Phylogenetic position of the ant genus *Acropyga* Roger (Hymenoptera: Formicidae) and the evolution of trophophoresy

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Trophophoresy is exhibited in two ant genera: *Acropyga* (Formicinae), in which all 37 species are thought to be trophophoretic, and *Tetraponera* (Pseudomyrmecinae), in which it has been observed in only one species, *T. binghami*. This study analyses a dataset comprised of both morphological and molecular (D2 region of 28S rRNA and EF1-alpha) data. Evidence is presented in favor of *Acropyga* being monophyletic, hence trophophoresy has evolved only once within the Formicinae and twice within the ants overall. The data further suggests that *Acropyga* belongs within a clade containing *Anoplolepis*, *Aphomomyrmex*, and *Petalomyrmex*. *Aphomomyrmex* and *Petalomyrmex* were found to be the sister group to *Acropyga*. The results indicate that the Lasiini and Plagiolepidini are not monophyletic and are in need of reexamination. Given the extant pantropical distribution of *Acropyga* it is speculated that *Acropyga* maybe of Gondwanan origin and that trophobiosis was the first form of agriculture to evolve in the ants.

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Introduction

The evolution of agriculture in ants conforms to two broad patterns: "farming" as practiced by fungus-growing ants and "herding" as practiced by trophobiont-tending ants. Fungiculture, practiced by the attine ants (tribe Attini), has evolved only once within the ants (for reviews, see Hölldobler and Wilson 1990; Mueller et al. 1998; Schultz et al. 2005). In a complex agricultural symbiosis that appears to be the product of ancient coevolutionary interactions, attine fungiculture is the product of a mutualism between the ants, their fungal cultivars, and an antibiotic-producing filamentous bacterium (Schultz et al., 2005).

Trophobiosis, in contrast, has evolved multiple times in ants, most commonly in the subfamilies Formicinae, Dolichoderinae, and Myrmicinae subfamilies, and more rarely in the Ponerinae, Ectatomminae, and Pseudomyrmecinae (Way 1963; Hölldobler and Wilson 1990). In trophobiosis, ants acquire honeydew, the sugar-rich fluid excreted by sap-feeding insects (the trophobionts: aphids and other sternorrhynchan insects in the order Hemiptera or certain lycaenid and riodinid caterpillars in the order Lepidoptera); in return, the ants protect these insects from parasites and predators (Hölldobler and Wilson 1990). For most ants, trophobiosis is facultative (Hölldobler and Wilson 1990). In a very few ant species, however, trophobiosis is obligate. Some obligately trophobiotic ant species have acquired particular adaptations for ensuring the transfer of trophobionts from parent to daughter colonies. Among the nomadic herding *Dolichoderus* species of Southeastern Asia, for

example, colonies reproduce by budding (Dill and Maschwitz 1998), in which a portion of the colony, including a newly mated daughter queen, workers, and mealybugs (Hemiptera: Pseudococcidae), separate from the parent colony and thereafter lead an independent existence. Colony budding may also occur in some *Pseudolasius* spp. that also depend on trophobionts (Acleridae, Aphidae, and Pseudococcidae) (Malsch et al. 2001; Kaufmann et al. 2003). In other obligately trophobiotic ant species, virgin queens carry trophobionts when they depart from the nest to mate and found new colonies. In the case of the plant-ant, *Aphomomyrmex afer*, mealybugs of the species *Paraputo anomala* cling to the virgin queens body when it leaves its birth nests, and these mealybugs subsequently serve as foundresses for a new mealybug "herd" in the new ant colony (Gaume et al. 2000). In another form of carrying behavior, the virgin queen departs on its mating flight carrying the trophobiont between its mandibles.

This latter form of trophobiosis has been termed trophophoresy (LaPolla et al. 2002). Only two ant genera are known to contain trophophoretic species: *Acropyga* (Formicinae), in which all 37 species are thought to be trophophoretic, and *Tetraponera* (Pseudomyrmecinae), in which trophophoresy has been observed in only one species, *T. binghami*. The details of the behavior remain largely unknown (Ward 2001). These two genera are members of demonstrably distantly related subfamilies (Baroni Urbani et al. 1992; Ward & Brady 2003). In both *Acropyga* and *T. binghami*, virgin queens depart from their parent nests carrying between their mandibles a trophobiont (in both genera a mealybug) that will serve as the progenitor of a new "herd" in their newly founded nests (Klein et al. 1992; LaPolla et al. 2002).

Acropyga are small ants (typically between 1-2 mm in total length), and live almost entirely underground where they place rhizoecine mealybugs (Hemiptera: Pseudococcidae) on roots to feed. The ants and mealybugs are apparently mutually dependent on each other for survival (Flanders 1957; LaPolla et al. 2002). The degree of species specificity between ant and mealybug remains unclear, but there is evidence that co-evolution has occurred between the two groups (LaPolla 2004). Fossil *Acropyga* are known from Dominican amber, indicating that trophophoresy is at least 15-20 mya old (fig. 1; Johnson et al. 2001; LaPolla 2004; LaPolla 2005).

The taxonomy of *Acropyga* has until recently been confused (LaPolla 2004), and the monophyly of the genus has been questioned. It has been suggested the genus was either paraphyletic or polyphyletic (Agosti 1991; Bolton 1994), with either scenario having important implications for the evolution of trophophoresy (i.e., implying that trophophoresy may have arisen more than once within the Formicinae and more than twice within the ants overall). While LaPolla (2004) presented seven putative morphological synapomorphies supporting *Acropyga* monophyly, that study did not examine the placement of the genus within the subfamily Formicinae. Here we provide evidence for the phylogenetic position of *Acropyga* drawn from both morphological and molecular data.

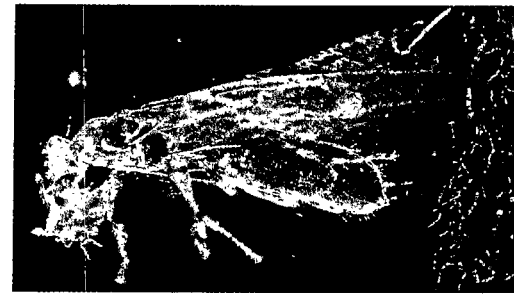

Fig. 1. Ancient trophophoresy. *Acropyga glaesaria* queen in Dominican amber with a mealybug (*Electromyrmococcus abductus*) held in her mandibles (see LaPolla 2005).

Materials and Methods

Source of Material

Twenty-six taxa were examined (table 1) for analysis. The sampling of the ingroup, *Acropyga* spp., was constrained by the availability of specimens for DNA sequencing. However, of the four species included in the analysis, *A. arnoldi* is the putative sister group to the rest of the genus, and the remaining species represent different, distantly related clades in the phylogeny of *Acropyga* (LaPolla 2004). The sampling of the diversity of the genus is therefore considered sufficient for the purposes of the present study.

Specimens were examined from a number of research collections, and below follows the list of the institutions and individuals' collections that

contributed to this study. Voucher specimens for every species used in DNA work are deposited in the National Museum of Natural History (USNM).

INBC: Instituto Nacional de Biodiversidad, Santo Domingo de Heredia, Costa Rica
JSLC: J.S. LaPolla Collection, private collection
JTLC: J.T. Longino Collection, Evergreen State College, Olympia, WA, USA
LACM: Natural History Museum of Los Angeles County, Los Angeles, CA, USA
MCZC: Museum of Comparative Zoology, Harvard University, Cambridge, MA, USA
PSWC: P.S. Ward Collection, University of California at Davis, CA, USA
SAMC: South African Museum, Cape Town, South Africa
SYPC: S. Yamane Collection, Kagoshima University, Kagoshima, Japan
UGBC: Centre for the Study of Biological Diversity, University of Guyana, Georgetown, Guyana
USNM: National Museum of Natural History, Washington DC, USA

Morphological Data

Characters were examined using a light microscope (Nikon SMU-Z) or a scanning electron microscope (Hitachi S-510). Morphological terminology employed throughout follows Bolton (1994), with modifications where noted.

Morphological Characters

The morphological characters and their alternative states are listed below. Table 2 lists the states assigned to each taxon. Character state designations (0, 1, 2) have no significance with respect to polarity.

1) Antennal segment number: (0) 12 segments; (1) 11 or fewer segments.
2) Antennal torulae (fig. 2a-b): (0) not tubular; (1) tubular.
3) Eye size: (0) 30 or more ommatidia; (1) 30 or fewer ommatidia.
4) Eye position: (0) posterior end of eye fails to reach the midline; (1) posterior end of eye surpasses approximate midline of head.

