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Morphological differentiation between species of *Myrmelachista* Roger (Formicidae: Formicinae) in Atlantic Forest areas of the Alto Tietê (São Paulo)

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Abstract

Myrmelachista is an exclusively Neotropical ant genus. The ants from this genus nest and forage in live or dead trees, but data on its life cycle are relatively scarce. The taxonomy of this genus is considerably complex. The morphological characters of taxonomic importance are not well defined, and combining characteristics from the largest possible number of castes is thus essential. The goal of the present study was to present the results of multidimensional morphological analyses conducted based on morphometric measurements of workers and alates to help with the identification of *Myrmelachista* species. For that purpose, we gathered data from 277 specimens (workers, males and gynes) from eight species cohabiting Atlantic Forest areas. Eighteen morphometric variables were measured on workers and 20 on reproductive ants. Measurements related to eye size and mandible width were the variables more strongly associated with the two morphological clusters obtained based on the morphology of workers. However, the morphospace described by workers cannot be used to delimit the species of *Myrmelachista*. Mandible width, petiole height and eye size (eye length and ocelli width) were the most informative variables associated with the four clusters of morphology that clearly delimit males of *M. arthuri*, *M. ruszkii* sp.7, and in part, *M. catharinae*. Finally, petiole length and head length were the best descriptors of the six morphological clusters recognized for gynes. *M. catharinae*, *M.* sp.7, *M. nodigera* and *M. gallicola* were clearly delimited in the gyne morphological space. *M. ruszkii* showed high phenotypic variability, and this species was classified in two morphological clusters based on gyne specimens. Species identification employing multiple gyne morphological traits exhibited the best results. Our results show that morphospace analysis can be useful for delimitation of *Myrmelachista* species.

Introduction

Myrmelachista is an arboreal genus with 69 described species (Longino, 2006; Bolton, 2013). Its distribution is restricted to the Neotropical region, and 41% of the species occur in Brazilian territory (Kempf, 1972; Fernández & Sendoya, 2004). It is characterized by a visible antennal club with one or two segments, a character that is absent in the remaining Neotropical Formicinae (Fernández, 2003). *Myrmelachista* species have antennae with nine or ten segments; most species have nine-segmented antennae and are found mainly in Central America (only two have been described in South America), whereas ten-segmented species are mainly found in South America (only three species have been described in Central America and Mexico) (Longino, 2006).

Workers have been observed in association with 20 families and 53 species of angiosperms (see review by Nakano et al., 2013) and in leaf litter (Suguituru et al., 2011, 2013). Nests are located in the trunks of live trees (Longino, 2006). Colonies with immature and reproductive ants also occupy twigs dispersed on the leaf litter (Nakano et al., 2012,

2013). The genus *Myrmelachista* is characterized by forming mutualistic associations with some myrmecophytes (Renner & Ricklefs, 1998; Edwards et al., 2009), where workers use formic acid to protect the host plant (Frederickson, 2005), as well as with Coccidae and Pseudococcidae (Kusnezov, 1951; Stout, 1979; Ketterl et al., 2003; Longino, 2006). Although some studies on this genus have been published recently (Longino, 2006; Nakano et al., 2012, 2013), information on the biology of *Myrmelachista* species is still scarce. Ants from this genus feed on extrafloral nectaries (Frederickson, 2005; McNett et al., 2010), but workers may take plant, fungal and animal fragments (eggs, feces and larvae) into the nests (Torres, 1984).

The taxonomy of this genus is considered extremely complex (Wheeler, 1934; Snelling & Hunt, 1975; Longino, 2006). Morphological characters of taxonomic importance are not well defined, and it is necessary to combine information from all castes (workers and reproductive ants). Nakano et al. (2014) showed that some species may be separated using molecular characters, despite their high morphological similarity (Nakano, 2010). However, no detailed morphological study has been performed to evaluate species delimitation in *Myrmelachista*.

It is well known that many species can be difficult to diagnose and delimit, especially when using single operational criteria such as morphological characters or DNA markers (Cardoso et al., 2009; Ezard et al., 2010). Morphological data are useful for delimiting species, and arguments exist to refine approaches to species delimitation (Barr et al., 1985; Ezard et al., 2010; Seifert et al. 2014a). Some studies have suggested that multivariate analysis based on morphology provides further insights into species alpha-taxonomy and distribution of organisms (Seifert, 2002; Seifert et al. 2014ab). In this study, we present the results of multidimensional morphological analyses performed based on morphometric measurements of workers and alates (males and females) to help identify *Myrmelachista* species.

