Intertwined evolution of swimming, morphology and microhabitat in tree frogs from the subfamily Hylinae

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Abstract

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Multidisciplinary studies including phylogenetic inference, functional morphology and phylogenetic comparative methods are imperative to understand how closely related species evolved. By combining those three approaches we aim to disentangle the evolution of morphological traits and their influence on swimming behavior and performance, both within multiple microhabitats for Neotropical tree frogs. The subfamily Hylinae includes 163 species; they have three main locomotion modes with a direct impact on species fitness: they jump, swim and climb. Convergences in morphotype and locomotion are primarily driven by microhabitat, but in some cases, morphology is the result of long-term phylogenetic constraints. Swimming is a critical behavior during life history for predator avoidance, reproduction, and early developmental stages. However, it is still poorly understood how swimming evolved in closely related species with multiple locomotion modes. I included 225 individuals from 16 localities in Mexico, corresponding to 31 species and 14 genera. Our data set includes linear velocity, forelimb and hind limb behavior, and we categorized aquatic and arboreal microhabitats. I estimated the correlations among the evolution of traits along the phylogeny by using the threshold model. I found that Hylinae species use the two types of swimming reported for frogs, alternating gait (considered the primitive condition in frogs) and simultaneously gait (the derived mode). In addition, there is a high rate of inter- and intra- specific variation in swimming behavior and performance. Therefore, our results have important implications for the study of tree frog evolution and habitat selection.

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I would like to dedicate this work to my mom.

The most extraordinary, strongest, kindest, bravest woman.

I also want to dedicate this to every international student pursuing a PhD (or any) degree outside your home country. Leaving the life as we knew it behind is not easy, it takes courage to make the leap and resilience to survive it.

You got this!

Chapter 1: A phylogenetic approach for neotropical tree frog conservation

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Abstract

Amphibian decline has been related to habitat destruction, pollution, climate change and emergent diseases. However, less is know about the distribution of threatened species on the amphibian tree of life, and the effect that phylogeny has on the threatened status of closely related amphibian species. Accounting for phylogenetic signal in extinction risk can inform strategies aimed at reducing the disproportionate loss of lineages with long evolutionary histories. We implemented comparative phylogenetic methods and multivariate regression to test which factors are influencing species threatened status in a large subfamily of frogs in the Neotropics, the Hylinae. Members of the subfamily Hylinae inhabit multiple microhabitats across a large latitudinal and altitudinal gradient. In the last decade, major taxonomic changes have been made within the family Hylidae. Every year, new species are described, other species are reduced to synonymy, and species complexes are uncovered. The present study is the most updated molecular phylogeny for the group, including 139 species, 265 individuals (representing 72% of the species in the family), and contains 12 species that were not previously studied. We sequence three genes (*16s*, *Rag-1* and *Rhodopsine*), and together with data from Genbank we estimate the phylogeny for 139 species using a Bayesian approach in Beast and Beast2. For each species, our data set includes 1) conservation status on the IUCN red list; 2) evolutionary distinctiveness (ED); 3) species distribution in square kilometers (km2); and 4) elevation profiles. We implemented a Phylogenetic General Linear Model to test the relationships among these variables. We found a significant correlation only between threatened status and elevation. The threatened status of the species is not correlated with ED within the subfamily Hylinae, inconsistently with larger studies that included multiple families. By taking evolutionary history into account and testing for relationships between IUCN threatened status and phylogeny, we aim to contribute to conservation efforts. We provided information at a regional scale at a species level, which can be used for countries with high Hylinae diversity to improve and redirect tree frog conservation.

Introduction

Amphibians are one of the most endangered groups within vertebrates. Within amphibians, tree frogs are particularly susceptible (Stuart et al., 2004). It has been extensively documented that frogs are threatened due to habitat destruction, pollution, climate change and emergent diseases (Lips et al., 2005). But less is known about which factors are correlated with threat status for closely related species at a local scale. Amphibian conservation efforts have been focused historically on species richness and species abundance as a measure for species population declines (Gascon, 2007). However, drastic environmental changes such as habitat loss and fragmentation also result in genetic bottlenecks, especially in species with limited gene flow and low dispersal capabilities (Fischer and Lindenmayer, 2007). Declines in genetic diversity within isolated populations have a direct negative impact on species fitness (Beebee, 2005). A reduced genetic diversity diminishes the capability of species to respond to environmental change and selection. Besides the amount of genetic variation among species, phylogenetic history provides an indication of phylogenetic diversity in each clade. Species with a high evolutionary distinctiveness and low diversification rates are particularly vulnerable; they respond to evolutionary or environmental constraints, and in some cases the species are highly specialized to a particular habitat. Therefore, in order to assure species persistence in the future, it is important to consider genetic diversity and phylogenetic history when determining conservation priorities (Jetz and Pyron, 2018).

The implementation of a phylogenetic approach in amphibian conservation has been done mostly at large scales across the amphibian tree of life. For example, Nowakowski et al (2018) evaluated losses in phylogenetic diversity from over 400 species of amphibians in five continents, by testing how amphibian assemblages respond to habitat conversion. A different approach implemented by Jetz and Pyron (2018) examined over 2,700 species of amphibians and tested for correlations between the species threatened status and their evolutionary distinctiveness (ED), estimated as the proportion of phylogenetic diversity in each clade. Taking into account the ED of each species has been considered as an indicator of how much "evolutionary information" will be loss if a species becomes extinct, and thus is relevant for conservation. These studies concluded that the loss of species with high ED would result in more rapid loss of taxonomic diversity. A decrease in genetic diversity and a consequent taxonomic homogenization means that species will be less capable to face and overcome rapid environmental changes. Thus, they identified the clades that are more vulnerable to extinction, and determined the most critical species requiring conservation around the world.

By including phylogenetic approaches, large-scale studies are highly beneficial to worldwide conservation efforts (Issac et al., 2012). In addition, they have also uncovered phylogenetic uncertainty in some clades, reinforcing the need to update and complete some sections of the Amphibian tree of life (Frost et al., 2006). This is particularly relevant for tropical clades, where new species are constantly being described and most species have limited data on their population status. However, generalizing conservation priorities based on global biodiversity can also have negative consequences and misguide conservation efforts at a local scale. For example, conclusions based on the amphibian tree of life suggested that species with rapid diversification are less prone to decrease by habitat modifications than lineages that have remained unchanged through evolutionary time (Jetz and Pyron, 2018). However, those results might not be applicable or advantageous at a regional level. For Hylinae, species that have high diversification rates correspond mostly to highland species where habitat destruction occurs at fast rates; whereas some species considered priority based only on evolutionary distinctiveness already inhabit protected areas. In addition, some of the species evaluated by the IUCN Red List are the most charismatic and common species, whereas 10.5 percent of the species are still considered Data deficient (IUCN, 2019). This causes a bias on the number of species categorized as threatened without a strong biological support. Conservation priority recommendations at local scale could be improved if they are based on fine-scale phylogenies.

This study seeks to understand the importance of species relationships and phylogenetic history on species decline and persistence among closely related species. This study also identifies clades that are already the focus of conservation efforts versus those requiring more attention (for which evolutionary history has not been considered while determining conservation efforts). We thus aim to contribute to the development of accurate conservation programs, which can be applicable at regional levels.

Study system

For the present study we estimated a phylogeny for the subfamily Hylinae. Robust phylogenetic trees with comprehensive sampling are needed to test for evolutionary correlations and ED among species. The clade Hylinae is composed of 193 species and 20 genera (Amphibia Web, 2019). Most of the members of the subfamily inhabit mountain areas, but some species have wide altitudinal and latitudinal distributions (Wiens et al., 2006, Pyron and Wiens, 2013). In the last decade, major taxonomic changes have been made within the family Hylidae (Duellman et al., 2016; Faivovich et al., 2018). Molecular phylogenetic data has invalidated previous hypotheses based exclusively on morphology, exposing a high rate of morphological homoplasy (Faivovich et al., 2005). Molecular data has also increased number of new species being described, species that are synonyms and the discovery of species complexes that were previously thought to be one species. Paradoxically, the most recent phylogenetic study of the family Hylidae only included 127 of the 193 species of Hylinae (Faivovich et al., 2018), suggesting that more taxonomic changes are likely to come. The fast pace of taxonomic changes for the group as well as the underrepresented diversity emphasizes the need to assess conservation status on the phylogeny. An incomplete or unresolved Hylinae phylogeny could mislead comparative studies especially with regard to trait evolution. Thus we present here an updated phylogeny for Hylinae.

Materials & Methods

Taxon sampling

Our current sampling includes 139 species representing 20 genera (Table 1), 12 species more than the current published phylogeny for tree frogs (Faivovich et al., 2018). This increased sampling was achieved by extensive fieldwork and donations from herpetology collections. Animals were collected with approval from UW Animal care and use committee (IACUC protocol # 4209-01) and deposited in the Herpetology and Genetic Resources collections at the Burke Museum of Natural History and Culture.

Molecular data and phylogenetics

We sequenced three genes widely used in frog phylogenetics for 12 new species: one mitochondrial (*16s*) and two nuclear genes (*Rag-1* and *Rhodopsin*). These genes have been used in previous studies to resolve species relationships of Hylinae (Pyron and Wiens, 2011). We extracted total genomic DNA from the liver following the MacManes Salt Extraction Protocol (Fetzener, 1999). We performed standard polymerase chain reaction (PCR) (Wiens et al., 2005) using a set of six primers and altered the temperature for the annealing stage for each gene (Caviedes-Solis and Nieto Montes de Oca, 2018.). PCR products were sent to Genewiz, Inc. for Sanger sequencing. We edited the sequences from the forward and reverse reads using Geneious 5.1.7 (Kearse et al., 2012), and we aligned them using Mafft (Katoh, and Standley, 2013).

Genbank data

We used the python tool kit SuperCRUNCH to filter large datasets from Genbank (Portik and Wiens, 2019). Instead of performing individual searches per gene, SuperCRUNCH allowed us to download all the data available in Genbank for the family Hylidae. We then selected five loci for the species within Hylinae: three mitochondrial (16s, 12s and ND1) and two nuclear (Rag-1 and Rhodopsine). The genes 12s and ND1 were included in order to incorporate in the analyses relevant samples that do not have sequences for the mitochondrial gene 16s. After creating a database for each gene, we used SuperCRUNCH to blast the Genbank sequences and select only those that overlapped with the fragments that we sequenced and the ones that were available for

12s and ND1. We used --max_hits 300 to indicate the maximum number of blast matches allowed per input sequence. We trimmed and align the sequences per gene using 'mafft' (Katoh, and Standley, 2013), which is incorporated in the SuperCRUNCH pipeline. We use the Genbank accession numbers to extract the voucher number from Genbank database online using the R packages 'Rentrez' (Winter, 2017), 'ape' (Paradis et al., 2004) and 'seqinr' (Charif and Lobry, 2007). Due to the large size of the dataset we implemented the 'web_history' searching function. Once all the sequences were re-labeled with the specimen voucher number, we used Sequence Matrix software (Vaidya et al., 2011) to concatenate the matrices for all the genes and to visualize the number of loci available per individual. We also implemented Sequence Matrix to export one fasta file per gene including the missing data, all of which are labeled with the voucher for subsequent species tree analyses.

Phylogenetic analyses

Gene trees. We estimated a Maximum likelihood (ML) phylogeny in RaxML v.8 (Stamatakis, 2014) for the mitochondrial gene 16s. The RaxML phylogeny included 996 individuals from the subfamily Hylinae: 130 sequences generated for this study and 866 samples from Genbank that share the fragment that we sequenced. Mitochondrial phylogenies have fast substitution rates, a relatively good resolution and have been used to infer population genetics or barcoding for multiple groups of tree frogs (Zarza et al., 2019). Therefore, mitochondrial sequences are the most abundant sequences in Genbank for the subfamily Hylinae. Subsampling individuals from Genbank to estimate species trees is not a trivial task. Studies comparing species across the world randomly choose one individual per species (Jetz and Pyron, 2018; Nowakowski et al., 2018), however this can underestimate the genetic diversity within species, and can mislead the phylogenetic inference. We used our tree estimated for 16s for a more detailed approach to subsample Genbank sequences. We subsampled within species to remove identical samples or samples with low genetic distance that were sequenced for large phylogeographic studies. We select the subsamples based on either their position in previous published phylogenetic analyses when they were available. For the genera that lacked current detailed molecular phylogenies, we subsampled individuals based on their position in the 16s RaxML tree that we estimated. After subsampling the Genbank sequences as described above, we used the final dataset to estimated a ML tree in RaxML for each of the five genes (16s n=379, 12s n=279, ND1 n=161, Rag-1 n=164, and Rhod = 180) and for the five genes concatenated (n=470). For all the RaxML analyses, we implemented a GTR gamma model and performed a bootstrap with 1000 replicates.

Species tree. We estimated a species tree by using a multispecies coalescent model to account for the discordances among the gene trees. A species tree is a representation of all the genes' evolutionary history and estimates branch lengths proportional to time, which are necessary to calculate the evolutionary distinctiveness among species. To perform the species tree analyses, we sampled two to four individuals per species. We performed the analyses in StartBEAST2 (Ogilvie et al., 2017) for 139 species and 265 individuals. We estimated different trees and site models for the mitochondrial and nuclear genes, we fixed the mean substitution rate, and used a GTR substitution model with four gamma categories and empirical frequencies. We estimated a strict clock model, which was linked for the five genes, and used analytical population size integration (Ogilvie et al., 2017). We performed three independent runs of 200 million generation each sampling every 10,000 steps. We used tracer to visualize the results and log combiner with a 10% of burn in and treeannotator to summarize the trees and to generate a consensus tree.

Divergence dating. Due to the large scale of the analyses and difficulties with reaching convergence in the species tree analysis, we performed divergence dating analyses for a total of 139 tips (one per species) using BEAST v. 1.8 (Drummond et al. 2013). All of the site models and clock models were unlinked. For each gene, we used a GTR substitution model with four gamma categories and estimated base frequencies. We implemented an uncorrelated lognormal clock and Birth-Death Incomplete sampling with a beta shape accounting for the 25% of missing species. We calibrated the root (including outgroups) with a mean and 95% Highest Posterior Density (HPD) of 73.5 (55.5-93.4) Ma, and included a calibration for the ingroup node (excluding the outgroup *Dendropsophus marmoratus*) with a mean and 95% HPD of 62.85 (42.3-81.4) Ma (Jetz and Pyron, 2018). We used a lognormal distribution for *ucld.mean* with a mean of -4.6 and sigma of 0.587405. We performed three independent runs of 100 million generation each and we sampled every 10,000. We used tracer to visualize the results and log combiner with a 10% of burn in and treeannotator to summarize the trees and to generate a maximum clade credibility (MCC) consensus tree.

Evolutionary distinctiveness

We calculated the evolutionary distinctiveness (ED) for each of the 139 species in the phylogeny using the *evol.distinct* in the R package ‘picante’ (Kembel et al., 2010). The ED of a species is a measure of the degree of lineage isolation with respect to other lineages in a phylogenetic tree.

Values of ED are estimated based on the branch lengths with respect to the closest speciation event. Thus, high ED values correspond to lineages that remain as one lineage for a long period of time after the last speciation event. Low ED corresponds to lineages that have undergone multiple recent speciation events throughout evolutionary history. We estimated ED values using the time-calibrated phylogeny estimated using BEAST v1.8. (Drummond et al., 2013). Each ED value was estimated as the mean per species across the posterior distribution of 27,003 trees, which combine the distribution for the three independent Beast runs after burn in.

Conservation status, distribution and altitude range

We downloaded the complete IUCN Red List assessment and distribution maps for species of Hylinae. We selected the threatened status and the altitudinal range (min and max values) for all species represented in the phylogeny. For the species with missing data for elevation, we included the altitudinal ranges from Amphibian Species of the World (Frost, 2019) or other sources (Duellman, 2001). For species lacking distribution data in the IUCN database, we estimated ranges using museum records obtained from Global Biodiversity Information Facility (GBIF. org, 2019). We extracted the distribution area in square kilometers from the shape files using the function area in the R package 'raster' (Hijmans et al., 2015). For the species altitudinal distribution, we used the highest point on their altitudinal range (meters) as an indicator of lowland versus highland species. We used a cut-off value of 900 to distinguishing between these two categories, which is the limit between lowland forest (evergreen and semi-evergreen forest) and highland forest (cloud forest and pine-oak forest). We also determined the absolute altitudinal range for each species by subtracting the lowest elevation value from the highest elevation value.

Testing for evolutionary correlations

We mapped the traits on the phylogeny and performed ancestral state reconstructions under Brownian motion using the R package ‘phytools’ (Revell, 2012). We estimated a Phylogenetic Generalized Linear Model (PGLM) with Brownian motion to test for correlations among the variables accounting for the phylogeny using the R packages ‘’phytools’, ‘ape’ (Paradis et al., 2004) and ‘geiger’ (Harmon et al., 2007). We tested for correlation between the threatened status in the IUCN and each of the next factors: evolutionary distinctiveness, area of distribution and species elevation range. For each species we categorized the threatened status according to the IUCN Red list scale. Continuous variables were log transformed and elevation range was used as binary: low (<900m) and high (>900m) elevation. We removed the outgroup species from the analyses to avoid bias in the branch length estimates.

