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Phylogeny, species delimitation and convergence in the South American bothriurid scorpion genus *Brachistosternus* Pocock 1893: Integrating morphology, nuclear and mitochondrial DNA ☆

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ABSTRACT

A phylogenetic analysis of the scorpion genus *Brachistosternus* Pocock, 1893 (Bothriuridae Simon, 1880) is presented, based on a dataset including 41 of the 43 described species and five outgroups, 116 morphological characters and more than 4150 base-pairs of DNA sequence from the nuclear 18S rDNA and 28S rDNA gene loci, and the mitochondrial 12S rDNA, 16S rDNA, and Cytochrome c Oxidase Subunit I gene loci. Analyses conducted using parsimony, Maximum Likelihood and Bayesian Inference were largely congruent with high support for most clades. The results confirmed the monophyly of *Brachistosternus*, the nominal subgenus, and subgenus *Ministernus* Francke, 1985, as in previous analyses based only on morphology, but differed in several other respects. Species from the plains of the Atacama Desert diverged basally whereas the high altitude Andean species radiated from a more derived ancestor, presumably as a consequence of Andean uplift and associated changes in climate. Species limits were assessed among species that contain intraspecific variation (e.g., different morphs), are difficult to separate morphologically, and/or exhibit widespread or disjunct distributions. The extent of convergence in morphological adaptation to life on sandy substrata (psammophily) and the complexity of the male genitalia, or hemispermatophores, was investigated. Psammophily evolved on at least four independent occasions. The lobe regions of the hemispermatophore increased in complexity on three independent occasions, and decreased in complexity on another three independent occasions.

1. Introduction

The resolution of species relationships by phylogenetic reconstruction is fundamental to the study of evolution, yet data and methods were fiercely debated in the past (Wagner, 2001). Whereas much energy was expended by some in attempting to determine the most reliable sources of data, others emphasized the benefits of combining diverse sources of data, which may yield new hypotheses that are not otherwise recovered (e.g., Baker et al., 1998; Farias et al., 2000; Lopardo et al., 2011). This integrative approach is increasingly used, in some cases with all data sources leading to similar results (e.g., Prendini et al., 2005; Crews and Hedin, 2006; Agnarsson et al., 2007; Talal et al., 2015) and, in others, revealing discordances which point to interesting underlying evolutionary mechanisms, such as the convergence of traits, often but not exclusively resulting from directional selection (e.g., Quicke and Belshaw, 1999; Mott and Vieites, 2009; Hedin and Thomas, 2010). The combination of data sources is especially advantageous in studies at the species level and is essential to satisfactorily determine the limits of species in complex and diverse taxa, common among arthropods.

The scorpion genus *Brachistosternus* Pocock 1893 currently includes 43 described species (Ojanguren-Affilastro and Pizarro-Araya, 2014), and is the most diverse genus of the family Bothriuridae Simon, 1880 (Kovařík and Ojanguren-Affilastro, 2013).

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Brachistosternus occurs in most arid to semi-arid habitats of southern and western South America from extreme southern Patagonia to Ecuador, from the Pacific to the Atlantic, and from sea level to over 4500 m in the Andes. *Brachistosternus* species are medium-sized scorpions that are usually very abundant, and in most cases the dominant scorpion taxa in areas where they occur (Agusto et al., 2006; Nime et al., 2013, 2014). All species of the genus are fossorial, constructing simple burrows usually in bare, dry substrata, the upper layers of which are loose (semi-consolidated). Although most species of the genus thrive on soils rich in loam, silt or clay, many inhabit softer, sandy substrata including stable or shifting sand dunes, dry river beds, and alluvial fans. The species of *Brachistosternus* may be defined as semi-psammophilous to ultrapsammophilous (Prendini, 2001) based on the presence of ecomorphological adaptations for burrowing in loose, dry (semi-consolidated to unconsolidated) substrata of varying hardness and texture, including dorsoventrally compressed basitarsi, laterally compressed telotarsi, combs of elongated macrosetae on the pro- and especially retrolateral margins of the basitarsi and the dorsal margins of the telotarsi, and setiform pro- and retroventral setae on the telotarsi (Prendini, 2000).

Besides the specialized psammophilous adaptations, many species of *Brachistosternus* possess the most complex hemispermatophores among scorpions in which these structures have been studied (Ojanguren-Affilastro and Ramírez, 2009). The hemispermatophores are the two internal halves of the spermatophore, the secondary reproductive structure of male scorpions, which fuse just prior to extrusion from the male genital aperture, attachment to the substrate and subsequent partial introduction into the female genital aperture during mating. The hemispermatophores of *Brachistosternus* exhibit diverse spines, lobes, and other structures which, in some cases, are asymmetrical (e.g., the cylindrical apophysis).

Brachistosternus is among the best known scorpion genera in South America, from taxonomic and geographical perspectives, and a suitable model for fine-scale analysis of morphological evolution. The taxonomy of *Brachistosternus* has been extensively revised in the past decade (Ojanguren-Affilastro and Roig-Alsina, 2001; Ochoa and Acosta, 2002; Ojanguren-Affilastro, 2002a, 2002b, 2002c, 2003a, 2003b, 2004; Ochoa and Ojanguren-Affilastro, 2007; Ojanguren-Affilastro et al., 2007; Ojanguren-Affilastro and Ramírez, 2009; Ojanguren-Affilastro and Pizarro-Araya, 2014), more than doubling the number of described species listed by Lowe and Fet (2000). The marked increase in known diversity is attributed to intensive sampling across the geographical distribution of the genus, aided by ultraviolet (UV) light detection, and the application of modern concepts of species. In spite of extensive work on the taxonomy of *Brachistosternus*, species diagnosis remains challenging as most species lack unique diagnostic characters, and must be separated using a limited combination of characters. Although minimal in most Andean species, intraspecific variation may be extensive among the lowland plains species with widespread distributions, resulting in taxonomic challenges that cannot be resolved with morphology alone.

Recent phylogenetic analyses of family Bothriuridae (Prendini, 2000, 2003; Mattoni and Prendini, 2007), based on exemplar species, unambiguously recovered the monophyly of *Brachistosternus*, but did not assess its internal relationships. Ojanguren-Affilastro and Ramírez (2009) performed the first phylogenetic analysis of species-level relationships in the genus, based on morphological data, on the basis of which subgenus *Ministernus* Francke, 1985 was upheld and subgenus *Leptosternus* Maury, 1973 synonymized with *Brachistosternus*. Two major, monophyletic groups of species, i.e., an Andean group and a plains group, were identified in the nominotypical subgenus. The low support values for the groups recovered (Ojanguren-Affilastro and Ramírez, 2009) suggested that the characters on which the analysis was based, were less informative for phylogenetic reconstruction than for species delimitation, or suffered from concerted convergence that biased the results. The availability of other sources of data (i.e., DNA sequences) provides an opportunity to produce a more robust phylogeny on which to explore the evolution of morphological characters.

The present contribution provides the first phylogenetic analysis of *Brachistosternus* based on molecular and morphological data. More than 4150 base-pairs (bp) of DNA sequence from two nuclear gene loci, 18S rDNA (18S) and 28S rDNA (28S), and three mitochondrial gene loci, 12S rDNA (12S), 16S rDNA (16S), and Cytochrome c Oxidase Subunit I (COI), were combined with 116 morphological characters from Ojanguren-Affilastro and Ramírez (2009) for a dataset including 41 of the 43 described species of *Brachistosternus* and five outgroups, including four other bothriurid genera. Analyses were conducted using parsimony, Maximum Likelihood and Bayesian Inference. The aims were to resolve the internal relationships among the species of *Brachistosternus* and test the monophyly and validity of its subgenera and species groups; to assess the limits of species that contain intraspecific variation (e.g., different morphs), are difficult to separate morphologically, and/or exhibit widespread or disjunct distributions; and to investigate the extent of convergence in morphological adaptations to life on sandy substrata (psammophily) and the complexity of the male hemispermatophore.

