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As relações filogenéticas de *Hyloxalus* Jiménez de la
Espada 1870 (Anura: Dendrobatidae)

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EXEMPLAR CORRIGIDO

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Resumo

Com 63 espécies, *Hyloxalus* é o gênero mais diverso de rãs venenosas neotropicais da superfamília Dendrobatoidea. As espécies deste gênero habitam diversos ecossistemas desde o nível do mar até os ecossistemas alto-andinos, da Colômbia, Equador e Peru. Há vários problemas na sistemática e na filogenia deste grupo, como por exemplo: taxonomia não resolvida de diferentes complexos de espécies, falta de sinapomorfias fenotípicas para o gênero, inconsistências entre filogenias propostas quanto às relações de parentesco, variação fenotípica subestimada mesmo se conhecendo variação notável na morfologia (e.g., espécies menores sem membrana no pé, e com linhas laterais, ou espécies maiores com membrana no pé, mas sem linhas laterais), além de que o 50% das espécies (31 espécies) atribuídas ao gênero tentativamente. Além disso, há déficit no conhecimento da diversidade, na filogenia, e na evolução de *Hyloxalus*, o que se torna evidente quando se compara este grupo com outros gêneros de dendrobatoídeos (e.g., *Allobates*). Assim, neste estudo são avaliadas as relações filogenéticas de *Hyloxalus*, e também se tenta resolver alguns desses problemas mencionados, utilizando como fontes de evidência caracteres de adultos e de girinos, e também sequências de DNA. Para isso, a morfologia e a anatomia da maioria de espécies do gênero e de outros dendrobatoídeos foi explorada e qualificada para procurar variação independente, a fim de se testar caracteres e hipóteses de homologia já propostos, e além de formular novos caracteres filogenéticos. Novas sequências de DNA foram geradas tanto para cinco espécies até então não amostradas, como para novas amostras de espécies já conhecidas deste ponto de vista. Sendo assim, essa evidência foi montada em um dataset junto com outros dados de evidência molecular e fenotípica disponível de representantes de espécies de *Hyloxalus* e outras rãs venenosas. Este dataset foi explorado para testar a monofilia de *Hyloxalus* e suas relações filogenéticas em uma análise de evidência total utilizando a máxima parcimônia como critério de otimalidade.

De acordo com os objetivos e os resultados, esta tese foi dividida em três capítulos: O primeiro deles discorre sobre a posição filogenética e avaliação do grupo de *Hyloxalus edwardsi* e sinapomorfias do mesmo. Os resultados mostram que, como foi suposto, o grupo é parte de *Hyloxalus*, irmão da espécie cis-Andina *H. picachos*. São propostas novas sinapomorfias fenotípicas para o grupo além da descrição de uma nova espécie para o mesmo. No segundo capítulo, é realizada a mais completa análise filogenética de *Hyloxalus* até hoje. A análise inclui 326 terminais em total, dos quais o grupo interno, *Hyloxalus*, inclui 189 como representantes de 38 espécies nominais e de 24 de espécies não descritas do gênero. O grupo externo inclui 137 terminais como representantes de 110 espécies de outros dendrobatoideos bem como de outras famílias. A hipótese recupera algumas relações monofiléticas bem suportadas dentro do grupo interno, mas demonstra que *Hyloxalus* é parafiletico. Os clados internos de *Hyloxalus* estão sustentados com sinapomorfias fenotípicas não ambíguas, além de outros caracteres fenotípicos que otimizam como sinapomorfias em distintos clados em Dendrobatoidea. Também é revelado uma diversidade críptica em espécies de ampla distribuição no gênero. Nestes dois primeiros capítulos, também foi examinado o efeito de grupo externo na topologia e nas hipóteses de homologia do grupo interno, utilizando o método de expansões sucessivas do grupo externo (ou successive outgroup expansion em inglês). Para isso foram feitas seis e nove análises no primeiro e segundo capítulo, respetivamente. Nestas análises, o grupo externo foi ampliado gradativamente com a adição de terminais selecionados que tenham a capacidade de refutar as hipóteses. Em cada rodada a topologia do grupo interno é afetada em diferentes níveis de inclusão, e em alguns casos, a monofilia de *Hyloxalus* é rejeitada. No terceiro capítulo, é estudada a histologia do tegumento e a homologia da glândula preta do braço em seis espécies do gênero. Essa glândula pode ser vista como um inchaço no braço, um caracter homoplástico historicamente relevante para a sistemática do gênero, com a hipótese de ter a função associada ao amplexo nupcial. Os cortes histológicos revelam glândulas exócrinas especializadas e modificações na derme e na hipoderme na região do

