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Short Communication

A nuclear DNA based phylogeny of endemic sand dune ants of the genus *Mycetophylax* (Emery, 1913): How morphology is reflected in molecular data

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ABSTRACT

Molecular methods have substantially advanced our knowledge about ant systematics in the past few years. Here, we infer the molecular phylogeny of sand dune ants of the genus *Mycetophylax*, Emery 1913 (Formicidae: Myrmicinae: Attini) using 730 base pairs of DNA sequences of the two nuclear genes longwave rhodopsin and wingless. Our analyses indicate that *Mycetophylax* is monophyletic, as suggested by its morphological characters. *M. morschi*, previously considered a species of *Cyphomyrmex* due to a scrobe-like impressed area on the head, forms a well-supported cluster with the two other species of *Mycetophylax*, *M. conformis* and *M. simplex*. Our analysis yields the first comprehensive phylogeny of *Mycetophylax* based on molecular data and includes specimens from localities within a wide distributional range as well as all species belonging to the genus following the recent taxonomic revision.

1. Introduction

Ants are a large and ecologically successful group of insects ubiquitously occurring in diverse ecosystems and habitats throughout the world. Over 12,500 species are currently known (Agosti and Johnson, 2013), all belonging to the monophyletic family Formicidae. Ant taxonomy and systematic has advanced in recent years, providing us good clues about phylogenetic relationships. Moreover, taxonomic reviews have provided a more comprehensive picture of the number of species within genera due to the description of new species or new synonyms (Mayhé-Nunes and Brandão, 2007; Rabeling et al., 2007; Klingenberg and Brandão, 2009; Sosa-Calvo and Schultz, 2010).

Mycetophylax Emery 1913 is a genus of the tribe Attini (Formicidae: Myrmicinae), which, like another genera of this tribe, grows Basidiomycota fungi and utilizes them as their main food source. Until recently, more than 15 species and subspecies (plus four synonyms) had been described as members of the *Mycetophylax* genus (Bolton et al., 2006). However, Klingenberg and Brandão (2009) synonymized most of these or transferred them to other genera, so that the only remaining species form a relatively homogenous group, characterized by a distinctly smooth mesosoma without spines or only rounded protuberances and a subtriangular head without psammophore. Considering these criteria, only three species remained in the genus, *M. morschi* (Emery, 1888), *M. conformis* (Mayr, 1884) (type species) and *M. simplex* (Emery, 1888) (Klingenberg and Brandão, 2009). Based on comparative morphological traits, the authors suggested that the *Mycetophylax* genus is monophyletic.

As previous molecular phylogenetic reconstructions of the Attini did not include *M. simplex* or specimens of the three species from different localities, we here use a comprehensive phylogeny of *Mycetophylax* based on molecular data to test the proposed monophyly of the genus.

2. Materials and methods

2.1. Taxon sampling and DNA extraction

Samples of 17 colonies of the three species of *Mycetophylax* were collected along the South Atlantic coast, based on their previously published distribution area (Klingenberg and Brandão, 2009; Cardoso and Cristiano, 2010; Cardoso et al., 2012). Additionally, we sampled specimens of the *Apterostigma* sp. *pilosum* complex, *Apterostigma steigeri* and *Sericomyrmex parvulus* in Viçosa, MG, Brazil, and *Mycocepurus goeldii* in Araranguá, SC, Brazil. Samples of *Trachymyrmex fuscus* from Rio Claro, SP, Brazil, were kindly provided by Prof. Dr. Odair C. Bueno. Additional sequences of other

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Attini genera and out-group were obtained from GenBank. Sample size, locations, and accession numbers are listed in Table 1. All collected ants were preserved in ethanol and our species determinations were confirmed by Rodrigo Feitosa, at the Museu de Zoologia da Universidade de São Paulo (MUZSP), where vouchers were also deposited.