Results

Taxon sampling and Phylogenetic inference

The phylogenetic analyses included sequences for five genes, three mitochondrial (*16s* 579, *ND1*, 1381 bp and *12S*, 910 bp) and two nuclear genes (*Rag-1*, 454 bp; *Rhod*, 338 bp). The concatenated matrix for the RAxML tree includes 3,662 bp for 470 individuals for 141 species. The phylogeny estimated with BEAST v.1.8 has the highest resolution and the highest posterior probability values. The species tree estimated with StarBEAST2 has considerable uncertainty (low posterior probability support) due to the large number of individuals. The posterior probabilities for most relationships were lower than those estimated with RaxML or Beast v1.8. The tree estimated with StarBEAST2 is also the most discordant with respect to the rest of the analyses in the present study as well as with respect to phylogenies previously published for Hylidae or Hylinae estimated with Parsimony (Faivovich et al., 2018) and ML (Duellman et al., 2016).

The relationships among the species were mostly consistent with previous phylogenetic hypotheses for the subfamily Hylinae. Genera that were consistently recovered as clades were: 1) *Acris* and *Pseudacris*; 2) *Sarcohyla, Plectrohyla* and *Exerodonta*; 3) *Rheohyla, Ecnomiohyla* and *Ptychohyla* (including *Duellmanohyla* and *Bromeliohyla*); 4) *Anotheca, Triprion, Diaglena* and *Smilisca*; 5) *Dryophytes* and *Hyla.* However, the relationships among those five clades were variable and they are discordant with previously published hypotheses (Duellman et al., 2016; Faivovich et al., 2018). We used the phylogeny estimated with BEAST v.1.8 (Drummond et al. 2013) for the subsequent analyses (Figure 1). The BEAST v.1.8 phylogeny has high posterior probabilities, divergence times and HD 95% interval for each node. The age for the node including all the members of Hylinae was 31.82 Ma (20.67-43.37 HD 95%). All the detailed node ages and corresponding HD 95% are in the Appendix A-1.

Evolutionary distinctiveness

We calculated the ED for the 141 species represented in the phylogeny. The mean and median for species members of Hylinae are ED = 9.60 and ED = 8.52, respectively. The minimum ED value was 3.88 for *Sarcohyla calthula* and *Sarcohyla pentheter*, the maximum ED value was 22.65 for *Smilisca baudinii*. The ED values per species are listed table 1 and ED values per species are not consistent within genera. Some genera contain many species with predominantly high ED, such as *Tlalocohyla, Ecnomiohyla, Smilisca* and *Triprion.* Some genera contain species with relatively low ED such as *Sarcohyla, Exerodona* and *Dryophytes.* Other genera contain species with high ED that diversify early, followed by a rapid burst of diversification leading to species with low ED, such as *Isthmohyla*, *Ptychohyla, Hyla* and *Pseudacris.*

Conservation status, distribution and altitudinal range

From the 193 species of tree frogs of Hylinae, 181 have been evaluated in the IUCN Red List as follow: Critically Endangered (27.6%), Endangered (17.7%), Vulnerable (5.5%), Near Threatened (4.4%), Least Concern (34.3%), Data Deficient (10.5%), and 6.2% have not been evaluated yet. The present study includes 139 of the 181 species that are included in the IUCN Red List. The proportion of the 139 species that are threatened is: Least Concern (57.5%), Critically Endangered (12.4%), Endangered (9.8%), Vulnerable (4.7%), Near Threatened (4.1%), Data Deficient (4.1%) and 7.3% have not yet been evaluated. A higher proportion of species rated “least concern” are included here. Those species are more frequently encountered and easily collected (Table 1).

Most species of Hylinae are microendemic with limited distributions and altitudinal ranges (Table 1). The average distribution area for species of Hylinae was 321,223 km^2, with a median of 5,381 km^2. The species with the narrowest areas are known only from their type localities such as *Ecnomiohyla rabborum, Ptychohyla zoque* and *Charadrahyla sperancensis*, whereas the species most widely distributed is *Pseudacris maculata* with an area of 5,606,939 km2. The average maximum elevation for the species of Hylinae was 1,666 m, with a median of 1,600 m. The average altitudinal interval that a species can cover, independently of the minimum and the maximum altitude where they can be found is 949 meters with a median of 900 meters. The species most widely distributed was *Pseudacris maculata*, both in area and elevation. Some species were restricted to low elevations, such as members of *Dryophytes*, *Pseudacris* and *Smilisca*, whereas some species were mostly restricted to high elevations such as members of *Charadrahyla, Megastomatohyla* and *Sarcohyla.*

Testing for evolutionary correlations

The values for the correlation obtained with the PGLM between IUCN threatened status and each of the other variables were not significant, with the exception of max elevation. Red List threatened status is not correlated with the ED (p-value 1), the size of their distribution area (p-value 0.98) or the altitudinal range that the species covered (p-value 0.24). However, max elevation was positively correlated with the IUCN threatened status (p-value 0.0094). Large p-values indicate that the predictors are not associated with the response variable, in this case the threatened status. Meaning that species of tree frogs of Hylinae that live in the highlands are more susceptible to be threatened independently of the size of their distribution. Ancestral state reconstruction for the IUCN threatened status and ED and max elevation is shown in Figure 2 and 3. The correlation graphs and their corresponding p-values and slopes are in Figure 4.

Discussion

Correlation among threatened status, ED, elevation and area

Our study did not show a correlation between the ED and threatened status, which confirms previous studies that found the same pattern at a global scale for all orders of amphibians (Jetz and Pyron, 2018). The same study reported that distribution area was the single-most important variable correlated with amphibian threatened status. However, we found no support for a correlation between area and threatened status in neotropical tree frogs. Most of the species in the subfamily Hylinae are microendemic or have restricted distributions, which make them most vulnerable to habitat loss. The two variables from the complete dataset that had a significant correlation was ED an altitude. Species that are highly threatened live in mountainous areas (Lips et al., 2004). Highland species have low connectivity among populations, thus low dispersal capabilities and a high fidelity to breeding sites (Amphibia web, 2019). Most of the species have no known or recorded threat, while smallholder farming, housing development and urban areas threaten some species. By focusing on endangered species distributed on high elevations we can evaluated the causes of threatened status for each particular area in order to make more informed recommendations for conservation efforts.

Threatened status is not correlated with ED, which indicates that species with a long evolutionary history are not currently threatened according to the IUCN Red List. This result gives hope for the persistence of tree frog species that have a high ED. However, there are still species that have not yet been evaluated by the IUCN and species that were evaluated but have insufficient data to determine if they are threatened. Since threatened species are the focus of conservation efforts. Not many species with high ED are currently under protection. This needs to be considered when threat status to species are updated or evaluated for the first time. Another factor to consider when accounting for ED is the completeness of the phylogeny. A phylogeny that is not complete can influence or dramatically change ED estimates (Colston and Pyron, 2019). Clades within Hylinae have experienced a high rate of diversification and are one of the most species-rich clades of aurans (Pyron and Wiens, 2011). Species of this subfamily are also known for their low abundance and their preference for pristine and remote habitats (Amphibia web, 2019). All these factors make species of Hylinae difficult to sample for molecular phylogenies.

One example of the effect that an incomplete phylogeny has on ED are *Megastomatohyla mixe* and *Megastomatohyla pellita,* two species considered in the top 100 focal amphibian species for the Evolutionary Distinct and Globally Endangered program (EDGE, ZSL 2019). EDGE program protects species with the highest ED within each vertebrate group. The amphibian species selected for this program are based on a large study that incorporated 5,713 species with ED values between 190-47 (Issac et al., 2012). For the species *Megastomatohyla mixe* and *Megastomatohyla pellita* the program website reports an ED of 24.77, the original source reported an ED of 7.77 (Issac et al., 2012), whereas the present study –which included more closely related species– estimated an ED of 14.58. These discordances show the high sensitivity that ED estimates have depending on the completeness of the phylogeny, the accuracy of divergence dating and branch length values. Therefore, if ED values are highly variable due to any of the aforementioned reasons, ED should not be used to assign the conservation priority given to a species.

Species level taxonomy can also influence ED estimates. *Smilisca baudinii*, a species widely distributed in Middle America, which has the highest ED among all the species in the current phylogeny, and may be composed of multiple independent evolutionary lineages (I.W.C.S. observation). Consequently, the high ED estimated for *Smilisca baudinii* could be an overestimate if new research finds multiple cryptic species. Misleading cryptic diversity is present along the subfamily Hylinae. For example, *Sarcohyla bistincta* was considered one widespread species with a threatened status of Least Concerned (LC). Recently, *Sarcohyla bistincta* was divided in to three species (Caviedes-Solis and Nieto Montes de Oca, 2018), two of which still lack an IUCN evaluation; all of them with narrower distributions and lower ED than if it was still considered one species. Therefore, it is necessary to increase the sampling effort to include rare species in the phylogeny. Which will allow for a more accurate evaluation of species threatened status using a phylogenetic approach.

Discordance in phylogenetic trees

Phylogenetic inference for Hylinae has been challenging. The taxonomy at species and genus levels using molecular data has been constantly updated in the past decade. The addition of new species to the phylogeny results on changes among species relationships and some clades still remain unstable or unsolved (Faivovich et al., 2005, Wiens et al., 2011, Duellman et al., 2016; Faivovich et al., 2018). Implementation of phylogenetic inference methods for large data sets also represents a challenge. Species tree analyses estimated with StartBEAST2 are the most current robust phylogenetic method, which accounts for incomplete lineage sorting (Ogilvie et al., 2017). However, due the large size of the data set and despite multiple attempts to modify the parameters, a tree calibration was not feasible using StarBEAST2, and the attempts did not result in high posterior probabilities. Therefore, we based the analyses on the posterior distribution of trees estimated with BEAST v.1.8. Our results validate the monophyly for 16 genera including: *Acris*, *Pseudacris*, *Sarcohyla, Plectrohyla*, *Exerodonta*, *Rheohyla, Ecnomiohyla, Anotheca, Triprion, Diaglena, Charadrahyla, Megastomatohyla, Isthmohyla, Tlalocohyla, Dryophytes* and *Hyla.* This result is concordant with previous phylogenetic hypotheses. However, detailed phylogenies with higher resolution are needed to disentangle the relationships among species. Efforts are needed specially to complete clades with a large percentage of missing species.

The genera that were not monophyletic include *Smilisca, Ptychohyla, Duellmanohyla* and *Bomeliohyla*. Faivovich et al. (2018) re-defined *Bromeliohyla* and re-assigned members from *Ptychohyla* to *Duellmanohyla* and two new genera: *Alantihyla* and *Quilticohyla.* The present study supports the monophyly of *Atlantihyla* and *Bromeliohyla* including *B. melacaena*, previously assigned to *Isthmohyla*. However, our results do not support the monophyly for *Quilticohyla, Ptychohyla* and *Duellmanohyla* according to the species composition described by Faivovich et al (2018). Instead, we found that the green coloration used as a diagnostic trait for *Quilticohyla* evolved multiple times in these groups. Surprisingly, the species within the genus

Smilisca were not recovered as monophyletic, the species *Smilisca baudinii* was recovered as the sister species for the clade composed of the rest of the species in *Smilisca*, *Diaglena, Anotheca* and *Triprion.* This might represent a sampling error, since *Smilisca baudinii* is the sister species of the rest of the species in *Smilisca* when more individuals per species are included in the phylogeny. This reinforces the fact that more detailed population level phylogenetic studies are needed for some genera.

Conclusion

Our study contributes to the systematics of tree frogs of Middle America and helps provide a basis for their conservation assessment and management. It approaches the estimate of evolutionary distinctiveness at a regional scale and evaluates multiple factors that can be influencing the threatened status for neotropical tree frogs. However, correlations estimated using phylogenetic general lineal models need to be considered a continuing process. Further analyses need to take in to account all the factors having an effect on the species threatened status and ED, including the addition of species missing on the phylogeny, the accuracy of voucher identification in museums and Genbank, the species delimitation, and population genetic analyses.

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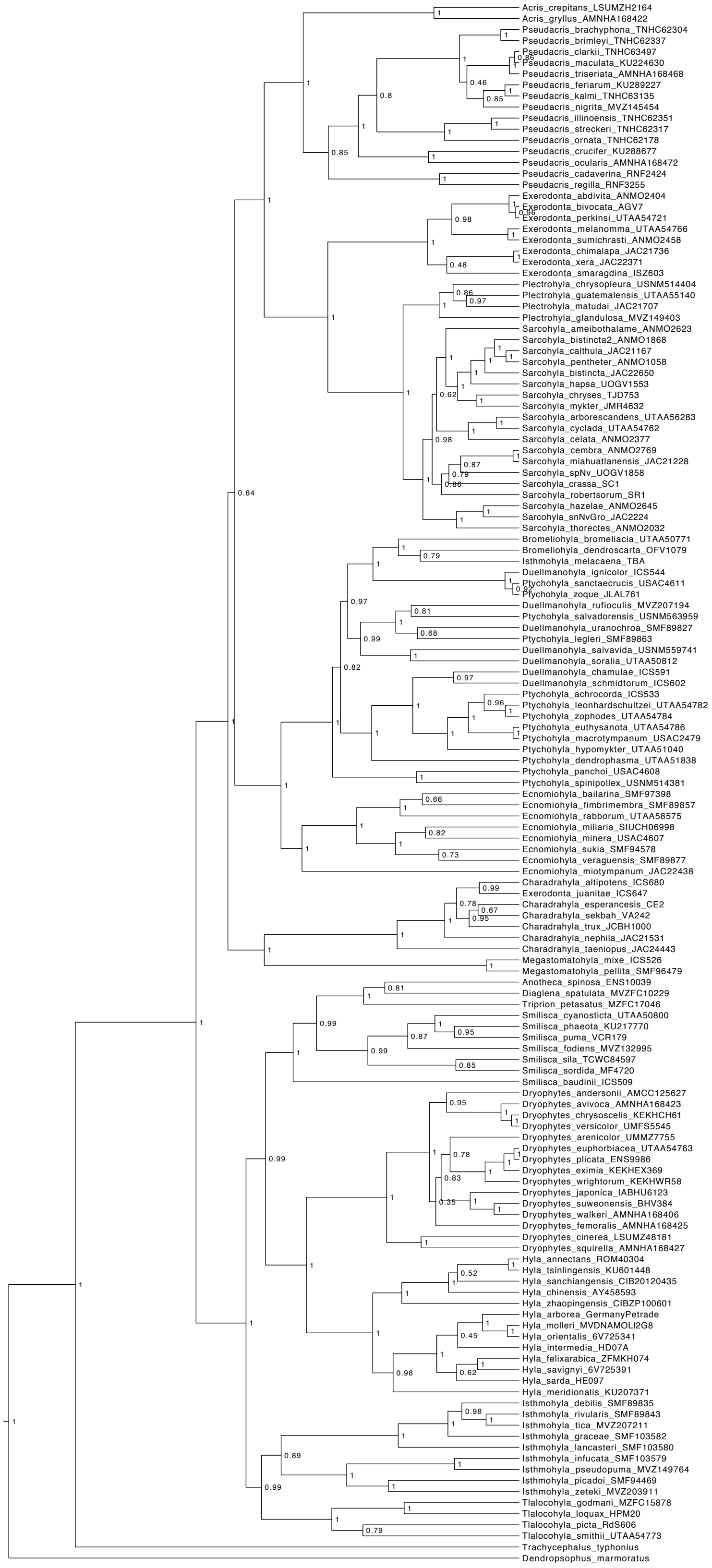

Figure 1. Phylogenetic inferences for the 139 species of the subfamily Hylinae, node values indicate posterior probability.

