Genitalic variation and taxonomic discrimination in the semi-aquatic spider genus *Paratrechalea* (Araneae: Trechaleidae)

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Abstract.   For spiders, morphological differentiation within genitalic traits is the main diagnostic criterion of a species. Beside some well-described exceptions of genitalic polymorphism and crypticity, spider genitalic variation is seldom quantitatively analyzed. Using geometric morphometrics landmark analysis, we report clear evidence of quantitative interspecific divergence and intraspecific variation in the genital shape of three species of the genus *Paratrechalea* (*P. azul*, *P. ornata* and *P. galianoae*). The genitalic species recognition was very consistent with our quantitative data for both sexes. Interspecific variation suggested a character displacement pattern between two syntopic populations of *P. azul* and *P ornata*, and also a possible case of species crypticity in *P. ornata* that will involve splitting the Uruguayan populations from the Brazilian ones.

Keywords: Morphological evolution, geometric morphometrics, character displacement

The line between what distinguishes a true species from a simple case of intraspecific variation is not only subtle, but also dependent on the species definition assumed (Bond et al. 2001; Huber 2004; Mutanen 2005). Within this fuzzy zone lie a large number of separate biological entities that remain undetected because of their morphological similarities (Adams & Funk 1997). Therefore, morphological species delimitation may pose a problem for some specific groups with a high degree of resemblance.

Systematic practice is based strongly upon the correlation of morphology and the recognition of the limits of true species, and there is no exception for spiders (Huber 2003, 2004; Huber et al. 2005). Spiders are one of the most diverse metazoan groups, with 40,998 described species (Platnick 2009). The structures with the most diagnostic traits for spider species are the genitalia, which in general have a high degree of specificity associated with a faster evolutionary pace that may be guided mainly by mechanisms of sexual selection (Eberhard 1985; Arnqvist 1997; Hosken & Stockley 2004; Mutanen 2005; but see also Costa & Capocasale 1984; Pérez-Miles 1989; Jocqué 2002; Huber 2003; Huber et al. 2005).

Leaving processes aside, two opposing cases of genitalic phenotypic expression may pose a problem for the usual taxonomic species recognition: cryptic species and polymorphism. Both cases have been described for spiders (crypticity: Ramirez & Chi 2004; Johannesen et al. 2005; Huber et al. 2005; polymorphism: Pérez-Miles 1989; Huber & González 2001; Jocqué 2002), suggesting that neither may be an exception. Cryptic species complexes can be found in several organisms (Bond & Sierwald 2002; Muster et al. 2004) and they are often revealed by integration of molecular and/or behavioral tools, and not by the definition of morphological apomorphies (Adams & Funk 1997). On the other hand, genitalic polymorphism can be discovered by several methods,

the best evidence being the hatching of different morphs from a single egg-sac (Jocqué 2002).

During recent decades the establishment of geometric morphometrics as a new morphometric paradigm has made a significant contribution to the study of shape variation (Rohlf & Marcus 1993). For example, using geometric morphometrics, cryptic species complexes can be not only quantitatively described by their morphological properties, but also can allow the testing of differences in shape consensus for the observed groups. This approach has already been applied in some spider studies (Bond & Beamer 2006; Costa-Schmidt 2008; Crews 2009), and despite the limitations of using morphometric data as phylogenetic characters (Zelditch et al. 2004; Bond & Beamer 2006), geometric morphometric tools can improve the capacity of taxonomic discrimination.

Here we present quantitative analyses of genitalic variation within three species of the semi-aquatic spider genus *Paratrechalea* Carico 2005 (Araneae: Trechaleidae): *P. azul* Carico 2005, *P. ornata* (Mello-Leitão 1943) and *P. galianoae* Carico 2005. The taxonomic status of the first two species can be questioned, since they share strong morphological and ecological crypticity and considerable intraspecific genitalic variation (Carico 2005; Silva et al. 2006). This last feature means that the formal descriptions are difficult to apply to some populations of those species, and for this reason we include in our analysis two samples from Uruguay representing the intraspecific variation within *P. ornata*.

