An integrative approach for species delimitation in the spider genus *Grammostola* (Theraphosidae, Mygalomorphae)

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The mygalomorph genus *Grammostola* (family Theraphosidae) is endemic to South America. The species *Grammostola anthracina* is one of the largest spiders in Uruguay and reputed to be the longest lived tarantula in the world. This nominal species has two distinct colour morphs comprising black and reddish-brown forms with controversial taxonomic status. Here, we present a phylogenetic study based on molecular characters (cytochrome *c* oxidase subunit I) of haplotypes of *G. anthracina* and closely related species. Our analysis together with new morphological data and biogeographical information indicates that the two morphs of *G. anthracina* constitute different species that are not sister to each other. Consequently, a new species, *Grammostola quirogai* is described, diagnosed and illustrated to encompass the black morph. Phylogenetic relationships and new taxonomic characters for *Grammostola* species included in this study are discussed.

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Introduction

Although many species concepts (e.g. Coyne & Orr 2004) exist, a general consensus that considers species as differently evolving metapopulation lineages (de Queiroz 1998, 2005, 2007) has developed. Typically referred to as the Lineage Species concept, it treats other species concepts as operational criteria used to identify and delimit species level lineages (Wiens & Penkrot 2002; Sites & Marshal 2003; de Queiroz 2005, 2007). An integrative approach to taxonomy, based on multiple lines of evidence, is thought to be necessary due to the complexity inherent to species delimitation (Dayrat 2005; Agnarsson & Kuntner 2007; Padial *et al.* 2010; Schlick-Steiner *et al.* 2010), which is at least in part due to the variable as well as non-stationary nature of the species (see Hey *et al.* 2003).

In spiders, as in other groups, morphological characters have been historically used to delimit species boundaries (Bertani 2001), but in several cases relying exclusively on morphology has proven to be insufficient. As such, molecular data are sometimes used in spider taxonomy (Starret & Hedin 2007; Bond & Stockman 2008; Aguilera *et al.* 2009; Hendrixon *et al.* 2013; Hamilton *et al.* 2014) to delimit species in particularly difficult groups like mygalomorph spiders. Morphologic characters used in taxonomy of the family Theraphosidae mostly come from the genitalia (e.g. Pérez-Miles *et al.* 1996; Bertani 2000); however, in the theraphosid genus *Grammostola* Simon, 1892; genitalia are extremely homogenous across species, making species delimitation based on these characters a difficult, if not impossible, task (Bücherl 1957; Schiapelli & Gerschman 1979).

Grammostola is endemic to South America and comprises 20 nominal species (World Spider Catalog 2015). Taxonomic studies of this genus are scarce and most were published decades ago (e.g. Bücherl 1951; Schiapelli &

Gerschman 1979; but see Ferretti *et al.* 2011, 2013). Although seven species of the genus have been cited for Uruguay (World Spider Catalog 2015), Pérez-Miles (1985) has disregarded the presence in Uruguay of *G. grossa* (Ausserer 1871) and *G. actaeon* (Pocock 1903). In addition, *G. alticeps* is only known from its type material referred as from "Loc. Uruguay; without further history" (Pocock 1903). Therefore, only four species are well documented in Uruguay: *G. andreleetzi* Vol 2008, *Grammostola anthracina* (Koch 1842), *Grammostola burzaquensis* Ibarra 1946 and *Grammostola iheringi* (Keyserling 1891) (Montes de Oca & Pérez-Miles 2009). *Grammostola anthracina* is the only one that has a wide distribution in the country. Unfortunately, this species is threatened by habitat fragmentation and illegal traffic to European countries associated with the pet trade (Costa & Pérez-Miles 2007). As other theraphosids, this species is large sized, has low vagility and is long-lived, with a lifespan of more than 30 years (Criscuolo *et al.* 2010). Females and juveniles are burrowers, while the males are the more vagile sex, dispersing in search of females during the mating season. *Grammostola anthracina* has two known allopatric colour morphs: black populations in the north of Uruguay and brown-reddish in the south of the country, both apparently separated by the Negro river; however, Postiglioni & Costa (2006) suggested that the isolation of both morphs is due to ecological factors. Previous studies have shown behavioural differences between these colour morphs in copulatory duration and insertion patterns (Costa & Pérez-Miles 2002; Postiglioni & Costa 2006). Although both morphs do not occur in sympatry, when individuals of each morph are experimentally place together, they can copulate. Moreover, they can mate with *G. iheringi*, another species that occurs in a restricted area of Uruguay. Notwithstanding, differences in distribution, sexual behaviour and colour between the two morphs suggest that they may be different species. First, to test the hypothesis that *G. anthracina* as currently understood (hereafter *G. anthracina* s.l.) comprises more than one species, we first conducted an exhaustive analysis of the distribution of each of the morphs to investigate for patterns or correlations with spatial variables. Second, we performed a molecular analysis based on the cytochrome oxidase I (COI) gene to determine the degree of genetic and genealogical differentiation of both morphs; this gene has been successfully used in spiders to elucidate the status of cryptic species (e.g. Starret & Hedin 2007; Bond & Stockman 2008; Aguilera *et al.* 2009; Hamilton *et al.* 2014). We used the genealogy herein as a guide to delimit and identify candidate species. Finally, in the light of the genealogical results, we use a process of reciprocal illumination to search for diagnostic characters by re-examining the external morphology of both morphs. As such, this contribution provides a first attempt to evaluate the taxonomy of *Grammostola* using an integrative approach that employs molecular and morphologic data; based on the gathered results, we formally describe the species *Grammostola quirogai* n. sp.

Materials and methods

Morph geographic distribution

We studied specimens housed in the Entomology Collection of the Facultad de Ciencias, Universidad de la República, Uruguay, and specimens kept alive in the Ethology, Ecology and Evolution Laboratory, of the Instituto de Investigaciones Biológicas Clemente Estable, Uruguay. We also performed fieldwork; we explored 26 hilly-rocky sites in Uruguay during the years 2011–2013 and collected 65 specimens (22 and 43 brown-reddish and black individuals, respectively) at 17 sites (Table 1). All specimens were deposited in the Entomology Collection of Facultad de Ciencias (FCE-MY), Universidad de la República, preserved in ethanol. Collection localities for each individual were georeferenced, to generate a spatial distributional map with the software DIVA-GIS (Hijmans *et al.* 2005) including an ecoregion layer for Uruguay (Panario 1988). To test an association between distribution and ecoregions, a logistic regression was carried out using the package PAST (Paleontological Statistics version 2.05, Hammer *et al.* 2001).