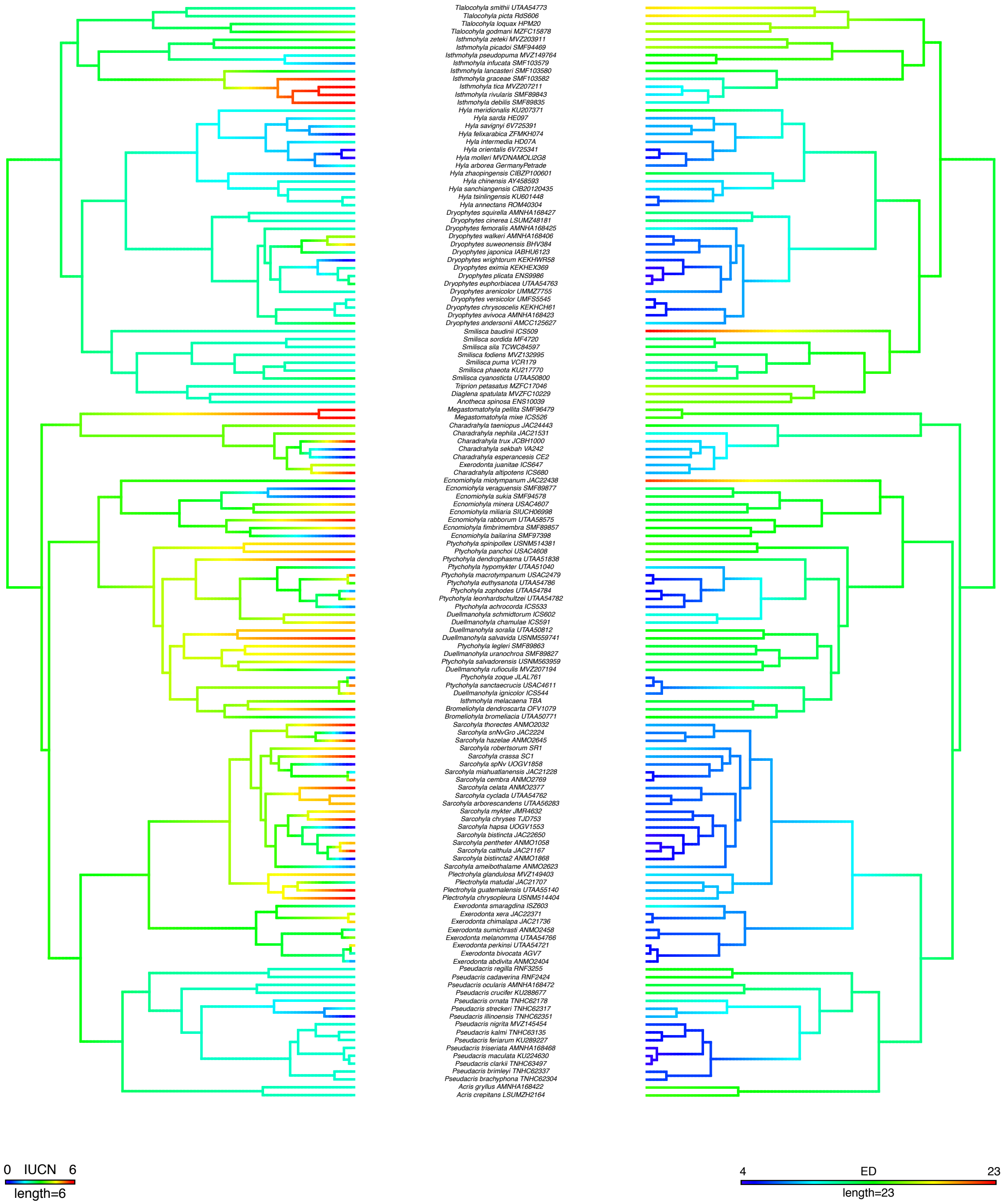

Figure 2. Character mapping for IUCN threatened status from 0=Data deficient, to 6=Endangered, and Evolutionary distinctiveness (ED).

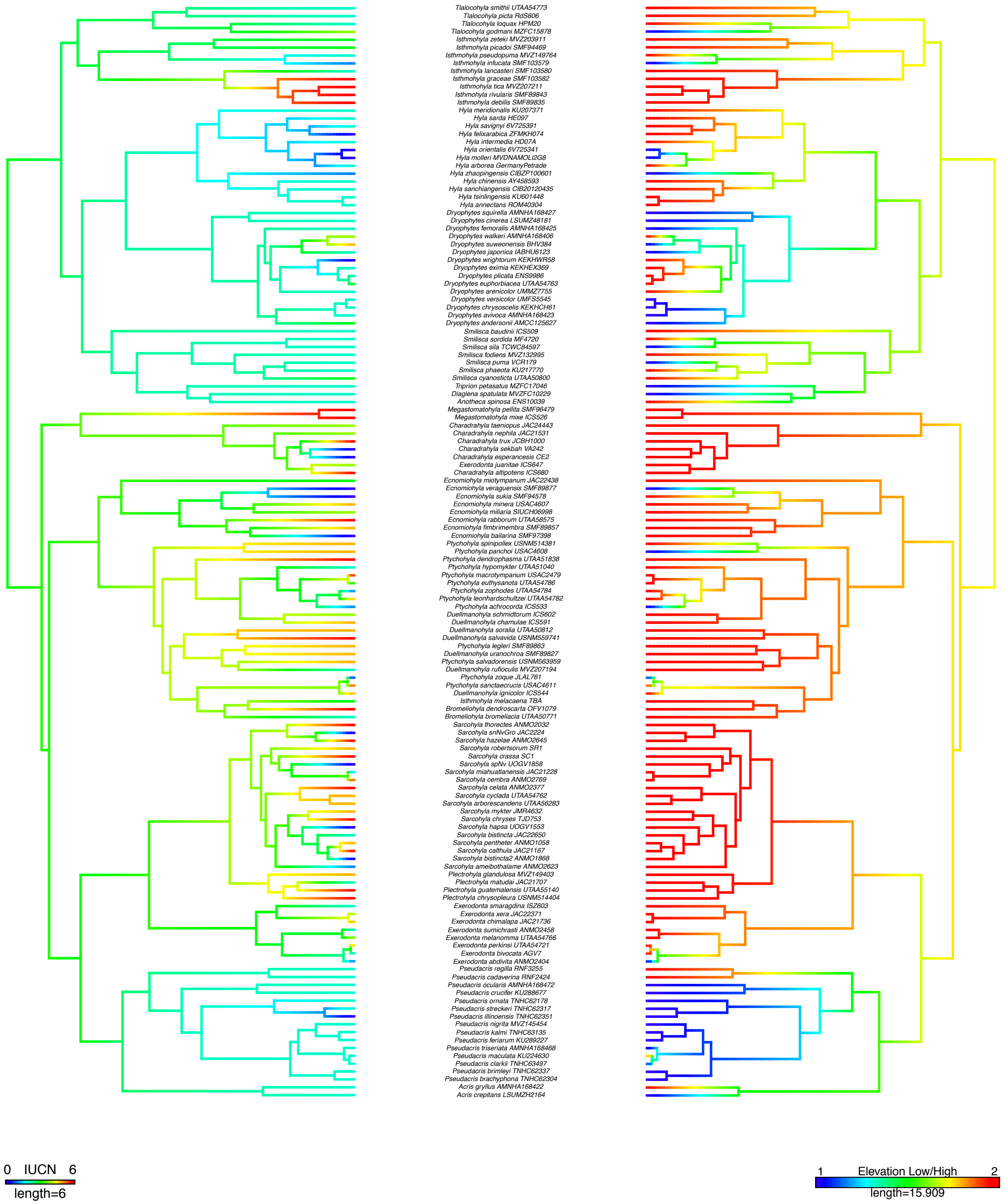

Figure 3. Character mapping for IUCN threatened status from 0=Data deficient, to 6=Endangered, and max elevation.

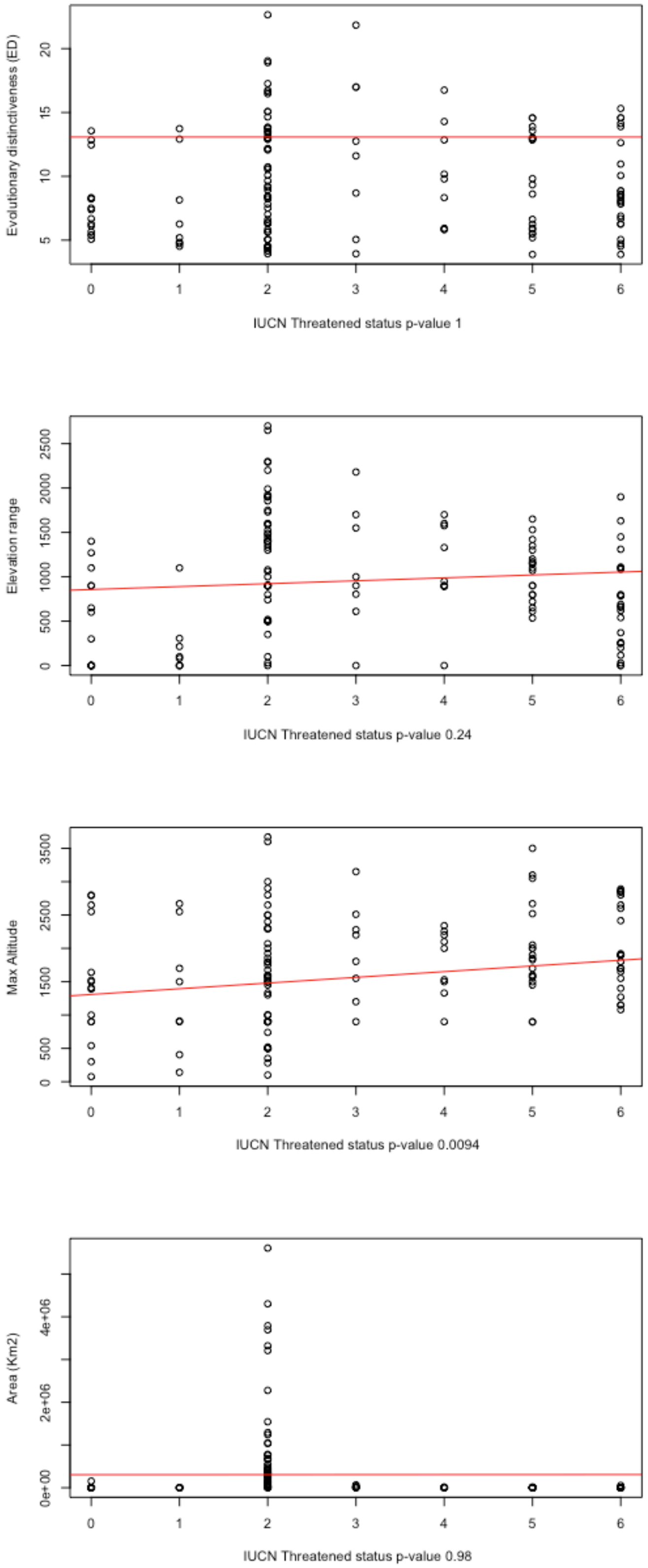

Figure 4. Phylogenetic General Linear model to test for correlations among IUCN threatened status and ED, Distribution area, Elevation range and Maximum elevation

Table 1. Species included in the study, IUCN threatened status and Evolutionary distinctiveness (ED), Distribution area, Elevation range and Maximum elevation.

Species	IUCN	ED	Area (km2)	Elevation (m) (min)	Elevation (m) (max)
Acris crepitans	LC	15.07	3209419.26	250	280
Acris gryllus	LC	15.07	667451.55	500	1000
Anotheca spinosa	LC	16.46	8659.60	95	2000
Bromeliohyla bromeliacia	LC	13.5	6748.89	350	1790
Bromeliohyla dendroscarta	CR	12.63	1829.59	450	1900
Charadrahyla altipotens	CR	8.33	10.84	1100	1900
Charadrahyla esperancesis	NotE	8.33	6.61	1640	1640
Charadrahyla nephila	VU	10.17	1690.37	680	2256
Charadrahyla sekbah	NotE	8.26	1.50	1390	1390
Charadrahyla taeniopus	VU	14.29	7545.05	1200	2100
Charadrahyla trux	CR	8.59	79.91	1760	2415
Diaglena spatulata	LC	16.61	108199.32	0	350
Dryophytes andersonii	NT	8.69	67727.64	0	900
Dryophytes arenicolor	LC	8.41	1241870.12	300	3000
Dryophytes avivoca	LC	5.06	446462.53	0	900
Dryophytes chrysoscelis	LC	4.54	3693367.54	0	900
Dryophytes cinerea	LC	12.16	1290871.14	0	900
Dryophytes euphorbiacea	NT	3.93	10183.06	1600	3150
Dryophytes eximia	LC	4.43	357544.04	910	2900
Dryophytes femoralis	LC	9.65	482056.23	0	900
Dryophytes japonica	LC	7.03	3321952.04	0	900
Dryophytes plicata	LC	3.93	25310.33	1400	3600
Dryophytes squirella	LC	12.16	772871.75	0	900
Dryophytes suweonensis	EN	5.84	7246.41	0	900
Dryophytes versicolor	LC	4.54	2278017.48	0	900
Dryophytes walkeri	VU	5.84	2789.85	1450	2340
Dryophytes wrightorum	LC	5.66	155568.32	2000	2650
Duellmanohyla chamulae	EN	9.82	473.41	350	1700
Duellmanohyla ignicolor	EN	6.63	183.57	680	1850
Duellmanohyla rufioculis	LC	12.93	17763.27	500	1580
Duellmanohyla salvavida	CR	13.89	325.31	90	1400
Duellmanohyla schmidtorum	VU	9.79	13870.82	500	2200
Duellmanohyla soralia	EN	13.89	1025.17	40	1570
Duellmanohyla uranochroa	EN	12.85	15102.03	300	1450

Ecnomiohyla bailarina	NotE	13.56	9.50	400	1500
Ecnomiohyla fimbrimembra	EN	13.56	1040.06	750	1900
Ecnomiohyla miliaria	VU	12.85	4479.59	0	1330
Ecnomiohyla minera	EN	13.01	155.57	630	1830
Ecnomiohyla miotympanum	NT	21.84	22082.60	100	2280
Ecnomiohyla rabborum	CR	14.14	2.50	900	1150
Ecnomiohyla sukia	NotE	12.85	13.50	400	1000
Ecnomiohyla veraguensis	NotE	12.45	1414.50	540	540
Exerodonta abdivita	DD	4.83	15.59	405	405
Exerodonta bivocata	DD	4.52	464.48	1600	1700
Exerodonta chimalapa	EN	5.93	251.18	850	1500
Exerodonta juanitae	VU	8.33	2682.39	580	1530
Exerodonta melanomma	VU	5.83	1328.47	400	2000
Exerodonta perkinsi	CR	4.52	33.35	1050	1080
Exerodonta smaragdina	LC	10.09	162830.79	100	1500
Exerodonta sumichrasti	LC	5.83	63393.38	200	1675
Exerodonta xera	VU	5.93	6116.72	1500	1500
Hyla annectans	LC	6.4	1032929.52	600	2500
Hyla arborea	LC	6.61	3791364.16	0	2300
Hyla chinensis	LC	9.24	657177.96	200	1000
Hyla felixarabica	NotE	7.51	10.00	2790	2790
Hyla intermedia	LC	8.46	225299.12	0	1855
Hyla meridionalis	LC	13.5	684017.87	0	2650
Hyla molleri	NotE	5.39	450.00	0	300
Hyla orientalis	NotE	5.39	700.00	0	900
Hyla sanchiangensis	LC	9.13	442581.78	500	1560
Hyla sarda	LC	8.38	31503.52	0	1750
Hyla savignyi	LC	7.51	780172.29	400	1800
Hyla tsinlingensis	LC	6.4	89298.64	600	2500
Hyla zhaopingensis	DD	12.91	1422.20	140	140
Isthmohyla debilis	CR	10.06	1982.87	910	1700
Isthmohyla graceae	CR	10.96	1874.72	1110	1650
Isthmohyla infucata	DD	13.73	49.65	830	910
Isthmohyla lancasteri	LC	14.65	4720.28	90	1450
Isthmohyla melacaena	NT	12.75	214.86	1550	1550
Isthmohyla picadoi	NT	16.99	1504.46	1900	2510
Isthmohyla pseudopuma	LC	13.73	5381.23	1000	2400
Isthmohyla rivularis	CR	8.86	6939.89	1210	2840
Isthmohyla tica	CR	8.86	6069.51	835	1920
Isthmohyla zeteki	NT	16.99	1474.52	1000	1804
Megastomatohyla mixe	CR	14.58	32.59	1800	1800

Megastomatohyla pellita	CR	14.58	1087.24	1500	1700
Plectrohyla chrysopleura	CR	8.52	110.34	930	1550
Plectrohyla glandulosa	EN	9.34	5553.32	2400	3500
Plectrohyla guatemalensis	CR	7.83	55082.04	900	2800
Plectrohyla matudai	LC	7.83	7723.69	700	2300
Pseudacris brachyphona	LC	5.64	285439.84	0	900
Pseudacris brimleyi	LC	5.64	135121.33	0	500
Pseudacris cadaverina	LC	13.84	63750.89	0	2290
Pseudacris clarkii	LC	4.17	541654.42	0	100
Pseudacris crucifer	LC	13.02	4302856.56	0	900
Pseudacris feriarum	LC	5.06	759360.33	0	900
Pseudacris illinoensis	NotE	8.23	98.00	0	900
Pseudacris kalmi	LC	5.06	34400.23	0	900
Pseudacris maculata	LC	4.16	5606939.54	3670	3670
Pseudacris nigrita	LC	6.28	384813.61	0	500
Pseudacris ocularis	LC	13.02	341591.48	0	500
Pseudacris ornata	LC	10.55	347129.31	0	500
Pseudacris regilla	LC	13.84	1543993.63	0	1000
Pseudacris streckeri	LC	8.23	549823.84	0	900
Pseudacris triseriata	LC	4.38	409563.54	0	900
Ptychohyla achrocorda	DD	6.26	1213.75	594	900
Ptychohyla dendrophasma	CR	15.31	44.34	1270	1270
Ptychohyla euthysanota	NT	5.05	8573.08	500	2200
Ptychohyla hypomykter	LC	8.92	20701.09	340	2070
Ptychohyla legleri	EN	12.91	805.70	700	1600
Ptychohyla leonhardschultzei	EN	5.19	1123.88	350	2000
Ptychohyla macrotympanum	CR	5.05	52.08	600	1700
Ptychohyla panchoi	EN	14.57	219.04	100	895
Ptychohyla salvadorensis	EN	13.01	3681.95	1440	2050
Ptychohyla sanctaecrucis	CR	6.28	73.84	366	1150
Ptychohyla spinipollex	EN	14.57	469.89	160	1580
Ptychohyla zophodes	DD	5.19	3866.73	400	1500
Ptychohyla zoque	NotE	6.27	3.00	76	76
Sarcohyla ameibothalame	DD	8.15	31.24	2455	2670
Sarcohyla arborescandens	EN	5.51	3286.81	1800	3100
Sarcohyla bistincta	LC	4.45	54.00	1400	2800
Sarcohyla bistincta2	NotE	5.07	100.00	1400	2800
Sarcohyla calthula	CR	3.88	1326.61	1780	1896
Sarcohyla celata	CR	6.89	51.85	2640	2890
Sarcohyla cembra	CR	4.73	202.59	2160	2850
Sarcohyla chryses	CR	6.25	232.60	2340	2600