The general aim of this work is to improve our knowledge of the morphometric properties of shape and size for genital traits of these species based on quantitative analyses. The specific aim is to quantify the visual genitalic variation observed within each species, identifying the main differences among them, and to infer the degree of intraspecific variation observed among three *P. ornata* samples.

METHODS

Study species and sampling design.—Three *Paratrechalea* species were analyzed in this work: *P. azul* Carico 2005, *P. ornata* (Mello-Leitão, 1943), and *P. galianoae* Carico 2005.

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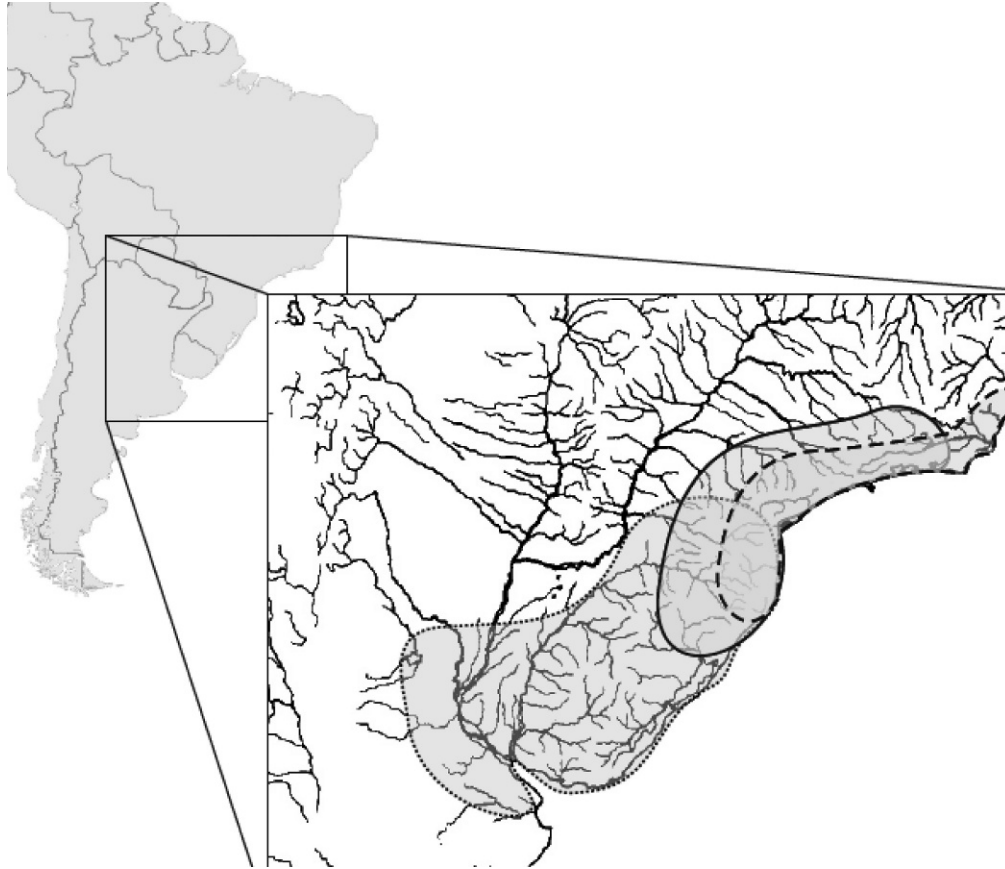
Figure 1.—Estimated geographic distribution of the study species based on the sampling data from Carico (2005) and Silva *et al.* (2006). Continuous line: *Paratrechalea azul*, dotted line: *P. ornate*, dashed line: *P. galianoae*.

Beside the cryptic aspect of somatic characters among these species, *P. azul* and *P. ornata* have a marked genital resemblance in both males and females. *P. galianoae* is easily recognized based on genital morphology, thus the insertion of this species within the data set will serve as a control group for the levels of divergence found between the two other species.