DNA-based analyses

Sampling design. All specimens collected by us (Table 1) were sequenced. We also sequenced one specimen of *G. andreleetzi* and *G. burzaquensis* from Uruguay, six *Grammostola pulchra* de Mello-Leitão 1921 from Brazil, and five *Grammostola rosea* (Walckenaer 1837) from Chile. All specimens, preserved in ethanol, were deposited in the Entomology Collection of Facultad de Ciencias (FCE-MY), Universidad de la República. The ingroup was completed with available GenBank haplotypes of specimens belonging to the subfamilies Theraphosinae (*Aphonopelma seemanni* (Pickard-Cambridge 1897): JN0181241; *Eupalaestrus weijemberghi* (Thorell 1894): JQ412446) and Selenocosmiinae (*Chilobrachys huahini* Schmidt & Huber 1996: JN018125; *Coremiocnemis cunicularia* (Simon 1892): JN018198). Trees were rooted with haplotypes of specimens of the subfamily Ornithoctoninae: *Cyriopagopus schioedtei* (Thorell 1891) (JN0181126) and *Haplopelma schmidti* von Wirth 1991 (JN018127 and AY309259). As such, the analysed matrix comprises a total of 86 sequences.

DNA isolation, PCR and sequencing. Muscle tissue was extracted from the right leg IV of each spider, removing 25 mg of tissue and stored in 100% ethanol at −80 °C. Total genomic DNA was isolated using the DNeasyTissue

Table 1 List of collection localities of *G. anthracina* s.l. (see also Fig. 3). The ID represents the abbreviation for each locality

# Locality	Locality	ID	Latitude	Longitude	Morph	FCE-MY	Accession Nos.
1	Masoller, Artigas	Mas001	−31.092	−56.016	Black	0947	KT965200
1	Masoller, Artigas	Mas002	−31.092	−56.016	Black	0948	KT965275
1	Masoller, Artigas	Mas007	−31.092	−56.016	Black	0953	KT965201
2	Sepulturas, Artigas	Sep094	−30.830	−56.054	Black	1246	KT965229
2	Sepulturas, Artigas	Sep095	−30.830	−56.054	Black	1242	KT965234
2	Sepulturas, Artigas	Sep096	−30.830	−56.054	Black	1245	KT965233
2	Sepulturas, Artigas	Sep097	−30.830	−56.054	Black	1244	KT965230
2	Sepulturas, Artigas	Sep098	−30.830	−56.054	Black	1243	KT965231
3	Arroyo Catalán, Artigas	Art064	−30.816	−56.350	Black	1204	KT965235
4	Baygorria, Río Negro	Bay035	−32.864	−56.827	Black	0935	KT965254
4	Baygorria, Río Negro	Bay036	−32.864	−56.827	Black	0936	KT965198
4	Baygorria, Río Negro	Bay037	−32.864	−56.827	Black	0937	KT965253
4	Baygorria, Río Negro	Bay038	−32.864	−56.827	Black	0938	KT965274
4	Baygorria, Río Negro	Bay040	−32.864	−56.827	Black	0940	KT965269
5	Cuchilla de Navarro, Río Negro	RNe061	−32.666	−57.026	Black	1235	KT965270
5	Cuchilla de Navarro, Río Negro	RNe062	−32.666	−57.026	Black	1203	KT965271
6	Cuchilla Negra, Rivera	CNe076	−31.074	−55.976	Black	1227	KT965266
6	Cuchilla Negra, Rivera	CNe081	−31.074	−55.976	Black	1230	KT965276
6	Cuchilla Negra, Rivera	CNe075	−30.951	−55.626	Black	1008	KT965242
6	Cuchilla Negra, Rivera	CNe077	−30.951	−55.626	Black	1231	KT965244
6	Cuchilla Negra, Rivera	CNe078	−30.951	−55.626	Black	1229	KT965240
6	Cuchilla Negra, Rivera	CNe079	−30.951	−55.626	Black	1228	KT965243
6	Cuchilla Negra, Rivera	CNe080	−30.951	−55.626	Black	1226	KT965241
7	Arerunguá, Salto	Are058	−31.462	−56.714	Black	1212	KT965251
7	Arerunguá, Salto	Are093	−31.462	−56.714	Black	1199	KT965272
8	Daymán, Salto	Day051	−31.401	−57.691	Black	1215	KT965256
8	Daymán, Salto	Day052	−31.401	−57.691	Black	1232	KT965205
8	Daymán, Salto	Day053	−31.401	−57.691	Black	1201	KT965221
9	Achar, Tacuarembó	Ach011	−32.399	−56.116	Black	0912	KT965239
9	Achar, Tacuarembó	Ach012	−32.399	−56.116	Black	0915	KT965212
9	Achar, Tacuarembó	Ach013	−32.399	−56.116	Black	0910	KT965264
9	Achar, Tacuarembó	Ach014	−32.399	−56.116	Black	0914	KT965265
9	Achar, Tacuarembó	Ach015	−32.399	−56.116	Black	0920	KT965273
9	Achar, Tacuarembó	Ach016	−32.399	−56.116	Black	0913	KT965203
9	Achar, Tacuarembó	Ach017	−32.399	−56.116	Black	0955	KT965268
10	Valle Edén, Tacuarembó	VaE022	−31.817	−56.167	Black	0944	KT965214
10	Valle Edén, Tacuarembó	VaE023	−31.817	−56.167	Black	0945	KT965252
10	Valle Edén, Tacuarembó	VaE024	−31.817	−56.167	Black	0946	KT965216
10	Valle Edén, Tacuarembó	VaE025	−31.817	−56.167	Black	0925	KT965215
10	Valle Edén, Tacuarembó	VaE026	−31.817	−56.167	Black	0924	KT965213
11	Arerunguá, Tacuarembó	Are054	−31.644	−56.306	Black	1205	KT965204
11	Arerunguá, Tacuarembó	Are055	−31.644	−56.306	Black	1200	KT965199
11	Arerunguá, Tacuarembó	Are057	−31.644	−56.306	Black	1377	KT965217
12	Isla Patrulla, Treinta y Tres	TyT084	−33.059	−54.543	Brown-reddish	1202	KT965223
12	Isla Patrulla, Treinta y Tres	TyT085	−33.059	−54.543	Brown-reddish	1218	KT965224
12	Isla Patrulla, Treinta y Tres	TyT086	−33.059	−54.543	Brown-reddish	1208	KT965225
12	Isla Patrulla, Treinta y Tres	TyT087	−33.059	−54.543	Brown-reddish	1213	KT965227
12	Isla Patrulla, Treinta y Tres	TyT088	−33.059	−54.543	Brown-reddish	1234	KT965228
13	Cerros de San Juan, Colonia	CSJ041	−34.187	−57.927	Brown-reddish	0956	KT965247
13	Cerros de San Juan, Colonia	CSJ042	−34.187	−57.927	Brown-reddish	0922	KT965202
13	Cerros de San Juan, Colonia	CSJ043	−34.187	−57.927	Brown-reddish	0923	KT965250
13	Cerros de San Juan, Colonia	CSJ044	−34.187	−57.927	Brown-reddish	0921	KT965245
13	Cerros de San Juan, Colonia	CSJ046	−34.187	−57.927	Brown-reddish	0918	KT965238
13	Cerros de San Juan, Colonia	CSJ047	−34.187	−57.927	Brown-reddish	0917	KT965255
14	Nueva Palmira, Colonia	NPa074	−33.850	−58.412	Brown-reddish	1240	KT965246
15	Grutas del Palacio, Flores	Flo082	−33.849	−56.970	Brown-reddish	1219	KT965232
15	Grutas del Palacio, Flores	Flo083	−33.849	−56.970	Brown-reddish	1223	KT965248