Sarcohyla crassa	CR	8.1	78.90	1540	2650
Sarcohyla cyclada	EN	5.51	3061.84	1600	2670
Sarcohyla hapsa	NotE	6.11	200.00	1280	2550
Sarcohyla hazelae	CR	6.68	497.92	2200	2865
Sarcohyla miahuatlanensis	DD	4.73	76.42	2550	2550
Sarcohyla mykter	EN	6.25	1062.91	1985	2520
Sarcohyla pentheter	EN	3.88	2297.21	1280	2000
Sarcohyla robertsorum	EN	8.61	147.29	2250	3050
Sarcohyla snNvGro	NotE	6.68	3.00	1409	1409
Sarcohyla spNv	NotE	7.39	3.00	1520	1520
Sarcohyla thorectes	CR	7.99	339.65	1530	1900
Smilisca baudinii	LC	22.65	1050505.42	0	1925
Smilisca cyanosticta	NT	11.6	28868.08	200	1200
Smilisca fodiens	LC	13.26	252651.47	0	1490
Smilisca phaeota	LC	10.73	321261.85	0	1600
Smilisca puma	LC	10.69	13817.15	0	520
Smilisca sila	LC	12.89	169790.18	0	500
Smilisca sordida	LC	12.06	50630.84	0	1525
Tlalocohyla godmani	VU	16.75	8441.64	0	900
Tlalocohyla loquax	LC	16.75	348676.24	0	1585
Tlalocohyla picta	LC	19.05	313542.73	0	1300
Tlalocohyla smithii	LC	18.9	260451.42	0	1332
Triprion petasatus	LC	17.26	153656.13	0	740

Chapter 2: Neotropical tree frog ecomorphology is predicted by hindlimb variation

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Introduction

Environmental factors are among the main causes for increasing diversification rates and rapid adaptive radiations (Ruber and Adams, 2001). Besides environmental pressures, phenotypic evolution is simultaneously mediated by phylogenetic constraints and genotypic variation (Vidal-Garcia et al., 2014). Studies focusing on a wide variety of organisms have consistently shown environmental changes are the primarily factors driving trait convergence. Therefore, morphological convergence is a widespread phenomenon across vertebrate clades (Losos, 2011). To understand how morphological characteristics converged in similar shapes, it is also important to consider that same shape can be attained in different ways. For example, elongation of morphological features can be attained by increasing length of a bone or the number of bones (Parra and Wake, 2001). Each trait has an independent evolutionary rate, is under multiple selective pressures, and it is being influenced by (and influencing) other traits. Therefore, specific morphotypes are the result of the independent evolution of multiple traits.

Among vertebrates, frogs present an interesting case of convergent evolution. Frog body plan has been consistent throughout evolutionary time, and the same characters allow multiple locomotion modes with minimal changes in their body plan. Distantly related species of frogs have converged on similar traits independently of their geographic origin or the evolutionary time they had to acquire those traits after speciation (Moen et al., 2013). But more interestingly, convergence of morphological traits also occurs among closely-related species. External features such as general body shape, limb proportions and webbing area are among the traits historically used for species diagnosis in tree frogs (Duellman, 2001). However, for closely related species of frogs, molecular phylogenies have revealed high rates of morphological convergences that invalidate diagnostic traits previously used to estimate relationships among species (Faivovich et al., 2005). For example, body size in *Sarcohyla ephemera* and hind limb length in *Sarcohyla cyclada* were considered diagnostic traits for those species, until molecular phylogenetic revealed that populations that differed in size for those traits were not genetically distinct (Caviedes-Solis et al., 2018).

Identifying morphotypes that can be used to diagnose closely related species of frogs requires an evaluation of subtle differences in body proportions in a phylogenetic context. An accurate characterization and categorization of morphotypes is the baseline for answering functional morphology questions, which enable tests of how performance varies according to differences in body plans (Vidal-Garcia and Keogh, 2015). Limb proportions are distinctive among different families of frogs, and are related to their primarily locomotion mode (Emerson and Inger 1988). For example, frogs from the family Ranidae have long hind limbs with a large muscle mass for jumping and swimming, which are their primary locomotion modes (Reilly et al., 2016). Tree frogs from the subfamily Hylinae have more slender hind limbs and longer forelimbs that increase climbing performance. However, tree frogs also rely on jumping and swimming during different life stages, and their morphotype is the result of selection for each locomotion type, often at the same time (Emerson and Koehl, 1990).

This project aims to characterize morphotypes and to evaluate morphological variation in limb proportions among Neotropical tree frogs in the family Hylidae. We focused on subfamily Hylinae, a diverse clade of closely related frogs containing 193 species. According to previous studies using secondary calibrations the clade has an age of ~62 Ma (Pyron and Jertz, 2018).

Species of Hylinae that are distributed in the lowlands have wide continuous distributions across some major biogeographical barriers, whereas species living in the highlands have restricted distribution and low dispersal capabilities. Hylinae also includes sister species with sympatric distributions (Amphibia Web, 2019), which is important for this study, because species coexisting in the same areas occupy multiple environments and have different degrees of niche partitioning throughout the forest canopy. Niche partitioning has been associated with ecomorphs according to different locomotion modes (Moen et al., 2013), which in the case of tree frogs might be influencing the evolution of forelimb and hind limb proportions.

Study system

Neotropical tree frogs are an ideal study system to answer macroevolutionary questions about morphology and locomotion. Hylinae (family Hylidae) is one of the most diverse Anuran subfamilies. It includes 193 species in 20 genera that are distributed throughout Central America, Eurasia, Japan and North Africa (Amphibia Web, 2019). In Mesoamerica it occupies a wide range of ecosystems and microhabitats, from tropical rainforests in the lowlands (sea level) to pine-oak forests in the highlands (>2500 meters elevation). Some allopatric species have conserved morphotypes and occupy similar microhabitats, while parapatric or sympatric species exhibit more variation in morphology and partition resources by microhabitat or seasonality (Faivovich et al., 2005). Morphology in Hylinae has been evaluated mostly in the context of phylogenetic inference. Less is known about how traits evolved in relationship to their functionality or habitat.

Methods

Data collection for morphological traits

Data collection included 234 specimens corresponding to 14 genera and 29 species (Table 1). All specimens were deposited in the Burke Museum of Natural History and Culture at the University of Washington (UWBM). We took pictures from the specimens using a Canon EOS camera from the Mammalogy collection at the Burke Museum. We use artificial lights and including a scale in centimeters as a reference. We measured eight morphological traits for each individual using Image J (Schneider et al., 2012): snout-vent, humerus, radio-ulna, hand (metacarpals-carpals-phalanges), femur, tibio-fibula, astragalus-calcaneum and feet (metatarsals-phalanges) (Figure1). For the limb measurements we measured right and left side and performed a t-test to corroborate if they were significantly different. After determining that there was no significant difference between the right and the left side, we used only the right-side measurements for subsequent analyses. All measurements were transformed to correct for body size and allometry effects.

Principal Component Analysis

To evaluate the patterns among the morphological data we performed a Principal Component Analysis (PCA). PCA minimize the distance and maximize the variance within and between individual morphological measurements. PCA included the eight morphological measurements for all the 234 specimens. We implemented two transformation methods for the data. First, we log transformed the values, a method recommended for data sets including a different number of individuals per species, some of which have a small sample size. We also performed a PCA based on the residuals using the function *principal* in the R package ‘psych’ (Revelle, 2018). For the PCA approaches using the log transformed values as well as the residuals, we estimated the proportion of variance that each of the principal components explained and we calculated the eigenvalues to test which components were contributing the most to that variation. We calculated the eigenvectors in order to determine the magnitude and the direction of the contribution from each of the original morphological variables. We plot the results color coding the individuals by genus and by species using the R package ‘ggbiplot’ (Wickham, 2016).

Phylogenetic Principal Component Analysis

For each of the 29 species, we removed the SVL measurements and we calculated the averages for seven linear measurements among all the individuals within species using the function *ddply* in the R package ‘plyr’ (Wickham, 2011). We used the R package ‘phytools’ (Revell, 2012) to 1) prune the phylogenetic tree estimated for 139 species of the subfamily Hylinae using the function *drop.tree,* and we kept the 29 species with morphological data; 2) to estimate a phylogenetic PCA using the function *phyl.pca*, and 3) to visualize the phylogeny and the phylogenetic PCA using the function *phylomorphospace.* To evaluate the effect that incorporating the phylogeny had on the PCA estimated with the average values for the 29 species, we also estimated a regular PCA.

Linear discriminant and cluster analyses

We implemented a Linear Discriminant Analyses (LDA) in the R package “mass” to test the probability of each individual to be placed in their correct genus and species based on the morphological data. LDA also informs the combination of variables used to predict the probability that each individual has to belong within a group. To determine the number of ecomorphs present in our data set independently of their genus and species, we realized a model-based cluster analyses using the R package “Mclust” (Fraley et al., 2012). Model-based clustering estimates the most likely number of clusters using a likelihood and Bayesian criteria approach. We visualize the clusters obtained with model-based on the principal component one and two obtained from the principal component analyses.

Results

Average values in millimeters corresponding to each morphological trait per species are indicated in Table 1. The proportion of variance contributed by each principal component and their corresponding eigenvalues are summarized in Table 2 for the PCA estimated using the log transformed data and on Table 3 for the PCA estimated with residuals. The contribution that each of the morphological variables has on the PCA analysis is different depending on the type of transformation. When the measurements were log transformed the two traits contributing the most were tibia and femur length, while the carpals and the tibia contributed the most to the PCA estimated using the residuals. The contribution from each trait to the first three principal components for each transformation is summarized in Table 4.

The PCA plots for the log transformed data and the residuals are summarized in Figure 2. To visualize the species distribution in the PCA, we plotted separately the genus (Figure 3) and the species (Figure 4) on the PCA biplot obtained based on the log-transformed data. Most of the genera and the species do not form defined clusters based on morphology. The LDA results indicate that the average probability to place an individual in the correct genus is 0.45. The highest probability is for the genus *Smilisca* and *Ptychohyla* with 0.96, whereas the lowest probability was zero for the genus *Megastomatohyla*, and for the genera that included one individual. The second LDA analysis indicates that the average probability to place an individual in the correct species is 0.30, the highest probability is for the species *Tlalocohyla picta* with 0.91, the probability was zero is for the species *Ptychohyla euthysanota, Megastomatohyla mixe* , *Charadrahyla nephila,* and *Duellmanohyla schmidtorum*, and for the species that included one individual.

The number of ecomorphs present in our data based on the model-based cluster analyses was five with over half of the individuals clustered in the same group (Figure 5). The clusters recovered do not correspond to the genus nor the species that each individual belongs to. The PCA color coded by cluster shows that clusters 2-5 (Figure 5) were distributed along the first principal component corresponding to tibia length, which encompasses most of the variation (95%) (Figure 5). Individuals from cluster number one are distributed along the PC 1 and PC2 indistinctively, suggesting that the cluster might be separated from the rest using another axis of variation driven from a different morphological characteristic.

We summarized the results for the PCA and the phylogenetic PCA estimated using the average per species in Table 5 and Table 6, respectively. The phylomorphospace can be visualized in Figure 6. The morphological variables that contributed the most to the variation for both the PCA of the log transformed data and the phylogenetic PCA were tibia and foot length. Across all the analyses the linear measurements for the hind limbs (femur, tibio-fibula, astragalus-calcaneum, and feet) are the ones contributing the most to the variation among the species. High eigenvector values for the tibia were consistent across all the PCA analyses. However, the order of contribution for the rest of the traits is not consistent among the results obtained when we analyzed the data using all the individuals, the average for each species, or when the phylogeny was incorporated.

Discussion

Species of frogs with similar morphologies have been reported across distantly related clades, and for multiple families of frogs around the globe. Similar morphologies have been found to be a consequence of both evolutionary convergence and evolutionary conservatism (Moen et al., 2013). We incorporated phylogenetic information to evaluate limb morphology variation using a phylogenetic PCA. Our results suggest that tibia was the character driving the variation, independently of the transformation of the data or when phylogeny was incorporated in the analyses. Our results based on the average data per species found the same relationships as when we included all the individuals, with the hind limb and particularly the tibio-fibula contributing to the variation among groups. We can infer that tibia has an important role in frog morphology.

Our results corroborate previous studies that also found a relationship between the hind limb and locomotion in a phylogenetic context (Gomes et al, 2009).

It has been previously reported for frogs that the length of the tibio-fibula has a large influence in locomotion performance, particularly for walking and burrowing frogs (Gomes et al., 2009; Moen et al., 2013). However, the contribution of the hind limb and the tibia to limb morphology variation is highly scale-dependent. For example, Jorgensen and Reilly (2013) did a large-scale study focusing on skeletal morphometrics and concluded that limb morphology was not significantly different among frog families. Whereas Emerson and Inger (1988) evaluated the relationships among of post-cranial skeleton and locomotion modes and found that the variation is driven by hind limb length. Our results are concordant with Emerson and Inger (1988) and reinforced the importance of hind limb evolution.

Besides the tibio-fibula, studies have reported that at a family level, femur and tarsus were the next traits contributing the most to the morphological variation (Emerson, 1976; Emerson and Inger, 1988). The second morphological trait driving variation in the present study was either femur length, carpal length, or foot length, depending on the data transformation technique (Table 4-6). Differences found in the PCA results according to the two transformations can be explained based on the small sample size for some of the species. The discordance regarding which hind limb structure was the second most influential is particularly relevant considering that foot size varies according to locomotion. This could be driven by trade-offs in multiple locomotion modes. Previous studies have reported large surface areas in the foot increasing swimming performance, especially if it is combined with interdigital webbing (Johansson and Lauder, 2004).

The high rates of morphological convergences in tree frogs have made it challenging to incorporate morphological traits in phylogenetic studies in Hylinae (Faivovich et al., 2005). It is still unclear what internal and external factors are causing morphological convergence in closely related species in Hylinae. Multiple members of Hylinae included in the present study have overlapping distributions and individuals cannot be distinguished to species level based only on morphological traits. The results from the cluster analyses suggest that there are five different ecomorphs within the 29 species in the present study. The clusters do not correspond to a phylogenetic placement and further experiments are needed to test what is driving the ecomorph variation. More research is needed to test if limb morphology is associated with a specific type of behavior and if there are trade-offs among locomotion modes for species performing more than one (Nauwelaerts, et al., 2007).

