Editor's Choice Article

Dated phylogenetic studies of the southernmost American buthids (Scorpiones; Buthidae)

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ABSTRACT

A dated molecular phylogeny of the southernmost American species of the family Buthidae, based on two nuclear and two mitochondrial genes, is presented. Based on this study, analyzed species of the subgenus *Tityus (Archaeotityus)* are neither sister to the remaining species of the genus *Tityus*, nor are they closely related to the New World microbuthids with decreasing neobothriotaxy. Analyzed species of the subgenus *Tityus* do not form a monophyletic group. Based on ancestral area estimation analyses, known geoclimatic events of the region and comparisons to the diversification processes of other epigean groups from the area, a generalized hypothesis about the patterns of historical colonization processes of the family Buthidae in southern South America is presented. Furthermore, for the first time, a Paleogene-African ingression route for the colonization of America by the family Buthidae is proposed as a plausible hypothesis.

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1. Introduction

The scorpion family Buthidae can be found in all continents except Antarctica, and is present in most of the tropical and temperate areas of the world. With more than 90 genera and 1100 species, this family is the largest of the scorpion families (Rein, 2016). In Africa and Asia, Buthidae is most highly diversified in arid areas, while in the Neotropics it is more diverse and abundant in tropical humid regions, with fewer records in arid environments. Due to the distribution and diversity of Buthidae, its presence in America is thought to have a Cretaceous-Gondwanic origin (Sissom, 1990; Fet et al., 2005), before the final fragmentation of Gondwana, about 110 Ma.

Taxonomic divisions of Buthidae are numerous and in many cases contradictory, since they are usually based in different sets of characters (Fet and Lowe, 2000). However, two recent contributions including phylogenies based on completely different kinds of characters (morphological vs. molecular), have reached very similar results that can shed some light to the higher phylogenetic division of the family. Fet et al. (2005) performed a phylogenetic analysis of the family using morphological characters, and divided the family into six groups of genera. These groups are: *Ananteris*, *Buthus*, *Charmus*, *Isometrus*, *Tityus* and *Uroplectes*. Sharma et al. (2015) performed a phylogenomic study of most extant groups of scorpions, and the relationships between species of different groups of Buthids that they recovered match, in general, with the suggested relationships between groups presented by Fet et al. (2005). Other more restricted molecular phylogenies of Buthidae (Soleglad and Fet, 2003; Fet et al., 2003) using mitochondrial genes also recovered similar phylogenetic relationships as in the more general studies.

In America, only two of the groups of Buthidae suggested by Fet et al. (2005) are present: *Tityus* and *Ananteris*.

The *Tityus* group is restricted to America. It includes 10 described genera, most of which are highly diversified. This group inhabits a wide range of environments, being only absent in the colder areas, and strikingly in the Atacama Desert. In the southern part of America only two genera of the *Tityus* group are present: *Tityus* Koch 1836, and *Zabius* Thorell 1893.

Tityus is the most diverse genus of the order, with more than 200 described species (Rein, 2016). It occurs in part of the Antillean islands, Central and South America, being especially diversified in tropical areas. In the southern and temperate part of South America the presence of this genus is comparatively marginal, with only a few species present in the area (Ojanguren-Affilastro, 2005). Recently Lourenço (2006) suggested a sub-generic division of *Tityus*, in which he separated the genus in five subgenera: *Archaeotityus* Lourenço 2006, *Atreus* Gervais 1843, *Brazilotityus* Lourenço 2006, *Caribetityus* Lourenço 1999, and *Tityus* Koch 1836.

In southern South America, south of the Tropic of Capricorn, only the subgenera *Archaeotityus* and *Tityus* are present. The subgenus *Archaeotityus* presents several conspicuous diagnostic characters, and all of its species are morphologically very similar (Lourenço, 1999, 2006). It includes the species from the *clathrattus* group (Lourenço, 1984a). This subgenus occurs in a wide range of environments, but in southern South America it is only present in areas of the humid Chaco. According to Lourenço (1999) it is supposed to include the most "primitive" species of the genus, in other words the species that maintained several ancestral characters.

The subgenus *Tityus*, as it is currently defined, presents a very high internal diversity, and includes several species complexes which could correspond to monophyletic groups (De-Souza et al., 2009; Lourenço, 1980, 1981, 1984a,b, 2002; Lourenço and Maury, 1985; Lourenço and da-Silva, 2006, 2007). In the southern part of the continent five of these complexes are present: *bahiensis*, *bolivianus*, *confluens*, *trivittatus* and *stigmurus;* the last one, however, is only present as synanthropic. In this area, the *bahiensis* complex is restricted to the Paranaense subtropical forests (Maury, 1969). The *trivittatus* and *confluens* complexes are present in arid areas of the Chaco phytogeographic province and related environments (Lourenço and da-Silva, 2007; Maury, 1970, 1974, 1984) as defined by Cabrera and Willink (1980). The *bolivianus* complex is remarkable because it presents a disjunct distribution, with 14 described species occurring at intermediate altitudes in the Andes from Ecuador to Argentina, and only one described species occurring in plains of eastern Argentina, southern Brazil and Uruguay (Lourenço and Maury, 1985).

The genus *Zabius* is the southernmost genus of Buthidae, reaching even the cold arid steppes of northern Patagonia (Acosta et al., 2008). It inhabits the semiarid areas of Central and Northern Argentina, Paraguay, and southern Brazil. It is part of the so called "New World microbuthids with decreasing neobothriotaxy" (Francke et al., 2014) which corresponds to the *proto-Tityus* of Lourenço (1999), plus other closely related genera. All these genera, except for *Zabius*, belong to northern lineages and occur in Northern South America, Central America and the Antillean area. *Zabius* currently contains only three described species, but in recent surveys four additional undescribed species of this genus from arid areas of Chaco and Espinal of central and northern Argentina were discovered (AAOA and CIM pers. obs.).

The *Ananteris* group occurs not only in America, but also in Africa and Asia, where it is most diversified. It includes 9 extant genera, and at least one extinct genus from Baltic amber (Lourenço, 2011; Rossi and Lourenço, 2015). Only two genera of this group occur in America: *Microananteris* Lourenço, 2003 with only one known species from French Guiana, and *Ananteris* Thorell, 1891 with about 80 described species from South and Central America (Botero-Trujillo and Noriega, 2011; Lourenço, 1985,

2015). Despite its high number of described species, *Ananteris* presents relatively scarce morphological interspecific variability, and all of its species occur in quite similar environments of tropical forests and related savannahs. Only the southernmost species of the genus, *Ananteris balzanii* Thorell 1891 reaches the semi-arid areas of northern Argentinean Dry Chaco (Ojanguren-Affilastro and Vezzani, 2000).