Genomic DNA extraction from one worker per colony was performed according to the standard CTAB/chloroform techniques (Sambrook and Russell, 2001) or following a modified phenol–chloroform protocol (Fernandes-Salomão et al., 2005). Nuclear sequences were obtained for the wingless (WG) and longwave rhodopsin (LW) genes, using previously published primers (Ward and Downie, 2005; Brady et al., 2006). These loci have been successfully sequenced in previous phylogenetic studies on ants and particularly the wingless locus was shown to be informative at the species- and genus-levels (Schultz and Brady, 2008; Mehdiabadi et al., 2012).

2.2. DNA amplification, sequencing and phylogenetic analysis

Polymerase chain reaction (PCR) was performed in a final volume of 25 μL (2U of GoTaq® Flexi DNA Polymerase (Promega), dNTPs (0.25 mM each), $MgCl_2$ (2.5 mM), reaction buffer (1×), a pair of primers (0.48 μM each) and 1 mL of DNA). The thermocycler conditions during the amplification reaction were 2 min denaturation at 94 °C, followed by 35 cycles of 94 °C for 1 min, 60 °C (for LW) or 55 °C (for WG) for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. Purified PCR products were sequenced directly using the same primers for amplification by Macrogen Inc., South Korea (www.macrogen.com).

The chromatograms were evaluated and edited using the program Consed (Gordon et al., 1998). Table 1 lists the species used and their respective sequence accession numbers in GenBank and the sequences obtained in this study are underlined. All sequences of LW and WG were separately aligned. Therefore, sequences were concatenated and analyzed by translation into amino acids using the program MEGA 5.0 (Tamura et al., 2011) to inspect for premature stop codons and identify the intron of LW. The intron of the LW gene was excluded from the alignment. Then, the edited sequences were aligned using ClustalW (Thompson et al., 1994) and returned to the nucleotide sequences, which were used in further phylogenetic analyses.

In order to select the substitution model of DNA evolution that fitted best to each gene under Akaike's Information Criterion (AIC) and Bayesian Information Criterion (BIC) we used jModeltest (Posada, 2008). Taking into account these parameters, a maximum likelihood (ML) tree was constructed with PAUP 4.0 (Swofford, 2003), with boot-strapping of 1000

Table 1
Nuclear DNA phylogeny of the genus *Mycetophylax*. Species, codes, collecting localities and accession numbers of the sequences included in the phylogenetic analysis.

Species	Code	Locality of sampled specimens	GenBank accession number	
			LW rhodopsin	Wingless
Mycetophylax morschi	CRS	Chuí/RS – Brazil	KC964626	KC964647
Mycetophylax morschi	MRS	Mostardas/RS – Brazil	KC964625	KC964645
Mycetophylax morschi	ASC	Araranguá/SC – Brazil	KC964621	KC964646
Mycetophylax morschi	LSC	Laguna/SC – Brazil	KC964622	KC964648
Mycetophylax morschi	SSC	São Franc. do Sul/SC – Brazil	KC964624	KC964644
Mycetophylax morschi	ARJ	Angra dos Reis/RJ – Brazil	KC964623	KC964643
Mycetophylax conformis	ARJ	Angra dos Reis/RJ – Brazil	KC964616	KC964638
Mycetophylax conformis	MRJ	Mambucaba/RJ – Brazil	KC964617	KC964639
Mycetophylax conformis	RRJ	Maricá/RJ – Brazil	KC964618	KC964640
Mycetophylax conformis	QRJ	Quissamã/RJ – Brazil	KC964619	KC964641
Mycetophylax conformis	CSP	Caraguatatuba/SP – Brazil	KC964620	KC964642
Mycetophylax simplex	CRS	Cassino/RS – Brazil	KC964631	KC964653
Mycetophylax simplex	SRS	São José do Norte/RS – Brazil	KC964632	KC964654
Mycetophylax simplex	MSC	Arroio do Silva/SC – Brazil	KC964629	KC964652
Mycetophylax simplex	ASC	Araranguá/SC – Brazil	KC964627	KC964649
Mycetophylax simplex	ASC	Araranguá/SC – Brazil	KC964628	KC964650
Mycetophylax simplex	CRJ	Cabo Frio/RJ – Brazil	KC964630	KC964651
Apterostigma sp. *pilosum complex*	–	Viçosa/MG – Brazil	KC964637	KC964658
Apterostigma steigeri	–	Viçosa/MG – Brazil	KC964636	KC964659
Mycocepurus goeldii	–	Araranguá/SC – Brazil	KC964635	KC964655
Sericomyrmex parvulus	–	Viçosa/MG – Brazil	KC964633	KC964656
Trachymyrmex fuscus	–	Rio Claro/SP – Brazil	KC964634	KC964657
Acromyrmex heyeri	–	–	EU204529/EU204286[a]	EU204210
Acromyrmex balzani	–	–	EU204490/EU204247	EU204170
Atta laevigata	–	–	EU204481/EU204238	EU204161
Cyphomyrmex rimosus	–	–	EU204466/EU204223	EU204146
Cyphomyrmex cornutus	–	–	EU204521/EU204278	EU204202
Cyphomyrmex cornutus			EU204532/EU204289	EU204213
Cyphomyrmex costatus	–	–	EU204488/EU204245	EU204168
Trachymyrmex septentrionalis	–	–	EU204503/EU204260	EU204184
Mycetophylax conformis	TRI	Trinidade e Tobago	EU204486/EU204243	EU204166
Mycetophylax morschi	BRZ	Brazil	EU204531/EU204288	EU204212
Kalathomyrmex emeryi	–	–	EU204478/EU204235	EU204158
Kalathomyrmex cf. emeryi	–	–	EU204524/EU204281	EU204205
Wasmannia auropunctata	–	–	EU204483/EU204240	EU204163