Table 1. Species used in analysis with GenBank accession numbers.

	28S rRNA	*Ef1-alpha*	*Origin of Specimen*
Acropyga acutiventris	DQ226030		Malaysia
Acropyga arnoldi	DQ226031	DQ226013	South Africa
Acropyga donisthorpei	DQ226032	DQ226014	Brazil
Acropyga epedana	DQ226033	DQ226015	USA
Amblyopone pallipes	DQ226034	DQ226016	USA
Anoplolepis gracilipes	DQ226035	DQ226017	USA
Anoplolepis steingroeveri		DQ226018	South Africa
Brachymyrmex depilis	DQ226036	DQ226019	USA
Camponotus chromaiodes	DQ226037	DQ226020	USA
Cladomyrma maryatiae	DQ226038	DQ226021	Malaysia
Dorymyrmex sp.	DQ226039	DQ226022	USA
Formica subsericea	DQ226040	DQ226023	USA
Gigantiops destructor	DQ226041		Guyana
Lasius sp.	DQ226042		USA
Lepisiota sp.	DQ226043		South Africa
Linepithema humile	DQ226044		USA
Melophorus sp.	DQ226045	DQ226024	Australia
Myrmecocystus mimicus	DQ226046		USA
Oecophylla smaragdina	DQ226047		Malaysia
Opithopsis respiciens	DQ226048	DQ226025	Australia
Paratrechina longicornis	DQ226049		USA
Petalomyrmex phylax	DQ226050	DQ226026	Cameroon
Plagiolepis alluaudi	DQ226051		USA
Pogonomyrmex sp.	DQ226052	DQ226027	USA
Polyrhachis sp.	DQ226053	DQ226028	Equatorial Guinea
Prenolepis imparis	DQ226054	DQ226029	USA

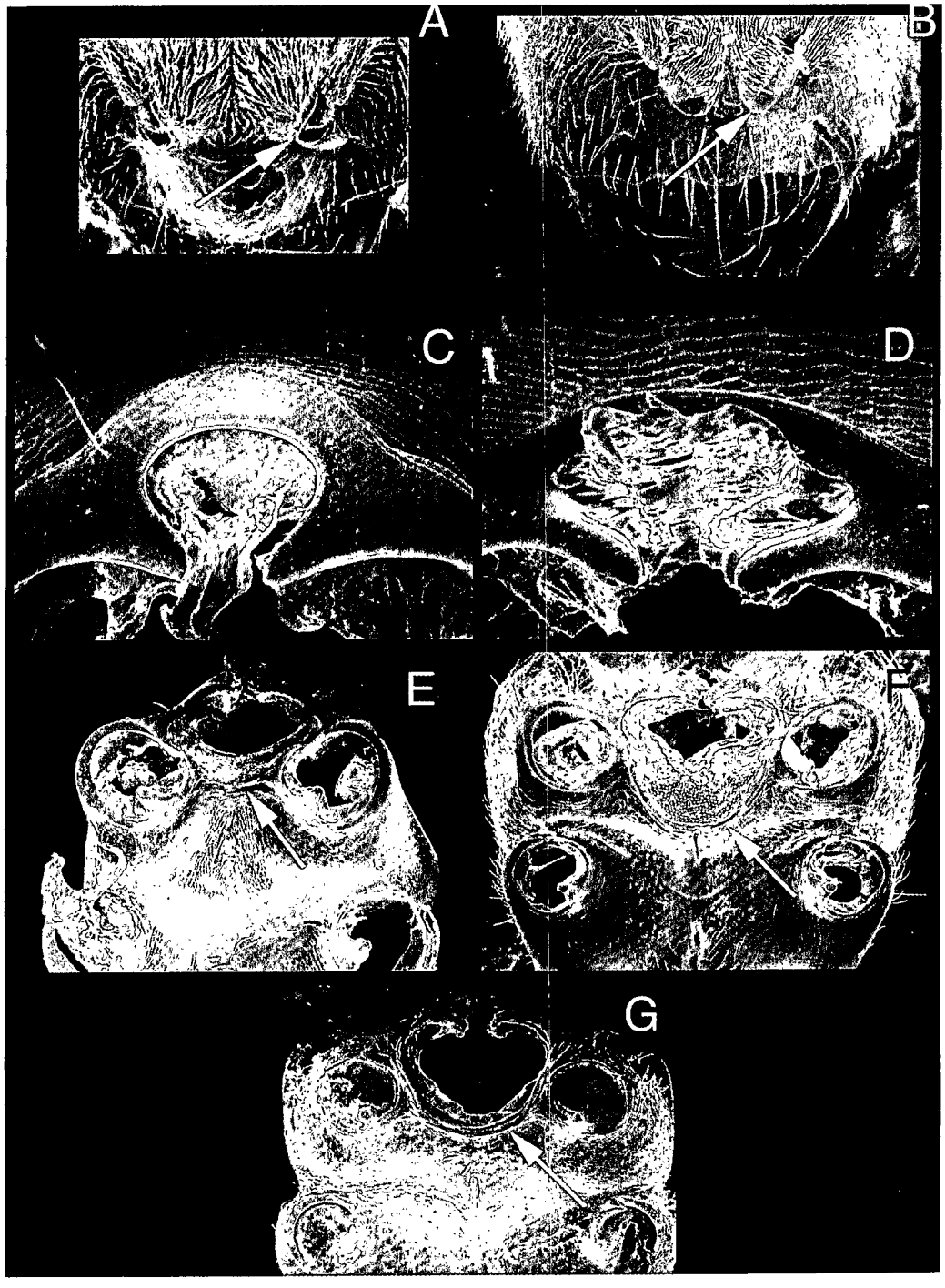

Fig. 2. Morphological characters used in phylogenetic analysis. Antennal torulae: A) not tubular, B) tubular; petiolar muscle orifice: C) round, D) oval; Petiole foramen: E) fails to reach anterior of metacoxal cavities, F) surpassess anterior of metacoxal cavities, G) reaches anterior of metacoxal cavities.