referido inchaço da maioria das espécies estudadas. A variação da morfologia externa e do tegumento foi codificada em novos caracteres. Os caracteres de morfologia externa foram otimizados na filogenia obtida no anterior capítulo e revelaram aquisições independentes em *Hyloxalus* e, portanto, homoplasias. Entretanto, um dos tipo de inchaço do braço encontrados é derivado e compartilhado pelo grupo de espécies *H. puchellus*. A análise dessas modificações tegumentárias dá suporte para a hipótese funcional de o inchaço participar no amplexo nupcial. Outros tipos de estudo tais como histoquímica, bioquímica e de história natural são necessários para entender a evolução e função do inchaço. Os resultados demonstram que a sistemática de *Hyloxalus* é uma área rica em evidencia para compreender as relações filogenéticas, a evolução e a biologia deste gênero e das rãs venenosas em geral.

Palavras-chave: Caracteres fenotípicos, Evidencia total, Expansões sucessivas do grupo externo, Dendrobatoidea, Hyloxalinae.

Abstract

With 63 species, *Hyloxalus* is the most speciose genus of the superfamily Dendrobatoidea, which comprises the neotropical poison-dart frogs and their relatives. Species of this genus inhabit diversity of environments from almost sea level to high-Andean ecosystems in Colombia, Ecuador, and Peru. The genus has several problems regarding its systematics and phylogeny. For example, unresolved taxonomy of species complexes, lack of the phenotypic synapomorphies for the genus, inconsistencies in the phylogenetic relationships between previous studies, underestimated phenotypic variation despite there are notorious morphological differences (e.g., small species without toe webbing and with lateral stripes, or large species with toe webbing and without lateral stripes), and a half of species (31 species) being assigned to the genus only tentatively. Moreover, progress about our knowledge about its diversity, phylogeny, and evolution is slow, which is evident when contrasting our knowledge of the group with other dendrobatoid genera (e.g., *Allobates*). Therefore, the present study evaluates the phylogenetic relationships of *Hyloxalus* and attempts to solve some of these problems, employing as evidence phenotypic characters of both adult and larvae as well as molecular DNA sequences. Accordingly, the morphology and anatomy of most species of this genus and several other dendrobatoids species were explored and assessed to search for independent variation. This variation was used to test characters and homology hypotheses previously formulated, and to propose new characters. Also, I generated molecular DNA data from five species that have never been sequenced before, and from additional populations of the other nominal species. All evidence was analyzed in a phylogenetic analysis together with the available phenotype and genotype data of

Hyloxalus and the poison frogs in general, in a total evidence analysis with parsimony as optimality criteria, to test the monophyly and the relationships of this genus.

Based on the aims and the results, this study was divided into three chapters, as follows: The first chapter evaluate the phylogenetic position and test the monophyly of the *Hyloxalus edwardsi* group and its synapomorphies. Results shows that this group is embedded within *Hyloxalus*, as proposed, sister to the cis-Andean species, *H. picachos*. New phenotypic synapomorphies for this group are provided, and a new species is described. The most comprehensive phylogeny of *Hyloxalus* to date is displayed in the second chapter, based on a total evidence approach and the direct optimization of genotypical and phenotypic evidence. The largest hypothesis contains 326 terminals, which 189 belong the ingroup, *Hyloxalus*, representing 38 nominal species (from a total of 63) plus 24 undescribed species of the ingroup. The outgroup has 137 terminals representing 110 species of Dendrobatidae and other anuran families. The resulting phylogeny shows a well-supported and monophyletic ingroup, where the known *Hyloxalus* is paraphyletic. Internal clades are supported by unambiguously optimized phenotypic synapomorphies, and other phenotype synapomorphies optimized for different taxonomical levels for other clades in Dendrobatoidea. Hidden diversity within this genus is recognized. Additionally, for the first and second chapters, the outgroup effect on the ingroup homology and topology hypotheses is examined by means of the successive outgroup expansion method. Either six and nine analysis of the sequential outgroup terminals addition were performed on each chapte, respectively. Ingroup topology is impacted at different inclusiveness levels in each round, even compromising *Hyloxalus* monophyly in some cases. In the third chapter, the homology and the integumentary structure of the homoplastic, dimorphic black arm gland—a relevant character for

