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Análise filogenética do grupo de espécies *Tityus clathratus* e outros grupos e subgêneros de *Tityus* (Scorpiones: Buthidae) baseada em caracteres moleculares e morfológicos

Phylogenetic analysis of the *Tityus clathratus* species group and other *Tityus* groups and subgenera (Scorpiones, Buthidae) based on molecular and morphological characters

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RESUMO

Entre os gêneros de bútideos, *Tityus* é o gênero mais diverso (>220 species). No entanto, apesar de sua riqueza, ampla distribuição e importância médica, pouca atenção tem sido dada ao estudo de suas relações filogenéticas. Nosso estudo teve como objetivo testar a monofilia de *Tityus* e seus subgêneros e grupos de espécies, com ênfase especial no grupo de espécies *Tityus clathratus*. Para isso, usamos 162 caracteres fenotípicos e ~3618 pares de bases de nucleotídeos de três marcadores mitocondriais (12S, 16S e COI) e dois nucleares (18S e 28S). Além disso, incluímos 161 terminais (141 terminais de *Tityus*/ 88 terminais do grupo de espécies *Tityus clathratus*), representando 58 espécies de *Tityus* (25% da riqueza do gênero). Os outros 20 terminais foram compostos por gêneros de butídeos do grupo externo distribuídos principalmente na América do Sul, cada um dos quais foi representado por duas espécies (exceto *Ananteris*). Matrizes moleculares e de evidencia total foram analisadas individualmente usando máxima verossimilhança (IQTREE) e máxima parcimônia com homologia estática (TNT) e homologia dinâmica (POY) para dados moleculares. Em geral, as hipóteses obtidas foram muito semelhantes, independentemente dos critérios de otimalidade e do esquema de homologia molecular empregados. Em todas as árvores: i) *Tityus* foi monofilético e teve dois clados principais, ii) *Mesotityus* foi sinônimo de *Tityus*, iii) *Microtityus* foi o grupo irmão de *Tityus* (exceto na árvore MP-SH-TE), iii) *Tityus* (*Mesotityus*) foi monofilético, e vi) *Ananteris* foi o grupo irmão dos demais gêneros analisados. Adicionalmente, observamos um aumento nos valores de suporte na análise de evidencia total comparada com a análise de dados moleculares, em ambos os critérios de otimalidade. Curiosamente, as relações dos gêneros do grupo externo, relações internas de *Tityus* (*Mesotityus*) e as posições de alguns terminais de *Tityus* (*Atreus*) e *Tityus* (*Tityus*) foram sensíveis ao critério de otimalidade e ao esquema de homologia molecular empregados. Além disso, com base em nossos resultados, propusemos um novo subgênero; três novos grupos de espécies; uma nova espécie de *Tityus* (*Mesotityus*): *Tityus crypticus* **sp. n.**; dois grupos de espécies sinônimos, e sete sinônimos de espécies do grupo de espécies *Tityus clathratus*. No total, 14 grupos de espécies de *Tityus* foram recuperados em nossas análises. Finalmente, para permitir o uso taxonômico de nossa filogenia, propusemos uma chave de identificação para gêneros de butídeos do Novo Mundo e grupos de espécies de *Tityus*. Este estudo foi a terceira análise de evidência total já feita na família Buthidae e a primeira a se concentrar nos terminais *Tityus*. No entanto, estudos filogenéticos adicionais, incluindo mais evidências (fenotípicas e genotípicas) e mais terminais de *Tityus*, são necessários para propor uma futura hipótese filogenética mais abrangente deste diverso gênero Neotropical.