2. Materials and methods

2.1. Taxon sample

Exemplar species of four bothriurid genera were selected as outgroup taxa based on recent phylogenetic analyses of family Bothriuridae (Prendini, 2000, 2003; Mattoni and Prendini, 2007): *Thestylus aurantiurus* Yamaguti and Pinto-da-Rocha, 2003; *Bothriurus flavidus* Kraepelin, 1911; *Cercophonius sulcatus* Kraepelin, 1908; and *Urophonius brachycentrus* (Thorell, 1876). The tree was rooted on *Scorpio fuscus* (Ehrenberg, 1829), an exemplar species of family Scorpionidae Latreille 1802, representing superfamily Scorpionoidea Latreille 1802, the putative sister-group of Bothriuridae (Prendini, 2000).

Forty-one of the 43 nominal species of *Brachistosternus* were included as ingroup taxa. *Brachistosternus castroi* Mello-Leitão, 1941 and *Brachistosternus holmbergi* Carbonell, 1923 were not included because they are of dubious validity (Ojanguren-Affilastro and Ramírez, 2009; Kovařík and Ojanguren-Affilastro, 2013). Samples of *Brachistosternus mattonii* Ojanguren-Affilastro, 2005, could not be obtained for DNA isolation. Eighteen species were represented by a single specimen, whereas 22 species were represented by two or more specimens (seven species by two specimens, five by three, two by four, three by five, three by seven, and two by eight), each usually from a different locality (a proxy for different populations), in order to test species limits. In total, 107 terminals belonging to 40 species were included.

Species with known intraspecific variation (e.g., different morphs, defined as a local variety of a species, morphologically distinguishable from other populations of the species) were represented by at least one specimen each, and species with widespread distributions, by specimens from central and peripheral points of the known distribution, to the extent possible.

Brachistosternus angustimanus Ojanguren-Affilastro and Roig-Alsina, 2001 occurs in a wide area of central Patagonia. Northwestern populations exhibit minor morphological differences from typical south-eastern populations (Ojanguren-Affilastro and Roig-Alsina, 2001). Five different populations were compared to test whether distant populations of this species are conspecific.

The typical form of *Brachistosternus cekalovici* Ojanguren-Affilastro, 2005 inhabits shrub steppes with a clay substratum. However, some populations, inhabiting coastal dunes that are usually isolated from each other by several kilometres of shrub steppe, exhibit minor morphological differences from one another and from the typical form (Ojanguren-Affilastro et al., 2007). Five specimens from different populations of *B. cekalovici* were compared to test whether they are conspecific.

Brachistosternus ehrenbergii (Gervais, 1841) occupies a wide area from northern Chile to central Peru, and exhibits some intraspecific differences among populations (Ochoa and Ojanguren-Affilastro, 2007). Putative records from Ecuador (Brito and Borges, 2015) are erroneous and correspond to *B. pegnai*. Seven specimens from across the known distribution were compared to test whether these populations are conspecific.

Brachistosternus ferrugineus (Thorell, 1876) occupies a large area, including central and northern Argentina, Bolivia, Paraguay and, probably, southern Brazil. Despite its broad distribution, morphological differences between distant populations of *B. ferrugineus* are minor, and cytogenetic studies revealed no differences (Rodriguez-Gil et al., 2009). The identity of *Brachistosternus simoneae* Lourenço, 2000 remains questionable. Diagnostic characters provided in the original description (Lourenço, 2000) overlap with those of *B. ferrugineus* (Kovařík and Ojanguren-Affilastro, 2013) and examination of specimens of the putative species failed to identify consistent differences with *B. ferrugineus*. Five specimens from widely separated populations of *B. ferrugineus* were compared, together with one specimen identified as *B. simoneae* because it was collected close to the type locality of the latter, to test whether geographically distant populations of *B. ferrugineus* are conspecific with one another, and with *B. simoneae*.

Brachistosternus intermedius Lönnberg, 1902 occurs at very high altitudes from northern Argentina to central Bolivia, where it is replaced by *Brachistoternus titicaca* Ochoa and Acosta, 2002, a morphologically similar species which occupies a similar habitat and niche (Ochoa and Acosta, 2002; Kovařík and Ojanguren-Affilastro, 2013). Seven specimens of *B. intermedius* from across the distribution were compared with one another, and with *B. titicaca*, to test whether they are conspecific. *Brachistosternus piacentinii* Ojanguren-Affilastro, 2003, *Brachistosternus kovariki* Ojanguren-Affilastro, 2003, and *Brachistosternus zambrunoi* Ojanguren-Affilastro, 2002 were also included in these analyses, because they grouped among populations of *B. intermedius* in some of the phylogenetic analyses.

Brachistosternus kamanchaca Ojanguren-Affilastro et al., 2007 is distributed throughout most of the coastal transitional desert of Chile. Different populations exhibit some morphological variation (Ojanguren-Affilastro et al., 2007). Four specimens from different populations, as well as the closely related species, *Brachistosternus barrigai* Ojanguren-Affilastro, 2014, were compared to test whether they are conspecific.

Brachistosternus montanus Roig-Alsina, 1977 occurs at high altitudes in central Argentina. Northern populations of this species differ morphologically from typical southern populations and were previously suggested to be a different species (Ojanguren-Affilastro, 2003b). Seven specimens from different populations across the distribution were compared to test whether they are conspecific.

Brachistosternus multidentatus Maury, 1984 is known from only two, apparently disjunct populations, ca. 1000 km apart, and without any known intermediate populations. One population inhabits dunes in central-western Argentina, and the other inhabits dunes on the Atlantic coast of central-eastern Argentina (Maury, 1984; Ojanguren-Affilastro, 2005). These populations do not exhibit conspicuous morphological differences. However, due to their apparent isolation, specimens of each were included to test whether they are conspecific.

Brachistosternus paulae Ojanguren-Affilastro, 2003 inhabits a wide area of southern Patagonia, from the coast to the eastern slopes of the austral Andes. Three specimens from geographically distant populations were compared to test whether they are conspecific.

Brachistosternus pentheri Mello-Leitão, 1931 inhabits a vast area of central-western Argentina. Northern populations differ from typical southern populations (Rodríguez-Gil et al., 2009) morphologically as well as in chromosome number (Roig-Alsina and Maury, 1984). Eight specimens from geographically distant populations were compared to test whether they are conspecific.

Brachistosternus roigalsinai Ojanguren-Affilastro, 2002 is widely distributed in the coastal transitional desert of north-central Chile (Ojanguren-Affilastro et al., 2007). Different populations exhibit some morphological variation (Ojanguren-Affilastro, 2002a). Eight specimens from different populations, as well as the closely related species, *Brachistosternus paposo* Ojanguren-Affilastro and Pizarro-Araya, 2014, were compared to test whether they are conspecific.

Brachistosternus weijenberghii (Thorell, 1876) inhabits an area approximately 1000 km long and 150 km wide at intermediate altitudes of the central and northern Andes of Argentina. Two slightly different morphs occur at the northern and southern extremes of the distribution, the southern of which was recognized as a different species, *Brachistosternus borellii* Kraepelin, 1911 (Roig-Alsina and Maury, 1981), until it was synonymized with *B. weijenberghii* (Ojanguren-Affilastro, 2002b). Specimens from two northern populations and two southern populations were compared to test whether they are conspecific, and to evaluate the validity of the synonymy.

2.2. Material examined

Most specimens were hand collected by the authors at night using portable UV lamps, comprising mercury vapor tubes attached to a chromium reflector, and powered by a 12 V, 7 A/h battery, or Maglite flashlights modified with UV light-emitting diode (LED) attachments. Some specimens were collected during the day by excavating burrows. Most specimens were preserved in 80% ethanol for morphological study. One or two juvenile specimens and, when available, an adult conspecific from the same collection event, were preserved in 95% ethanol for DNA isolation, following standard procedures (Prendini et al., 2002, 2003). Tissue samples from which DNA was extracted are stored (in the vapor phase of liquid nitrogen at −150 °C) in the Ambrose Monell Collection for Molecular and Microbial Research (AMCC) at the American Museum of Natural History (AMNH), New York (Appendix A). Additional material, deposited in the Argentine National Arachnological Collection, Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia', Buenos Aires, was examined for the morphological character matrix (Appendix B).