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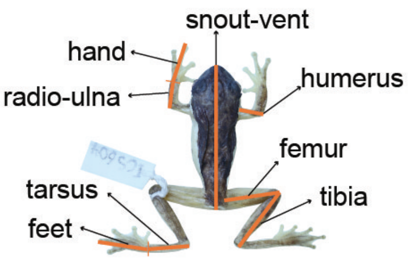

Figure 1 Linear measurements incorporated in the analyses

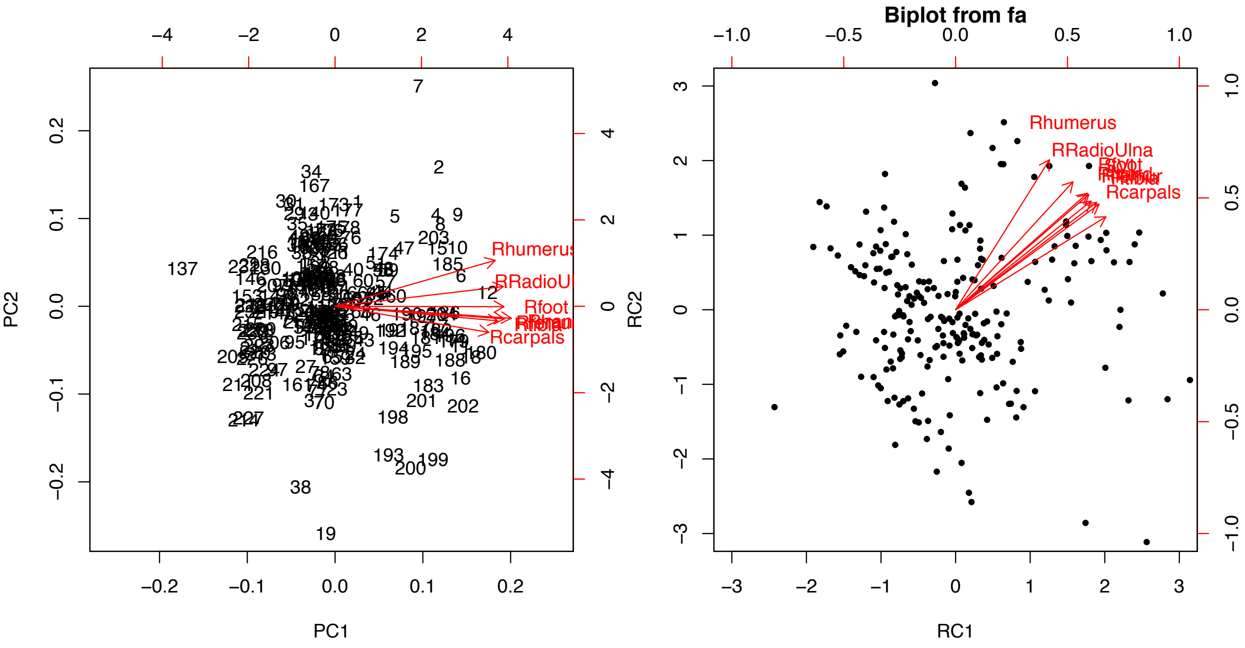

Figure 2. PCA for the log transform data and the residuals are summarized

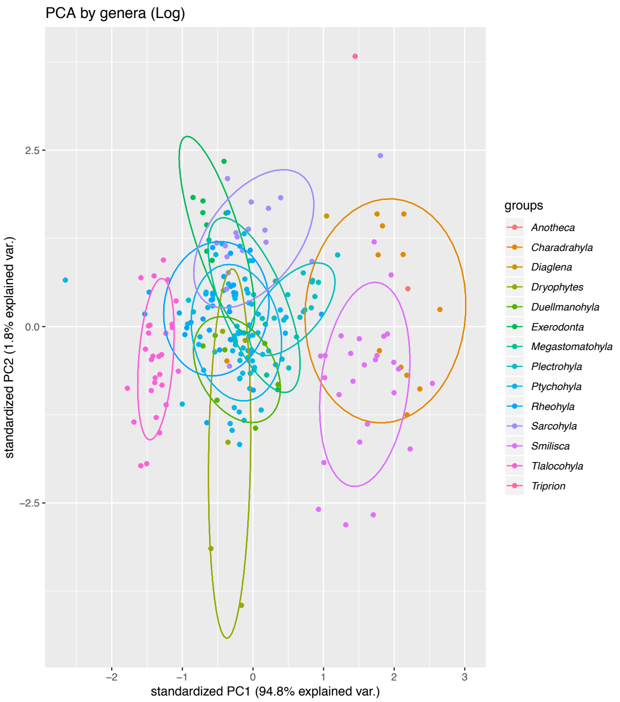

Figure 3. PCA based on the log transformed values with the individuals color coded by genus.

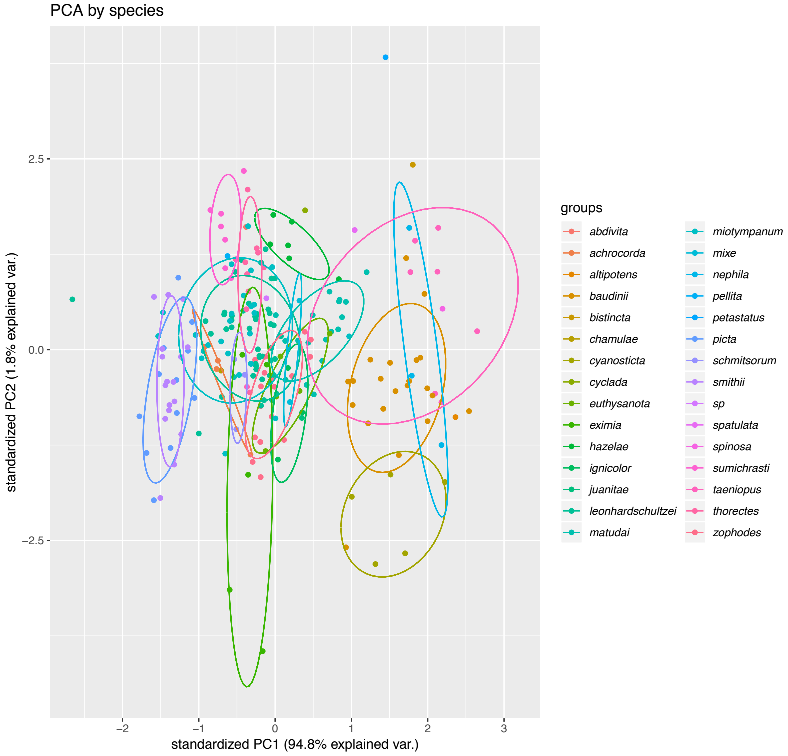

Figure 4. PCA based on the log transformed values with the individuals color coded by species.

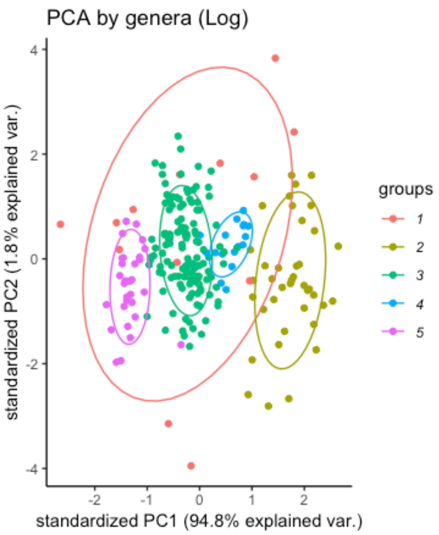

Figure 5. Five clusters obtained with model-based cluster analyses plotted on the principal component one and two obtained from the principal component analyses with the log data.

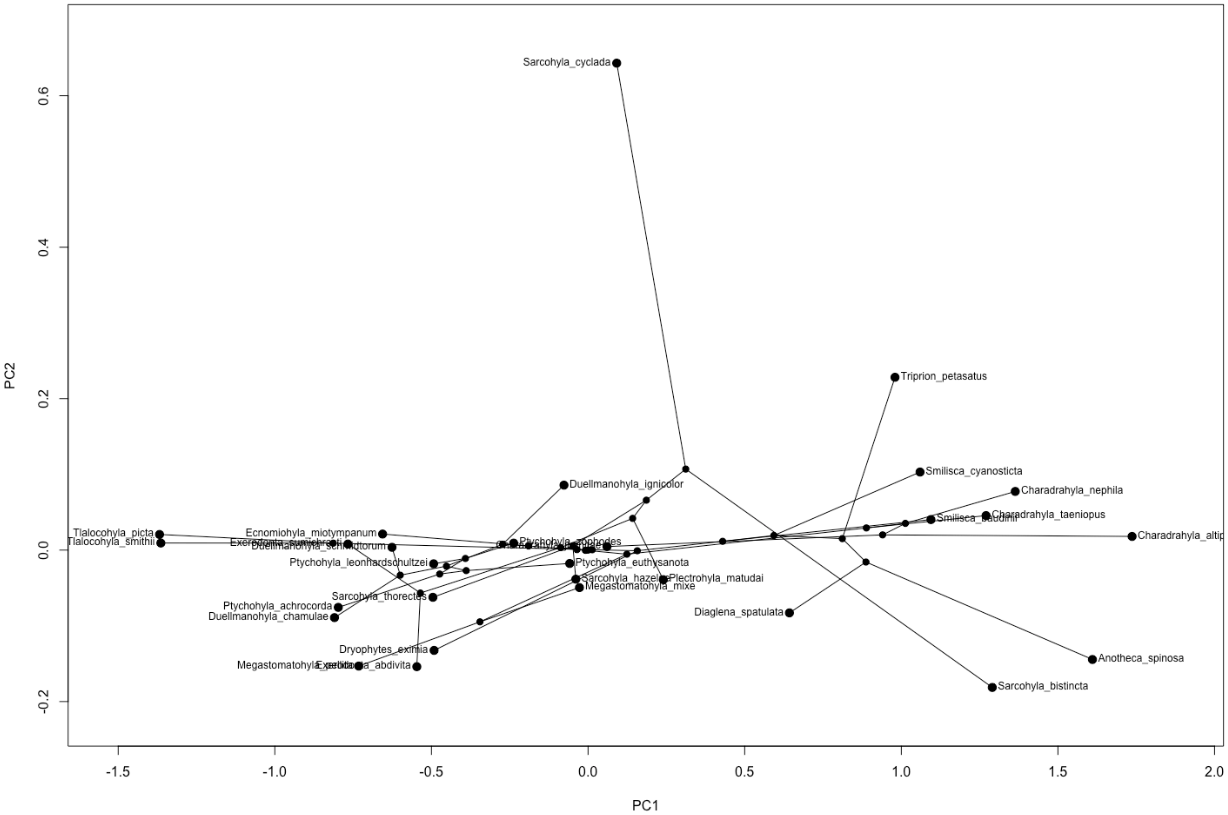

Figure 6. Phylomorphospace for the phylogenetic PCA based on the average measurements for 29 species.

Table 1. Average values in millimeters for morphological traits measured per species.

Species	SVL	Hand	Radio-ulna	Humerus	Femur	Tibio-fibula	Tarsal	Foot
Anotheca spinosa	74.97	22.43	15.50	10.22	30.05	31.88	13.77	32.44
Charadrahyla altipotens	72.53	21.34	13.66	11.07	30.81	37.45	19.51	31.52
Charadrahyla nephila	59.19	19.78	11.15	10.77	26.77	31.23	15.61	28.07
Charadrahyla taeniopus	59.74	18.32	12.04	11.17	26.32	31.01	15.90	26.31
Diaglena spatulata	64.96	9.85	12.25	7.67	22.74	22.83	10.64	24.45
Dryophytes eximia	32.10	8.37	6.23	4.15	13.98	15.91	8.61	15.32
Duellmanohyla chamulae	28.47	8.18	5.54	4.43	11.89	13.29	7.41	11.29
Duellmanohyla ignicolor	37.14	10.16	6.47	6.14	16.35	19.21	10.80	14.22
Duellmanohyla schmidtorum	30.72	8.51	5.57	4.92	13.12	14.78	8.37	11.70
Ecnomiohyla miotympanum	28.10	8.34	5.60	5.24	13.02	14.67	7.78	12.19
Exerodonta abdivita	34.92	6.81	7.89	5.31	12.94	16.35	7.47	12.25
Exerodonta juanitae	34.32	10.61	7.27	6.18	16.44	20.13	11.59	15.16
Exerodonta sumichrasti	28.29	7.77	5.58	5.61	11.59	13.59	7.02	11.40
Megastomatohyla mixe	36.97	11.13	7.18	6.06	15.60	17.65	9.36	16.78
Megastomatohyla pellita	26.88	8.28	6.09	4.92	11.23	13.24	6.54	13.11
Plectrohyla matudai	39.57	13.06	8.12	7.02	17.59	19.57	10.24	17.13
Ptychohyla achrocorda	28.44	8.03	5.47	4.46	11.81	13.73	7.76	11.48
Ptychohyla euthysanota	37.31	10.74	7.01	5.97	15.92	18.45	9.93	15.48
Ptychohyla leonhardschultzei	32.34	8.93	6.11	5.36	13.60	15.51	8.21	13.02
Ptychohyla zophodes	34.37	10.05	6.35	5.59	15.17	17.15	9.34	14.47
Sarcohyla bistincta	62.92	19.14	14.34	10.97	23.11	22.89	13.02	31.43
Sarcohyla cyclada	49.01	9.72	5.19	9.75	24.16	16.60	9.28	16.56
Sarcohyla hazelae	36.98	11.15	7.51	7.05	14.88	17.05	9.17	15.45
Sarcohyla thorectes	30.96	9.02	6.24	5.69	12.41	14.84	7.85	13.34
Smilisca baudinii	57.98	16.44	11.16	9.06	26.32	28.78	14.86	24.01
Smilisca cyanosticta	55.41	16.75	9.71	8.11	27.34	30.41	16.19	23.14
Tlalocohyla picta	24.46	5.94	4.03	3.72	10.05	11.34	6.24	9.94
Tlalocohyla smithii	23.65	6.12	4.09	3.79	9.85	11.55	6.28	9.34
Triprion petasatus	78.36	9.98	11.72	11.37	28.60	27.47	8.96	32.28

Table 2. PCA summary based on the log transformed data for all the individuals in the study. Numbers in bold indicate the traits with the highest values.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Variance(eigenvalue)	0.690	0.01	0.01	0.01	0	0	0
Proportion of Variance	0.950	0.02	0.01	0.01	0.01	0	0
Cumulative Proportion	0.950	0.97	0.98	0.99	1	1	1
Broken-stick value	2.590	1.59	1.09	0.76	0.51	0.31	0.14

Table 3. PCA summary based on the residuals for all the individuals in the study.

	RC1	RC2
SS loadings	4.340	3.4
Proportion Var	0.540	0.42
Cumulative Var	0.540	0.97
Proportion Explained	0.560	0.44
Cumulative Proportion	0.560	1

Table 4. Contribution of each morphological trait to the principal components one and two for the log transformed data (PC1 and PC2) and the residuals (RC1 and RC2). Numbers in bold indicate the traits with the highest values.

	PC1	PC2	RC1	RC2
SVL	0.979	-0.007	0.740	0.64
Hand	0.973	-0.066	0.760	0.61
Radio-ulna	0.965	0.113	0.660	0.71
Humerus	0.949	0.272	0.520	0.84
Femur	**0.986**	-0.073	0.790	0.6
Tibia	**0.991**	-0.088	**0.800**	0.59
Tarsals	0.973	-0.165	**0.840**	0.52
Foot	0.981	-0.002	0.740	0.65

Table 5. Summary results for the PCA based on the average values for the 29 species.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Variance (eigenvalue)	0.8	0.03	0.02	0.01	0.01	0	0
Proportion of Variance	0.91	0.04	0.03	0.02	0.01	0	0
Cumulative Proportion	0.91	0.95	0.97	0.99	1	1	1
Broken-stick value	2.59	1.59	1.09	0.76	0.51	0.31	0.14

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Hand	-0.94	0.276	0.039	-0.183	0.054	-0.028	0.024
Radio-ulna	-0.944	-0.132	0.289	-0.002	-0.072	0.022	0.034
Humerus	-0.941	-0.181	-0.213	-0.156	-0.105	0.001	-0.023
Femur	-0.968	-0.083	-0.187	0.111	0.056	0.015	0.072
Tibia	**-0.978**	0.067	0.007	0.167	-0.051	-0.094	-0.025
Tarsals	-0.927	0.339	-0.047	0.109	-0.053	0.083	-0.038
Foot	**-0.975**	-0.167	0.059	0.002	0.122	0.012	-0.055

Table 6. Summary results for the Phylogenetic PCA based on the average values for the 29 species.

PC1	PC2	PC3	PC4	PC5	PC6	PC7
0.200669824	0.048	0.039	0.026	0.014	0.011	0.009

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Hand	0.947	-0.119	-0.238	0.179	-0.006	0.019	-0.024
Radio-ulna	0.905	-0.373	0.183	-0.067	-0.047	-0.028	-0.029
Humerus	0.928	0.281	0.177	0.126	-0.103	-0.029	0.039
Femur	0.937	0.327	0.029	-0.077	0.034	-0.009	-0.079
Tibia	**0.978**	0.026	-0.052	-0.154	-0.044	0.117	0.030
Tarsals	0.937	0.018	-0.295	-0.154	0.007	-0.089	0.047
Foot	**0.977**	-0.037	0.161	0.049	0.123	0.007	0.037

Chapter 3: To kick or not to kick. Swimming behavior and performance in tree frogs (Anura: Hylinae)

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Introduction

The ability to swim provides terrestrial organisms with access to aquatic environments, which increases the number of niches that a species can occupy (Carroll et al., 2011). Within anurans, some species rely almost entirely on aquatic environments throughout their life, whereas others are predominately terrestrial, arboreal or burrowing (Pough, 2016). Anurans have evolved specialized behaviors and morphological adaptations associated with each of these environments (Moen et al., 2013, Herrel et al., 2016). However, independent of the primary environment and locomotion mode that frogs have as adults, most species have an aquatic stage during their life cycle, either during breeding season or during the larval stage (Duellman and Trueb, 1994). Therefore, swimming remains a critical locomotor strategy with important fitness consequences in anurans.

In frogs, swimming kinematics has been categorized into three phases including propulsion, gliding, and recovery (Nauwelaerts et al., 2001). The type of hind limb gait used for propulsion has been divided in two main categories: simultaneous or alternating (Abourachid and Green, 1999). A simultaneous gait involves the rapid and synchronous extension of both hind limbs, whereas an alternating gait is the extension of one of the hind limbs while the other remains retracted. The gliding and recovery phases also depend on the type of gait; a simultaneous gait leaves the legs extended during gliding and retracted during recovery, whereas the gliding and recovery phases happen at synchrony during an alternating gait with one leg extended while the other is being retracted. For each phase of swimming there are differences in the way each section of the limb moves, the limb orientation with respect to the body and the direction at which they are moving. The distance that a frog can cover, and the speed attained during each phase of swimming also change (Gal and Blake, 1988). However, the relationship between the speed and distance that a frog can achieve for each of the swimming phases, as well as those differences for the two types of gaits still remains poorly understood.

