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Phylogeny of leafcutter ants in the genus *Atta* Fabricius (Formicidae: Attini) based on mitochondrial and nuclear DNA sequences

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ABSTRACT

Leafcutting ants of the genus *Atta* are the most conspicuous members of the tribe Attini, the fungus-growing ants. *Atta* species have long attracted the attention of naturalists, and have since become a common model system for the study of complex insect societies as well as for the study of coevolutionary dynamics due to their numerous interactions with fungi and other microbes. Nevertheless, systematics and taxonomy of the 15 species in the genus *Atta* have proven challenging, due in part to the extreme levels of worker polymorphism these species display, leading to disagreements about the validity of as many as five different subgenera and calling into question the monophyly of the genus. Here, we use DNA sequence information from fragments of three mitochondrial genes (COI, tRNA leucine and COII) and one nuclear gene (EF1-αF1), totaling 1070 base pairs, to reconstruct the phylogenetic relationships of *Atta* species using maximum parsimony, maximum likelihood and Bayesian inference techniques. Our results provide support for monophyly of the genus *Atta*, and suggest that the genus is divided into four monophyletic groups, which correspond to four of the five previously erected *Atta* subgenera: *Atta sensu stricto* and *Archeatta*, each with species composition identical to earlier proposals; *Neoatta* and *Epiatta*, with major differences in species composition from earlier proposals. The current geographic ranges of these species suggest that the historical separation of South America from Central and North America has played a role in speciation within this genus.

1. Introduction

Leafcutter ants of the genus *Atta* Fabricius, 1804 (Formicidae: Attini) are among the most recognizable fauna of the Neotropics. Many visitors to this part of the world, naturalists and tourists alike, have been fascinated by the sight of thousands of worker ants carrying freshly cut pieces of vegetation back to their massive, subterranean nests. Likewise, residents of areas inhabited by *Atta* species are well aware of the capability of these ants to defoliate crops and ornamental plants, often very quickly (Cherrett, 1986a,b,c; Cherrett and Peregrine, 1976). A testament to the reputation of these ants is the ubiquity and diversity of common names by which they are known: saúvas (Brazil), arrieras (Mexico), zompopas (Costa Rica), bibijagua (Cuba), weewee (Nicaragua), bachacos (Venezuela) and many others (Weber, 1972).

Indeed, throughout their geographic distribution, which ranges from the southern United States to northern Argentina (Hölldobler and Wilson, 1990), *Atta* species are considered major pests (Cherrett, 1986a,b,c; Hernandez et al., 1999; Robinson and Fowler, 1982; Varon et al., 2007). Estimates of the economic damage caused by *Atta* species have been calculated to be as much as several million dollars per year in Texas (Cameron and Riggs, 1985) and US$130 million per year in the state of São Paulo, Brazil (Fowler et al., 1986).

Despite their reputation as pests, *Atta* species are important contributors to ecosystem functions in the various habitats in which they occur (Brener and Silva, 1995; Garrettson et al., 1998; Moutinho et al., 2003; Sternberg et al., 2007; Wirth et al., 2003). Like all members of the tribe Attini, *Atta* species cultivate a symbiotic fungus that serves as the primary food source for ants larvae and alates as a major source of enzymes for work adult ants (Mueller et al., 1998; Siqueira et al., 1998; Silva et al., 2003). Leafcutter ants (including the genus *Acromyrmex* Mayr) have been described as the dominant herbivores of the Neotropics (Hölldobler and Wilson, 1990; Wirth et al., 2003) and play a major role in nutrient cycling as they bring organic material deep into their subterranean nests (Garrettson et al., 1998; Moutinho et al., 2003).

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Furthermore, *Atta* nests are host to a diversity of associated organisms, ranging from reptiles and amphibians (Weber, 1972) to arthropods (Moser and Neff, 1971; Steiner, 2004; Waller and Moser, 1990) and microbes (Bacci et al., 1995; Carreiro et al., 2004; Currie, 2001; Rodrigues et al., 2005).

As a result of both their importance as agricultural pests and their diverse ecological interactions, *Atta* species are among the best studied tropical insects (Mueller and Rabeling, 2008). Nevertheless, the phylogenetic relationships among the fifteen currently recognized species (Bolton et al., 2006) are essentially unknown (Schultz and Brady, 2008) and the last taxonomic revisions are outdated (Borgmeier, 1959; Gonçalves, 1942). This is due in part to the difficulties associated with morphological studies of the genus, primarily because of the extreme levels of polymorphism they display (Borgmeier, 1950, 1959). It is nearly impossible to distinguish minor workers of different *Atta* species, and collections containing a full nest series ranging from the smallest minima workers to the largest soldiers from a single nest are rare (Solomon, personal observation). Some species can be differentiated using morphological characters of the male genitalia, as recognized by Emery (Emery, 1913). The highly distinctive genitalia of *Atta mexicana*, *Atta insularis* and *A. mexicana* males compared to some other *Atta* species suggested that these three species may form a basal group within the genus or even outside the genus, therefore bringing into question its monophyly (Borgmeier, 1950). The clarification of this question based on a complete analysis of male genitalia throughout every *Atta* species and other related genera, such as *Acromyrmex*, has never been possible, probably because a comprehensive collection of males is too difficult to assemble due to the fact that males are generally produced only once per year for nuptial flights, are short-lived outside the nest (less than a day) and the timing of these flights differs between species and geographical regions (Moser, 1967; Moser et al., 2004).

Although subgenera are not currently recognized within *Atta* (Bolton et al., 2006), we review here the extensive history of attempts to establish morphological groups of species within the *Atta* genus (summarized in Table 3). Based on the analysis of male genitalia of five species, Emery (1913, 1922) recognized three major groups called *Archeatta*, *Atta* s.str. and *Neoatta*. By considering major worker morphology, two other species were included in *Archeatta* and each group received the status of subgenus (Gonçalves, 1942). The subgenera *Archeatta* and *Atta* s.str., as well as their species composition, were confirmed by every further classification until all subgenera were synonymized by Bolton et al. (2006). However, the remaining nine *Atta* species have proven more controversial in terms of their classification into subgenera. The major questions have been (1) whether these species should represent a single subgenus (*Neoatta*) or divided into distinct subgenera and (2) in case of division, what would be the species composition of each group. Table 3 summarizes the attempts to address these questions by Gonçalves (1942, 1986), who grouped the nine species into a single subgenus, and by Borgmeier (1950, 1959), who proposed that these species be divided into as many as three subgenera.