2.3. Morphological data

The morphological character matrix of Ojanguren-Affilastro and Ramírez (2009), comprising 116 characters, was adopted and modified. Five outgroup taxa from the previous analysis were omitted, while two (*C. sulcatus* and *S. fuscus*) were added. The states of some characters were adjusted to accommodate these omissions and additions to the taxon sample. For example, the number of states in character 34 was reduced from 11 to 8 (Appendices C and D). Hemispermatophores were dissected from surrounding tissues and observed in 80% ethanol.

2.4. DNA sequencing

Five gene loci were selected to reconstruct the phylogeny of *Brachistosternus* because they evolve at different rates and provide phylogenetic resolution at different, overlapping taxonomic levels (Prendini et al., 2003, 2005; González-Santillán and Prendini, 2014; Santibáñez-Lopez et al., 2014). Approximately 513 base-pairs (bp) of the D3 region of the large-subunit ribosomal RNA (28S rDNA) gene, and 1761 bp of the small-subunit ribosomal RNA (18S rDNA) gene were sequenced from the nuclear genome, as well as ca. 485 bp of the large-subunit ribosomal RNA (16S rDNA) gene, ca. 335 bp of the small-subunit ribosomal RNA (12S rDNA) gene and 1078 bp of the Cytochrome c Oxidase Subunit I (COI) gene, incorporating the DNA barcoding fragment (Hebert et al., 2003), from the mitochondrial genome.

Genomic DNA was extracted from muscle tissue taken from the leg or pedipalp of each specimen using the Qiagen DNeasy Blood and Tissue Kit. DNA was amplified using PureTaq-Ready-To-Go PCR Beads (GE Healthcare), 1 μl of each primer (Appendix E), 2–4 μl of DNA template, and 19–21 μl of molecular grade water for a total reaction volume of 25 μl. The following adjuvants were added for samples that proved difficult to amplify: 2–4 μl of Magnesium Chloride, 0.5–1 μl of Fisher BioReagents Bovine Serum Albumine (BSA), and/or 0.5–1 μl of 5% Dimethyl Sulfoxide (DMSO), adjusting the total reaction volume to 25 μl with molecular grade water. Alternatively, illustra Hot Start Mix RTG beads (GE Healthcare) were used without adjuvants. All amplifications were performed in an Eppendorff Mastercycler thermocycler using the following thermal profile: 94 °C for 3–5 min; 35–40 cycles of 94–95 °C for 15–30 s, 42–52 °C for 15–30 s, 72 °C for 15–30 s; 72 °C for 10 min. PCR products were verified on a 1% agarose-TBE electrophoresis gel stained with SYBR Safe (Invitrogen). Amplified products were purified using an Ampure Magnetic Beads Purification System (Agentcourt) and re-suspended in 40 μl of molecular grade water using a Biomek NX robot (Beckman-Coulter).

Amplification products were sequenced in both directions. Two optimized protocols were used, depending on the length of the fragment. Whereas each 8 μl sequencing reaction mixture included 0.3 μl of Big Dye, 1 μl of Big Dye Terminating buffer, 1 μl of 3.2 pm primer, 5 μl of gene amplification product and 0.7 μl of molecular grade water for fragments less than 500 bp, the 8 μl mixture included 0.5 μl of Big Dye and 0.5 μl of molecular grade water for fragments greater than 500 bp. Samples were sequenced in an Eppendorff Mastercycler thermocycler using 35 cycles of the following thermal profile: 96 °C for 15 s, 50 °C for 15 s, 60 °C for 4 min. Cycle-sequenced products were cleaned using CleanSeq magnetic beads on the Biomex NX robot. Products were re-suspended in EDTA and 33 μl were sequenced on an Applied Biosystems, Inc. (ABI) 3730xl automated capillary DNA sequencer.

The accuracy of sequences was verified by independently amplifying and sequencing the complementary strands of all fragments. Primer sequences were removed and complementary strands of DNA assembled into consensus sequences, edited, and checked for quality using Sequencher 5.0 (Gene Codes Co.). If complementary strands disagreed (besides minor mismatches), the sample was reamplified and sequenced to resolve discrepancies.

In total, 570 sequences were generated from 114 samples for the study (Appendix A; Table 1). All except one ingroup COI sequence (*Brachistosternus alienus* 2256) of 376 bp, were complete. The 12S fragment varied from 330 to 339 bp: 330 bp (1 outgroup), 332 bp (7 ingroups), 333 bp (33 ingroups), 334 bp (3 outgroups, 28 ingroups), 335 bp (32 ingroups), 336 bp (2 ingroups), 337 bp (1 outgroup, 6 ingroups), or 339 bp (1 ingroup). The 16S fragment varied from 481 to 486 bp: 481 bp (2 outgroups, 9 ingroups), 482 bp (2 outgroups, 47 ingroups), 483 bp (1 outgroup, 29 ingroups), 484 bp (22 ingroups), 485 bp (1 ingroup), or 486 bp (1 ingroup). Length variation was minimal in 28S: 513 bp (3 outgroups, all ingroups), 514 bp (1 outgroup), or 517 bp (1 outgroup). All complete COI sequences were 1078 bp and all 18S sequences were 1761 bp.

Table 1
Nucleotide diversity and composition of aligned nuclear 18S rDNA (18S) and 28S rDNA (28S), and mitochondrial 12S rDNA (12S), 16S rDNA (16S) and Cytochrome c Subunit I (COI) sequences used for phylogenetic analysis of the South American bothriurid scorpion genus *Brachistosternus* Pocock, 1893: number of haplotypes; unaligned base-pairs; aligned base-pairs, excluding five hypervariable regions which were recoded as presence/absence (Appendix F); number of variable positions (VP); number of parsimony-informative (PI) positions; and percentage GC content (GC). Numbers for COI represent total, first, second and third codon positions.

Marker	Haplo.	Unalign.	Align.	VP	PI	GC
18S	6	1761	1761	8	7	51.1
28S	36	513–517	517	26	15	60
12S	90	330–339	324	139	104	21.8
16S	94	481–486	463	143	117	27.2
COI	102	1078	1078	143	122	35.7
COI codon pos 1	28	359	359	22	13	47.6
COI codon pos 2	12	359	359	7	6	41.4
COI codon pos 3	102	359	359	114	103	18.2

2.5. Multiple sequence alignment and phylogenetic reconstruction

Edited sequences of the three gene loci with length variation (28S, 12S and 16S) were aligned prior to phylogenetic analysis. Alignment of the 28S sequences, conducted in the online version of MAFFT v.7 (Katoh and Standley, 2013), by applying the "Auto" strategy and a gap opening penalty of 1.53, was trivial. Alignment of the 12S and 16S ribosomal sequences, which contained regions of ambiguous alignment representing hypervariable regions (HVRs) unlikely to evolve on a per-site nucleotide substitution basis, was conducted in the online version of MxScarna (http://mxscarna.ncrna.org/), by applying a secondary structure model with a stem candidate length of 2 and a threshold of base pairing probability of 0.01. Regions of the 12S and 16S alignments comprising more than two continuous gaps in at least 5% of the taxa were excised and re-coded as multi-nucleotide character states in a presence/absence matrix per haplotype (Appendix F), following Lutzoni et al. (2000). Alignment was not required for the COI sequences, in which codon positions were determined using Bioedit v. 7.2.5 (Hall, 1999).

Nucleotide composition homogeneity tests were conducted separately on the alignments of each locus (and codon position for COI) using Tree-Puzzle v. 5.2 (Schmidt et al., 2002) to verify, based on a chi-squared test, whether all partitions were appropriate for phylogenetic reconstruction (Rosenberg and Kumar, 2003). Nucleotide characteristics of each locus in *Brachistosternus* were calculated using DnaSP v. 5.10.1 (Librado and Rozas, 2009).