ABSTRACT

Among the buthid genera, *Tityus* is the most species-rich genus (>220 species). Nevertheless, despite its richness, wide distribution, and medical importance, insufficient attention has been paid to the study of its phylogenetic relationships. Our study aimed to test the monophyly of *Tityus* and its subgenera and species groups, with a special emphasis on the *Tityus clathratus* species group, using a total evidence approach. To do so, we used 162 phenotypic characters and ~3618 nucleotide base pairs from three mitochondrial (12S, 16S, and COI) and two nuclear (18S and 28S) markers. Also, we included 161 terminals (141 terminals of *Tityus*/ 88 terminals of the *Tityus clathratus* species group), representing 58 *Tityus* species (25% of the genus richness). The other 20 terminals were composed of outgroup buthid genera mainly distributed in South America, and each genus was represented by two species (except for *Ananteris*). Molecular-only and total evidence matrixes were individually analyzed using maximum likelihood (IQTREE), and maximum parsimony with static homology (TNT) and dynamic homology (POY) for the molecular data. In general, the hypotheses obtained were very similar, independently of the optimality criteria and molecular homology scheme employed. In all the trees: i) *Tityus* was monophyletic and had two main clades, ii) *Mesotityus* was a synonym of *Tityus*, iii) *Microtityus* was the sister group of *Tityus* (except for the MP-SH-TE tree), iii) *Tityus* (*Mesotityus*) was monophyletic, and vi) *Ananteris* was the sister group of the other genera analyzed. Additionally, we observed an increase in the support values in the total evidence dataset analysis compared to the molecular-only dataset analysis, in both optimality criteria. Interestingly, outgroup genera relationships, *Tityus* (*Mesotityus*) internal relationships, and the positions of a few terminals of *Tityus* (*Atreus*) and *Tityus* (*Tityus*) were sensible to the optimality criteria and molecular homology scheme employed. Also, based on our results, we proposed: a new subgenus; three new species groups; a new species of *Tityus* (*Mesotityus*): *Tityus crypticus* **sp. n.**; two species groups synonyms, and seven species synonyms of the *Tityus clathratus* species group. In total, 14 *Tityus* species group were recovered in our analyses. Finally, to allow the taxonomic use of our phylogeny, we proposed an identification key to New World buthid genera and *Tityus* species groups. This study was the third total evidence analysis ever done in the family Buthidae and the first to focus on *Tityus* terminals. Nevertheless, additional phylogenetic studies, including more evidence (phenotypic and genotypic) and *Tityus* terminals, are needed to propose a future comprehensive phylogenetic hypothesis of this diverse Neotropical genus.

INTRODUCTION

Scorpiones is an order of widely distributed arachnids with 2557 valid species (extant and fossil) grouped into >233 genera (Rein 2021). The phylogeny and classification of scorpions have been historically controversial at suprageneric levels (Stockwell 1989; Soleglad & Fet 2003; Prendini & Wheeler 2005). The number of families recognized by different authors range from 18 to 22, most not supported by phylogenetic evidence but authority (Prendini & Wheeler 2005). Despite this, the use of phylogenetic systematics in scorpion classification has demonstrated to be quiet useful to the understanding of phenotypic evolution (e.g., Stockwell 1989; Prendini 2004b; Prendini & Wheeler 2005; Mattoni *et al.* 2012; Ochoa *et al.* 2013; Santibáñez-López *et al.* 2014; Ojanguren-Affilastro & Mattoni 2017).

Such diversity is also expressed in terms of phenotypic characters associated with metasoma and pedipalp carination, hemispermatophore morphology, and trichobothrial patterns have demonstrated informative to unveiling historical relationships among scorpions (Prendini 2000; Soleglad & Fet 2003; Prendini & Wheeler 2005). More recently, with the availability of massive DNA sequences and genomes, some studies have focused solely on the genotype (e.g., Teruel *et al.* 2006; Borges *et al.* 2010; Sharma *et al.* 2015; Ojanguren-Affilastro *et al.* 2017b). However, few studies have combined molecular sequences with morphological data (Santibáñez-López *et al.* 2014; Esposito *et al.* 2017, 2018) and this is particularly true in contributions dealing with the family Buthidae Koch, 1837. A family that holds 44 species of the medically relevant species to humans worldwide (Borges & Graham 2016).