In the present investigation, we present the first estimation of the phylogenetic relationships among *Atta* species from all proposed subgenera based on DNA sequence information. The goals of this study were (1) to test the monophyly of the genus *Atta*; (2) to determine whether the phylogenetic relationships of *Atta* species based on DNA sequence information correspond to the species groups originally outlined by Emery (1913, 1922) and later modified by Gonçalves (1942, 1944, 1982, 1986) and Borgmeier (1950, 1959) and (3) to determine whether mtDNA and nDNA sequence information provide consistent reconstructions of the evolutionary relationships between *Atta* species.

2. Materials and methods

2.1. Overview of material

A total of 62 specimens were obtained for molecular analyses, of which 57 correspond to the genus *Atta*, representing 13 of the 15 currently recognized species (Table 1). The species not included are *Atta cubana* Fontenla and *Atta goiana* Gonçalves, because despite extensive effort, no fresh material (i.e. preserved in alcohol) could be obtained for these two species. *A. cubana* occurs only on the small Cuban island of Isla de la Juventud (Fontenla Rizo, 1995) and *A. goiana* is known only from the Brazilian state of Goiás (Borgmeier, 1959; Gonçalves, 1942). The species selected as outgroups were chosen based on their close phylogenetic affinity to *Atta*, as determined by other studies (Schultz and Brady, 2008; Schultz and Meier, 1995).

Mitochondrial DNA sequence information was obtained for 40 ingroup specimens and three outgroup specimens, spanning three genes: cytochrome oxidase I (COI), tRNA leucine (tRNAleu) and cytochrome oxidase II (COII) as well as a highly variable intergenic spacer (IGS) between COI and tRNAleu. Nuclear DNA sequence information corresponding to the intron 1 region of the F1 copy of the gene elongation factor 1-α (EF1∝F1) was obtained for 29 ingroup specimens and four outgroup specimens. Voucher specimens were deposited in the insect collection at the Laboratory of Molecular Evolution at São Paulo State University (UNESP-Rio Claro, SP, Brazil) and the Entomology Collection at the Smithsonian Institution (NMNH, Washington, DC, USA). DNA sequences were deposited in GenBank (Accession Nos. as shown in Table 1).

2.2. DNA extraction

Genomic DNA was extracted from a single individual from each colony using either of the following methods. (1) Single entire ant specimens were frozen in liquid nitrogen inside a microtube, disrupted with a pestle and incubated with 0.55 ml lysis buffer (250 mM Tris, pH 5.0, 2 M NaCl, 100 mM EDTA, 2% SDS) at 55 °C for 3 h, after which proteins were precipitated by the addition 0.25 ml 5 M NaCl followed by centrifugation; the supernatant was then collected and added to 250 ml of isopropanol. The mixture was then centrifuged for precipitation of DNA, which was washed with 70% ethanol and solubilized in TE buffer. (2) Single, entire ant specimens or parts of specimens (e.g. the head) were frozen in liquid nitrogen inside a microtube, disrupted with a pestle and then extracted using the AccuPrep Genomic DNA Extraction Kit (Bioneer, Inc.) following the manufacturer's recommendations.

2.3. PCR reactions

Three sets of mtDNA primers (Table 2) were used to amplify two fragments of mtDNA (COI (part.); and COI-IGS-tRNAleu-COII) and one section of nDNA (EF1∝F1). For the section COI-IGS-tRNAleu-COII, a single ~600 kb mitochondrial DNA segment was amplified in a 25 μl PCR reaction containing the Ready-to-Go Kit (Amersham 27-9555-01), ~100 ng genomic DNA and 6 pmol of the primers C1-J-2828 and C2-N-3386, which were designed using GenBank sequences of other attine and non-attine ants and named according to Simon et al. (1994). PCR conditions included a 94 °C for 3 min denaturing followed by 30 cycles (94 °C for 10 s, 50 °C for 1 min, 72 °C for 3 min) and a 70 °C for 15 min final extension.

For the other two loci (COI and EF1∝F1) PCR reactions contained 1 μl each of genomic DNA (approximately 10 ng), 1× reaction buffer, dNTPs and $MgCl_2$, forward primer (5 pmol/μl),

Table 1
Summary of specimens used in this study and the accession numbers for sequences deposited in GenBank.