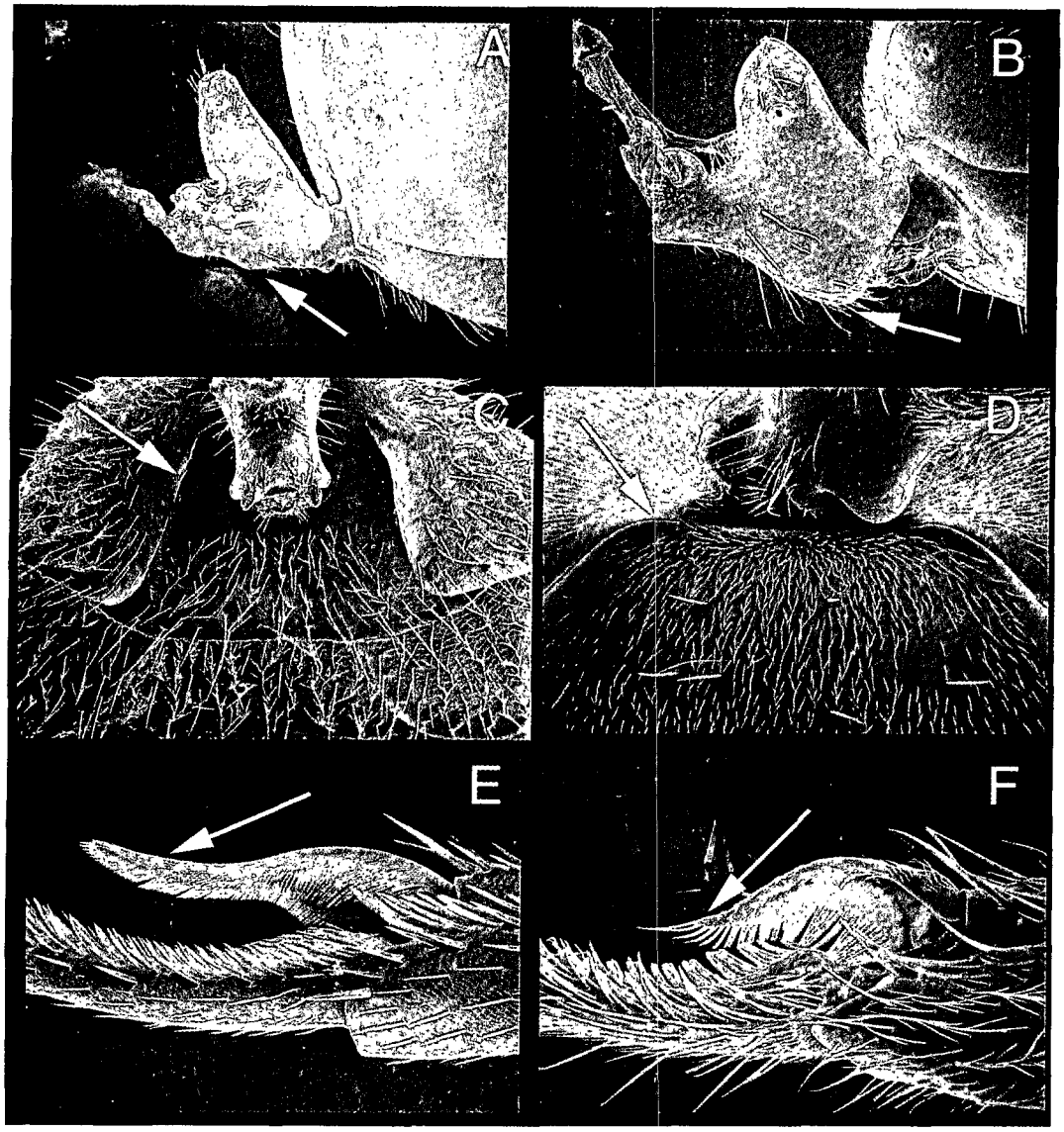

Fig. 3. Morphological characters used in phylogenetic analysis. Petiolar ventral surface: A) with ventral extension, B) without ventral extension; first gastral sternite: C) surpasses petiole articulation, D) does not surpass petiole articulation; protibial spur: E) long; F) short.

5) Palp formula: (0) 6:4; (1) less than 6:4.
6) Mandibular teeth number: (0) 6 teeth or greater; (1) fewer than 6 teeth.
7) Propodeal spiracle: (0) small and round; (1) large and round; (2) oval-shaped or slit-like.
8) Metapleural gland: (0) present; (1) absent.
9) Petiole muscle orifice (fig. 2c-d): (0) round; (1) oval.
10) Petiole foramen (adapted from Bolton, 2003) (fig. 2e-g): (0) fails to reach anterior of metacoxal cavities; (1) surpasses anterior of metacoxal cavities; (2) reaches anterior of metacoxal cavities.
11) Petiole ventral surface (fig. 3a-b): (0) with

ventral extension; (1) without ventral extension.

12) First gastral sternite (third abdominal) (fig. 3c-d): (0) surpasses petiole articulation; (1) does not surpass petiole articulation.
13) Proventriculus: (0) asepalous; (1) sepalous.
14) Protibial spur (fig. 3e-f): (0) long; (1) short.
15) Acidopore: (0) absent; (1) present

Molecular Data

For DNA extraction a whole ant was selected from among several nestmates in a vial; the remaining specimens are deposited as vouchers in the USNM. When possible specimens were identified to species. Samples were placed in labeled Eppendorf tubes and ground under liquid nitrogen, using microtissue grinders (Phoenix Research). DNA was extracted with sodium dodecyl sulfate, Proteinase-K, and phenol/chloroform, as described in Kjer et al. (2001). Dried DNA pellets were resuspended in 50-250 ml of Tris-EDTA buffer. Material was separated out into two tubes, one of which was added to a stock DNA collection and kept at -70°C, and the other kept in a freezer for amplification by polymerase chain reaction (PCR).

Table 2. Morphological character matrix. Numbers of characters and character states correspond to those found in the Material and Methods.

	000000000111111 123456789012345
Acropyga acutiventris	11101110010011
Acropyga arnoldi	11101(01)100100011
Acropyga donisthorpei	11101110010011
Acropyga epedana	11101110010011
Anoplolepis gracilipes	10010000011001
Brachymyrmex depilis	10000100010011
Camponotus chromaiodes	00010021101101
Cladomyrma maryatiae	10010000020011
Dorymyrmex sp.	00000000010000
Formica subsericea	10010020101101
Gigantiops destructor	00010020001101
Lasius sp.	00011000010011
Lepisiota sp.	10010100010211
Linepithema humile	01000000010000
Melophorus sp.	00010120121001
Myrmecocystus mimicus	00010020010101
Oecophylla smaragdina	00011021001101
Opithopsis respiciens	00010120101101
Paratrechina longicornis	00010100010211
Petalomyrmex phylax	10011010020?11
Plagiolepis alluaudi	10000000010211
Polyrhachis sp.	00010021101101
Prenolepis imparis	00010000010211

D2 region of 28S ribosomal RNA Protocol

D2 sequences were amplified on a thermal cycler using the reaction conditions described in Kjer et al. (2001). Primer sequences for the D2 were: 5'-GAGTTCAAGAGTACGTGAAACCG-3' paired with 5'-CCTTGGTCCGTGTTTCAAG -AC-3'. PCR conditions were 95°C, 30s; 52°C, 60s; 72°C, 60s for 35-45 cycles. Amplified DNA was separated on a 1.5% low-melting-point agarose gel (NuSieve 3:1; FMC Bioproducts). Bands of DNA were cut from the agarose gel, purified with GeneClean (Bio101), and sequenced on an ABI 377 automated sequencer (Applied Biosystems). Sequences were completed in both strands and edited manually with the assistance of Sequence Navigator (Applied Biosystems). During the editing of each strand, nucleotides that were readable but either showed irregular spacing between peaks or had some important competing background peak were coded in lowercase letters. These letters were: 1) converted to uppercase if the complementary strand strongly confirmed them; 2) left lowercase when both strands were lowercase; 3) changed to Ns (nucleotide undermined) when strands were contradictory. Sequences are deposited at Genbank under accessions DQ 226030-DQ 226054.

EF 1-alpha

EF 1-alpha sequence data (F1 copy) were generated for an approximately 1.2KB fragment (representing 364 amino acid residues and an intron of variable size, approximately 0.1KB in length). Extracted DNA was generally amplified in two steps: In the first step, a fragment approximately 2KB in length was amplified using the primers M3 (forward) and TRS10R (reverse) with a 55°C annealing temperature. Then, using the product of the initial amplification as template, 4 shorter overlapping fragments were reamplified with a variable (58°C to 64°C) annealing temperature, using as primers (i) For1 and Rev1.1, (ii) U377.1 and TRS1R, (iii) Cho7.1 and L53 or U52.1 and L53, and (iv) TRS4F and TRS9.1R. Primer sequences are listed in Table 3. Sequences are de-

Table 3. EF 1-alpha primer sequences.

M3	5' CAT ATW AAC ATT GTS GTS ATY GG 3'
TRS10R	5' ACG GCS ACK GTT TGW CKC ATG TC 3'
For1	5' GCA TCG ACA AGC GTA CCA TCG 3'
Rev1.1	5' CGT CTT ACC ATC GGC ATT GCC 3'
U377.1	5' TTG GCG TGA AGC AGC TGA TCG 3'
TRS1R	5' ACC TGG TTT YAA GAT RCC GGT 3'
Cho7.1	5' CTT CAG GAT GTC TAT AAG ATT GG 3'
U52.1	5' CCG CTT CAG GAT GTC TAT AA 3'
L53	5' CC GCG TCT CAG TTC YTT CAC 3'
TRS4F	5' GCG CCK GCG GCT CTC ACC ACC GAG G 3'
TRS9.1R	5' GGA AGG CCT CGA CGC ACA TMG G 3'

posited at Genbank under accessions DQ 226013-DQ 226029.

Phylogenetic Analyses Methods

After eliminating the intron sequence, alignment of the 1092 bp of EF 1-alpha nucleotide sequence was trivial, as EF 1-alpha is a protein-coding gene and amino acid number is highly conserved across all ants (TRS, unpublished). DNA sequence alignments are available upon request. The D2 region of the 28S rDNA gene was aligned according to secondary structure (Gutell 1994; annotation following Kjer 1995). Our alignment is reproduce in appendix 1.

Six data subsets were separately analyzed: (1) the D2 region of the 28S rDNA gene (25 species), (2) EF 1-alpha (26 species), (3) morphology (23 species), (4) combined 28S, EF 1-alpha, and morphology (26 species), (5) maximum combined EF 1-alpha and 28S (26 species, 10 incomplete for one or the other gene), and (6) minimum combined EF 1-alpha and 28S (16 species, complete for both genes).