Materials and Methods

Collection sites and species

Collections were performed at three sites: (1) Francisco Affonso de Mello Municipal Natural Park, 23°31'22" S, 46°11'16" W, 807–1140 m, in the municipality of Mogi das Cruzes; (2) Barragem de Ponte Nova, 23°31'85" S, 45°50'77" W, 783 m, in the municipality of Salesópolis; and (3) Nascentes do Tietê State Park, 23°34'19" S, 45°44'10" W, 1027 m, in the municipality of Salesópolis. All sites were located in the Alto Tietê region, which belongs to the Brazilian Atlantic Forest and is characterized by dense ombrophilous forest (Fiaschi & Pirani, 2009; Colombo & Joly, 2010). Eight species of *Myrmelachista* can be found in this region (Suguituru et al., 2015), corresponding to 11% of the species recorded in the Neotropical Region and to 29% of the Brazilian species. Therefore, our study area exhibited high local species richness of the genus *Myrmelachista*, which is adequate to evaluate species discrimination tools, considering that these species are seldom recorded (Deyrup, 2003; observations on different published lists of ant species recorded in Brazil).

The species analyzed were *Myrmelachista arthuri* Forel, 1903, *M. catharinae* Mayr, 1887, *M. gallicola* Mayr, 1887, *M. nodigera* Mayr, 1887, *M. reticulata* Borgmeier, 1928, *M. ruszkii* Forel, 1903, *Myrmelachista* sp.4 and *Myrmelachista* sp.7 (Suguituru et al., 2015). A more detailed description of the collection sites, methods used, and specimen identification can be found in Nakano et al. (2012, 2013, 2014).

Species (or morphospecies) were identified by comparing the examined specimens with specimens deposited in the reference collection of the Museum of Zoology of the University of São Paulo. Vouchers were deposited in the myrmecofauna collection of the Alto Tietê Myrmecology Laboratory of the University of Mogi das Cruzes and in the Museum of Zoology of the University of São Paulo.

Number and morphology of specimens

The number of specimens per species is shown in Table 1. The morphological parameters were measured using a micrometer ruler coupled to a stereoscopic microscope. Eighteen morphological parameters were selected for workers and 20 for reproductive ants according to Longino (2006) and Silva and Brandão (2010).

Table 1. Total number of specimens per *Myrmelachista* species subjected to morphological analysis.

Species	Number				Total/specie
	Number of nests	Worker/nest	Males	Gynes	
Myrmelachista arthuri	6	5	7	-	37
Myrmelachista catharinae	10	5	7	9	66
Myrmelachista gallicola	5	5		2	27
Myrmelachista nodigera	5	5	-	10	35
Myrmelachista reticulata	1	5	-	-	5
Myrmelachista ruszkii	5	5	3	19	47
Myrmelachista sp.4	5	5	-		25
Myrmelachista sp.7	4	5	4	11	35

Data Analysis

We used a method developed to delimit species morphometrically in a multivariate space without using an *a priori* definition. This approach for species delimitation identifies clusters in the morphospace when two or more well-separated groups are a better way of describing a given sample than one group (Ezard et al., 2010). The approach to species delimitation consists of four steps (based on Ezard et al., 2010): (1) extraction of orthogonal axes with robust covariance estimators (Croux et al., 2007); (2) reduction of the dimensionality of the orthogonal axes to only those with significant explanatory power (Jackson, 1993); (3) identification of the optimal number, shape and orientation of groups within the rotated dimension-reduced data (Fraley & Raftery, 2002). In step 1, each morphometric variable was centered on the median and scaled by percentile variability prior to reducing the dimensionality (i.e., the rotation was based upon the robust covariance estimates). In step 2, the number of components retained was determined using the broken stick criterion (Peres-Neto et al. 2005). In step 3, the dimension-reduced space was split into groups using cluster analysis, with optimal delimitation by K-means approaches (Klingenberg & Froese, 1991) combined with methods to estimate the shape and orientation of clusters in the morphospace, and Gaussian mixture models and a Bayesian approach were used to estimate the support for particular arrangements of clusters using iterative Expectation-Maximization methods for maximum-likelihood (Fraley & Raftery, 2002). The volume and shape can be equal or variable among axes (elliptical, round, diagonal or univariate), and the choice between competing models is made through the Bayesian Information Criterion (BIC). All analyses were performed in R version 3.1.1 (R Core Team, 2014) and used the mclust (Fraley & Raftery, 2002), pcaPP (Filzmoser et al., 2014) and mvoutlier (Filzmoser & Gschwandtner, 2014) packages.

