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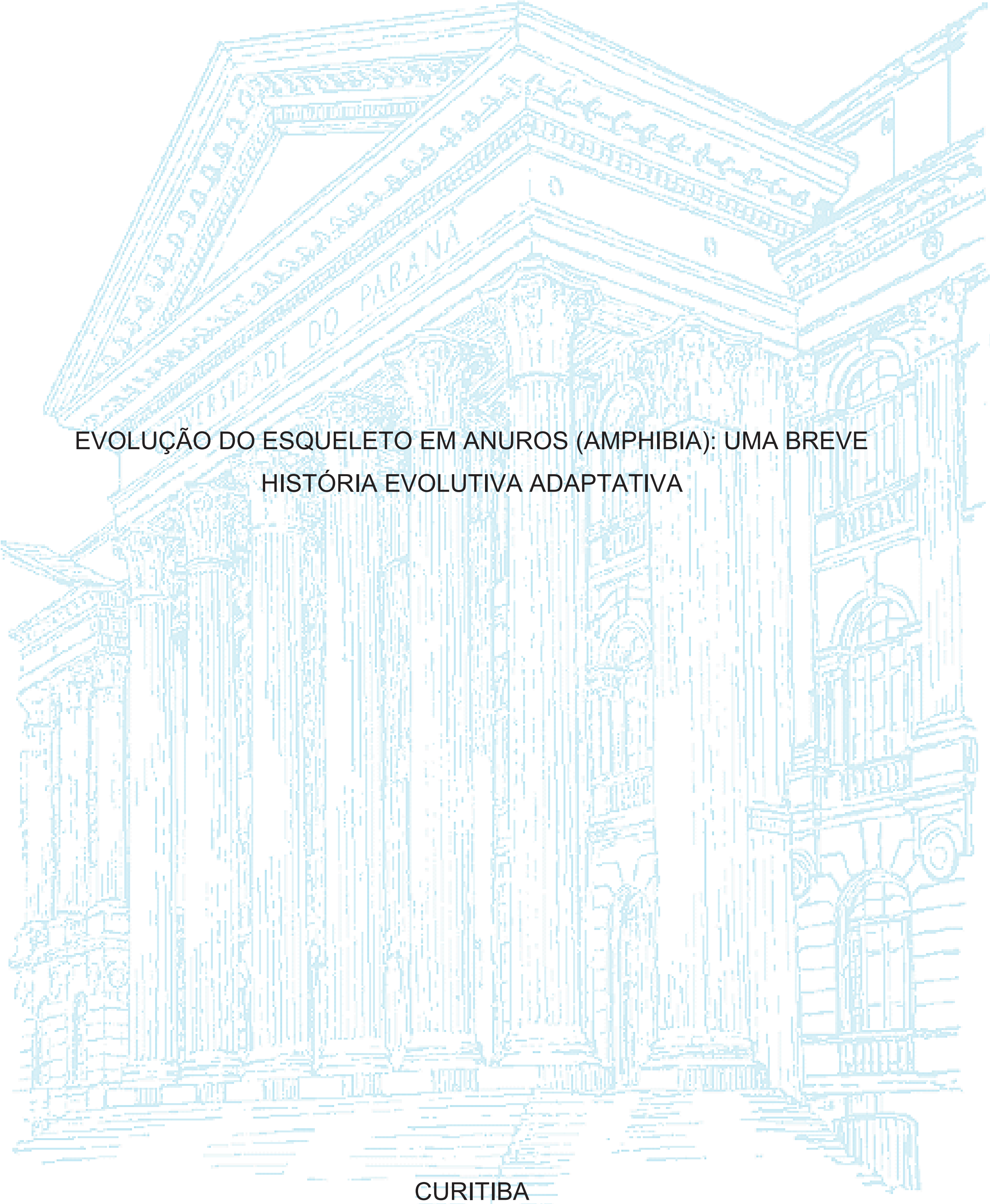

EVOLUÇÃO DO ESQUELETO EM ANUROS (AMPHIBIA): UMA BREVE HISTÓRIA EVOLUTIVA ADAPTATIVA

CURITIBA
2023

ANDRÉ EDUARDO CONFETTI

EVOLUÇÃO DO ESQUELETO EM ANUROS (AMPHIBIA): UMA BREVE HISTÓRIA EVOLUTIVA ADAPTATIVA

Tese apresentada ao Programa de Pós-Graduação em Zoologia do Departamento de Zoologia, Setor de Ciências Biológicas da Universidade Federal do Paraná como requisito parcial para a obtenção do título de Doutor em Zoologia.

Orientador: Prof. Dr. Marcio Roberto Pie

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação ZOOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **ANDRÉ EDUARDO CONFETTI** intitulada: **Evolução do esqueleto em Anuros (Amphibia): Uma breve história evolutiva adaptativa**, sob orientação do Prof. Dr. MARCIO ROBERTO PIE, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 30 de Março de 2023.

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PRA MIM O MELHOR AMIGO DO SER HUMANO É O SAPO!
MAS O CACHORRO CUIDA DA CASA! É COMPANHEIRO NOS PASSEIOS, NA ALEGRIA E NA TRISTEZA!
O QUE UM SAPO FAZ POR VOCÊ?!
EU NÃO TENHO AMIGOS POR INTERESSE, MINHA SENHORA!
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RESUMO

Os anuros são um grupo diverso de animais vertebrados, com 7533 espécies descritas e ampla distribuição em todo o mundo, exceto pelos polos. Eles se distinguem de outros anfíbios por perderem a cauda na fase adulta e apresentarem uma morfologia variada, incluindo fusões ósseas nos membros locomotores e vertebras caudais. O gênero *Brachycephalus* é um exemplo de anuros miniaturizados, com 39 espécies encontradas nas matas de altitude da floresta atlântica do Brasil, apresentando encurtamento dos membros, redução de dígitos e estruturas mineralizadas em sua derme. A grande diversidade morfológica desses animais reflete os ambientes aos quais eles se adaptaram ao longo do tempo, incluindo água, solo e árvores. É descrito na literatura que mudanças morfológicas presentes nos diferentes gêneros de anuros são fortemente relacionados a pressões evolutivas do meio. Nesse sentido, para avaliar os aspectos evolutivos dos membros locomotores e a evolução dos dígitos dos anuros, assim como investigar como se aprofundou a mineralização na derme de Brachycephalus, foram utilizados de métodos filogenéticos comparativos, assim como do uso de imagens geradas através de micro-tomografia computadorizada. Em nossos resultados vimos a influência da ecologia na forma dos anuros, enfatizando a importância do meio em que vivem na mudança da forma dos membros locomotores dos animais. Além do mais, a evolução dos dedos ocorre de forma modular, com alta taxa de modificação nas falanges distais. Por fim nossos resultados demostram que a adaptação em anuros pode ser observada através de condições específicas em algumas espécies, como a deposição de estruturas mineralizadas em sua derme, que pode ser influenciada pelas condições climáticas, como é o caso dos animais do gênero *Brachycephalus*. O nosso trabalho destaca o uso de modelos 3D por meio de imagens de micro-tomografia computadorizada, que permite a ampla exploração do esqueleto dos animais sem danificá-los, tornando assim possível uma maior liberdade ao se trabalhar com animais de museu e coleções científicas.

Palavras-chave: MicroCT; Osteologia; Morfologia; Modularidade; *Brachycephalus*

ABSTRACT

Anurans are a diverse group of vertebrate animals, with 7533 described species and a wide distribution worldwide, except for the poles. They are distinguished from other amphibians by losing their tail in the adult stage and presenting varied morphology, including bony fusions in the locomotor limbs and caudal vertebrae. The genus *Brachycephalus* is an example of miniaturized anurans, with 39 species found in the high-altitude forests of the Brazilian Atlantic Forest, presenting limb shortening, digit reduction, and mineralized structures in their dermis. The great morphological diversity of these animals reflects the environments to which they have adapted over time, including water, soil, and trees. It is described in the literature that morphological changes present in different genera of anurans are strongly related to evolutionary pressures from the environment. In this sense, comparative phylogenetic methods and the use of images generated through computerized micro tomography were used to evaluate the evolutionary aspects of locomotor limbs and digit evolution in anurans, as well as to investigate how mineralization in the dermis of *Brachycephalus* has deepened. Our results showed the influence of ecology on the shape of anurans, emphasizing the importance of the environment in which they live in changing the shape of their locomotor limbs. Moreover, digit evolution occurs in a modular way, with a high rate of modification in the distal phalanges. Finally, our results demonstrate that adaptation in anurans can be observed through specific conditions in some species, such as the deposition of mineralized structures in their dermis, which can be influenced by climatic conditions, as is the case with animals of the genus *Brachycephalus*. Our work highlights the use of 3D models through images of computerized micro-tomography, which allows for the extensive exploration of the animals' skeletons without damaging them, thus making it possible to work more freely with museum animals and scientific collections.

Key words: MicroCT; Osteology; Morphology; Modularity; *Brachycephalus*

SUMÁRIO

1. INTRODUÇÃO

Os anuros são, dentre os vertebrados terrestres, o grupo que apresenta a maior diversidade de espécies, sendo descritas cerca de 7533 espécies (Frost, 2023), além é claro, de possuir uma ampla distribuição no globo terrestre, sendo possível encontrar esses animais em todos os continentes com exceção dos pólos (Duelmann, 1999). Dentre essas espécies a grande maioria se encontra nos Neotrópicos, região que estende do norte do México até o sul da Argentina, englobando por total a América central e quase totalmente a América do Sul (Duelmann, 1999; Young, 2004).

Os anuros se distinguem dos demais anfíbios por conta de várias características morfológicas, entre elas a perda da cauda no estágio adulto, sendo ela perdida durante o seu desenvolvimento quando girino (Wake & Koo, 2018), assim como sua conspícua morfologia. Entretanto, nem todos os anuros se desenvolvem passando pela fase de girino, algumas espécies apresentam desenvolvimento direto. Essas espécies que englobam o grupo dos Terrarana, são conhecidas por seu desenvolvimento ocorrer exclusivamente dentro dos ovos, esses altamente dependentes da umidade do ambiente, tendo em vista que não são depositados em corpos d'água (Hedges et al., 2008).

Referente a sua morfologia, os anurtos apresentam as mais variadas formas, cores e tamanhos. Algumas características que os distinguem dos demais tetrápodes está intimamente relacionado com seu esqueleto, onde é possível encontrar fusões entre a tíbia e a fíbula, assim como a fusão do rádio e a ulna; além das fusões dos ossos dos membros locomotores, há a fusão das últimas vertebras caudais, originando-se assim o urostilo; há também um prolongamento do ílio, na cintura pélvica (Stokely & List, 1955; Jenkins & Shubin, 1998; Pugener & Maglia, 2009). Dentro dos anuros ainda, é possível encontrar animais com mais peculiaridades referentes a sua morfologia, como é caso dos animais hiper ossificados da família Ceratophryidae (Wild, 1997; Quinzio & Fabrezi, 2012); ou os animais com adaptações específicas ao ambiente ao qual estão inseridos, como o achatamento do corpo nos animais da família Pipidae (Trueb & Cannatella 1986; Cannatella &Trueb 1988) ou os sapos de hábitos fossoriais (Garg & Biju, 2017; Keeffe & Blackburn, 2020). Dentre todas as condições morfológicas peculiares adquiridas ao longo das linhagens de anuros, há de se destacar a miniaturização. O processo de miniaturização não ocorre somente com anuros, sendo relatado em vertebrados em geral, e consiste na brusca diminuição do corpo dos animais em um curto período de tempo na escala evolutiva, que acarreta em modificações severas em sua morfologia interna e externa (Hanken, 1985; Hanken & Wake, 1993; Rüber et al. 2007).

Dentre os anuros miniaturizados, um gênero que chama atenção é *Brachycephalus*. Esse gênero compreende 39 espécies, que são encontrados em matas de altitude da floresta atlântica, no Brasil (Frost, 2023; Bornschein et al., 2016). Os animais pertencentes ao gênero apresentam algumas condições morfológicas únicas, tais quais: o encurtamento dos membros; redução no número de dígitos; deposição de estruturas mineralizadas em sua derme; e, hiper ossificação e fusão de alguns ossos, que em casos mais extremos é encontrado uma grande estrutura ossificada única, formando uma espécie de carapaça (Clemente-Carvalho et al., 2008; Ribeiro et al., 2015; Folly et al., 2021; Nunes et al., 2021; Mari et al., 2023). Além do mais, *Brachycephalus* possui uma diversidade morfológica interessante dentro do gênero, podendo ser separado em dois grupos morfológicos: grupo *B. ephippium* e grupo *B. pernix*, sendo que cada um dos grupos se distingue com base em seu formato de corpo (Ribeiro et al., 2015; Folly et al., 2023).

Essa grande diversidade morfológica dos anuros é reflexo da gama de ambientes aos quais eles ocuparam ao longo das linhagens, sendo possível encontrar animais aquáticos, cavadores, escaladores, saltadores e caminhantes (Stepanova & Womack, 2020). A pressão seletiva é facilmente identificada em grupos que apresentam tantos hábitos ecológicos e morfologias distintas, como visto nos anuros. Essa pressão seletiva é responsável por mudanças sutis como a diferenciação de dedos, até a adaptação de membros inteiros para desempenhar determinada função no meio (Přikryl et al. 2009; Reilly & Jorgensen 2011; Stepanova & Womack 2020). E para entender melhor o papel desempenhado pelo meio na diferenciação de estruturas morfológicas, diversos trabalhos vieram sendo realizados ao longo dos anos dentro do campo da morfologia funcional (Wainwright & Reilly, 1994; Forsberg & Ranta, 2004; Richmond et al., 2005; Kilbourne 2017; Kilbourne & Hutchinson, 2019).

Tendo em vista a ampla diversidade morfológica de anuros, assim como seus vastos hábitos ecológicos, principalmente relacionados a locomoção, se vê necessário um maior aprofundamento e entendimento do processo evolutivo por trás de tais mudanças. Embora tenha na literatura uma gama de trabalhos voltados ao estudo da morfologia, poucos nos contam de fato a história evolutiva por trás dos membros locomotores, assim como das falanges dos anuros, estruturas essas desempenhando um papel fundamental na conquista do território ao qual possibilitou a diversificação das espécies ao longo dos anos.

Com isso, o nosso objetivo foi entender um pouco mais os mecanismos envolvidos na diferenciação da morfologia dos anuros, focando especificamente na anatomia dos membros locomotores e dedos. O presente trabalho é divido em três capítulos, na forma de artigo científico, os quais se propõe a contar uma história evolutiva e adaptativa do esqueleto apendicular dos anuros. O primeiro capítulo se intitula: "*The evolution of anuram limbs*" e tenta entender melhor o processo evolutivo por trás da diferenciação dos membros locomotores dos anuros, assim como o papel do meio em sua diferenciação. O segundo capítulo, por sua vez, é intitulado: "*The evolution of the anuran phalanx: rates, phylogenetic signal, and modularity*" onde tentamos entender os padrões de diferenciação das falanges dos anuros, estruturas que carregam uma grande carga evolutiva, que por consequência, apresenta uma grande variação e desempenham um papel fundamental na adaptação dos animais. Por fim, o terceiro capítulo explora um pouco mais do processo adaptativo e evolutivo do gênero *Brachycephalus*, onde focamos nossos esforços na compreensão nas estruturas mineralizadas em sua derme. O terceiro capítulo é intitulado: "*Environmental correlates of osteoderm wall thickness in Brachycephalus (Anura: Brachycephalidae): an adaptation to cope with miniaturization?*".

Além do mais, a presente tese tem como objetivo adicional mostrar a importância do emprego da micro-tomografia computadorizada em trabalhos de biologia comparada. O uso de imagens gerados através de *microCT* não é uma novidade, embora pouco utilizado, e nos permite abordar melhor os animais com mais detalhes, já que com o modelo 3D obtido através das imagens provenientes da micro-tomografia, é possível dar zoom e observas as estruturas que, muitas vezes, não são possíveis com animais fixados. Além do mais, a técnica de micro tomografia nos permite trabalhar com animais de museu de forma segura, sem que haja nenhum dano na amostra.

2. ARTIGO CIENTÍFICO 1

The evolution of anuran limbs

Abstract

Anurans are among the most diverse clades within terrestrial vertebrates, showing remarkable variation in ecology, life-history, and reproductive mode. However, the extent to which this diversity is reflected in their skeletal morphology is still poorly known. Using a dataset obtained from microCT images, we used morphometric, statistical, and phylogenetic analyses to assess the role of locomotion mode on the shape of anuran locomotor limbs. Although burrowing anurans showed a certain correspondence between their mode of locomotion and the shape of their limbs, that was not the case for other modes of locomotion. However, there is a strong correspondence of evolutionary characters in the forelimbs, showing us a high rate of evolution. Furthermore, our results showed that the evolution of anuran locomotor limbs can be modular.

