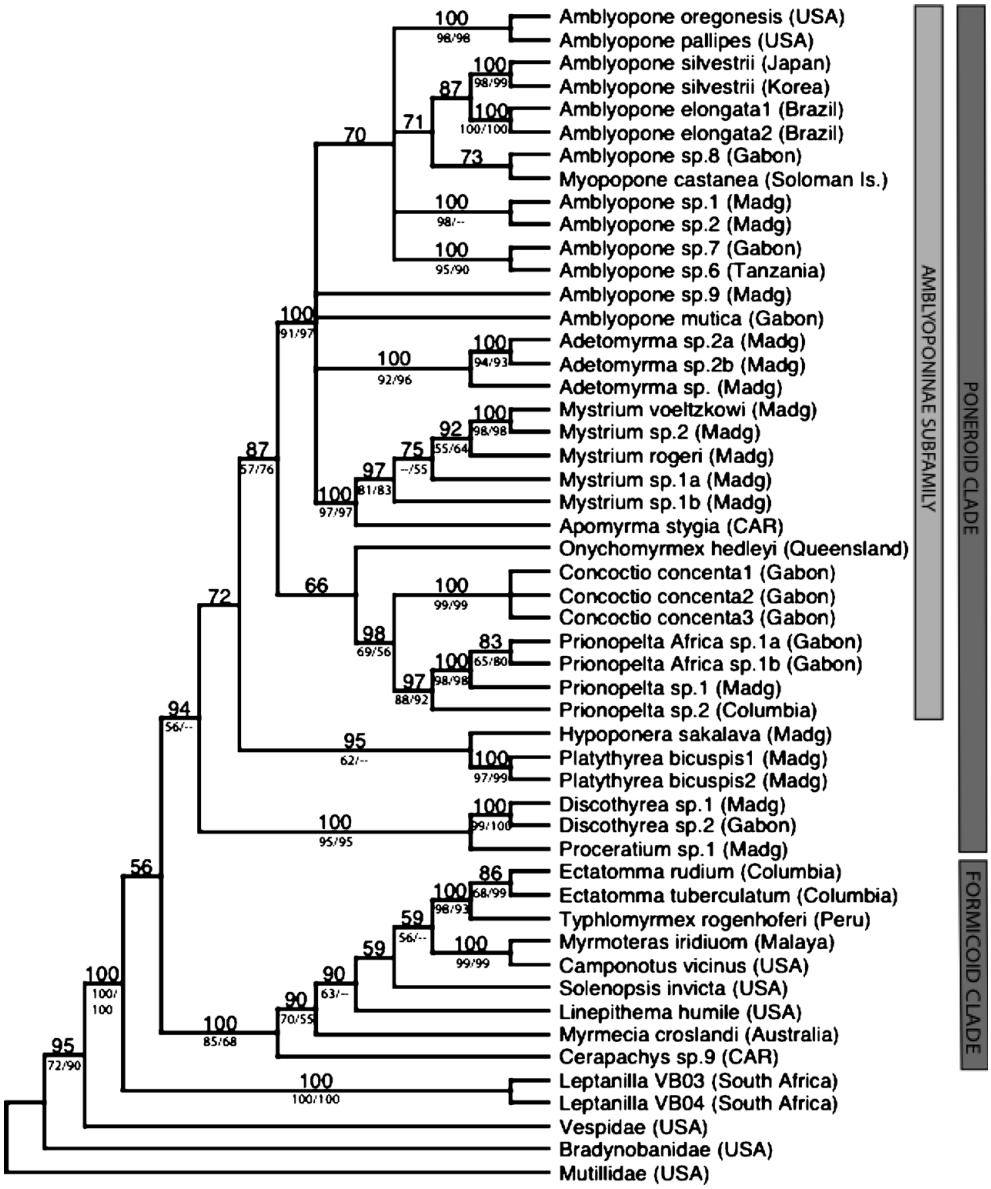

Fig. 3. Maximum likelihood tree recovered in a Bayesian framework (2,000,000 generations) with a GTR + Γ + I (PINVAR) model of sequence evolution with all characters within the 28S data set. Values above the branches represent support values from Bayesian analysis greater than 50% (bpp). Values below the branches represent maximum likelihood (1000 replicates) and parsimony (500 replicates) bootstrap percentages greater than 50% (ML/PS) [(—) denotes lack of support for the clade in the respective analysis method].

