Molecular systematics of basal subfamilies of ants using 28S rRNA (Hymenoptera: Formicidae)

Gary D. Ouellette [a,b], Brian L. Fisher [a], Derek J. Girman [b,*]

[a] *Department of Entomology, California Academy of Sciences, 875 Howard Street, San Francisco, CA 94103, USA*
[b] *Department of Biology, Sonoma State University, 1805 E. Cotati Ave, Rohnert Park, CA 94928, USA*

Abstract

For many years, the ant subfamily Ponerinae was hypothesized to contain the basal (early branching) lineages of ants. Recently the Ponerinae were reclassified into six poneromorph subfamilies based on morphological analysis. We evaluate this new poneromorph classification using 1240 base pairs of DNA sequence data obtained from 28S rRNA gene sequences of 68 terminal taxa. The molecular tree supported the monophyly of the ant family Formicidae, with 100% parsimony bootstrap (PB) support and posterior probabilities (PP) of 1.00, with the ant subfamily Leptanillinae as a sister group to all other ants (PB = 62, PP = 93). However, our analyses strongly support the polyphyly of the Poneromorph subfamilies (sensu Bolton). The Ectatomminae and Heteroponerinae are more closely related to the Formicoid subfamilies than to the rest of the poneromophs (PB = 96, PP = 100). The Amblyoponinae (PB = 52, PP = 96), Paraponerinae (PB = 100, PP = 100), Ponerinae (PB < 50, PP = 71), and Proceratiinae (PB = 98, PP = 100) appear as distinct lineages at the base of the tree and are identified as a poneroid grade. Monophyletic origins for the poneroid subfamilies Amblyoponinae, Paraponerinae, Ponerinae and Proceratiinae are supported in our analysis. However, the genus *Platythyrea* forms a distinct sister group to the Ponerini within the Ponerinae. The Heteroponerinae, based on our sample of *Heteroponera*, are associated with the subfamily Ectatomminae (PB = 98, PP = 100). Furthermore, our data indicate the genus *Probolomyrmex* belongs to the Proceratiinae as suggested by recent morphological analysis (PB = 98, PP = 100).
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1. Introduction

Ants, aculeate wasps in the family Formicidae (order Hymenoptera), as a whole are a well-defined clade. Cladistic analyses of morphological data (Baroni Urbani et al., 1992), molecular data (Astruc et al., 2004; Saux et al., 2004; Ward and Brady, 2003; Ward and Downie, 2005; Ward et al., 2005), and numerous diagnostic characteristics (Bolton, 2003), unequivocally support a monophyletic origin for ants. Relationships among the ants, however, are not well understood. This lack of taxonomic resolution has deterred progress in understanding why ants have become so successful; in terms of biomass, they now dominate terrestrial habitats throughout the world (Grimaldi et al., 1997; Hölldobler and Wilson, 1990; Wilson and Hölldobler, 2005a,b). One major obstacle has been the inability to clearly define the early branching lineages of ants and the sequence of their major evolutionary radiations. Identifying basal lineages within the ants (i.e. sister group(s) to remaining ants) would help to solve this mystery.

The poneromorphs (Ponerinae sensu lato), consist of a heterogeneous assemblage of taxa considered "primitive" based on their behavior and morphology. Bolton (2003) recently defined the Poneromorph subfamilies to include six subfamilies (Amblyoponinae, Ectatomminae, Heteroponerinae, Paraponerinae, Ponerinae, Proceratiinae). Both

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* Corresponding author. Fax: +1 707 664 1281.
  E-mail address: girman@sonoma.edu (D.J. Girman).

morphological (Grimaldi et al., 1997; Hashimoto, 1996; Keller, 2000; Ward, 1994) and molecular studies (Saux et al., 2004; Sullender and Johnson, 1998; Ward and Brady, 2003; Ward and Downie, 2005) suggest the poneromorphs are paraphyletic but include only a subset of lineages that are thought to have diverged early on, near the base of the formicid tree. Bolton's work represents the most current and comprehensive morphological study of this group. His findings provide a series of working hypotheses about ant relationships that can be tested with molecular data. Testing the monophyly of his classification using DNA sequence data is an important next step in the study of ant relationships.

The objective of this study is to reconstruct subfamily-level phylogenetic relationships among the Poneromorph ants using sequence data from the 28S ribosomal RNA gene. The results should identify the relative positions of the putative basal lineages of the ants. The 28S rRNA locus employed here has been used frequently to reveal phylogenetic relationships for a variety of taxonomic groups (Belshaw et al., 1998; Cameron and Mardulyn, 2001; Linares et al., 1991; Morgan et al., 2002; Sullender and Johnson, 1998; Whiting et al., 1997). The molecular sequence analysis also provides an opportunity to test how well the morphological characters used to establish the poneromorph subfamilies delineate actual phylogenetic relationships.

2. Poneromorph subfamilies

The Poneromorph subfamilies represent a large and diverse group of ants with a worldwide distribution. The species themselves range from small and cryptic to large and conspicuous. Based on their morphology and social organization, all are considered relatively primitive among the Formicidae (Hölldobler and Wilson, 1990; Wheeler, 1928; Wilson and Hölldobler, 2005b). The extant representatives of the Poneromorphs (Bolton, 1994, 2003; Xu, 2000) comprise 44 genera consisting of approximately 1600 described species (Bolton, 1995). The poneromorphs are traditionally recognized by the tergo-sternal fusion of the fourth abdominal segment (Bolton, 1990, 2003; but see Ward, 1994).

The Poneromorph subfamily Amblyoponinae is distributed worldwide and consists of the tribe Amblyoponini and nine extant genera (Bolton, 1994, 2003; Brown, 1960; Saux et al., 2004; Xu, 2000). These ants are mostly cryptobiotic obligatory predators that nest in soil or ground litter. The Amblyoponinae have long been considered a "primitive" group of ants, with numerous character states that are thought to be relatively ancestral (plesiomorphic) (Brown, 1960; Hölldobler and Wilson, 1990; Wilson, 1971). But recent morphological and molecular analyses (Hashimoto, 1996; Saux et al., 2004; Ward, 1994) have challenged the basal placement of the tribe. Resolving the phylogenetic position of the Amblyoponinae will provide insights into the evolution of the Poneromorph families will provide insights into the evolution of the Poneromorph subfamilies.