In this contribution, the dispersal processes of Buthidae in the southern part of the Neotropics are studied, by inferring a dated molecular phylogeny which includes all known species of the area, as well as four undescribed Chacoan species of *Zabius* and an undescribed species of *Tityus* endemic from Paraje Tres Cerros, an area of isolated hills of eastern Argentina. Several outgroups of America, Asia and Africa are also included. Based on the results of this study, the proposed relationships between groups and subgenera of *Tityus*, as well as between other genera of Buthidae, are revised. Furthermore, ancestral area estimations coupled with historical geoclimatic information from South America give rise to hypotheses on dispersal patterns of several groups of this family, as well as a Paleogene-African origin for the dispersal of the family Buthidae to America, proposed here for the first time.

2. Materials and methods

2.1. Taxon sampling

Most specimens used in this study were manually collected by the authors at night using UV lamps, or during the day under stones, or logs, or in the basses of large grasses. Permits for legal collection from Argentina, Bolivia, Brazil, Cuba and Ecuador were obtained in each case. All specimens are deposited in the Museo Argentino de Ciencias Naturales Arachnological collection (MACN-Ar), Buenos Aires, Argentina. Newly-generated sequences from this study were deposited in GenBank.

Sequences of some terminals were obtained from GenBank. These terminals were chosen based on their putative relationships with the focal group, their presence in previous phylogenies of the family, and availability. In three cases there was insufficient data from both nuclear and mitochondrial genes from the same species (*Parabuthus* Pocock, 1890, *Buthacus* Birula 1908, and *Centruroides* Marx, 1890), therefore, a combination of genes for two clearly co-generic species was used. Although concatenating sequences from different species (i.e. chimeric sequences) is not ideal, in the absence of a full dataset for the same species, and considering the terminal at genus level, the approach taken here is valid, and has been used before (e.g. Hedtke et al., 2013). Chimeric sequences pose the threat of creating reticulate histories if the taxon groupings are incorrect, however, in the case of this study there is no risk of mis-grouping the taxa in the chimeric sequences, as they definitely belong to the same genera.

For the phylogenetic analyses of *Tityus*, sequences for all known species of the area of study (including the type species of the genus) were used: *Tityus argentinus* Borelli, 1899, *Tityus bahiensis* (Perty, 1833), *Tityus confluens* Borelli, 1899, *Tityus paraguayensis* Kraepelin, 1895, *Tityus trivittatus* Kraepelin, 1898, *Tityus uruguayensis* Borelli, 1901, and an undescribed species of *Tityus* (*Tityus* sp1). GenBank sequences (16S, COI, and 28S) from *Tityus serrulatus* Lutz & Mello, 1922 (a species occasionally cited in the area as synanthropic (Camargo and Ricciardi, 2000; Bortoluzzi et al., 2007)) were also included. To add support to the phylogenetic hypothesis presented in this study, data from the following related species available from nearby areas were included: *Tityus carvalhoi* Mello-Leitão, 1945, from southern Brazilian Cerrados, belonging to the subgenus *Tityus*, *Tityus mattogrossensis* Borelli, 1901 from Southern Brazilian Cerrados, *Tityus bastosi* Lourenço,

1984 from tropical forests of Ecuador, and the type species of the subgenus, *Tityus clathrattus* Koch, 1845, all belonging to the subgenus *Archaeotityus*, using GenBank sequences (16S). For the *bolivianus* group of the subgenus *Tityus*, sequences from two Andean Bolivian species *Tityus soratensis* Kraepelin, 1912, and *Tityus andinus* Kraepelin 1911 were included. This last species was considered a synonym of *T. argentinus* by Lourenço and Maury (1985), but we revised the material studied by these authors and consider it a valid species with clear diagnostic characters; its re-description however, will be part of a further contribution (Ochoa & Ojanguren-Affilastro in prep.). GenBank (16S) sequences were included from the following representatives belonging to the subgenus *Atreus* from northern South America: *Tityus nematochirus* Mello-Leitão, 1940, *Tityus pachyurus* Pocock, 1897, *Tityus perijanensis* González-Sponga, 1994, and *Tityus discrepans* (Karsch, 1879).

In total, sequences from 22 individuals belonging to *Tityus* were used, of which one individual for the nominal species *T. argentinus*, *T. bahiensis*, *T. confluens*, *T. paraguayensis*, *T. trivittatus*, *T. carvalhoi*, *T. mattogrossensis*, *T. soratensis*, *T. andinus* and *T. bastosi*, and six individuals each for *Tityus* sp1 and *T. uruguayensis*. The specimens of *T. uruguayensis* used for molecular studies belong to the only known population of the species in Argentina, from an area around the ruins of an old human settlement in El Palmar National Park. We have extensively surveyed similar areas nearby, and we could not find this species. Therefore, due to its characteristics, we consider that this population of *T. uruguayensis* has an anthropic origin and that this species is not part of the native epigean fauna of the west side of the Uruguay River basin (Ojanguren-Affilastro, 2005). *Tityus* sp1 belongs to the *bolivianus* complex and is endemic from Paraje Tres Cerros. This is an isolated low altitude hilly area of subtropical western Argentina corresponding to the Botucatu stratigraphic formation (Aceñolaza, 2007). It formed about 10–5 million years ago (Ma), in a process which is probably related to the final rapid uplift of the Andes (Ghosh et al., 2006; Garzione et al., 2008).

For the inclusion of *Zabius* in the phylogenetic analyses, sequences from one individual each of both known species of Argentina (including the type species of the genus) were used: *Zabius fuscus* Thorell 1893 and *Zabius birabeni* Mello-Leitão 1938. In addition, sequences were obtained from one individual each of four undescribed species from northern and central Argentina. *Zabius* sp1 from Chacoan areas of central Argentina, *Zabius* sp2 from Chacoan salt-flat areas of central Argentina, *Zabius* sp3 from Chacoan-Espinal hilly areas in central Argentina, and *Zabius* sp4 from Chacoan areas of northern Argentina.

Sequences from the following representatives of three genera within the *Tityus* group, which are not present in southern South America, were also included. Firstly, one individual of the Cuban species *Alayotityus sierramaestrae* Armas, 1973 since the genus *Alayotityus* is most closely related to *Zabius*, based on the phylogenetic analyses of Francke et al. (2014). Secondly, two more distantly related representatives of the American buthid fauna, which belong to the other two more diversified genera of the *Tityus* group besides the genus *Tityus*. Of these, sequences were included from one individual of the Cuban species *Rhopalurus junceus* (Herbst, 1800), and a terminal taxon for the genus *Centruroides*, combining mitochondrial genes 16S & COI from the North American species *Centruroides limpidus* (Wood, 1863) and the nuclear gene 28S of the North American species *Centruroides hentzi* (Banks, 1900).

Furthermore, the only representative of the *Ananteris* group from the area of study, *A. balzanii*, and one congeneric representative, *Ananteris* sp., from Southern Brazilian Cerrados were included. As a sister genus within the *Ananteris* group, sequences from the Asian species *Lychas mucronatus* (Fabricius, 1798), which is the only species of the group included in the analysis of Sharma et al. (2015), were included.