Swimming performance can be measured as the distance reached with a determined movement, the time taken to swim a specified distance, or the maximum achieved (burst speed) (Arnold, 1983, Irschick, 2003, Careau et al., 2014). Previous research has shown that frogs using a simultaneous gait will have a larger force per kick, and they will reduce the drag during the gliding phase when both legs are extended, resulting in a higher speed (Gal and Blake, 1988). Whereas frogs using an alternating gait will generate propulsion force only with one limb, while the other limb is still creating drag during the gliding and the recovery phases (Abourachid and Green, 1999; Nauwelaerts and Aerts, 2002). Besides gait type, swimming performance in frogs is also influenced by other behaviors, such as the rotation of the ankles and the angles of the legs during each phase of the gait (Rovovska-Havelkova et al, 2014).

A swimming behavior using alternating gait is considered the ancestral state in anurans, because it is present in the earliest diverging lineages (Leiopelmatidae and Ascaphidae; Abourachid and Green, 1999) and some species of *Rana* (Nauwelaerts and Aerts, 2002). The remaining species have been reported to use a simultaneous gait, which has been considered the derived state. The evolution of swimming behavior and performance has been mostly studied in distantly-related aquatic species (Moen et al, 2013), and the forelimb behaviors have been understudied. In addition, less is known about how swimming evolved in species dependent on multiple locomotion modes; for example, species that jump and swim.

The subfamily Hylinae is one of the most diverse groups of frogs (193 species). Hylinae belongs to the family Hylidae, one of the frog families with the highest diversification rates and a relatively recent rapid radiation (~60Ma). Tree frogs have three main types of locomotion, they jump, climb and swim; and each species has behavioral and morphological adaptations for each locomotion type (Manzano et al., 2008). During previous field expeditions we found that species in the subfamily Hylinae have the two types of swimming gaits reported for frogs (simultaneous and alternating). In addition, we observed a wide variety of forelimb behaviors. The present study aims to 1) describe the swimming kinematics in tree frogs from the subfamily Hylinae, 2) understand the relationship between swimming behavior and performance, 3) Evaluate if there are significant differences in swimming performance among genera and species. Detailed studies about kinematics are the base to understand functional morphology in tree frogs. The broader goal of this research is to incorporate both kinematics and morphological data on a comparative phylogenetic framework, which will be addressed on chapter 4 of this dissertation.

Methods

Data collection

We sampled 17 localities in eight states in Mexico during July 2014, August and September 2015 and August and September 2016. To increase our chances of including a larger number of clades within the subfamily Hylinae we targeted localities with high species abundance, but also visited type localities to increase the probability of finding rare species. Our field trips covered a wide altitudinal range (0 to 2,300 m), along different vegetation types, including tropical rain forest, semi-deciduous forest, cloud forest, pine forest and pine oak forest. We collected frogs at night, and behavioral experiments were performed within 24 hours after capture. Swimming performance trials were video recorded in a transparent water tank (20 cm wide and 100 cm long) with a temperature between 20-22 degrees Celsius. Each frog was placed in the tank and encouraged to swim by gently pushing their backs. All video footage was collected in the field using a Casio High Speed Exilim Ex-ZR700 Digital Camera (EX-ZR700WE) with a video quality of 720 pixels and 60fps. All animal research was approved by the UW Institutional Animal Care and Use Committee (IACUC #4367-01). We analyzed the swimming video recordings with the software Kinovea (www.kinovea.org). Swimming trials that did not include at least one complete full stroke cycle were excluded from the analyses. All the raw footage and the files tracked using the software Kinovea are deposited online in the following repository: https://osf.io/.

We categorized the swimming behaviors qualitatively for the type of hind limb gait, and the movement and position of the forelimbs (Table 1, Figure 1). We separate the hind limb gait in three categories/states: using both legs simultaneously, alternating legs, or using both types of hind limb gait (simultaneous and alternating) during the same swimming trial and without stopping. We separate the fore limb behaviors in six categories/states, three when the fore limbs were moving: rowing alternating the arms, rowing with both arms simultaneously, or using a combination of both; and three categories when species maintained the forelimbs in a still position: arms still and straight positioned posteriorly, arms still positioned anteriorly in 90 degrees, or a combination of both. The quantitative dataset includes: the burst of speed produced in the first frame after the kick; the maximum speed during the swimming trial; the number of kicks per trial (either alternating or simultaneous); the average distance per kick; and speed for the complete swimming trial. A description of the quantitative dataset is summarized in table 2.

Statistical analyses

All the statistical analyses were estimated after log transforming the data to correct for body size. We log transformed as recommended for data sets including a different number of individuals per species, some of which have a small sample size. We implemented a t-test to determine if the burst of speed in the first gait was significantly faster than the maximum speed during a swimming trial. This comparison allowed us to evaluate if the maximum speed in each trial was due to the stress caused of the stimuli applied to make the frogs swim. We implemented an Analyses of variance (ANOVA) to test for significance among variables. For the cases where the ANOVA had a significant p-value, we then implemented a single step multiple comparison Tukey HSD, which determines which pairs of variables are significantly different from each other. We used the ANOVA and the Tukey test in three cases: 1) to test if each of the states for forelimb and hind limb behaviors had a significant effect on the speed of a swimming trial; 2) if the hind limb behavior had an effect in the distance the frogs can displace with one kick and the number of kicks they can do per swimming trial; and 3) to test for significant differences in speed among the genera and species included in the study.

Results

Tree frogs from the subfamily Hylinae exhibit a wider variety of swimming behaviors than previously described. Species have intra and interspecific variation including the two types of hind limb gaits and different forelimb behaviors. The complete data set includes 198 video recordings corresponding to 14 genera and 29 species (Supplementary table 1). The percentage of each forelimb and hind limb behavior used per species is represented in figure 2.

Hind limb kinematics

Among the 29 species we studied, four species used exclusively an alternating gait, whereas fourteen use exclusively a simultaneous gait (Figure 2A; Supplementary table 2). The remaining 11 species use a combination of both simultaneous and alternating gaits. There were also seven species that used both types of gait during the same swimming trial (*Exerodonta abdivita, Exerodonta sumichrasti, Sarcohyla hazelae, Plectrohyla achrocorda, Sarcohyla bistincta,* and *Sarcohyla thorectes*). Swimming speed was significantly different depending on the hind limb behavior (p-value 0.000996). Simultaneous gait is significantly faster than alternating gait (p-value 0.00912, Figure 3A). Distance traveled is also significantly larger using simultaneous gait (p-value <2e-16, Figure 3B), and the number of kicks performed using a simultaneous gait is significantly lower (p-value 2.31 e-14, Figure 3C).

Forelimb kinematics

The rowing behavior was also variable, which has not been previously reported in tree frogs. The most common type of forelimb behavior is keeping the arms still (pressed against the body), with rowing second (Figure 2B). Forelimb behavior for 11 species was exclusively maintaining the arms still in the same position, which involved muscle force to keep them next to the body or to maintain them extended in the front of the body at 90-degree angles. Maintaining the forelimbs in 90 degrees in front of the body has only been reported in the earliest diverging lineages of frogs (*Ascaphidae* and *Liopelmatidae)*. In addition, seven species exclusively row using the arms alternating or simultaneously, and three species changed from one position to another in the same swimming trial without apparent external stimuli. The results for the ANOVA and Tukey tests supported that the mean speed that individuals can achieve was not significantly different depending on the forelimb behavior (p-value 0.106).

Speed among species of Hylinae

The t-test comparing burst speed from the first kick with maximum speed of a swimming trial per individual was significant (p-value < 2.2e-16, Figure 4). Differences among swimming speeds for the 29 species are visualized in figure 5, respectively. When we compare speeds at a species level we found that the speed for five pairs of species are significantly different (Table 3). *Smilisca baudinii* is the fastest species and is significantly faster than species independent of the type of gait used. For example, species with alternating gait (*Megastomatohyla mixe*), or species with simultaneous gait (*Exerodonta abdivita* and *Charadrahyla juanitae*), are still slower than *Smilisca baudinii*, which uses a simultaneous gait.

Discussion

Our research contributes to the knowledge of swimming kinematics in frogs and reports a larger diversity of swimming behaviors for tree frogs than previously described. Previous research on swimming behavior and performance has focused mostly on aquatic species (Peters et al., 1996, Richards, 2010). For tree frogs, climbing has traditionally been the primary focus of performance due to their unique morphology (e.g. enlarged toepads) (Mazano et al., 2008; Herrel et al., 2013). However, swimming is a critical behavior during tree frog life history. Swimming behavior is relevant during breeding season for species that reproduce in aquatic habitats, when the most expensive metabolic cost is reproduction (McLister, 2003). Swimming is also important for predator avoidance, specially during early stages of development when froglets are primarily aquatic (Duellman, 2001).

Hind limb kinematics

We found that a simultaneous gait is the most common gait in frogs from the subfamily Hylinae, but alternating gait was a more prevalent behavior than expected. Previous studies suggested that an alternating gait reduces the energetic cost and it is slower but steadier (Rovovska-Havelkova et al, 2014). However more research is necessary to evaluate if the energetic costs is significantly different when using a simultaneous or an alternating gait. We found that an alternating gait is significantly slower than a simultaneous gait. This corroborates the conclusion that speed differs when the limbs remain flexed due to an increase in drag (Goal and Blake, 1988). In addition, species that kick using a simultaneous gait can go further on fewer kicks. This is related to the longer time and distance that species can travel during the gliding phase after a simultaneous gait (Gal and Blake, 1988). The gliding phase reduces drag and is less energetically expensive when the limbs are not moving, saving energy for the next propulsion phase (Nauwelaerts, 2005). Previous hypotheses suggested that species using more than one gait might change the gait in the same swimming trial to minimize metabolic energy consumption (Rovovska-Havelkova et al, 2014).

Species of frogs with multiple locomotion modes have similar behaviors. For example, jumping has hind limb movements similar to swimming; both require a large muscle mass, and simultaneous gait for propulsion (Kamel et al., 1996; Reilly and Jorgensen, 2011). An alternating gait has been mostly tested for climbing and walking behaviors, where the animals move faster by increasing the frequency of the gait (Ahn et al, 2003). Our results did not support the hypothesis that an alternating gait increases speed for swimming when the frequency of the kicks increases, as happens in walking. The relationship between performing a gait alternating the limbs for walking and an alternating gait for swimming has not been tested, and it is unknown if similar morphologies increase the performance for both. For species spending more time in arboreal microhabitats there might be a constraint in the way frogs can move the legs, alternating the forelimbs and hind limbs is indispensable for climbing (Manzano et al., 2008). Therefore, the selection for climbing, as the primary locomotion of arboreal species can be larger than the pressure for jumping, thus having a negative consequence on swimming performance.

Forelimb kinematics

The relationship between the types of forelimb behaviors used different locomotion modes has not been tested. For jumping, moving the forelimbs from back to front during the gliding phase is indispensable for reducing the impact during landing (Essner et al., 2010). However, according to our results they do not have an influence on swimming performance. Another question that remain unanswered is why a 90 degrees arms position is still prevalent in derived species of tree frogs. The forelimb position does not cause a significant reduction of swimming speed, but it is still unknown if it is energetically expensive or detrimental to maintain the forelimbs contracted in one position against the current. One possible explanation for the 90 degrees forelimb position might be a phylogenetic constraint, and the ancestral state was retained in more derived species. Therefore, it is important to incorporate a phylogenetic component to test these ideas.

Speed among species of Hylinae

The study of behavior in closely related organisms can uncover patterns previously not considered. Selecting one species as a representative for an entire family can be misleading. Therefore, it is important to study locomotion in a more integrative way, particularly for species that perform multiple locomotion modes. Research focused on the kinematics of eight species with different ecologies found that species from different clades converge on the same swimming kinematics (Robovska-Havelkova et al., 2014). In the present study, species within one subfamily also converged on similar locomotion strategies; however, some behaviors were also consistent among monophyletic clades corresponding to some genera. Some clades swim using exclusively an alternating gait or a simultaneous gait, whereas some species use a combination of both. Finally, the highest speed achieved during a swimming trial was significantly different than the speed of the first kick, therefore we can hypothesize that maximum speed at the beginning of the trials was not triggered by an escape response.

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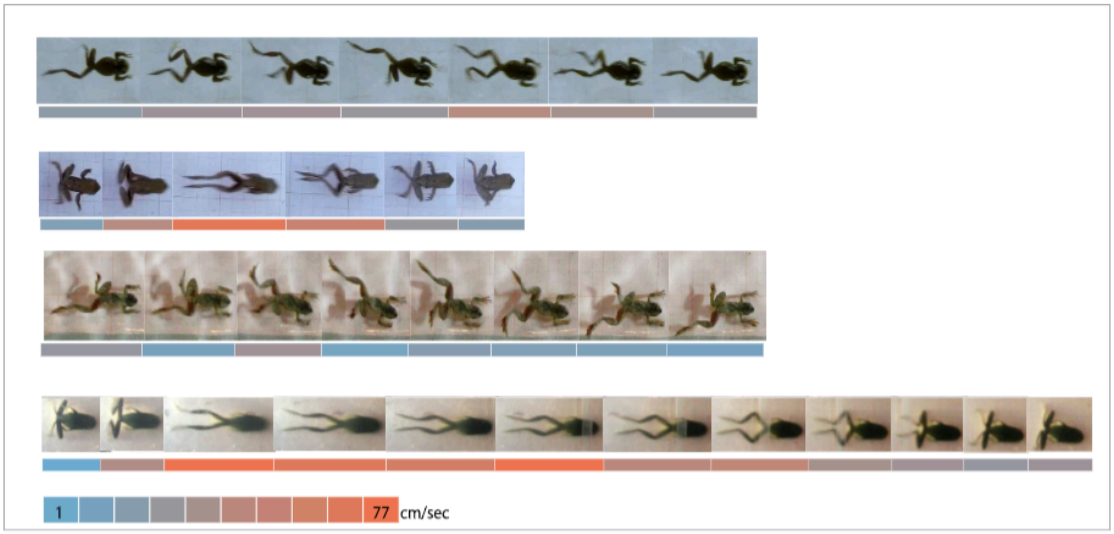

Figure 1. Hind limb behavior: alternating gait (A,C), simultaneous gait (B,D). Forelimb behavior: arms still in the front at 90 degrees(A, C), arms still in the back (D), rowing with both arms simultaneously (B). *Plectrohyla matudai* (A), *Ptychohyla leonhardschultzei* (B), *Megastomatohyla mixe* (C), and *Smilisca baudinii* (D)

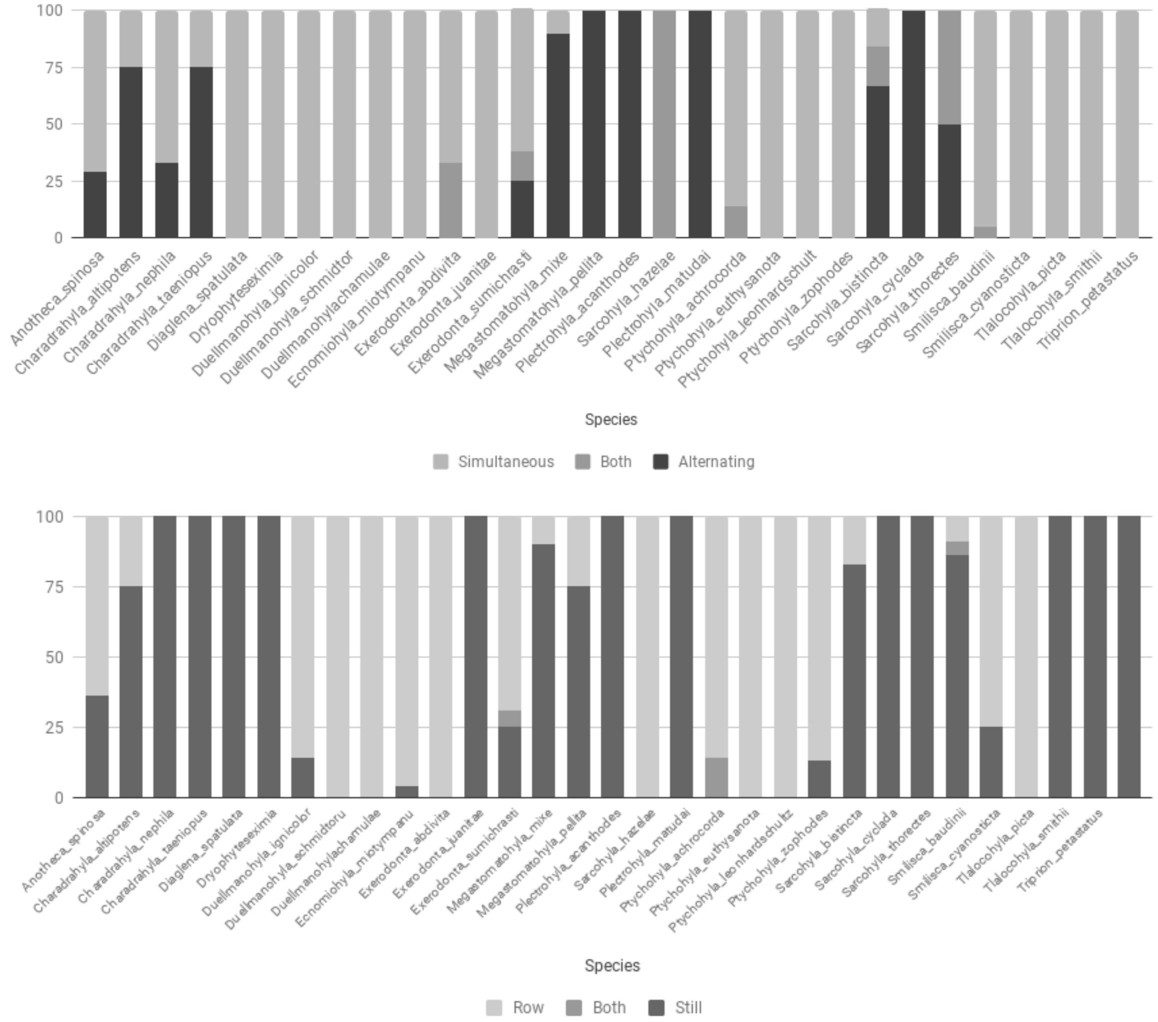

Figure 2. Percentage of behaviors used for each species. The upper graph corresponds to the hind limb gait and the lower graph correspond to the forelimb behavior.