1.1. About the systematics of Buthidae and *Tityus*

Buthidae is the most diverse family of the order, with more than 1216 species (extant and fossil) and 95 genera (Rein 2021). Their species are Worldwide distributed (except Antarctica) (Borges & Graham 2016). However, contributions that have used phenotypic data to infer phylogenetic relationships of buthid genera are surprisingly scarce. During the last 20 years, a period comprising a vast majority of the published phylogenies dealing with buthid taxa (41 papers; 60.98% published between 2011–

2020), near a half included evidence of a single marker (46.34%), 21.95% included two markers (usually 16S and COI), and 12.20% included three or more than three markers (Appendix 1; Figure 1B). However, only 14.6% of the papers codified morphology (Appendix 1; Figure 1A, B). These works corresponded to Prendini (2001b, 2003, 2004a; b) and Prendini & Esposito (2010) who analyzed two African genera, and Francke *et al.* (2014) that studied New World microbuthid genera (i.e., *Alayotityus*, *Chaneke*, *Microtityus*, *Tityopsis*, and *Zabius*) using 30 characters.

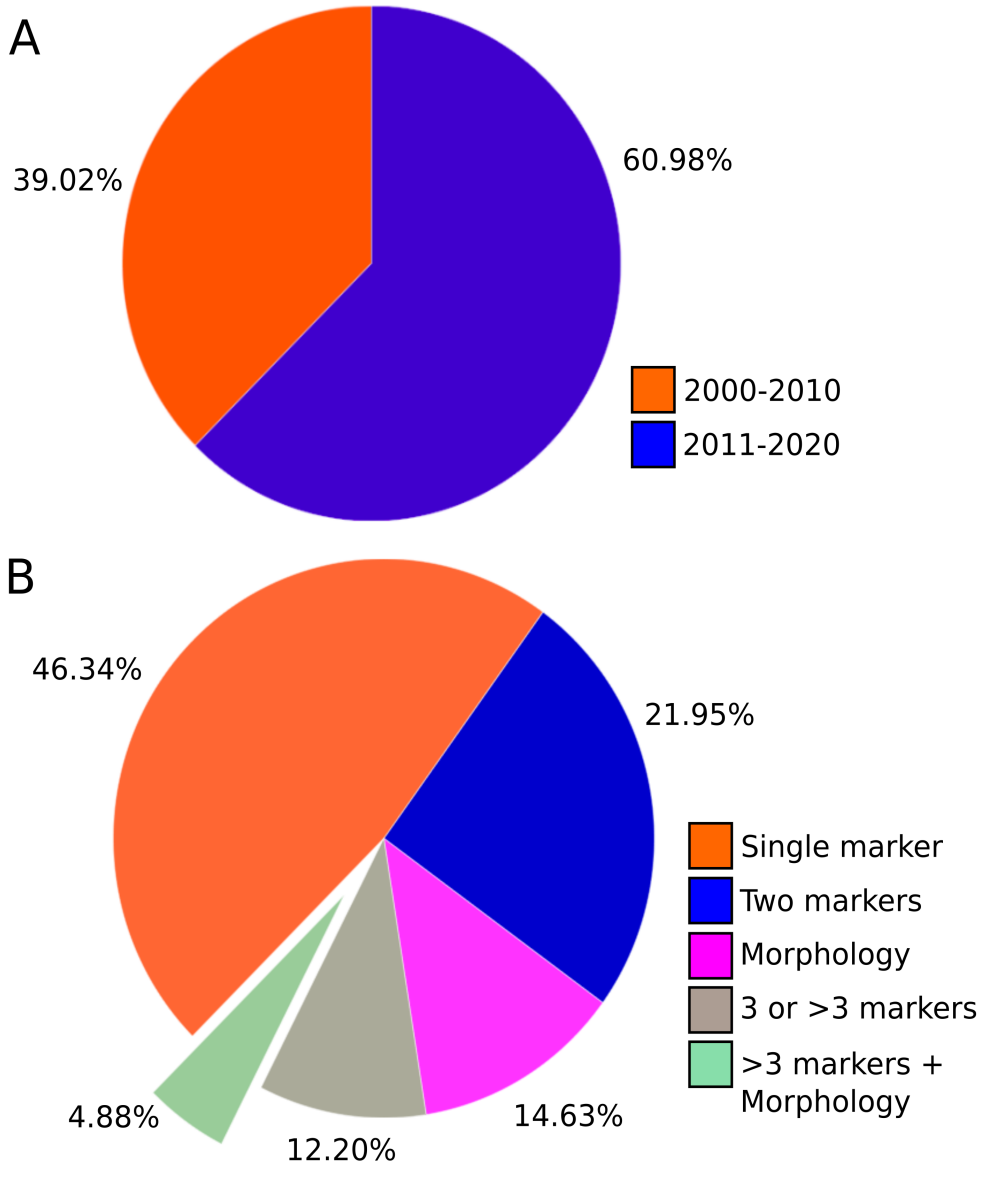

Figure 1. Compilation of 41 publications dealing with buthid taxa phylogenies of 2000–2020. **A.** Proportion of publications between 2000–2010 and 2011–2020. **B.** Proportion of sources of evidence used. **Observation=** morphology was only considered if codified into a matrix.

On the other hand, only 5% of the publications codified morphology together with molecular evidence, and this percent corresponded to Esposito *et al.* (2017, 2018). In these papers, Esposito *et al.*, codified 90 morphological characters and sequenced five Sanger loci (12S, 16S, 18S, 28S, and COI) for Neotropical club-tailed scorpion genera (i.e., *Heteroctenus*, *Ischnotelson*, *Jaguajir*, *Physoctonus*, *Rhopalurus*, and *Troglorhopalurus*), then analyzed both sources of evidence using a total evidence approach using three different optimality criteria (i.e., bayesian inference, maximum likelihood, and maximum parsimony).