Results

Workers

The robust PCA of 18 morphometric variables from 205 worker specimens from the Atlantic Forest populations revealed a continuum of variation but no discrete clusters within the *Myrmelachista* morphospace (Fig 1). The clustering on the dimension-reduced morphospace found weak evidence to reject the null hypothesis of homogeneous data in the worker measurements. The best model found two clusters of morphologies (ellipsoidal, varying volume, shape, and orientation) and did not delimit the worker individuals in the data set. The broken stick criterion suggested that two principal components should be retained, which represented 94% of the variation in the original data (Table 2). As expected, the first component is a size component, with variables loading relatively weakly and positively into this component. In the second component, mandible width and eye size loaded positively and relatively strongly with axis II (Table 2).

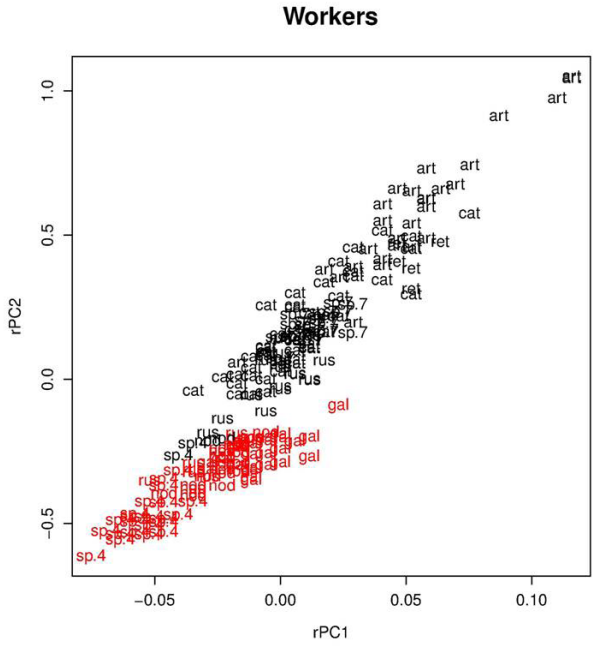

Fig 1. Point clouds for workers of *Myrmelachista* species in the Brazilian Atlantic Forest. The two clusters identified in the robust PCA are shown in different colors. Symbols: art= *M. arthuri*, cat = *M. catharinae*, gal= *M. gallicola*, rus= *M. ruszkii*, sp.4 = *M.* sp.4, sp.7= *M.* sp.7.

Males

The robust PCA of 20 morphometric variables from 21 male specimens suggested that five groups can be characterized mostly by their distinctive size and shapes in the plane of the first and second PCA axes (Fig 2). The clustering on the dimension-reduced morphospace found evidence in male measurements to reject the null hypothesis of homogeneous data in favor of the alternative hypothesis of more than one species of males. The best model found five clusters of morphology (univariate, equal variance) and delimited mainly males of *M. catharinae* (71%; N=7) and *M.* sp.7 (100%, N=4). *M. arthuri* was split in two groups, although very close in the morphospace (Fig 2). By contrast, individuals of *M.* sp.7 and *M. ruszkii* were equal in the morphospace; two individuals of *M. catharinae* were classified as a particular group. The broken stick criterion suggested that one principal component should be retained; PCI and PCII represented 89% of the variation in the original data (Table 2). The first component is a size component, with variables loading relatively weakly and positively into this component. Mandible width and petiole height were the variables with higher loads in axis I. Principal component II showed some allometry with positive loading of head width, maximum eye length, scape length, size, petiole size (length and width), femur length, distance between ocelli and ocelli width (the latter being strongly associated with PC II) (Table 2).

