Molecular Phylogenetics and Evolution 51 (2009) 427–437

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

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

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ARTICLE INFO

Article history:
Received 23 June 2008
Revised 24 October 2008
Accepted 6 November 2008
Available online 13 November 2008

Keywords:
Cytochrome oxidase I
Cytochrome oxidase II
tRNA leucine
Elongation factor 1-α
Molecular systematics

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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doi:10.1016/j.ympev.2008.11.005

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),