Parsimony (MP) analyses

MP analyses were conducted in PAUP 4.0b10 (Swofford 2002) using the heuristic search option with TBR branch-swapping and 1000 random-taxon-addition replicates; successive-approximations weighting (SW) analyses used 500 replicates. Heuristic-search bootstrap analyses (Felsenstein 1984) used TBR branch-swapping and consisted of 1000 pseudoreplicates, with 10 random-taxon-addition replicates per pseudoreplicate. In all cases gaps are treated as missing. MP analyses of the 28S data set and the combined (28S, EF 1-alpha, and morphology) data utilized four step-matrix characters representing four D2 variable regions as coded in the INNASE computer application (Lutzoni et al. 2000).

Maximum-likelihood (ML) analyses

Nucleotide substitution models for ML analyses were evaluated with the relevant nucleotide data and the MP (EF 1-alpha) or SW (28S, combined) tree using the Akaike Information Criterion (AIC) calculated in ModelTest 3.06 (Posada and Crandall 1998). ML analyses were conducted in PAUP 4.0b10 (Swofford 2002). Heuristic searches employed the adopted model and an optimal MP tree as the branch-swapping starting tree and consisted of five iterative sub-searches, each iteration employing updated model parameter values and topologies based on the results of the preceding iteration and each using successively more intensive branch-swapping regimes (Currie et al. 2003; Sallum et al. 2002; Mueller et al. 1998; Villesen et al. 2004). Heuristic ML bootstrap analyses employed TBR branch-swapping and consisted of 1000 pseudoreplicates in which parameter values were constrained to those estimated on the optimal ML tree.

Bayesian analyses

Bayesian analyses were conducted in MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). Analyses of the EF 1-alpha data and the combined data employed multiple character partitions governed by separate, unlinked models based on the model-fitting analyses described above and subject to the limitations of models available in MrBayes. Two character partitions were employed in analyses of EF 1-alpha: (i) first and second codon positions and (ii) third codon positions. Four character par-

titions were employed in analyses of the combined data set: (i) 28S D2, (ii) EF 1-alpha first and second codon positions, (iii) EF 1-alpha third codon positions, and (iv) morphology. Analysis of each data set included four separate runs, each consisting of one million MCMC generations and four simultaneous MCMC chains (three heated), and each with a "burn-in" of 200K generations. For each analysis, post-burn-in trees from all four runs were pooled to calculate posterior probabilities.

Results and Discussion

Phylogenetic Analyses

Morphology

MP analyses of the morphology data produced 90 equally parsimonious trees (MPTs) with parsimony-informative length = 38, C.I. = 0.474, R.I. = 0.804; successive approximations weighting favored a subset of 12 of these trees (fig. 4).

28S D2. MP analyses of the 28S D2 nucleotide data produced 2510 MPTs with parsimony-informative length = 220, C.I. = 0.668, R.I. = 0.610; successive approximations weighting favored a subset of three of these trees. For one of these successive approximations weighting trees, the AIC favored the TVM+G model. ML analysis with this model identified a single tree with log likelihood of -1928.44657. Bayesian analyses using the GTR+G model (the closest model available in the MrBayes application) identified a tree similar to the ML tree.

EF 1-alpha. MP analyses of the EF 1-alpha data produced a single MPT with parsimony-informative length = 590, C.I. = 0.595, R.I. = 0.493. For this tree and the complete EF 1-alpha sequence data, the AIC favored the TrN+I+G model. ML analysis using this model identified a single tree with log likelihood of - 4280.97199. For this ML tree and the subset of EF 1-alpha sequence data consisting of codon positions 1 and 2, the AIC favored the TrN+I model; for this ML tree and codon positions 3, the AIC favored the TVM+G model. Bayesian analyses using character partitions governed by the closest available models in the mrbayes application, GTR+I and GTR+G, respectively, identified a nearly identical tree, differing only with regard to the relationships of BRA, CMM, and PRE.

Combined morphology, EF 1-alpha, and 28S D2, 26 species

MP analyses of the full 26-taxon data set, including all four MP character data partitions (morphology, 28S D2, EF 1-alpha, and the four INNASE step matrices), produced 176 MPTs with parsimony-informative length = 749, C.I. = 0.526, R.I. = 0.533; SW favored a subset of 7 of these trees. For one of these trees and the combined 28S D2 and EF 1-alpha sequence data, the AIC favored the GTR+I+G model. ML analysis using this model identified a single tree with log likelihood of -6331.4013. Based on the preceding analyses, Bayesian analyses of the 26-species data set utilized the following character partitions and models: (i) morphology with Tuffley-Steele parsimony; (ii) 28S D2 with GTR+G; (iii) EF 1-alpha first and second codon positions with GTR+I; and (iv) EF 1-alpha third codon positions with GTR+G (fig. 5).

Combined morphology, EF 1-alpha, and 28S D2, 16 species

Because the 26-species data set contained 10 species lacking sequences for one of the two genes, and because these missing data adversely affected branch support in the resulting trees, analyses were carried out for the 16-species data set for which all sequence data were complete. MP analyses of the 16-species data set, including all four MP character data partitions (morphology, 28S D2, EF 1-alpha, and the four INNASE step matrices) produced 3 MPTs with parsimony-informative length = 643, C.I. = 0.533, R.I. = 0.502 (fig. 6A); SW favored one of these trees. For this tree and the combined 28S D2 and EF 1-alpha sequence data, the AIC favored the GTR+I+G model. ML analysis using this model identified a single tree with log likelihood of -5839.43443. Based on the preceding analyses, Bayesian analyses of the 16-species data set utilized the following character partitions and models: (i) morphology with Tuffley-Steele parsimony; (ii) 28S D2 with GTR+G; (iii) EF 1-alpha first and second codon positions with GTR+I; and (iv) EF 1-alpha third codon positions with GTR+G (fig. 6B).

The combined analyses of both morphological and molecular (D2 and EF-la; Fig. 6) datasets (with a reduced number of taxa, excluding those without both molecular datasets [16-taxa]) recovered a well-supported monophyletic *Acropyga*