The subfamily Ectatomminae, as presently constituted (Bolton, 2003), includes the tribes Ectatommini and Typh-lomyrmecini and four genera largely confined to tropical and warm temperate climates of New World, Old World and Indo-Australian Regions. Members of the Ectatommini are defined by two synapomorphies: empodia are absent, and a narrow band of thin lamella borders the anterior clypeal margin (Lattke, 1994). The tribe Typhlomyrmecini is comprised of *Typhlomyrmex* (Mayr) and has a restricted distribution from southern Mexico to northern Argentina (Bolton, 1994; Brown, 1965). Brown (1965) noted close morphological similarities with members of *Gnamptogenys* and suggested that *Typhlomyrmex* may have evolved from ectatommine ancestors. Following a phylogenetic study based on morphological characters, Lattke (1994) suggested a close relationship between *Typhlomyrmex* and Ectatommini. More recently, Bolton (2003) placed this tribe among the Ectatomminae.

The newly established subfamily Heteroponerinae is currently comprised of the genera *Acanthoponera*, *Aulacopone*, and *Heteroponera*. This group has a Palearctic, neotropical, and Australasian distribution and contains 20 described species (Bolton, 1995). However, no apomorphy definitively describes this group (Bolton, 2003).

Paraponerinae is a monotypic subfamily comprised of the tribe Paraponerini and the neotropical species *Paraponera clavata* (Fabricius). This species was traditionally placed within the current subfamily Ectatomminae based upon morphological similarities (Brown, 1958; Emery, 1895). Following a comprehensive study of larval morphology, Wheeler and Wheeler (1971) agreed with the placement of *Paraponera clavata* within the Ectatomminae, despite differences in mandible structure. However, a series of more recent phylogenetic studies have refuted this conclusion (Bolton, 2003; Brady, 2003; Keller, 2000; Lattke, 1994), providing strong support for separating *Paraponera clavata* from the Ectatomminae.

Ponerinae is the largest subfamily of Poneromorphs. This group is represented by the tribes Platythyreini, Ponerini and Thaumatomyrmecini (Bolton, 2003). The tribe Platythyreini contains the genus *Platythyrea* with 38 described species (Bolton, 1995). Their distribution is primarily tropical, though they extend into the subtropical regions of all major continents (Brown, 1952, 1975). The tribe Ponerini contains 23 genera which are found on all continents and major islands (Bolton, 1994; Hölldobler and Wilson, 1990). Despite representing the majority of ponerine species, the tribe Ponerini has received relatively little attention and undergone few taxonomic changes since it was established in 1911 (but see Bolton, 2003). The tribe Thaumatomyrmecini is a neotropical group containing the genus *Thaumatomyrmex* Mayr (Bolton, 1994) and eight described species (Baroni Urbani and De Andrade, 2003).

The subfamily Proceratiinae as defined by Bolton (2003) contains the tribes Proceratiini and Probolomyrmecini. The Proceratiini are comprised of the genera *Discothyrea* and *Proceratium* and have a world distribution that is largely restricted to tropical and subtropical climates. Observations of these ants indicate they specialize in consuming various arthropod eggs (Brown, 1979). The genus *Probolomyrmex* was originally placed among the tribe Proceratiini.

Following an observation by Mann (1923) that their sculpture and pilosity is similar to the *Platythyrea*, Brown (1952, 1975) moved *Probolomyrmex* to the Ponerinae tribe Platythyreini. He considered *Probolomyrmex* to represent a direct descendent of *Platythyrea* and described their morphological similarities with the Proceratiini as convergent characters arising from adaptations to a hypogaeic life. Brown (1975) also indicated a close relationship with the Ponerinae based on both behavioral and morphological characteristics. These similarities included general habitus, down-curved pygidial spine shared by the male caste, and primitive wing venation. Perrault (2000), based on a detailed study of cephalic and mesosomal characters, removed *Probolomyrmex* from Platythyreini (Ponerinae) and defined a new subfamily Probolomyrmecinae. Bolton (2003) demoted the subfamily Probolomyrmecinae to tribe rank within the subfamily Proceratiinae.

3. Methods

3.1. Taxa

Selection of poneromorph and non-poneromorph species for molecular analysis was based primarily on major groups recognized by Bolton (1994, 2003). A total of 66 ant taxa comprising 40 genera and 14 ant subfamilies were investigated in this study as shown in Table 1. Since the monophyly of Poneromorph ants was being tested, representatives from six poneromorph ant subfamilies (Amblyoponinae, Ectatomminae, Heteroponerinae, Paraponerinae, Ponerinae, and Proceratiinae) and eight non-poneromorph ant subfamilies (Cerapachyinae, Dolichoderinae, Ecitoninae, Formicinae, Myrmicinae, Myrmeciinae and Pseudomyrmecinae) were included in the analysis. Two additional Hymenopteran families were included as outgroups (Vespidae, Scoliidae). Outgroups were chosen following recent phylogenies of the vespoid families (Brothers and Carpenter, 1993; Carpenter, 1990). All voucher specimens have been deposited with the California Academy of Sciences, Department of Entomology Collection (CASC).

3.2. DNA amplification and sequencing

DNA was extracted from specimens stored in 95% ethanol. Extractions were conducted using a Qiagen DNeasy™ Tissue Kit (Qiagen Inc., Valencia, CA) following the protocol for animal tissues. Individual ants were homogenized using a disposable pestle, then digested over night using 20 μL of 20 mg/mL Proteinase K at 55 °C. The lysate was then pipetted onto a silica-gel-membrane and purified with a series of ethanol washes using supplied DNeasy™ Buffers. The DNA was resuspended with 200 μL of 10 mM Tris buffer.

A partial region of the 28S rRNA gene, including the expansion segments D1, D2, D3 and intervening core regions (Hancock et al., 1988; Hillis and Dixon, 1991), was PCR-amplified following standard protocol (Saiki et al.,

1988). The primers used in this study are listed in Table 2. The total amplified region spans positions 3318–4414 corresponding to coordinates in the *Drosophila melanogaster* 28S gene, using the numbering of Tautz et al. (1988). Double-stranded PCR products were generated in 25 μL reactions comprised of 10× buffer (100 mM Tris–HCL, pH 9.0, 500 mM KCl, 1% Triton X-100), 2.5 mM MgCl$_2$, 0.8 mM dNTPs, 0.1 mM of each primer, 1.25 units of Taq polymerase (GibcoBRL), 1 μL DMSO, and 3 μL extracted DNA. Reactions were performed using a PTC-200 programmable thermocycler (MJ Research, Inc.) using the following cycle parameters: one cycle of 95 °C for 2 min; 34 cycles of 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 1 min. These cycles were followed by a prolonged step of 10 min at 72 °C and then 4 °C. Amplified PCR products were purified using the Promega Wizard PCR Preps DNA Purification System (Promega Inc., Madison, WI) following the manufacturer's instructions. Products were cycle-sequenced in both directions using a 7-deaza-dGTP sequencing kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Cycle sequenced products were separated in 5% acrylamide gels using a LI-COR Gene ReadIR 4200 automated sequencer (LI-COR Inc., Lincoln, NE). All sequencing was accomplished at the Core Sequencing Facility located at Sonoma State University. All new DNA sequences generated for this project have been submitted to the NCBI GenBank database (see Table 1 for accession numbers). Sequence data for 14 previously sequenced samples were obtained from GenBank (Table 1).