ID	Species	GenBank Accession #		
		COI (part.)	COI-IGS-tRNAleu-COII	EF1∝F1
A1	*Atta sexdens piriventris*	FJ547432	FJ599547	FJ599514
A2	*Atta sexdens piriventris*	FJ547433	FJ599548	FJ599515
A3	*Atta sexdens piriventris*	FJ547434	FJ599549	–
A4	*Atta sexdens rubropilosa*	FJ547435	FJ599550	–
A5	*Atta sexdens rubropilosa*	FJ547436	FJ599551	–
A6	*Atta sexdens rubropilosa*	FJ547437	FJ599552	FJ599516
A7	*Atta sexdens rubropilosa*	FJ547438	FJ599553	FJ599517
A8	*Atta sexdens sexdens*	FJ547439	FJ599554	–
A9	*Atta sexdens sexdens*	FJ547440	FJ599555	–
A10	*Atta sexdens sexdens*	FJ547441	FJ599556	–
A11	*Atta opaciceps*	–	FJ599557	–
A13	*Atta robusta*	FJ547442	FJ599558	–
A14	*Atta robusta*	FJ547443	FJ599559	–
A15	*Atta robusta*	FJ547444	FJ599560	FJ599518
A16	*Atta capiguara*	FJ547445	FJ599560	FJ599519
A17	*Atta capiguara*	FJ547446	FJ599561	–
A18	*Atta capiguara*	FJ547447	FJ599562	FJ599520
A19	*Atta capiguara*	FJ547448	FJ599563	–
A20	*Atta capiguara*	FJ547449	FJ599564	–
A21	*Atta laevigata*	–	–	FJ599521
A22	*Atta laevigata*	FJ547450	FJ599565	–
A23	*Atta laevigata*	FJ547451	FJ599566	–
A24	*Atta laevigata*	FJ547452	FJ599568	FJ599522
A25	*Atta laevigata*	–	–	FJ599523
A26	*Atta vollenweideri*	FJ547453	FJ599569	FJ599524
A29	*Atta bisphaerica*	FJ547454	FJ599570	FJ599525
A30	*Atta bisphaerica*	FJ547455	FJ599571	FJ599526
E677	*Atta laevigata*	FJ547456	FJ599572	FJ599527
E523C	*Atta columbica*	FJ547457	FJ599573	–
E523D	*Atta columbica*	FJ547458	FJ599574	–
E521	*Atta texana*	FJ547459	FJ599575	–
E524	*Atta saltensis*	–	FJ599576	–
E678	*Atta laevigata*	–	–	FJ599528
E692	*Atta laevigata*	–	–	FJ599529
SES020113-01	*Atta cephalotes*	FJ547460	FJ599577	–
SES040131-04	*Atta cephalotes*	FJ547461	FJ599578	–
SES040125-03	*Atta cephalotes*	FJ547462	FJ599579	–
SES040123-07	*Atta cephalotes*	FJ547463	FJ599580	–
SES040123-01	*Atta cephalotes*	FJ547464	FJ599581	–
SES040208-10	*Atta cephalotes*	FJ547465	FJ599582	–
SES050801-06	*Atta cephalotes*	–	–	FJ599530
CR050316-02	*Atta insularis*	FJ547466	FJ599583	FJ599531
MEX10	*Atta mexicana*	FJ547467	FJ599584	–
MEX1	*Atta mexicana*	FJ547468	FJ599585	–
SALT F	*Atta saltensis*	FJ547469	–	–
UGM050723-03	*Atta sexdens sexdens*	–	–	FJ599532
SES050814-06	*Atta sexdens sexdens*	–	–	FJ599533
SALT S	*Atta saltensis*	–	–	FJ599534
VOLL	*Atta vollenweideri*	–	–	FJ599535
SES050814-04	*Atta laevigata*	–	–	FJ599536
IS060307-01	*Atta opaciceps*	FJ547470	–	FJ599537
AGH020623-14	*Atta columbica*	–	–	FJ599538
BFL2	*Atta texana*	–	–	FJ599539
UGM060402-06	*Atta texana*	–	–	FJ599540
MEXQIV	*Atta mexicana*	–	–	FJ599541
SES060821-03	*Atta mexicana*	–	–	FJ599542
AcroCoro	*Acromyrmex coronatus*	–	–	FJ599543
AcroBalz	*Acromyrmex balzani*	FJ547471	FJ599586	–
AcroOctoGuad	*Acromyrmex octospinosis*	FJ547472	FJ599587	FJ599544
AcroOctoCuba	*Acromyrmex octospinosis*	FJ547473	FJ599588	FJ599545
T24	*Trachymyrmex* sp.	–	–	FJ599546

reverse primer (5 pmol/μl), 0.04 μl of Taq polymerase and 5.96 μl of water for a total reaction volume of 10 μl. Average PCR conditions were as follows, with slight modifications depending on the annealing temperatures of individual primer pairs: Initial denaturation at 95 °C for 3 min was followed by 35 cycles of 95 °C for 5 s and an annealing temperature that increased by 0.5 °C for each successive round of amplification, beginning at 45 °C, for 20 s each round, with a final elongation step of 68 °C for 15 s.

2.4. DNA sequencing

For the gene segment COI-IGS-tRNAleu-COII, PCR products were purified and sequenced as follows. Purification was accomplished using the Wizard PCR Preps Kit (Promega A7170). Because of non-specific amplification in *Atta cephalotes* (Martins et al., 2007), purified PCR products needed to be cloned using the pGemT Vector System I Kit (Promega A3600). The clones were transfected

Table 2
Primer pairs used to specifically amplify mitochondrial or nuclear DNA loci of *Atta* species.

Primer pair (5′ → 3′ primer sequence)	Locus	Reference
C1-J-2828[a] (ATTCATTCTTATCTTGAAATATTATTTC)	COI-IGS-tRNAleu-COII	Martins et al. (2007)
C2-N-3386[a] (TTCATAAGTTCAGTATCATTGGTG)	COI-IGS-tRNAleu-COII	Martins et al. (2007)
Ben (GCTACTACATAATAKGTATCATG)	COI	Moreau et al. (2006)
Jerry (CAACATTTATTTTGATTTTTTGG)	COI	Simon et al. (1994)
U52.1 (CCGCTTCAGGATGTCTATAA)	EF1∝F1	Lapolla et al. (2006)
L53 (CCGCGTCTCAGTTCYTTCAC)	EF1∝F1	Lapolla et al. (2006)

[a] C1-J-2828 and C2-N-3386 are were previously called ANT-F and ANT-R, respectively, in Martins et al. (2007).

by heat shock (Sambrook and Russel, 2001) in DH5α *Escherichia coli* cells, and positive clones were selected and subjected to a mini-prep procedure (Sambrook and Russel, 2001). Sequencing reactions contained 100 ng amplicon or 500 ng plasmids, 6 pmol primer (the same as for the amplification of a given amplicon to direct sequencing or the vector primers T7 or SP6 for sequencing recombinant plasmids), 4 μl Big Dye (PE Applied Biosystems 4303153) and were carried out with an initial denaturation (96 °C for 90 s) followed by 25 PCR cycles (96 °C for 12 s, 50 °C for 6 s, 60 °C for 4 min). Sequencing products were purified following the manufacturer's guidelines and analyzed using an ABI377 automated sequencer (PE Applied Biosystems).

For the gene segments COI and EF1∝F1, PCR products were analyzed by running 3 μl of the product on a 1.5% agarose gel and subsequently visualized with ethidium bromide staining. For samples that successfully amplified, the remaining PCR product were purified by polyethylene glycol (PEG) precipitation, using a 1:1 PCR product/20 % PEG mixture which was incubated for 15 min at 37 °C followed by a 10-min centrifugation at 2688*g* and two washes with 80% ethanol. Cycle-sequencing reactions were performed for both forward and reverse sequences using the ABI BigDye Terminator Kit (version 3.1). Sephadex column purification was used to clean the cycle-sequencing product, which was then analyzed on a PRISM 3100 genetic analyzer (Applied Biosystems).

Forward and reverse sequences were assembled into individual contigs for each locus using SeqMan II v.5.05 (DNASTAR). Alignments between sequences were created initially using Clustal X (Thompson et al., 1999) and then adjusted manually in MacClade v. 4.06 (Maddison and Maddison, 2000). The IGS region between COI and tRNAleu was too variable to be unambiguously aligned across species, and was therefore not included in phylogenetic analyses.