Table 1 Continued

# Locality	Locality	ID	Latitude	Longitude	Morph	FCE-MY	Accession Nos.
16	Cuchilla de Polanco, Lavalleja	Lav089	−34.024	−55.305	Brown-reddish	1198	KT965222
16	Cuchilla de Polanco, Lavalleja	Lav090	−34.024	−55.305	Brown-reddish	1197	KT965267
16	Cuchilla de Polanco, Lavalleja	Lav091	−34.024	−55.305	Brown-reddish	1239	KT965226
16	Cuchilla de Polanco, Lavalleja	Lav092	−34.024	−55.305	Brown-reddish	1237	KT965219
17	Pan de Azúcar, Maldonado	PAz029	−34.645	−55.247	Brown-reddish	0931	KT965236
17	Pan de Azúcar, Maldonado	PAz030	−34.645	−55.247	Brown-reddish	0932	KT965237
17	Pan de Azúcar, Maldonado	PAz032	−34.645	−55.247	Brown-reddish	0933	KT965258
17	Pan de Azúcar, Maldonado	PAz034	−34.645	−55.247	Brown-reddish	0934	KT965263

Kit (Qiagen) and stored at −20 °C prior to amplification. Concentration quality of extracted DNA was quantified with a spectrophotometer (Nanodrop 1000). COI was amplified using the primers designed by Folmer *et al.* (1994): LCOI 1490 (5′-GGTCAACAAATCATAAAGA TATTGG-3′) and HCOI 2198 (5′-TAAACTTCAGGGT GACCAAAAAATCA-3′) following the PCR conditions from Petersen *et al.* (2007). PCR products were purified and sequenced by Macrogen Inc. (Korea). All sequences were manually edited using the program PROSEQ v.3 (Filatov 2002) and have been deposited in GenBank (Accession numbers: KT965198–KT965276).

Data analyses. Sequences were aligned with CLUSTAL X v.2 using the default parameters (Thompson *et al.* 1997). Uncorrected genetic distances (uncorrected p-distance) were calculated between pair of haplotypes, population and clades using MEGA v.5.0.5 (Tamura *et al.* 2011). An AMOVA was performed in ARLEQUIN v. 3.5 (Excoffier & Lischer 2010) to quantify the geographic structure of the genetic variation within and between morphs of *G. anthracina* s.l. Haplotypes were grouped by morph colour and locality. We also calculated standard indexes of molecular diversity, including the nucleotide diversity $\prod$ (Nei 1978), also with ARLEQUIN v. 3.5.

Phylogenetic analyses were performed using maximum parsimony (MP), maximum-likelihood (ML) and Bayesian inferences (BI). We used the program TNT v. 1.1 (Goloboff *et al.* 2003) to generate MP trees, through heuristic search, from 1000 replicates, TBR branch swapping algorithm and retaining five trees per replicate. Clade support was evaluated with Jackknife (Farris *et al.* 1996). Model-based analyses were conducted with the GTR + I + G model of DNA substitution, which was chosen via Bayesian information (BIC) and Akaike (AIC) criteria with the program MODELTEST v.2.1 (Posada & Crandall 1998). The ML tree was inferred using the program PHYML (Guidon & Gascuel 2003) implemented in Phylogeny.fr (Dereeper *et al.* 2008). Clade support was calculated with 500 Bootstrap replicates (Felsenstein 1985). Bayesian analysis was carried out with BEAST v.1.8.0 (Drummond *et al.* 2012). Tree search consisted of three independents runs of 60 000 000 generations sampled every 10 000. The program TRACER v.1.5 (Rambaut & Drummond 2007) was used to explore convergence of the runs. After reaching convergence, the first 15% of the samples were discarded as burn-in. Post-burn-in annotated trees of the three runs were combined with LOGCOMBINER v1.8.0. The maximum clade credibility tree was reconstructed with the program TREEANNOTATOR v.1.8.0 included in the BEAST package (Drummond *et al.* 2012). As another way of exploring relationships among haplotypes of *G. anthracina* s.l., we constructed a haplotype network though MJ algorithm (Bandelt *et al.* 1999) using NETWORK (fluxus-engineering.com).

