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**Análise filogenética das rãs de
desenvolvimento direto (Anura, Terrarana,
Craugastoridae)**

**Phylogenetic analysis of the direct-developing
frogs (Anura, Terrarana, Craugastoridae)**

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Resumo

Uma grande tendência em estudos de sistemática filogenética é a reintegração de dados morfológicos em análises de evidência total. Até o momento, poucos estudos exploraram os efeitos da incorporação de dados morfológicos conjuntamente com dados moleculares em análises de evidência total. No entanto, aqueles que o fizeram concluíram que mesmo uma pequena base de dados fenômicos pode ter grandes e desproporcionais impactos nos resultados. Dada a demonstrada importância de caracteres morfológicos em testes de relacionamentos filogenéticos, aqui apresento um caso de exploração dos efeitos da incorporação de evidência morfológica como uma fonte independente de evidência filogenética e como importante “framework” para testar hipóteses suportadas por evidência molecular na superfamília Brachycephaloidea. Nesse estudo, 338 espécies de anfíbios foram incluídas, das quais 318 correspondem a espécies da superfamília Brachycephaloidea. Todos os gêneros conhecidos da superfamília foram amostrados. A base de dados completa inclui 13.686 caracteres moleculares (marcadores mitocondriais e nucleares) e 185 caracteres morfológicos, que foram analisados conjuntamente. Ademais, realizei análises adicionais modificando as bases de dados para avaliar os efeitos de amostragem de caracteres e táxons. Como resultado, encontrei que a superfamília Brachycephaloidea não é monofilética, como anteriormente considerada. Da mesma forma, a filogenia resultante da análise de evidência total mostrou que vários outros táxons também não são monofiléticos: as famílias Brachycephalidae e Craugastoridae, assim como os gêneros *Craugastor*, *Psychrophrynella* e *Pristimantis*. Através do exame detalhado do impacto de diferentes amostragens de caracteres e táxons sobre as relações filogenéticas na superfamília Brachycephaloidea, encontrei que a inclusão de dois táxons-chaves (*Atopophrynus syntomopus* e *Dischidodactylus duidensis*) tiveram grandes e desproporcionais impactos na topologia das árvores. Finalmente, eu apresento uma nova taxonomia para as rãs de desenvolvimento direto, reconhecendo cinco famílias, das quais três são morfológicamente diagnosticáveis (Eleutherodactylidae, Hypodactylidae e Strabomantidae), enquanto as outras duas são diagnosticáveis apenas com evidência molecular (Ceuthomantidae e Craugastoridae). Da mesma forma, 12 dos 26 gêneros inclusos são morfológicamente diagnosticáveis.

Abstract

A major recent trend in systematics is the re-integration of morphological data into total evidence analysis. To date, few studies have explored the effects of incorporating morphological and molecular data into total evidence analyses; however, those that have done so have found that even a comparatively small phenomic dataset can have disproportionately large impacts on results. Given the demonstrated importance of morphological characters in testing the phylogenetic relationships, herein I provide a case in point for the exploration of the effects of incorporating morphological evidence as an independent source of phylogenetic evidence, and an important framework to test hypotheses supported by molecular evidence, in the superfamily Brachycephaloidea. In this study, 338 amphibian species are included, of which 318 species correspond to the superfamily Brachycephaloidea. All known genera within the superfamily were sampled. The complete dataset included 13,686 molecular characters (mitochondrial and nuclear markers) and 185 morphological characters, which were analyzed together. In addition, I performed additional analyses modifying the complete datasets to evaluate the effects of character and taxon sampling. As results, I found that the superfamily Brachycephaloidea does not represent a monophyletic as previously thought. Likewise, the phylogeny resulting from this analysis showed several taxa to be nonmonophyletic: Brachycephalidae and Craugastoridae as well as genera *Craugastor*, *Psychrophynella*, and *Pristimantis*. From the detailed examination of the impact of both character and taxon sampling on the phylogenetic relationships of the superfamily Brachycephaloidea, I found that the inclusion of a comparatively small phenomic dataset (185 character) as well as the inclusion of two key taxa (*Atopophrynus syntomopus* and *Dischidodactylus duidensis*) had disproportionately large impacts on the tree topology. Finally, I provide a new taxonomy for this group. In this, I recognize five families, of which three are morphologically diagnosable (Eleutherodactylidae, Hypodactylidae, and Strabomantidae) whereas the other two are diagnosable solely with molecular evidence (Ceuthomantidae and Craugastoridae). Likewise, 12 out of 26 genera included are morphologically diagnosable.