Phylogenies were reconstructed using parsimony, Maximum Likelihood (ML) and Bayesian Inference (BI), on the morphological data (parsimony), the molecular data (parsimony, ML and BI), and the combined dataset (parsimony, ML and BI). Parsimony analyses were conducted with TNT v. 1.1 (Goloboff et al., 2008a) under equal weights and implied weights, varying the constant of concavity, *k*, between 5 and 100, to select a range with stable results that maximized congruence between the morphological and molecular data, as well as with the tree obtained by BI. The tree search was set to hit the minimum cost 100 times using default parameters of the "new technology search" (Goloboff, 2002). Nodal support was assessed with the bootstrap, calculated using a heuristic search, set to hit the minimum cost three times using default parameters of the "new technology search" for each of the 1000 pseudoreplicates, the consensus of each pseudoreplicate calculated by collapsing with tree-bisection-reconnection.

ML and BI analyses were conducted via the CIPRES Science Gateway v. 3.3 (Miller et al., 2010) on four different data matrices: DNA excluding re-coded HVRs, henceforth 'DNA'; DNA including re-coded HVRs, henceforth 'DNA + HVRs'; morphology and DNA excluding re-coded HVRs, henceforth 'mor + DNA'; morphology and DNA including re-coded HVRs, henceforth 'mor + DNA + HVRs'. The best partitioning scheme and substitution model for each DNA partition was chosen with the Bayesian Information Criterion (Schwarz, 1978), deemed less generalist and more realistic with adequate sample sizes than the Akaike Information Criterion (Akaike, 1973; see Burnham and Anderson, 2004) using the "greedy" search strategy in PartitionFinder v. 1.1.1 (Lanfear et al., 2014).

ML analyses were conducted with RAxML v. 8.0.24 (Stamatakis, 2006), using the rapid bootstrapping algorithm and GTRGAMMA substitution model for DNA, and the MULTIGAMMA model for mor + DNA + HVRs, as this has been shown to provide accurate results (Stamatakis et al., 2007) when applied to a mixed model as determined by PartitionFinder.

BI was conducted using Markov Chain Monte Carlo (MCMC) simulations in MrBayes v. 3.2.1 (Ronquist et al., 2012) with two parallel runs of four simultaneous chains for 10 million generations, sampling every 1000 generations. The partitioning scheme applied and the nucleotide substitution models set as priors for each partition are listed in Appendix G. The *Mk* model for morphology (Lewis, 2001), with a variable ascertainment bias, a dirichlet prior assuming equal frequencies for all states, and a gamma rate prior was applied to the morphological characters. Due to differences among the DNA fragments, the substitution rates were set to vary, and the character state frequencies and gamma shape parameters unlinked across partitions. The first two million generations were discarded as burn-in on generating a consensus tree, based on the likelihoods reaching stationarity, and whether the effective sample size of all parameters was >200, using Tracer v. 1.5 (Rambaut and Drummond, 2007). Nodal support was assessed with 1000 non-parametric bootstrap replicates (ML; Felsenstein, 1985) and posterior probabilities (BI).

2.6. Species delimitation

The limits of the abovementioned twelve species of *Brachistosternus* were tested with a General Mixed Yule-Coalescent (GMYC) model (Pons et al., 2006), implemented in the R v. 3.0.2 (R core team, 2013) package "splits" v. 1.0-19 (Ezard et al., 2009). Both single and multiple rate GMYC models were implemented for the phylogeny obtained from MrBayes (DNA + HVRs), converted to an ultrametric tree using the penalized likelihood (PL) method (Sanderson, 2002) in r8s v. 1.8 (Sanderson, 2003) with the truncated Newton (TN) algorithm, as recommended by the developer, and setting the rates to gamma (Appendix H). As node ages were not required, the age was set relative to one taxon (in this case, *Brachistosternus andinus* Chamberlin, 1916). In addition to the GMYC model, a Bayesian implementation thereof was applied, using the R package "bGMYC" v. 1.0.2 (Reid and Carstens, 2012), to a random sample of 100 of the last 500 trees from each of the two Bayesian runs (again converted to ultrametric trees using the PL method with the TN algorithm in r8s), setting the MCMC simulation at 50,000 generations with a burn-in of 40,000, sampling every hundredth generation. After initial tests with varying Yule and coalescent rate change parameters on a single tree, the upper and lower bounds of the Yule and coalescent rate change parameters were set, respectively, to 1 and 0.05, and the upper bound of the threshold parameter to 103 (number of tips in the trees, for ingroup taxa only).

Whereas several recent studies praised the GMYC method as an affective method for species delimitation (e.g., Monaghan et al., 2009; Brewer et al., 2012; Ceccarelli et al., 2012) others criticized its efficacy (e.g., Carstens et al., 2013; Miralles and Vences, 2013). Bayes Factor Species Delimitation (BFD; Grummer et al., 2014) was therefore also applied as a likelihood-based hypothesis testing method for identification of individuals whose assignment to particular species was uncertain. An advantage is that BFD can detect "misplaced" individuals (due to topological errors) in addition to "lumped" or "split" individuals. BFD also takes incongruence between gene trees and species trees (Degnan and Rosenberg, 2009) into account by applying multispecies coalescence analysis.

The limits of the twelve species were independently tested using the coalescent species tree algorithm *BEAST (Heled and Drummond, 2010) in BEAST v. 1.8.0 (Drummond et al., 2012), while estimating the marginal likelihood (MLE) by path-sampling (PS; Lartillot and Philippe, 2006) and stepping-stone sampling (SS; Xie et al., 2011). PS and SS can be used as a means of comparing the MLEs of the runs, also taking into account the importance of proper priors (Baele et al., 2012, 2013), such that when the same informative priors are set for the parameters of the runs, the changes in MLE between runs are due to the alternative "species" groups set as inputs. Each coalescent species tree analysis was conducted on the complete dataset and again with some of the partitions (18S, 28S, or both) removed, when these contained no variable sites among the terminal taxa in particular analyses.

Molecular clock rates were estimated, nucleotide substitution models simplified to HKY (Hasegawa et al., 1985) for all partitions to avoid over-parametrization, the prior for the species tree set as a Birth–Death process, and the piecewise linear and constant root used for the population size model. Lognormal priors with initial values of 1, mean values of 0.01 and 1 standard deviation were set as priors for mean uncorrelated lognormal clock, population hyper-parameter and Yule process birth rate parameters. The priors set for the remaining parameters were informative and constant for all runs. MCMC chains were set to run for 20 million generations, sampling every 1000 generations. Additionally, the MLE chain was set to run for 5 million generations with 200 path steps. Each postulated species tree analysis was performed twice to check whether the runs converged and the ESS values were greater than 200. Bayes Factors (2*ln*Bf) were estimated from the MLE (combined from the two runs) to compare species group scenarios and select the most likely scenario (Kass and Raftery, 1995): 2*ln*Bf = 0–2, "not worth more than a bare mention"; 2*ln*Bf = 2–6, "positive" support; 2*ln*Bf = 6–10, "strong" support; and 2*ln*Bf >10, "decisive" support in distinguishing between competing hypotheses.

2.7. Analysis of morphological convergence

As the topological results of the molecular phylogenetic analysis data differed markedly from the previously published analysis based only on morphology (Ojanguren-Affilastro and Ramírez, 2009), two suites of morphological characters associated with psammophily (the adaptation to sandy habitats) and with the complexity of the male genitalia, were investigated in more detail to determine whether they may be biased by concerted evolution.

Prendini (2001) defined three increasingly specialized ecomorphotypes among scorpions usually inhabiting loose gravelly or sandy substrata (semi-psammophilous, psammophilous, and ultra-psammophilous) according to the presence of particular morphological character states which confer improved locomotor or burrowing efficiency on these substrata and are hypothesized to be selectively advantageous, or adaptive. Eight character states (Appendix I) contributing to the ecomorphotypes defined by Prendini (2001), were identified among the species of *Brachistosternus*, on the basis of which an ensemble psammophily index, representing the sum of these character states present in each terminal

or internal node, was calculated. The range of ecomorphotypes was scaled as follows: pelophilous (psammophily index = 0–1); semipsammophilous (2–4); psammophilous (5–7); ultrapsammophilous (8–9). Two species with different psammophilous character states may possess the same psammophily index, e.g., *Brachistosternus pegnai* Cekalovic, 1969 and *Brachistosternus artigasi* Cekalovic, 1974, both of which received a score of 3, but which possess character states 0, 38 and 43, and 38, 40 and 43, respectively.