Morphological characters and species description

After a global morphological assessment, we focused our attention on the morphology of the male tibial apophysis (leg I), which have proven to be useful to diagnose some species of the genus (Ferretti *et al.* 2011). We examined, using an Olympus SZH stereomicroscope, six specimens of the black morph, six of the brown-reddish morph, two of *G. burzaquensis*, four of *G. iheringi*, two of *G. rosea*, and two of *G. pulchra*. Except for carapace and legs, which were measured with a dial calliper (0.05 mm, scale resolution), all measurements were taken with an ocular micrometre. Photographs were taken with a Lumenera Infinity Lite camera adapted to a stereomicroscope. Abbreviations: FCE-MY, Facultad de Ciencias collection, Montevideo; m, male; f, female; RB, retrolateral branch from tibial I apophysis; AME, anterior median eyes; ALE, anterior lateral eyes; PME, posterior median eyes; PLE, posterior lateral eyes; OQ, ocular quadrangular; P, proventral; V, Ventral; R, retrolateral; D, dorsal; PMS, posterior median spinnerets; PLS, posterior lateral spinnerets.

Results

Geographic distribution

The black morph is distributed in northern Uruguay, whereas the brown-reddish morph has a southern distribu-

tion, extending towards the north by the Uruguay River coast and along the Santa Ana Sierra (Fig. 1). Both morphs were recorded nearby at two areas. In the area of Queguay, Paysandú in north-western Uruguay, they were recorded to be 14 km from each other. The brown-reddish record is based on a specimen collected in 2010 (Route 3 and Queguay River); in spite of an intense effort in the field, we could not find other specimens of this morph in that area, but we did find a black individual in a locality (Route 26 near Araujo Stream) 14 km away. The second area where both morphs were found is the confluence of the Santa Ana Sierra and Negra Sierra. The black morph is on Negra Sierra, 7 km apart from the Puntas del Cuñapirú, Santa Ana Sierra locality, where a specimen of the brown-reddish morph was collected in 2001. This latter locality has been modified by human activity, and during our field work, we did not find any specimens. Association analyses indicated a significant relationship of both morphs with distinct Uruguayan ecoregions. The black morph associates with the Basaltic basin, whereas the brown-reddish morph is found in the Laguna Merin formation, Santa Lucía formation and Eastern Sierras ($\chi^2 = 49.37$, $P < 0.01$). Gondwana and West sedimentary basins shared both morphs (Fig. 1).

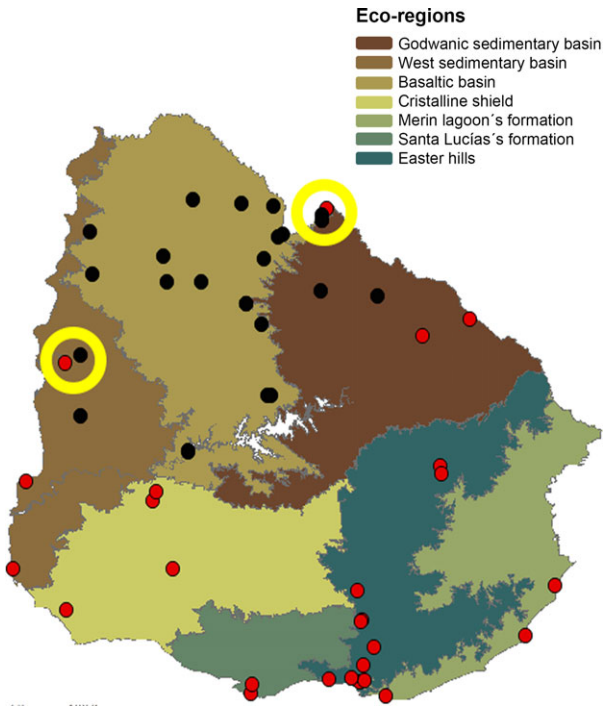

Fig. 1 Distribution in Uruguay of the morphs of *Grammostola anthracina*. Yellow circles show the areas where black and brown-reddish morphs become closer. Delimitation of ecoregions, according to the dominant type of rock, follows Panario (1988).

DNA-based results

Population and genetic divergence analyses. The analysed matrix consisted of 568 bp, with 225 variable sites, of which 181 were parsimoniously informative, and includes 50 distinct haplotypes. The sample of 65 sequences of *G. anthracina* s.l. shows a high haplotypic diversity (Hd = 0.979; 42 distinct haplotypes). Nucleotide diversity $\prod$ for the sample of *G. anthracina* s.l is 0.067, whereas the sample of the brown-reddish and black morphs is 0.053 and 0.014, respectively. Average genetic divergence between black and brown-reddish morphs is 10.3%. For interspecific comparisons, the minimum genetic distance is 10% for the pair brown-reddish *G. anthracina* and *G. burzaquensis*, and the maximum value, 14.6%, corresponding to the comparison of *G. pulchra* (Brazil) and *G. andreleetzi* (Uruguay).

From the 42 haplotypes of *G. anthracina* s.l., 28 were recovered from specimens of the black morph and 14 from individuals of the brown-reddish morph. An AMOVA indicated that 56.23% of the total variation observed in *G. anthracina* s.l. is due to differences between morphs ($F_{st} = 0.86$, $P < 0.001$). Both morphs show considerable geographic structure; in most cases, each locality studied was represented by a unique haplotype not shared with other populations. Intrapopulation genetic differentiation was lower in the brown-reddish morph (0.33%) than in the black morph (2.3%). Pairwise distances between localities were low when comparing localities of the same morph (brown-reddish: 0.017 ± 0.009; black: 0.054 ± 0.2).

Phylogenetic analysis. The topologies derived from the three phylogenetic analyses (MP: consensus of 64 shortest trees of length: 1718, CI: 32, RI: 79; log-likelihood: −3605.08) are highly congruent; one important discrepancy is noted below. Therefore, only the Bayesian-derived topology is shown (Fig. 2). *Grammostola* was recovered as monophyletic (JK < 50, BML: 0.69, PP: 0.99), but *G. anthracina* s.l. was not recovered as monophyletic. Haplotypes of the black morph of *G. anthracina* form a clade (JK < 50, BML: 0.56, PP: 0.96) as well as those of the brown-reddish morph (JK: 99, BML: 0.59, PP: 0.82), which are most surprisingly not sister lineages. The black morph clade was sister (JK < 50, BML: 0.62, PP: 0.96) to *G. pulchra*. In the MP and BI analyses, the brown-reddish morph of *G. anthracina* was recovered as sister (JK < 50, PP: 0.82) to *G. burzaquensis*. Meanwhile, in the ML tree *G. burzaquensis* was recovered as sister (BML: 0.57) to the clade black morph of *G. anthracina* and *G. pulchra*. Within the black morph, clade haplotypes form three groups, which are not allopatric. Meanwhile, the brown-reddish shows geographic structure with haplotypes falling into two clades that are geographically segregated, one in the west (localities:

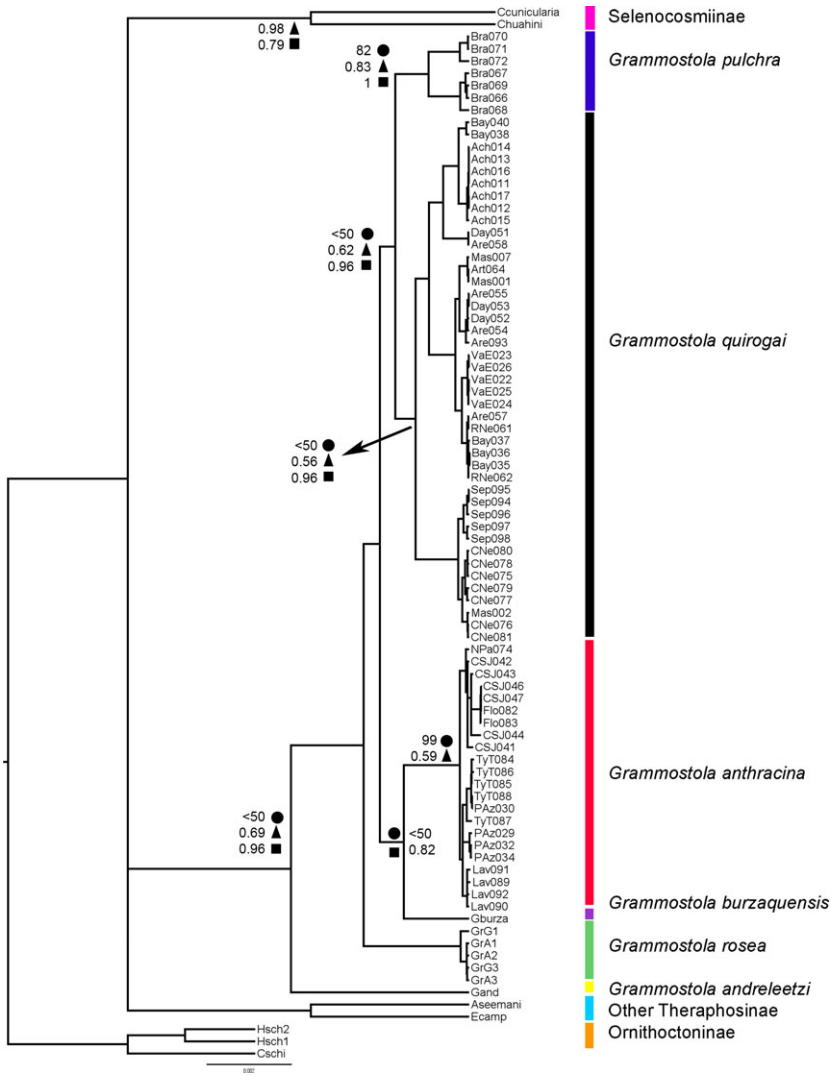

Fig. 2 Genealogical relationships among haplotypes of the mitochondrial COI gene of *Grammostola* as recovered in the Bayesian analysis. Support for selected nodes is indicated as follows: ● Jackknife ▲ Bootstrap ■ Posterior probability. Non-significant supported nodes are without data. Terminal labels correspond to localities as indicated in Table 1.

Nueva Palmira, Cerros de San Juan and Estancia el Timote) and another towards the east (localities: Pan de Azúcar, Isla Patrulla and Cuchilla de Navarro); the same structure is also seen in the haplotype network (Fig. 3).

Morphological characters

Male tibial apophyses of the studied species differ in the presence and distribution of spines and setae on the prolateral (PB) and retrolateral (RB) branches. The brown-reddish morph has on the PB and RB a group of subapical macrosetae, a mega-spine on the inner side of RB and an apical retrolateral spine on tibia I (Fig. 4A), whereas the black morph and the other studied species lack the group of subapical macrosetae and each have a small spine on the inner side of RB and 0–2 apical retrolateral spine on tibia I (Fig. 4B). In addition, *G. iheringi* has a spine on the inner side of PB and an apical retrolateral mega-spine on tibia I (Fig. 4C). Males of *G. burzaquensis* have a spine on the inner side of RB, and two apical retrolateral spines on tibia I (Fig. 4D). *Grammostola pulchra* has on the RB a subapical short spine and a spine in its inner side, a group of subapical macrosetae in PB, and a long apical retrolateral spine on tibia I (Fig. 4E). *Grammostola rosea* alternatively has a spine on the inner side of PB, and 1–2 long apical retrolateral spines on tibia I (Fig. 4F). All species have a short apical spine on RB (Fig. 4A–F).

Taxonomic description

The mitochondrial data show that both morphs of *G. anthracina* s.l. are genealogical distinct (i.e. each one is monophyletic) and that on average their haplotypes diverge by a value (10.3%) that falls within the range of values observed for all other species pairs of *Grammostola* (Table 2). In addition, the mitochondrial gene tree shows that both

morphs are not sister to each other. However, given that there are several known causes (including the fact that the mitochondrial genome is only inherited by females) for which a gene tree may depart from the species tree (Pamilo & Nei 1988), the analysis of nuclear genes is needed to test the obtained topology. Nonetheless, the phenome may be considered as a good proxy of the nuclear genome because most characters have genetic basis. Both morphs of *G. anthracina* s.l. not only differ from each other in coloration, but also in leg morphology (Fig. 4) and behaviour. As such, it is the integration of the genetic, phylogenetic and phenotypic results, including behavioural differences (Costa & Pérez-Miles 2002; Postiglioni & Costa 2006) which suggests that *G. anthracina* as currently understood is composed of two species; one corresponding to the black morph and other to the brown-reddish morph. Even when the black morph and the brown-reddish morph clades show internal structure, most of these subclades lack significant support and, more importantly, there are not morphologic differences among them. As such, we consider these internal clades represent intraspecific variation. Therefore, we consider further splitting of *G. anthracina* s.l. is not warranted at the light of current data. Nonetheless, we acknowledge that the direct assessment of the variation of nuclear loci represents a step needed to further test our taxonomic hypothesis.