Introduction

Among the most representatives anurans found in the new world are the frogs of the superfamily Brachycephaloidea, which counts with almost 1118 species and is the larger anuran superfamily in the world (Frost et al. 2018). The majority of the species in the superfamily are characterized by having direct development of terrestrial eggs rather than a free-living larval stage (Lynch 1971; Joglar 1989; Hedges et al. 2008), being an exception actually known the ovoviviparity in *Eleutherodactylus jasperi* (Drewry and Jones 1976) and *Craugastor laticeps* (McCranie et al. 2013). For many years, most species of Brachycephaloidea were placed in a single genus, *Eleutherodactylus*; however, with the implementation of multi-gene molecular phylogenies analyses, several arrangements have been proposed (e.g., Hedges et al. 2008).

Molecular data started to be employed to assess relationships among terraranan frogs with the Darst and Cannatella (2004). In that work, the author found that *Brachycephalus* was more closely related to members of the leptodactylid tribe Eleutherodactylini rather than to other hylids. Izecksohn (1988) had already argued that *Brachycephalus* and *Euparkerella*, another member of the tribe Eleutherodactylini, were closely related given their hook-like lateral processes in terminal phalanges. Posteriorly, Frost et al. (2006) also found *Brachycephalus* to be embedded within Eleutherodactylinae (previously tribe Eleutherodactylini), and therefore they transferred all recognized genera of subfamily Eleutherodactylinae to the family Brachycephalidae. Heinicke et al. (2007) performed the first comprehensive molecular phylogenetic analysis of terrarana, including a total of 276 species. That way, Heinicke et al. (2007) found big clades; *Eleutherodactylus* distributed mainly in Caribe, *Craugastor* distributed on Central American, and *Pristimantis* with a South American distribution. Too, some species from southeast Brazil were grouped within of the genus *Ischnocnema* whereas the broad-headed eleutherodactylid species were assigned to the genus *Limnophys*.

Hedges et al. (2008) increased the molecular sampling of Heinicke et al. (2007) to 362 species, resulting on a cladogram containing four major clades that were recognized as the families Brachycephalidae, Craugastoridae, Eleutherodactylidae, and Strabomantidae.

These families were grouped within of the clade Terrarana, which is an unranked taxon that corresponds to the more inclusive family Brachycephalidae of Frost et al. (2006). Hedges et al. (2008) also recognized the subfamilies of Eleutherodactylidae named Eleutherodactylinae and Phyzelaphryninae, each of which with two genera. These authors also recognized the subfamily Holoadeninae (six genera) and Strabomantinae (ten genera). Later, Pyron and Wiens (2011) used a slightly smaller amount of data for the same species in Hedges et al. (2008) and found that the strabomantid genera *Hypodactylus* and *Strabomantis* are more closely related to craugastorids (*Haddadus*, *Craugastor*) than to the other sampled strabomantid genera. Thus, Pyron and Wiens (2011) synonymized the family Strabomantidae to Craugastoridae and propose a new subfamily Pristimantinae. Furthermore, their results also indicated that the family Craugastoridae is more closely related to Eleutherodactylidae rather than to Brachycephalidae, contradicting the previous by Hedges et al. (2008) and Heinicke et al. (2009).

More recently, Padial et al. (2014) conducted a phylogenetic analysis of a dataset containing 431 species that corroborated most findings in Pyron and Wiens (2011), with some key differences. One of the most striking differences is the position of *Ceuthomantis*. Padial et al. (2014) recovered *Ceuthomantis* embedded within the family Craugastoridae as the sister group to *Pristimantis* and *Yunganastes* rather than as sister taxon to all other Terrarana as in previous works (e.g., Hedges et al. 2008, Heinicke et al. 2009, Pyron and Wiens 2011). Another important discordant result was the recovered clade of Brachycephalidae and Craugastoridae, conflicting with the clade of Craugastoridae and Eleutherodactylidae found by other authors. Accordingly, Padial et al. (2014) recognized three families (Brachycephalidae, Craugastoridae, and Eleutherodactylidae) grouped within the superfamily Brachycephaloidea. Moreover, Padial et al. (2014) also corroborated the monophyly of all genera, transferring *Atopophrynus* and *Geobatrachus* to incertae sedis within their Brachycephaloidea.