In a similar manner, character states that contribute to the complexity of the male genitalia, or hemispermatophores, were identified (Appendix I), and an ensemble male genital complexity index was calculated. The ensemble indices for terminals were calculated directly from the morphological dataset and for the internal nodes from parsimony optimizations, using a node-driven approach described by Ramírez and Michalik (2014). Ambiguous ancestral reconstructions were presented as the range of minimum and maximum values.

3. Results

3.1. Phylogenetic reconstructions

Nucleotide composition and site-specific information of the DNA data matrices used for phylogenetic reconstructions is outlined in Table 1. The results of parsimony, ML and MB were mostly congruent topologically, with minor differences limited to weakly supported groups (Fig. 1; supplementary figs. in Appendix J), except as discussed below. The tree obtained by simultaneous analysis of the mor + DNA + HVRs with BI (Fig. 1) is preferred. The tree obtained with implied weighting stabilized at values of the constant of concavity, *k*, ranging from 55 to 100. The tree recovered by this range of mild *k* values was maximally congruent topologically with the preferred BI tree, and with the trees obtained by separate analyses of the morphological and molecular datasets (Appendix J), in agreement with the suggestion by Goloboff et al. (2008b) that mild concavities are preferable for DNA sequence data. Bootstrap percentages were calculated for $k = 100$.

The monophyly of *Brachistosternus*, the nominal subgenus, and *Ministernus* were well supported (synapomorphies in Appendix K). Subgenus *Ministernus* comprised two monophyletic groups, one including *B. andinus* and *Brachistosternus peruvianus* Toledo-Piza, 1974, the other including *B. ferrugineus* and *B. simoneae*. Both groups received high support.

Subgenus *Brachistosternus* comprised the remaining 37 species of the genus, grouped into four major clades. The clade placed sister to the rest, referred to as the "**Pacific Coastal Desert Clade**", comprised *B. ehrenbergii*, *B. paposo*, *B. pegnai*, and *B. roigalsinai*, which occur in the Pacific coastal desert from northern Chile to southern Ecuador. *Brachistosternus ehrenbergii* was placed sister to *B. pegnai*, and *B. roigalsinai*, sister to *B. paposo*.

The next clade of the nominal subgenus, referred to as the "**Atacama Desert Clade**", comprised four species from the lowlands of the Atacama Desert in northern Chile and southern Peru: *B. mattonii*; *Brachistosternus ochoai* Ojanguren-Affilastro, 2004; *Brachistosternus turpuq* Ochoa, 2002; and *Brachistosternus sciosciae* Ojanguren-Affilastro, 2002.

The remaining species of the genus were divided into two different clades. The first clade, referred to as the "**Argentine Plains Clade**", comprised all species from the plains of Argentina, i.e., *Brachistosternus alienus* Lönnberg, 1898, *B. angustimanus*, *B. multidentatus*, *B. pentheri*, and *Brachistosternus telteca* Ojanguren-Affilastro, 2000, and was well supported. *Brachistosternus pentheri* was consistently placed sister to the other species of this clade.

The second clade, referred to as the "**Andean-Pacific Clade**", comprised all species from high and intermediate altitudes of the Andes, as well as the remaining species from the plains of Chile and Peru excluded from the clades mentioned previously, and was also well supported. This clade comprised several smaller subclades, the first of which, consistently recovered and well supported in the analyses, comprised five Chilean species, consistently arranged as follows: (*Brachistosternus cepedai* Ojanguren-Affilastro et al., 2007 (*Brachistosternus negrei* Cekalovic, 1975 (*Brachistosternus chango* Ojanguren-Affilastro et al., 2007 (*Brachistosternus aconcagua* Ojanguren-Affilastro and Scioscia, 2007 *Brachistosternus chilensis* Kraepelin, 1911)))). The second subclade of the Andean-Pacific Clade, consistently recovered and well supported, comprised another three Chilean species, arranged as follows: (*Brachistosternus coquimbo* Ojanguren-Affilastro et al., 2007 (*B. artigasi B. cekalovici*)).

The remaining species of the Andean-Pacific Clade were consistently divided into two well supported groups. The first group included species from the Andes and the plains of Bolivia, Chile, and Peru, i.e., *Brachistosternus barrigai* Ojanguren-Affilastro and Pizarro-Araya, 2014, *Brachistosternus donosoi* Cekalovic, 1974, *Brachistosternus galianoae* Ojanguren-Affilastro, 2002, *B. kamanchaca*, *Brachistosternus ninapo* Ochoa, 2004, *Brachistosternus perettii* Ojanguren-Affilastro and Mattoni, 2006, *Brachistosternus prendinii* Ojanguren-Affilastro, 2003, and *Brachistosternus quiscapata* Ochoa and Acosta, 2002. The second group was restricted to Andean species from high and intermediate altitudes in Argentina, Bolivia and Chile, i.e., *B. intermedius*, *B. kovariki*, *B. montanus*, *B. piacentinii*, *B titicaca*, *B. weijemberghii*, and *B. zambrunoi*.

3.2. Species delimitation

The results of the species delimitation analyses based on the GMYC and bGMYC methods are dubious. Whereas the single method GMYC and bGMYC "over-split" (i.e., identified more "species" than realistically present), the multiple method GMYC tended to "lump" together individuals belonging to different nominal species. The questionable results of the GMYC and bGMYC analyses (Appendix L) will not be considered further.

In contrast, the results of the BFD analyses were plausible. Based on the BFD analyses with PS and SS (Table 2), the inland and coastal dunes populations of *B. cekalovici* are conspecific, despite morphological differences, as are the populations of *B. ehrenbergii*, which formed at least two distinct clades; the geographically disjunct coastal and inland populations of *B. multidentatus*; and the geographically distant populations of *B. paulae*. However, the north-western populations of *B. angustimanus*, the northern populations of *B. montanus*, *B. roigalsinai* and *B. pentheri*, and the southern populations of *B. kamanchaca* and *B. weijenberghii* are not conspecific with typical populations. The north-western populations of *B. angustimanus*, the northern populations of *B. montanus* and *B. pentheri*, and the southern populations of *B. kamanchaca* represent four undescribed species, whereas the southern populations of *B. weijenberghii* corresponding to *B. borellii*, may also be distinct. *Brachistosternus barrigai* is conspecific with the typical, northern populations of *B. kamanchaca*.

According to the BFD analysis with SS, the northern populations of *B. roigalsinai* represent an undescribed species, distinct from *B. paposo*, whereas, according to the BFD analysis with PS, these populations are conspecific with *B. paposo*, which was placed within the northern clade of *B. roigalsinai* in most phylogenetic analyses.

Both BFD analyses also suggested that *B. intermedius* comprises two species, but differed regarding which populations were assigned to each. According to the analyses with SS, Argentine populations are conspecific with *B. intermedius*, central Bolivian populations represent an undescribed species, and *B. titicaca* is a distinct species whereas, according to the analyses with PS, most northern Argentine populations and some central Bolivian populations