However, if compared to the number of buthid genera and species, phylogenetic works that have been made are far from enough. For example, only four publications have dealt with *Tityus,* the most species-rich genus of the order, but they are usually based on a few sanger markers and a handful of species. Borges *et al.* (2020) (21 *Tityus* spp.) used venom sodium channel-active toxin (NaTx) and Borges & Graham (2016) (26 spp.) and Román *et al.* (2018) (27 spp.) used 16S and COI sequences to reconstruct their phylogenetic hypotheses, while Ojanguren-Affilastro *et al.* (2017b) (18 *Tityus* spp.) included more than three markers (16S, 28S, COI, D3, H3a). Thus, to improve our knowledge on the morphological evolution and phylogeny of *Tityus*, it is essential to perform additional phylogenetic analyses using a total evidence approache.

1.2. The confused taxonomy of *Tityus*

Among the Neotropical buthid genera, *Tityus* represent the most species-rich, with more than 220 species (Francke & Stockwell 1987; Fet & Lowe 2000; Souza *et al.* 2009; Lourenço 2006, 2015). Its distribution is broad, ranging from the Dominican Republic to Central Argentina (Francke & Stockwell 1987; Armas & Antún 2004; Souza *et al.* 2009). *Tityus* also contains several species (23 spp.) that have been considered as dangerous to humans due to their potent venoms and involvement in scorpionism cases (Lourenço 2011, 2015; Borges & Graham 2016). Nevertheless, despite their wide distribution and medical importance, insufficient attention has been paid to the detailed phenotypic study of *Tityus* species. As a result, *Tityus* is considered as one of the most taxonomically confused genera (Kraepelin 1911; Fet &

Lowe 2000; Souza *et al.* 2009; Ojanguren-Affilastro *et al.* 2017a; Moreno-González *et al.* 2019).