Introduction

Anurans are the most diverse group of amphibians, consisting of 7,553 species worldwide (Frost 2023). They are found on every continent except for the poles, with the highest concentration of species in the Neotropical region (Duelmann 1999; Young 2004). Anurans differ from other amphibians by not having a tail as adults, which is lost in the tadpole stage, during larval development (Wake and Koo 2018). However, this condition is not shared among all frogs, given that some species go directly to adult stage when hatch the eggs, as is the case of the Neotropical frog group, the Terrarana, where development is direct (Hedges et al. 2008). Although anurans have a similar bone structure to most other tetrapods, most of the morphological variation inthe group is related to their osteology (Stokely and List 1955; Pugener and Maglia 2009).

The primary differences in the osteological structure of anurans compared to most tetrapods are found in the following bones: radioulnar fusion, tibia-fibula fusion, fusion of the last caudal vertebrae forming the urostyle, and ilium extension (Stokely and List 1955; Jenkins and Shubin 1998; Pugener and Maglia 2009). Furthermore, many species of frogs have unique osteological conditions, such as frogs of the genus *Brachycephalus* (Ribeiro et al. 2015; Ribeiro et al. 2017; Pie et al. 2018). In this genus, the animals show reduced digits, as well as fusion in the bones of the skull, and in extreme cases, the presence of a large bone plate resulting from the fusion of the vertebrae (Clemente-Carvalho et al. 2008; Nunes et al. 2021; Folly et al. 2021). In addition to *Brachycephalus*, there are other species with distinct osteology patterns due to miniaturization or some specific ecological condition. Several studies have focused efforts on understanding and describing these osteological conditions over the last few years (Paluh et al. 2020; Paluh et al. 2021; Essner et al. 2022; Folly et al. 2023). Many species shows specific morphological variations for their locomotion, as an adaptive response to the environment in which they live (Kilbourne 2017; Kilbourne and Hutchinson 2019; Stepanova and Womack 2020).

Anurans are known to be adapted to a wide range of habitats and have mastered different types of locomotion (Přikryl et al. 2009; Reilly and Jorgensen 2011; Stepanova and Womack 2020). The vast majority of anurans have more than one locomotor habit, which makes it possible to explore habitats with greater success. Among the methods of locomotion, one can categorize frogs according to their main locomotor mode, namely aquatic, arboreal, terrestrial, and burrowing (Stepanova and Womack 2020). However, it is known that they can show the association of more than one of these habits, such as the genus *Rhinella*, which is primarily terrestrial/walker animals, but there are records of being excellent jumpers (Reilly et al. 2016). This range of ways of moving makes these animals highly successful when it comes to adapting to the environment. Therefore, morphology plays a very important role in the adaptability of frogs to the environment in which they inhabit.

In ecology, studies of functional morphology have been gaining prominence over the years (Lauder 1990; Wainwright and Reilly 1994; Forsberg and Ranta 2004; Richmond et al. 2005). This area of biology has the main goal to understand the role of the environment in the evolution of animals, whether at a genetic, physiological or even morphological level. Recently, Kilbourne (2017) showed the influence of locomotor mode in relation to the morphology of mustelid limbs, showing that there is clear evidence that the environment can modify the shape of animals. This work (Kilbourne, 2017) also underscores the importance and need of more comparative biology works in this field. With this in mind, we see the importance of studying and expanding the range of knowledge in functional morphology field, in order to better understand the evolutionary process and the differentiation of morphology. Given the importance of functional morphology studies in understanding evolution, this work aims to investigate the role of the environment in the differentiation of the anuran skeleton, being highly adapted and dependent on their environment. It is expected to find a positive relationship between the mode of locomotion and limb differentiation, providing insight into the evolutionary trends in anuran locomotor structures.

Materials and methods

Data collection

Morphological measurements were obtained from specimens that were digitized using microcomputed tomography and made available on MorphoSource (Table S1). A total of 105 anuran species were measured, including representatives from all four main types of anuran locomotion, namely arboreal, terrestrial, aquatic, and burrowing. We performed linear measurements of fore- and hindlimb bones, presents in Figure 1. Forelimb measurements included humerus length (HL), deltoid ridge length (DR), epicondylar breadth (EB), mediolateral diameter (MLh) and anteroposterior diameter (APh) for humerus; radio-ulna length (RUL), mediolateral diameter (MLru), anteroposterior diameter (APru) and olecranon process length (OL) for radio-ulna; scapula length (SL); and metacarpal III length (MC3L). Hindlimb measurements included: femur length (FL), mediolateral diameter (MLf) and anteroposterior diameter (APf) for femur; tibiofibula length (TFL), mediolateral diameter (MLtf) and anteroposterior diameter (APtf) for tibiofibula; ilium length (IL) and acetabulum diameter (ACE) of the ilium; metatarsus length (MT3L); urostile lenght (UL), urostile ridge lenght (URL) and urostile ridge height (URH) for urostile.. Phylogenetic relationships of the studied species were obtained from Jetz & Pyron (2018), as made available in the VetLife portal (https://vertlife.org, accessed on 1 September 2020). To account for uncertainty in their phylogenetic relationships, all analyses were repeated using 1000 alternative trees.

Data management

All data downloaded in morphosource was decomposed in MorphoDig (Lebrun, 2018) software using “Decompose surface” tool. After decompose process all individual bones was saved as .ply format to measurements. The measurements was taken in 3D Slicer software (Fedorov et al., 2012) using the “MarkupsLine” tool in “Murkups” module.

Univariate analyses

We began our analyses by focusing on variation in body size between modes of locomotion, which was inferred the score of each species along the first axis of a PCA based on the covariance matrix of the log-transformed linear data. Alternative proxies for body size, such as the geometric means of trait values, provided nearly indistinguishable results and were not considered further. Differences in body size between modes of locomotion were tested using the phylANOVA function in phytools 0.9-99 (Revell 2012). Given that the dominant influence of body size on frog morphological variation might obscure important interspecific differences, we performed major axis regressions between each of the 23 traits and our measure of body size using smatr 3.4-8 (Warton et al. 2012). Regression residuals for each trait were then used to estimate their phylogenetic signal (λ of Pagel [1999], as estimated in the phylosig function in phytools) and evolutionary rate (σ^2, as estimated in the fitContinuous function of a λ model in geiger v. 2.0.6.4 [Pennell et al. 2014]).

Multivariate analyses

Regression residuals for each trait were used in a PCA based on the correlation matrix, and the broken stick model was used to determine the number of PCs to be retained, as implemented in vegan 2.5-5 (Oksanen et al. 2019). Unless otherwise indicated, all analyses were carried out in R 3.6.1 (R Core Team 2019).

Results

Our results showed that there were no significant differences in body size among species with different modes of locomotion according to a phylogenetic ANOVA (Figure 2, F= 0.8498, p=0.783). The broken-stick test of the size-free PCA selected two axes for further analysis. The PCA loadings are given in table 1. PC1 accounted for 22,1% of the variation in the dataset and reflected differences in femur length and tibia-fibula length. PC2 accounted for 17.5% of the variation, and reflected differences majority in the bones of fore limbs traits, especially in humerus length, radio-ulna length and deltoid ridge. Our principal components analysis does not form groups by locomotor modes, although there is a relationship between certain habits with specific measures (Figure 5). In particular, the PCA seems to reflect specially measures of fore limb bones. Although our PCA does not show structured clusters, this analysis showed us that forelimb bones can explain the major variance in our data.

As it is evident that there is clear relationship between the osteological structure of frogs and the locomotor habit, a phylogenetic signal test was performed to see if there is a phylogenetic signal in the locomotor bones of frogs. The phylogenetic signal analysis (Pagel's λ) showed intriguing differences between the bones of fore and hind limbs (Figure 4A). In particular, the phylogenetic signal of hind limb bones was consistently higher than forelimb bones (Figure 4A). Interestingly, this difference is not simply a reflection of overall variation in rates (Figure 4B). Indeed, faster rates of evolution were found in the radius in the ulna in the case of the forelimb, whereas hindlimb evolution was associated with faster rates in the femur (Figure 4B).

Discussion

Our analyses demonstrated considerable variation in measured structures across the species, yet this variation is not directly related to their mode of locomotion several studies have shown that the variation of the locomotor limbs of tetrapods are related to several factors and not only with the variation in the size of the animals, being mainly related to some ecological condition that exerts evolutionary pressures (Kilbourne 2017; Stepanova & Womack, 2020).

Assuming that the variation in the size of the animals is not the main cause of the variation in our data, one could expect that there is an evolutionary relationship between the measurements of the bones with either the environment or the locomotor behavior of the animals. However, our analyses do not indicate that there is a clear evolutionary relationship with the locomotor mode. Kilbourne (2017), using a similar methodology that we used in this present work, found a clear relationship between the evolution of the shape of the locomotor limbs of mustelids in relation to the locomotor mode of the animals. Although there were some differences between species, our PCA makes it clear that there are clusters of animals with burrowing habits, however, we cannot in fact assume that statistically there is a relationship between the influence of the environment and the shape of the skeleton, since our PCA indicates the variation of 21.3% and 13.4% respectively in PC1 and PC2. However, when we perform the PCA with the broken stick criteria, we observe that the first 2 PCs, if interpreted by this method, can tell us a little more about the variation in our data. The first 2 PCs tell us that most of the variation in our data is concentrated in the fore limbs of anurans. Stepanova and Womack (2020), similarly, investigated the influence of microhabitat on the differentiation of lower and upper limbs in Anurans. In their work, Stepanova and Womack (2020) saw that there is a great influence on the form caused by the habit, mainly for species with digging habits, something similar to what we found in our analyses, as well as uncertainty in relation to the other habits. This reinforces that different habits can suffer different adaptive pressures.

The consistently higher phylogenetic signal in the hindlimb found in our study is particularly intriguing and might be related to the fact that changes in the forelimbs may be related to the constant search for adaptation to the environment by the species. Rothier et al. (2023) found patterns of differentiation of mammalian locomotor limbs, revealing that the evolution of limbs involves different patterns in terms of morphological diversification. As found in our work, Rothier et al. (2023) saw that there is a large concentration of evolutionary characters in the distal portion of the locomotor limbs compared to their proximal part, indicating that the phalanges and the carpals and metacarpals concentrate the most of the diversification of the locomotor limbs. In addition to the presence of evolutionary rate, there is a large concentration of characters shared by relative species, evidenced by the phylogenetic signal, however, more evident in the lower limbs, although not ruled out in certain measures in the forelimbs. The presence of a phylogenetic signal in different measures in both locomotor limbs may indicate a possible evolution in modules, not just as a single structure.

Conclusions

Ecology plays a crucial role in shaping morphological structures. Natural selection acts upon the pressures that the environment exerts on organisms, resulting in differentiation. In anurans, although the phylogenetic signal is evident, it cannot be unequivocally stated that locomotor habit is directly related to this differentiation.

Nevertheless, burrowing animals tend to have highly adapted locomotor limbs for their function. We also found that there is a tendency towards differentiation in the locomotor limbs, with the highest concentration of variation occurring in the extremities of the forelimbs. This suggests that phalanges and metacarpals tend to differentiate more than the other bones. We also showed that bones evolve independently of each other and without a clear pattern, indicating possible modular evolution. Our work underscores the importance of comparative biology studies in understanding the evolution of morphological structures. Understanding the relationship between ecology and morphological differentiation can lead to a better comprehension of organismal evolution and ecology.

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Tables and figures

Table 1. Loadings of the principal component analysis. HL = humerus length, HDR = humerus deltoid ridge length, HAP = anteroposterior diameter of humerus, HML = mediolateral diameter of humerus, EB = epicondylar breadth, RUL = radio-ulna length, RUAP = anteroposterior diameter of radio-ulna, RUML = mediolateral diameter of radio-ulna ,OL = olecranon process, SL = scapula length, MC3L = metacarpal III length, FL = femur length, FAP = anteroposterior diameter of femur, FML = mediolateral diameter of femur, TLF = tibia-fibula length, TFAP = anteroposterior diameter of tibia-fibula, TFML = mediolateral diameter of tibia-fibula, MT3L = metatarsal III length, IL = ilium length, ACE = acetabulum diameter, UL = urostyle length, URL = urostyle ridge length and URH = urostyle ridge height.

Trait	PC1	PC2
HL	0.04	0.48
HDR	-0.13	0.20
HAP	-0.26	0.11
HML	-0.31	0.04
EB	-0.27	0.11
RUL	0.04	0.48
RUAP	-0.20	-0.03
RUML	-0.12	0.19
OP	-0.28	0.05
SL	-0.15	0.17
MC3L	0.28	0.18
FL	0.39	0.11
FAP	-0.12	-0.20
FML	0.00	-0.18
TFL	0.39	0.10
TFAP	0.13	-0.16
TFML	-0.11	-0.31
MT3L	0.25	-0.15
IL	0.00	0.07
ACE	-0.08	-0.22
UL	0.25	0.00
URL	0.14	-0.21
URH	0.02	-0.19
% Variance explained	22.10%	17.50%

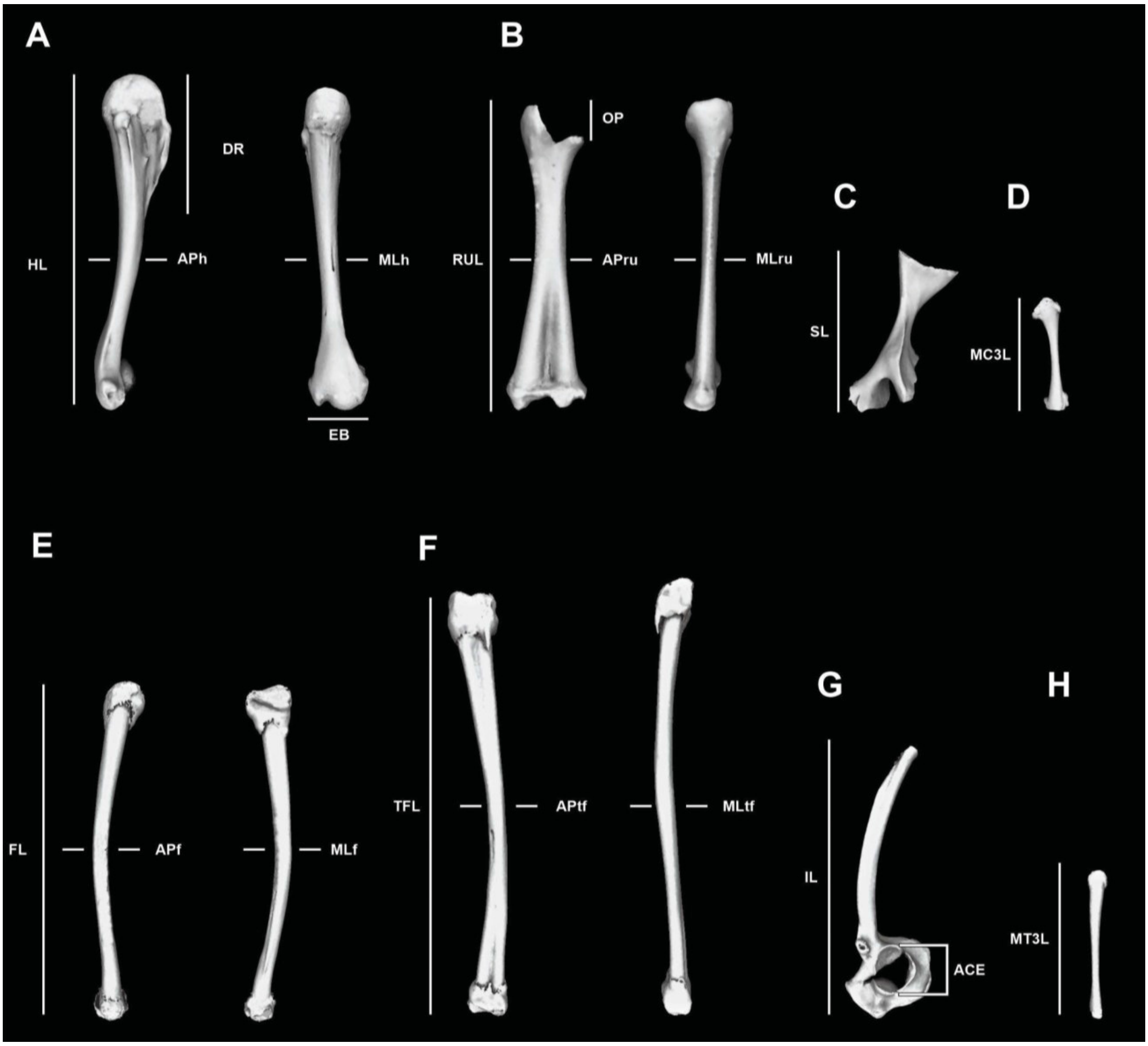

Figure 1. Bones and linear dimensions included in the present study. A = humerus, B = radio-ulna, C = scapula, D = metacarpal III, E = femur, F = tibia-fibula, G = ilium, H = metatarsus III. HL = humerus length, DR = deltoid ridge length, EB = epicondylar breadth, MLh = mediolateral diameter of humerus, APh = anteroposterior diameter of humerus, RUL = radio-ulna length, OL = olecranon process, MLru = mediolateral diameter of radio-ulna, APru = anteroposterior diameter of radio-ulna, SL = scapula length, MC3L = metacarpal III length, FL = femur length, MLf = mediolateral diameter of femur, APf = anteroposterior diameter of femur, TLF = tibia-fibula length, MLtf = mediolateral diameter of tibia-fibula, APtf = anteroposterior diameter of tibia-fibula, IL = ilium length, ACE = acetabulum diameter and MT3L = metatarsus length.