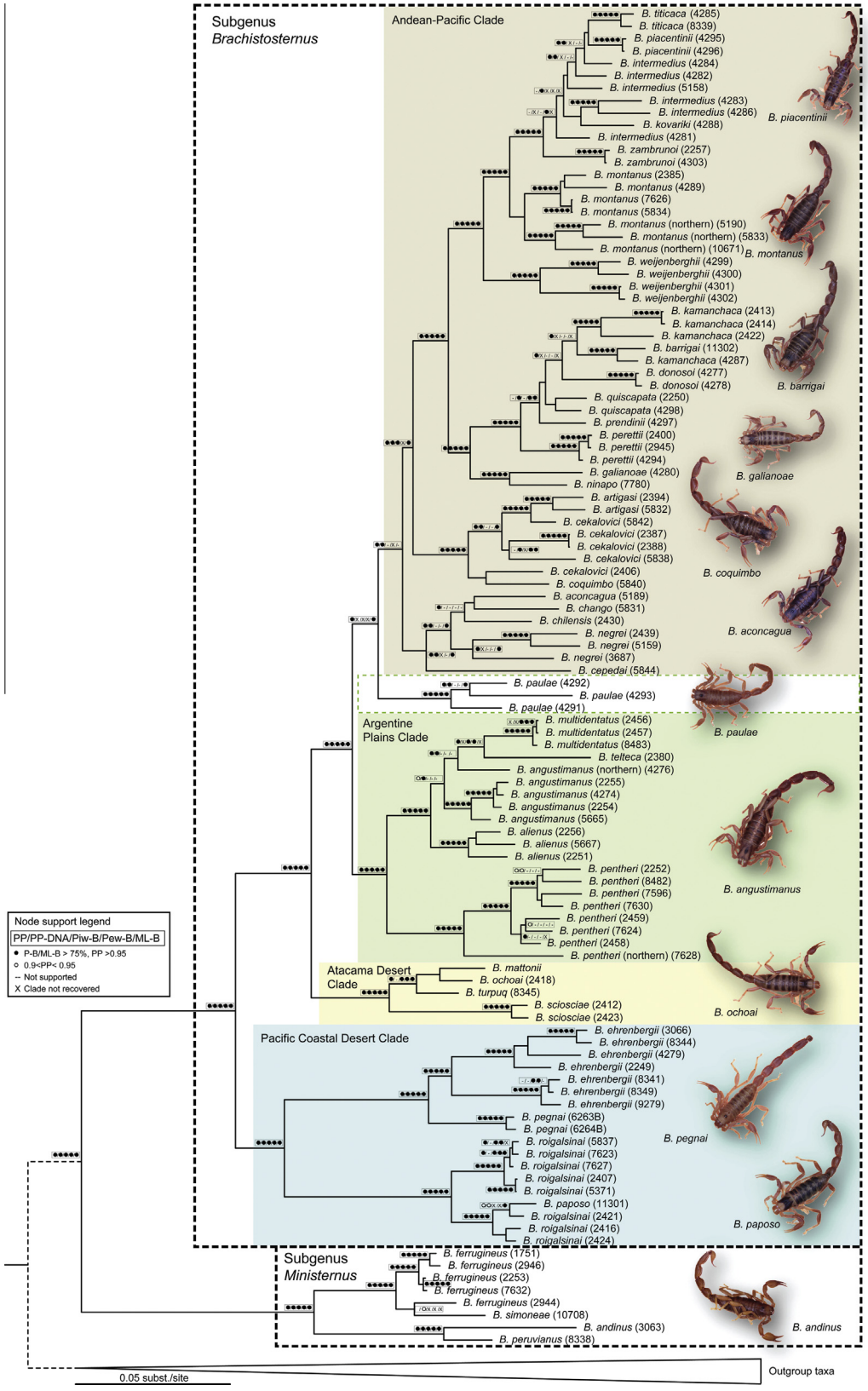

Fig. 1. Preferred phylogeny of the South American bothriurid scorpion genus *Brachistosternus* Pocock, 1893 obtained by Bayesian Inference of 4142 aligned nucleotides from two nuclear (18S rDNA, 28S rDNA) and three mitochondrial (12S rDNA, 16S rDNA, Cytochrome c Oxidase Subunit I) gene loci (Appendix A), and 116 morphological characters (Appendices C and D). Support values for Bayesian posterior probabilities with DNA + morphology (PP), Bayesian posterior probabilities with DNA only (PP-DNA), parsimony with implied weights (bootstrap; Piw-B, concavity constant, *k*, from 55 to 100), parsimony with equal weights (bootstrap; Pew-B) and Maximum Likelihood (bootstrap; ML-B) shown at nodes, symbols explained in legend. *Brachistosternus paulae* Ojanguren-Affilastro, 2003, considered most closely related to the Argentine Plains Clade, is colored as different from the Andean-Pacific Clade (see discussion for details). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Marginal likelihood estimation (MLE) values recovered by path-sampling (PS) or stepping-stone sampling (SS) for null (H0) and alternative (HA1–A6) hypotheses with Bayes Factor Species Delimitation of selected samples of the South American bothriurid scorpion genus *Brachistosternus* Pocock, 1893. Bayes Factors (2*ln*Bf) were estimated from the MLE to compare species group scenarios and select the most likely scenario. Preferred hypotheses in boldface. Lists of individuals and putative species groupings in Appendix H.

	H0	HA1	HA2	HA3	HA4	HA5	HA6
Brachistosternus angustimanus and *B. pentheri*							
MLE by PS (2*ln*BF)	**−11171.32 (∗∗∗)**	−11172.26 (1.88)	−11177.14 (11.64)				
MLE by SS (2*ln*BF)	−11098.41 (28.06)	−11088.11 (7.46)	**−11084.38 (∗∗∗)**				
Brachistosternus cekalovici							
MLE by PS (2*ln*BF)	**−5324.79 (∗∗∗)**	−5339.23 (28.88)	−5329.13 (8.68)	−5333.91 (18.24)			
MLE by SS (2*ln*BF)	**−5222.63 (∗∗∗)**	−5287.32 (129.38)	−5251.57 (57.88)	−5299.32 (153.38)			
Brachistosternus ehrenbergii							
MLE by PS (2*ln*BF)	**−8496.49 (∗∗∗)**	−8506.67 (20.36)					
MLE by SS (2*ln*BF)	**−8416.32 (∗∗∗)**	−8481.97 (131.3)					
Brachistosternus ferrugineus							
MLE by PS (2*ln*BF)	−7554.59 (34.74)	−7540.95 (7.46)	**−7537.22 (∗∗∗)**				
MLE by SS (2*ln*BF)	−7538.56 (61.62)	**−7503.07 (∗∗∗)**	−7507.75 (9.36)				
Brachistosternus intermedius							
MLE by PS (2*ln*BF)	−7959.04 (52.92)	−7941.81 (18.46)	−7933.78 (2.4)	−7937.1 (9.04)	**−7932.58 (∗∗∗)**	−7935.26 (5.36)	−7938.45 (11.74)
MLE by SS (2*ln*BF)	−7953.88 (121.24)	−7924.06 (61.6)	−7894.94 (3.36)	−7902.14 (17.76)	−7909.02 (31.52)	−7899.45 (12.38)	**−7893.26 (∗∗∗)**
Brachistosternus kamanchaca							
MLE by PS (2*ln*BF)	−8581.32 (22.04)	**−8570.3 (∗∗∗)**	−8581.24 (21.88)				
MLE by SS (2*ln*BF)	−8559.07 (34.88)	**−8541.63 (∗∗∗)**	−8577.25 (71.24)				
Brachistosternus montanus and *B. weijenberghii*							
MLE by PS (2*ln*BF)	−8333.24 (26.2)	−8346.25 (52.22)	**−8320.14 (∗∗∗)**				
MLE by SS (2*ln*BF)	−8321.91 (38.12)	−8332.21 (58.72)	**−8302.85 (∗∗∗)**				
Brachistosternus multidentatus							
MLE by PS (2*ln*BF)	**−3840.44 (∗∗∗)**	−3847.27 (13.66)					
MLE by SS (2*ln*BF)	**−3832.35 (∗∗∗)**	−3845.37 (26.04)					
Brachistosternus paulae							
MLE by PS (2*ln*BF)	**−8437.78 (∗∗∗)**	−8466.07 (56.58)	−8465.19 (54.82)	−8446.87 (18.18)	−8457.27 (38.98)		
MLE by SS (2*ln*BF)	**−8408.08 (∗∗∗)**	−8453.81 (91.46)	−8455.24 (94.32)	−8421.77 (27.38)	−8434.92 (53.68)		
Brachistosternus roigalsinai							
MLE by PS (2*ln*BF)	−8194.53 (18.86)	−8197.56 (24.92)	**−8185.1 (∗∗∗)**	−8185.28 (0.36)			
MLE by SS (2*ln*BF)	−8183.75 (29.5)	−8180.71 (23.42)	−8178.98 (19.96)	**−8169 (∗∗∗)**			

∗∗∗ = preferred hypothesis.

represent *B. intermedius*, some Argentine populations and some Bolivian populations represent an undescribed species, and some central Bolivian populations, presently regarded as *B. intermedius*, are conspecific with *B. titicaca*. *Brachistosternus kovariki*, *B. piacentinii*, and *B. zambrunoi* were recovered as distinct species in both analyses.