3.3. Sequence alignment

Sequences were initially assembled and examined thoroughly using the program Sequencher 3.0 (Gene Codes Corporation Inc.). All sequences were confirmed and adjusted by visual inspection of chromatograms. Sequence alignment was conducted using the multiple alignment program CLUSTAL X (Thompson et al., 1997) applying the default settings of gap opening and extension penalty for both pairwise and multiple alignments. The resulting alignment was further modified by hand, using the program Sequencher v.4.5 (Gene Codes), to correct obvious alignment errors. After manual inspection, we excluded a set of 163 sites that were ambiguously aligned. We removed sites from indel-rich regions that were hypervariable within the ingroup since this has been found to be a less stringent, but effective mechanism for removing ambiguous sites (Brady, 2003; Ward and Downie, 2005). Gaps in the non-excluded sequence regions were coded as "missing." Bias in nucleotide base composition across taxa was analyzed using a chi-square test of heterogeneity of base frequency (Irwin et al., 1991).

3.4. Phylogenetic analyses

The reconstruction of phylogenetic relationships among taxa was conducted using two phylogenetic approaches

Table 1
DNA samples examined

Family	Subfamily	Genus	Species	Locality	Source	Accession number
Formicidae	Amblyoponinae	*Adetomyrma*	*sp. Ma-02*	Madagascar	CASENT0500384	DQ400974
Formicidae	Amblyoponinae	*Adetomyrma*	*sp. Ma-04*	Madagascar	CASENT0500505	DQ400975
Formicidae	Amblyoponinae	*Amblyopone*	*sp. Ma-01*	Madagascar	CASENT0500015	DQ400976
Formicidae	Amblyoponinae	*Amblyopone*	*sp.*	Madagascar	CASENT0500385	DQ400977
Formicidae	Amblyoponinae	*Myopopone*	*castanea*	Solomon Islands	CASENT0501682	DQ400999
Formicidae	Amblyoponinae	*Myopopone*	*castanea2*	Solomon Islands	Saux et al. (2004)	AY325929
Formicidae	Amblyoponinae	*Mystrium*	*mysticum*	Madagascar	CASENT0500386	DQ401000
Formicidae	Amblyoponinae	*Mystrium*	*mysticum2*	Madagascar	CASENT0500392	DQ401001
Formicidae	Amblyoponinae	*Mystrium*	*mysticum3*	Madagascar	CASENT0500395	DQ401002
Formicidae	Amblyoponinae	*Prionopelta*	*sp. Ma-04*	Madagascar	CASENT0501708	DQ401016
Formicidae	Amblyoponinae	*Prionopelta*	*sp. Ma-03*	Madagascar	CASENT0501695	DQ401017
Formicidae	Amblyoponinae	*Concoctio*	*concenta*	Gabon	Saux et al. (2004)	AY325940
Formicidae	Amblyoponinae	*Onychomyrmex*	*hedleyi*	Australia	Saux et al. (2004)	AY325938
Formicidae	Ectatomminae	*Ectatomma*	*ruidum*	Ecuador	CASENT0500163	DQ400987
Formicidae	Ectatomminae	*Ectatomma*	*ruidum*	Columbia	CASENT0500450	DQ400986
Formicidae	Ectatomminae	*Ectatomma*	*sp. Col-01*	Columbia	CASENT0500472	DQ400988
Formicidae	Ectatomminae	*Gnamptogenys*	*sp. Per-01*	Peru	CASENT0501440	DQ400990
Formicidae	Ectatomminae	*Gnamptogenys*	*sp. Bur-01*	Myanmar	CASENT0501210	DQ400989
Formicidae	Ectatomminae	*Rhytidoponera*	*sp. Sol-01*	Solomon Islands	CASENT0501452	DQ401023
Formicidae	Ectatomminae	*Typhlomyrmex*	*rogenhoferi*	Peru	CASENT0501441	DQ401026
Formicidae	Heteroponerinae	*Heteroponera*	*sp. Ec-01*	Ecuador	CASENT0513587	DQ400991
Formicidae	Paraponerinae	*Paraponera*	*clavata*	Peru	CASENT0501765	DQ401008
Formicidae	Paraponerinae	*Paraponera*	*clavata*	Brazil	Ward and Downie (2005)	AY703556
Formicidae	Ponerinae	*Platythyrea*	*bicuspis*	Madagascar	CASENT0500170	DQ401010
Formicidae	Ponerinae	*Platythyrea*	*sp. Ma-01a*	Madagascar	CASENT0510129	DQ401011
Formicidae	Ponerinae	*Platythyrea*	*sp. Ma-01b*	Madagascar	CASENT0516801	DQ401012
Formicidae	Ponerinae	*Anochetus*	*katonae*	Gabon	CASENT0500363	DQ400978
Formicidae	Ponerinae	*Asphinctopone*	*sp. Gb-01*	Gabon	CASENT0500364	DQ400979
Formicidae	Ponerinae	*Dinoponera*	*sp. Pr-01*	Peru	CASENT0501213	DQ400983
Formicidae	Ponerinae	*Hypoponera*	*sp. Ma-56*	Madagascar	CASENT0500165	DQ400993
Formicidae	Ponerinae	*Hypoponera*	*sp. PNG-01*	Papua New Guinea	CASENT0518808	DQ400994
Formicidae	Ponerinae	*Hypoponera*	*sp. Ec-01*	Ecuador	CASENT0513467	DQ400992
Formicidae	Ponerinae	*Leptogenys*	*sp. Gb-02*	Gabon	CASENT0500368	DQ400995
Formicidae	Ponerinae	*Leptogenys*	*intermedia*	South Africa	CASENT0501752	DQ400996
Formicidae	Ponerinae	*Loboponera*	*vigilans*	South Africa	CASENT0500369	DQ400998
Formicidae	Ponerinae	*Odontomachus*	*coquereli*	Madagascar	CASENT0500168	DQ401005
Formicidae	Ponerinae	*Odontomachus*	*troglodytes*	Congo	CASENT0510497	DQ401004
Formicidae	Ponerinae	*Pachycondyla*	*cambouei*	Madagascar	CASENT0500169	DQ401007
Formicidae	Ponerinae	*Pachycondyla*	*tarsata*	Gabon	CASENT0500373	DQ401006
Formicidae	Ponerinae	*Phrynoponera*	*bequaerti*	Gabon	CASENT0500374	DQ401009
Formicidae	Ponerinae	*Plectroctena*	*mandibularis*	South Africa	CASENT0500375	DQ401013
Formicidae	Ponerinae	*Plectroctena*	*mandibularis*	South Africa	CASENT0500376	DQ401014
Formicidae	Ponerinae	*Psalidomyrmex*	*procerus*	Gabon	CASENT0501757	DQ401022
Formicidae	Ponerinae	*Streblognathus*	*aethiopicus*	South Africa	CASENT0501205	DQ401024
Formicidae	Proceratiinae	*Probolomyrmex*	*sp. Gb-01*	Gabon	CASENT0500378	DQ401018
Formicidae	Proceratiinae	*Discothyrea*	*sp. Ma-05*	Madagascar	CASENT0500162	DQ400985
Formicidae	Proceratiinae	*Discothyrea*	*sp. Gb-01*	Gabon	CASENT0500366	DQ400984
Formicidae	Proceratiinae	*Proceratium*	*sp. Ma-02*	Madagascar	CASENT0500379	DQ401019
Formicidae	Proceratiinae	*Proceratium*	*sp. Ma-01*	Madagascar	CASENT0501211	DQ401020
Formicidae	Proceratiinae	*Proceratium*	*sp. Ma-01*	Madagascar	CASENT0501212	DQ401021
Formicidae	Proceratiinae	*Proceratium*	*stictum*	Australia	Ward and Downie (2005)	AY703557
Formicidae	Pseudomyrmecinae	*Tetraponera*	*aethiops*	Gabon	CASENT0501761	DQ401025
Formicidae	Cerapachyinae	*Cerapachys*	*sp. Gb-02*	Gabon	CASENT0501756	DQ400982
Formicidae	Cerapachyinae	*Cerapachys*	*sp. 9*	Central African Rep.	Saux et al. (2004)	AY325959
Formicidae	Cerapachyinae	*Cerapachys*	*larvatus*	Australia	Ward and Downie (2005)	AY703558
Formicidae	Dolichoderinae	*Linepithema*	*humile*	USA	CASENT0500524	DQ400997
Formicidae	Dolichoderinae	*Leptomyrmex*	*erythrocephalus*	Australia	Ward and Downie (2005)	AY703561
Formicidae	Ecitoninae	*Neivamyrmex*	*sp. USA-01*	USA	CASENT0501768	DQ401003
Formicidae	Formicinae	*Camponotus*	*sp. Ma-13*	Madagascar	CASENT0500416	DQ400980
Formicidae	Formicinae	*Camponotus*	*vicinus*	USA	Saux et al. (2004)	AY325957
Formicidae	Formicinae	*Myrmoteras*	*iriodum*	Malaya	Saux et al. (2004)	AY325956
Formicidae	Myrmeciinae	*Myrmecia*	*picta*	Australia	Ward and Downie (2005)	AY703565
Formicidae	Myrmicinae	*Solenopsis*	*invicta*	USA	Saux et al. (2004)	AY325955
Formicidae	Myrmicinae	*Myrmica*	*tahoensis*	USA	Ward and Downie (2005)	AY703562