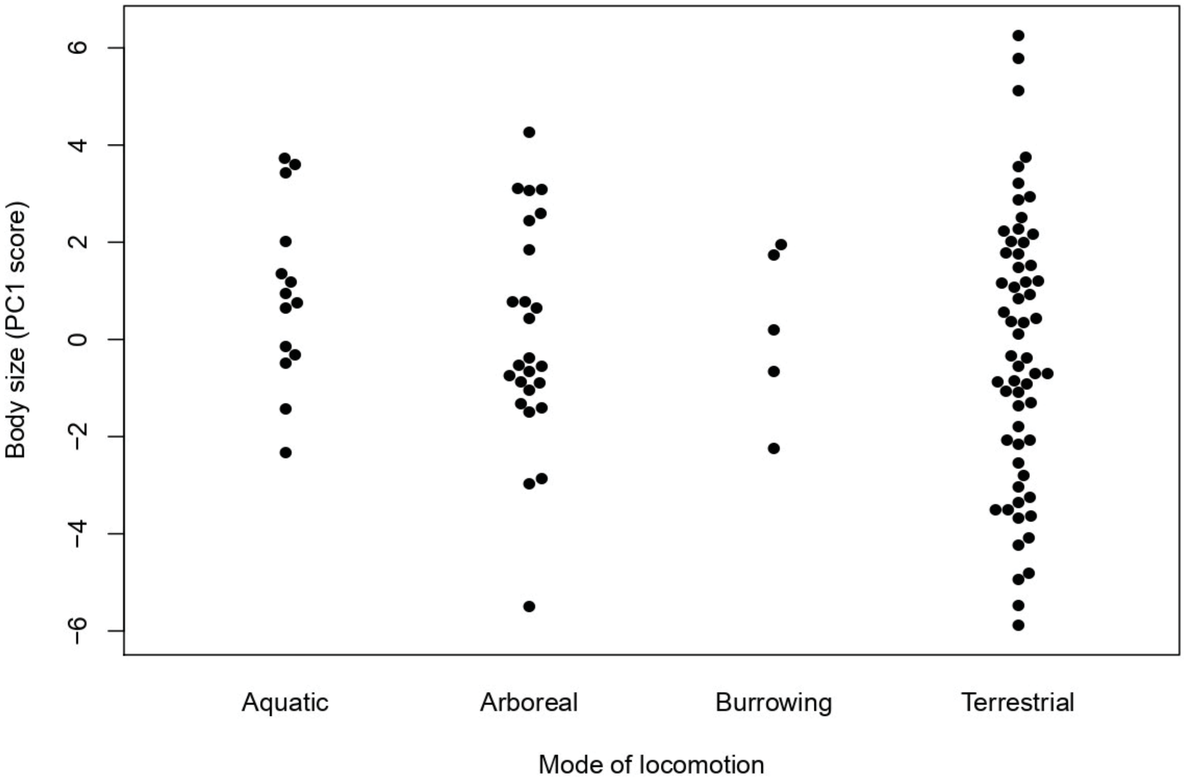

Figure 2. Swarmplot of body size (PC1 score of a principal component analysis of the covariance matrix of log-transformed measurements) of the studied frog species according to their corresponding mode of locomotion.

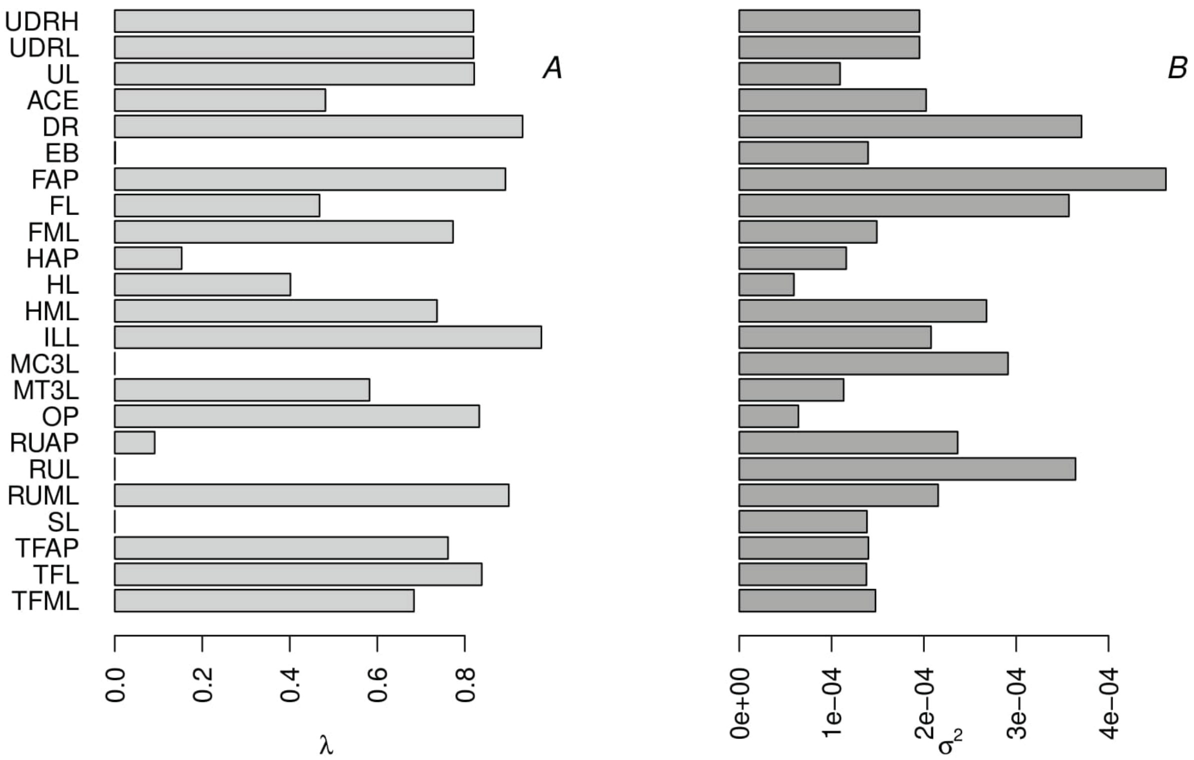

Figure 3. Estimates of phylogenetic signal (*A*) and evolutionary rate (*B*) for each of the studied measures. HL = humerus length, DR = deltoid ridge length, EB = epicondylar breadth, MLh = mediolateral diameter of humerus, APh = anteroposterior diameter of humerus, RUL = radio-ulna length, OL = olecranon process, MLru = mediolateral diameter of radio-ulna, APru = anteroposterior diameter of radio-ulna, SL = scapula length, MC3L = metacarpal III length, FL = femur length, MLf = mediolateral diameter of femur, APf = anteroposterior diameter of femur, TLF = tibia-fibula length, MLtf = mediolateral diameter of tibia-fibula, APtf = anteroposterior diameter of tibia-fibula, IL = ilium length, ACE = acetabulum diameter and MT3L = metatarsus length.

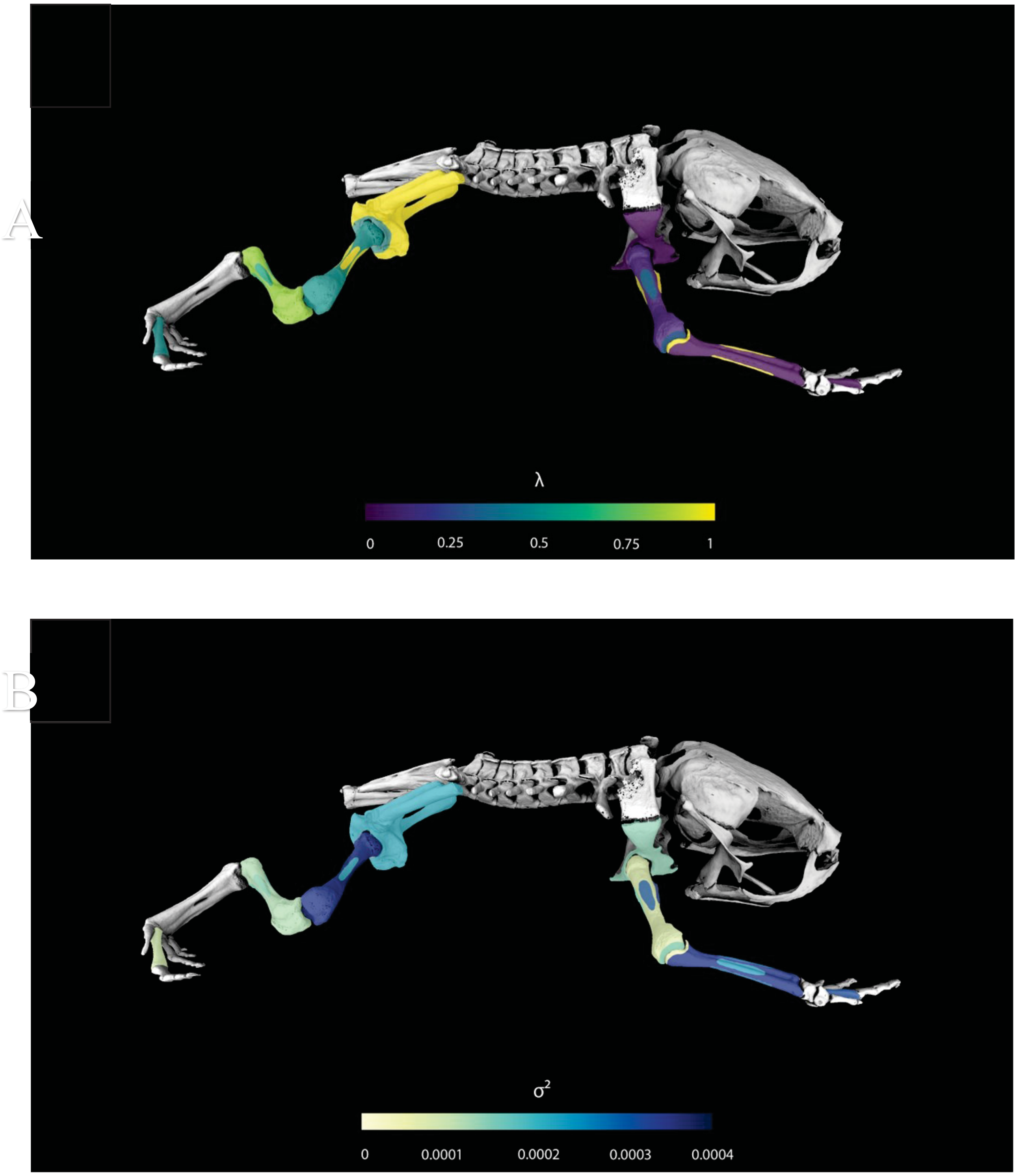

Figure 4. Graphical representation of estimates of phylogenetic signal (*A*) and evolutionary rate (*B*) for each of the studied measures. In A, warm color represents more phylogenetic signal and colder color is less phylogenetic signal in structures. In B, warm colors represent lower evolutionary rates and cold colors represent higher evolutionary rates.

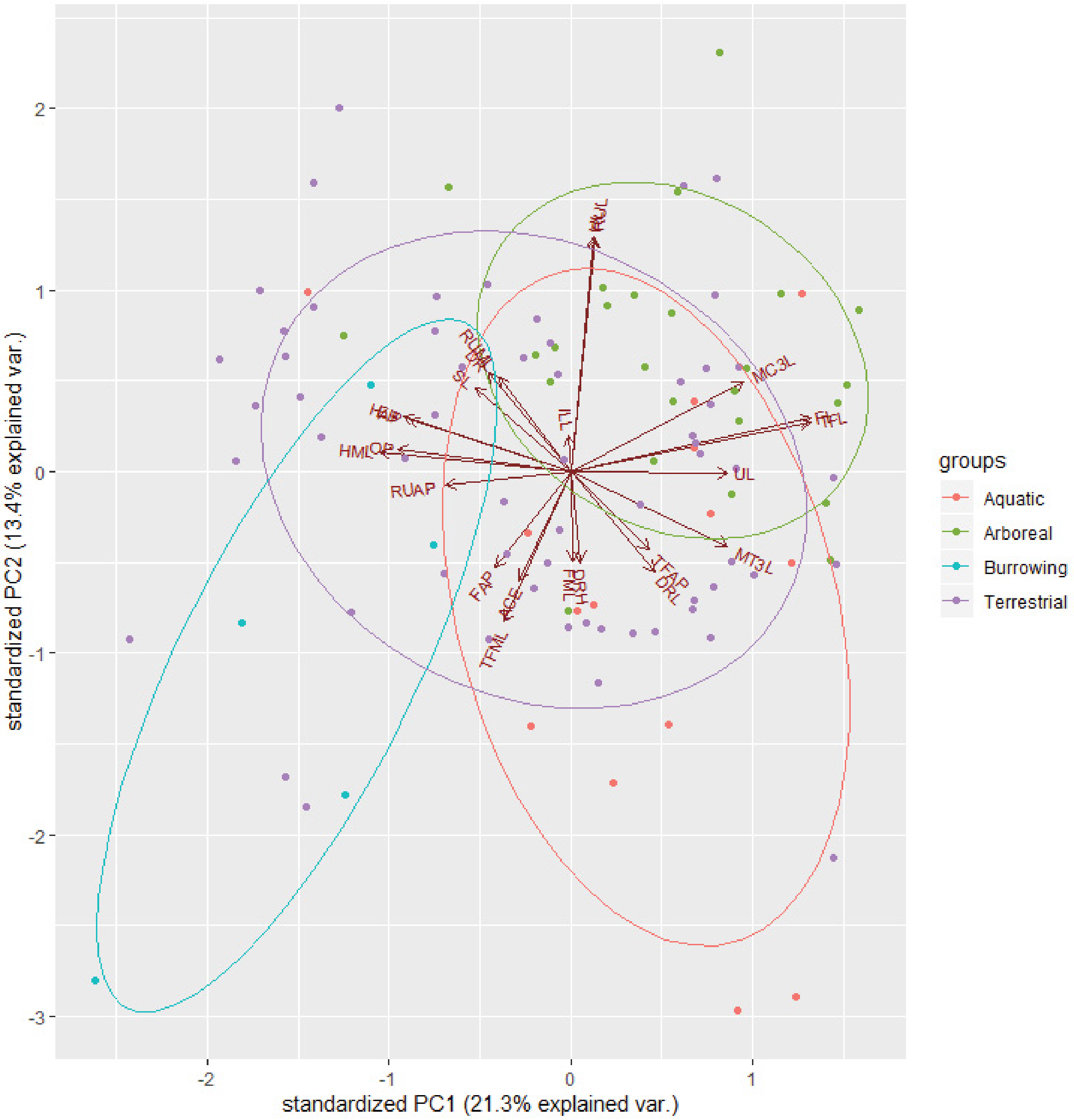

Figure 5. PCA Plot with ellipses representing the groups based on mode of locomotion.