2.5. Phylogenetic analyses

Phylogenetic analyses were performed separately for each gene segment and for all genes together in a concatenated dataset using maximum parsimony (MP), maximum likelihood (ML) and Bayesian optimality criteria. To avoid problems resulting from missing data, incomplete sequences were excluded from the concatenated dataset. However, because the same individual samples were not always available for all three loci, sequences from different individuals from the same species were in some cases concatenated to provide a complete sequence for a species. Mitochondrial DNA analyses used *Acromyrmex balzani* and two populations of *Acromyrmex octospinosus* (one from Cuba and one from the Caribbean island of Guadeloupe) as outgroups. Nuclear DNA analyses used as outgroups the same two populations of *A. octospinsosus* as well as *Acromyrmex coronatus* and an unidentified species of *Trachymyrmex*. The appropriate model of nucleotide substitution was selected for ML and Bayesian searches using the Akaike Information Criterion (AIC) implemented in Modeltest 3.7 (Posada and Crandall, 1998) for each gene (COI, tRNAleu, COII, EF1∝F1), as well as for the concatenated dataset.

MP heuristic searches were conducted in PAUP* (Swofford, 2003) using 10 random sequence addition replicates and TBR branch swapping. Nodal support was assessed using 1000 non-parametric bootstrap replicates with identical heuristic search criteria. ML searches were conducted in GARLI 0.951 (Zwickl, 2006) using default conditions, as recommended in the User's Manual. 100 non-parametric ML bootstrap replicates were performed to estimate support for each node.

Bayesian searches were conducted in MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). Initially, four separate runs were conducted, each with four incrementally heated chains and uninformative, default priors and with nucleotide substitution parameters partitioned by gene; convergence and optimal burn-in were assessed as described in Brown and Lemmon (2007) using the program MrConverge v1.2b (written by A.R. Lemmon; http://www.evotutor.org/MrConverge). In order to better evaluate distinct tree islands sampled by initial searches, subsequent searches consisted of two groups of four runs each, with each group beginning with a starting tree taken from one of the distinct tree islands. After discarding burn-in, a majority rule consensus tree was calculated from the posterior samples of runs from each ending tree island using PAUP* to determine the posterior probabilities of each node.

3. Results

The mtDNA alignment was 550 base pairs in length, excluding the IGS region (which varied in length from 131 base pairs in *Atta laevigata* to 207 base pairs in *A. mexicana*) because it was too variable to be unambiguously aligned across all species; the nDNA (EF1∝F1) alignment was 520 base pairs in length, for a combined total of 1070 base pairs. The models of nucleotide substitution selected using the Akaike Information Criterion (AIC), as implemented in Modeltest 3.7 (Posada and Crandall, 1998), were as follows: GTR + I + Γ, GTR + Γ and K81uf + Γ for the genes COI, tRNAleu and COII and EF1∝F1, respectively.

Phylogenetic reconstructions yielded similar topologies for mitochondrial (Fig. 1) and nuclear (Fig. 2) genes except for the position of the group (*A. cephalotes* + *A. columbica*), which varied between mtDNA and nDNA reconstructions. In reconstructions using mtDNA, (*A. cephalotes* + *A. columbica*) was sister to the clade consisting of *A. mexicana, A. texana* and *A. insularis* (*Archeatta* sensu Borgmeier, 1950), which together formed a clade that was sister to the remaining species of *Atta*. In reconstructions using nDNA, (*A. cephalotes* + *A. columbica*) did not group with *A. mexicana, A. texana* and *A. insularis*, but instead formed a separate clade, sister to the remaining *Atta* species. As a result of this equivocal position, in analyses of the concatenated dataset (mtDNA + nDNA) the group (*A. cephalotes* + *A. columbica*) fell out as sister to the remaining *Atta* species (i.e. not part of the clade consisting of *A. mexicana, A. texana* and *A. insularis*) but with relatively low bootstrap support and Bayesian posterior probability (Fig. 3).

Bayesian searches using only mtDNA sequences reached convergence after 1.3 million generations, with an optimal burn-in of 1252 generations, as determined by MrConverge v1.2b (http://www.evotutor.org/MrConverge). Bayesian searches using the concatenated dataset became stuck in two distinct areas of parameter space, resulting in MCMC runs that failed to adequately converge. The parameter that appeared to most influence convergence was