Table 1 (*continued*)

Family	Subfamily	Genus	Species	Locality	Source	Accession number
Formicidae	Leptanillinae	*Leptanilla*	*VB03*	South Africa	Saux et al. (2004)	AY325961
Formicidae	Leptanillinae	*Leptanilla*	*VB04*	South Africa	Saux et al. (2004)	AY325962
Scoliidae	Campsomerinae	*Campsomeris*	*sp.*	Madagascar	CASENT0501206	DQ400981
Vespidae	Polistinae	*Polistes*	*sp. GD501*	USA	CASENT0501451	DQ401015

Sources with CASENT numbers refer specimens sequenced for this study using numbering assigned by the California Academy of Sciences, Department of Entomology.

Sequences used from previous studies are indicated by their reference. GenBank numbers for all sequences from the National Center for Biotechnology Information (NCBI) are provided.

Table 2
Sequences of 28S rRNA primers used in this study

Designation	Sequence (5′–3′)	Position	Reference
M06	CCCCTGAATTTAAGCATAT	3318–3337	Schmitz and Moritz (1994)
D2Br	GCACTCTCAAGCAACCCGAC	3568–3549	Saux et al. (2004)
D2B	GTCGGGTTGCTTGAGAGTGC	3549–3568	Saux et al. (2004)
28SC	CGGTTTCACGTACTCTTGAA	3692–3673	Brady (2003)
D3A	GACCCGTCTTGAAACACGGA	4046–4065	Chen et al. (2003)
D3Ar	TCCGTGTTTCAAGACGGGTC	4065–4046	Modified Chen et al. (2003)
D3B	TCGGAAGGAACCAGCTACTA	4414–4395	Chen et al. (2003)

Position refers to coordinates in the *Drosophila melanogaster* 28S gene, using the numbering of Tautz et al. (1988).

Primer combinations are as follows, with the forward primer listed first for each pair: M06–28SC, M06–D2Br, D2B–D3Ar, M06–D3Ar, D3A–D3B, D2B–D3B.

provided in the software programs PAUP* version 4.0b10 (Swofford, 1999) and MRBAYES v.3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Maximum parsimony and Bayesian inference analysis were used. For the model-based inference of phylogeny, model parameters were determined using the program ModelTest 3.06 (Posada and Crandall, 1998).

Maximum parsimony (MP) analyses with equally weighted characters were computed via heuristic search using tree bisection-reconnection (TBR) branch swapping with 100 random stepwise addition replicates to reduce the chance of missing the most optimal solution due to being isolated within a tree island (Page, 1993). If analyses produced more than one most parsimonious tree, a strict consensus was generated. Branch support was assessed using the non-parametric bootstrap (Felsenstein, 1985) under the same search conditions described above for MP with 1000 TBR replicates.