On the basis of mitochondrial genomes and including one terminal per each family (*Brachycephalus brunneus*, *Craugastor augusti*, and *Eleutherodactylus atkinsi*), Pie et al. (2017) corroborated that Brachycephalidae is more closely related to Craugastoridae than to

Eleutherodactylidae, as it had been found by Hedges et al. (2008), Heinicke et al. (2009) and Padial et al. (2014).

Finally, Heinicke et al. (2018) provided a phylogenetic analysis of a massive molecular dataset for 30 species of Brachycephaloidea, which corroborated the results previously found by Hedges et al. (2008). Nevertheless, its low number of ingroup taxa compared to those included by Hedges et al. (2008), Pyron and Wiens (2011), and Padial et al. (2014) renders their conclusions problematic inasmuch as taxon sampling density is hugely important (Frost et al. 2018).

Historical background of the Craugastoridae

Craugastoridae was described by Hedges et al. (2008) to join 113 species in two genera. The genus *Craugastor* distributed in southwestern USA, Mexico, Central America and the northwestern South America is composed of three subgenera, *Campbellius*, *Craugastor*, and *Hylactophryne*, of which *Craugastor* has eight informal taxa (species groups and two species series) whereas *Hylactophryne* has two species series. On the other hand, the genus *Haddadus* distributed in southeastern Brazil includes only two species (“*Eleutherodactylus*” *binotatus* and “*E.*” *plicifer*). Later, Pyron and Wiens (2011) using a slightly smaller amount of data per species than Hedges et al. (2008), found that the strabomantid genera *Hypodactylus* and *Strabomantis* are more closely related to craugastorids (*Haddadus*, *Craugastor*) than they are to the other sampled strabomantid genera. Thus, they incorporated Strabomantidae into Craugastoridae.

Recently, Padial et al. (2014) performed a parsimony analysis under direct optimization of nucleotide sequences including a broad sample of GenBank and found results in broad agreement with Pyron and Wiens (2011) regarding the status of Craugastoridae, except for the inclusion of *Ceuthomantis*. Considering this, Craugastoridae is composed of three subfamilies Craugastorinae, Holoadeninae, and Pristimantinae. From those, the subfamily Craugastorinae is composed of three genera—*Craugastor*, *Haddadus*, and *Strabomantis*. In the case of Holoadeninae, it composes all genera placed in Holoadeninae by Hedges et al. (2008)—*Barycholos*, *Bryophryne*, *Euparkerella*, *Holoaden*,

Noblella, and *Psychrophrynella*, plus a clade previously recognized within Strabomantidae by Hedges et al. (2008)—*Lynchius*, *Oreobates*, *Phrynopus*, and *Pristimantis*, and the genus *Hypodactylus* as sister of all other genera. Additionally, Holoadeninae also includes to “*Eleutherodactylus*” *bilineatus*, a species previously considered within of *Ischnocnema* (Cannedo and Haddad 2012). More recently, De la Riva et al. (2017) described the genus *Microkayla* for placed all Bolivian species formerly in *Psychrophrynella* plus five species from southern Peru. This new genus was also was placed in the subfamily Holoadeninae. Lastly, the subfamily Pristimantinae was considered as a junior synonym of Ceuthomantinae (Padial et al. 2014b), encompassing the genera *Ceuthomantis*, *Dischidodactylus*, *Pristimantis*, and *Yunganastes*.