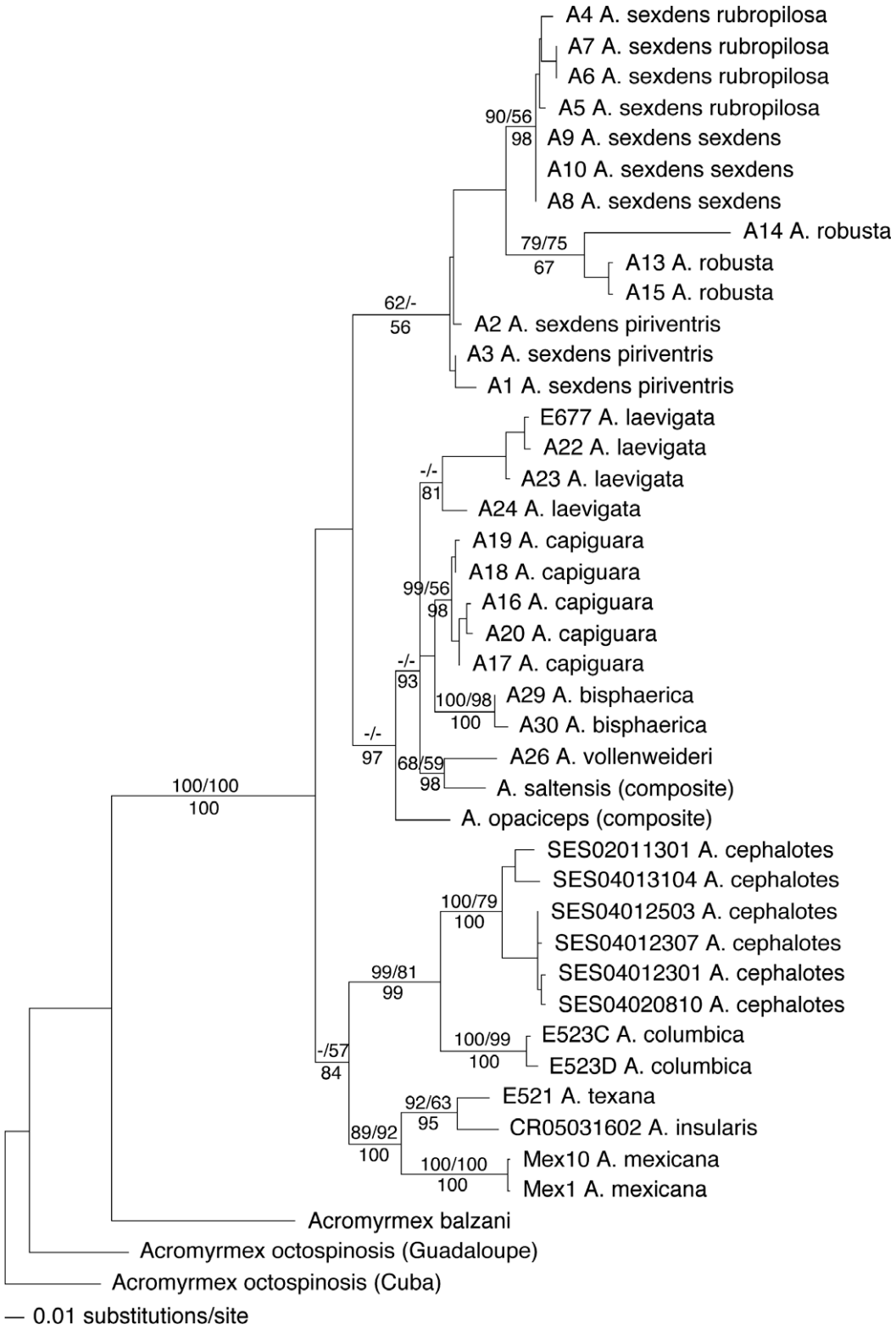

Fig. 1. Maximum likelihood phylogram of *Atta* species reconstructed using sequence information from the mtDNA genes cytochrome oxidase I, tRNA leucine and cytochrome oxidase II. Support values (shown only for ingroup branches only) above branches represent 1000 non-parametric parsimony bootstrap replicates (left) and 100 non-parametric ML bootstrap replicates (right) and below branches represent Bayesian posterior probabilities (Bpp); "–" indicates bootstrap values or Bpp less than 50. The tree was rooted using sequence information from *Acromyrmex balzani* and *Ac. octospinosis* from Cuba and from the Caribbean island of Guadaloupe.

tree topology. Specifically, the MCMC runs converged on two tree topologies that differed in (1) whether *A. robusta* or *A. sexdens piriventris* is the sister to the other two *A. sexdens* subspecies and (2) whether *A. laevigata* or *A. opaciceps* is sister to the group containing *A. bisphaerica*, *A. capiguara*, *A. opaciceps*, *A. saltensis* and *A. vollenweideri*. To attempt to reach convergence, two groups of four

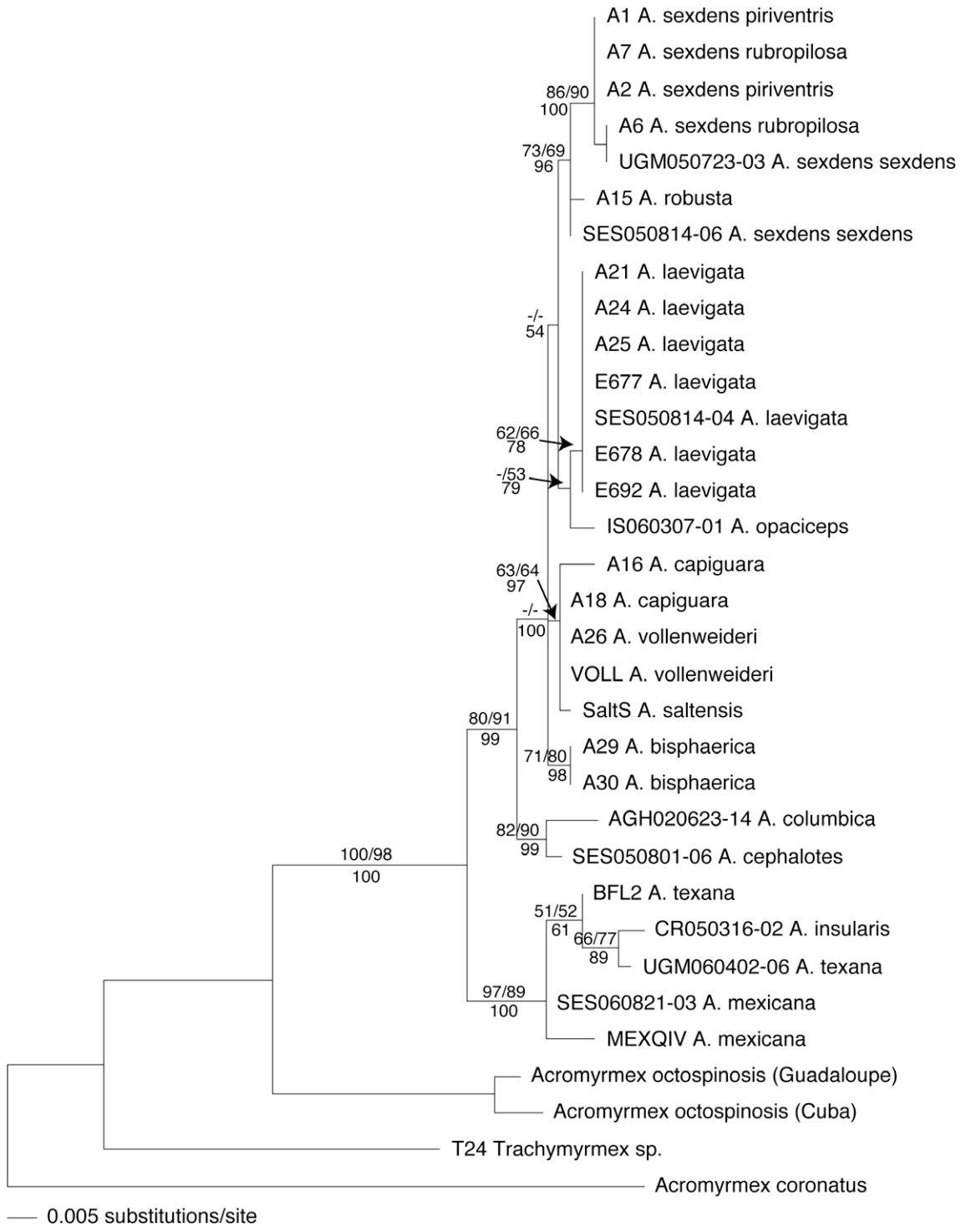

Fig. 2. Maximum likelihood phylogram of *Atta* species reconstructed using EF1-α (nDNA) sequence information. Support values (shown only for ingroup branches only) above branches represent 1000 non-parametric parsimony bootstrap replicates (left) and 100 non-parametric ML bootstrap replicates (right) and below branches represent Bayesian posterior probabilities (Bpp); "–" indicates bootstrap values or Bpp less than 50. The tree was rooted using sequence information from *Acromyrmex coronatus*, *Ac. octospinosis* from Cuba and from the Caribbean island of Guadaloupe and from an unidentified species of *Trachymyrmex*.