and Simon, 1999; Rannala and Yang, 1996). Although all analyses agree on overall topology, support values varied between methods. The topology recovered using Bayesian techniques of the Amblyoponinae subfamily of ants allows for direct comparison of support values for the clades among all analysis techniques by allowing the authors to present support values recovered by maximum parsimony, maximum likelihood, and Bayesian inference on one topology (Fig. 3).

4.1. Reclassification of the Amblyoponinae

All parsimony, maximum likelihood, and Bayesian inference tree topologies show support for a monophyletic Amblyoponinae, as long as *Apomyrma* is included in the subfamily. The clade containing members of the genera *Adetomyrma*, *Amblyopone*, *Myopopone*, *Mystrium*, and *Apomyrma* consistently show strong support across all analysis performed (>90%) within the Amblyoponinae clade. In addition, the data presented here robustly support (100% bpp; 97% ML bs; and 97% MP bs) *Apomyrma* as a sister to *Mystrium* and is therefore a member of the Amblyoponinae. Based on these results, we suggest that *Apomyrma* be placed in the subfamily Amblyoponinae. Our proposed new classification of the subfamily follows Bolton (2003) but includes *Apomyrma* as a genus-rank taxon of the Amblyoponinae (see

Bolton, 2003 for a complete list of synonymies and taxonomic histories). In our study, the resolution within this subfamily is not sufficient to address relationships among the genera of Amblyoponinae and therefore we follow the single tribe-rank classification of Bolton (2003).

Interestingly, *Apomyrma* was first described as a member of the Amblyoponini tribe within the Ponerinae subfamily based on worker morphology (Brown et al., 1970) and larval morphology (Wheeler and Wheeler, 1970). Bolton (1990) transferred the genus from the Ponerinae subfamily to the Apomyrmini tribe within the subfamily Leptanillinae based on abdominal morphology. Finally Baroni Urbani et al. (1992) elevated *Apomyrma* to subfamily status based on cladistic analysis of these morphological characters. Ward (1994) made important observations concerning the morphological characters of *Amblyopone* and *Adetomyrma* that suggested a close relationship between *Apomyrma* and Amblyoponini. He observed that: (1) abdominal segment IV in *Adetomyrma* lacks tergosternal fusion as in *Apomyrma* but unlike all other amblyoponine genera and (2) a few Australian *Amblyopone* species (*A. australis*, *A. gingivalis*, *A. hackeri*, and *A. longidens*, as well as an undescribed species from north Queensland) possess dentiform teeth on the labrum as in *Apomyrma*. In Ward (1994, p. 163, Fig. 9) the undescribed *Amblyopone* species was mistakenly noted as *Onychomyrmex doddi* (P.S. Ward, pers. comm.). As noted by Ward (1994), most of the diagnostic characters of the Apomyrminae could be derived from those seen in the Amblyoponinae.

4.2. Diagnosis

The inclusion of *Apomyrma* within the Amblyoponinae requires only minor changes to the diagnosis presented in Bolton (2003) and Ward (1994). The newly defined subfamily can be distinguished from all other subfamilies by the following combination of worker- and queen-based features:

1. Dentiform seta present on clypeus, labrum or both (absent in *A. mutica* and *mutica*-like species in Madagascar).
2. Waist of a single segment (petiole), with articulation to abdominal segment III very broad, petiole without a distinctly descending posterior face (petiole with a distinctly descending posterior face in *Apomyrma*).