Based on the BFD analysis with SS, *B. simoneae* is conspecific with *B. ferrugineus* whereas, based on analysis with PS, *B. simoneae* is distinct from *B. ferrugineus*, the northern populations of which represent an undescribed species, distinct from the typical populations of central Argentina.

3.3. Analysis of morphological convergence

Optimization of the ensemble psammophily index revealed the ancestral ecomorphotype of *Brachistosternus* to be semi-psammophilous, consistent with occurrence of the basal species of the genus on loose (semi-consolidated) substrata in arid to semi-arid habitats. Further specialization for life on softer substrata, including sand dunes, occurred independently on four occasions, in the psammophilous species, *B. scioscciae* and *B. zambrunoi*, the ultra-psammophilous species, *B. cepedai*, and the sister-group comprising the ultra-psammophilous species, *B. multidentatus* and *B. telteca* (Fig. 2). Five species possessed only three or four of the six potential ecomorphological adaptations to life on sand, representing another four convergences, in *B. galianoae*, *B. pentheri*, and the monophyletic groups comprising *B. artigasi* and *B. cekalovici*, and *B. ehrenbergii* and *B. pegnai*.

Optimization of the ensemble hemispermatophore complexity index indicated that a marked increase in complexity (from 0 to at least 9) was ancestral for the genus (Fig. 2). The complexity of the hemispermatophore further increased on three independent occasions, among the species related to *B. barrigai*, *B. ehrenbergi*, and *B. multidentatus*, respectively, reaching its greatest complexity in *B. ehrenbergii*. A marked decrease in the complexity of the hemispermatophore also occurred on three independent occasions, among *B. cepedai*, *B. scioscciae*, and, to a lesser extent, *B. paulae*.

4. Discussion

4.1. Brachistosternus *phylogeny and species delimitation*

The monophyly of *Brachistosternus* was well supported in all analyses, as in previous phylogenetic studies (Prendini, 2000, 2003; Mattoni and Prendini, 2007; Ojanguren-Affilastro and Ramírez, 2009). The genus is defined by 15 unambiguous synapomorphies. Two basal clades, corresponding to subgenera *Brachistosternus* and *Ministernus*, were also recovered, as previously (Ojanguren-Affilastro and Ramírez, 2009). Both subgenera were also well supported, with 11 and 9 synapomorphies, respectively.

Subgenus *Ministernus* includes four described species, which consistently formed two monophyletic groups, one, including *B. andinus* and *B. peruvianus*, from the inter-Andean valleys of south-central Peru, and the other, including *B. ferrugineus* and *B. simoneae*, from semi-arid areas of central South America.

The species delimitation analyses were equivocal concerning the status of the different populations of *B. ferrugineus*, and the validity of *B. simoneae*. Accordingly, the known populations of *B. ferrugineus* are regarded as a single species, distinct from *B. simo-*

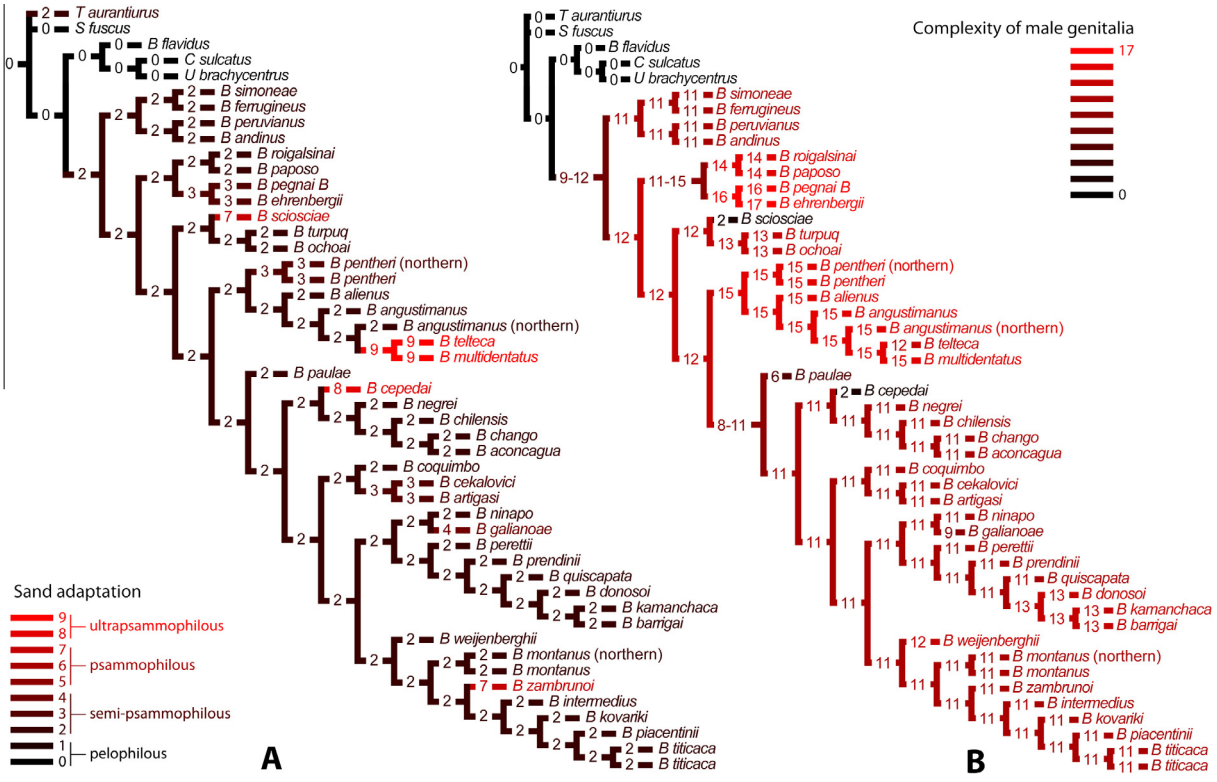

Fig. 2. Morphological convergence revealed by character optimization on the preferred phylogeny of the South American bothriurid scorpion genus *Brachistosternus* Pocock, 1893 (Fig. 1). (A) Psammophily optimized as an ensemble index, indicating that extreme specialization evolved on at least four independent occasions. (B) Complexity of male hemispermatophore optimized as an ensemble index, indicating that the lobe regions increased in complexity on three independent occasions, and decreased in complexity on another three independent occasions.

neae, pending further investigation. Subgenus *Brachistosternus* comprised 37 described species, as reported by Ojanguren-Affilastro and Ramírez (2009). However, neither the "Plains" group of species nor the "Andean" group, reported by Ojanguren-Affilastro and Ramírez (2009), were monophyletic in the analyses presented here, which instead recovered four well supported clades, the Pacific Coastal Desert Clade; the Atacama Desert Clade; the Argentine Plains Clade; and the Andean-Pacific Clade.

Although the Pacific Coastal Desert Clade was well-supported, the limits of some of its component species were ambiguous. All species delimitation tests consistently separated northern from southern populations of *B. roigalsinai*, despite the apparent absence of morphological differences among them. Further evaluation of the disparity between genetic and morphological variation in this species, sampling additional gene loci and populations from the extremes and intermediate parts of the distribution, and from offshore islands, is underway (Ojanguren-Affilastro et al., in preparation).

Three species of the Atacama Desert Clade, *B. mattonii*, *B. ochoai* and *B. turpuq*, are morphologically similar to species of the Argentine Plains Clade, with which they formed a monophyletic group in the analyses of Ojanguren-Affilastro and Ramírez (2009). According to the analyses presented here, however, these species diverged earlier than the Argentine species. The fourth species of the Atacama Desert Clade, *B. sciosciae*, was previously considered to be related to *B. cepedai* and *B. paulae*, two small species with similar external morphology, and simplification of the lobe region (internal spines, basal spines, spines in a row and basal triangle) of the hemispermatophore (Ojanguren-Affilastro and Ramírez, 2009). According to most of the analyses presented here, these three species belong to different clades, and their hemispermatophores became simplified independently.