runs each were performed, in which each group was given one of the two topologies as a starting tree. Although convergence was not achieved in either of these groups since one run in each group began sampling trees from the other topology, convergence was achieved when the runs were grouped by ending tree island. Since these topologies appear to have similar likelihoods based on our data, and since the differences in tree topology are not important for the purposes of this study, we did not explore these alternative topologies any further, and instead chose to represent the uncertainty with polytomies in Fig. 4. These uncertainties are also represented by the low bootstrap and Bayesian posterior probabilities of the relevant nodes in Fig. 3.

Support for terminal taxa was generally stronger for mtDNA analyses, while basal nodes were better supported in nDNA

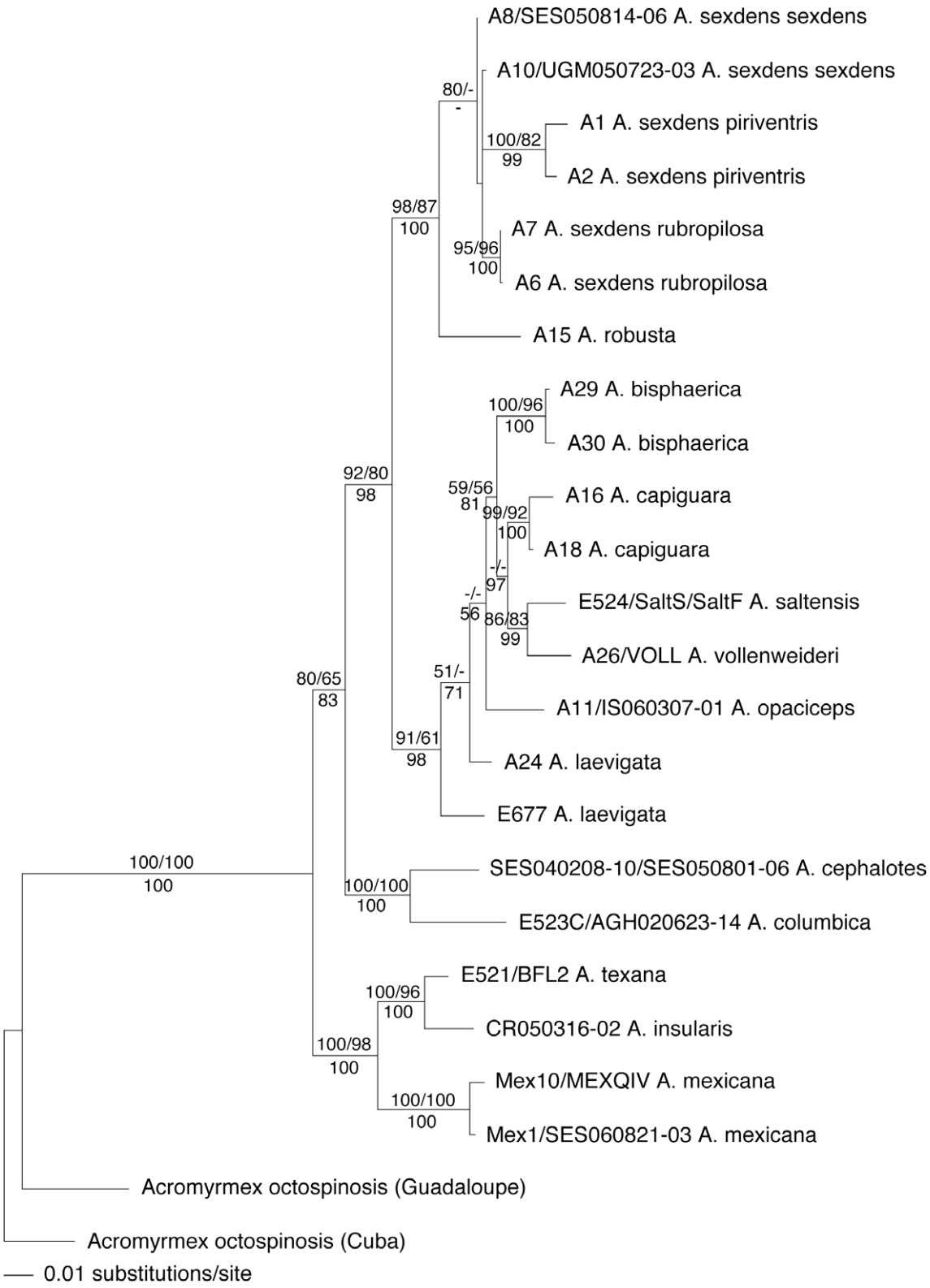

Fig. 3. Maximum likelihood phylogram using a concatenated dataset (mtDNA + nDNA). Support values above branches represent 1000 non-parametric parsimony bootstrap replicates (left) and 100 non-parametric ML bootstrap replicates (right). Support values below branches are Bayesian posterior probabilities (Bpp). Nodes without support values or with a "–" had bootstrap values or Bpp less than 50. Taxon names that include a "/" indicate that sequences from multiple individuals of the same species were concatenated to provide a complete sequence. The tree was rooted using sequence information from *Acromyrmex octospinosis* from Cuba and from the Caribbean island of Guadaloupe.

reconstructions, most likely due to differences in evolutionary rates between these parts of the genome (Lin and Danforth, 2004). All analyses had strong support for monophyly of the genus *Atta* with respect to the outgroups used.