Attempts to organize started with the pioneering work of Kraepelin (1911) who presented the first proposal for species groups. He established three main morphological species groups based on coloration patterns, total length, and the dilatation degree of the female pectinal basal middle lamellae: *Tityus bolivianus* Kraepelin, 1895; *Tityus cambridgei* Pocock, 1897, and *Tityus columbianus* (Thorell, 1876) species groups (Table 1). However, later on, Kraepelin observed a high degree of variation in color patterns and concluded that color was an unsatisfactory character to establish groups and at the same accepted he was unable to find new morphological characters (Lourenço 2015). More than 20 years later, Mello-Leitão (1931; 1939; 1945) proposed 15 species groups (Table 1) using some of the characters proposed by Kraepelin (1911) but adding dorsolateral and ventral metasomal carinae development. This classification remained settled until González-Sponga (1996), who recognized, for the Venezuelan fauna, the groups *Tityus androcottoides* and *Tityus nematochirus,* and created the *Tityus discrepans* species group (Table 1) to accommodate some species that presented a single ventromedian carina on metasomal segments II–IV.

Then, Fet & Lowe (2000) presented a updated list of *Tityus* species group classifications (i.e., *T. androcottoides*, *T. asthenes*, *T. bahiensis*, *T. bolivianus*, *T. clathratus*, *T. crassimanus*, *T. magnimanus*, *T. melanostictus*, *T. stigmurus*, and *T. trivittatus*) (Table 1). However, Lourenço (2002a; b) ignored most of these classifications and returned to the proposal of Kraepelin (1911), using his same characters, adding the subaculear tubercle development and ignoring the metasomal carinae characters proposed by Mello-Leitão (1945) and González-Sponga (1996). In his classification, Lourenço recognized three main groups each one named after the oldest described species in each one: *Tityus asthenes* Pocock, 1893, *Tityus bahiensis* Perty, 1933 and *Tityus clathratus* C. L. Koch, 1844. Posteriorly, Lourenço (2006) formalized his new classification into five subgenera, using coloration, total size, dilation degree of the basal middle lamellae of female pectines, the shape of the subaculear tubercle, and development of the pectinal fulcra: *Tityus* (*Archaeotityus*) Lourenço, 2006; *Tityus* (*Atreus*) Gervais, 1843; *Tityus* (*Brazilotityus*) Lourenço,

2006; *Tityus* (*Caribetityus*) Armas & Marcano-Fondeur, 1992; and *Tityus* (*Tityus*) Koch, 1836 (Table 1). However, for 14 years, this subgeneric classification was not tested under a phylogenetic framework, and the use of species groups prevails (e.g., Borges *et al.* 2010; Ojanguren-Affilastro *et al.* 2017a).

Nowadays, the taxonomy of *Tityus* faces great challenges. The large number of *Tityus* species, their high phenotypic similarity, and the few somatic characters that have been proposed to delimit species-groups and species, make *Tityus* one of the most challenging genera to work with. *Tityus* species have traditionally been delimited based on differences in **i)** total body size; **ii)** coloration patterns; **iii)** pectinal tooth count; **iv)** number of chela movable finger oblique rows of denticles; **iv)** morphometric proportions of male pedipalp and metasoma; **v)** vertical development and/or array of the metasoma and pedipalp carinae, and **vi)** the shape of the subaculear tubercle (e.g., Lourenço 1984, 2002a; b). However, some of these somatic characters, such as counts and morphometric proportions, frequently overlap among different species, which makes it even harder to set boundaries among species (Prendini 2001a; Teruel & García 2008a; b; Moreno-González *et al.* 2019).

Therefore, *Tityus* faces considerable taxonomic impediment due to the lack of studies that explore new phenotypic characters. Though, it can be also attributable to the absence of funding to taxonomic studies in countries where this genus is distributed and the reduced number of systematic and taxonomic revisions published by scorpiologists, whom concentrated their efforts mainly on the descriptions of new taxa during the last 70 years. Other factors that have significantly contributed to this impediment are **i)** the use of juveniles to describe new species (e.g., *T. adisi* Lourenço & Pézer, 2002; *T. canopensis* Lourenço & Pézer, 2002; *T. prancei* Lourenço, 2000; *T. rionegrensis* Lourenço, 2006), and the lack of **ii)** detailed phenotypic descriptions; **iii)** genotypic data, and **iv)** comparative diagnoses. Also, another factor that contribute to this impediment is the poor quality and low number of images depicting character states, for a vast majority of the described species.