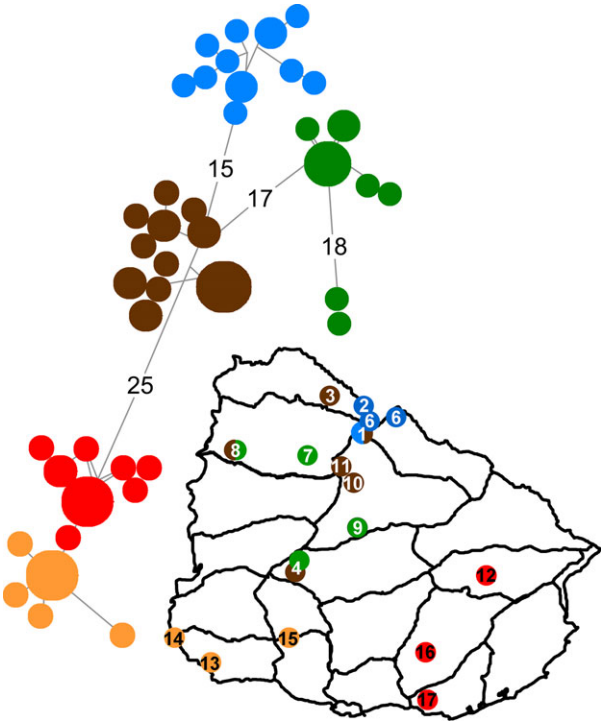

Fig. 3 Network of haplotypes of the COI gene of *Grammostola anthracina* s.s. (orange and red) and *G. quirogai* n. sp. (brown, green and blue). Locality numbers as those of Table 1.

The type locality of *G. anthracina* is in Montevideo, Uruguay; even though the original description does not consider the colour and tibial apophysis characters, we can infer that it belongs to the brown-reddish morph according to the distribution of this morph. As such, we restrict the name *G. anthracina* to the brown-reddish morph and describe the black morph as a new species below:

Genus *Grammostola* Simon, 1892

Grammostola quirogai n. sp. (Figs 4B, 5A–C, 6A,B)

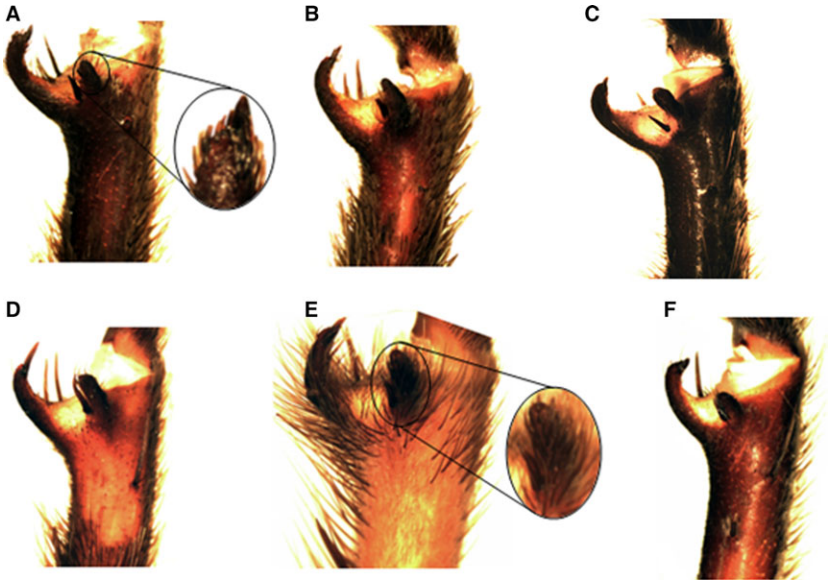

Fig. 4 Males right tibial apophysis on leg I. —A. *Grammostola anthracina* s.s. —B. *Grammostola quirogai* n. sp. —C. *Grammostola iheringi*. —D. *Grammostola burzaquensis*. —E. *Grammostola pulchra*. —F. *Grammostola rosea*.

Holotype. m, URUGUAY, Cuchilla del Daymán, Route 31 km 24, 31°24′02″ S, 57°41′29″W, 14 February 2013, F. Costa, F. Pérez-Miles & L. Montes de Oca (FCE-MY 1215).

Paratypes. Artigas, Subida de Pena (1m FCE-MY 0195, 1f FCE-MY 1249), Rivera, Camino a Portones Negros (1m FCE-MY 1261, 1f FCE-MY 1264), Salto, Route 31 km 24 (1f FCE-MY 1201), Tacuarembó, Pozo Hondo-Tambores (1m FCE-MY 0163, 2ff FCE-MY 0167), Tacuarembó, Route 43 km 18 (1m FCE-MY 0626).

Etymology. The specific epithet is a patronym dedicated to the memory of Horacio Quiroga, an Uruguayan poet, playwright and master of short story writing, born in Salto where the new species has been registered.

Diagnosis. This species can be distinguished from congeners by its dark coloration with some white-grey hairs. Male differs from those of other species by the spine combination of the tibial apophysis of leg I, having a small spine on the inner side of RB, and 0–2 apical retrolateral spine on tibia I.

Description

Male (holotype). Total body length, 39.24. Carapace length 19.02, width 18.14. Anterior eye row recurve, posterior procurve. Eyes sizes and interdistances: AME 0.25, ALE 0.5, PME 0.47, PLE 0.5, AME–AME 0.5, AME–ALE 0.375, PME–PME 0.95, PME–PLE 0.175, ALE–PLE 0.275, OQ length 1.325, width 2.175, clypeus 0.275. Fovea transverse, straight, width 2.98. Labium length 1.985, width 2.58 with 144 cuspules, maxillae with 248/252 cuspules in a

Table 2 Intra- and interspecific distances (p-distance) for species of the genus *Grammostola*

Genetic distances between groups	*G. quirogai* n. sp.	*G. anthracina*	*G. rosea*	*G. pulchra*	*G. burzaquensis*
G. anthracina	0.103	–	–	–	–
G. rosea	0.113	0.116	–	–	–
G. pulchra	0.102	0.107	0.112	–	–
G. burzaquensis	0.109	0.1	0.126	0.105	–
G. andreleetzi	0.137	0.146	0.129	0.15	0.135
Genetic distances within groups	0.0554	0.013	0.0129	0.0431	