[a] Opsin exon 1/opsin exon 2, note that the other sequences were deposited with introns.

replicates. The Bayesian analysis was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and consisted of two independent runs of ten million generations each, sampled every 1000 generations and an appropriate burn-in was determined using Tracer v1.4 (Rambaut and Drummond, 2007). A burn-in period, in which the initial 25% of the trees were discarded, were adopted to produce a single consensus topology that was visualized using the FigTree v1.4 program (Rambaut, 2009). The topologies inferred by both methods were tested to check the congruence using the Shimodaira-Hasegawa test implemented in PAUP 4.0.

3. Results

Partial sequences were obtained for the LW (384 bp without intron) and WG (346 bp) genes, resulting in an alignment of 730 base pairs comprising 19 sequences from specimens of *Mycetophylax* (17 obtained in this study plus two downloaded from the GenBank), 15 sequences of other Attini ants and one out-group. The alignment included 208 variable sites and 150 parsimony informative sites. The substitution models selected for LW and WG genes under AIC were HKY + G and GTR + G, respectively; whereas the substitution model K80 + G for both genes was selected under BIC criterion. We used both in independent Maximum Likelihood and Bayesian analysis. The topologies from both phylogenetic methods were statistically equivalent (S–H test, $p = 0.236$). Likewise, independent analysis carried out using the models of sequences evolution estimated under BIC or AIC criterion recovered identical topologies.

Fig. 1 shows the Bayesian consensus phylogeny based on the concatenated sequences. Our phylogenetic analysis unambiguously supports the monophyly of the genus *Mycetophylax* with higher statistical support as a sister group of the genus *Cyphomyrmex*. *M. morschi* is clearly nested within the genus *Mycetophylax*, as currently suggested based on morphological traits (Klingenberg and Brandão, 2009). This result was also supported when both nuclear markers were analyzed separately (data not shown) and by both methods of phylogenetic reconstruction.