systematic in *Hyloxalus*, because it is homoplastic, with hypothesize function related to the reproductive amplexus—is assessed in six species through the use of histological techniques. Serial cuts show that specialized hypertrophied exocrine glands together with other dermal and hypodermal modifications fill the arm swelling in most of the six species examined. New characters from the external morphology and the integument are proposed. Optimization of the external morphology character disclosed independent evolutionary acquisition of the arm swelling and therefore homoplasies. However, an external swelling type is derived and shared in the *H. pulchellus* group. Furthermore, the analysis of the integumentary features provide support for the proposed functional hypothesis, that is, the arm swelling is involved in the reproductive amplexus. Nevertheless, further histochemical, biochemical, and natural history studies are necessary to elucidate the evolution and function of the arm swelling. In general, all of these results highlight that *Hyloxalus* systematics is a rich source of evidence to understand the phylogenetic relationships, evolution, and biology of the genus and the poison frogs in general.

Key words: Phenotypic characters, Total evidence, Successive outgroup expansion, Dendrobatoidea, Hyloxalinae.

General Introduction

Hyloxalus Jiménez de la Espada, 1870 currently comprises 63 species (Frost 2021), making it the largest genus of the poison frogs, superfamily Dendrobatoidea. Most of the species of this genus exhibit a cryptic coloration (brown, black) with lateral stripes on their body; however, some species have bright coloration on the dorsal or ventral surface of the body. The species of this genus are distributed in northwestern South America, between Brazil, Colombia, Ecuador, and Peru countries. These occupy the Andes mountains and the adjacent Chocoan and Amazonian lowlands, occupying different habitats from the streams, ponds, grasslands, forest, and paramos, in almost sea level to above 4000 m. of elevation.

Hyloxalus is supported by abundant molecular synapomorphies, and their monophyly has been recovered recurrently since its resurrection (Grant et al. 2006, Santos et al. 2009, 2014, Pyron and Wiens 2011, Pyron 2014, Grant et al. 2017, Jetz and Pyron 2018, Guillory et al. 2019); however, internal relationships of the *Hyloxalus* have been reviewed partially (Páez-Vacas *et al.* 2010, Acosta-Galvis and Vargas-Ramírez 2018); moreover, there are inconsistencies in the topological position of some clades between previous studies. Thus for instance the topological placement of *H. nexipus*, *H. sylvaticus*, and *H. pulcherrimus* varies between phylogenies previously proposed (see Grant et al. 2006, 2017, Santos et al. 2009, 2014, Santos and Cannatella 2011, Pyron and Wiens 2011, Pyron 2014, Jetz and Pyron 2018, Guillory et al. 2019).

In this genus, some groups has been recognized by the shared presence of the morphological characters, but subsequent studies revealed that these are non-natural neither homologous. For example, the anal sheath in the *H. edwardsi* group (Lynch 1982), the black arm gland of the *H. ramosi* group (Grant and Castro 1998, Grant and

Ardila 2002, Grant et al. 2006, 2017), and the pale dorsolateral stripe of the *H. azureiventris* group (Grant et al. 2006). In other cases, some groups have been proposed on the basis of the genotype evidence; for example, the *H. bocagei* group (Páez-Vacas et al. 2010) and the *H. subpunctatus* group (Acosta-Galvis and Vargas-Ramírez 2018); nevertheless, the paraphyly was reported for the *H. bocagei* group (Grant et al. 2017).

Furthermore, in *Hyloxalus* some systematics problems are persistent. For example, more than one-third of the species (21 species of the genus) is known only from the original description and there are no additional data of these (e.g., *H. pinguis*, Rivero and Granados-Díaz 1990; see also Rivero 1991a, b; Rivero and Serna 1986, 2000 “1995” and Duellman 2004), complex species and species with a wide distribution has not been reviewed and their identity remains unclear (e.g., *Hyloxalus lehmanni*, *H. breviquartus*, *H. elachyhistus*, *H. littoralis*, *H. pulchellus*; Coloma 1995, Grant et al. 2006), and about half species of this genus are tentatively assigned to this based on phenotype resemblance only.

Added to this, unambiguously phenotypic synapomorphies are unknown for the genus, and various characters of adults of *Hyloxalus* (e.g., lateral stripes, extension of toe webbing, cryptic coloration, dorsal skin texture, swelling finger IV) are shared with species of the other genera, including of the family Aromobatidae. This difficult the assignation of the species to the genus without molecular data. However, the species of the *Hyloxalus* exhibit differences in external morphological (e.g., *H. chocoensis* have extensive toe webbing and lack of oblique, ventral, and dorsal lateral stripes, and *H. subpunctatus* with basal toe webbing and complete oblique lateral stripe), which can be used to test the *Hyloxalus* relationships.