As non-American outgroups inside Buthidae, sequences from representatives of the *Uroplectes* and *Buthus* groups (Appendix A) were included. According to Fet et al. (2005), the *Uroplectes* group is most closely related to the *Tityus* group. This is partially supported by the results of Sharma et al. (2015). The *Buthus* group is the most distantly related group to the American buthids in both mentioned contributions.

From the *Uroplectes* group, sequences were included which belong to *Grosphus flavopiceus* Kraepelin, 1900, and one terminal taxon for the genus *Parabuthus*, combining the mithochondrial genes COI & 16S from the African species *Parabuthus transvalicus* Purcell, 1899, and the nuclear gene 28S of the African species *Parabuthus laevifrons* (Simon, 1888). The only representative of the *Uroplectes* group included in previous molecular analyses of the Buthidae by Fet et al. (2003) and by Soleglad and Fet (2003), belongs to the genus *Grosphus* (*Grosphus madagascariensis* (Gervais, 1843)), and the only representative of the *Uroplectes* group in the analysis of Sharma et al. (2015) belongs to genus *Parabuthus* (*P. transvaalicus*).

From the *Buthus* group, sequences were included from four species of two genera: the Asian species *Mesobuthus martensii* (Karsch, 1879) which was included in the analysis of Sharma et al. (2015), and three African species of genus *Androctonus* Ehrenberg, 1828: *Androctonus hogarensis* (Pallary, 1929), *Androctonus crassicauda* (Olivier, 1807) and *Androctonus australis* (Linnaeus, 1758), the last one was also included in the analysis of Sharma et al. (2015). One terminal taxon of the genus *Buthacus* Birula 1908 was also included, by combining the genes COI & 28S from the African species *Buthacus occidentalis* Vachon 1953 and the gene 16S of the Asian species *Buthacus yotvatensis* Levy, Amitai & Shulov 1973.

Sequences from the representatives of groups *Isometrus* and *Charmus* were unavailable for this study.

The bothriurid species *Brachistosternus paposo* Ojanguren-Affilastro & Pizarro-Araya 2014 was used to root the buthid phylogeny.

A list of the studied material and their localities is provided in Appendix A, while the GenBank accession numbers of all sequences included in this study can be found in Table B.1, Appendix B.

Point locality records were georeferenced in the field with portable Global Positioning System devices (Garmin® GPS II Plus, Etrex, Etrex Vista and Etrex Vista C) or retroactively using the GeoNet Names Server (http://earth-info.nga.mil/gns/html/). A distribution map was generated using the web site www.simplemappr.net.

In this contribution, we will accept the concept of species groups and complexes used for the genus *Tityus*, but will consider these subdivisions as synonyms, and in general refer to them as "complexes". We will accept the sub-generic division of genus *Tityus* of Lourenço (2006) and will follow the generic group division of family Buthidae suggested by Fet et al. (2005). A priori we will consider all subgenera and genera as monophyletic.

2.2. DNA sequencing

Four gene fragments were selected to reconstruct the phylogeny of buthids 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; Ojanguren-Affilastro et al., 2015): 491 base-pairs (bp) of the D3 region of the nuclear large-subunit ribosomal RNA (28S rDNA) gene, 291 bp of the nuclear Histone 3-a gene fragment (H3a), ca. 330 bp of the mitochondrial large-subunit ribosomal RNA (16S rDNA) gene and 654 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 of each specimen using the Qiagen DNeasy Blood and Tissue Kit. The selected molecular markers were amplified by PCR in reactions with a total volume of 15 μl which contained 1.5 μl ×10 PCR Buffer (Invitrogen), 10 μmoles $MgCl_2$, 0.25 μmoles of each dNTP, 0.4 μmoles of each primer, 0.1 μlTaq Polymerase (Invitrogen), 0.5 μl BSA, 1–2 μl genomic DNA and ddH2O to bring the final volume to 15 μl. Four gene fragments (two mitochondrial and two nuclear) were amplified using the primers in Table B.2 of Appendix B. All amplifications were performed in a Bio Rad MyCycler thermal cycler using the following thermal profile: 94 °C for 3–5 min; 35 cycles of 95 °C for 15–30 s, 42–52 °C for 15–30 s, 72 °C for 15–30 s; 72 °C for 10 min. Amplified products were purified using Exo-SAP (Affymetrix) and sent for sequencing, in Applied Biosystems 3130xl and 3500xl Genetic Analyzers, to the Instituto Nacional de Tecnología Agropecuaria (INTA-Castelar-Argentina).

2.3. Multiple sequence alignment and phylogenetic reconstruction

The sequences of the molecular markers were edited in Sequencher 4.1.4. (GeneCodes Corp.). Alignment of the COI, H3a and 28S-D3 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 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 16S alignment comprising more than two continuous gaps in at least 5% of the taxa were excluded from the subsequent phylogenetic analyses. Nucleotide composition homogeneity tests were conducted separately on the alignments of each locus (and codon position for COI and H3a) 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).

Phylogenies were reconstructed using Bayesian Inference (BI), maximum likelihood (ML), and parsimony on a dataset with one individual per species.

BI analyses were conducted via the CIPRES Science Gateway V. 3.3 (Miller et al., 2010) and the best partitioning scheme and substitution model for each DNA partition was chosen with the Bayesian Information Criterion (Schwarz, 1978), using the "greedy" search strategy in Partition Finder v. 1.1.1 (Lanfear et al., 2012). Markov Chain Monte Carlo (MCMC) simulations were carried out in MrBayes v. 3.2.3 (Ronquist et al., 2012) with two parallel runs of four simultaneous chains for 20 million generations, sampling every 2000 generations. The partitioning scheme applied and the nucleotide substitution models set as priors for each partition can be found in Table B.3 of Appendix B. 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 based on posterior probabilities.

ML analyses were conducted with RAxML v. 8.0.24 (Stamatakis, 2014), using the rapid bootstrapping algorithm and GTRGAMMA substitution model for DNA. Nodal support was assessed with 1000 non-parametric bootstrap replicates (Felsenstein, 1985).

Parsimony analyses were conducted with TNT v. 1.1 (Goloboff et al., 2008) under implied weighting (concavity constant k = 10). 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 (1000 replicates), calculated using a heuristic search of ten random addition sequences (RAS) followed by TBR branch swapping; a pilot tree search found the optimal tree in 100% of the replicates of RAS+TBR.

2.4. Divergence time estimation

A time-calibrated tree was reconstructed for *Tityus* using BEAST v. 1.8.2 (Drummond et al., 2012). Two independent MCMC runs of 50 million generations each, sampling every 5000 generations, were conducted. The dataset was partitioned by marker and the substitution models unlinked, applying the appropriate model to each partition, as recommended by the Bayesian Information Criterion (Schwarz, 1978), implemented in Partition Finder v. 1.1.1 (Lanfear et al., 2012). Clock models were also unlinked, applying an uncorrelated lognormal relaxed clock prior to each partition. Default settings for estimated mean clock rates with lognormal distributions were applied, allowing for auto-optimisation as the runs progressed. Tree models were linked across all partitions and a birth-death tree prior was set.