It should be noted that the leptanilline genus *Protanilla* possesses a pair of stout setae on the labrum (Bolton, 1990). The Leptanillinae however can be easily distinguished from the Amblyoponinae by the presence of two distinct isolated segments (petiole plus postpetiole).

4.3. Tergosternal fusion

The discovery of the amblyoponine genus, *Adetomyrma*, was significant in the ant systematics community because this ant, based on morphological evidence, is a member of the amblyoponine ants, but does not exhibit apomorphic abdominal characters seen in all other poneroid subfamilies (Grimaldi et al., 1997; Ward, 1994). Our results support the placement of *Adetomyrma* in the Amblyoponinae as originally described by Ward (1994). Workers of *Adetomyrma* lack tergosternal fusion of abdominal segments III and IV, a character seen in all other Poneromorph genera, excluding *Apomyrma*, *which* lacks tergosternal fusion of abdominal segments IV. This study suggests that *Adetomyrma* has undergone secondary reversal of this character along with *Apomyrma*. Contrary to Ward's (1994) suggestion, it does not appear that *Adetomyrma* is "the sole survivor of an early lineage in the tribe" or even a basal member of the entire poneroid group.

4.4. Poneromorph subfamilies

Members of the poneromorph group of subfamilies as defined in Bolton (2003) appear to be a polyphyletic group although not strongly supported by bpp and bootstrap. Several previous studies based on morphology have suggested that the poneromorph subfamilies might not comprise a monophyletic lineage (Hashimoto, 1991; Grimaldi et al., 1997; Keller, 2000; Lévieux, 1972; Ward, 1994; Wheeler et al., 1999). The clade containing members of *Typhlomyrmex* and *Ectatomma* are the only poneromorph taxa included in this study that do not group with the remaining members of the poneromorph subfamilies. *Typhlomyrmex* repeatedly clustered with *Ectatomma* and all analyses place the *Ectatomma* separate from the clade containing *Discothyrea* and *Proceratium*. These results are consistent with Bolton's (2003) morphological analysis and classification of the Ectatomininae and Proceratiinae, as well as Lattke's (1994) cladistic analysis of the ectatommine ants.

The results of this present study place the included ant subfamilies into roughly two clades with the basal placement of *Leptanilla* unclear. One clade contains all the poneromorph taxa from the Amblyoponinae (including *Apomyrma*), Ponerinae, and Proceratiinae subfamilies, termed here the poneroid clade. The other clade contains members from subfamilies Cerapachyinae, Dolichoderinae, Ectatomminae, Formicinae, Myrmeciinae, and the Myrmicinae, termed the formicoid clade. Interestingly, *Leptanilla* is recovered as a basal lineage to either the formicoid clade (Fig. 1), both clades (Fig. 2) or as a basal group lacking resolution as to their relationship to the other ants (Fig. 3), among the ant taxa sampled in this study. This placement has not been

suggested in previous ant literature. It should be noted that the possibility that *Leptanilla* is the basal lineage of the poneroid clade, cannot be ruled out.

This study demonstrates the use of 28S rDNA sequence data in defining a major monophyletic lineage within ants. Though the monophyly of the Amblyoponinae subfamily is resolved, the relationships among the major lineages are less clear. Most importantly, the basal lineage of ants is still not resolved. *Amblyopone* and *Myrmecia* are not recovered as basal clades as previously proposed (Wilson, 1971). *Leptanilla* is a possible candidate, even though this would have many implications for ant systematics. As Ward (1994) noted, if *Leptanilla* is actually a basal lineage of ants, then this would imply that tergosternal fusion of abdominal segment III occurred early in the ants and has been secondarily lost in many lines. Findings in this study and the lack of stability in previous morphological studies (Baroni Urbani et al., 1992; Grimaldi et al., 1997; Hölldobler and Wilson, 1990; Ward, 1994) suggest that our definition of ancestral ant morphology and behaviors needs further attention. The use of DNA sequence data, however, provides the most promising avenue to begin unraveling the relationships among major ant lineages, which will lead to a better understanding of ant evolution.

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