Brachistosternus paulae is the southernmost species of the genus, reaching the extreme south of the Patagonian plains at latitude 47°S (Ojanguren-Affilastro, 2003a). Although placed in the plains group of species, forming a clade with *B. sciosciae* and *B. cepedai*, in the morphological phylogeny of Ojanguren-Affilastro and Ramírez (2009), its phylogenetic position differed among the various analyses presented here. The most biogeographically plausible position for this species is in the Argentine Plains Clade, as obtained by analyses of the molecular data with ML, BI, and parsimony with implied weighting and *k* values between 55 and 100.

The species delimitation analyses indicated that the northern populations of *B. angustimanus* represent a different species from the typical southern populations, a finding supported by morphological differences that were previously dismissed as intraspecific variation (Ojanguren-Affilastro and Roig-Alsina, 2001). This discovery also suggests the possible importance of the Río Negro and Río Colorado rivers, in northern Patagonia, as an agent of vicariance in scorpions (Ojanguren-Affilastro et al., in preparation).

Brachistosternus multidentatus is among the few ultrapsammophilous species of the genus, known from only two populations inhabiting almost vegetationless dunes, about 1000 km apart. Several attempts to find additional populations of this species in the intervening area were unsuccessful. Despite the large distance between these apparently disjunct populations, specimens from both are morphologically similar, and were considered conspecific in the species delimitation analyses presented here. Presumably, the two populations were connected until recently, before grassland and steppe habitats developed in the intervening area. The possibility that additional populations occur in some suitable, yet inaccessible habitat in the arid regions of central Argentina, cannot be ruled out either.

Although northern populations of *B. pentheri* differ morphologically and in chromosome number (Rodriguez-Gil et al., 2009) from typical central and southern populations, neither difference was previously considered sufficient justification to recognize the northern populations as a different species. However, the species delimitation analyses presented here, taken together with previously noted differences in chromosome number and morphology, justify the recognition of a new species for these populations (Ojanguren-Affilastro et al., in preparation).

The Andean-Pacific Clade is the most complex and diverse group of *Brachistosternus*. The high diversity of this group, the minor differences among its component species, and the relatively new habitats, especially at high altitude, that they occupy, are consistent with a rapid and recent radiation.

Most species of the subclade comprising *B. aconcagua*, *B. cepedai*, *B. chango*, *B. chilensis*, and *B. negrei*, occur in plains, or at intermediate altitudes, in shrub steppes, grasslands and occasionally along sandy watercourses in forested habitats of central and southern Chile. However, *B. cepedai* inhabits arid dunes, almost 300 km north of the geographically closest species of the clade. This ultrapsammophilous species was previously considered to be closely related to *B. sciosciae* due to their similar external morphology and hemispermatophores (Ojanguren-Affilastro and Ramírez, 2009). Based on the analyses presented here, however, the morphological character states that supported the previous sister-group hypothesis between *B. cepedai* and *B. sciosciae*, appear to be convergent. The two species are evidently not closely related and *B. cepedai* may be a northern relict of a once more widely distributed clade.

According to the species delimitation analyses presented here, *B. barrigai* is conspecific with the typical, northern populations of *B. kamanchaca*, with which it should be synonymized, whereas the southern populations of *B. kamanchaca* represent an undescribed species. These results, which conflict with the morphological diagnoses for these species (Ojanguren-Affilastro and Pizarro-Araya, 2014), require further evaluation before decisions are taken regarding the taxonomic status of *B. barrigai* and the southern populations of *B. kamanchaca*.

Also according to the species delimitation analyses, the southern populations of *B. weijenberghii*, which correspond to *B. borellii*, are not conspecific with the typical, northern populations, suggesting that *B. borellii* should be revalidated. Although slight morphological differences in pigmentation pattern and in the shape and length of the distal lamina of the hemispermatophore are evident between the northern and southern populations (Ojanguren-Affilastro, 2002b), this variation is bridged by populations from intermediate locations, suggesting clinal variation in a single species. Specimens from intermediate populations should therefore be included in future analyses, before decisions are taken regarding the taxonomic status of *B. borellii*.

The species delimitation analyses confirmed the earlier suggestion by Ojanguren-Affilastro (2003b) that the northern populations of *B. montanus* represent an undescribed species, to be described elsewhere (Ojanguren-Affilastro et al., in preparation), which differs from typical, southern populations in minor, but consistent morphological differences.

The identity of several populations of *B. intermedius* also remains equivocal. Various phylogenetic analyses recovered the different populations of *B. intermedius* as polyphyletic, comprising several smaller clades, and including other Andean species. The species delimitation analyses obtained with SS are considered more plausible, however, because they are independently supported by some morphological differences among the populations. On the basis of these analyses, the typical populations from northern Argentina and southern Bolivia are regarded as *B. intermedius*, whereas the populations from central Bolivia may represent an undescribed species to be confirmed with additional samples and analyses. *Brachistosternus titicaca*, restricted to the Titicaca Basin of Bolivia and Peru, is considered distinct from *B. intermedius*. *Brachistosternus kovariki*, *B. piacentinii*, and *B. zambrunoi* are also considered valid species.

Due to the nature of the multispecies coalescent, which provides more reliable results with larger sample sizes, the results of the BFD analyses for species that were sparsely sampled, must be interpreted with caution. A wider range of molecular markers may also be desirable when thoroughly investigating species limits, where factors such as gene flow among populations should be considered (Carstens et al., 2013).

4.2. Morphological convergence

All species of *Brachistosternus* scored at least 2 for the ensemble psammophily index, based on the presence of at least two ecomorphological adaptations to life on loose (semi-consolidated) substrata, i.e., laterally compressed telotarsi with setiform pro- and retroventral setae (Prendini, 2000). These adaptations are consistent with the semi-psammophilous ecomorphotype (Prendini, 2001), a precursor for the more stenotopic psammophilous and ultrapsammophilous ecomorphotypes of some species of *Brachistosternus*, which scored more than 2 for the ensemble psammophily index, based on the presence of additional adaptations for life on still softer, sandier substrata.

Brachistosternus ehrenbergii, *B. pegnai*, and *B. pentheri* each scored 3 for the ensemble psammophily index, although they inhabit the full range of substrata on which species of the genus may occur. *Brachistosternus galianoae*, known only from dark volcanic sands near the Sajama Volcano in the central Andes of Bolivia (Ojanguren-Affilastro, 2002c), and characterized by dark infuscation rather than the pale, immaculate coloration typical of species that inhabit sandy substrata, also scored 4 for the index. The absence of pigmentation would presumably be deleterious on the dark sands inhabited by this species.

The morphological characters identified as psammophilous adaptations in *Brachistosternus* evolved in a similar manner among many distantly related scorpion taxa (Prendini, 2001). In contrast, characters concerning the complex lobe region of the male genitalia, or hemispermatophore of *Brachistosternus* have not been observed in other scorpion taxa, and may be unique to the genus (Ojanguren-Affilastro and Ramírez, 2009). The ultimate, evolutionary reason for these structures is unknown, but they are probably involved in reproductive behavior and may be sexually selected (Eberhard, 1996; Arnqvist, 1998). Species of subgenus *Brachistosternus* from the plains, that diverged basally, possess more complex internal structures of the hemispermatophore, e.g., well developed internal spines, basal spines and spines in a row, a large and chitinous basal triangle, and a large, usually clavate cylindrical apophysis, the plesiomorphic condition in the subgenus. On the other hand, Andean species, which appear to have radiated recently, possess less complex internal structures, e.g., internal spines are absent and the remaining spines weakly developed, the basal triangle and cylindrical apophysis are medium sized, and the apex of the apophysis is never thickened. It is unclear whether the decrease in complexity of the hemispermatophore is associated with life on softer, sandier substrata, or with some other aspect of the biology of these scorpions. These internal structures are absent or, if present, extremely reduced, in *B. cepedai*, *B. paulae*, and *B. sciosciae*, three small psammophilous species from the plains of Argentina and Chile, in which this simplification of the hemispermatophores appears to have evolved independently, but well developed in other psammophilous species, e.g., *B. multidentatus*.

Further investigation is necessary to better understand the factors promoting the evolution of these scorpions which are

ubiquitous throughout the arid zone of southern South America. Exceptional adaptations for life on sandy substrata, and complex genitalia offer an excellent model system for such studies.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.08.007.

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