Three separate analyses were carried out, one with no constraints on monophyly and no node age priors ("unconstrained"), another with constraints on topology based on the results of Sharma et al. (2015) and fossil-based node age priors ("constrained") and a third with the same topological constraints as the second, but to corroborate node ages, instead of setting node age priors, substitution rates for COI were used ("constrained + rates"), setting a normal distribution with a mean of 0.008125 and a standard deviation of 0.0005, resulting in a distribution with values within the ranges obtained in previous studies on scorpions (Gantenbein et al., 2005; Ceccarelli et al., 2016a,b). The topological constraints on the two "constrained" analysis forced (1) a sister group relation between the *Buthus* group and the remaining buthids, and (2) the *Ananteris* group sister to the *Tityus* + *Uroplectes* groups. In addition, for the fossil-based node age estimation, three fossil calibration points were used as priors for node age estimation, setting the fossil ages on stem lineage of the constrained clades. The first prior was set as a uniform distribution with minimum age of 15 million years (Myr) for the most recent common ancestor (mrca) of *T. mattogrossensis*, *T. clathrattus*, *T. paraguayensis* and *T. bastosi*, based on a fossil found in Chiapas amber belonging to said group of taxa (Riquelme et al., 2015). The estimated age of a specimen found in Dominican amber, assigned to the genus *Tityus* (Santiago-Blay and Poinar, 1988) was used to set another uniform prior with a minimum age of 20 Myr to the mrca of all *Tityus* specimens in our study. The third calibration point was set as a uniform distribution on the mrca of all buthids with a minimum age of 44 Myr, based on the oldest known fossil belonging to this family, found in Baltic amber (Dunlop and Penney, 2012). After verifying the correct "mixing" of chains in Tracer v. 1.5, and establishing that the effective samples sizes of the parameters were greater than 200, Log Combiner v. 1.8.2. (part of the BEAST package) was used to combine the trees from the two runs. Tree Annotator v.1.8.0 (Drummond et al., 2012) was used to choose the maximum clade credibility (mcc) tree with the "mean node heights" option applied to the 20,000 output trees from the two combined BEAST runs.

2.5. Ancestral area estimation

The mcc tree from the fossil-based constrained analysis in BEAST was used as an input for ancestral area estimations in the R v. 3.3.2 (R core team, 2016) package BioGeoBEARS v. 0.2.1

(Matzke, 2013). For a more detailed account of how BioGeoBEARS can be applied to biogeographical studies, readers are directed elsewhere (e.g. Ceccarelli et al., 2016a). A total of 10 areas were assigned to the terminal taxa. The terminals from the *Ananteris*, *Buthus* and *Uroplectes* groups were reduced to a single terminal each and all known areas for the entire groups were assigned to avoid negative area-bias from the under-sampling of those groups. Similarly, the bothriurid representative was designated the label "outgroup" and the areas assigned correspond to the distributional range of the family. The maximum range size was set to four areas and where a terminal taxon was found in more than four areas, the label "widespread" was assigned post-analysis. BioGeoBEARS was run with a time-stratified model, divided at 0–5, 5–20 and 20–60 Ma, to set lower dispersal probabilities through the Chacoan area, based on information regarding marine ingressions during the Miocene (Donato et al., 2003). The dispersal multiplier matrices can be found in Appendix B, Matrix B1. The DEC, DEC+J, DIVALIKE, DIVALIKE+J, BAYAREALIKE and BAYAREALIKE+J algorithms were run on the data and their likelihoods compared by Akaike Information Criterion tests (AIC; Akaike, 1973).

3. Results

3.1. Phylogenetic inferences and node age estimates

Nucleotide composition and site-specific information of the DNA data matrices used for phylogenetic reconstructions is outlined in Table B.4 of Appendix B. The trees obtained with Bayesian inference using MrBayes and BEAST, maximum likelihood, and parsimony under implied weights K = 10, have, in general, similar topologies (Figs. 1, and C.1; C.2, C.3, C.4, C.5, C.6 of Appendix C). In most cases the relative positions of the clades representing the *Ananteris* and *Uroplectes* groups do not coincide with most previous analyses on the group. However, since the data in this study provided insufficient phylogenetic signal for resolving deep relationships, as can be seen from the low posterior probability values, the tree herein presented (Fig. 1) corresponds to the dated tree obtained by molecular phylogenetic analyses with Bayesian inference using BEAST v. 1.8.2, enforcing the sister relationship of species of groups *Uroplectes* and *Tityus* based on the results of Sharma et al. (2015). Node age estimates were similar whether the tree was calibrated with fossil node age priors or with the substitution rate of COI (Fig. 1 and Fig. C.6 of Appendix C), therefore the results presented here will be based on the fossil-calibrated phylogeny.

The most recent common ancestor (mrca) of the Buthidae in this analysis diverged between 44 and 53.29 Ma (95% Highest Posterior Density), separating species of the old world genera *Androctonus*, *Buthacus* and *Mesobuthus*, all three belonging to the *Buthus* group, from the remaining buthid groups.

The following two diverging ancestors gave rise to the clades which include species belonging to the *Ananteris* and *Uroplectes* groups, whose separation takes place in a comparatively narrow temporal succession. The second divergence, between 32.18 and 49.15 Ma, gave rise to a clade which includes an Asian species of genus *Lychas* and two species from genus *Ananteris*, all belonging to the *Ananteris* group. The genus *Lychas* separated from the genus *Ananteris* between 25.84 and 44.54 Ma. The two species from the genus *Ananteris* included in this study diverged around 6 Ma (2.12–7.2). The third divergence, between 30.79 and 47.53 Ma, resulted in a clade which includes *Grosphus flavopiceus* and genus *Parabuthus* both belonging to the *Uroplectes* group.

The final clade diverging between 26.04 and 42.17 40 Ma forms a well supported monophyletic group in all analyses and only includes species belonging to the American *Tityus* group. These species form two major clades. One includes all species of genus

Tityus of the current analysis, and the other includes species of four different genera: *Rhopalurus*, *Centruroides*, *Alayotityus* and *Zabius*.

The species of the genus *Tityus* studied here are grouped in two major clades (Fig. 1). The mrca of the first clade to diverge, around 30 Ma (95% HPD: 22.9–37.6), gave rise to (1) a clade comprising all species of this analysis belonging to subgenus *Archaeotityus*, and (2) a clade comprising five species included in subgenus *Tityus*. The subgenus *Archaeotityus* diverged between 7.16 and 15.89 Ma and includes: ((*T. mattogrossensis*, *T. paraguayensis*), (*T.bastosi*, *T. clathrattus*)). The clade comprising the five species included in the subgenus *Tityus*: ((*T. serrulatus*, *T. bahiensis*), (*T. carvalhoi*, (*T. confluens*, *T. trivittatus*))) diverged between 5.85 and 13.57 Ma; the species of this subgenus belong to four species complexes: *stigmurus* complex (*T. serrulatus*); *bahiensis* complex (*T. bahiensis*); *confluens* complex: (*T. confluens*); and *trivittatus* complex (*T. trivittatus* and *T. carvalhoi*), (Lourenço, 1980, 2002; Lourenço and da-Silva, 2006, 2007). In this contribution, it will be referred to as the "*bahiensis* clade". In all phylogenetic analyses carried out here, *T. trivittatus* is more closely related to *T. confluens* than to *T. carvalhoi*, therefore both groups will be considered indistinct and referred to as *trivittatus* complex.