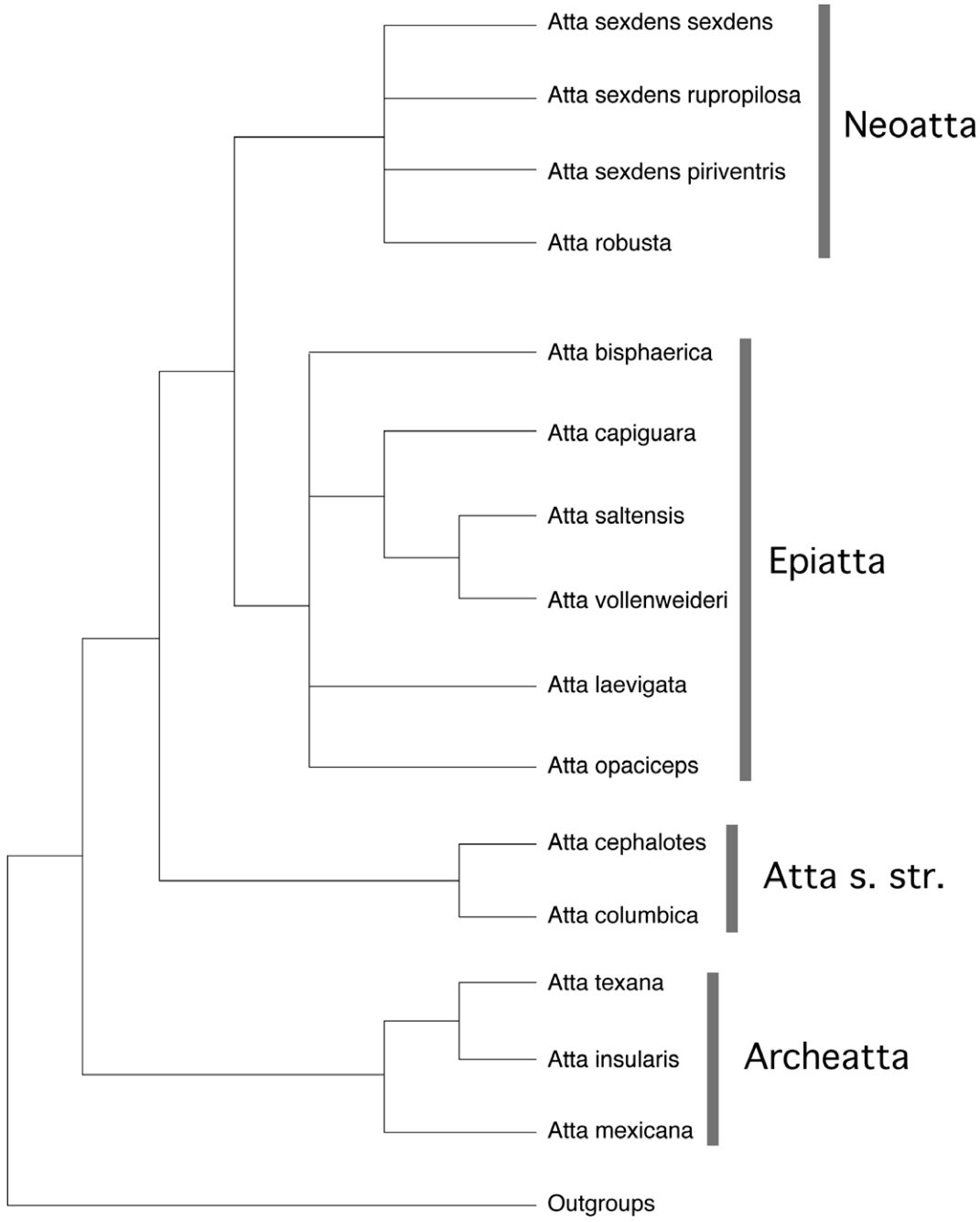

Fig. 4. Majority-rule consensus cladogram based on mtDNA and nDNA. Polytomies indicate insufficient resolution (non-parametric bootstrap and/or Bayesian posterior probabilities less than 50) based on the current analysis. Subgeneric names are given as described in Section 4.

4. Discussion

Combining DNA sequence data from mitochondrial and nuclear genes for 13 of the 15 recognized species of *Atta*, the analyses presented here provide the most complete and best resolved phylogenetic estimate for the genus to date (Gonçalves, 1986; Schultz and Brady, 2008). Our analyses provide support for the monophyly of *Atta* (Fig. 3), although a conclusive test of monophyly for the genus must await a phylogenetic analysis that includes all species of *Acromyrmex*, the genus which is the sister group of *Atta* (Bacci et al., in preparation). Additionally, our results reveal four major clades within *Atta* that can be compared to previous subgenus proposals based on morphological characters (Fig. 4 and Table 3).

The name "*Archeatta*" was first created by Emery (1922) for the species *A. insularis* and later expanded into a subgenus by Gonçalves (1942) by adding *A. mexicana* and *A. texana*, but has since been synonymized with *Atta* (Bolton et al., 2006) (Table 3). Our molecular phylogenetic analyses showed strong support for the monophyly of these three species, which form a clade that is sister to the remaining species of the genus (Fig. 3). Within this clade, *A. texana* and *A. insularis* are strongly supported as sister species in the concatenated dataset.

The subgenus "*Atta* s. str.", consisting of only the species *A. cephalotes* and *A. columbica*, was proposed by Emery (1922) (Table 3). Our molecular phylogenetic analyses showed strong support for the monophyly of these two species, although their relative

Table 3
List of the currently recognized species of the genus *Atta* and their division into subgenera according to different propositions.

Species	Subgenera propositions					
	Emery (1922)	Gonçalves (1942)	Borgmeier (1950)	Borgmeier (1959)	Gonçalves (1986)	This work
A. sexdens	*Neoatta*	*Neoatta*	*Neoatta*	*Neoatta*	*Neoatta*	*Neoatta*
A. robusta	—	*Neoatta*	*Epiatta*	*Neoatta*	*Neoatta*	*Neoatta*
A. bisphaerica	—	*Neoatta*	*Palaeatta*	*Palaeatta*	*Neoatta*	*Epiatta*
A. saltensis	—	—	—	*Palaeatta*	*Neoatta*	*Epiatta*
A. goiana	—	*Neoatta*	*Palaeatta*	*Palaeatta*	*Neoatta*	—
A. capiguara	—	—	*Epiatta*	*Neoatta*	*Neoatta*	*Epiatta*
A. vollenwiederi	—	*Neoatta*	*Epiatta*	*Neoatta*	*Neoatta*	*Epiatta*
A. laevigata	*Neoatta*	*Neoatta*	*Epiatta*	*Neoatta*	*Neoatta*	*Epiatta*
A. opaciceps	—	*Neoatta*	*Epiatta*	*Neoatta*	*Neoatta*	*Epiatta*
A. cephalotes	*Atta*	*Atta*	*Atta*	*Atta*	*Atta*	*Atta*
A. columbica	*Atta*	*Atta*	*Atta*	*Atta*	*Atta*	*Atta*
A. mexicana	—	*Archeatta*	*Archeatta*	*Archeatta*	*Archeatta*	*Archeatta*
A. insularis	*Archeatta*	*Archeatta*	*Archeatta*	*Archeatta*	*Archeatta*	*Archeatta*
A. texana	—	*Archeatta*	*Archeatta*	*Archeatta*	*Archeatta*	*Archeatta*
A. cubana	—	—	—	—	—	—