Supporting Information

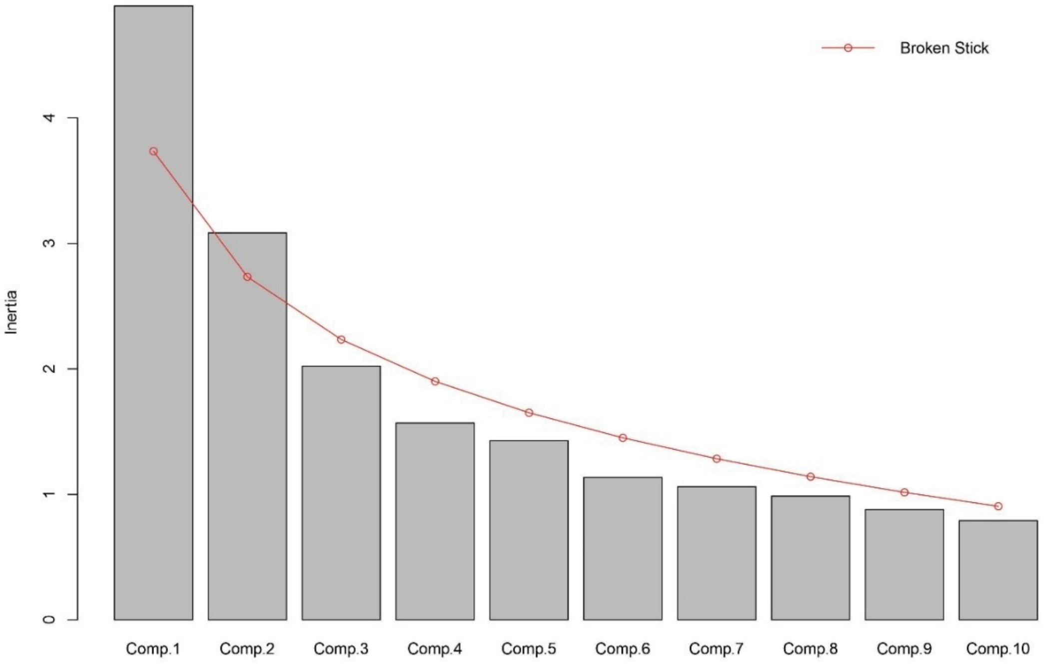

Figure S1. Scree plot and broken stick model to help compare the real eigenvalues with null model values generated by the broken stick model (red dot line).

3. ARTIGO CIENTÍFICO 2

The evolution of the anuran phalanges: rates, phylogenetic signal, and modularity

Abstract

Variation in limb structure constitutes the basis of several ecomorphological adaptations across tetrapod lineages. The tetrapod limb can be broadly divided into three regions - the stylopod, the zeugopod, and the autopod. Stylopod and zeugopod bones are readily recognizable across the fish-amphibian transition, yet the autopod has been altered substantially in several lineages. Indeed, it has been argued that phalanx lengths change as a developmental module that is separate from the rest of the autopod. The main goal of this study was to characterize major evolutionary trends in phalanx length evolution in Anura. We compiled a dataset from microCT data available in the MorphoSource platform, including a total of 130 frog species. Although ancestral character reconstructions suggest a relatively constant rate of evolution of all phalanx lengths along the anuran history, estimates of phylogenetic signal and evolutionary rate detected substantial differences in these parameters among different phalanx types. In particular, in the manus, proximal and medial phalanges showed similarly high phylogenetic signal and evolutionary rates, whereas the distal phalanx evolved slower and with lower phylogenetic signal. On the other hand, differences in tempo and mode of phalanx length evolution were more complex in the pes. Finally, the correlational structure of manus and pes phalanges suggests that the medial phalanx in the manus is actually homologous to the penultimate phalanx of the pes, rather than the medial phalanx of the pes. Our results

highlight a complex and highly modular evolutionary history of the phalanges in Anura, and provide valuable insights into the proximate causes of phalanx development.

Introduction

The tetrapod limb is usually divided into three main regions: the stylopod (humerus/femur), the zeugopod (radius and ulna/tibia and fibula), and the autopod (carpal, tarsal, metacarpal and metatarsal bones, and digits). The bones of the stylopod and zeugopod are readily recognizable across the fish-amphibian transition, but the autopod has been drastically remodeled (Carroll & Holmes 2007), forming an evolutionary novelty that is apomorphic for tetrapods (Wagner & Larsson 2008). Indeed, there have been several important events in the evolution of the autopod. For instance, it has been argued that phalanx sizes change as an integral developmental module that is separate from the rest of the autopod (Kavanagh et al. 2013, Hiscock et al. 2017). Paleontological studies have explored potential drivers of variation in the number of phalanges (phalangeal formula) (e.g. Holder 1983, Hopson 1995, Shapiro et al. 2007, Gallis et al. 2001), yet the determinants of the evolution of variation in phalanx size are still poorly known, even though most of the ecomorphological changes in the tetrapod limb take place in the autopod, including adaptations for running, grasping, perching, paddling and digging (e.g. Richardson & Chipman 2003, DeGusta & Vrba 2005, Sustaita et al. 2013, Toussaint et al. 2020).

Considerable advances in our understanding of autopod development have been achieved in the past few decades. Digit development begins with the formation of interdict areas and digital rays through a Turing-like patterning mechanism (Sheth et al. 2012, Raspopovic et al. 2014, Hiscock et al. 2017). The proximal region of the autopod (i.e. metacarpals and metatarsals) begin to condense early, whereas the formation of the digits begins to take place as outgrowths at the tip of the autopod, and this difference seems to reflect distinct developmental processes. For instance, *Bmpr1b* knockout mice showed loss of phalanx development, whereas their metacarpals remained unaltered (Yi et al. 2000). Digit formation at the end of the autopod results from unidirectional growth along a proximal-distal axis in the distal mesenchyme located just beneath a specialized epithelial structure, known as the apical ectodermal ridge (AER). The epithelial cells within the AER play an important role in mediating overall limb growth by secreting FGF signals that promote cell proliferation in the underlying mesenchyme (Mariani et al. 2017). This region of mesenchyme growth at the end of the autopod is called the "digital crescent" or the "phalanx-forming region" (PFR) (Montero et al. 2008, Suzuki et al. 2008, Witte et al. 2010). Indeed, digit elongation at the PFR results from a FGF gradient that specifies the distal domain of growth competency (Montero et al. 2008, Suzuki et al. 2008, Hiscock et al. 2017, Feregrino et al. 2019). And, particularly important in our context, FGF signaling from the AER appears to play a role in both digit length and phalanx numbers. For instance, Sanz-Ezquerro & Tickle (2003) examined the expression of *Fgf8* in developing chicken feet and found a correlation between the duration of Fgf8 expression at the tip of the digit and the resulting number of phalanges. Moreover, experimentally prolonging its expression at the tip of the digit induces the formation of an additional phalanx, whereas the inhibition of an FGF receptor prevented the formation of the most distal phalanx (Sanz-Ezquerro & Tickle 2003). Although these advances provide a model to explain phenomena such as hyperphalangy in cetacean flippers (Richardson & Oelschläger 2002), the determinants of individual phalanx length are still poorly known. In one of the few studies addressing modularity and evolution in the autopod, Kavanagh et al. (2013) showed that, within populations of two bird species, the size of successive phalanges within a digit exhibit predictable relative proportions, which themselves varied from nearly equal sizes to more distinct gradients from large to small. In addition, Kavanagh et al. (2013) measured phalanx proportions in 132 vertebrate species, including birds, early sarcopterygians, cetaceans, lissamphibians and ichthyosaurs, and argued that the same scaling rules apply to all lineages.

The main goal of this study is to characterize evolutionary trends in phalanx evolution using anurans as a model system, given their high species richness, and diversity of ecological adaptations. To that end, we integrate measurements from microcomputed tomography across a broad sample of species, phylogenetic comparative methods and graphical modelling. Our results demonstrate several intriguing differences rate and phylogenetic signal among phalanges, providing valuable insights into the mechanisms of phalanx development and differentiation.

Methods

Measurements were obtained based on microtomography data available on MorphoSource (https://www.morphosource.org/) as part of the Frog Diversity project. We included a total of 130 species from a variety of anuran lineages. All species were downloaded in .stl 3d object format or in raw data image stack files. The raw data stack files were converted into .nii (NIfTI files) using the NIfTI Input/Output plugin in Fiji (Schindelin et al., 2012). Measurements were performed in 3D Slicer (https://www.slicer.org/) using the Model (for .stl files), Volume (for .nii files), and Markup modules. We selected the longest digit in the manus (digit III) and pes (digit IV) to measure phalanx lengths (figure 1). In the manus, we measure the proximal phalanx (Mpp); medial phalanx (Mmp), and distal phalanx (Mdp), whereas in the pes we measured the proximal phalanx (Ppp); medial phalanx (Pmp); penultimate phalanx (Pptp) and terminal phalanx (Ptp). All measurements were ln-transformed prior to the analyses. The phylogenetic relationships of the studied species were obtained from Jetz & Pyron (2018), as available on the VertLife portal (https://vertlife.org/, accessed on 19 April 2021). To account for uncertainty in their phylogenetic relationships, all analyses were repeated using 100 alternative trees.

We used five analytical approaches to explore anuran phalanx evolution. First, we assessed the extent to which variation in phalanx length is isometric. We used the geometric mean of all measurements as a proxy for body size, and compared it to the length of each phalanx of the corresponding individual to test whether their relationship showed a slope significantly different from 1. To assess to what extent that conclusion was based on our particular proxy for body size, we also regressed all traits against each other. Second, we explored general trends in phalanx length evolution across lineages using ancestral state reconstruction, as implemented in the traitgram function in 'phytools' v. 1.0-1 (Revel, 2012). Third, we estimated the level of phylogenetic signal in each phalanx using Pagel's λ (1999) implemented in the phylosig function in ‘phytools’. Fourth, we used the fitContinuous function in geiger v. 2.0.6.4 (Pennell et al. 2014) to estimate their rates of evolution. The latter was measured as the σ^2 parameter of a Brownian motion (BM) model, which assumes that variance accumulates among lineages as a linear function of elapsed time since divergence. The main goal of these analyses is not to interpret absolute estimated parameter values, but rather to compare them across phalanges, both within and between manus and pes, as a means to assess general long-term trends in their evolution during the history of Amphibia. Finally, we explored patterns of trait covariation in phalanx size using the graphical modelling approach of Magwene (2001). We began by computing the inverse correlation matrix of log-transformed phalanx length data. Using this matrix, we calculated, for each trait, the coefficient of multiple determination, which is a measure of how much of the variance in a given trait is jointly explained by all of the other traits in the dataset. In other words, it measures how much one could predict a given phalanx length based on the knowledge of all other phalanges. The inverse correlation matrix was scaled to have ones on the main diagonal, generating a partial correlation matrix (i.e. the degree of correlation between each pair of traits, given for all other traits). We used the absolute values of partial correlation, given that there is no distinction between negative and positive partial correlations (Magwene 2001). We then excluded near-zero partial correlations using their edge exclusion deviance, such that all edges with deviances less than 3.84 (the 5% point on the χ^2 distribution with df=1) were omitted. The final matrix was then represented as a graph in which edge widths were proportional to their partial correlation. Finally, we calculated edge strengths, which are a measure of the level of information between two traits, conditioning on all other traits in a conditional independence graph (see Magwene 2001 for formal definitions of these metrics).

Results

Patterns of interspecific variation in phalanx length showed that all phalanges showed negligible departures from isometry (Figure 2 Table 1). This is remarkable, given the considerable differences in autopod morphology, as well as ecological characteristics, of the sample of studied species. Rather, variation in phalanx length seems to be largely driven by changes in intercepts, rather than in allometric coefficients.

There was a general pattern based on ancestral character reconstruction of a relatively constant accumulation of variation across lineages, both in the manus (figure 3A) and the pes (figure 3B). In particular, different phalanges vary according to their size, being larger on the proximal phalanx and decreasing towards more distal phalanges. However, this seemingly congruent pattern of evolution belies intriguing differences uncovered in further analyses. First, in the manus, the distal phalanx tended to show both lower phylogenetic signal than the medial and distal phalanges, which themselves showed relatively similar phylogenetic signal (Figure 4A). Although many mechanisms might lead to lower phylogenetic signal, they usually are caused by heterogeneity in evolutionary rates across lineages. Therefore, one could expect that the observed differences in phylogenetic signal are due to a higher evolutionary rate in distal phalanges, yet the obtained estimates are precisely the opposite of that expectation (Figure 4B). This might suggest that the lower phylogenetic signal in distal phalanxes could be due to fine-scale adaptations on finger tips that might not involve particularly fast evolutionary rates. Interestingly, patterns in the pes are more complex, with differences among phalanges, but not in a simple proximal-distal axis (Figure 4C, D). One limitation of that graphical representation is that one cannot easily visualize the extent to which the represented variation is due to differences in estimates within versus between trees. To make this inference easier, in Figure 5 we use lines to represent estimates for each of the tested trees. The difference between distal versus proximal and medial phalanges is still clear in the manus, but the incongruence in the pes becomes more apparent, with a decrease in phylogenetic signal and increase in evolutionary rate in the medial phalanx (Figure 5).

Finally, our graphical modelling analysis showed intriguing patterns regarding the relationships between phalanges across manus and pes (figure 6). As expected, there was high integration within the manus and pes, yet partial correlation coefficients were progressively lower from proximal to distal phalanges, which might suggest that they become increasingly less integrated, as expected given their relatively low phylogenetic signal and evolutionary rate (Figures 4, 5). There were also relatively strong partial correlation coefficients between proximal phalanges in the manus and pes, as well as between their most distal phalanges. Interestingly, there was no significant partial correlation between medial phalanges in the manus and pes, yet there was moderate partial correlation between the medial phalanx in the manus and the penultimate phalanx in the pes, suggesting that they are actually serial homologs.

Discussion

The results of the present study provide valuable insight into the mechanisms driving phalanges evolution in Anura. In particular, we showed differences in the tempo and mode of phalange evolution in the manus and pes which could not have been easily predicted given current knowledge about tetrapod autopod development. The development of phalanges requires the specification of two distinct cell types. In particular, as soon as the proliferation of the PFR progenitor cells displaces distally the source of the FGF gradient, the proximally located cells lose their progenitor state and undergo a divergent cell type specification and differentiate accordingly into either chondrocytes (the cellular building blocks of the phalanges themselves) or prospective interzone cells that eventually form the synovial joints to connect the digit bones (Shwartz et al. 2016, Hiscock et al. 2017). Dahn & Fallon (2000) demonstrated in chicken embryos that the forming digits have their segmentation pattern specified by the interdigit mesenchyme that is located immediately posterior to them. In particular, when the newly-formed cartilage reaches a critical length, a joint is initiated behind the growing tip, establishing a phalanx behind the new joint, and growth of the digit ray continues distal to the new joint (Suzuki et al. 2008). Based on these mechanisms, one could expect that all phalanges share largely similar developmental mechanisms, particularly during the segmentation of the autopod. Indeed, Kavanagh et al. (2013) showed that, within populations of two bird species, the size of successive phalanges within a digit exhibits predictable relative proportions, which themselves varied from nearly equal sizes to more distinct gradients from large to small. In addition, Kavanagh et al. (2013) measured phalanx proportions in 132 vertebrate species, including birds, early sarcopterygians, cetaceans, lissamphibians and ichthyosaurs, and argued that the same scaling rules apply to all lineages. This result is intriguing, given that it contradicts the previous hypothesis that each phalanx was ascribed a uniquely specified identity to each phalanx (Lewis and Wolpert 1976), and suggests that digit development is actively integrated across each digit as a single developmental module.

In some respects, our results agree with Kavanaugh et al. (2013), given that we indeed found significant partial correlations between adjacent phalanx lengths, such that they are unlikely to vary independently. The largely isometric variation in phalanx length (Figure 2), as well as the relatively constant evolutionary rates (Figure 3), seem to confirm that scenario. However, there is one fundamental aspect that our results contradict the hypothesis of Kavanaugh et al. (2013): based on the ratios between phalanx size, they argue that the predictability in phalanx size increases as from proximal to distal phalanges ("With knowledge of this relationship, one can predict the size of P3 accurately by knowing the sizes of P1 and P2"). However, we show that predictability actually decreases from proximal to distal phalanges, as shown by the decreasing partial correlations (Figure 4). Indeed, our results suggests that parts of the autopod, particularly the distal phalanges, might experience additional regulation in their development, yet the underlying mechanisms for this phenomenon is still not understood. The distal region in anuran manus is often specialized, especially in species with unique niches (Gray et al. 1997, Manzano et al. 2008), which might explain the evolutionary pressures that led to this pattern.

Our analyses revealed a relatively constant, but still complex, pattern of Anuran phalanx evolution. These changes are the result of the combination of evolutionary forces, mainly the phylogenetic history of the species in our analyses, and their ecology. One can also consider that the fingers of anurans present a very interesting pattern of differentiation, being constant, however, with a low evolutionary speed, yet a high rate of modification in the tip of the digits of the manus, in the pes we did not find any accentuated pattern as it was in the case of the manus. Furthermore, our results showed us that the development of the phalanges is more complex than simply a single structure, indicating a possible evolution in modules. In addition, there is a strong relationship between the manus and pes digits. Finally, our data showed us important insight evolutionary history of frogs digits, however, it is evident the need for further studies of their underlying developmental mechanisms to better understand these evolutionary patterns.

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Tables and Figures

Table 1. Statistical values of isometric regression analysis. M_pp: Manus proximal phalanx; M_mp: Manus medial phalanx; M_dp: Manus distal phalanx; P_pp: Pes proximal phalanx; P_mp: Pes medial phalanx; P_ptp: Pes pesnultimate phalanx; P_tp: Pes terminal phalanx. All values was log-transformed before the regression.