4. Discussion

The purpose of this study was to assess the monophyly of the genus *Mycetophylax* as presently recognized by morphological traits. Our results clearly supported the findings based on morphological traits described by Klingenberg and Brandão (2009). All species and specimens of the genus *Mycetophylax* from different localities throughout their distributional range fell into a well-supported monophyletic clade. This result is in agreement with previous molecular phylogenetic hypotheses based on a study with *M. morschi* (denominated *Cyphomyrmex*) and *M. conformis*, though only one specimen from each species was included in the study (Schultz and Brady, 2008). Our molecular phylogenetic reconstruction included six additional specimens of *M. morschi* and five of *M. conformis* from different localities, and six specimens of *M. simplex*,

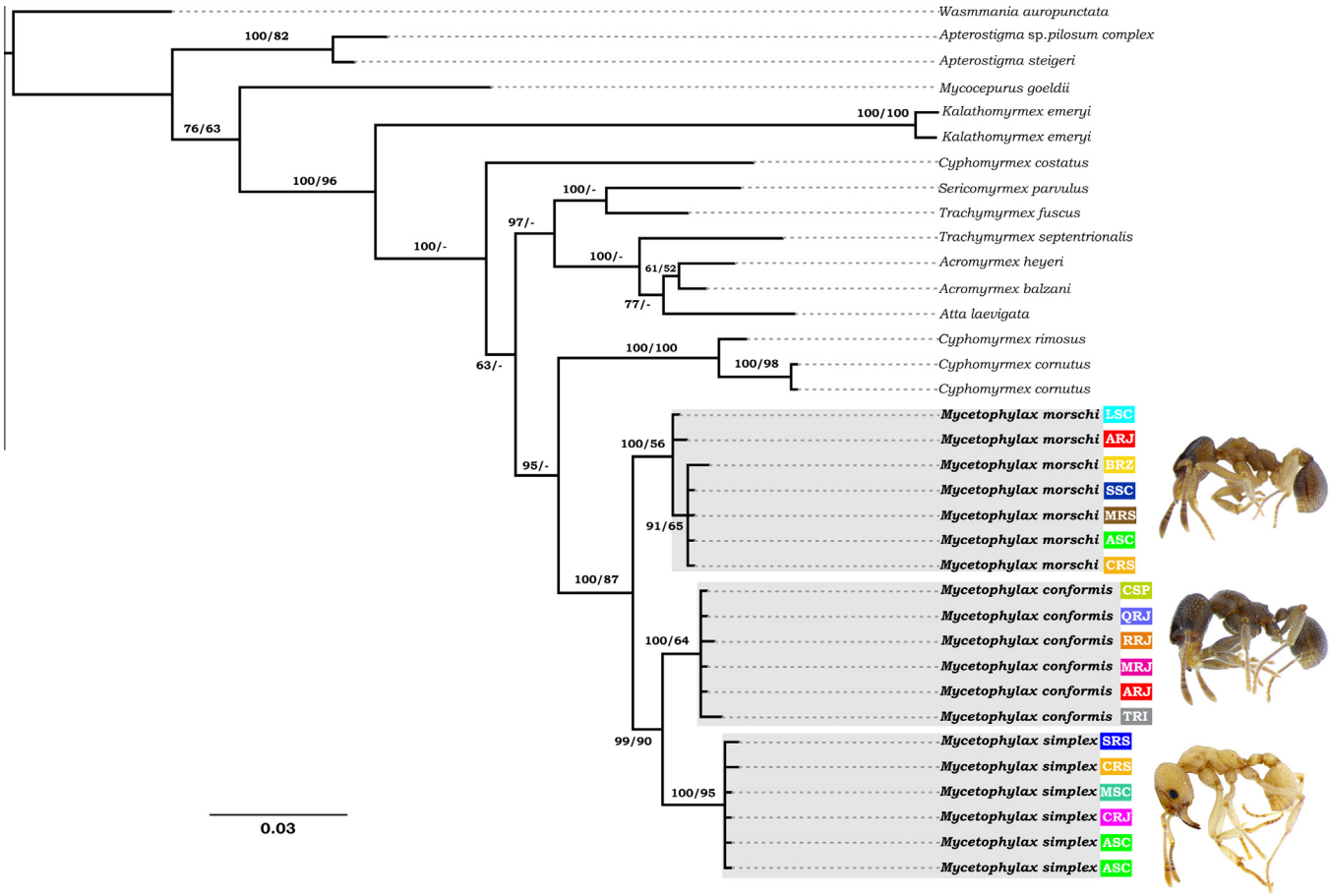

Fig. 1. Bayesian phylogenetic consensus tree of combined wingless and long-wave rhodopsin gene sequences. The first numbers at branches are Bayesian posterior probabilities followed by bootstrap values calculated from the maximum likelihood analysis of 1000 sequence replicates. Three branches are proportional to mutational events sampled in sequences alignment (scale bar). Codes at the tips indicate samples localities as given in Table 1.