A maximum likelihood analysis was performed using MRBAYES v.3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Bayesian inference (BI) analysis was performed using the general time reversible model with a proportion of sites invariant and gamma distributed rates (GTR + I + G; nst = 6, rates = invgamma) identified as the best nucleotide substitution model with ModelTest 3.06. A random start tree was generated and four independent chains (three hot and one cold) were used with a "temperature" parameter of 0.2. Each MCMC run went for 5,000,000 generations with sampling every 100 generations, allowing the standard deviation of split frequencies between simultaneous runs to approach zero, and the potential scale reduction factor (PRSF) values for each parameter to reach or approach 1.0. The burn-in value was determined based on preliminary runs to ensure that the

likelihoods reached a steady state. The equilibrium samples (trees retained after burn-in) were used to generate a 50% majority rule consensus tree. We estimated the Bayesian posterior probability (PP) values, which represent the percentage of trees sampled after burn-in that recover any particular clade on the tree (Huelsenbeck and Ronquist, 2001).

4. Results

DNA sequence data for 68 taxa were analyzed (Table 1) for the region encompassing domains D1–D3 of the nuclear 28S rRNA gene (Hancock et al., 1988). Once aligned, the total data set consisted of 1405 characters, including numerous gaps to accommodate alignment of sequences from such divergent lineages. Exclusion of ambiguously aligned characters produced a data set of 1240 characters, of which 381 sites were variable and 236 characters were phylogenetically informative (19%). Nucleotide frequency was biased toward G + C, averaging 59.5% for all sites with empirical base frequencies of: A = 22.22%, C = 27.52%, G = 32.30%, and T = 17.96%. Base composition heterogeneity among taxa was non-significant (χ^2 = 54.39, df = 201, P = 1.0).

4.1. Maximum parsimony analyses

Parsimony analysis using equally weighted characters resulted in a strict consensus tree of six equally most parsimonious trees of 1096 steps (CI = 0.5018, RI = 0.7156) (Fig. 1). The topological differences among these equally parsimonious trees primarily involve relationships within the subfamily Ponerinae. The ants form a well-supported clade distinguishing the Formicidae from the outgroup taxa. Within the formicids, the distinctions of the
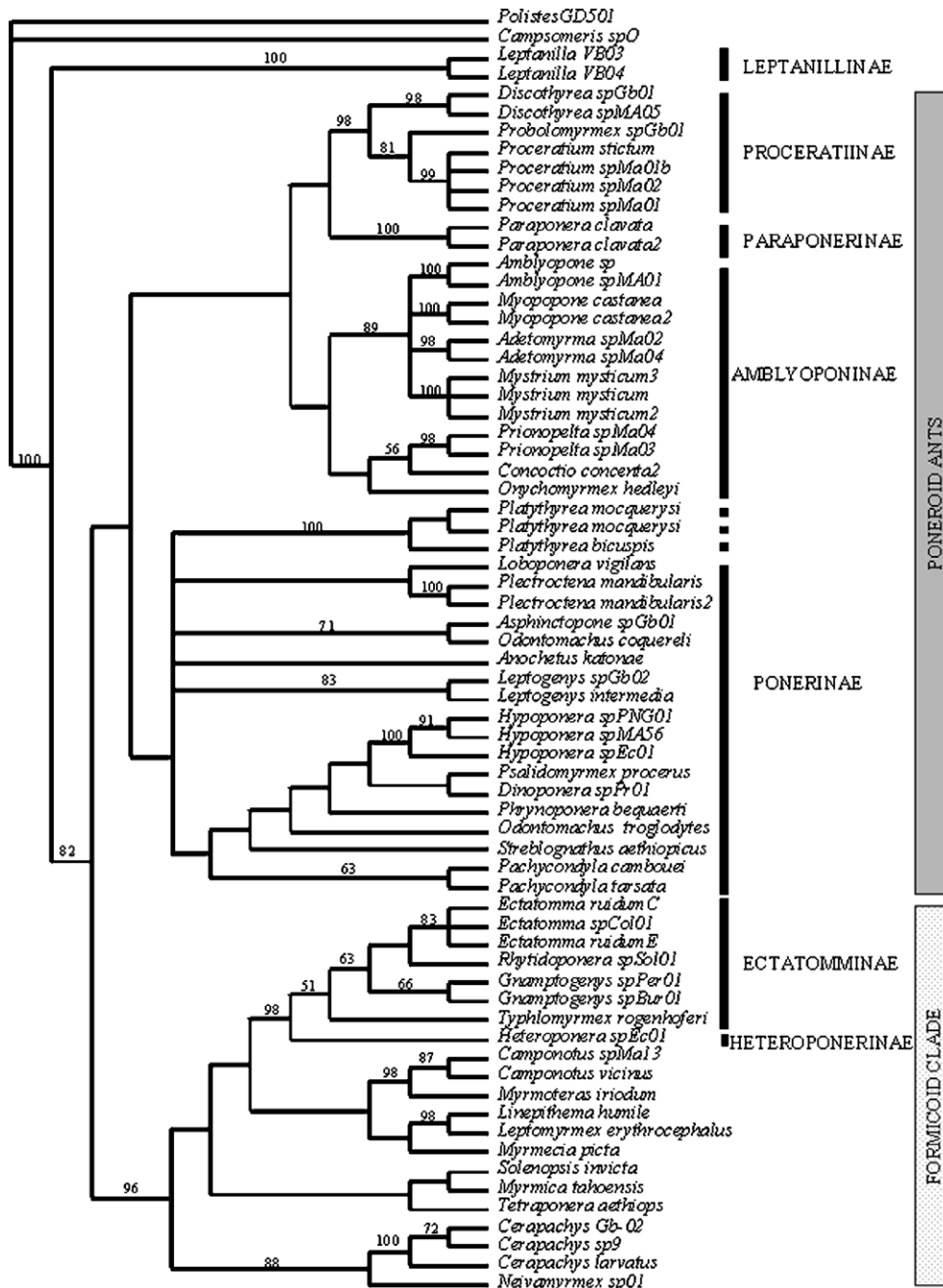
Fig. 1. A strict consensus of six unweighted maximum parsimony trees (L = 1096, CI = 0.5018, RI = 0.7156). Numbers above branches represent bootstrap proportions, greater than 50%, based on 1000 replications. Subfamily names follow the classification herein.