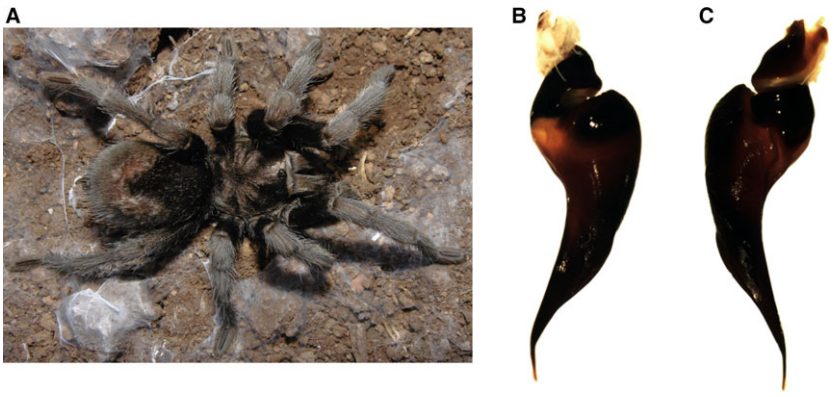

Fig. 5 *Grammostola quirogai* n. sp. —A. Male, habitus. —B. Left palpal bulb, retrolateral. —C. Left palpal bulb, prolateral.

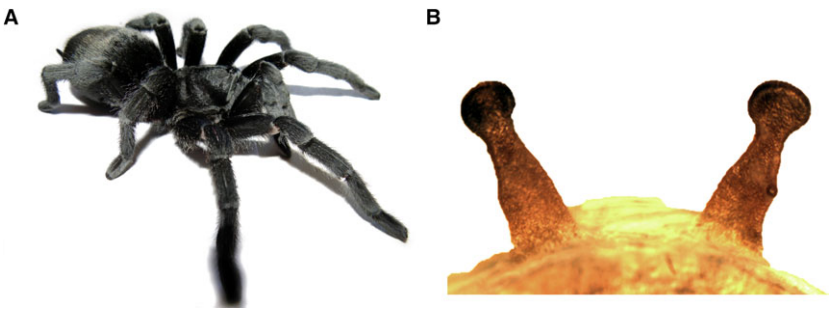

Fig. 6 *Grammostola quirogai* n. sp. —A. Female, habitus. —B. Spermatechae, dorsal view.

triangular group with base on the proximal edge. Sternum length 7.4, width 6.5. Posterior sigillae submarginal. Chelicerae with 11 promarginal teeth (second basal shorter) and 4 little retromarginal. Tarsi I-IV densely copulate, scopula entire. Metatarsi I-II completely scopulate, III scopula on apical third, IV absent. Tibia I with paired distal proventral apophyses. Present one apical spine on the retrolateral branch, one spine at the basis of the proventral branch, and 0–2 retrolateral tibial spines (Fig. 4B). Flexion of metatarsus retrolateral to tibial apophyses. Palpal organ pyriform, curved and long embolus (Fig. 5B,C). Length of leg and palpal segments, in Table 3. Spination: Femora: palp 1P, I-IV 0. Patela: palp 1-1P, I-II 0, III 1P, IV 1R. Tibia: palp 2-1 P, 1V; I 1-1 P, 1-1 R; II1-1-1-2 V, 1-1 R; III 1-1 P, 1.1 V, 1-1-1 R; IV 1-1-2 D, 1-1 P. Metatarsus: I1 V; II 2 V; III 2-1-1 P, 1-1-2 V, 1-1-1-2 R; IV 1-1-2 P, 1-1-1-2 V, 3R. Tibia: palp and I-IV 0. Colour: Cephalothorax, abdomen and legs black with some grey hairs (proximal to moult, the spider lost the intensity and become brownish) (Fig. 5A). Type III-IV urticating hairs present. PMS monoarticulated, PLS triarticulated, apical segment digitiform.

Variation (range (mean ± standard deviation)): Total length 39.2–51.2 (42.56 ± 4.97), carapace length 19.2–23.3 (21.18 ± 1.48), width 18.4–23.1 (20.76 ± 1.93). AME 0.25–0.38 (0.32 ± 0.05), ALE 0.5–0.64 (0.57 ± 0.05), PME 0.38–0.47 (0.39 ± 0.04), PLE 0.48–0.68 (0.55 ± 0.08), AME–AME 0.50–0.63 (0.59 ± 0.06), AME–ALE 0.3–0.43 (0.38 ± 0.05), PME–PME 0.97–1.88 (1.34 ± 0.35), PME–PLE 0.13–0.3 (0.22 ± 0.07), ALE–PLE 0.28–0.40 (0.33 ± 0.06), OQ length 1.33–2.50 (2.04 ± 0.45), width 2.18–2.8 (2.59 ± 0.26), clypeus 0–0.63 (0.26 ± 0.24). Fovea width 1.8–3.1 (2.66 ± 0.55). Labium length 1.7–2.8 (2.26 ± 0.45), width 2.28–3.38 (3.05 ± 0.46). Sternum length 7.4–9.1 (8.54 ± 0.69), width 6.1–7.5 (6.94 ± 0.62). Legs I 56.14–64.1 (60.23 ± 3.44), II 21.95–27.6 (25.09 ± 2.24), III 58.8–57.8 (53.6 ± 3.72), IV 58.2–70.8 (64.46 ± 5.03), palp 27.08–31.77 (29.69 ± 2.38).

Table 3 Length (mm) of legs and palpal segments of the holotype and one female paratype of *Grammostola quirogai* n. sp.