which was not included in the previous molecular phylogenetic analysis (see Wetterer et al., 1998; Schultz and Brady, 2008). *M. morschi*, of which some taxonomists still believe that it should be considered as member of the genus *Cyphomyrmex*, was unambiguously placed in the genus *Mycetophylax*.

We also observed that the inclusion of *M. simplex* in the analysis changed the relationship between *M. conformis* and *M. morschi*. In the Bayesian consensus phylogeny reconstructed with these three species, *M. morschi* appeared as sister group of a well-supported cluster comprising *M. simplex* and *M. conformis* (posterior probability (PP) = 0.99). Early during the evolutionary time, the genus *Mycetophylax* seems to have diverged into two different lineages. One of these lineages evolved into *M. morschi*, while the other diversified into *M. conformis* and *M. simplex*. This relationship is also supported by the morphological similarity between *M. conformis* and *M. simplex* that can be easily distinguished from *M. morschi* that bears a scrobe-like depression on head (see Klingenberg and Brandão, 2009).

The genus *Cyphomyrmex* is likely the sister group of the genus *Mycetophylax*. Although this cluster does not show statistical support in Maximum Likelihood analysis, it is well-supported by Bayesian analysis and is in agreement with a previous report (Schultz and Brady, 2008). Our analysis and previous phylogenetic hypothesis (Schultz and Brady, 2008) confirmed that the genus the *Cyphomyrmex* is paraphyletic in relation to other Attini genera. However, we included only a small set of species from this genus and the relationship among *Cyphomyrmex* species needs further examination with molecular data. The inclusion of species from *C. strigatus* group may help to shed light on the sister group of the genus *Mycetophylax*, but unlikely will present potential impact on the relationships of the genus *Mycetophylax*. Moreover, the species *Kalathomyrmex emeryi*, which previously was included in the genus *Mycetophylax*, clearly branches off early in the tree, even with the inclusion of *M. simplex* in the analysis. Considering the morphological distinctiveness of *K. emeryi* due to psammophore setae on the clypeus (Klingenberg and Brandão, 2009) and its basal position on the tree, we agree that *K. emeryi* should be considered a distinct monotypic genus, as defined in the taxonomic revision conducted by Klingenberg and Brandão (2009). The other species removed from the genus *Mycetophylax* was not included in the