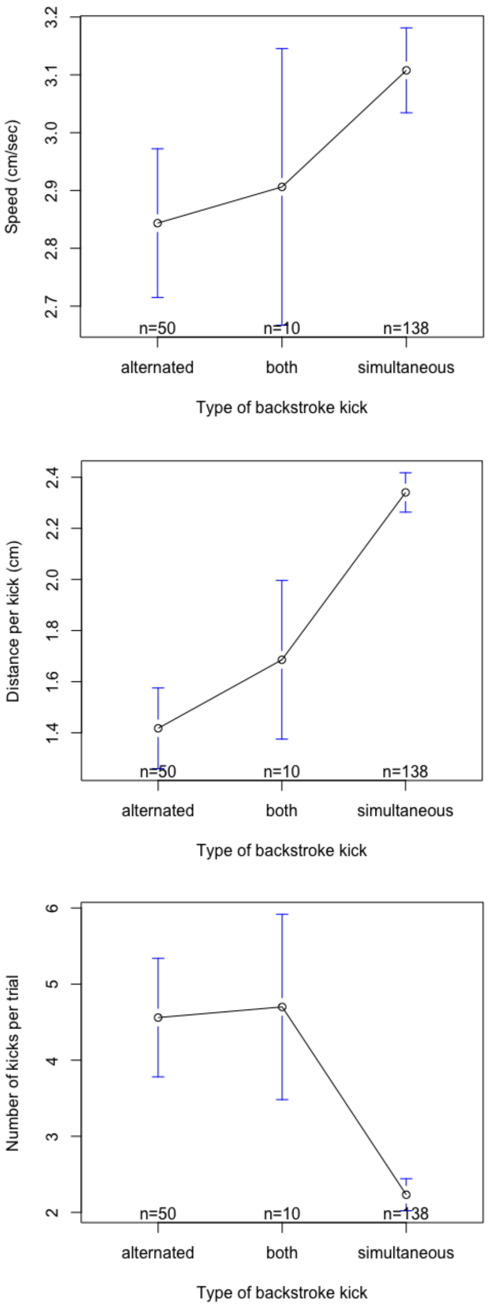

Figure 3. Type of hind limb gait compared to A) Speed of a swimming trial, comparison between alternating and simultaneous hind limb gait p-value = 0.009; B) distance per kick, comparison between alternating and simultaneous hind limb gait p-value <2e-16; and C) number of kicks per trial, comparison between alternating and simultaneous hind limb gait p-value 2.31 e-14.

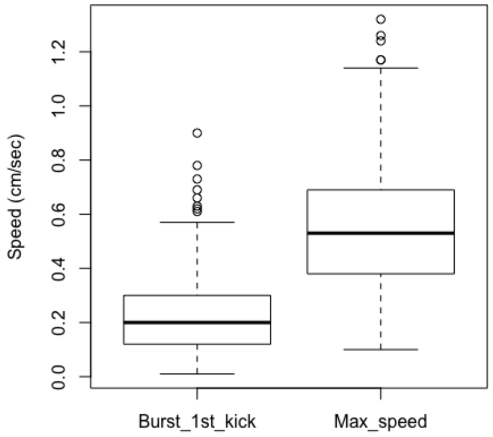

Figure 4. Burst of speed in the first and maximum speed during a swimming trial (cm/sec). T-test p-value < 2.2e-16

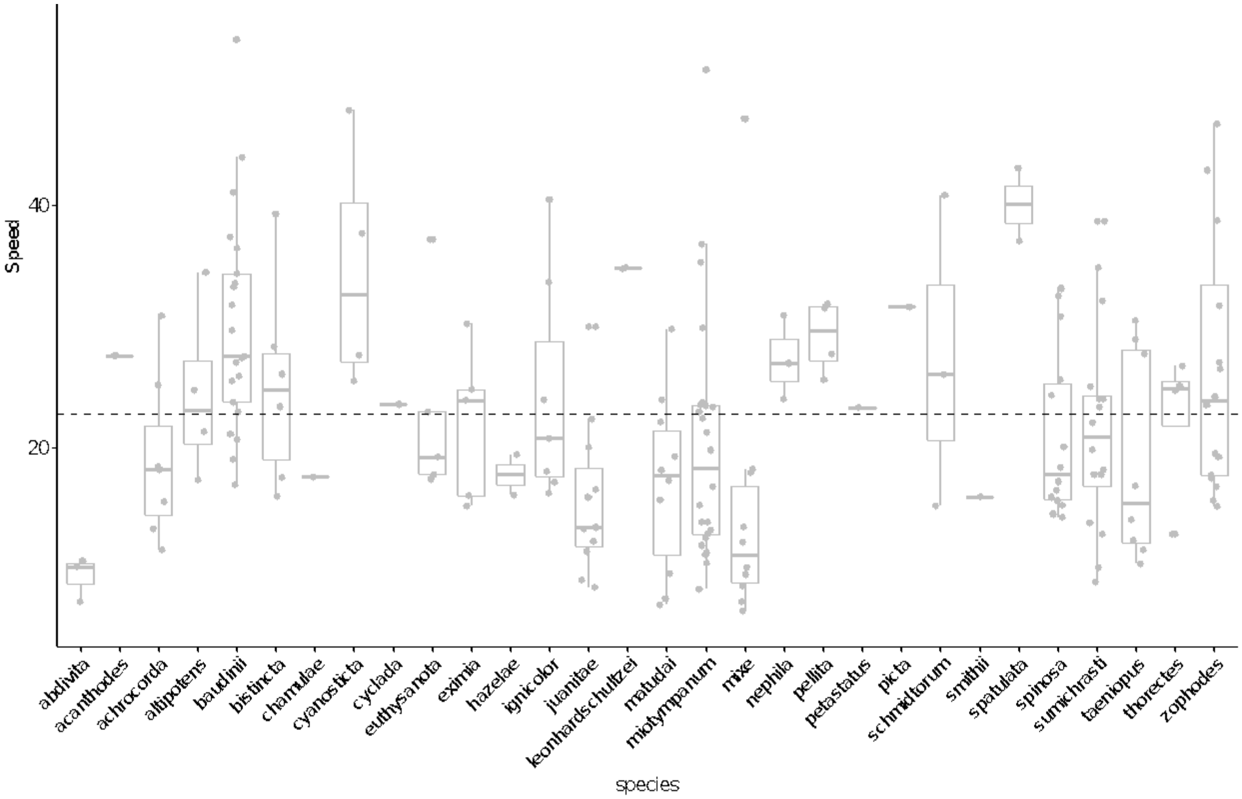

Figure 5. Speed (cm/sec) for the 29 species included in the present study.

Table 1. Description of qualitative swimming behaviors and their corresponding states.

Character	State	Description
Hind limb Gait	Simultaneous	Starts with a propulsion generated by rapid and synchronous extension of both hind limbs, followed by a gladding phase when both legs remain extended during, and it finishes with the retraction of both legs simultaneously during recovery.
	Alternating	Starts with the extension of one of the hind limbs while the other remains retracted, gliding and recovery phases happen at synchrony. One leg is extended while the other is being retracted, and they alternate these positions for each kick.
	Both	A combination of alternating and simultaneous gaits. Both present during the same swimming trial (without stopping).
Forelimb behavior	Still	Arms still and straight pointing posteriorly and pressed against the body Arms still positioned anteriorly to the body in 90 degrees. A combination of arms still pressed against the body or anterior to the body in 90 degrees during the same swimming trial (without stopping).
	Moving	Rowing alternating the right and the left arm. Rowing with both arms simultaneously. A combination of rowing alternating and simultaneously during the same swimming trial (without stopping).

Table 2. Description of quantitative swimming behaviors and their corresponding states.

Character	State
Speed	Speed (cm/sec) of a complete swimming trial
Burst of speed	Speed produced in the first frame after the kick (either simultaneous or alternating).
Max speed	Maximum speed during the swimming trial (either simultaneous or alternating)
Number of kicks	Number of kicks in a swimming trial. We counted the leg movements starting from a static position, including the propulsion, gliding and recovery phase as a kick. We counted both legs as one kick for simultaneous gaits and each leg independently as a kick for alternating gait.
Average distance (per kick)	We divided the total distance covered in a in a swimming trial by the number of kicks. We consider one kick as described above (*number of kicks)*.

Table 3. Significant results for Tukey test among species.

Species pairs	diff	lwr	upr	p adj
Smilisca baudinii – Megastomatohyla mixe	-0.83	-1.40	-0.26	4.20E-05
Smilisca baudinii – Exerodonta abdivita	1.140	0.22	2.05	0.0016
Smilisca baudinii – Charadrahyla juanitae	-0.67	-1.23	-0.12	0.0021
Ptychohyla zophodes – Megastomatohyla mixe	0.70	0.10	1.30	0.0046
Smilisca cyanosticta – Exerodonta abdivita	1.28	0.15	2.42	0.0082

Chapter 4: Integrative evolution of swimming, morphology and microhabitat in tree frogs (Anura: Hylidae: Hylinae)

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Introduction

The evolution of multiple locomotion modes in frogs has allowed them to conquer a wide variety of microhabitats. Frogs are capable of performing exceptional behaviors ranging from burrowing deep into the ground to gliding long distances from tree to tree. Differences in locomotion performance can have direct impacts on the fitness of species by influencing the use of resources and the likelihood of survival and reproduction (Arnold, 1983). Convergent evolution is common in the order Anura, where species from unrelated families have converged on similar morphotypes and locomotion modes (e.g., swim, burrow, climb, glide) (Moen et al., 2013). These convergences are mostly correlated with the microhabitat that frogs occupy (i.e., aquatic/semi-aquatic, terrestrial and arboreal) (Bossuyt et al., 200; Bossuyt et al., 2006; Moen et al., 2016), but in some cases similar morphologies are the result of phylogenetic constrains (Futuyma, 2010; Zug, 1978; Nauwelaerts et al., 2007). To evaluate the processes that cause "initially dissimilar species to become similar" (Zug, 1978), it is necessary to disentangle the influence of 1) the relationships between form and function of morphological traits, 2) environmental variables, and 3) how phylogenetic constraints limit the evolutionary pathways of characters.

Species in Hylinae use three main types of locomotion to move along one or more different types of microhabitats: jumping, climbing, and swimming. Each locomotor type provides an advantage during different life stages and different seasons. However, given the different functional demands of these locomotor modes, trade-offs in performance might occur (Essner et al., 2010). Jumping has traditionally been the primary focus of studies on performance in all frogs. Several studies have measured force and speed of jumping and found associations with muscle mass and the use of energy stored in elastic tissues (Abdala et al., 2018). Climbing has traditionally been the primary focus of performance in arboreal frogs, related to their unique morphology (e.g. enlarged toepads) (Herrel et al., 2013; Federle et al., 2006; Lee et al., 2001). However, swimming is a critical behavior during tree frog life history; swimming is important for predator avoidance, during the breeding season for species that reproduce in aquatic habitats, and especially during early stages of development when froglets are primarily aquatic (Duellman, 2001).

Swimming behavior in frogs has been described as involving three phases: propulsion, gliding and recovery (Nauwelaerts et al., 2001). Each phase has a different combination of forelimb and hindlimb movements. Hindlimb kinematics are grouped into two distinctive gaits, simultaneously moving both legs, or alternating one leg at a time (Abourachid and Green, 1999). Swimming performance and the speed the species can achieve is related to the force of propulsion and the strategies used to reduce drag during swimming (Gal and Blake, 1988). Research has focused on the details of each type of gait (simultaneous or alternating), such as the angles formed by the legs during the gait (Richards, 2008; Richards, 2010), and the rotation of the hindlimb articulations (Rovovska-Havelkova et al, 2014). Less is known about how forelimb behavior influences swimming performance. Forelimb kinematics have been mostly studied in the context of jumping, where the forelimbs are used to reduce the landing impact, and studies based on *Rana pipiens* show that arm movement is also present during swimming (Peters et al., 1996). Species in the families Ascaphidae and Leiopelmatidae do not use the forelimbs to land when they jump and have an alternateing hindlimb gait while they swim, both considered the ancestral states in frogs. A hypothesis proposed to explain the lack of forelimb movement for landing combined with an hindlimb alternating movement, suggested that the ability of rotating the limbs in the pectoral and pelvic girdles is reduced or missing. Therefore, the capability to move the forelimbs and the hindlimbs synchronously was a key feature that evolved to control jumping, as well as to enable rapid repetition of jumping and swimming in recently derived frogs (Essner et al., 2010)

Besides swimming kinematics, morphological and ecological factors also influence swimming performance (Nauwelaerts et al., 2005; Emerson, 1982; Soliz et al., 2016). Fast swimming has been related to morphologies that increase acceleration and reduce drag. For example, a large inter digital membrane will increase propulsion by displacing a larger volume of water per kick, and a flat body shape will reduce drag while moving through the water. Morphological characteristics related to locomotion also include limb proportions as well as variation in osteology and musculature (Emerson, 1983; Emerson and Jongh 1980; Fabrezi et al., 2014). More recently, research has started to consider multiple frog species with different ecologies to gain a more integrative view of swimming locomotion (Soliz et al., 2016). Understanding the patterns of swimming kinematics in a phylogenetic context is important for understanding the evolution of species with multiple locomotion modes. Robovska-Havelkova et al., (2014) analyzed swimming performance for eight species with different ecologies. They found that hindlimb kinematics and speed of hindlimb extension are associated with species' ecological preferences and not constrained by the phylogeny. The relationship between ecology and locomotion was also studied in frog assemblages across three continents by Moen et al., (2013). Species from different continents sharing the same habitat also share similar morphologies and performance; however, some differences in performance and morphology across these species were due to evolutionary conservatism.

Previous studies have focused on distantly related species, and more information about swimming performance is available for species that are aquatic or semiaquatic. However, arboreal frogs remain an understudied, yet fascinating system for integrative studies of locomotion. Tree frogs from the subfamily Hylinae use three different types of locomotion (jumping, swimming and climbing), they inhabit multiple microhabitats (forest floor, understory, canopy, lentic and lotic) and species are generally conserved morphologically despite obvious differences in body size and limb dimensions. Tree frogs spend most of their times in the trees, and therefore they are typically classified as arboreal ecomorphs rather than exclusively terrestrial or aquatic. Understanding how locomotion evolved is crucial for macroevolutionary studies. Locomotion modes allowed frogs successfully colonize multiple niches. Niche differentiation can lead to high diversification rates and species richness communities living in small areas. A comprehensive study is needed to compare locomotion performance among closely related species instead of across distantly related groups, convergences happen in closely related species and despite having similar ecomorphs tree frogs have a wide variety of behaviors that can influence locomotion performance. **My goal is to understand the evolution of morphological traits and their influence on swimming behavior and performance in the Hylinae subfamily of Neotropical tree frogs.** This comparative study takes an integrative approach to understand the evolution of locomotor performance, while accounting for inter- and intraspecific variation in a phylogenetic context, to investigate the relationships among locomotion, morphology and microhabitat.

Methods

Data collection

Swimming performance and behavior. We recorded swimming performance trials for 29 species of tree frogs from the subfamily Hylinae (Table 1). We video recorded in the field and we analyzed the swimming video recordings with the software Kinovea (www.kinovea.org) (See chapter 3 for details). All animal research was approved by the UW Institutional Animal Care and Use Committee (IACUC #4367-01). We recorded the average speed from a swimming trial (cm/sec). We code the qualitative data for swimming behavior as binary states for the comparative phylogenetic analyses. We separate the hindlimb gait into two states: 1) using both legs simultaneously and 2) alternating the legs. We separate the forelimb behaviors in two states, 1) forelimbs in a still position: including arms pointing posteriorly and pressed against the body, arms positioned anteriorly in 90 degrees, or a combination of both; and 2) forelimbs moving: rowing alternating the arms, rowing with both arms simultaneously, or using a combination of both. We categorize the forelimbs and hindlimb movements that each species performed exclusively, and when species performed more than one we categorized them as the most common behavior for each species.