Regression	Slope	p	R^2
M_pp ~ Size	1.03447	2.00E-16	0.9528
M_mp ~ Size	1.04554	6.16E-16	0.9204
M_dp ~ Size	0.90197	2.00E-16	0.8639
P_pp ~ Size	1.01275	2.00E-16	0.9185
P_mp ~ Size	1.03997	2.00E-16	0.9347
P_ptp ~ Size	1.04244	2.00E-16	0.9503
P_tp ~ Size	0.92286	2.00E-16	0.8955
M_pp ~ M_mp	0.94583	2.00E-16	0.9453
M_pp ~ M_dp	1.00032	2.00E-16	0.8371
M_mp ~ M_dp	1.05149	2.00E-16	0.8753
P_pp ~ P_mp	0.96869	2.00E-16	0.9722
P_pp ~ P_ptp	0.94103	2.00E-16	0.9064
P_pp ~ P_tp	0.95687	2.00E-16	0.7779
P_mp ~ P_ptp	0.97823	2.00E-16	0.9455
P_mp ~ P_tp	0.98849	2.00E-16	0.8013
P_ptp ~ P_tp	1.00854	2.00E-16	0.8443

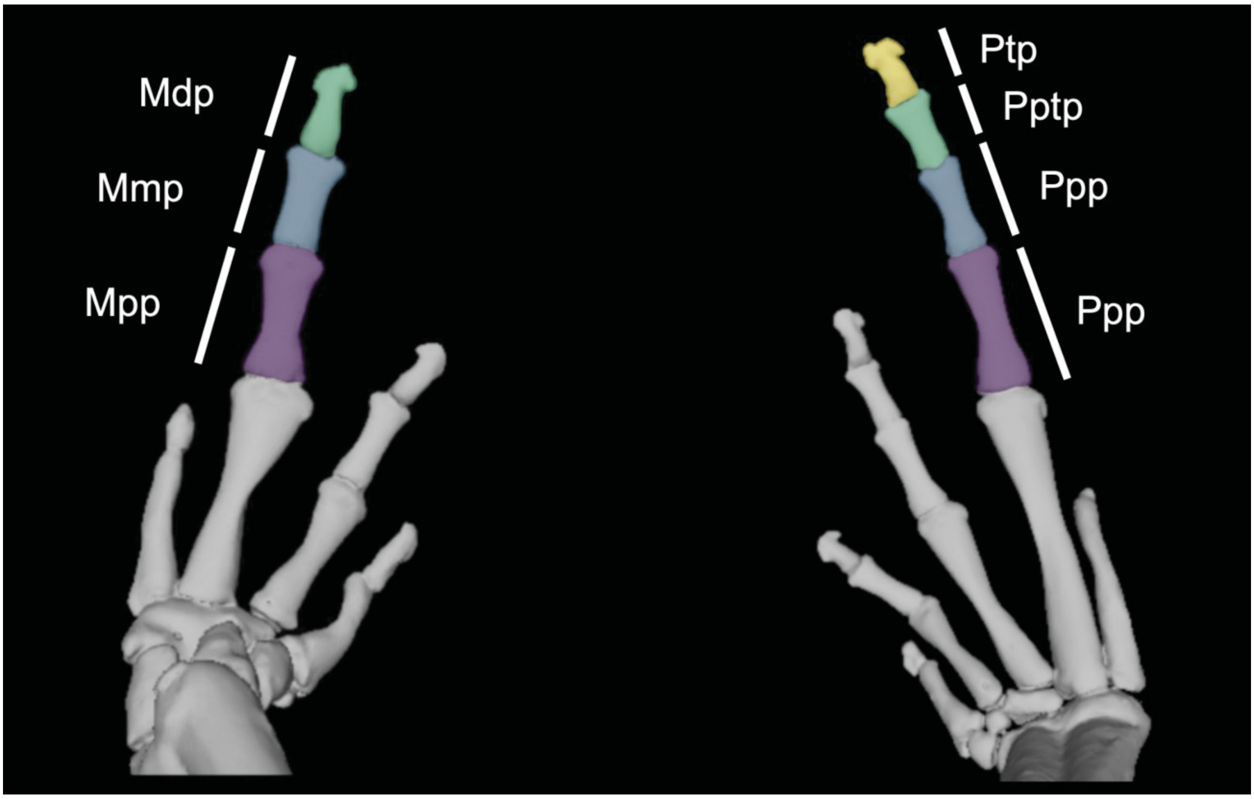

Figure 1. Illustration of the phalanges measured in the present study in the manus (digit III, left) and pes (digit IV, right): proximal phalanx (Mpp); medial phalanx (Mmp), and distal phalanx (Mdp), whereas in the pes we measured the proximal phalanx Ppp); medial phalanx (Pmp); penultimate phalanx (Pptp) and terminal phalanx (Ptp).

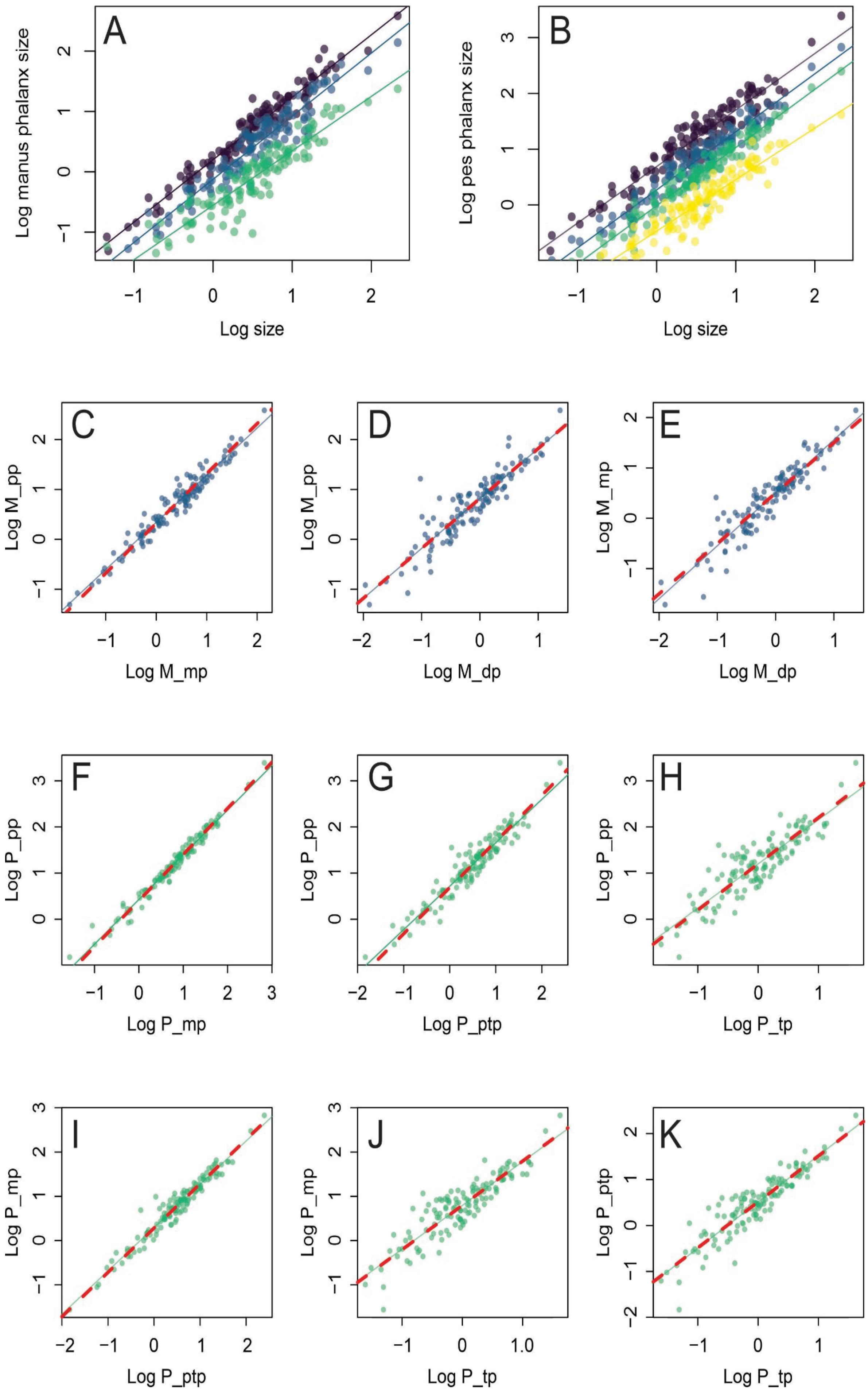
A
Log manus phalanx size
Log size
−1
0
1
2
B
Log pes phalanx size
Log size
0
1
2
3
C
Log M_pp
Log M_mp
D
Log M_pp
Log M_dp
E
Log M_mp
Log M_dp
F
Log P_pp
Log P_mp
G
Log P_pp
Log P_ptp
H
Log P_pp
Log P_tp
I
Log P_mp
Log P_ptp
J
Log P_mp
Log P_tp
1.0
K
Log P_ptp
Log P_tp
−2

Figure 2. Tests assessing potential departures from perfect isometry (red dashed lines) in different phalanges. In figure A and B are represented the regression of the phalanx sizes of manus (A) and pes (B) with size. In figure A, dark blue dots are values of proximal phalanx; blue dots are medial phalanx and green dots distal phalanx. In figure B the colors dark blue, blue, green and yellow are representing proximal, medial, penultimate and termina phalanx of pes, respectively. Figures C, D and E are regression between manus phalanx; Figures F, G, H, I, J and K are regression between pes phalanx. M_pp: Manus proximal phalanx; M_mp: Manus medial phalanx; M_dp: Manus distal phalanx; P_pp: Pes proximal phalanx; P_mp: Pes medial phalanx; P_ptp: Pes penultimate phalanx; P_tp: Pes terminal phalanx. The red line in figures C to K represents the ideal line of variation to discard an influence of the size in our results.

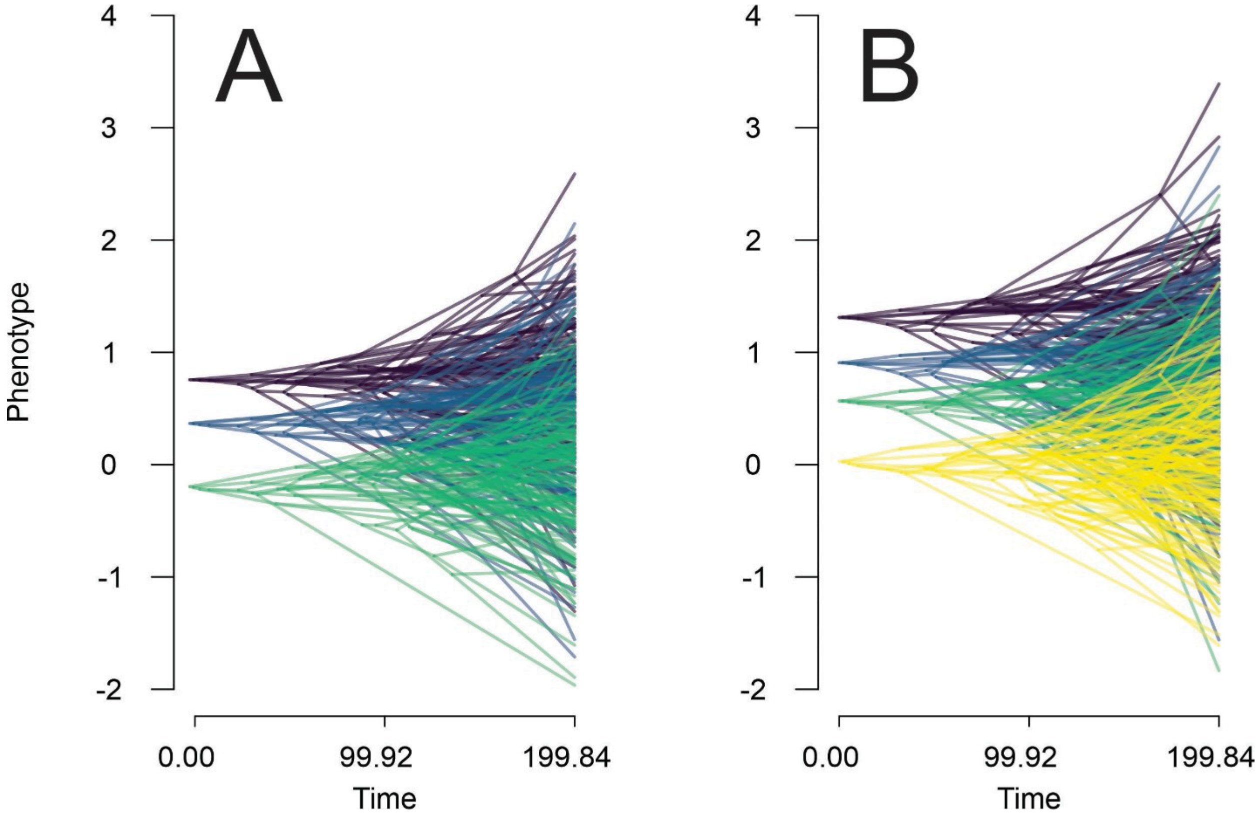

Figure 3. Phenogram representing the ancestral state reconstruction of phalanx size in the manus (A) and pes (B). Is evident an accumulation of variation specially on the most terminal phalanges in both manus and pes.

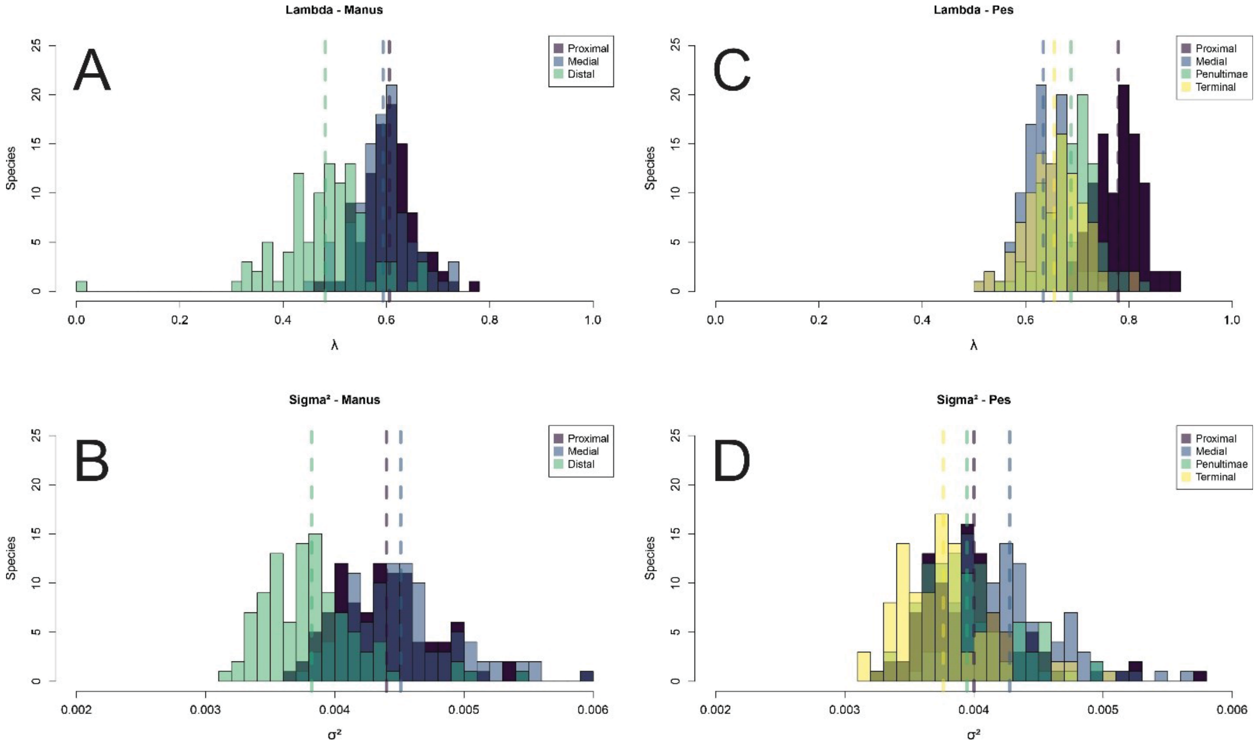

Figure 4. Histograms of phylogenetical signal and evolutionary rates analysis of both manus and pes. The observed distributions represent variation in estimated rates across different alternative topologies to account for phylogenetic uncertainty.