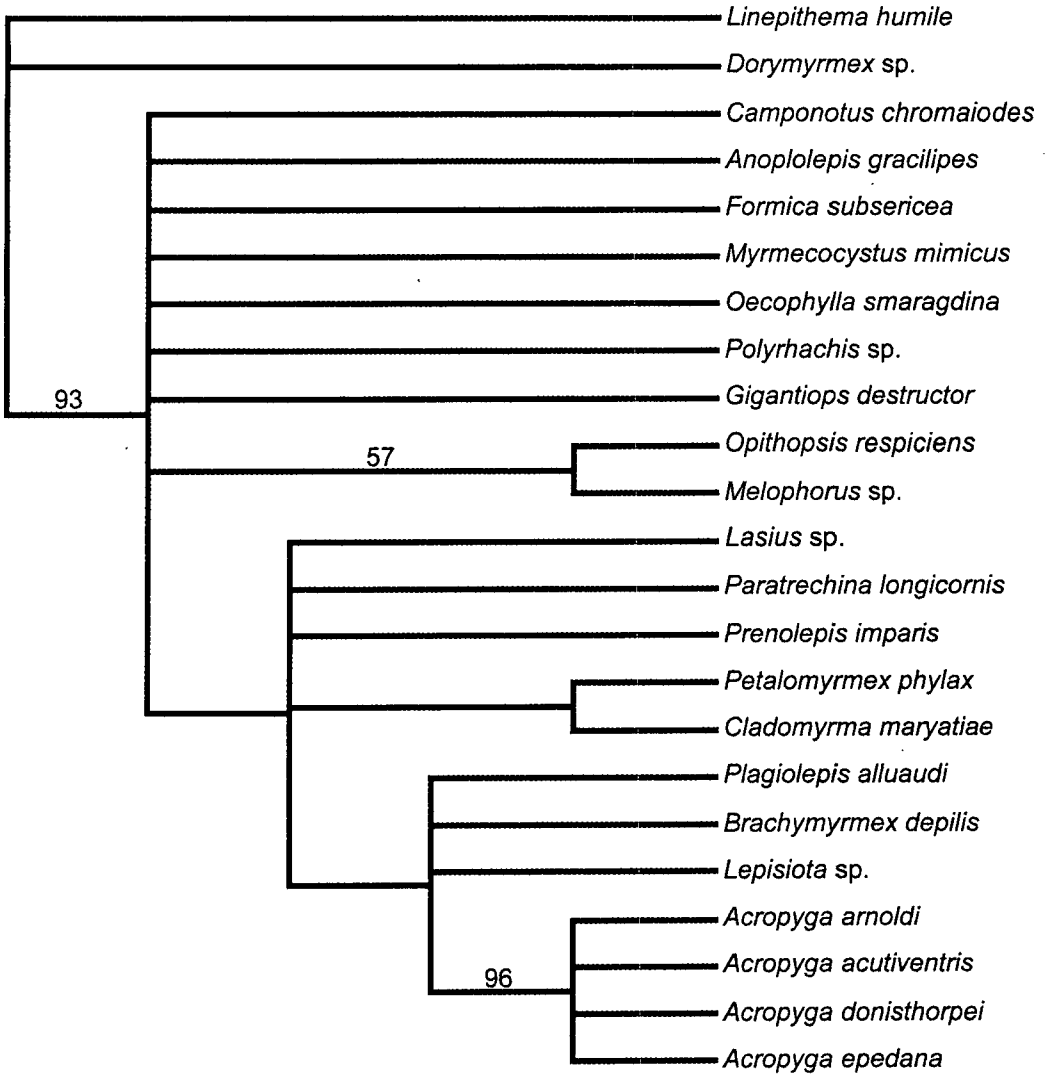

Figure 4. Cladogram of strict consensus tree resulting from a heuristic search of morphological data summarizing the 90 shortest trees to estimate relationships of Formicinae (tree length= 38 steps).

(100% bootstrap values [parsimony and likelihood] and a 100% posterior probability). *Acropyga* was found within a clade containing *Anoplolepis* and *Petalomyrmex*. The sister group for *Acropyga* was found to be *Petalomyrmex*, with high support (98% MP bootstrap, 96% ML bootstrap, and 100% posterior probability).

Our combined morphological and molecular dataset of three *Acropyga* (for the 16-taxon dataset), while relatively limited, is significant because it contains *A. arnoldi*, the sister taxon to all other *Acropyga* (LaPolla 2004), and a member of the derived *decedens* species-group, *A. epedana* (LaPolla 2004), thereby providing phylogenetic depth to the analysis. We can therefore state that *Acropyga* is monophyletic, corroborating the morphology-based study of LaPolla (2004). We can also state that trophophoresy has probably evolved

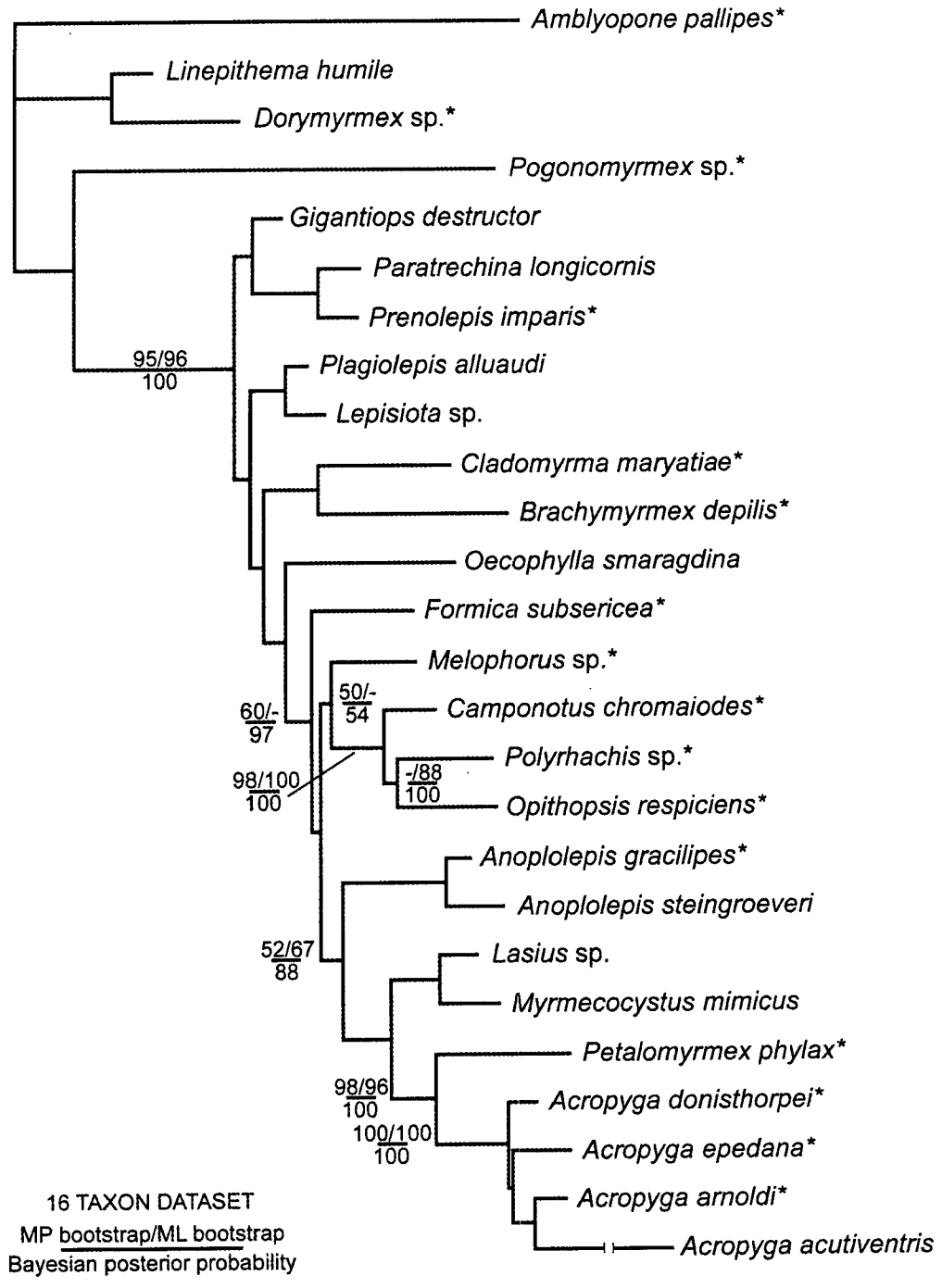

Fig. 5. Phylogram of Formicinae resulting from a Bayesian analysis of the full 26-taxon dataset. Asterisks (*) indicate taxa for which data were available from both genes (EF1-alpha and D2), i.e. those taxa constituting the "16-taxon dataset." Support values from the analysis of the 16-taxon dataset are indicated. The break in the line for *Acropyga acutiventris* represents a long branch that has been shortened for image size constraints.