In the present year, Heinicke et al. (2018) employed a dataset containing 389 loci and over 600,000 nucleotides for 30 terraranan. The phylogeny of Heinicke et al. (2018) found similar results to those presented by Hedges et al. (2008) at the same time it provided additional resolution at short internodes. Between the results of Heinicke et al. (2018) was recovered to *Ceuthomantis* as sister-taxon to all other terraranans, rather than deeply embedded within the group. Furthermore, Strabomantidae was recovered as a monophyletic group rather than paraphyletic with respect to Craugastoridae (contra Pyron and Wiens 2011, and Padial et al. 2014). Additionally, Heinicke et al. (2018) described a new subfamily for the genus *Hypodactylus* and recovered the genus *Tachiramantis* (Heinicke et al. 2015) as most closely related to *Craugastor* and *Haddadus*.

Given the above, two opposing hypotheses are available to explain the phylogenetic relationships of 824 species currently placed in seventeen genera. These are; first, recognize both Craugastoridae and Strabomantidae as valid families (Hedges et al. 2008; Heinicke et al. 2009; Heinicke et al. 2015). Second, recognize Strabomantidae as part of Craugastoridae (Pyron and Wiens 2011; Padial et al. 2014). Either of these two hypotheses have implications on the position of the genus *Ceuthomantis* because although Pyron and Wiens (2011) and Padial et al. (2014) agree on the paraphyletic condition of Craugastoridae with respect to Strabomantidae, they have discordant hypotheses on the position of *Ceuthomantis*. In Pyron and Wiens (2011), *Ceuthomantis* is shown to be the sister taxon to

all other terraranans as in Hedges et al. (2008); Heinicke et al. (2009); Heinicke et al. (2015) whereas in Padial et al. (2014) the genus *Ceuthomantis* is deeply embedded within of Craugastoridae.

To attempt resolve this disagreement on the phylogenetic status of the family Craugastoridae, I performed a phylogenetic analysis of the superfamily Brachycephaloidea with special emphasis in the family Craugastoridae sensu Padial et al. (2014). To date, this is the first phylogenetic study with both molecular and morphological evidence analyzed together in this group.

Re-integration of morphological data into total evidence analysis

Morphology, understood as the description and analysis of organismal form, is one of the oldest biological disciplines and has contributed significantly to our understanding of how animals function and how the overwhelming diversity of phenotypes evolved (Wanninger 2015). However, with the arrival of molecular techniques in the evolutionary sciences, the ever faster and cheaper generation of gene sequences resulted in an explosion of data for phylogenetic and developmental analyses in the past 30 years. As a consequence, morphology has received progressively lesser attention, resulting in a noticeable crisis of morphological approaches toward phylogenetic systematics.

Recently, an older idea so-called “total evidence” analysis, originally proposed by Kluge (1989) is being recovered (Wanninger 2015; Giribet 2015; Pyron 2015, 2016; Sanchez et al. 2017; Gavryushkina et al. 2017; Martin et al. 2017). This approach proposes that phenomic data (e.g., data from morphology, fossils, behavior, ontogeny, etc.) provides a rich, independent source of phylogenetic evidence and an important framework to test hypotheses supported by molecular evidence by combining all evidence in a simultaneous analysis to identify the hypothesis that best explains all the evidence. To date, several studies of amphibians have already incorporated this trend (e.g., Grant et al. 2006; 2017; de Sá et al. 2014; Castroviejo-Fisher et al. 2015). Moreover, such studies have found that inclusion of morphological evidence has a disproportionately large impact on the results of the total evidence analyses, at least under the parsimony optimality criterion (e.g., de Sá et

al. 2014). In a similar fashion, large impact on the results has been also found in studies of insects based in model-based approaches (Nylander et al. 2004).

Given the demonstrated importance of morphological characters in testing the phylogenetic relationships, this study provides a case in point for the exploration the effects of incorporating morphological evidence as an independent source of phylogenetic evidence and an important framework to test hypotheses supported by molecular evidence.

Conclusions

Detailed examination of the impact of both character and taxon sampling on the phylogenetic relationships of the superfamily Brachycephaloidea reveals that the inclusion of a relatively small phenomic dataset (with 185 characters) in comparison to the molecular data (13,686 characters), as well as the inclusion of key taxa (*Atopophrynus syntomopus* and *Dischidodactylus duidensis*) have disproportionately large impacts on the tree topology and nodal support. Among this, it is striking to note that the superfamily Brachycephaloidea sensu Padial et al. (2014) or Terrarana sensu Hedges et al. (2008) does not represent a monophyletic group as previously thought.