The second clade that diverged from the genus *Tityus*'s mrca gave rise to a further two diversifying clades, which separated between 11.49 and 23.38 Ma, one including all species of this analysis belonging to the subgenus *Atreus* (*T. nematochirus*, *T. pachyurus*) *T. perijanenesis*) *T. discrepans*), and the other comprising five species included in the subgenus *Tityus*, and in the *bolivianus* complex (Lourenço and Maury, 1985). It includes: (*T. uruguayensis*, *Tityus* sp1, (*T. soratensis*, (*T. andinus*, *T. argentinus*))). Andean species of this clade: *T. soratensis*, *T. andinus*, and *T. argentinus*, diverged from the lowland species *T. uruguayensis* and *Tityus* sp1, between 9.72 and 19.46 Ma.

The remaining clade of the *Tityus* group includes two subclades, whose mrca diverged between 17.43 and 32.26 Ma, one formed by the North American *Centruroides* plus the Cuban *R. junceus*, and the other including the Cuban *A. sierramaestrae*, plus all analyzed *Zabius*, with a split between these two genera between 12.71 and 27.3 Ma.

The divergence of the Argentinean species of *Zabius*'s mrca occurred about 5.28 Ma (95% HPD: 3.29–7.38), resulting in a group including *Z. fuscus* and two closely related new species, and another group including *Z. birabeni* and two closely related new species. Each group subsequently underwent two chronologically coincidental internal splits, the first one around 3.7 Ma, and the most recent one around 1.73 Ma. *Zabius fuscus* is most closely related to a new lowland species from central Argentina (sp2), and to another new montane species (sp3) from a hilly area of central Argentina. The Patagonic species *Z. birabeni* is most closely related to a new Chacoan species from northern Argentina (sp4), and to another new Chacoan species from central Argentina (sp1).

3.2. Ancestral area estimations

Based on the likelihoods and AIC values returned by the different biogeographical methods in BioGeoBEARS, the results presented here are based on the DEC+J analyses (see Appendix C, Fig. C.7. all other results are not shown). Due to the undersampling of buthid sister-groups to the *Tityus* group, the biogeographical events and areas estimated for most deep nodes are not reliable, even though the analysis returned a widespread ancestor for the family Buthidae. The first discernable ancestral area, with 0.713 relative probability, is Africa, for the mrca of the *Tityus* and *Uroplectes* groups (estimated between 30.79 and 47.53 Ma). The mrca of the *Tityus* group is then estimated to have occupied the Amazonas-Guyana area of northern South America (0.402 relative probability), from where the genus *Tityus* diversi-
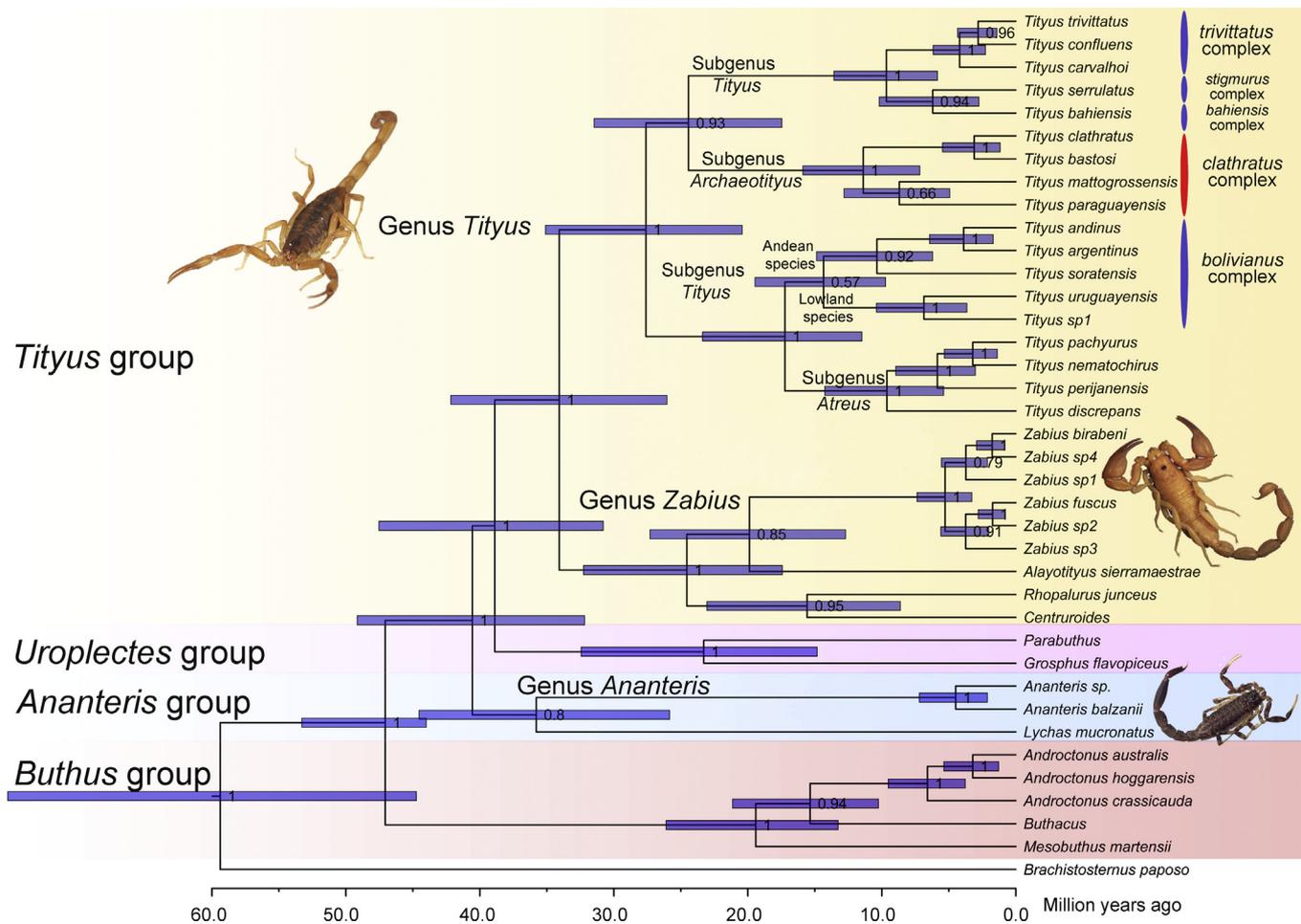
Fig. 1. Phylogenetic tree for the southernmost American buthids with node age estimates inferred by BEAST, enforcing the sister relationship of species of groups *Uroplectes* and *Tityus* based on the results of Sharma et al. (2015). 95% Highest Posterior Density of node heights are shown by blue bars. Major clades are indicated on left of the tree. Time scale is indicated below the tree.