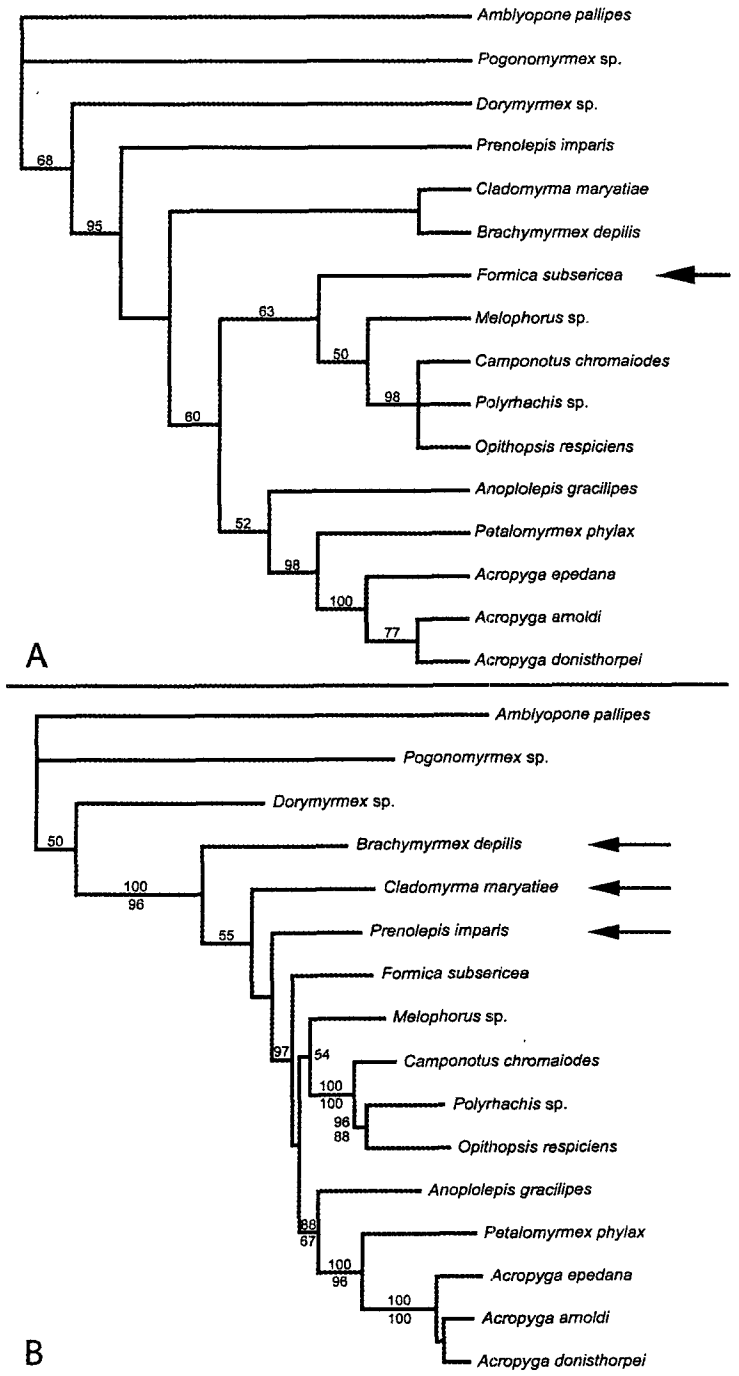

Fig. 6. Analysis of the16-taxon data set; arrows indicate taxa that differ in position from the 26-taxon dataset. A) Consensus of 3 MPTs resulting from the parsimony analysis; numbers above branches are bootstrap values. B) Phylogram resulting from the Bayesian analysis; numbers above branches are Bayesian posterior probabilities, below are bootstrap values from the maximum likelihood analysis.

only once among the formicine ants (there is always the possibility the behavior will be observed in another group), and possibly twice among the ants overall (Klein et al., 1992; Ward, 2001).

In all analyses, except morphology only, *Petalomyrmex phylax* was found to be the sister group of *Acropyga*. The only exception is the result of the morphology-only analysis, in which all conclusions about genus-level relationships lacked statistical support. *Petalomyrmex* is a monotypic genus whose sister group is *Aphomomyrmex* (Snelling 1979; Chenuil and McKey 1996), another monotypic genus. Both genera are plant-ants restricted to the Afrotropical region. A close relationship between *Petalomyrmex* (and, by implication, *Aphomomyrmex*) and *Acropyga* is intriguing because, whereas *Petalomyrmex phylax* is not trophobiotic, *Aphomomyrmex afer* is obligately trophobiotic (Gaume et al. 2000) and practices an unusual method for acquiring mealybugs. Mealybugs of the species *Paraputo anomala* cling to the bodies of virgin *Aphomomyrmex afer* queens when those queens depart from the natal nest to mate and found new colonies. This phoretic behavior on the part of the mealybug is thought to benefit the ants because, first, it provides a mealybug that will serve as a "seed individual" for producing a mealybug "herd," and, secondly, because the queen does not have to carry the mealybug in her mandibles, she is free to use her mandibles to excavate an opening for her nest chamber (Gaume et al. 2000). Gaume et al. (2000) speculated that, because the mealybug clings to the queen's body, there is little possibility that it will wander off and become separated from the queen. It bears noting that *Acropyga* queens, which carry the mealybug in their mandibles, are presumably presented with this same problem, and that perhaps the mealybug does not wander when put down by the queen after all. It is known that *Acropyga* queens sometimes return to their birth nests after mating (Bünzli 1935); in such cases they do not need to excavate a nest cavity. But independent nest founding has also been observed in *Acropyga* (Bünzli 1935; LaPolla et al. 2002) and independently-founding queens obviously need to excavate nest cavities. Morphological characters of male genitalia (the placement of the penis valve apodemal ridge) of *Acropyga*, *Aphomomyrmex*, and *Petalomyrmex* also support a close relationship between these genera (LaPolla and Longino, 2006).

Origins of Acropyga

Acropyga is at least 20 million years old based on the presence of Dominican amber fossils (Johnson et al. 2001). However, the genus is certainly much older than these fossils. For example, the current distribution of *Acropyga* is pantropical. This is at least consistent with a Gondwanan origin of the genus, especially because the species-level phylogeny of LaPolla (2004) found all Old World species to be paraphyletic from which arises a clade of New World species. This suggests a vicariant pattern, preceded by a Gondwanan distribution predating the complete separation of the southern continents, especially because *Acropyga* species are poor dispersers (Bünzli 1935). Under a scenario in which extant *Acropyga* distribution is due to vicariance a more ancient origin for *Acropyga*, perhaps in the mid to late Cretaceous, is plausible. Additional data are required to confidently test this hypothesis.

Certainly, honeydew from trophobionts was readily available in the Cretaceous, with scale insects making up nearly 10% of all insect samples from New Jersey and Canadian amber (Grimaldi and Agosti 2001). The recent discovery of a fossil formicine ant from New Jersey amber (92 mya), *Kyromyrma neffi*, suggests that the subfamily originated fairly early in ant evolution (Grimaldi and Agosti 2001). An ancient origin for *Acropyga* is thus not impossible. Contrasting *Acropyga* agriculture with other forms of ant agriculture may provide insight into general patterns of agricultural evolution. *Acropyga* fossils from Dominican amber represent the oldest definitive record of trophobiosis (Johnson et al. 2001; LaPolla 2005). Therefore, we can infer that *Acropyga* are certainly among the oldest "agricultural ants," however, the question of just how old still remains to be answered with the collection of more data. Trophophoresy is probably older than fungiculture in ants. Attine ants are known only from the New World, and there is no evidence that they have ever occurred outside of this region. There are fossil attines from Dominican amber. Thus, *Acropyga* could have entered trophobiotic relationships very early in the course of ant evolution. Quaintly put, this could mean that ants were herdsmen before they were farmers.

Comments on the Lasiine Tribe Group

Bolton (2003) recently proposed the lasiine tribe group which consists of three Formicinae tribes: Lasiini, Myrmoteratini, and Plagiolepidini. While Bolton acknowledged that the Lasiini and Plagiolepidini might need to be combined in the future, this study indicates that even combination of the two would not results in monophyly. With strong support for the *Acropyga/Anoplolepis/Petalomyrmex* clade at the apex of the tree, recovery of the two tribes as monophyletic seems very unlikely even with the addition of taxa. A thorough review of the Lasiini and Plagiolepidini is needed in order to redefine the tribes in light of the current lack of monophyly. In fact, across the Formicinae a phylogenetic review of tribal classification is much needed. We recovered a monophyletic Camponotini, but our dataset cannot address the status of the other tribes. Perhaps as Shattuck (1992) concluded after analyzing the Dolichoderinae, the time has come to seriously reconsider the use of the tribal rank within the Formicinae as well, but this can only be considered after a well supported phylogeny of generic relationships within the subfamily has been proposed.