The three species have nocturnal semi-aquatic habits, and are easily found living next to streams and rivers. *P. azul* and *P. ornata* have a syntopic distribution in the northeastern region of Rio Grande do Sul (Brazil) (Fig. 1). The entire zone of overlap is not yet established. Sampling efforts did not find any isolated populations of *P. azul* in the Rio Grande do Sul State, even though there are large sampling gaps. We assume that they are sibling species, based on ecological requirements (Costa-Schmidt & Araújo, pers. observ.), on genitalic resemblance (Carico 2005; Silva et al. 2006) and by quantitative morphological analyses of non-genitalic characters (Costa-Schmidt 2008).

Within their known distributions (Fig. 1), *P. azul* and *P. ornata* can be found in second to higher order streams at altitudes ranging from sea level to 200 meters. *P. galianoae* seems to be strongly associated with dense riparian vegetation, typically being found along first and second order streams in an altitudinal level starting at ca 300 m. There is a clear checkerboard distribution between *P. ornata* and *P. galianoae* (Costa-Schmidt, unpub. data), and we assume that this distribution is a direct response of the habitat structure and altitudinal restrictions.

Comparisons were made of field-collected adult males and females from four different locations (Table 1, Fig. 1). We considered each sampled population as a single level of a classification factor. All specimens were preserved in 80% alcohol. Voucher specimens are deposited in the Museu de Ciências Naturais – Fundação Zoobotânica do Rio Grande do Sul (MCN-FZB/RS), and in the Facultad de Ciências (Montevideo – Uruguay).

Geometric morphometrics procedures.—Digital images were made by attaching a camera (Nikon Coolpix 5400) to the ocular of a stereomicroscope (Nikon SMZ600), using the same magnification for each structure. An ocular scale was subsequently used to make pixel/millimeter conversions. All images were arranged in a single folder, and tpsUtil v. 1.33 (Rohlf 2004) was used to create a file containing the sequence of pictures to be further analyzed.

We have chosen to analyze the genital structures used in species diagnoses. The generic diagnosis was presented by Carico (2005), emphasizing the ventral division of the median apophysis of the male pedipalpus and the external posterior-median scape of the female epigynum. Additionally, the same author and Silva et al. (2006) used the ectal division of the retrolateral tibial apophysis (Fig. 2A, RTA) as a component of the male diagnosis. Thus, based on this and also on Huber

Table 1.—Sampling location, city, country, geographic coordinates, and sample size of each studied population.

Sampling location	City, country	Coordinates	Sample size					
			P. azul		P. ornata		P. galianoaez	
			F	M	F	M	F	M
Pedra de Amolar River, Barra do Ouro	Maquiné, Brazil	29°32'20.52''S, 50°14'46.83''W	38	35	35	37	–	–
Santa Lucia River, Paso del Molino	Minas, Uruguay	34°16'40.10''S, 55°14'00.80''W	–	–	21	14	–	–
Yerbal Chico River, Quebrada de los Cuervos	Treintay y Tres, Uruguay	32°55''30.50''S, 54°27''33.10''W	–	–	20	19	–	–
Pedras Brancas River, Pedra Branca Fall	Itati, Brazil	29°23''45.59''S, 50°02'42.44''W	–	–	–	–	34	17

(1995), we assumed that the analyses of the ectal division of the RTA and the epigynal scape (Fig. 2B) present the most diagnostic information needed to delimit the species. Since males have two symmetrical palps (assumption made visually), we chose to analyze only the RTA of the left palp.

Only Type II landmarks (Slice et al. 1996); i.e., those defined by local properties such as maximum curvatures, were plotted in each image. The already created tpsUtil file was loaded with the tpsDig 2.04 software (Rohlf 2005) for plotting of landmarks, consisting the rough data. Landmarks descriptions for each genital structure are presented in Table 2.