Recently, Ojanguren-Affilastro *et al.* (2017a) presented an integrative taxonomical approach of focal *Tityus* species, using different evidence sources (i.e., sanger

sequences, phenotypic characters, and karyotypes) to describe a new species, *Tityus curupi* (Paraje Tres Cerros, Northeastern Argentina). Lately, an attempt to contribute to the taxonomic downfalls of *Tityus* was presented by Moreno-González *et al.* (2019), who tested traditional and new phenotypic characters as well as morphometric ratios to set boundaries among *Tityus clathratus* species group members (as *Tityus* (*Archaeotityus*)) from Colombia. However, additional efforts that incorporate new phenotypic characters and genotypic evidence are urgently needed to improve our current taxonomic understanding of *Tityus.*

1.3. Briefly history about the systematics and taxonomy of *Tityus clathratus* species group

The species that gave the name to what is nowadays known as the *Tityus clathratus* species group (previously *Tityus* (*Archaeotityus*) and now *Tityus* (*Mesotityus*)) was described from Cabo da Boa Esperança (Cape of Good Hope) in South Africa as *Tityus clathratus* C. L. Koch, 1836. However, this locality was traditionally attributed to a mislabelling (e.g., Mello-Leitao 1945). And only recently, Lourenço (2012) estimated, based on a detailed study of the field expeditions performed in the early years of 19 century by European naturalists to Northern South America, that the most probably type locality of *Tityus clathratus* is somewhere near the coast of Venezuela.

In Venezuela, however, González-Sponga (1981) described a monotypic genus, *Mesotityus vondageli*, based on several apparently exclusive putative synapomorphies, despite its close phenotypic similarity to *Tityus clathratus*. After it, Lourenço (1984) presented the first taxonomic revision of the *Tityus clathratus* group. In it, he included a list of valid species (6 spp.), phenotypic characters to species distinction (e.g., subaculear tubercle shape, dilatation degree of the basal middle lamellae of female pectines, etc.), three synonyms of *Tityus clathratus* (i.e., *Tityus guianensis* Di Caporiacco, 1947; *T. quelchi* Pocock, 1893, and *Tityus fahrenholtzi* Roewer, 1943), and the description of a new species: *Tityus bastosi.* Later on, in between 1980–2000 some species were described and included into the *Tityus clathratus* species group: *Tityus ocelote* Francke & Stockwell, 1987 from Costa Rica (Francke & Stockwell 1987); *Tityus tayrona* Lourenço, 1991, *Tityus betschi* Lourenço 1992, and *Tityus erikae* Lourenço 1999 from Colombia (Lourenço 1991, 1992, 1999).

While others, such as *Tityus parvulus* Kraepelin, 1914 were redescribed (Lourenço 2000).

During the first decade of the 2000s, the tendency was pretty much the same, with an increasing number of species descriptions using the same phenotypic characters proposed by Lourenço (1984): *Tityus julianae* from Ecuador (Lourenço 2005); *Tityus kaderkai* Kovarik, 2005 from Bolivia (Kovarik 2005); *Tityus mraceki* from Colombia (Kovarik 2007); *Tityus maranhensis* from Brazil (Lourenço *et al.* 2006); *Tityus rondonorum* and *Tityus wayuu* from Venezuela (Rojas-Runjaic & Armas 2007). Also, the morphology of *Mesotityus vondageli* was reassessed by Armas & Rojas-Runjaic (2006) who argued that despite some putative exclusive synapomorphies (e.g., pedipalp trichobothrial patterns, shape of spiracles, and distoterminal macrosetae on pedipalp tips) *Mesotityus* was highly similar to members of the *Tityus clathratus* species group. Nevertheless, Rojas-Runjaic & Armas kept it as a valid genus until more analyses were performed. Finally, during the second decade of 2000, efforts to describe new species continued with the description of *Tityus mana* Lourenço, 2012 from French Guiana; *Tityus grahami* Lourenço, 2012 from Brazil (Lourenço 2012), and *Tityus wachteli* Kovarik, Teruel, Lowe a & Friedrich, 2015 from Peru (Kovarik *et al.* 2015). In general, the tendency was a growing number of species descriptions without any improvement in the phenotypic characters to diagnose species.