Leptanillinae and Formicoid clades were well supported by the parsimony analysis with parsimony bootstrap values (PB) of 100 and 96, respectively. In contrast, there was no bootstrap support for a clade of poneromorph ants; rather, the specific relationships among these lineages remain unresolved in the maximum parsimony analysis. Among the classically defined poneromorph ants (sensu Bolton, 2003), the Ectatomminae and the Heteroponerinae were tightly associated with each other, but were well within the strongly supported Formicoid clade of ants rather than with the other poneromorph ants. Though the

Proceratiinae and the Paraponerinae formed distinct, well-supported clades, their associations with other poneromorph clades were not supported by the bootstrap analyses. The genera *Onychomyrmex*, *Concoctio*, and *Prionopelta* did not show a strong affiliation with the other members of the Amblyoponinae, but the remaining Amblyoponinae (*Amblyopone*, *Adetomyrma*, *Myopopone*, and *Mystirum*) were well supported (PB = 89) as a clade. The subfamily Ponerinae are not well resolved with this data set although they all fell within the same clade in all six shortest topologies.

4.2. Bayesian analyses

Preliminary BI analyses converged on similar log likelihood scores (standard deviation of split frequencies between simultaneous runs less than 0.01) and reached a steady state at approximately 100,000 generations. To be conservative, burn-in was set at 250,000 generations in an analysis with 5,000,000 generations using the GTR $+ I + \Gamma$ model identified by ModelTest. The rate matrix parameters estimated were: R(a) [A–C] $= 0.87$, R(b) [A–G] $= 2.00$, R(c) [A–T] $= 1.34$, R(d) [C–G] $= 0.34$, R(e) [C–T] $= 4.83$, R(f) [G–T] $= 0.62$. The estimated base compositions were A $= 0.208$, C $= 0.285$, G $= 0.311$, T $= 0.197$. The proportion of invariable sites (PINVAR) was 0.422 and the alpha shape parameter (α) of the gamma (Γ) distribution was 0.455. These analyses produced a majority-rule consensus tree topology that shares many characteristics with the parsimony results, but also produced some distinct contradictions.

The BI analysis also strongly supported the monophyly of the ant taxa relative to the outgroups and the basal position of the Leptanillinae (Fig. 2). In addition, this analysis also supported the distinction of a Formicoid clade that included both Ectatomminae and the Heteroponerinae. Thus both analyses fail to support a clade defined by
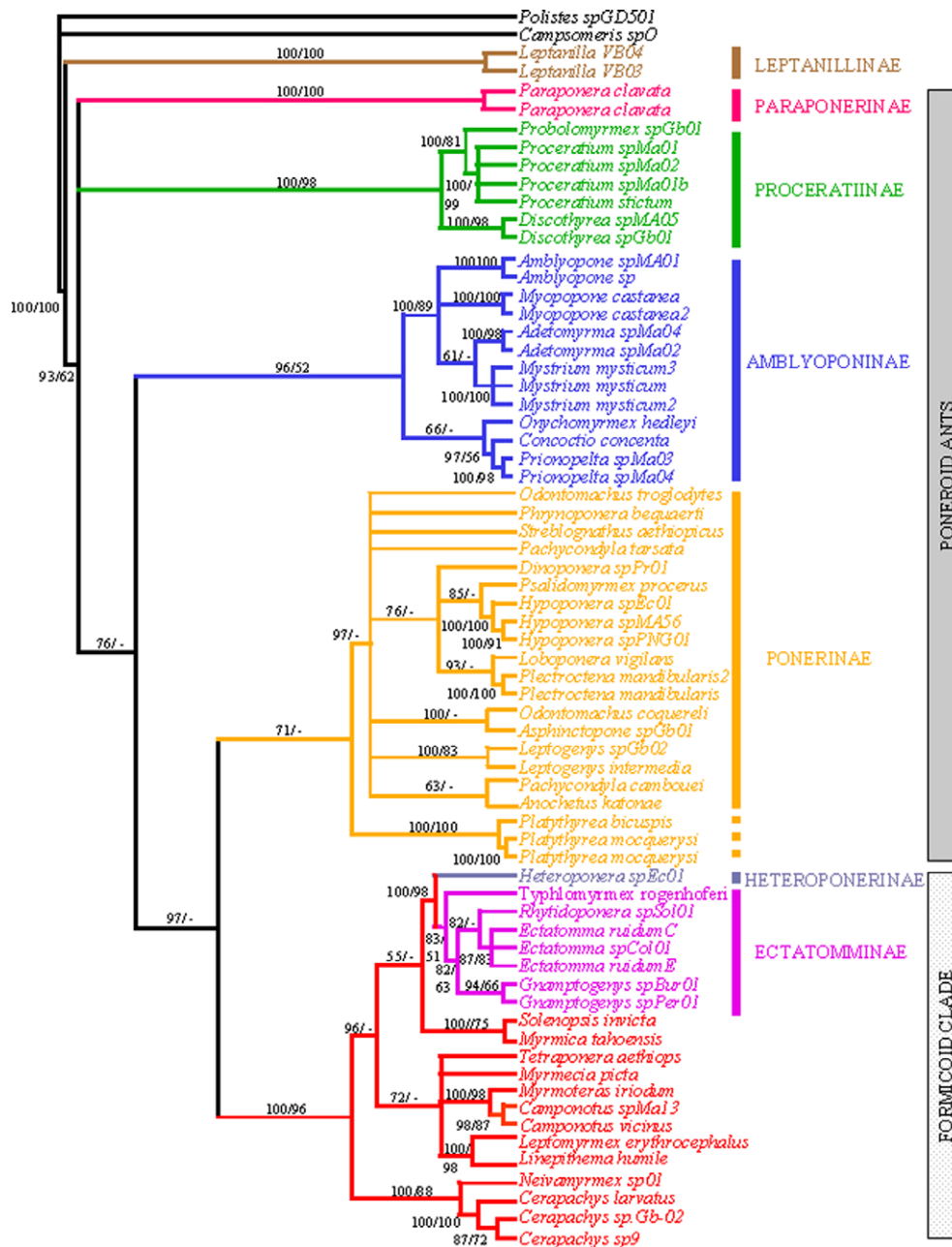

Fig. 2. The majority rule consensus tree inferred by BI analysis under the GTR $+ \Gamma + I$ substitution model. The numbers at the interior branches indicate the posterior probability ($\times 100$) values to the left of the slash and maximum parsimony bootstrap support to the right of the slash. Dashes indicate bootstrap support of less than 50%. Subfamily names follow the classification herein.