Using a simple morphological formula, "form = shape + size", we first isolated these two morphological components (also addressed here as morphological properties). To isolate all landmark configurations, we applied a General Procrustes Superimposition analysis (GPS). Each landmark configuration was translated, rotated and scaled to the unit centroid size, using a least squares procedure. After this treatment, each configuration was described by a single point within a multidimensional space, called the pre-form space. Because of its multidimensionality and non-Euclidian properties, the pre-form residuals were projected into a plane that is tangent to the mean pre-form shape, where Euclidian properties are fulfilled and conventional statistical analyses were applied. After this projection, a Principal Component Analysis was made over the scores of this projection, composing the shape variables. More information about these methods can be found elsewhere (Rohlf & Slice 1990; Zelditch et al. 2004; Crews in press). These morphometric procedures and the further analyses and graphic outcomes were made using the RMorph library (Baylac 2007) developed for R environment (R Development Core Team 2007).

Size component was estimated by centroid size, which is defined as the square root of summed squared distances of landmarks from their centroid (Swiderski 2003). The advantage of this method is that it takes an overall size measurement of the form, while linear estimates of size underestimate other two-dimensional variables of the same structure.

Shape analysis.—Differences in genital structures were tested by a one-way MANOVA using species and populations as a descriptor factor composed by the following levels: *P. azul*, *P. galianoae*, *P. ornata* (MaqB) (*Maquine* population), *P. ornata* (PasU) (Paso del Molino population), and *P. ornata* (QueU) (Quebrada de los Cuervos population), where "B" means Brazil and "U" means Uruguay. Pairwise comparisons were performed between all possible combination levels using the same model in the case of significant analysis. The probability results of these pairwise tests were corrected with a Bonferroni correction for multiple comparisons. Discriminant analysis based on shape variables was applied in order to verify the percent of correct classifications achieved by the tested factor, which is a way to confirm whether the informed classification within the factor levels does represent variation among groups.
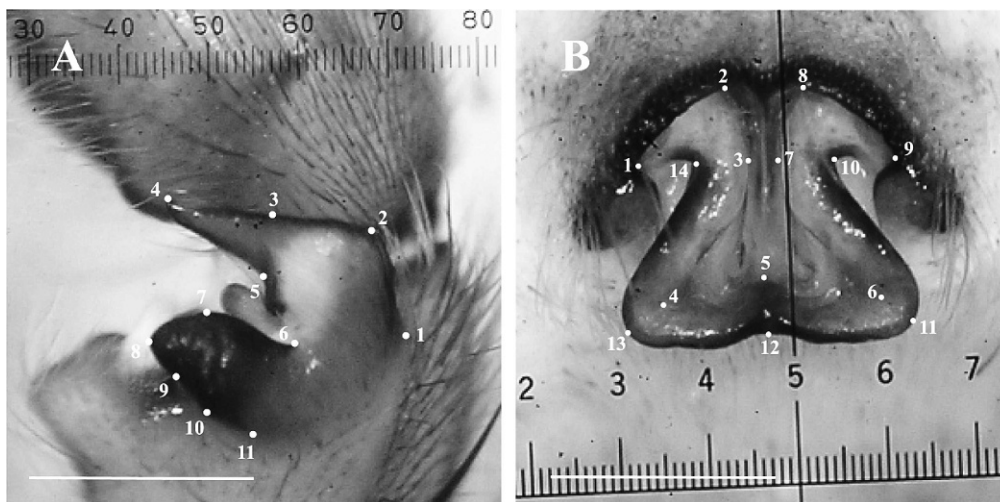

Figure 2.—Landmark positions within each analyzed structure. A. Ectal division of the retrolateral tibial apophysis (11 landmarks); B. Epigynum scape (14 landmarks). Photos from *Paratrechalea ornata* specimens. Scale = 1 mm.

Table 2.—Morphological landmarks used in this study for each genital structure analyzed.