		Femur	Patella	Tibia	Metatarsus	Tarsus
Male (FCE-MY 1215)	palp	11.14	5.44	8		2.5
	I	16.28	8.57	12.1	11.13	8.06
	II	15.54	8.39	11.28	10.54	7.28
	III	13.71	7.75	10.71	12.4	7.03
	IV	16.39	7.81	13.05	15.75	7.79
Female (FCE-MY 1201)	palp	11.68	6.82	7.27		6.5
	I	15.41	9.75	11.64	9.13	6.93
	II	14.41	8.51	9.94	8.8	6.1
	III	12.36	7.7	8.18	9.55	5.81
	IV	15.52	8.11	11.05	13.96	6.03

Female (paratype FCE-MY: 1201). Total body length 46.21, carapace length 20.94, width 19.73. Anterior eye row recurve, posterior procurve. Eyes sizes and interdistances: AME 0.325, ALE 0.8, PME 0.5, PLE 0.575, AME–AME 0.6, AME–ALE 0.25, PME–PME 1.25, PME–PLE 0.225, ALE–PLE 0.275, OQ length 2.32, width 2.56, clypeus 0.925. Fovea transverse, straight, width 5.2. Labium length 2.65, width 3.14 with 117 cuspules, maxillae with 224/209 cuspules. Sternum length 8.27, width 8.75. Chelicerae with nine promarginal teeth (from distal to proximal, decreasing in size and the 8 is smaller than other); three retromarginal. Tarsi I-IV and palp densely scopulate, entire. Scopula on metatarsi I 3/4, II 2/3, III 1/3 apical, IV absent. Length of legs and palpal segments in Table 3. Spination: Femur: palp 1P; I 1P; II-IV 0. Patella: palp and I-IV 0. Tibiae: palp 1-1-3V, 1R; I 1-1-2V, II 1-1-1P, 1-1-2V, III 1-1-1P, 1-2V, 1-1R; IV 1P, 1-1V, 1-1R. Metatarsus: I 1-1-1V; II 1-3V; III 1-1-1P, 1-1-2V, 1-1R; IV 1P, 1-1-1-1-1-1-2V, 1-1-1-1-1R. Tarsus palp and I-IV 0. Colour: as in male (Fig. 6A). Type III-IV urticating hairs present. PMS mono-articulated, PML triarticulated, apical segment digitiform. Two straight spermathecal receptacles (Fig. 6B).

Variation (range [mean ± standard deviation]): Total length 36.7–52.8 (47.2 ± 6.26), carapace length 17.4–23.27 (20.28 ± 2.34), width 16.9–21.5 (19.8 ± 2.01). AME 0.28–0.45 (0.35 ± 0.07), ALE 0.48–0.8 (0.61 ± 0.13), PME 0.28–0.5 (0.34 ± 0.1), PLE 0.4–0.58 (0.5 ± 0.08), AME–AME 0.55–0.75 (0.65 ± 0.08), AME–ALE 0.25–0.48 (0.35 ± 0.09), PME–PME 0.97–1.5 (1.29 ± 0.22), PME–PLE 0.13–0.26 (0.2 ± 0.05), ALE–PLE 0.25–0.4 (0.54 ± 0.3), OQ length 1.88–2.9 (2.31 ± 0.4), width 2.17–2.8 (2.53 ± 0.23), clypeus 0.25–0.93 (0.54 ± 0.3). Fovea width 3.3–5.2 (3.91 ± 0.76). Labium length 1.9–3.40 (2.7 ± 0.59), width 3.1–3.3 (3.17 ± 0.08). Sternum length 7.25–9.25 (8.26 ± 0.78), width 5.8–8.77 (7.71 ± 1.16). Legs I 40.56–53.36 (49.22 ± 5.22), II 14.8–22.3 (18.85 ± 2.74), III 33.1–49 (43 ± 6.27), IV 41.8–58.6 (52.98 ± 6.51), palp 25.05–33 (30.64 ± 3.22).

Discussion

Grammostola taxonomy has been problematic due to the morphological homogeneity of its species, which is reflected in a number of critical changes of species composition over time (Bücherl 1951; Schiapelli & Gerschman 1961; World Spider Catalog 2015). The integration of morphological, behavioural and molecular characters, considered together with distributional data, provides evidence that has allowed us to uncover a new species herein described as *G. quirogai*. This result shows the power of integrating multiple lines of evidence in taxonomic studies.

Previous studies using DNA barcoding in Theraphosidae have shown an average of 6% of divergence between

congeneric species (Hamilton *et al.* 2011). Ours comparisons of species of *Grammostola* range between 10% and 15%. Morphological evidence found in spines and setae from the male tibial apophysis as well as coloration also support the discrimination among all the *Grammostola* species, including *G. anthracina* s.s. and *G. quirogai* n. sp.

Grammostola anthracina shows low level of genetic variation; meanwhile, the genetic diversity seen in *G. quirogai* n. sp is larger. In fact, the sample of *G. quirogai* n. sp is the most variable of those here analysed (Table 2). This species shows a relatively deep genealogy, but which is not geographically structured; clades show a large degree of geographic overlap, including localities were pairs of clades are found in sympatry. The average observed variation between the internal clades of *G. quirogai* n. sp (6.8–7.2%) is much lower than of those values corresponding to comparisons between species pairs of *Grammostola* (10–14.3%; Table 2). Given these results and the fact that those clades do not differ morphologically, we treat them as representing within species variation. However, we acknowledge that until nuclear DNA sequences are analysed, we cannot completely rule out that those clades represent in fact distinct isomorphic species. Having say that, if our taxonomic scenario (i.e. *G. quirogai* n. sp is a species with relatively deep mitochondrial genealogical structure) probes to be correct, it would be of interest to understand the reasons why this species appears at the mitochondrial level more variable than its congeners so far analysed. Under a scenario of neutrality, distinct demographic and historical scenarios could produce the observed pattern (Avise 2000; see also Leffler *et al.* 2012).

After exhaustive sampling, we did not find *G. anthracina* s.s. and *G. quirogai* n. sp. coexisting at the same locality. Postiglioni & Costa (2006) suggested that the absence of overlapping is mainly due to ecological factors. We also consider that usually theraphosids have poor vagility (Ferretti *et al.* 2014) limiting the gene flow. The reported areas of closer occurrence (Fig. 1) likely represent the distributional limits of each taxon; in these areas, individuals occur in low density and are difficult to find. Our present data are not sufficient enough to determine whether these distributional ranges are stable or whether one species is displacing the other.

Finally, our analyses also highlight additional areas of study worth exploring. For example, most relationships among species of *Grammostola* are poorly supported; as such, the addition of taxa missing in our sampling together with the analysis of nuclear DNA sequences is required to more effectively investigate phylogenetic relationships of these spiders. Such study is desirable before advancing a scenario of historical biogeography accounting for the diversification of *Grammostola*.

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