poneromorphs. In contrast to the parsimony analyses, the BI analysis did not suggest a distinct clade defining only poneroid ants. In fact, the results were relatively ambiguous as to the basal relationships among most poneroid lineages. This analysis did support a more derived position for the Formicoid ants and a strongly supported sister relationship with the subfamily Ponerinae (PP = 97). This analysis suggests a grade of Poneroid ants as opposed to a clade. Within the subfamily groups of poneroid and ectatommine ants, the BI analysis was typically in agreement with the parsimony analyses. Almost all clades supported by parsimony bootstrap (PB) values of 75% or more were also supported by posterior probabilities (PP) of 0.95 or greater in the Baysian Inference analysis, with the exception of the genus *Ectatomma*. The associations of genera within the same subfamilies of Formicoid ants were well supported in both analyses, including the Dolichoderinae (*Leptomyrmex* and *Linepithema*), the Formicinae (*Myrmoteras* and *Camponotus*), and the Myrmicinae (*Solenopsis* and *Myrmica*).

Several distinctions strongly supported by high posterior probability values in the BI analysis were not strongly supported in the parsimony analysis. A distinction depicting the Ecitoninae and Cerapachyinae forming a sister clade to the remaining Formicoid subfamilies in the analysis (the Dolichoderinae, Formicinae, Myrmeciinae, Myrmicinae, and Pseudomyrmecinae), were strongly supported in the BI analysis; the same distinction was present, but not supported, in the MP analysis. Relationships among the remaining Formicoid subfamilies examined remain ambiguous, with the exception of the sister group relationships of the Ectatomminae and the Heteroponerinae.

Within the Poneroid lineages, the BI analysis found strong support for a clade defining the entire Amblyoponinae subfamily (PP = 0.96) as well as a close association of *Concoctio* and *Prionopelta* (PP = 0.97) within that larger group. In addition, although the the BI analysis did not strongly support a clade defining the entire subfamily Ponerinae, there was substantial support for two clades, one consisting of the genus *Platythyrea* (PP = 1.00) and the other consisting of the remaining genera in the Ponerinae (PP = 0.97).

5. Discussion

5.1. Formicidae

Our molecular data supported the monophyly of the Formicidae. The family formed a strong clade with respect to the sampled outgroups. An important component of this study is derived from the range of taxa examined in these analyses. Our analysis included 14 of the 21 subfamilies (sensu Bolton, 2003) and included all major extant lineages. Those subfamilies not analyzed include only a fraction (2.5%) of the known extant species. Our results are congruent with analyses of portions of the major ant lineages found in recent molecular studies (Astruc et al., 2004; Brady, 2003; Saux et al., 2004; Ward and Downie, 2005;

Ward et al., 2005). Morphologically, the monophyly of this family is supported by unique synapomorphies presented by Bolton (2003). The results reveal that the principal lineages of Formicidae include a basal grade of five subfamilies followed by a well-supported Formicoid clade (sensu Saux et al., 2004). The nine Formicoid subfamilies examined represent the bulk of ant species diversity.

5.2. Early branching lineages

As reported by Saux et al. (2004), the Leptanillinae clade is sister to all other ants (PB = 82, PP = 1.00). This suggests that extant ants belong to two major groups, the Leptanillinae, represented by 50 extant species, and a second group composed of the Poneroid (~1350 species) and Formicoid ants (~10,500 species). Leptanillinae ants are small, blind, and apparently specialized predators on geophilomorph centipedes, and are restricted to subterranean habitats (Ogata et al., 1995). Their specialized morphology and behavior are not thought to represent early ant morphology and behavior (Wilson and Hölldobler, 2005b). Their specialized attributes and subterranean habitat, however, may have contributed to the persistence of this lineage over time. This surprising find warrants further investigation using additional loci and samples from leptanilline genera such as *Anomalomyrma*, *Noonilla*, *Phaulomyrma*, *Protanilla* and *Yavnella*. The inclusion of additional leptanilline genera would likely eliminate any long branch attraction as one potential explanation of this result (Anderson and Swofford, 2004).

The results also confirm the polyphyly of the poneromorph subfamilies. There was no support for a clade containing the poneromorph subfamilies Paraponerinae, Proceratiinae, Amblyoponinae, and Ponerinae, and the subfamilies Ectatomminae and Heteroponerinae were allied with the remaining ant subfamilies within the well-supported Formicoid group. Therefore, these analyses support a basal grade of poneromorph subfamilies, which we refer to as the "poneroid grade." Future studies with additional loci may reveal that these lineages form a clade and not a grade. Nevertheless, whether grade or clade, the term 'poneromorph' (sensu Bolton, 2003) should be abandoned as a term to define major ant lineages. Poneroid can be used to refer to Paraponerinae, Proceratiinae, Amblyoponinae, and Ponerinae, and formicoid ectatommines can be used to refer to Ectatomminae and Heteroponerinae.

Interestingly, the character used to traditionally define the poneromorph subfamilies, tergo-sternal fusion of the IV abdominal segment, has come under increasing scrutiny (Bolton, 2003; Ward, 1994). Outside the poneromorphs, this fusion was found in *Tatuidris* (Agroecomyrmecinae) and *Anklyomyrma* (Myrmicinae). In addition, the genus *Adetomyrma* (Ward, 1994) lacked tergosternal fusion on abdominal segment IV, but was still assigned to the Amblyoponinae because it possessed all of the other synapomorphies of Amblyoponinae (see Ward, 1994). The fact that poneromorphs are not a monophyletic group, and that

tergosternal fusion is present outside this group yet absent among the Amblyoponinae (*Adetomyrma*), indicate that fusion is not an informative character at this level.

5.3. Poneroid subfamilies

The monophyly of subfamily Proceratiinae and the placement of the Paraponerinae as a distinct lineage within the Poneroid ants were strongly supported in all analyses. The Amblyoponinae were strongly supported in the BI analysis and weakly supported in the MP analysis. The Ponerinae were weakly supported in both analyses. However, a clade supporting all of the Ponerinae excluding *Platythyrea* was strongly supported in the BI analysis. The BI analysis also supported an association between the Ponerinae and the Formicoid clade, suggesting what could be a more derived position in a grade of poneroid subfamilies. However, this association was not supported by the parsimony analysis, which lacked bootstrap support for any of the distinctions among poneroid subfamilies.