RTA landmark	Description
1	Insertion point of the distal lobe into the palpal tibia
2	Maximum curvature of the distal lobe anterior margin
3	Mid-point of the distal lobe anterior margin
4	Tip of the distal lobe
5	Mid-point of the distal lobe posterior margin
6	Maximum curvature between the distal and basal lobes
7	Point of maximum inflection of the basal lobe anterior margin
8	Tip of the basal lobe
9–10	Equidistant inflexion points along the basal lobe posterior margin
11	Insertion point of the basal lobe into the palpal tibia

Epigynum landmark	Description
1–2–8–9	Connection points of the scape with the posterior margin of the anterior field
3–4–6–7	Maximum inflexion points on the margin of the inner fold
10–11–13–14	Points of maximum inflexion on the rim of the scape
12	Midline point on the posterior rim of the scape
5	Midline point on the margin of the inner fold

Because we could describe an average shape for each considered level using shape variables only, we were able to estimate the Mahalanobis distance as a dissimilarity measure among the average shapes under analysis. This estimate is directly related to morphological divergence; i.e., the higher this estimated value, the bigger the morphological divergence. Permutation tests among individuals of each pairwise group comparison were done in order to verify whether the estimated distances between those groups could not be achieved by random sampling.

RESULTS

The analyses suggest that shape is the most informative morphological property in assigning groups. Size showed different patterns within each genital structure, demanding different interpretations of their size variation. The overall findings are summarized below. First, analyses of both structures resulted in similar groupings of species and populations. Second, *P. galianoae* was the most divergent in all analyses, thus corroborating the assumption of its use as an outgroup for analysis of the *P. azul* and *P. ornata* relationship. Third, there is an expected shape difference among species for both genital structures, with *P. azul* and *P. ornata* populations being closer within the shape space. Fourth, all shape analyses indicate that two populations, initially presumed to be *P. ornata*, can be interpreted as being a new species.

Shape analysis.—Genital structures showed strong differentiation among groups for all analyses. Principal Component Analysis (PCA) indicated the presence of at least three groups based on the first two components (PC1 and PC2, Figs. 3, 4), corresponding to the three studied species. The addition of the third PC allows us to distinguish another group, composed of the Uruguayan populations of *P. ornata* (Fig. 5).

Discriminant analysis confirms that the assumed classification explains the observed shape variation for both RTA and epigynum shape variables (Table 3); i.e., genital structures are good predictors of species classification. This analysis also suggests that the group formed by the Uruguayan populations is consistent with a few misclassifications, including a single female specimen from Paso del Molino that was initially placed as being from Maquine.

MANOVA models were highly significant for both genital structures (Table 4). All pairwise comparisons showed the same highly significant levels; i.e., the samples have different shapes of RTA and epigynum (Table 4). Again, we observed a splitting behavior between Uruguayan *P. ornata* populations from the Brazilian population. The comparison between the Paso del Molino and Quebrada de los Cuervos samples was also significant for both structures, but with contrasting lower significant levels in relation to other comparisons (Table 4, last row).
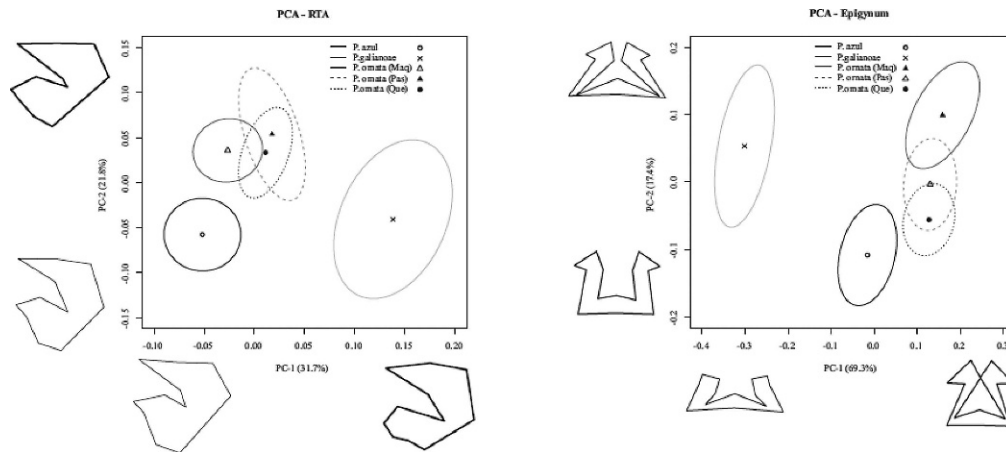