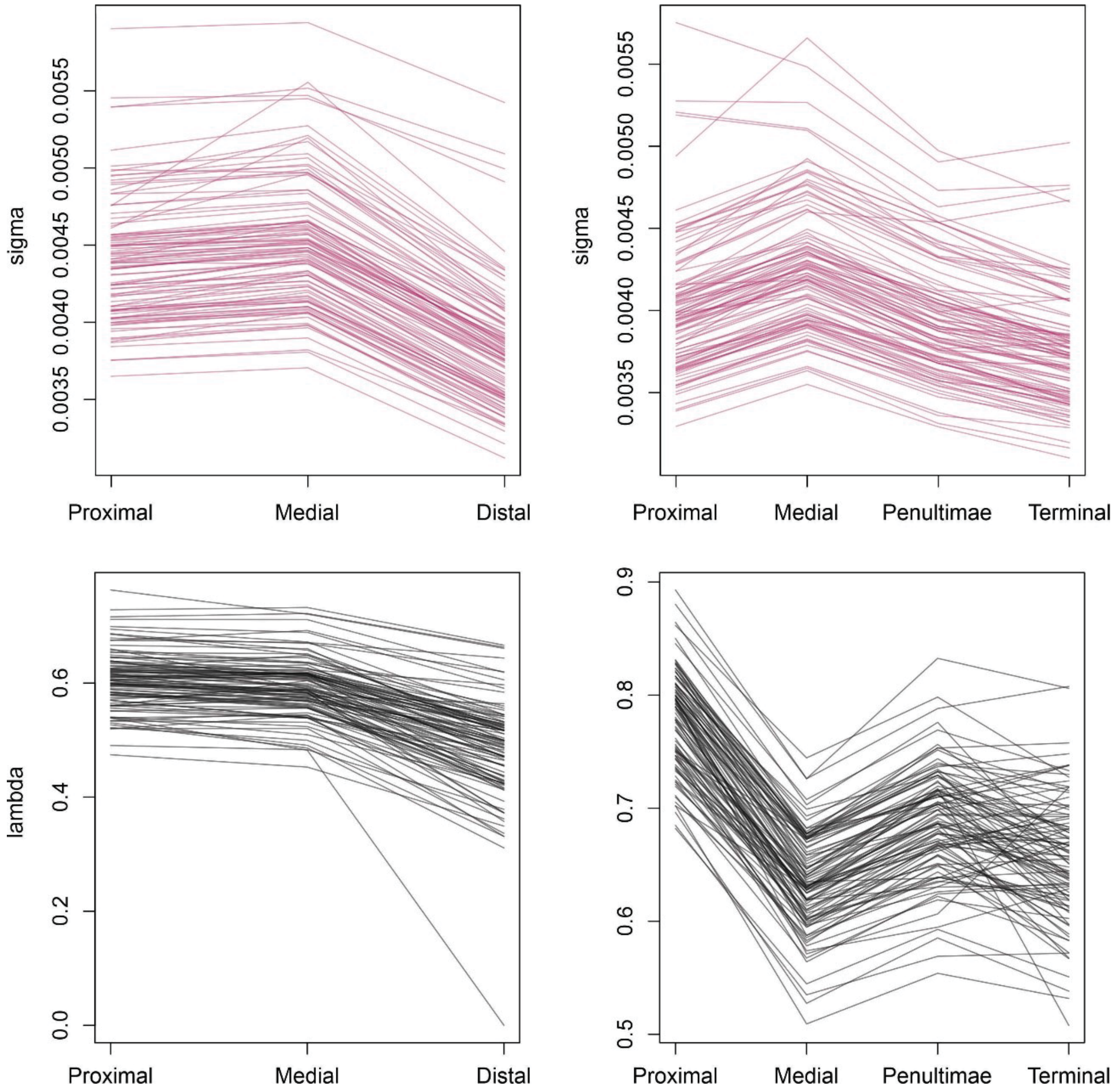

Figure 5. Estimates of phylogenetical signal and evolutionary rates of phalanges in the manus and pes. Lines indicate estimates from the same alternative topology.

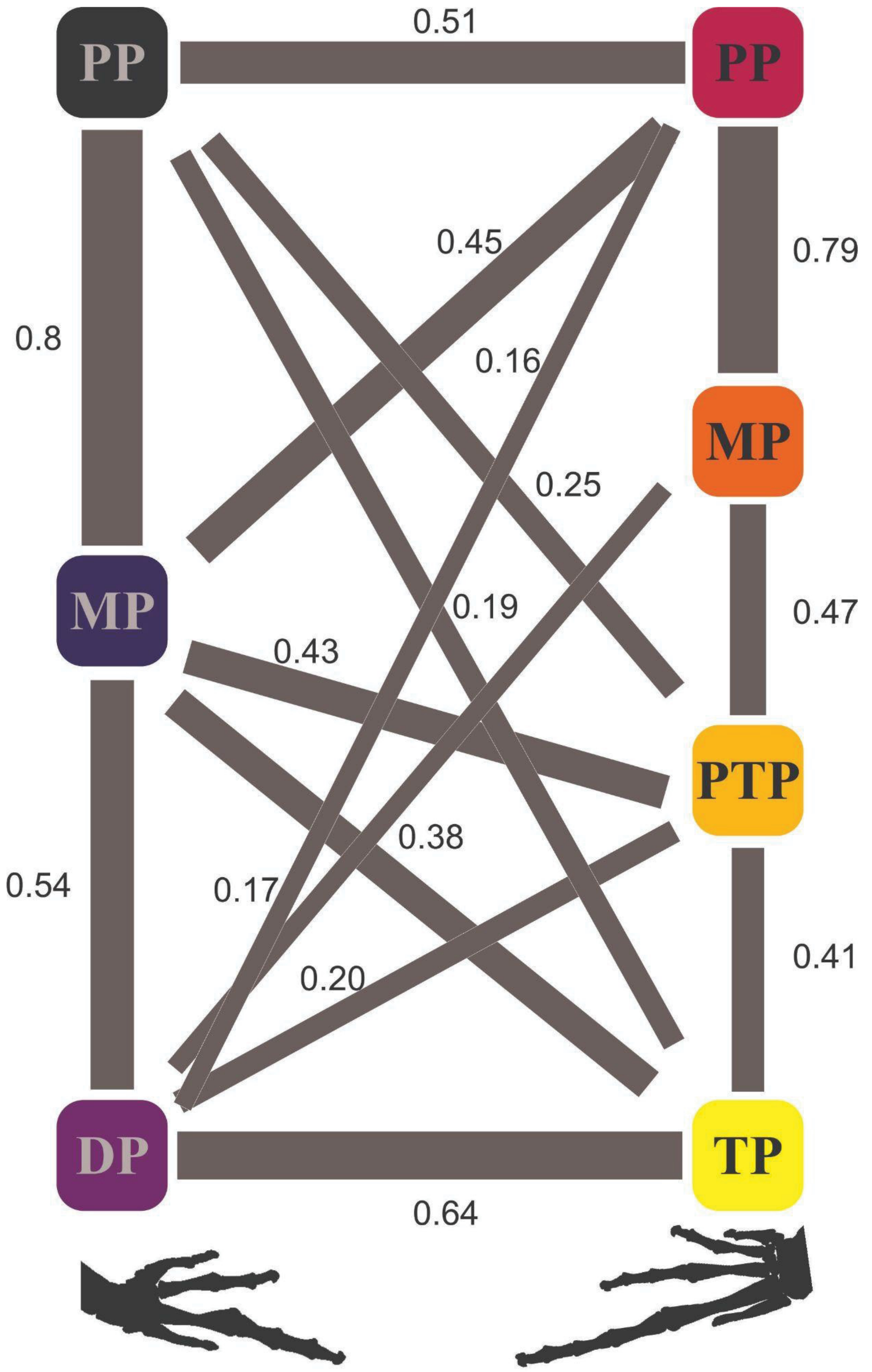

Figure 6. Correlational analysis using Magwene graphical model. Line with corresponds to variation in partial correlation between the lengths of the corresponding phalanges. Number values indicate partial correlation values obtained in our analysis. PP: proximal phalanx; MP: medial phalanx; DP: distal phalanx; PTP: penultimate phalanx; TP terminal phalanx.

4. ARTIGO CIENTÍFICO 3

Environmental corelates with osteoderm wall thickness in *Brachycephalus* (Anura: Brachycephalidae): an adaptation to cope with miniaturization?

Abstract

Brachycephalus is a fascinating genus of anurans endemic to the Atlantic forests of Brazil. Their most obvious feature is its miniaturization, with many of its species barely exceeding 1 cm in total body length. Such small body size, possibly close to the absolute lower limit for the Bauplan of a terrestrial vertebrate, is likely to impose several important challenges, such as a surface area:volume ratio that leaves them particularly prone to desiccation. One potential adaptation to meet this challenge is the evolution of calcium accumulation in their skin through the formation of osteoderms. In this study we explore this hypothesis by precisely measuring osteoderm wall thickness for a broad sample of *Brachycephalus* and comparing them to the climatic conditions where those species are found. We demonstrate that osteoderm wall thickness is significantly associated with both mean annual temperature and precipitation. In addition, the observed variation in osteoderm wall thickness showed a clear correspondence to different clades within the genus. These results demonstrate that osteoderms can represent an important adaptation to cope with the constraints of miniaturization.

Keywords: Anura; microCT; morphology; climate

Introduction

Miniaturization is an evolutionary process that involves the extreme reduction in size of organisms within a lineage, in which, although extremely small, they are derived from a larger-bodied ancestor (Hanken and Wake 1993). This process occurs in several groups of vertebrates, being observed in amphibians, reptiles, and fishes (Hanken 1985; Yeh 2002; Rüber et al. 2007; Glaw et al. 2012), and even in invertebrates (Schmidt-Rhaesa 2001; Rundell and Leander 2010; Polilov 2015). The implications of miniaturization on the body of organisms range from structural reduction and simplification to morphological novelty and the increase of morphological variability (Hanken and Wake 1993). In anurans, miniaturization is a common phenomenon, which was described by several studies (Yeh 2002; Maglia et al. 2007; Zimkus et al. 2012). Yeh (2002) described some implications of miniaturization in frogs skeletal morphology, such as skull bone loss and modification in limb morphology as reduction of phalanges. Furthermore, miniaturization in anurans morphology also implies in the reduction of brain and sense organs (Yeah 2002; Essner et al. 2022). In some cases, miniaturization is also related with terrestrialization in some anuran clades, which was evidenced in the reduction on pedal webbing in puddle frogs, where the smallest species can be found in leaf-litter and bigger ones have semiaquatic habits (Zimkus et al. 2012). In some species miniaturization comes with reduction of ossification too, an example of that is found in *Acris crepitans* (Maglia et al. 2007). In their study, Maglia et al. (2007) found support that *Acris crepitans* is a miniaturized species by the small size, reduced skull ossification and some morphological novelty. However, the results described by Maglia et al. (2007)

in ossification in *A. crepitans* goes in opposite way when compared with another group of miniaturized frogs, the *Brachycephalus* genus.

*Brachycephalu*s is a genus of miniaturized frogs that are endemic to the Brazilian Atlantic Forest, with their distribution ranging from the south of the state of Bahia to the north of the state of Santa Catarina (Figure 1). The distribution of *Brachycephalus* is restricted to the leaf litter layer of cloud forests (Bornschein et al. 2016). In addition to altitude, another interesting pattern observed in the distribution of these animals is their microendemism. It is quite uncommon to find more than one species cohabiting the same forest, making it possible to find only one species per mountain (Pie et al. 2013; Ribeiro et al. 2015) *Brachycephalus* exhibits significant morphological variability among species, and can be classified into two distinct groups: *B. ephippium* group and *B. pernix* group (Ribeiro et al. 2015, Folly et al. 2032). The *B. ephippium group* consists of animals with a bufoniform body, where the ribs and some vertebrae are fused, forming a carapace-like structure (Ribeiro et al. 2015). These animals are the largest representatives of *Brachycephalus*, measuring just over 2 cm in length (Ribeiro et al. 2015). The *B. ephippiu*m group is distributed from the state of Espírito Santo to the south of the state of São Paulo. We also have animals includes smaller-sized animals with a leptodactyliform body and no apparent bone fusion, which composes the *B. didactylus* group, however, recent studies do not classify this group as an independent group like *B. pernix* and *B. ephippium* groups. (Ribeiro et al. 2015, Folly et al., 2023). These animals have a broader distribution and can be found from the south of the state of Bahia to the north of the state of Santa Catarina. Lastly, the *B. pernix* group comprises animals with a bufoniform body, but without apparent bone fusion (Ribeiro et al. 2015). The *B. pernix* group is restricted to the states of Paraná and Santa Catarina. Overall, *Brachycephalus* is a genus of miniaturized frogs with several morphological peculiarities that distinguish them from other groups of frogs. In this genus, the animals show reduced digits, as well as fusion in the bones of the skull, and in extreme cases, the presence of a large bone plate resulting from the fusion of the vertebrae, a different pattern of other miniaturized frogs, as *Acris crepitans* (Maglia et al. 2007; Clemente-Carvalho et al. 2008; Ribeiro et al. 2015). Among all the characteristics that make this genus unique and a good group for evolution studies, ossification and mineralization in the skin of these animals deserves a greater focus, both because it is one of the morphological characteristics that distinguishes *Brachycephalus* from other anurans, and due to the lack of studies. Ossification in the skin is present in several groups of vertebrates, however it is more commonly reported in reptiles (Krmpotic et al. 2009; Delorme and Vickaryous 2010; Quinzio and Fabrezi 2012; Williams et al. 2022). Skin ossification consists of the deposition of mineralized elements in the layers of the dermis of animals. Several works report mineralization in the skin of anurans, and one of the possible explanations for this deposition of mineralized structures lies in the animal osmoregulation (Stiffler 1995; Azevedo et al. 2006; Witzmann et al. 2010). However, deposition in *Brachycephalus* skin was recently described only by Mari et al. (2022) In their work, Mari et al. (2022) found evidence that supports the presence of a layer of mineralized structures in *Brachycephalus* dermis, which was hypothesized that this condition is an adaptation of those animals to the environment.

Aiming on mineralization of *Brachycephalus* dermis, the objective of this work is correlate the influence of the environment in genus mineralized structures in dermis, going deeper on wall thickness of these structures, not just in the presence or absence. Furthermore, we expect to find a phylogenetical relation of the wall thickness of *Brachycephalus* skin throughout the genus. We also will seek to trace a possible relation of miniaturization and deposition of mineralized structures in the *Brachycephalus* dermis.

Materials and methods

Data collection and Wall thickness analysis

The high resolution images were obtained by high resolution microcomputed tomography (microCT) scans generated at the University of Florida's Nanoscale Research Facility, using a Phoenix v|tome|x M (GE's Measurement & Control business, Boston, MA, USA). Raw X-ray data were processed using GE's proprietary datos|x software v 2.3 to produce a series of tomography images. The wall thickness analysis was performed at VG StudioMax 3.0.3 (Volume Graphics, Heidelberg, Germany) using the tool with the same name. The wall thickness analysis can be made using two approaches: the ray method or the sphere method. Ray Method is indicated for quite simple objects with a good geometry. The sphere method is indicated for objects with more complexity, reaching points that ray method can miss. This method creates spheres at each position and tries to determine the largest sphere in every position, filling the spaces. In our analysis we use the sphere method to get the wall thickness of the *Brachycephalus* osteoderm. At least, how some scans were made using μm and others mm as scale, we converted after the wall thickness analysis all values to mm scale. We also use a simple equation to get an estimate value of skeleton surface area and skin surface area, which is presented in following equations.

$$Skeleton\ surface = \frac{Volume\ of\ Skeleton}{SVL\ of\ species}$$

$$Skin\ surface = \frac{Volume\ of\ Skin}{SVL\ of\ species}$$

Phylogenetic tree

To perform our phylogenetic comparative analyses, we built and phylogenetic tree with only the purposes to have a robust topology of *Brachycephalus* genus resolution. To make that tree, we obvained sequences of *Brachycephalus* species used in our analysis ribosomal genes, mostly 16S, in GenBank. After the selection of genes, we align they using Muscle algorithm in MEGA11 software (Tamura et al. 2021). With our sequences aligned, we create an ultrametric maximum likelihood phylogenetic tree statistical Tamura-Nei substitution model. As external group we added *Ischnocnema guentheri* gene sequence.

Univariate analyses

We began our analyses by focusing on the relationship of the climatic conditions and our wall thickness analyses results. We obtained our climatic data using getData function of Raster (Hijmans 2023) and sp (Bivand et al. 2013) packages in R. Those data were downloaded from Worldclim database and we used resolution of 30 seconds. We chose as condition the variables: BIO1 (Annual mean temperature); BIO5 (Max temperature of warmest month); BIO6 (Min temperature of coldest month); BIO12 (Annual precipitation); BIO13 (Precipitation of wettest month) and BIO14 (Precipitation of driest month). As variable of our wall thickness analyses, we used maximum wall thickness median value. Finally, we explored patterns of wall thickness evolution using ancestral trait reconstruction by maximum likelihood using the function contmap in PHYTOOLS 0.9-99 (Revell 2012) were was carried out using R 4.1.1 (R Core Team 2023).