fied and dispersed to the rest of the continent. Similarly, the mrca of the genera *Zabius*, *Alayotityus*, *Rhopalurus* and *Centruroides* was estimated to have occupied northern areas of the American continent. More fine-scale biogeographical area and events estimations would only be possible with denser taxon sampling. For the southernmost *Tityus* species from south-eastern South America and the central Andean region, the mid- to late Miocene dispersal route was estimated to have occurred from the Amazon-Guyana through the Andes eastwards to southern Brazil, the Argentine Mesopotamian region and Uruguay (0.682 and 0.582 relative probabilities, respectively).

4. Discussion

4.1. Buthid phylogeny

The results obtained in this study generally coincide with previous phylogenetic studies of the family Buthidae, especially with regards to the relationships within groups. The species belonging to the *Buthus* group were recovered as monophyletic. Also, *Ananteris* and *Lychas*, both from the *Ananteris* group, were recovered as monophyletic in most analyses. This is the first molecular phylogeny in which the Asiatic *Lychas mucronatus* and the American *Ananteris* are grouped in the same clade, confirming previous results of Fet et al. (2005), based on morphological characters. Similarly, these are also the first molecular phylogenetic

analyses in which two representatives of the *Uroplectes* group form a single clade, therefore providing further support for a close relationship between the species of this group. Nevertheless, all of these results must be viewed with caution, considering the scarce sampling of species (or even genera) within the different groups.

Species of the *Tityus* group form a single clade in our analyses, also confirming all previous phylogenetic studies of the family. The clade formed by *Centruroides* + *Rhopalurus junceus*, is coincident with most previous published bibliography of the group, which considers both genera closely related (Lourenço, 1979; Fet et al., 2003; Soleglad and Fet, 2003; Teruel et al., 2006). The clade formed by (*Alayotityus sierramaestrae* + all studied species of *Zabius*) supports, at least partially, the close relation between these genera proposed by Francke et al. (2014).

The species of the subgenus *Archaeotityus* studied here form a monophyletic group, which is not sister to the remaining species of the genus *Tityus* as suggested by Lourenço (1999); instead, the studied species of subgenus *Archaeotityus* (including its type species) and the *bahiensis* clade (which includes the type species of subgenus *Tityus*), are closely related to each other, forming a different clade with respect to the sampled species of the *bolivianus* complex of the subgenus *Tityus* and the species of the subgenus *Atreus*. The close relation between subgenera *Archaeotityus* and *Tityus* is consistent with previous results of Borges et al. (2010).

The results of this study support the monophyly of the genus *Tityus*, but not the monophyly of the subgenus *Tityus* as it is currently defined. However, increased taxonomic sampling of the genus,

including representatives from all subgenera, would be necessary to make any formal decision regarding the status of the subgenus.

4.2. Biogeographical processes in southern South American Buthidae

Based on the results of this study, there is a close phylogenetic relation between the analyzed species of the subgenus *Atreus*, and those of the subgenus *Tityus* belonging to the *bolivianus* group. This sister-group relation is also supported by several morphological characters shared by both groups (Lourenço, 1984b; Lourenço and Maury, 1985; Pinto-Da-Rocha and Lourenço, 2000). The results from this study are compatible with a common origin of both groups in the northern part of South America, where species of the subgenus *Atreus* are currently distributed, whereas species of the *bolivianus* complex are likely to have used the Andes as a corridor to disperse to the south. Between 20 and 16 Ma, when species of the *bolivianus* complex started to radiate, the Andes reached an average altitude of 2000–3000 m asl (Garzione et al., 2008; Ghosh et al., 2006); those comparatively lower Andes presented a warmer and more humid climate than nowadays, all being favorable conditions for the diversification of this group in the area. The disjunct distribution pattern of the *bolivianus* complex (part in the Andes and part in eastern South America: Uruguay, southern Brazil and Argentine Mesopotamia) could be explained either by dispersal plus extinction events, or by vicariant speciation, depending on how widespread the ancestor was. In both cases, the current-day disjunct distribution and the divergence between 9.72 and 19.46 Ma (mid- to late Miocene), of Andean and Lowland subclades, is likely be related to the temporally congruent marine ingressions that occurred in South America approximately between 20 and 5 Ma (Donato et al., 2003), which covered large areas of the modern-day Chacoan region. Assuming that the current distribution of the group reflects an ancient continuous distribution, ranging from the Andean area of northern and central South-America, to the Atlantic coast of Uruguay and southern Brazil, the long periods of flooding acted as a vicariant barrier, favoring the divergence of the two clades on either side through allopatric speciation. The ancient postulated continuous distribution of the *bolivianus* complex, would also suggest that the split between *Tityus* sp1 and *T. uruguayensis*, between 3.65 and 10.4 Ma, most likely occurred due to a process of allopatric speciation during the late Miocene. The species' divergence is temporally congruent with the uplift of the hills of Paraje Tres Cerros, to which *Tityus* sp1 is endemic, and coincides with a scenario in which the Uruguay River became a significant barrier for the epigean fauna, since the Uruguay River was already present in the Miocene (Montoya-Burgos, 2003) and presents the same course at least since the Pliocene (Panario and Gutierrez, 1999).

The comparatively recent diversification of the Chacoan-Cerrados species of the *trivittatus* complex, between 2.26 and 6.17 Ma, is congruent with the retrogression of the sea from actual Chaco during the late Pliocene, and the emersion of large areas of land. Those recently emerged areas became a newly available habitat for many epigean arthropods as scorpions, and were likely to have been occupied by species of the *trivittatus* complex from a northern lineage, while a subsequent allopatric speciation event between the Chaco and the Brazilian Atlantic Forest took place, as estimated by the biogeographical analyses. Our results provide further support for the Chaco-Cerrados-Caatinga corridor proposed by Lourenço (1986). It is intriguing why species of the *bolivianus* complex did not re-settle in the re-emerged areas of Chaco, but it could be related with ecological constraints of the subgenus, since most of its species are lithophilous (Prendini, 2001), occurring only in rocky habitats, which are extremely rare in the Chaco.