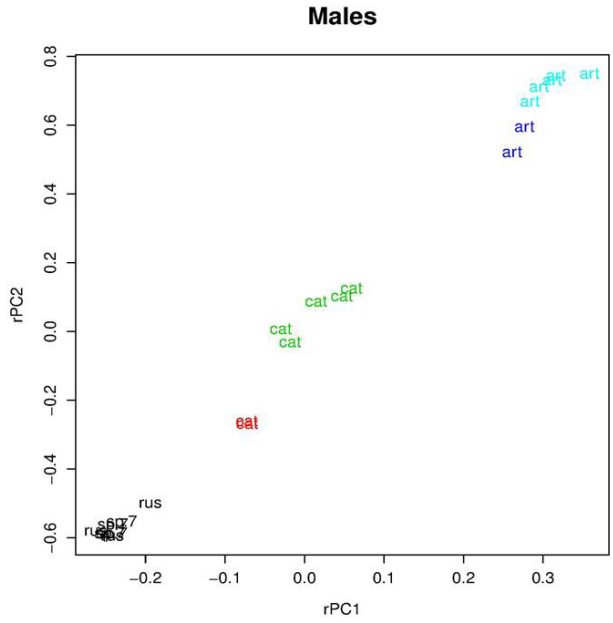

Fig 2. Point clouds for males of *Myrmelachista* species in the Brazilian Atlantic Forest. The five clusters identified in the robust PCA are shown in different colors. Symbols: art= *M. arthuri*, cat = *M. catharinae*, rus= *M. ruszkii*, sp.7= *M.* sp.7.

Gynes

The robust PCA of 20 morphometric variables from 51 gyne specimens showed that the species can be distinguished from each other with different degrees of consistency (Fig 3). The clustering on the dimension-reduced morphospace found evidence for more than one species in the gyne dataset. The best model found seven clusters of morphology (ellipsoidal, equal volume, shape and orientation) and delimited the gynes of *M. catharinae* (88%; N=9), *M.* sp.7 (100%, N=11), *M. nodigera* (100%, N=10) and *M. gallicola* (100%, N=2); individuals of *M. ruszkii* (N=19) were classified into two clusters suggesting high morphometric variability. The broken stick criterion suggested that two principal components should be retained, which represented 95% of the variation in the original data (Table 2). The first and second components explain 53% and 42%, respectively. The first component showed some allometry because petiole length loaded positively and relatively strongly (compared to the other variables loading negatively). Petiole length was the variable with higher loads in the first principal component; and head length and petiole length were the variables with higher loads in the second principal component (Table 2).

Table 2. The loadings onto the robust principal components for workers, males and gynes specimens of *Myrmelachista*; larger absolute values indicate more influential traits on the dimension-reduced space. The first or second higher loadings in each axis were highlighted in bold. Workers: 18 variables and 205 specimens; Males: 20 variables and 21 specimens; Gynes: 20 variables and 51 specimens.

	Workers		**Males**		**Gynes**	
	Component I	Component II	Component I	Component II	Component I	Component II
Standard Deviation	3.43	2.57	3.91	2.35	4.24	3.76
Proportion of Variance	0.60	0.34	0.66	0.24	0.53	0.42
Cumulative Proportion	0.60	0.94	0.66	0.90	0.53	0.95
Trait						
Head width	0.29		0.236	0.109	-0.251	
Head length	0.243		0.188			**0.454**
Mandible length	**0.357**				-0.209	0.35
Mandible width		**0.577**	**0.454**	-0.357	-0.158	0.122
Distance between mandibles	0.279		0.136		-0.266	
Scape length	0.162		0.234	0.13	-0.113	0.223
Eye length		**0.577**	0.195	**0.556**	-0.229	0.151
Eye width		**0.577**	0.255	-0.155	-0.142	0.105
Distance of eye to mandible insertion	0.324		0.249			0.199
Interocular distance	0.288		0.286		-0.252	
Pronotum width	0.229		0.178		-0.278	0.181
Weber's length	0.285		0.123		-0.238	0.123
Hind tibia length	0.217		0.124		-0.227	
Hind femur length	0.203			0.118	-0.218	
Petiole width	0.211		0.219	0.11	-0.108	
Petiole length	0.265			**0.386**	**0.487**	**0.642**
Petiole height	0.166		**0.489**		-0.152	0.175
Gaster width	0.244		0.125		-0.246	
Ocelli width				**0.452**	-0.267	
Distance between ocelli				0.324		0.182