Recently, some taxonomic works have been published such as Lourenço (2016) who synonymized *T. wachteli* under *T. silvestris* arguing the polymorphic nature of the second species, and Teruel *et al. (2017)* who revalidated *T. wachteli* based on the absence of compelling morphological arguments by Lourenço (2016). Also, not so long ago, Moreno-González *et al.* (2019), performed a comprehensive revision the *Tityus clathratus* species group from Colombia (as *Tityus* (*Archaeotityus*)) using traditional (e.g., pectinal tooth and movable finger denticle rows counts; morphometric ratios; development of dorsosubmedian carinae of the metasoma; and shape of subaculear tubercle) and new morphological characters (e.g., a glandular region in the pectinal basal plate in females and metasomal macrosetae) to establish species boundaries in the Colombian fauna. Finally, Ythier *et al.* (2020) published a

new species *Tityus kukututee* (Suriname) mainly based on coloration patterns, but ignored other phenotypic characters proposed by Moreno-González *et al.* (2019). So, considering this panorama and the lack of studies, a comprehensive taxonomic revision and phylogenetic analysis of the *Tityus clathratus* species group is essential and could significantly contribute to the knowledge of *Tityus*.

CONCLUSIONS

-Even though our phenotypic characters (162 chars.) only comprised around 5% of the total concatenate matrix (molecular matrix ~3618 bp), we observed an increase in overall support values in the total evidence analyses compared to the molecular-only analyses, in both optimality criteria (maximum likelihood and maximum parsimony).

-The outgroup genera relationships, the international relationships of the *Tityus clathratus* species group, and the positions of a few terminals within *Tityus* (*Atreus*) and *Tityus* (*Tityus*) were highly dependent on the optimality criteria (maximum likelihood or parsimony) and molecular homology scheme (dynamic homology vs. static homology) employed.

-The genus *Microtityus* is the sister group of *Tityus* and this relationship is supported by unambiguous molecular synapomorphies.

-The monotypic genus *Mesotityus* González-Sponga, 1981 **syn. n.** is a junior synonym of *Tityus*.

-*Tityus* and *Tityus* (*Mesotityus*) were recovered as monophyletic.

-Two species groups were synonimized and three new species groups were proposed.

-The *Tityus* clade was composed of two main clades (A and B).

-Species such as *Tityus michelii* and *T. smithii* still await species group designation. It was not possible to examine the morphology of both species, albeit their phylogenetic positions did not significantly varied between datasets and optimality criteria.

-Based on our hypotheses, a new species is described: *Tityus* (*Mesotityus*) *crypticus* **sp. n.**. This species is more closely related to *Tityus* (*Mesotityus*) *silvestris* than to any other species.

-Seven species of *Tityus* (*Mesotityus*) were considered as synonyms.

-*Tityus clathratus* was recovered as paraphyletic in MP-DO-TE and ML-SH-MOL

trees. But, was recovered as monophyletic in the MP-SH-MOL, MP-DO-MOL, MP-SH-TE, and ML-SH-TE.

-*Tityus aff. clathratus*, *Tityus aff. mattogrosensis*, and *Tityus aff. ocelote* constituted cryptic species only supported by unambiguous molecular synapomorphies.

-Almost all clade nodes within *Tityus* were supported by unique and homoplastic phenotypic synapomorphies. Besides, some species groups presented several internal nodes only supported by unambiguous molecular synapomorphies.

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