The diversification of *Zabius* species from Chaco, Espinal and Monte phytogeographic provinces (*sensu* Cabrera and Willink,

1980), between 7.38 and 0.8 Ma, is also coincident with the retrogression from the sea from these areas. Additionally, the genera most closely related to *Zabius*, occur exclusively in Central America, the Antillean area, and northern South America (Francke et al., 2014). All this evidence supports the north-to-south dispersal estimated by the biogeographical analyses for this genus, during the retrogression of the sea from actual Chaco. This dispersal route is also coincident to the Chaco-Cerrados-Caatinga corridor proposed by Lourenço (1986). The only known species of *Zabius* from Brazil, *Zabius gaucho* Acosta, Candido, Buckup & Brescovit 2008, was described from forested areas of Rio Grande Do Sul, that are apparently not related with this distribution pattern; however, all known records of this species are actually synanthropic, and the species was never collected in natural environments near the type locality (Acosta et al., 2008), therefore no biogeographical affinities regarding this species can be traced from these records. The isolated record of *Zabius* from Tucumán province in northwestern Argentina by Teruel (2002) does not contradict the distribution pattern herein suggested, since the area where it has been collected belongs to the Montane Chaco. Old records of this genus from Paraguay (Kraepelin, 1899), that have been highly debated (Maury, 1984; Mattoni and Acosta, 1997), now seem very plausible, since they were found very close to our new records from Formosa province in northern Argentina (*Zabius* sp4), and are also coherent with our proposed dispersal and colonization pattern for the genus.

The divergence of the two species of *Ananteris* studied here, from Brazilian Cerrados and Argentinean dry Chaco, took place between 2.12 and 7.2 Ma. This divergence time, and the distribution of all the remaining species of the genus extending far north through the continent, is also coherent with a settlement of this genus in Chaco through the Chaco-Cerrados-Caatinga corridor, after the Pliocene retrogression of the sea.

In a dated phylogeny of the Bothriurid scorpion genus *Brachistosternus* Pocock, 1893 calibrated with geological events, Ceccarelli et al. (2016a) found that two scorpion species from Chaco and Cerrados, *Brachistosternus ferrugineus* (Thorell, 1876) and *Brachistosternus simoneae* Lourenço 2000, also present a divergence time of about 4 Ma, similar to that of the Chacoan *Tityus*, *Zabius* and *Ananteris* species. However, the species of *Brachistosternus* that occupied the area belong to a lineage with an Andean origin (Ojanguren-Affilastro et al., 2015; Ceccarelli et al., 2016a).

Similar results to the ones here were also obtained in other epigean groups, as in the dated phylogeny of the new world genus gecko *Homonota* Gray 1845 by Morando et al. (2014). The divergence of the species from the *borelli* species group (*sensu* Morando et al., 2014), *Homonota uruguayensis* (Vaz-Ferreira & Sierra de Soriano 1961), from Uruguay and southern Brazil, and the ancestor of *Homonota taragui* Cajade et al., 2013 (a species endemic from Paraje Tres Cerros (Cajade et al., 2013)), occurred about 8 Ma. This estimated age of divergence is congruent with the age of separation of *Tityus* sp1 and *T. uruguayensis* (Fig. 1), both from the same areas as *H. taragui* and *H. uruguayensis* respectively. Morando et al. (2014) also obtained similar diversification times for the Chacoan species of the *borelli* group, as the ages estimated here for the species of the *trivittatus* complex, and the Chacoan species of *Zabius*, and *Ananteris* (between 4 and 5 Ma). Morando et al. (2014) suggest that after the Miocene marine ingressions, the species of *Homonota* belonging to the *borelli* group re-settled the area of Paraje Tres Cerros, as well as Chaco and Monte phytogeographic provinces, diverging from an ancestor which remained isolated on the emergent land from what is now the distribution area of *H. uruguayensis*. However, the eastern coastline of the Miocene marine ingressions occupied an area from southwestern Uruguay to northeastern Argentina, Paraguay, and southern Brazil (Aceñolaza, 2007; Herbst and Santa-Cruz, 1999; Hernández et al.,

2005), not covering the middle Uruguay River Basin in Corrientes Province, Argentina (Fig. 2). Thus, the ancestor of the *borelli* group, as well as the ancestor of *Tityus* sp1 and *T. uruguayensis*, could have been distributed in a region that includes not only Uruguay and Southern Brazil, but also the Argentine margin of the middle Uruguay River basin (including the area of Paraje Tres Cerros). Based on this, and contrary to the opinion of Morando et al. (2014), we consider it more likely that the ancestor of *H. taragui* originated due to a process of allopatric speciation, in a similar scenario to *Tityus* sp1. Based on this hypothesis, (which is also compatible with both phylogenetic trees obtained by Morando et al., 2014), Chacoan species of *Homonota* of the *borelli* group should derive from an ancestor of the area of Paraje Tres Cerros.

The distribution pattern of *T. paraguayensis* in humid Chaco is also compatible to a north-to-south ingression route; however, in this area *T. paraguayensis* occurs exclusively in habitats close to rivers, therefore we consider that its ingression route is more related to the course of these rivers than to the Chaco-Cerrados-Caatinga corridor.

The node age estimates for the divergence of the Antillean and continental species of *Tityus* group's mrca is compatible with the subduction of the Gaarlandia Land Bridge (Iturralde-Vinnent, 2006); however, more genera and species of this area are necessary to accurately trace the history and origin of the different groups of the diverse Antillean scorpion fauna.

Despite the important differences in the origin and diversification processes in the five Chacoan epigean groups herein mentioned (*Tityus*, *Zabius*, *Ananteris*, *Brachistosternus* and *Homonota*), their diversifications in the Chaco are temporally congruent with each other and with the end of the Marine ingressions which led to the emergence of newly available habitats in the area (Werneck, 2011). Additionally, both endemic species of Tres Cerros hills mentioned here (*Tityus* sp1 and *H. taragui*), share similar diversification times, which can be linked to the rising of these hills. All this provides strong support for our hypothesis of diversification of the order in the area, as well as for the obtained node age estimates for the dated phylogenetic events discussed in this contribution.

4.3. Final considerations and buthid dispersal to America

This study represents the first dated molecular phylogeny of Buthidae including such a diverse sample of American Buthids. It includes representatives of all major clades from America as well as representatives from the most closely related groups from the rest of the world. It is far from being complete since Buthidae contains more than 1100 described species and more than 90 described genera; nevertheless, this analysis provides a first insight to understand the dispersal patterns of the family in the study area. An overview of the results reveals that the node age estimates obtained for the diversification of the *Tityus* group in America, as well as its separation with the remaining groups of the family, are difficult to reconcile with the current hypothesis of a Cretaceous-Gondwanic origin of American buthids, although node age estimates carried out with a more complete taxon sample are likely to result in slightly older node age estimates. Nevertheless, the most straightforward interpretation of the results is compatible with ancestral dispersal events to America likely to have taken place sometime between the separation of the *Tityus* group and the old world Buthids between 30.79 and 47.53 45 Ma, and the initial divergence of the *Tityus* group in America, about 40 Ma (26.04–42.17 Ma, 95% HPD). This hypothesis implies the colonization of America by this group post-dating the Cretaceous-Gondwanic hypothesis by about 50–60 Myr. This is not contradicted by the ambiguity observed in the results of this study with respect to the sister group relationships of *Tityus* group compared to the results of Sharma et al. (2015) (*Uroplectes* group, vs. *Ananteris* group), since in both cases the node age estimates between new and old world buthids is quite similar.