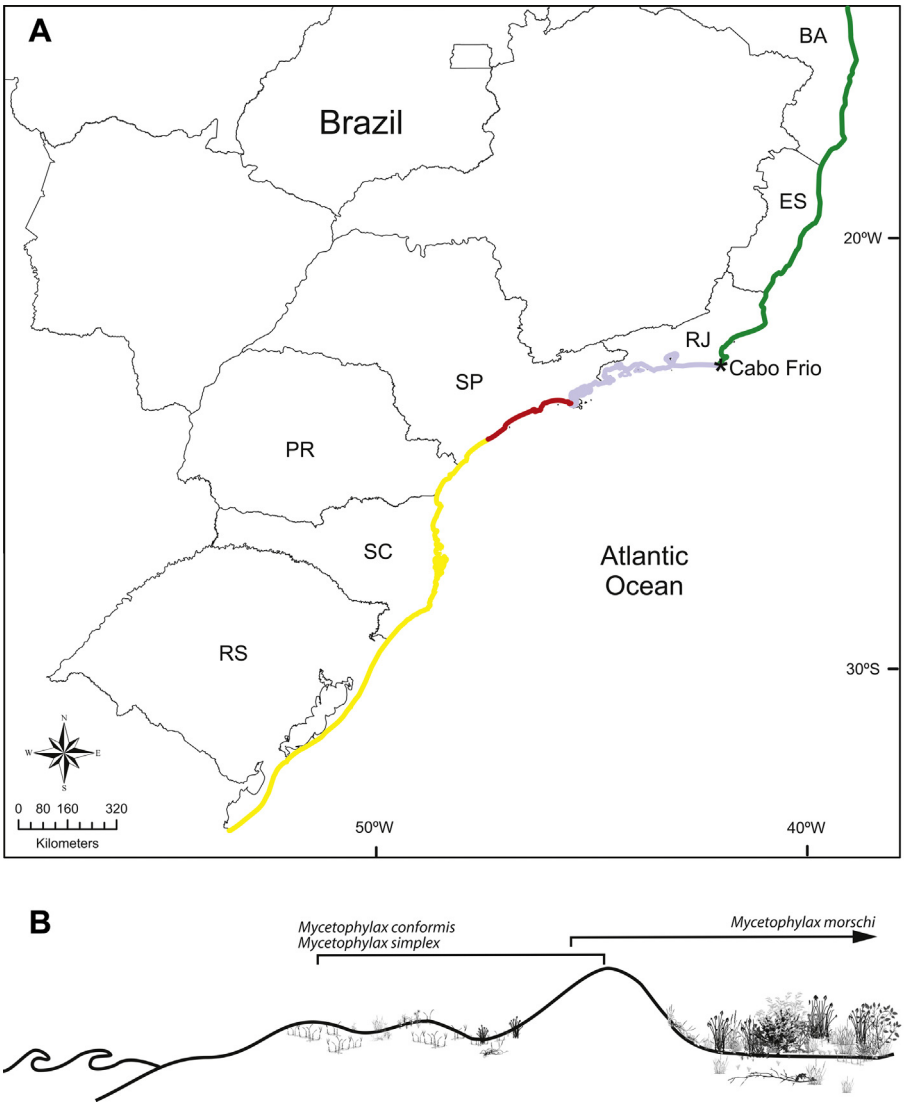

Fig. 2. Map showing distribution of *Mycetophylax* genera along Atlantic coast in Brazil (based in Cardoso et al., 2012) and nesting habits on sand dunes. (A) *M. simplex* together with *M. morschi* on yellow area. *M. morschi* only on red area. *M. conformis* and *M. morschi* together on purple area. *M. conformis* only on green area. Co-occurrence of the three species indicated by asterisk and (B) nesting sites partitioning of *Mycetophylax* species in the dunes along Atlantic Ocean in Brazil. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

analysis, however *Paramycetophylax bruchi* also presents some morphological distinctions from *Mycetophylax* (see Klingenberg and Brandão, 2009). Its phylogenetic position requires further analysis.

Mycetophylax seems to be a small genus with marked geographic distribution. It is restricted to the sand dune environments of the Atlantic coast. *M. conformis* and *M. simplex* are parapatric throughout most of their range (Fig. 2). *M. conformis* and *M. simplex* did not overlap in their geographic distribution along the Atlantic coast, but in Cabo Frio, RJ (Fig. 2). This pattern suggests that speciation between them could have been facilitated by vicariant events. The coast of Brazil is known to have been profoundly remodeled during the Quaternary (Dillenburg and Hesp, 2009). The sand dune fields along the coast were modified due to marine introgressions and regressions, which could have created islands that isolated populations and might have promoted speciation. Yet, *M. morschi* is sympatric with the other two species. However, their nests were positioned more inland, far from sea shore, where the vegetation is denser. This niche partitioning suggests that speciation between *M. morschi* and other *Mycetophylax* species could have been facilitated by habitat shift events (Fig. 2B). The high phylogenetic resolution (phylogenetic signal) among the *Mycetophylax* species may reflect that there has been sufficient time for the accumulation of shared derived nucleotide substitutions among lineages and that supports the observed monophyly.

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