Amblyoponinae is generally supported as a clade, which is congruent with recent diagnosis of Amblyoponinae (Bolton, 2003; Saux et al., 2004; Ward, 1994). The BI analysis provides marginal support for the classical placement of the Amblyoponinae as an earliest branching poneroid lineage (Brown, 1960). Our results confirm *Adetomyrma* as a member of Amblyoponinae (Saux et al., 2004; Ward, 1994). The results also support a sister-group relationship between *Prionopelta* and *Concoctio*, a hypothesis supported by shared morphological features such as antennal insertions at the posterior margin of the clypeus; the form of the petiole, including peduncle; and the form of the abdominal segment III. All of the analyses provide strong support for a subclade consisting of *Amblyopone*, *Adetomyrma*, *Myopopone*, and *Mystrium*. This result is concordant with previous work and suggests that further analyses with additional loci and a broader sampling of taxa from this subfamily should be pursued to evaluate the single tribe status (Amblyoponini) within this subfamily (Bolton, 2003; Saux et al., 2004).

Platythyrea is supported as a sister clade to the remaining Ponerinae taxa, a relationship first recognized by Brown (1975). Brown suggested *Platythyrea* is more closely related to the basal stock of Ponerinae based on some worker-queens possessing palp formula consisting of six maxillary and four labial palpomeres. These findings also corroborate Bolton's (2003) recognition of Platythyreini as a close relation to Ponerini but also question whether it should remain as a member of the subfamily Ponerinae. The sequence region encompassing the D1–D3 expansion segments alone is inefficient for resolving relationships between genera of Ponerini. The phylogenetic relationship between genera of Ponerinae is thus an entirely open question that needs further investigation employing denser taxon sampling within the subfamily and new molecular and morphological evidence.

All analyses showed *Probolomyrmex* within the Proceratiinae. This supports the decision by Perrault (2000) to remove *Probolomyrmex* from the Platythyreini (Ponerinae) and Bol-

ton's, 2003 decision to place the genus within the subfamily Proceratiinae. The placement of *Probolomyrmex* within the Proceratiinae is supported by several morphological characters, including the absence of the promesonotal suture (Brown, 1952) and stridulatory organ (Markl, 1973), vestigial to absent frontal lobes (Bolton, 1994), exposed and entirely visible antennal sockets (Bolton, 1994, 2003), simple tarsal claws (Wheeler, 1922), one or no spur on the mesotibia and metatibia (Wheeler, 1922), and subtriangular mandibles (Wheeler, 1922). Males of these genera possess large ocelli and elongated scapes and lack an anal lobe on the hind wing, cerci, and a terminal pygidial spine (Taylor, 1965).

The Paraponerinae is a subfamily made up of a single species and, as such, forms a distinct lineage within the Poneroid ants. *Paraponera clavata* was traditionally placed within the tribe Ectatommini on the basis of shared wing venation and male genitalia (Brown, 1958; Brown and Nutting, 1950; Emery, 1895; Weber, 1946). Our results support Lattke's (1994) removal of *Paraponera* from the Ectatommini and Bolton's (2003) elevation of this taxon to its own subfamily. Previous analysis of the sting apparatus anatomy also suggested *Paraponera clavata* is not related to the other Ectatommine genera (Kugler, 1991). Moreover, Bolton supported the resurrection of Paraponerinae and defined the subfamily by four apomorphies: (1) petiole possessing long anterior peduncle, (2) lateral margin of hypopygium armed with a row of spines, (3) abdominal segment III significantly reduced, and (4) antennal scrobes bipartite. Our results also support Lattke's (1994) revision where *Paraponera* (Paraponerinae), *Discothyrea*, and *Proceratium* (Proceratiinae) were removed from the Ectatomminae. These genera are clearly poneroid ants associated with subfamilies as defined by Bolton (2003).

6. Formicoid: Ectatomminae and heteroponerinae

Ectatomminae is strongly supported to be within the Formicoid ants, which is consistent with results from Saux et al. (2004). This is the first molecular study to conclude that the Heteroponinae also belong within the Formicoid clade and are closely related to the Ectatomminae. As noted by Bolton (2003), no unequivocal apomorphy exists for the Heteroponerinae. The placement of *Heteroponera* as a sister taxon to the Ectatomminae is strongly supported. Lattke (1994) also noted the close relationship between heteroponerine and ectatommine genera. Further studies using additional heteroponerine genera (*Acanthoponera* and *Aulacopone*) will help to characterize the close relationship between the Heteroponinae and Ectatomminae.

Results from this study are congruent with earlier treatments of the ectatommine genera. All molecular results strongly support the inclusion of *Typhlomyrmex* within the Ectatomminae as was shown in Saux et al. (2004). Emery (1911) recognized *Typhlomyrmex* as a member of a subtribe of Ectatommini. *Typhlomyrmex* was removed from Ectatommini after Brown (1953) raised the genus to tribe status based on differences in wing venation of the male caste

(vein Mf1 arising basad of cu-a) and larval mandible structure (inflated basal part, narrowing to an acute apical blade). Given the molecular phylogeny and our current understanding of Ectatomminae (Bolton, 2003; Lattke, 1994), we agree with the classification of Emery (1911) regarding the grouping of *Typhlomyrmex* within Ectatomminae.

Lattke (1994) has defined the Ectatomminae based on two synapomorphies: (1) empodia absent and (2) a narrow and thin lamella present bordering the anterior clypeal margin. As noted in the data matrix (Lattke, 1994), *Typhlomyrmex* possesses both these synapomorphies, further supporting its status as a member of Ectatomminae. More recently, Bolton (2003) transferred the tribe Typhlomyrmecini into the subfamily Ectatomminae.

Overall, this study provides the broadest molecular analysis of the poneromorph ant subfamilies to date. It should be noted that this study relies on analyses of a single locus, and some interpretations may reflect patterns of locus evolution that may differ from patterns of species evolution. Nevertheless, these results represent a critical step toward understanding the relationships among the subfamilies that comprise the major basal lineages of the ants. The results are congruent with many of the advances in classification based on morphological analysis (Bolton, 2003). Most notably, Bolton recognized the need to divide the Ponerinae (sensu lato) into distinct subfamilies. This work strongly supports the rationale for the breakup, agrees with the subfamily divisions, but demonstrates the need to abandon the poneromorph grouping. Further research with additional loci will be required to better characterize relationships among the subfamilies within the poneroid grade.

Acknowledgments

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