Figure 3.—Projections of the first and second scores of the Principal Component Analysis for RTA (left) and epigynum scape (right). Symbols represent the consensus shape of each species/population, with delimiting 95% confidence ellipses. Illustrations within the axes represent the shape variation along each axis.
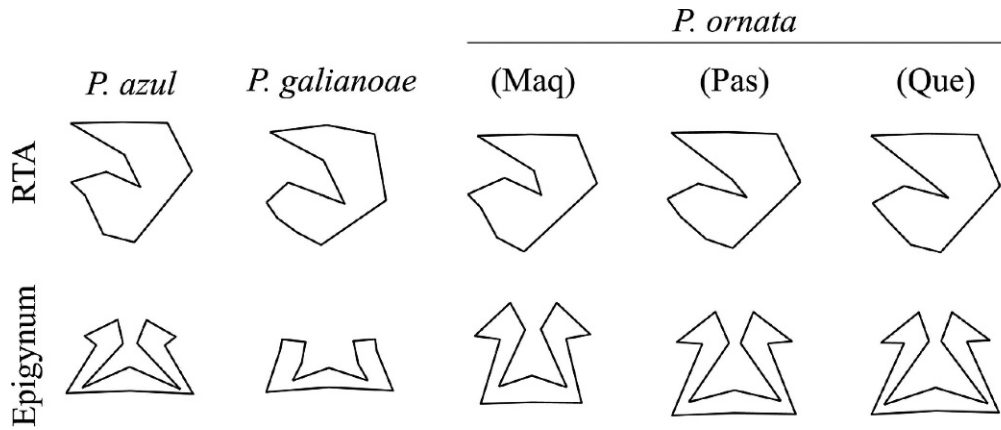
Figure 4.—Shape consensus observed for each species/population within the first component of a Principal Component Analysis.

The Mahalanobis distance between the samples' mean shapes also confirms the same diverging pattern of *P. ornata* between the Brazilian and the Uruguayan populations (Table 5). It is important to emphasize that the observed Mahalanobis distance for RTA between the Maquine and Uruguayan populations was even higher than that between *P. ornata* and *P. azul*. This was not observed for the epigynum, however, which showed lower Mahalanobis distances when comparing the Maquine population with the Uruguayan ones (Table 5). Permutation tests indicate that the estimated distances were significant for all comparisons and for both structures ($P \leq 0.0003$ for *P. ornata* (PasU) and *P. ornata* (QueU) comparisons; $P \approx 0$ for all other comparisons).

DISCUSSION

Here we report the application of a robust morphometric method to discriminate among a small group of samples representing three putatively related spider species. The outcome of such analyses ranged from the determination of species using their genital shape to the recognition of a splitting within what was once considered a single species. This latter conclusion has important and deep roots in the standard methodology used in spider systematics. Much of spider systematics has been based upon the assumption that genital

specificity (Huber 2003, 2004) is directly linked to the widespread pattern of a faster rate of genital evolution (Eberhard 1985; Hosken & Stockley 2004). So, for genital traits, we are facing the challenging task of distinguishing between interspecific shape divergence and intraspecific shape variation.

The application of geometric morphometrics proved to be highly important for the discrimination of cryptic spider species within two different taxonomic situations. The first one deals with two already-described taxonomic entities that have a strong niche overlap (*P. azul* and *P. ornata*), but with known behavioral reproductive isolation and non-genital morphometric differences (Costa-Schmidt 2008; Costa-Schmidt et al. 2008). The second case deals with our findings that within *P. ornata*, two cryptic entities exhibit clear spatial niche segregation (geographical allopatry).

Evolutionary explanations for the observed pattern are suggested below, based on available information about the species and populations, which opens the subject for further studies and/or interpretations. On the other hand, the observed split of *P. ornata* demands a taxonomic description based on consistent characters identified in each group.