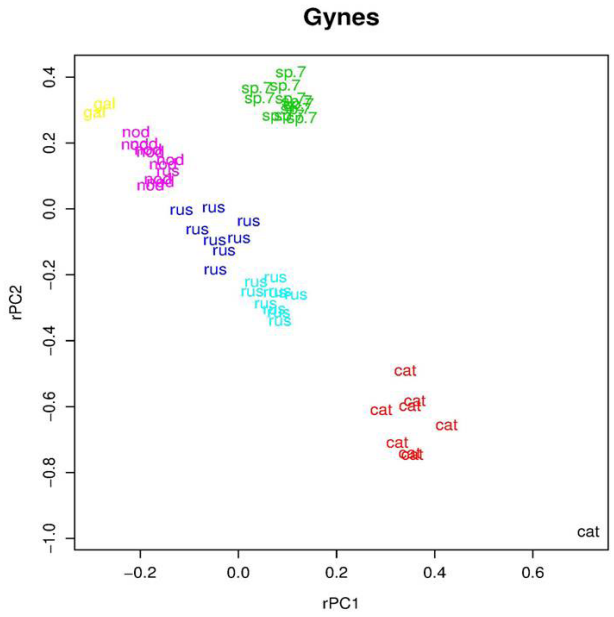

Fig 3. Point clouds for gynes of *Myrmelachista* species in the Brazilian Atlantic Forest. The seven clusters identified in the robust PCA are shown in different colors. Symbols: Symbols: cat = *M. catharinae*, gal= *M. gallicola*, nod = *M. nodigera*, rus= *M. ruszkii*, sp.7= *M.* sp.7.

Discussion

The *Myrmelachista* species analyzed in the present study exhibit ten-segmented antennae, a characteristic associated with a group considered to be very heterogeneous (Snelling & Hunt, 1975). Our results indicate that workers can not be identified based only on multivariate morphometric analyses. Separation of workers using morphometric data is a complex task because some studies show that the morphology of individuals of the same species originating from different colonies may vary to the point of being considered different species (Wheeler, 1934; Snelling & Hunt, 1975).

Surprisingly, the set of variables used to describe the morphometry of gynes was efficient at separating groups of species. The resulting groups of species were largely in agreement with the identification by comparison with worker specimens deposited in biological collections, the identity of which was confirmed through direct comparisons with type specimens.

Workers of *M. catharinae* and *M. arthuri* are genetically close (Nakano et al., 2014). In our analyses, these species exhibited high morphological overlap, similarly to workers from *M. gallicola* and *M. nodigera*. Other studies have also reported morphological (Quirán & Martinez, 2006), behavioral (Nakano et al., 2013) and genetic (Nakano et al., 2014) similarities between these species.

Genetic analysis of *M. catharinae* workers revealed two groups (Nakano et al., 2014), which was not confirmed by morphometry. However, males of *M. catharinae* were classified into two groups, which suggests some phenotypic variability retrieved in our morphometric analyses.

The multivariate analysis indicated that characters such as head length and the sizes of the eye, ocelli and petiole may help with the delimitation of some species. In terms of function, these morphometric variables are mainly related to predation and locomotion (see review by Silva & Brandão, 2010). *M. arthuri* workers have been observed in the field preying on soldiers and minor workers of *Atta sexdens* Linnaeus, 1758, as well as on small beetles from different species (see image in Suguituru et al., 2015; GHP Castro personal communication), although this type of behavior is not usually reported for *Myrmelachista* species.

Our results clearly show that, unlike workers, the analysis of morphometric characters of males and especially of gynes enables the distinction of morphological groups corresponding to five of the named species in the Atlantic Forest sites studied (Tab. 1).

Our observation that gynes of *M. nodigera*, *M. catharinae* and *M.* sp.7 are morphologically different corroborates the molecular data from Nakano et al. (2014). In contrast, the observation of different morphological groups for workers and gynes of *M. ruszkii* differs from the results of the molecular analyses (Nakano et al., 2014). The morphometric analysis indicated high phenotypic variability of *M. ruszkii*, and of males of *M. arthuri*.

Longino (2006) stated that *Myrmelachista* species are more easily identified using reproductive ants, which is in accordance with our results. However, males and gynes of *Myrmelachista* are rarely recorded in studies of ant fauna, and the data available on *Myrmelachista* reproductive biology are still scarce (Nakano et al., 2012, 2013), hindering the taxonomic resolution of this genus. Our results also indicate that the eyes, petiole and ocelli are important characters for species distinction when reproductive ants are collected.

The overlapping of morphometric characters of *M. catharinae* and *M. arthuri* workers could lead to the classification of these species under the same species name, which would then represent a morphologically and biologically highly variable species. This may also be the case for *M. nodigera* and *M. gallicola*. In both cases, the morphometric and molecular analyses produce consistent results.

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