Multivariate analyses

We performed a principal component analysis to determine the axes of variance in our dataset, where we used the morphometric data that was provided by wall thickness analyses and volumetric information obtained from our 3D models in VGStudio max software. The PCA was made using prcomp function in R 4.1.1

Results

Our measurements of osteoderm wall thickness revealed that *Brachycephalus* of *B. pernix* group have mineralized elements in their skin. Likewise, both *B. pitanga* and *B. nodoterga* of *B. ephippium* group showed the presence of larrge mineralized structures in the skin. *Brachycephalus sulfuratus* in the other hand, does not show the same patter. This analysis shows us that *B. fuscolineatus* have the thinner mineralized structures in the skin, in both maximum and mean wall thickness values. Except for *B. fusculinatus*, all members of *B. ephippium* group analyzed (*B. ephippium, B. nodoterga* and *B. pitanga*) showed the thicker structures, which is reflected in life, since the species of this group display super ossification. Figure 2 shows the wall thickness analyses results on our 3D models.

To understand the patterns of ossification in *Brachycephalus* dermis, we perform a series of linear regressions (figure 3). In linear regressions (figure 3) we observed a positive relation of wall thickness values with BIO1; BIO6 and BIO13 variables indicating that maximum wall thickness is related with temperature (BIO1 and BIO6) and with humidity (BIO13). We also observe that there is a negative relation with BIO14, indicating again, influence of precipitation in wall thickness of ossification structures in *Brachycephalus*.

A PCA was performed to understand the variation of morphometrics measurements in our dataset (figure 4). In our analysis PC1 can explain 74.46% of all variation, and tell us that both volumes (skin and skeleton) are responsible of all of this variation.

Finally, to understand the patterns of *Brachycephalus* ossification in dermis evolution, we performed an ancestral state reconstruction (figure 5). We used only the values which was more representative in our previously analysis: maximum wall thickness; mean wall thickness; skin and skeleton volumes. This analysis showed there is clear evidence of patterns in *Brachycephalus* groups; being all *B. ephippium* group different of all other species of the genus. This results reenforces that this pattern is observed since *Brachycephalus* common ancestor diversified into the actual groups and species. In other species groups (*B. didactylus* group and *B. pernix* group) did not show an evident pattern of evolution in their osteoderms (Figure 5).

Discussion

Our analysis shows a peculiar point of view of *Brachycephalus* osteoderm studies. Mari et al. (2022) did an interesting approach on how the climate can be related with ossified structures in *Brachycephalus* skin. In this work, Mari et al. (2022) describe that location with higher humidity and pluviosity tends to contain animals with higher concentration of mineralized structures in skin. We went deeper on that, and instead just investigate the presence or absence of those structures in the *Brachycephalus* dermis, our results showed that there is a pattern of thickness in those structures.

Furthermore, our results corroborate the hypothesis by Mari et al. (2022), in which they described the pattern of mineralized structures deposition in species which are influenced by climatic conditions. In our results we saw that there is a positive relation between the maximum wall thickness of the osteoderms with the mean temperature, where, animals exposed to higher temperatures tends to have thicker mineralized structures in their dermis. This can be confirmed by Seebacher and Franklin (2007); Clarac and co-workers (2017); Inacio Veenstra and Broeckhoven (2022). In those studies, the authors reach in a conclusion that mineralized structures are involved in thermal changes in crocodilians, not directly, but those structures are can be more related in blood vascularization, is responsible to do thermoregulation. With evidence in literature, we can assume that osteoderms in *Brachycephalus* may be responsible to execute the same function that found in crocodilian osteoderms (Seebacher and Franklin 2007; Clarac et al., 2017; Inacio Veenstra and Broeckhoven 2022).

In the same way of temperature, we found that precipitation has an important role on mineralization on *Brachycephalus* skin, especially in the extreme scenarios. We saw that in animals in sites with higher precipitation on wet seasons have thicker mineralized structures in their skins, which was opposite found in driest scenarios. In places where the driest month can have lowest precipitations, the mineralized structures is thinner. These results can show us the importance of the environmental equilibrium to these animals, where the climatic conditions have great influence in their morphology.

The deposition of mineralized structures in *Brachycephalus* skin shows us an interesting pattern as well, where was clear the differences of groups based in their morphology. This division was proposed by (Ribeiro et al. 2015) and in this work, the authors divided the genus in three morphological groups: *Brachycephalus didactylus* group, *B. epphipium* group and *B. pernix* group. Our ancestral state reconstruction showed that there is a pattern of evolution of these osteoderms in *Brachycephalus* and this was clear when we see the branch of *B. ephippium* group. Animals of *B. ephippium* group are known for their super-ossified structures in their skull and vertebrae (Clemente-Carvalho et al. 2008, Ribeiro 2015, Folly et al. 2023). In our analysis we saw that the group also have deposition in osteoderms in the skin, and this deposition is proportional to their ossification. On the other hand, *B. pernix* group do not show the same pattern, for instance, in this group of *Brachycephalus* we can assume that the climatic conditions have more influence in their patterns of mineralization in the skin than the evolutionary influence. One interesting evidence that we found in our analysis is related with *Brachycephalus* which shows white and black strips in their dorsum in live, such *B. albolineatus*, *B. auroguttatus* and *B. fusculineatus*. In our microCT images we can confirm that strips are calcified material in their dorsum.

Despites the whole influence of climatic conditions and the evolutionary component of our results, the morphology still have an important role in our variation. Our PCA analyses showed us that the volume of the bones and the volume of skin are responsible for great part of our variance, where we saw that smaller volumes presents thicker osteoderms, which can be a mechanism to compensate their size.

Conclusions

We focused in understand the mineralized structures in *Brachycephalus* skin, but different from some works whith focus in osteoderms deposition in vertebrates, we aimed in the wall thickness of those components. We found that there is an interesting relationship between environment, in special the climatic variables, with the wall thickness of mineralized structures of *Brachycephalus* dermis. There is relationship with temperature and precipitation with osteoderms deposition. This may be a mechanism to control the body temperature and a way to avoid dehydration.

We also can say that there is a pattern in the distribution of those mineralized structures in *Brachycephalus* phylogeny. We observed a clear formation of two distend groups in that aspect, when *B. ephippium* group is completely different from the other frogs from the genus, which reflects in their morphology. But, is not environmental and phylogenetical factors contributing to the variability in our results. This can be obvious, but the size appears to affect of wall thickness of osteoderms in *Brachycephalus*, where bigger the volume of skin and bones in our microCT images, thinner the osteoderms and vice-versa. This can be a directly response to compensate the size of these animals.

Finally, we could prove that microCT images is a great tool on studies of comparative biology, in special due the fact that technics can show us results that we cand get only with simple morphometrics and histology. More than that, microCT possibility to work with detailed morphometry without damaging the voucher, which is a viable tool to work with museum specimens.

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Figures

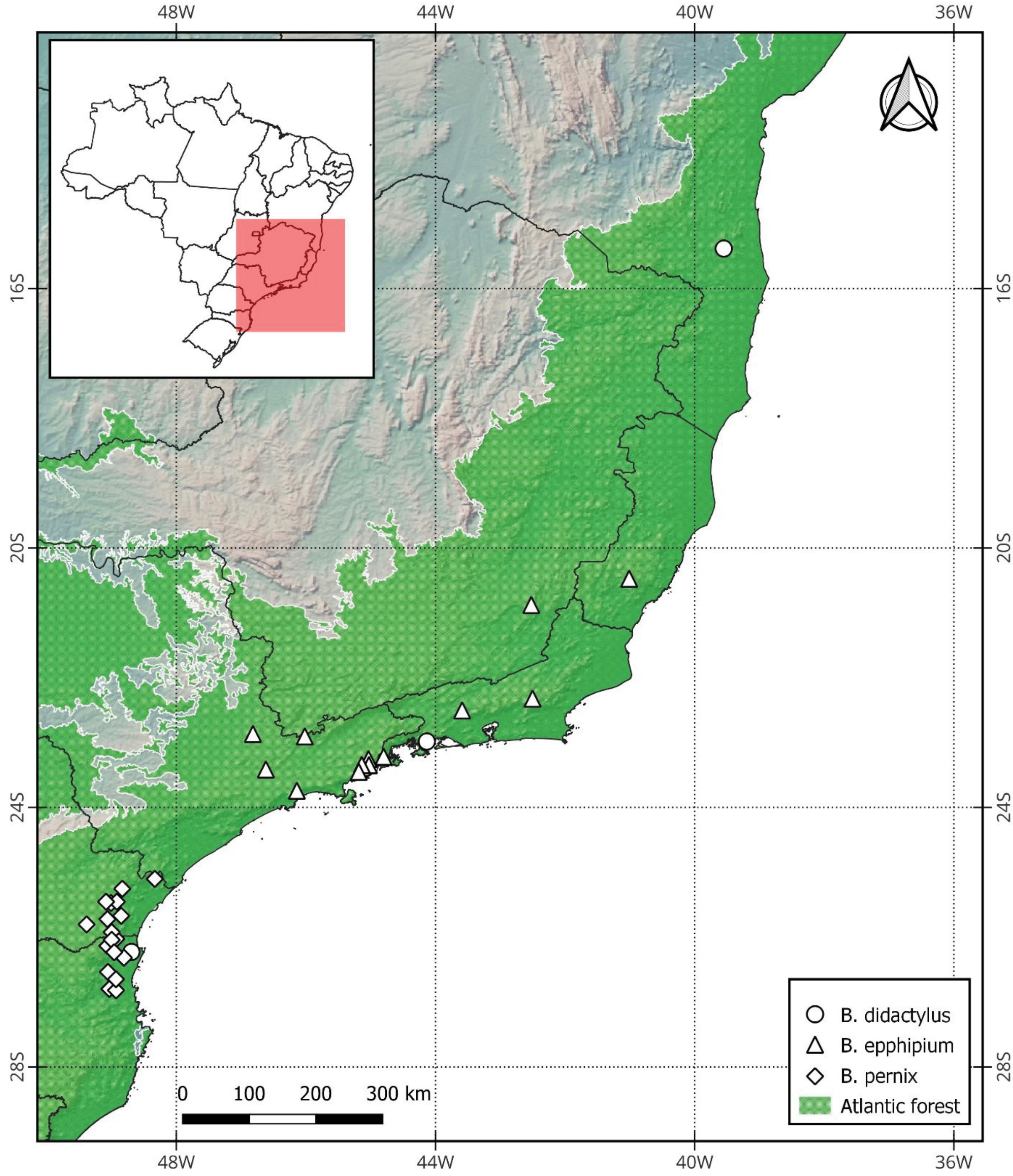
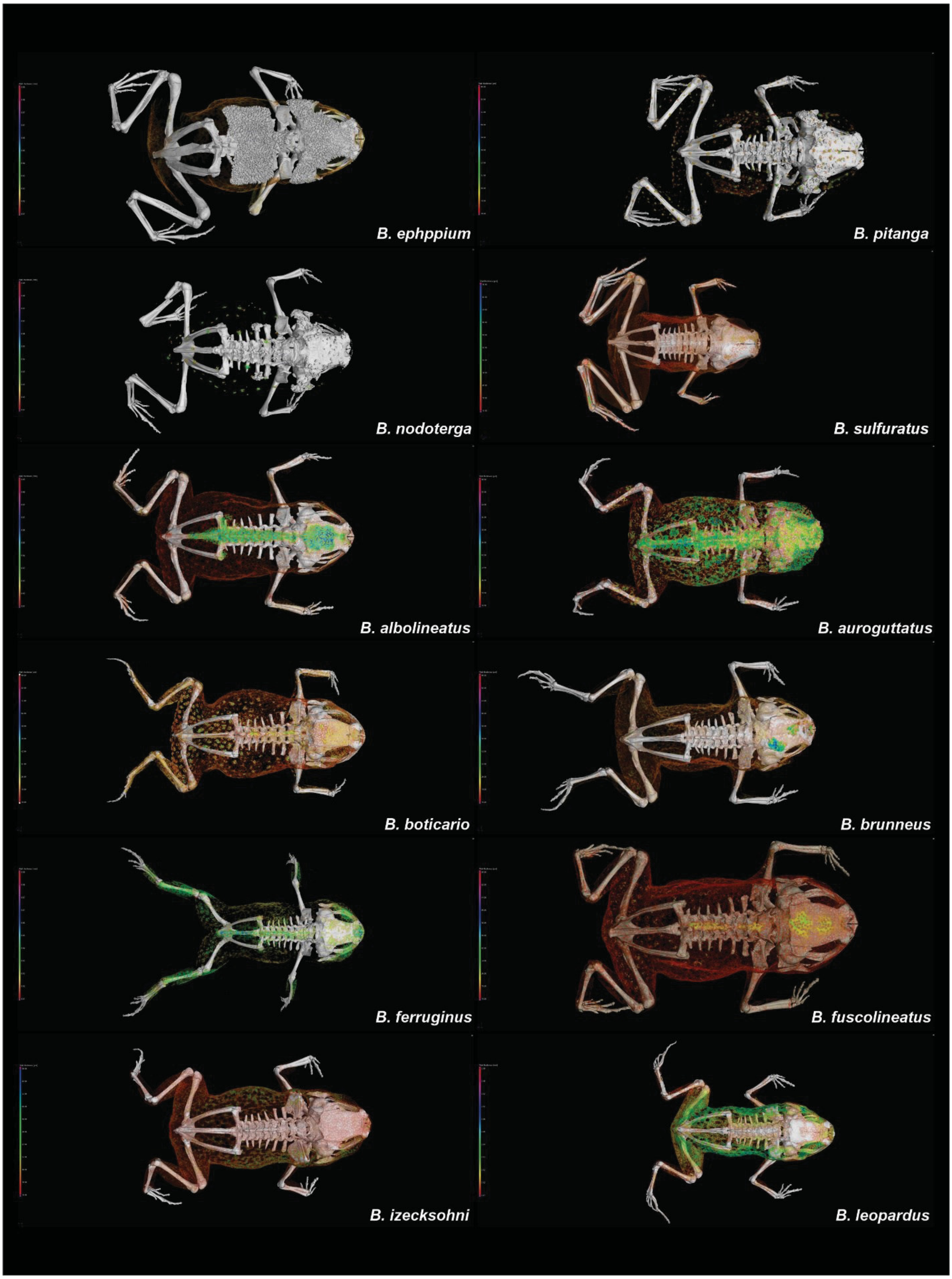


Figure 1: *Map of distribution of* Brachycephalus *genus. Circles represent* B. didactylus group*, triangles represent* B. ephippium *group and losangles represent* B. pernix *group. Atlantic forest is represented in green color.*


B. ephppium
B. pitanga
B. nodoterga
B. sulfuratus
B. albolineatus
B. auroguttatus
B. boticario
B. brunneus
B. ferruginus
B. fuscolineatus
B. izecksohni
B. leopardus

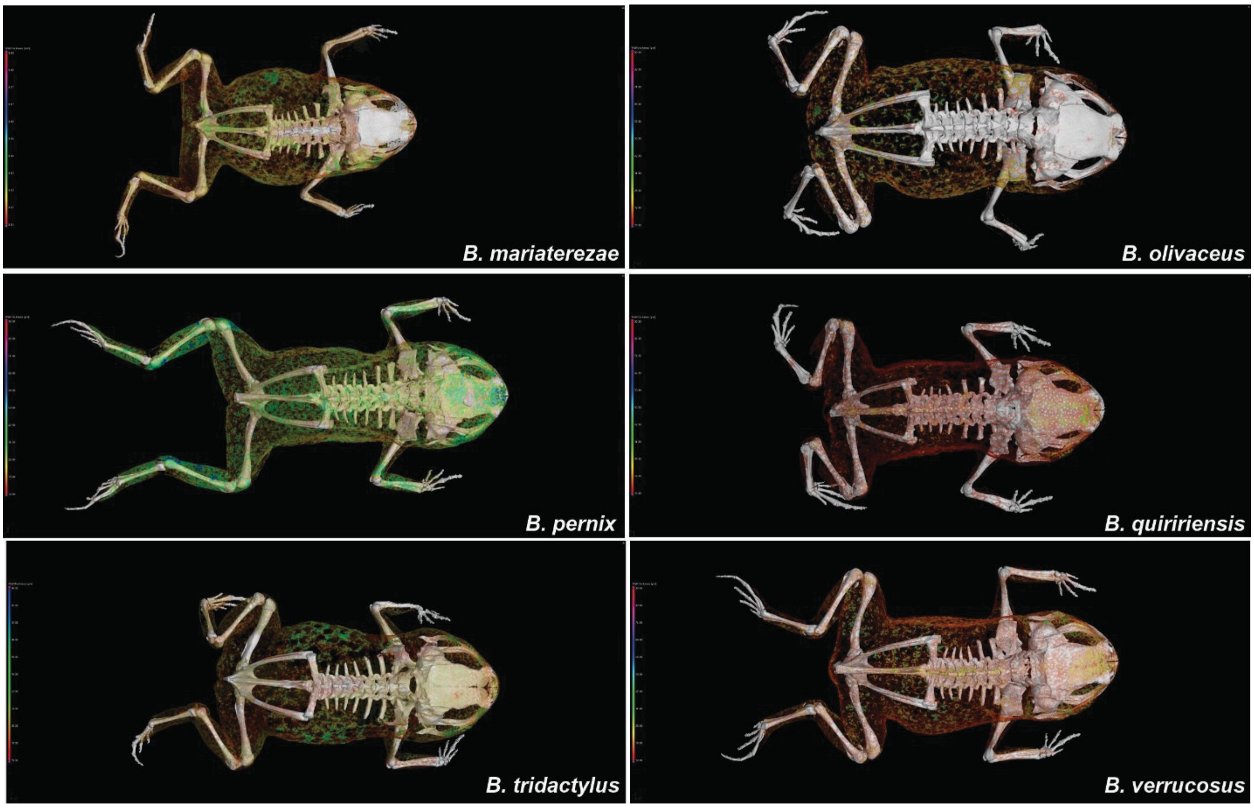


Figure 2: Wall thickness analysis from images obtained by microCT analysis. In those images, we created two objects to avoid that wall thickness analysis run in bones too. Wall thickness analysis was performed only in the skin object, bones was added to image after the analysis completion.