A plausible dispersal route compatible with the results presented here would be a trans-Atlantic ingression of the ancestors of *Tityus* group to America from Africa. This Paleogene-African dispersal to America is coincident with trans-oceanic dispersals from Africa to America by other groups, such as the ancestors of caviomorph rodents, plathyrrine monkeys, and some groups of geckoes (Bond et al., 2015; Pierre-Olivier et al., 2011; Takai et al., 2000; Gamble et al., 2011). This is also coherent with the available fossil
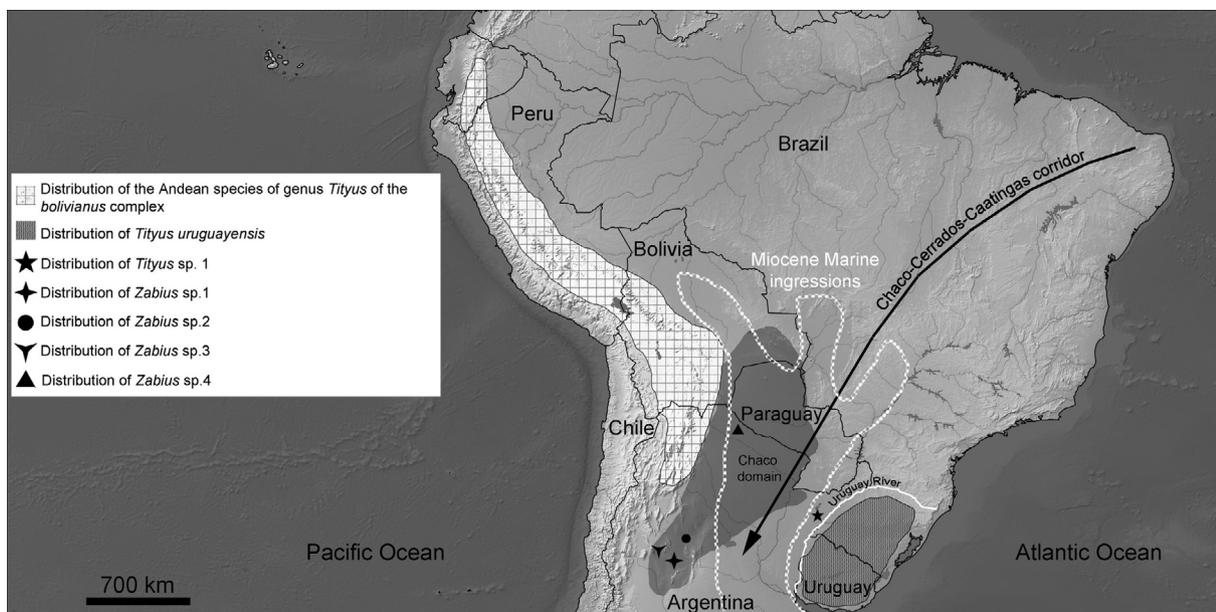
Fig. 2. Distribution map of the species of *Tityus* of the *bolivianus* complex, and the new species of *Zabius* mentioned in this contribution. Approximate area occupied by Miocene marine ingressions is indicated with a white relief. Chaco-Cerrado-Caatingas corridor is indicated with a dark arrow. Chaco Domain, (or phytogeographic province), is indicated in grey. Uruguay River is indicated with a white line.

record of Buthidae in America, as well as with the only previous dated *Tityus* phylogeny. According to Borges et al. (2010) the diversification process of the Venezuelan species of *Tityus* of the *Atreus* group, can be traced no further than 18 Ma. The age of buthid amber fossils from Central America and the Caribbean, several of which have been assigned to genus *Tityus*, ranges from 20 to 40 Ma (Santiago-Blay and Poinar, 1988, 1993; Santiago-Blay et al., 1990; Lourenço, 2009; Riquelme et al., 2015). The oldest known buthid fossil in America, *Uintascorpio haladrasorum* Perry, 1995, dates from a period no earlier than the early to mid-Eocene (Santiago-Blay et al., 2004).

Two other remarkable facts indirectly, but strongly, support the Paleogene-African dispersal of American buthids: (1) first is the intriguing absence of buthids from the deserts of the Pacific coast of South America, as the Atacama and Sechura deserts, which are among the oldest and more stable deserts in the world (Ceccarelli et al., 2016b). Temperate and subtropical arid environments are known for harboring a very high diversity of scorpions. This is also the case of the Pacific coastal deserts of South America, which present one of the highest diversity of scorpions of the continent (Agusto et al., 2006; Pizarro-Araya et al., 2014; Ojanguren-Affilastro et al., 2015). Remarkably, representatives of the family Buthidae are absent from this entire area, and from most of the area west of the Andes, with records only in the north-western part of the continent (Brito and Borges, 2015) or in high altitude forests and grasslands (Ochoa, 2005). If this family would have been present in South America before the Andean uplift, approximately 50 Ma, one would expect the presence of at least some representatives of Buthidae in the area between central Chile, and west central Peru, since at this latitude to the east of the Andes this family is well represented (Ojanguren-Affilastro, 2005). However, it seems that the central and southern Andes have acted as an effective barrier for Buthidae. An explanation for this may be that this family colonized the American continent after the uplift of the Andes. It is unlikely to think about a massive extinction process that would have only affected representatives of Buthidae that remained isolated on the west side of the Andes, and not Bothriuridae and Caraboctonidae. (2) Additionally, it is remarkable that no buthids have been found between the numerous scorpion fossils of the Crato *Lagerstätte* in northern Brazil (Carvalho and Lourenço, 2001). This area was a paleo-lake of the early cretaceous (110 Ma) from a Caatinga like environment (Menon, 2007), a kind of habitat where Buthidae is by far the dominant scorpion family nowadays. In this *Lagerstätte* only fossils of Chactids and Hemiscorpiids have been found (Menon, 2007), being both families also still present in similar environments of the area (Lourenço, 2002).

Our intention here is not to reject any previous hypothesis for the origin of the *Tityus* group, but to put forward for the first time, an alternative hypothesis, suggesting that this group could have followed the same Paleogene/trans-Atlantic ingression route followed by the ancestors of several other groups of American fauna.

The presence in America of *Ananteris* group buthids cannot be accurately explained historically with our current data. Our results only show a split between the Asian genus *Lychas* and the American genus *Ananteris* about 40 Ma, as well as a very recent split between two marginally distributed species of *Ananteris* about 5 Ma. These results may also be temporally congruent with a Paleogene-African ingression, but also with other scenarios. Broader taxon and molecular marker sampling is required to adequately test the hypotheses proposed in this study and to improve our understanding of the history of this group.

Acknowledgements

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.2017.02.018.

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