Nevertheless, a few studies reported variation in the hyoid musculature, urogenital system, integument of the ventral skin, and also in behavior in *Hyloxalus* and other dendrobatoids (Trewavas 1933, Bhaduri 1953, Myers et al. 2012, Grant et al. 2017, Quiguango-Ubillús and Coloma 2008). Likewise, in tadpoles of this genus and also in superfamily there is variation in its morphology and anatomy, which has been proposed as characters (Myers 1987, Haas 1995, Haas 2003, Grant *et al.* 2006, Sánchez 2013, Dias et al. 2021); however, the tadpoles of 33 species of *Hyloxalus* remain unknown, and larval morphology, both external and internal, of the genus is poorly understood (Haas 1995, Anganoy-Criollo 2013). In addition, the review of the homoplastic characters of the superfamily revealed new characters, resulting in new synapomorphies at distinct levels for this group (e.g., Cavalcanti et al. 2022). Therefore, the study of this variation could provide new characters useful to test the *Hyloxalus* relationships.

Despite this, the recent phylogenies in Dendrobatoidea used only genomic data (e.g., Guillory et al. 2019, 2020, Muell et al. 2022), and the phenotype characters are less used and studied. Therefore, based on the above mentioned, I review the phenotype of the adult and larvae of the *Hyloxalus* to define new characters and to test the *Hyloxalus* relationships together with the genotype evidence. Phylogenetic results are used to reevaluate the taxonomy of this genus and to understand the phenotype evolution in this anuran group. The main aim of this study is to test the phylogenetic relationships of *Hyloxalus* employing distinct sources of evidence by mean a parsimonious total evidence analysis.

In the first chapter, the homology of the cloacal sheath and the phylogenetic position of the *Hyloxalus edwardsi* group are evaluated. I examined the adult morphology and the anatomy associated with the cloacal sheath. The phylogenetic

position of this group is corroborated by means of the new genotype data of a new species of this group, which is also described. Topological position of this species was severely tested by the successive outgroup expansion method (Grant 2019).

In the second chapter, the *Hyloxalus* phylogeny is evaluated with the increase of terminals and characters, both phenotype and genotype. For this, I reviewed the morphology of adults and larvae from most species of this genus and also several species across Dendrobatoidea to test the characters previously proposed, identify new characters, and test phylogenetic relationships of *Hyloxalus*. Moreover, five mitochondrial and five nuclear molecular markers of the unsequenced species and the representatives of the other populations and species are amplified. All new evidence generated, together with the phenotype and genotype data available for this genus was analyzed in a total evidence analysis using parsimony as optimality criteria to infer the phylogenetic relationships. The homology and topology hypotheses of the ingroup (*Hyloxalus*) are severely tested as possible through the successive outgroup expansion to identify the outgroup with the greatest chances of refuting the hypothesis because is expected an impact on the phylogeny with the addition of the new evidence (i.e., terminals and characters). The phylogeny with the greatest explanatory power is used to infer the *Hyloxalus* relationship. New unambiguous phenotype synapomorphies are provided for different clades within *Hyloxalus*, and also for other Dendrobatoidea clades. Phylogeny is used to propose a monophyletic taxonomy in *Hyloxalus*, and to analyze the phenotype evolution.

In the third chapter, the homology of the black arm gland—a dark arm swelling of the adult males of nine species of *Hyloxalus*—is evaluated, given this character was found as homoplastic within *Hyloxalus* previously (see Grant et al. 2017). External morphology and histology are used to characterize the integumentary

structure. These data provide evidence to propose characters both external morphology and integument and also test the glandular nature of this arm swelling. The external morphology characters are optimized in the largest *Hyloxalus* phylogeny generate in the second chapter to study their evolution. The systematic and biological implications of the findings are analyzed.