Genital variation between *P. azul* and *P. ornata*.—This work fulfills an important aspect related to the morphological
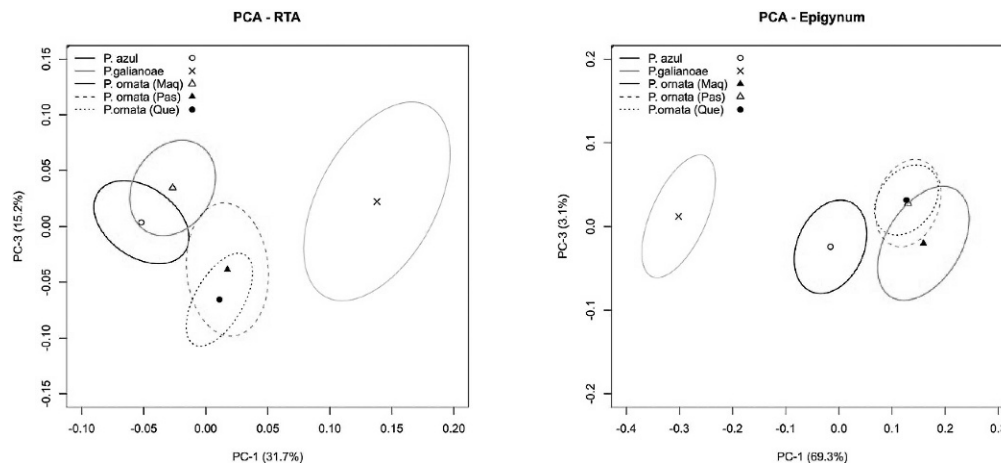

Figure 5.—Projections of the first and third scores of the Principal Component Analysis for RTA and epigynum scape. Symbols represent the consensus shape of each species/population, with delimiting 95% confidence ellipses.

Table 3.—Correct classification percentages achieved by discriminant analyses.

Species	RTA	Epigynum
P. azul	100%	100%
P. galianoae	100%	100%
P. ornata (Maq)	100%	100%
P. ornata (Pas)	85.7%	71.4%
P. ornata (Que)	100%	100%

variation of genital structures and the recognition of distinct species within the genus *Paratrechalea*. Since the beginning of our efforts in studying *P. azul* and *P. ornata*, we have been able to gather fundamental information supporting their taxonomic status as two different species (non-genital morphometry: Costa-Schmidt 2008; reproductive behavior: Costa-Schmidt et al. 2008), and this was also corroborated here by information gained from genitalic analysis.

The previously described genital variation within these two species shows quantitatively that the variation does not overlap along their shape space, mainly for the syntopic interspecific samples from Maquine. We believe that such divergence may be due to a reinforcement mechanism, such as character displacement (Brown & Wilson 1956), avoiding possible hybrid formation between the syntopic populations. The hypothesis of character displacement was also raised in the analysis of non-genital structures, especially for those populations sampled along the region of syntopy (Costa-Schmidt 2008).

Genital morphology species-specificity.—Another important aspect is the almost impossible discovery of genital polytypism within a species (Huber 2003), mainly if those morphs are dispersed along a geographical continuum where they can be easily misinterpreted as different species. In other words, genitalic differences found along the spatial distribution of a single species may lead to taxonomic inflation because of systematic methodological bias. Moreover, we cannot deny the huge influence that Mayr's non-dimensional definition has

Table 5.—Estimated Mahalanobis distances between the average shapes of the compared groups. Above diagonal: species/population comparisons for epigynum scape. Below diagonal: species/population comparisons for RTA.

	azul	gal	orn (Maq)	orn (Pas)	orn (Que)
azul	–	273.80	65.98	37.32	30.05
gal	207.19	–	374.06	357.83	367.72
orn (Maq)	56.37	160.14	–	16.34	23.92
orn (Pas)	147.95	124.33	68.58	–	5.16
orn (Que)	140.98	140.28	69.92	10.01	–

on taxonomic practice (Mayr 1963; Huber 2003), which is the basis of species-specific genitalic morphology.