position within the genus was ambiguous. Information from mtDNA suggested that these two species form a clade together with *A. insularis*, *A. mexicana* and *A. texana* (Fig. 1), while nDNA information suggested that they form a separate, sister clade to the remaining *Atta* species (Fig. 2). Reconstructions using the concatenated dataset favored the latter topology, although with relatively low support (Fig. 3).

The species composition we found within *Atta* s. str (*A.cephalotes*, *A. columbica*) and Archeattta (*A. texana*. *A. insularis*, *A. mexicana*) was identical to that previously proposed based on morphological characters (Table 3). There is therefore no conflict between morphological data and the molecular data reported here with respect to these groupings. However, since morphological data are inconsistent with respect to the remaining nine *Atta* species (Table 3), our molecular data allow us to confidently group these species.

Borgmeier (1950) proposed the subgenus "*Epiatta*", which included the species *A. capiguara*, *A. laevigata*, *A. opaciceps* and *A. vollenweideri*. This grouping was later abandoned by Borgmeier and others, and in his 1959 revision of the genus, Borgmeier (1959), assigned these species, along with *A. sexdens* and *A. robusta*, into the previously erected (Gonçalves, 1942) subgenus "*Neoatta*" (Bolton et al., 2006). Borgmeier (1950) also proposed the subgenus "Palaeatta" which originally consisted of *A. bisphaerica* and *A. goiana* and was expanded in his 1959 revision (Borgmeier, 1959) to also include *A. saltensis*. Gonçalves (1986) reduced the number of subgenera to three by eliminating Palaeatta, whose species were assigned to *Neoatta* (Bolton et al., 2006); the remaining three subgenera were later synonymized with *Atta* (Bolton et al., 2006).

Our analyses support the monophyly of Emery's (1922) and Gonçalves' (1942, 1986) *Neoatta*, but also indicate that this clade is subdivided into two distinct and well-supported monophyletic groups. *A. robusta* and *A. sexdens* together form a clade which we refer to as *Neoatta*, while another clade is formed by the species *A. bisphaerica*, *A. capiguara*, *A. laevigata*, *A. opaciceps*, *A. saltensis* and *A. vollenweideri* which we refer to as *Epiatta*. We did not find evidence for the existence of the Palaeatta subgenus, which was proposed by Borgmeier (1950, 1959) to include *A. bisphaerica*, *A. saltensis* and *A. goiana* (Table 3), since our results (Figs. 1–3) indicate that *A. bisphaerica* and *A. saltensis* are not sister species.

The phylogenetic relationships within the clades we refer to as *Neoatta* and *Epiatta* remain somewhat unclear. Our results suggest that some taxa (*Atta laevigata* and *A. sexdens sexdens*) may be paraphyletic (Fig. 3), although the support for these patterns is weak. Since we cannot confidently reconstruct the species-level relationships within these clades, we chose to portray them as soft polytomies (Fig. 4). Future phylogenetic analyses within these clades should include samples from multiple populations within each species, ideally spanning the geographic range of widespread taxa such as *A. sexdens sexdens* and *A. laevigata*.

Because there are no known fossils of *Atta* (except possibly for a Miocene trace fossil of an *Atta* nest described from Patagonian Argentina (Laza, 1982), it is not currently possible to estimate the dates of divergence events within this genus (Brady et al., 2006). However, a recent phylogenetic analysis of the Attini by Schultz and Brady (2008) indicate that the genus *Atta* originated approximately 8–13 million years ago, suggesting that the subsequent species radiations have been relatively recent. Interestingly, the most basal split in our phylogeny of *Atta*, between the *Archeatta* species group and the remaining species, divides the genus into those species that occur in South America (*Neoatta*, *Epiatta* and *Atta*) and those that do not (*Archeatta*) (Weber, 1972). This pattern, in which North or Central American species are the sister group to the remaining species, appears to be a repeated theme throughout the evolutionary history of the higher attines: recent analyses suggest that the exclusively North American species of *Trachymyrmex* (Rabeling et al., 2007) are the sister lineage to the leafcutter ants, and the North American species *Acromyrmex versicolor* is sister to the remaining *Acromyrmex* species (Schultz and Brady, 2008). Although all three of these divergences could not have occurred simultaneously since one predates the other two, it is possible that some ongoing geological phenomenon, such as the repeated appearance and disappearance of the Isthmus of Panama, (Collins et al., 1996; Haug and Tiedemann, 1998) is responsible for this biogeographical pattern.

Our results also suggest that *A. insularis*, which is now endemic to Cuba, is the sister species to *A. texana*, which currently ranges from just south of the Rio Grande river in northeastern Mexico, across southern and eastern Texas and into Louisiana (Cameron and Riggs, 1985; Mueller et al., unpublished; Sanchez-Peña, 2005). Paleogeographic data suggest that Cuba has not been connected to North America in the last 34 million years (Iturralde-Vinent, 2006), therefore the common ancestor of *A. insularis* and *A. texana* must have dispersed across the sea and into Cuba, where it subsequently diverged via allopatric speciation. A second *Atta* species from Cuba, *Atta cubana*, was described in 1995 (Fontenla Rizo, 1995), but no material could be obtained for use in this study. We anticipate that *A. cubana* is closely related to *A. insularis*, possibly even an isolated population of the latter.

We hope this study will lay the foundation for comparative studies of leafcutter ants within a phylogenetic framework. Further insight into the evolution of the leafcutter ants, especially concerning the origins of leafcutting behavior, extreme worker polymorphism, polyandry and other characteristics unique to this

group of attine ants will require phylogenetic analyses of the leaf-cutter ant genus *Acromyrmex* as well as the non-leafcutting, higher attine genus *Trachymyrmex*.

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