General Discussion and Conclusions

The revision of the *Hyloxalus* revealed that there are additional phenotypic characters in poison frogs. The variation found in this anuran group allowed me to reformulate some characters previously proposed, and also define new characters, which optimized as unambiguous synapomorphies at different clades of *Hyloxalus*, and of other clades of Dendrobatoidea (e.g., *Anomaloglossus*, *Ameerega*, Dendrobatoidea). For example, characters of the extrinsic musculature of the cloacal sheath are shared by the species of the *H. edwardsi* group; and the study of the arm swelling integument shows that there are heterospecific features in dermal and hypodermal layers and provided synapomorphies for a clade within of the *H. pulchellus* group. Thus, the phenotypic variation of *Hyloxalus* and Dendrobatoidea is a rich evidence source that can be useful both in systematic and phylogenetic studies, even more, in the present genomic era, where genotypic data tend to be common and widely used to infer relationships, while the phenotype evidence is increasingly limited.

The topological relationships within *Hyloxalus* were severely tested with the successive outgroup expansion. This revealed that with the addition of a single species the known *Hyloxalus* relationships are affected because the relationships of this species and its sister species changes topologically. This impact also was found when was incorporated more terminals and characters of the ingroup to the phylogeny; however, the impact was larger because some clades remain sensitive to the outgroup in the finals analysis rounds. Nevertheless, given most ingroup relationships become stable or insensitive in the three last expansion rounds, suggested that these changing clades in their topological position are due to the ingroup evidence of each clade (i.e., terminals and character) rather outgroup effect.

Thus the successive outgroup expansion allows identifying the weak regions of the tree where is necessary more evidence to unveil their relationships.

Three monophyletic large clades within *Hyloxalus* were identified: the *H. bocagei*, *H. pulchellus*, and the *H. subpunctatus* clades. These groups were recovered in the last three final rounds of the successive outgroup expansion, and also have been previously recovered (Grant et al. 2017). Likewise, other proposed Dendrobatoidea relationships were recovered. In addition to the molecular evidence, each of one these three groups are supported in phenotypic synapomorphies. Moreover, this study showed that there are additional undescribed species in each one of these clades, therefore their diversity is larger than formally described. On the other hand, with the available phenotype synapomorphies, some unsequenced species were allocated in one of these three clades, but in another case, this was not possible. Thus, on the basis of the phylogenetic evidence and the divrecuperatedersity of each clade, in Chapter 2 were proposed a new monophyletic taxonomy for *Hyloxalus* that mirror their phylogenetic relationships, recognizing *H. bocagei*, *H. pulchellus*, and *H. subpunctatus* clades as generic unit, as was predicted by Grant et al. (2006).

The evolutionary analysis of the phenotype shows that some synapomorphies are related to habitat. For instance, the *Hyloxalus* inhabiting riparian habitats have flat subarticular tubercles on hand and smooth skin on the ventrolateral body flanks; in change, those that occupy terrestrial habitats have projected subarticular tubercles on hand and granular ventrolateral body flanks. However, there are exceptions to this pattern outside *Hyloxalus*, because the terrestrial Dendrobatinae has flat subarticular tubercles. Unfortunately, the function for most phenotypic features in Dendrobatoidea is unknown or for a few cases, this is suspected. The life history is complex in poison frogs with elaborated reproductive behavior and social

interactions, but this knowledge come from a few groups (e.g., Oophaga, Dendrobates), and in *Hyloxalus* there is a larger gap in that because the behavioral data only have been described for four species, obfuscating the evolutionary study of the many phenotypic characters. To illustrate this, the reproductive function of the dimorphic arm swelling present in some *Hyloxalus* is suspected, given that the reproductive amplexus was documented for one species (*H. fascianigrus*) with arm swelling. Nonetheless, the use of the other tools and technicals can help to understand the character evolution, because in this case the histological characterization of the exocrine glands and the integument supply some insight into the reproductive function of this character. However, more natural history studies like phenotypic characters evaluations are recommended.

Finally, the *Hyloxalus* and dendrobatoids systematic is a fruitful field that supplies data to the continuous discussion in phylogeny and evolution of this anuran group and amphibians in general; additionally, these provide empirical evidence to the homology and characters theoretical debate. An interesting non-evaluated aspect in this study is the impact of the phenotypic evidence on phylogeny, which has provided arguments in favor of the phenotypic evidence in phylogenetic systematic. This is recommended for further studies. On the other hand, although this research treats to resolve some problems of the *Hyloxalus* systematic and phylogenetic, new questions arose from the results, invite to new and further tests in different aspects of poison frogs, such as taxonomic problems in complex species, relationships, phenotype evolution, natural history, and behavioral studies, among others biological aspect. Therefore, I expect that this study generated additional experiments and researches that enrich the knowledge of Dendrobatoidea.

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