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Appendix 1. *Alignment of Taxa.* Secondary structural symbols are as follows: "( )" represent hair-pin stem loops; "| |" represent simple stems; "[ ]" represent unalignable regions.

```
Large subunit rRNA               2 (1)              D2                   D2-2a              2b                      2c
Camponotus chromaiodes   [CAA] ?????????????????????????????????nncgaacn(---agaUUcAUCGUcAGCGGCGCUGGCuUCGnGUCGGUGGGCGAUGUCCCGCGGAG
Anoplolepis gracilipes   [ANL] ???????????????CCUAAGAAACCCAAAAG-AUCGAACG(GGGAGAUUCAUCGUCAGCGGCGCUGGCUUCGCGUCGGUGGGCGAUGUCCCGCGGAG
Petalomyrmex phylax      [PET] ccguucaggGGUAAACCUAAGAAACCCAAAAG-AUCGAACG(GGGAGAUUCAUCGUCAGCGGnGUUGGCUUCGCGUCGGUGGGCGAUGUCUCGCGGAG
Formica subsericea       [FOR] ?????????????????????????????????????????(???????????????????GCGCUGGCUUCGCGUCGGUGGgCGAUGUCCCGCGGAG
Polyrhachis sp.          [POR] ????????????????????????CCCAAAAG-AUCGAACG(GGGAGAUUCAUCGUCAGCGGCGCUGGCUUCGCGUCGGUGGGCGAUGUCCCGCGGAG
Opithopsis respiciens    [OPR] ???????????????????????????????G-AUCGAAAG(GGgAGAUUCAUCgguCASgACGCuuGCUUCGCGUCGGUGGGCGAUGUcccgCGGAG
Prenolepis imparis       [PRE] ???????????????????????????????g-aucgaacg(-ggagAUUCAUcgucAgcggCGCUGGcUUCGCGUCggucGGCGAUGUcccgcGGAG
Acropyga arnoldi         [ARN] ?????????????????????????????????????????(?????????ucgucancggcgcUGGCUUCGCGUCGGUGkGCGAUGUUCCGCGGAG
Acropyga donisthorpei    [ART] ????????????????????????????????????????G(GGnnnAUUCAUCGUCAGCGGCGCUGGCUUCGCGUCGGUGGGCGAUGUUCCGCGGAG
Acropyga epedana         [ARE] ?????????????????????????????????????????(??????????????????????????????????????gggcgauguuccgccgag
Acropyga acutiventris    [ARA] ?????????????????????????????????????????(?????????????????????????GCUUCGCGUCGGUGGGCnAUGUUACACGGAG
Cladomyrma maryatiae     [CMM] ?????????????????????????????????????????(????????????????????????????????????????????????????????
Brachymyrmex depilis     [BRA] ?????????????????????????????????????????(?????uu-aucgucagcgacgcuggcuucgcgucggugggcgauguccgcgcgg
Melophorus sp.           [MEL] ?????????????????????????????????????????(?????????aucgucagcgacgcuggcuucgcgucggugggcgauguccgcgGag
Paratrechina longicornis [PAL] CCGUUCAGGGGUAAACCUAAGAAACCCAAAAG-AUCGAACG(GNGAGAUUCAUCGUCAGCGGCGCUGGCUUCGCGUCGGUCGGCGAUGCCCCGCGGAG
Lasius sp.               [LAS] ?????????????????????????????????????????(GgGAGAuuuaUCGUCAGCGGCGCUGGCUUCGCGUCGGUCGGCGAUGUCCCGCGGAG
Gigantiops destructor    [GIG] ccguucaggggguaaaccuaAgAAaCCcAAAAG-AUCGAACG(GGGAGAUUCAUCGUCAGCGGCGCUGGCUUCGCGUCGGUGGGCGAUGUCCCGCGGAG
Lepisiota sp.            [LEP] ?????????????????????????????????????????(???A-AuuCAUggUCA-CGGCGCuGgcUUCGCGUCGGUG-GCgAUGUccGCGNAG
Plagiolepis alluaudi     [PLA] ??????????????ACCUAAGACACCCAaAAG-AUCGAACG(gg-AGAUUCAUCGUCAGCGGCGCUGGCUUCGCGUCGGUGGGCGAUGUCCCGCGGAG
Mymecocystus mimicus     [MRC] ?????????????????????????????????????????(???????????????????????????????????????????????cccgcggag
Oecophylla smaragdina    [OEC] ????????????????????????????????????GAAAG(GGAGA-UUcAUCGUCAGCGACGCUGGCUUCGCGUCGGuGGGCGAUGUUCCGCGGAG
Dorymyrmex sp.           [DOR] ???????????????????????????????G-AUCGAACG(GGGAGAUUCAUCGUCAGCGGCGCUGGCUUCGCGUCGGUGGGCGAUGUUCCGCGGAG
Amblyopone pallipes      [AMB] CCGUUCAGGGGUAAACCUAAGAAACCCAAAAG-AUCGAACG(GGGAGAUUCAUCGUCAGCGGCGUUGGCUUCGCGUCGGUGGGCGAUGUCCCGCGG-G
Pogonomyrmex sp.         [POG] CCGUUCAGGGGUAAACCUAAGAAACCCAAAAG-AACGAACG(GGGAGAUUCAUCGUCAGCGACGCUGGCUUCGCGUCGGUGCGCGAUGCUACGCGGGG

                 2c'                           2b'                 D2-2a'             D2-3
[CAA] -UCCUCGCGG-CUCG-CGCGCGGGCACGCUACCGUnnG-UCrAUGUCCGnCUGUCGUCGnCGUGCACU-UcUCCC)CAAGUAGAa|CGUCGC|GAC
[ANL] -UCCUCGCGG-cUCC-CGCGCGGGCACGCUACCGUGCG-UCGAUGUCCGGCGCUCGUCGUCGUGCACU-UCUCCC)CAAGUAGAA|CGUCGC|GAC
[PET] -UCCUCGCGG-CUCG-CGCGCGGGCACGCUACCGUGCG-UCGAUGUCCGGCGCUCGUCGUCGUGCACU-UCUCCC)CAAGUAGAA|CGUCGC|GAC
[FOR] --CCUCGCGG-CUCU-CGCGCGGGCACGCUACCGUGCG-UCGAUGUCCGGCGCUCGUCGUCGUGCACUUUCUCCC)CAAGUAGAA|CGUCGC|GAC
[POR] -UCCUCGCGG-CUCG-CGCGCGGGCACGCUACCGUGCG-CCGAUGUCCGGCUGUCGUCGUCGUGCACU-UCUCCC)CAAGUAGAA|CGUCGC|GAC
[OPR] -UCCUCGCGG-CUCG-CGCGCGGGCACGCUACCGUGCG-UCGAUGUCCGGCUGUCGUCGUCGUGCACU-UCUCCC)CAAGUAGAA|CGUCGC|GAC
[PRE] -UCCUCGCGG-CUCG-CGCGCGGGCACGCUACCGUGCG-CCGAUGUCCGGUGCUCGUCGUCGUGCACU-UCUCCC)CAAGUAGAA|CGUCGC|GAC
[ARN] -UCCUCGUGG-CUCG-CGCGCGGACACGCUACCGUGCG-UCGAUGUCCGGCGCUCGUCGUCGUGCACU-UCUCCC)CAAGUAGAA|CGUCGC|GAC
[ART] -UCCUCGUGG-CUCG-GGCGCGGACACGCUACCGUGCG-UCGACGUCCGGCGCUCGUCGUCGUGCACU-UCUCCC)CAAGUAGAA|CGUCGC|sAC
[ARE] -uccucgcgg-cucg-cgcgcggaCACgcuaccgugcg-ccgauguccggcgcucgucgucgugcacu-ucuccc)caaguagaa|cgucgc|gac
[ARA] -UCnUCGUwC-CUCG-CGCGCGGACACGCUnCUGUGCG-UCGAUGUCCnACGCUyGUCGUCGUGCACU-UcUCCC)CAAGUAGAA|AGUCGC|Gms
[CMM] ???????????????????????????????????????????????????????????????????????????)?????????|cgucgn|cgc
[BRA] guccucgcgg-cucg-cgcgcgggcacgcuaccgugcg-ucgacguccggcgcucgucgucgugcacu-ucucCC)CAAGUAAAA|CGUCnn|nnC