As we found sharp limits for the shape component of male and female genitalia, we must try to translate those differences into something that can be used by a taxonomist when analyzing a particular sample; i.e., we must apply some effort in order to describe which shape trait is shared in each group (Zelditch et al. 2004). Such information was necessarily proposed elsewhere (Carico 2005; Silva et al. 2006), consisting of formal descriptions of diagnostic characters of each species, even though our data demonstrate that the diagnosis of *P. ornata* is not capable of distinguishing the new split species. This effort will be presented elsewhere, when additional diagnostic characters that better describe the species studied will be followed with the description of a new species.

CONCLUSIONS

In a broader sense, the observed interspecific divergence patterns were expected in relation to empirical and theoretical data of genital traits used in taxonomy. The number of shape differences among species was lower between *P. azul* and *P. ornata*, which may induce us to believe that they are sister species, even though this assumption will only be resolved after a robust cladistic analysis. Intraspecific analysis showed an interesting divergent pattern, suggesting that we have two separate taxonomic entities within the *P. ornata* dataset.

Table 4.—MANOVA of shape principal components for species/population (the first 18 and 24 PCS were selected for RTA and epigynum scape respectively), and pairwise comparisons for all levels combinations.

	RTA[a]			Epigynum[b]		
	Wilks	F	P	Wilks	F	P
Samples (df = 4)	0.0003	38.804	$< 2.2 \, e^{-16}$	0.0007	26.501	$< 2.2 \, e^{-16}$
Residuals (df = 117)						
Pairwise comparisons						
P. azul vs. P. galianoae	0.0484	109.1597	$2.3 \, e^{-57}$	0.0463	103.0024	$1.3 \, e^{-68}$
P. azul vs. P. ornata (MaqB)	0.0828	61.5561	$7.7 \, e^{-46}$	0.1267	34.4585	$8.7 \, e^{-43}$
P. azul vs. P. ornata (PasU)	0.0598	87.3079	$8.2 \, e^{-53}$	0.2015	19.8172	$4.0 \, e^{-31}$
P. azul vs. P. ornata (QueU)	0.0564	92.9630	$4.4 \, e^{-54}$	0.2411	15.7367	$1.1 \, e^{-26}$
P. galianoae vs. P. ornata (MaqB)	0.0590	88.5747	$4.2 \, e^{-53}$	0.0217	225.5691	$3.0 \, e^{-88}$
P. galianoae vs. P. ornata (PasU)	0.1092	45.3268	$6.2 \, e^{-40}$	0.0299	162.4213	$5.9 \, e^{-80}$
P. galianoae vs. P. ornata (QueU)	0.0851	59.7632	$2.9 \, e^{-45}$	0.0300	161.9082	$7.2 \, e^{-80}$
P. ornata (MaqB) vs. P. ornata (PasU)	0.1438	33.0732	$4.4 \, e^{-34}$	0.4001	7.4973	$1.4 \, e^{-14}$
P. ornata (MaqB) vs. P. ornata (QueU)	0.1176	41.6779	$2.4 \, e^{-38}$	0.3196	10.6436	$7.7 \, e^{-20}$
P. ornata (PasU) vs. P. ornata (QueU)	0.5919	3.8304	$7.7 \, e^{-6}$	0.7302	1.8473	0.01648529

[a] Samples: num df = 72, den df = 395.6; Pairwise comparisons: df1 = 18, df2 = 100
[b] Samples: num df = 96, den df = 477.9; Pairwise comparisons: df1 = 24, df2 = 120

It is still early to state an evolutionary explanation in this system, since fundamental reproductive aspects (like the presence of polyandry) remain to be answered. Subsequent approaches must be applied in order to evaluate better the hypothesis raised. For example, careful sampling design associated with the analytical procedures presented here would allow us to understand whether character displacement did influence the evolutionary history of these spider species.

ACKNOWLEDGMENTS

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