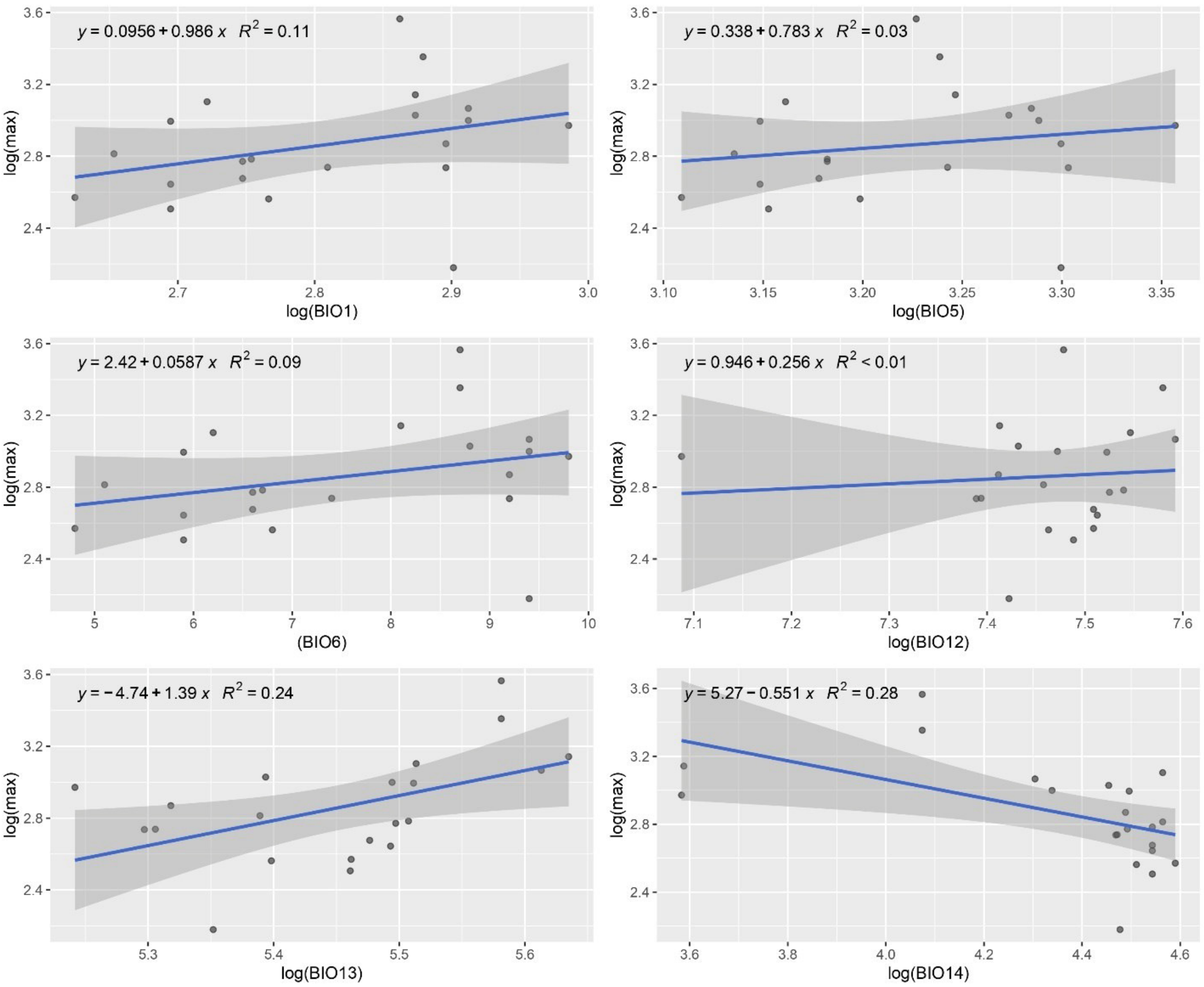


Figure 3: Linear regression of logarithmized values of maximum wall thickness and the climatic variables. BIO1 (Annual mean temperature); BIO5 (Max temperature of warmest month); BIO6 (Min temperature of coldest month); BIO12 (Annual precipitation); BIO13 (Precipitation of wettest month) and BIO14 (Precipitation of driest month).

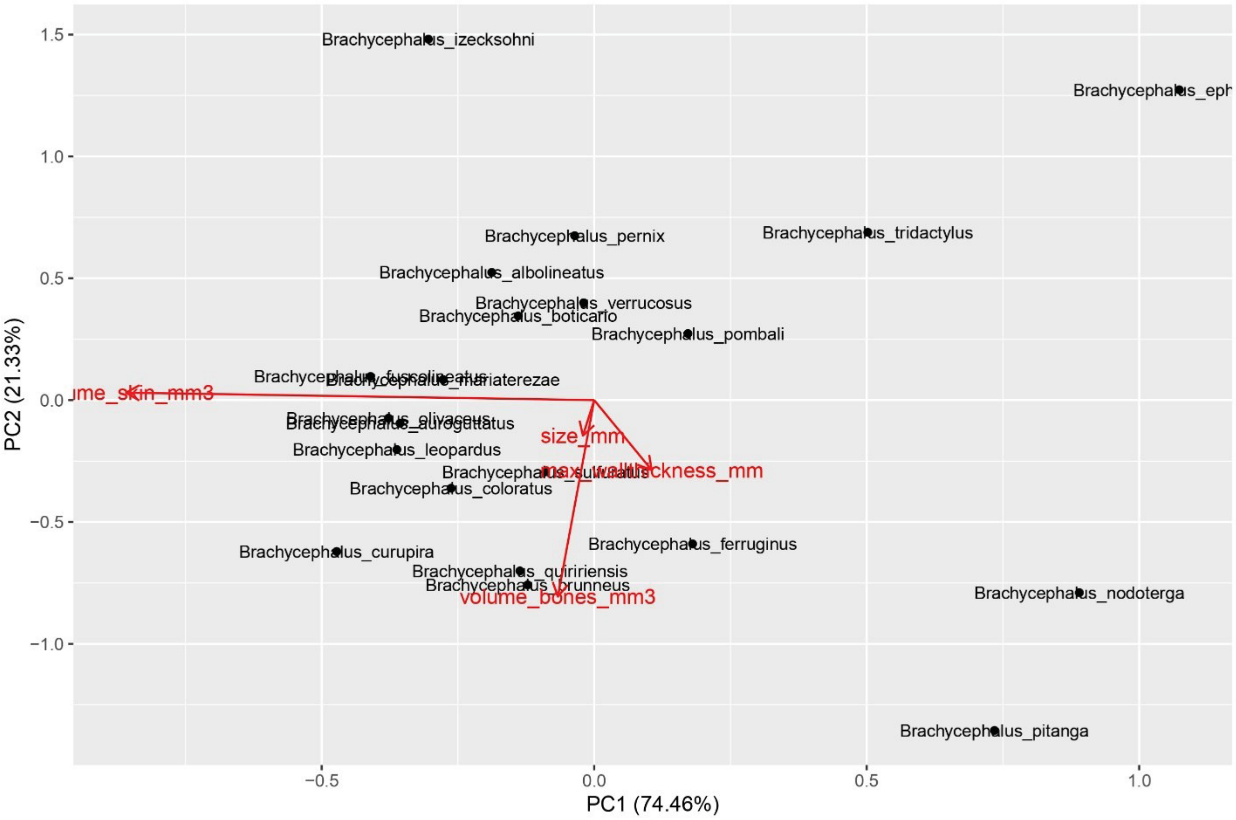


Figure 4: *PCA plot of the distribution of the variability of our dataset.*

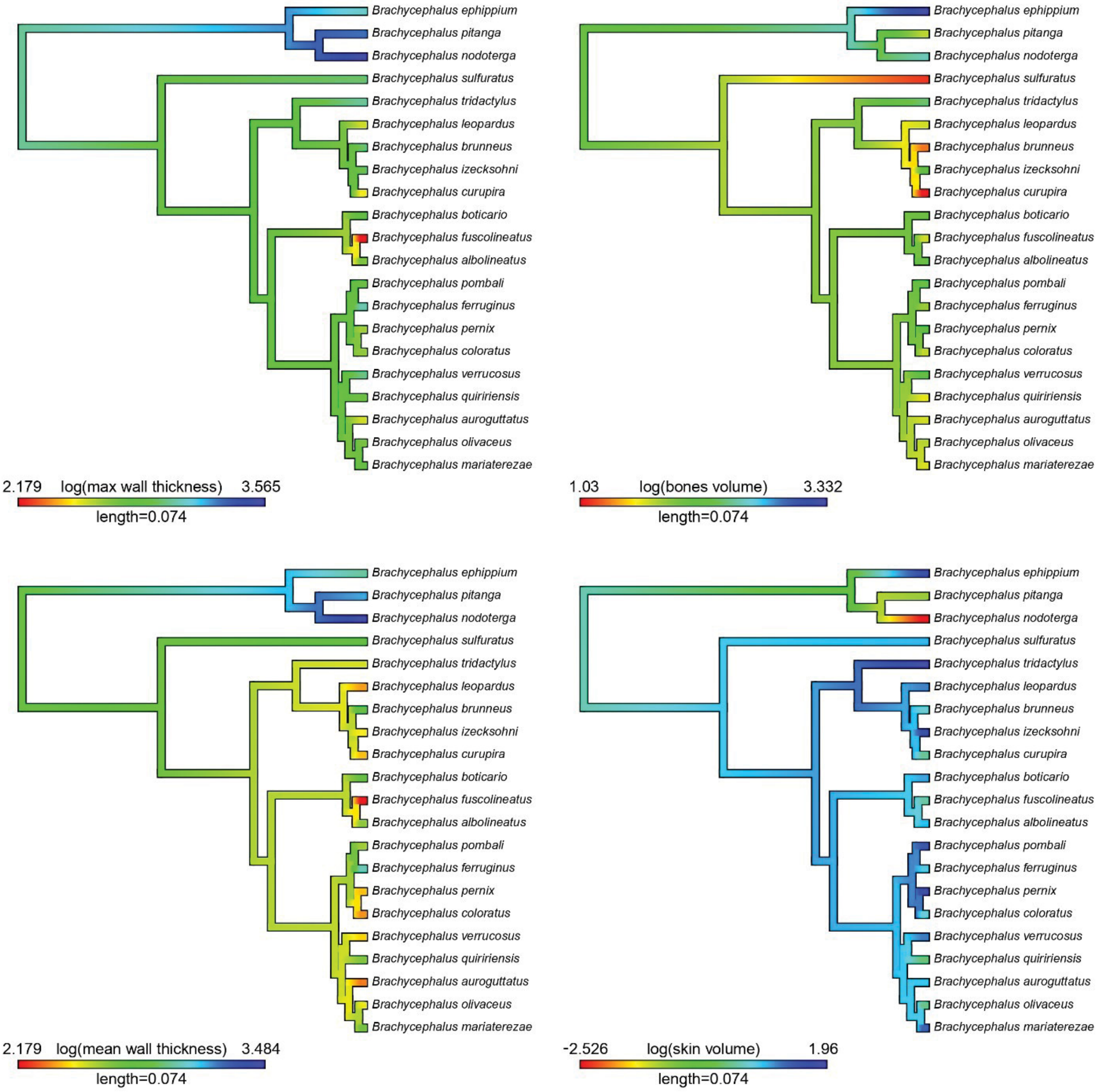


Figure 5: Ancestral state reconstruction analysis. Warm colors represent lower values than colder colors, which in turn, higher values, both for the maximum wall thickness, mean wall thickness, skeleton volume and skin volume values. All data was obtained after transform to logarithm our values.

5. CONSIDERAÇÕES FINAIS

Anuros são animais que apresentam uma diversidade ampla de formas e hábitos que estão interligados entre si. A ecologia desempenha um papel de destaque na diferenciação da forma dos animais, e nos anuros não é diferente. Foi observado na presente tese que em algumas circunstâncias o meio ao qual os animais estão inseridos podem ser cruciais nas mudanças dos mesmos. Entretanto, foi visto que não é apenas o meio o responsável palas modificações morfológicas nos esqueletos dos anuros. Foi visto que os membros locomotores podem variar de acordo com o hábito locomotor, entretanto, algumas formas de locomoção exercem uma pressão maior do que as outras, como é caso dos animais cavadores. Vimos que dentre todos os hábitos locomotores, os animais cavadores são os que mais sofreram influência do meio na diferenciação de seus membros locomotores. Nossos resultados também demonstraram que a diferenciação dos membros locomotores não acontece de forma simples e única, pelo contrário, há evidencias de que tais modificações ocorrem em módulos isolados, cada osso diferenciando-se de forma independente um do outro. Outro ponto de destaque que observamos, é que as modificações evolutivas se concentram mais nos membros superiores, se comparado com os membros inferiores, o que nos levou a tentar entender melhor os padrões evolutivos das falanges dos anuros.

Vimos que com os dígitos dos anuros apresentam uma constante taxa de diferenciação, e essa variabilidade é resultado da combinação de forças evolutivas, em especial a história filogenética desses animais e de sua ecologia. Podemos considerar as falanges dos anuros estruturas com um padrão de diferenciação interessante, sendo constante com uma velocidade de variação baixa, porém, apresentam uma alta taxa de modificação nas falanges distais, seja nas mãos ou nos pés dos anuros. Esse padrão evolutivo sugere de que a evolução dos dedos se dá por módulos, já que as estruturas tem padrões de diferenciação diferentes umas das outras, embora sejam necessárias novas abordagens com o foco exclusivamente em evolução modular. Por fim, podemos ver também, que as falanges apresentam uma forte correlação entre os ossos das mãos e dos pés, sendo fortemente visível nas falanges distal e terminal (mãos e pés respectivamente).

Vimos também a adaptação em anuros em relação ao meio ao qual estão inseridos, pode ser observada através de condições muito específicas, como foi observado em *Brachycephalus*. Animais do gênero *Brachycephalus* apresentam uma condição bastante interessante, a deposição de estruturas mineralizadas em sua derme. O conhecimento acerca de tal adaptação é um tanto quanto obscuro, e os nossos resultados nos ajudaram a clarear um pouco mais essa lacuna referente a mineralização na derme dos animais. Foi encontrado um padrão interessante referente a espessura desses oesteodermos, e esse padrão é levado ao longo das linhagens dos animais. Assim como é possível afirmar que as condições climáticas têm uma relação interessante com a espessura dessas estruturas. Esses resultados só reforçam a nossa ideia hipótese de que o meio tem um papel de importância na diferenciação de estruturas biológicas.

Para finalizar, essa tese colocou em lugar de destaque o uso de modelos 3D por meio de imagens de micro tomografia computadorizada. Essa técnica nos permite explorar o esqueleto dos animais de forma que sem ela, não seria possível, principalmente em animais miniaturizados, como é o caso de *Brachycephalus*. Além do mais, o uso de microtomografia computadorizada nos permite trabalhar como animais de museu sem que haja a danificação dos mesmos, assim como o acervo digital dos modelos 3D e das imagens de *microCT* nos permite trabalhar com coleções de todo o mundo, sem que haja gastos com viagem, de uma certa forma, tornando a ciência mais acessível.

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