[MEL] -uccucgCGG-CUCG-CGCGCGGGCACGnUACCGUGCG-UCGAUGUCCGGCGCUCGUCGUCGUGCACU-UCUCCC)CAAGUAGAA|CGUCGC|GAC
[PAL] -UCCUCGCGG-CUCG-CGCGCGGGCACGCUACCGUGCG-CUGAUGUCCGGCGCUcGUCGUCGUGCACU-UCUCCC)CAAGUAGAA|CGUCGC|GAC
[LAS] -UCCUCGUGG-CUCG-CGCGCGGGCACGCUACCGUGCGCUCGAUGUCCGGCGCUCGUCGUCGUGCACU-UCUCCC)CAAGUAGAA|CGUCGC|GAC
[GIG] -UCCUCGCGG-CUCG-CGCGCGGGCACGCUACCGUGCG-UCGAUGUCCGGCGCUCGUCGUCGUGCACU-UCUCCC)CAAGUAGAA|CGUCGC|GAC
[LEP] -UCCUCgCGG-CUCG-CGCGCGGGCACGcuuCCGUGCG-UCGAUGUCCGGCGCUcGUCGUCGUGCACU-UCuCCC)CAAGUAGAA|CGUCGC|GAC
[PLA] -UCCUCGCGGGCUCG-CGCGCGGGCACGCUACCGUGCG-UCGAUGUCCGGCGCUCGUCGUCGUGCACU-UCUCCC)CAAGUAGAA|CGUCGC|GAC
[MRC] -uccungugg-cucg-cgcgcgggcacgcuaccgugcg-ucgagggncggcgcucgangWcgggCACU-UCUCCC)CAAGUAGAA|CGUCGC|GAC
[OEC] -UCUUCGCGA-CUCG-CGCGCGGGCACGCUACCGUGCG-UCGAUGUCCGGCGCUCGUCGUCGUGCACU-UCUccc)cAAGUAGAA|CGUCGC|GAC
[DOR] -CCCUUGCGG-AUCCUACcGnnnnCACGCUACCGUGCG-CCGAUGUCCGGCGCUcGUCGUCGUGCACU-UCUCCC)CUAGUAGGA|CGUCGC|GAC
[AMB] ----U------UCGCU--CGCGGGCACGUCGCCGUGCG-AGCACGUCCGACG-UCGUCGUCGUGCACU-UCUCCC)CAAGUAGGA|CGUCGC|GAC
[POG] -CCCUUGCGG-CUCG-CGCGUGG-UACGCCGCCGUGCG-CCGAUGUCCGGCGUUCGUAGUCGUGCACU-UCUCCC)CUAGUAGAA|CGUCGC|GAC

       4a        4b         4c            4d                    4d'         4c'        4b'       4a'
[CAA] (CCGCUGGGUGUCGGUCUACGGCCCGGGnGCnGnGACUGuCGC-uCCGC-CG-UUAAACG-GCnCGCGGACAAACCCUCGGUCGCCCGGCCGGCUGCUCGgCGG)
[ANL] (CCGCUGGGUGUCGGUCUACGGCCCGGGUGUGUCGACUGUCGCGUC-GC-CGGUAAAACGGNCACGC-GACAAACCCCCGGUCGCCCGGCCGGCUGCUCGGCGG)
[PET] (CCGCUGAGUGUCGGUCUACGGCCCGGGUGCGGUGGCUGUCGCGUC-GC-CGGUAAAACG-GCACGC-GACAAACCCCCGGUCGCCCGGCCGGCUGCUCGGCGG)
[FOR] (CCGnUGGGUGUCGGUCUACGGCCCGGGUGCGGUGACUGUCGCGUC-GC-CGGUAAAACG-GCACGC-GACAAACCCCCGgUCGCCCGGCCGGCUGCUCGGCGG)
[POR] (CCGCUGGGUGUCGGUCUACGGCCCGGGUGCGGUGACUGUCGCGUC-GC-CGGUAAAACG-GCACGC-GACAAACCCUCGGUAGCCCGGCCGGCUGCUCGGCGG)
[OPR] (CCGCUGGGUGUCGGUCUACGGCCCGGGUGCGGUGACUGCCGCGUC-GC-CGGUAAAACG-GCACGC-GACAAACCCUCGGACGCCCGGCCGGCUGCUCGGCGG)
[PRE] (CCGCUGGGUGUCGGUCUACGGCCCGGGUGCGGUGCCUGUCGCGUC-GC-CGGUAAAACG-GCACGC-GACAAACCCCCGGUCGCCCGGCCGGCUGCUCGGCGG)
[ARN] (CCGCUGGGUGUCGGUCUACGGCCCGGGUGCGGUGACUGUCGCGUC-GC-CGGUAAAACG-GCACGC-GACAAACCCUCGGUCGCCCGGCCGGCUGCUCGGCGG)
[ART] (CCGCUGGGUGUCGGUCUACGGCCCGGGUGCGGUGACUGUCGCGUC-GC-CGGUAAAACG-GCACGC-GACAAACCCUCGGUCGCCCGGCCGGCUGCUCGGCGG)
[ARE] (cCGCUGGGUGUCGGUCUACGGUCCGGGUGCGGUGACUGUCGCGUC-GC-CGGUAAAACG-GmACGC-GAmAACCCUCCGGUCGCCCGGCCGGCUGCUCGGCGG)
[ARA] (CCGCUGAGUAUUUGUCUACGGCCUGGGUGCGCUGACUGUAAAC---GC-CGGUAAAACGGGCACGC-GACAnnnnCmCsGUCGCCnGGCCGGCAGCUCGGCGG)
[CMM] (ccgcuggguguccgGucUACGGCCCGGGkGCGGUGACUGUCgCGUC-GC-CGGUAAAACG-Gmacgc-gACAAACCCUCGgUAGCCCsGCCGGCUGCUCGGCGG)
[BRA] (sCGCUGGGUGUCGGUCUACGGCCCGGGUGCGGUGUCUGUCGCGUC-GCGCGGUAAAACG-GCACGC-GACAAACCCCCGGUCGCCCGGCCGGCUGCUCGGCGG)
[MEL] (CCGCUGGGUGUCGGUCUACGGCCCGGGUGCGGUGACUGUCGCGUC-GC-CGGUAAAACG-GCACGC-GACAAACCCCCGGUAGCCCGGCCGGCUGCUCGGCGG)
[PAL] (CCGCUGGGUGUCGGUCUACGGCCCGGGUGcGGUGUCUGUCGCGUC-GC-CGGUAAAACG-GCACGC-GACAAACCCCCGGUCGCCCGGCCGGCUGCUCGGCGG)
[LAS] (CCGCUGGGUGUCGGUCUACGGCCCGGGUGAGGUGACUGUCGCGUC-GC-CGGUAAAACG-GCACGC-GACAAACCCUCGGUCGCCCGGCCGGCUGCUCGGCGG)
[GIG] (CCGCUGGGUGUCGGUCUACGGCCCGGGUGCGGUGACUGUCGCGUC-GC-CGGUAAAACG-GCACGC-GACAAACCCUCGGUCGCCCGGCCGGCUGCUCGGCGG)
[LEP] (CCGCUGGGUGUCGGUCUACGGcCCGAGUGCGGUGACUGUCGCGUC-GC-CGGUAAAACG-GCACGC-GACAAACCCUCGGUCGCCCGGCCGGCUGCUCGgCGG)
[PLA] (CCGCUGGGUGUCGGUCUACGGCCCGAGUGCGGUGACUGUCGCGUC-GC-CGGUAAAACG-GCACGC-GUCAAACCCUCGGUCGCCCGGCCGGCUGCUCGGCGG)
[MRC] (CCGCUGGGUGnCGGUCUACGGnCCGGGUGAGGUGACUGUCGCGUn-GC-CGGUAAAACG-GCACGC-GACAAACCCUCGGUCGCCCGGCCGGCUGCUCGGCGG)
[OEC] (CCGCUGGGUGUCGGUCUACGGCCCGGAUGCGGUGACUGUCGCGUC-GC-CGGUAAAACG-GCACGC-GACAAACCUCCGAUCGCCCGGCCGGCUGCUCGGCGG)
[DOR] (CCGCUGGGUGUCGGUCUACGGCCCGGGUGCGGUGACUGUCGCGUC-GC-CGGUAAAACG-GCACGC-GGCAAACCCCCGGUCGCCCGGCCGGCUGCCCGGCGG)
[AMB] (UCGCUGGGUGUCGGUCUACGGCCCGAGUGCGGUAUCUGUCGCGUC-GC-CGGUAAAACG-GCACGC-GGCAAACCCUCGGUCGCCCGGCCGGCUGCCCGGCGG)
[POG] (CCGCUGGGUGUCGGUCUACGGCCCGGGUGCGGAGACUGACGCGUC-GC-CGGUAAAACG-GCACGC-GUCGACCCCCCGGUCGCCCGGCCGACUGCCCGGCGG)

            5a                                           5C
[CAA] UACGCGCAA|GGUAU|CAGGCCGCAC[UUC-------------------------------------------------AUACACGG]UGC
[ANL] UACGCGCAU|GGUAU|CAGGCCGCAC[UU------------------------------------------------------ACGG]UGC
[PET] UACGCGCAA|GGUAU|CAGGCCGCAC[UUAUUUUU-----------------------------------------------AACGG]UGC
[FOR] UACGCGCAA|GGUAU|CAGGCCGCAC[UGU--------------------------------------------------AUUACGG]UGC
[POR] UACGCGCAA|GGUAU|CAGGUCGCAC[CU-----------------------------------------------------AACGG]UGC
[OPR] UACGCGCAU|GGUAU|CAGGCCGCAC[UCUU-----------------------------------------------AUAACGAGA]UGC
[PRE] UACGCGCAA|GGUAU|CAGGCCGCAC[UA--------------------------------------------------AUUUACGG]UGC
[ARN] UACGCGCAA|GGUAU|CAGGCCGCAC[UCUU--------------------------------------------------AAGGCG]UGC
[ART] UACGCGCAA|GGUAU|CAGGCCGCAC[UC-----------------------------------------------------ACGUG]UGC
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continued