The experiments shown here, explicitly designed to evaluate the impact of character and taxon sampling, allowed identifying that the genus *Brachycephalus* is sensitive to the inclusion of *Atopophrynus syntomopus* and/or *Dischidodactylus duidensis*. As consequence of this sensitivity to taxon sampling, the genus *Brachycephalus* is placed outside of the superfamily, being positioned as sister clade of Nobleobatrachia sensu Frost et al. (2006) or Hyloidea sensu Pyron and Wiens (2011). Moreover, the results indicate that there is no evidence of a relationship between nodal support values and its refutation. Therefore, it is not possible to predict the refutation of clades in subsequent phylogenetic analyses by considering their nodal support values.

The phylogeny resulting from this analysis refuted the monophyly of several taxa: 1) the superfamily “Brachycephaloidea” as presently constituted is non-monophyletic and the species *Atopophrynus syntomopus*, *Dischidodactylus duidensis*, *Geobatrachus walker*

should no longer be retained within this superfamily; 2) the family Brachycephalidae is demonstrated to be polyphyletic since *Brachycephalus* is not closely related to *Ischnocnema*; 3) the family Craugastoridae is paraphyletic due to the nesting of *Ischnocnema*; 4) the genus *Craugastor* is paraphyletic due to the nesting of *Strabomantis zygodactylus* within the clade; 5) the genus *Psychrophynella* is paraphyletic due to the nesting of *Noblella pygmaea* within it; and 6) the genus *Pristimanits* is polyphyletic because it comprises two well-differentiated clades. Several interesting sister-group relationships include: *Ischnocnema* is closely related to a clade including *Haddadus*; *Ceuthomantis* clearly embedded within of the superfamily; *Hypodactylus latens* and *Niceforonia adenobrachia* forming a sister clade of family Eleutherodactylidae; and the genus *Brachycephalus* was placed as sister clade of Nobleobatrachia sensu Frost et al. (2006).

The phylogenetic hypothesis resulting from this study is used to propose a new taxonomy arrangement for this group. Five families including 26 genera are recognized (Ceuthomantidae, Craugastoridae, Eleutherodactylidae, Hypodactylidae, and Strabomantidae). From these five families, three are diagnosable morphologically (Eleutherodactylidae, Hypodactylidae, and Strabomantidae) whereas the other two are diagnosable solely by molecular evidence (Ceuthomantidae, Craugastoridae). At the generic level, twelve genera are diagnosable morphologically (*Ischnocnema*, *Tachiramantis*, *Craugastor*, *Strabomantis*, *Noblella*, *Microkayla*, *Holoaden*, *Barycholos*, *Niceforonia*, Genus new-Colombia1, *Eleutherodactylus*, *Diasporus*) whereas the other fourteen are defined uniquely by molecular evidence (*Yunganastes*, *Pristimantis*, *Haddadus*, *Ceuthomantis*, New genus-Colombia2, *Oreobates*, *Psychrophrynella*, *Phrynopus*, New genus-Brazil, *Lynchius*, *Euparkerella*, *Bryophryne*, *Phyzelaphryne*, *Adelophryne*).

Regarding perspectives toward posterior total evidence analyses and given the demonstrated importance of character and taxon sampling for the phylogenetic inference of the new world direct-developing frogs, the concerted efforts should focus in incrementing both the morphological evidence as well as taxon sampling. As suggestion, these efforts

should start by increasing the representativeness of hyloid families sensu Frost et al. (2006) since outgroup sampling used herein is not robust to propose closely relationships of *Atopophrynus syntomopus*, *Brachycephalus*, *Dischidodactylus duidensis*, and *Geobatrachus walkeri* with some other clade. Regarding the specific phylogenetic relationships of the genus *Brachycephalus*, it would also be important to include *Brachycephalus hermogenesi* and the morphology of *Brachycephalus didactylus* given that those species were already considered nonrelated to the genus *Brachycephalus* with support from morphology. Finally, I anticipate that further investigations focused in morphological features will provide new characters that will potentially increase our understanding on the evolutionary relationships within this diverse group.

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