Morphology. For each individual included in the swimming trials we took the measurements in the field when the specimens were not collected and from images using Image J (Schneider et al., 2012) when the specimen was collected. We measured seven morphological traits for the limbs: humerus, radio-ulna, hand (metacarpals-carpals-phalanges), femur, tibio-fibula, astragalus-calcaneum and feet (metatarsals-phalanges). Based on the seven linear measurements we calculate the length of the complete forelimb (humerus, radio-ulna and hand), and the length of the complete hindlimb (femur, tibio-fibula, astragalus-calcaneum and feet). We estimate the average value per species for each of the seven limb traits, forelimb length and hindlimb length. We log transformed the data to account for the low sample size, and to correct for body size variation in frogs due to the life stage, and sexual dimorphism.

Microhabitat. We assigned an aquatic and an arboreal microhabitat to each species based on literature and personal observations. We code the microhabitats as binary states for the comparative phylogenetic analyses. Aquatic microhabitats include two states: 1) still water (ponds and tree holes), and 2) flowing water (slow flow and rapidly moving streams). Arboreal microhabitats include two states: 1) vegetation close to forest floor (e.g. grass and secondary vegetation <2m), and 2) canopy (> 2m).

Phylogeny. For the comparative analyses we used time-calibrated phylogeny estimated using BEAST v1.8. (Drummond et al., 2013). The phylogeny was estimated including 139 species using five loci: three mitochondrial (*16s, 12s* and *ND1*) and two nuclear (*Rag-1* and *Rhodopsine*). We calibrated the tree with a mean and 95% HPD of 62.85 (42.3-81.4) Ma (Jetz and Pyron, 2018). We pruned the maximum clade credibility (MCC) consensus to include only the 29 species of interest using the function *drop.tree* in the R package 'phytools' (Revell, 2012)

Statistical analyses

Does limb length vary according to behavior?

We implemented an analyses of variance (ANOVA) to test: 1) if hindlimb length is significantly different depending on the type of hindlimb gait (simultaneous and alternating); and 2) if forelimb length is significantly different depending on the type forelimb behavior (still and moving).

Does speed vary according to behavior?

We implemented an Analyses of variance (ANOVA) to test if swimming speed is significantly different depending on 1) hindlimb gait (simultaneous and alternating) and 2) type of forelimb behavior (still and moving).

Does speed vary according to limb morphology?

We used a linear regression to test if swimming speed per species (dependent variable) varied according to the hindlimb and forelimb length (independent variables). We then implemented a multiple regression to test if swimming speed per species (dependent variable) varied in response to length variation on limb structures independently (humerus, radio-ulna, hand (metacarpals-carpals-phalanges), femur, tibio-fibula, astragalus-calcaneum and feet (metatarsals-phalanges).

Phylogenetic comparative methods

We compared how three models of character evolution fitted the swimming performance data (swimming speed (cm/sec): a Brownian Motion (BM) model, which assumes evolution is close to neutral; an Early-Burst model (EB) in which character change tends to be concentrated towards the base of the tree; and an Ornstein-Uhlenbeck model (OU), which assumes stabilizing selection with no specific trend [29,30]. Model selection will be estimated using Akaike Information Criteria (AIC) [31].

We implemented a Phylogenetic Generalized Least Squares (PGLS) to estimate the relationship between swimming speed (cm/sec) and three sets of characters: behavior (hindlimb and forelimb), microhabitat (aquatic and arboreal) and femur length, which is the morphological trait that based on the multiple regression analyses is driving the variation on swimming speed. PGLS estimates the covariance in traits across taxa, accounting for tree topology and the model of character evolution [32]. According to simulation analyses, PGLS outperform other comparative methods when the evolution is not Brownian (Rohlf 2001 and Revel, 2010) [29].

To estimate the evolutionary covariance among all the continuous and discrete traits included in the present study using a phylogenetic framework we used a "*Threshold model*" (Felsenstein 2005, 2012). We included the seven linear measurements, forelimb behavior, hindlimb gait and microhabitat. Threshold model was proposed for discrete traits. The value of the discrete phenotype is determined by its "liability, which is defined as an unobserved continuous character. When a character crosses the threshold value then the discrete character changes the state from one discrete character to the other. The evolutionary correlation then is the correlation of the liabilities.

Results

The complete data set includes 168 videos corresponding to 80 individuals from14 genera and 29 species. Each individual has a complete dataset including morphological measurements, swimming performance and swimming behavior data. Summaries of the morphological data and the swimming performance, and the binary states for the behavior and the microhabitat are summarized in table 1. The pruned phylogeny used for the comparative phylogenetic analysis with speed (cm/sec) mapped on the phylogeny is provided in Figure 1.

Does limb length vary according to behavior?

Hindlimb length was not significantly different depending on the hindlimb gait (p-value 0.24). Forelimb length was significantly different depending on the forelimb behavior (p-value 3.4e-11, Figure2). Species maintaining the arms in a still position have larger limbs compared to species that move the forelimbs during swimming.

Does speed vary according to behavior?

Swimming speed was significantly different depending on the hindlimb behavior (p-value 0.000996). Simultaneous gait is significantly faster than alternating gait (p-value 0.00912). Swimming speed was not significantly different depending on the forelimb behavior (p-value 0.106). The position of the arms with respect of the body as well as the movement does not affect the swimming speed.

Does speed vary according to limb morphology?

The length of the limbs did not predict the swimming speed. Speed was not significantly correlated with forelimb length (p-value 0.0739, Figure 3A), nor the hindlimb length (p-value 0.8764, Figure 3B). However, when the length of each limb structure was tested independently as a predictor for speed, there was a significant correlation between femur length and speed (p-value 0.00554). A scatterplot showing the relationship of femur length (log) and swimming speed (log), color coded by hindlimb gait behavior, is provided in Figure 4.

Comparative analyses

The model of character evolution that best fits swimming speed was the Ornstein-Uhlenbeck (OU) model, meaning that swimming speed its under stabilizing selection with no specific trend. The AIC values and their weights for each model of character evolution (BM, OU and EB) are summarized in Table 2. The results for the PGLS are summarized in Table 3. When the phylogeny was incorporated, swimming speed was not correlated with behavior (hindlimb and forelimb), microhabitat (aquatic and arboreal). However, swimming speed was significantly correlated with femur length.

The results for the evolutionary covariance among all the traits included in the present study are summarized in Figure 5. The evolutionary correlation among the morphological characters was significant, and each of the limb structures are positively correlated with each other. The swimming speed was not significantly correlated to the microhabitat or the behavior when the phylogeny is incorporated. Swimming speed is significantly correlated to the limb structures, and the morphological traits with the highest correlations are femur and tibia. The correlation is negative, meaning that swimming speed increases when tibia and the femur lengths decrease.

Discussion

Swimming speed is not consistent among species or along the phylogeny. The subfamily Hylinae does not contain clades of species that have exclusively fast or slow swimming speeds. Instead, swimming speed and behavior in Hylinae has more inter and intra specific variation than previously described. Among the 29 species included in the study there are species that only use one type of hindlimb gait and one type of forelimb behavior. But there are also species that combine multiple types of gait and forelimb behaviors. This supports the hypothesis that evolutionarily derived frogs have more than one type of swimming behavior (Nauwelaerts and Aerts, 2002), and that not all derived frogs have a simultaneous gait as it was previously suggested (Abourachid and Green, 1999).

Swimming speeds were significantly faster when species used a simultaneous gait rather than an alternating gait. This corroborates previous findings that showed that swimming with a simultaneous hindlimb gait creates a stronger force of propulsion, which results in a higher speed (Gal and Blake, 1988). This differs from species that use an alternating gait, which creates less propulsion by using just one hindlimb at a time, and it also increases drag while the hindlimb is being retracted (Nauwelaerts and Aerts, 2002). Contrary to what we expected, differences in forelimb behavior were not correlated with swimming speed. We expected an increase in speed for species that maintain the forelimbs pressed against the body, and thereby reducing drag, or, species that row with the forelimbs might also increase propulsion. We also expected a speed decrease for species that maintain the arms at a 90 degree angle in front of their body. However, forelimb positions and movements did not have an effect on swimming speed. More research is needed to evaluate the role that forelimbs play in swimming behavior and if those behaviors are related to other types of performance advantages/ trade-offs.

Differences in limb length have been associated with differences in swimming performance in frogs from the family Ranidae (Bossuyt F. and Milinkovitch M.C. 2000). A long tibio-fibula increases propulsion and has been related to an increase in swimming speed and jumping force

(Zug G.R. 1978). We did not find an association between hindlimb or forelimb length with swimming performance (speed) or swimming behavior (hindlimb gait or forelimb behavior). However, when testing each section of the hindlimb independently, we found a negative correlation between speed and femur length. Enriquez Urzelai et al. (2015) evaluated the differences in hindlimb length for multiple locomotion modes. They found that the rations of tibio-fibula to femur differ among species locomotion modes. Arboreal species have a larger tibio-fibula ratio than terrestrial species, which has been reported to be advantageous for arboreal species that are required to navigate among the vegetation (Vidal Garcia et al., 2014).

When the phylogeny was incorporated to test for correlations between swimming performance with respect to the other variables, we found that swimming speed was not correlated to behavior. These results differ from the analyses that did not account for a phylogenetic effect testing for variation, which suggested that an increase in swimming speed was correlated to a simultaneous swimming gait. This result can be attributed to species that are able to perform the two types of hindlimb gaits, or individuals that use them interchangeably.

Our results testing for evolutionary correlations were concordant whether we implemented a PGLS or whether we used the threshold model. For both analyses the swimming speed is correlated with limb length. However, using PGLS the only limb section that has a correlation was the femur, whereas with the threshold model the complete limb length was correlated. The evolutionary correlation between swimming speed and length of the hindlimb structures is negative, meaning that swimming speed increases as the limb length decrease. This correlation might be explained by the increase in drag that is incurred by a longer femur. This result differs from when the phylogeny is not incorporated, when only the femur and not the complete hind limb length is positively correlated to speed. Swimming speed was not correlated with aquatic and arboreal microhabitats. This result was surprising considering that locomotion performance was mostly related to microhabitat when distantly related species were compared in a phylogenetic context (Moen et al., 2013).

The present study represents a contribution to the understanding of frog kinematics, and it opens the possibility of more detailed studies of swimming locomotion in tree frogs, as well as possible tradeoffs with other types of locomotion. Differences in swimming behaviors such as hindlimb gait might not be related to speed in an evolutionary context, but they might be related to climbing and jumping behavior and performance (Enriquez Urzelai et al., 2015; Essner et al., 2010). There are also other factors that can be influencing swimming behavior such as buoyancy and maneuverability. Frogs that keep the arms in 90 degrees normally have an alternating gait, which correspond to a slower more constant speed. At a lower speed positioning the arms in the back the weight of the head might cause the frog to sink. However, if the arms are in the front at 90 degrees, then they can help stabilize the head and the direction of movement. Our results showed that swimming performance and behavior are highly variable in Hylinae. Swimming performance and behavior are not influenced by microhabitat, and swimming speed is negatively correlated to the length of hindlimb structures. Still, more research is needed to understand the intricate relationships between locomotion, morphology and microhabitat in tree frogs.

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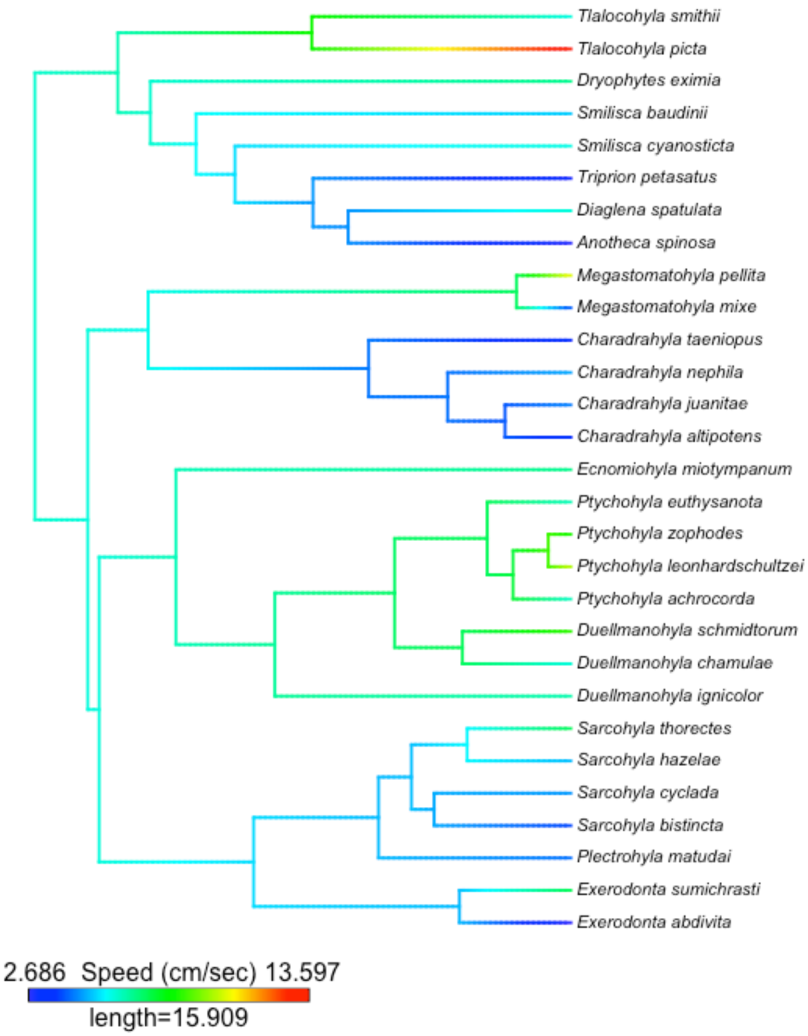


Figure 1. Pruned species tree (Fig 1, Chapter 1) including the 29 species in the study with the speed (cm/sec) mapped by estimating the internal nodes using ML, and interpolating the states along each edge ("contMap" function Phytools, Felsenstein, 1985).

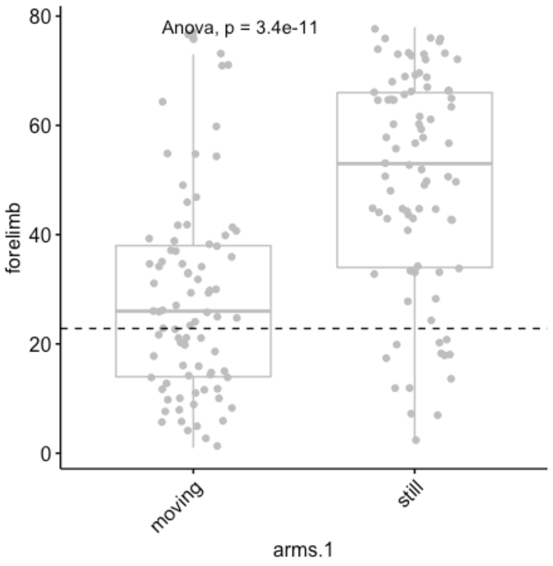


Figure 2. ANOVA results for the comparison between the forelimb length (y axis) and the forelimb behavior (arms moving and arms in a still position; x axis).

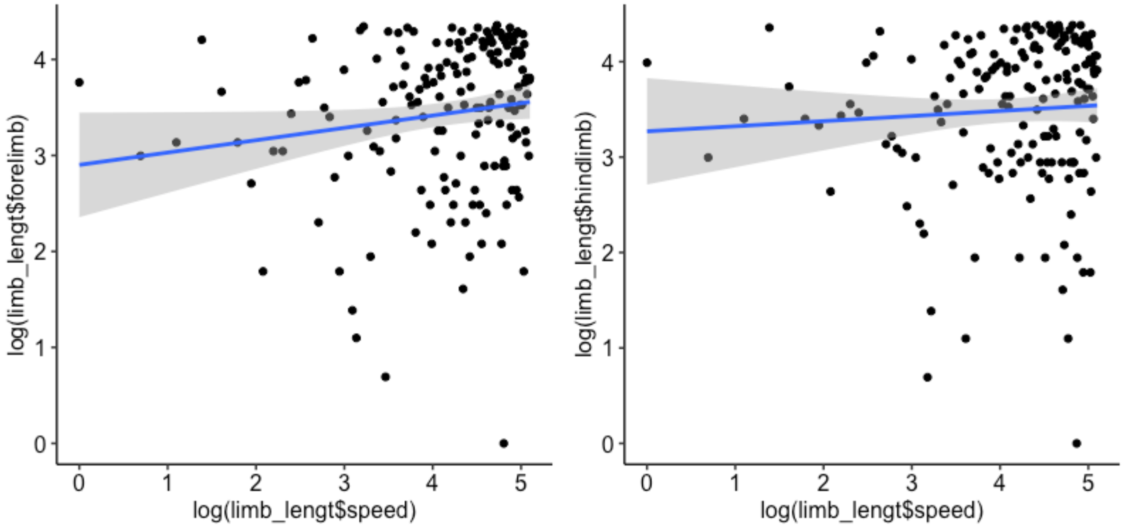


Figure 3. Linear regression between swimming speed log(cm/sec) and forelimb length (A) and hindlimb length (B).

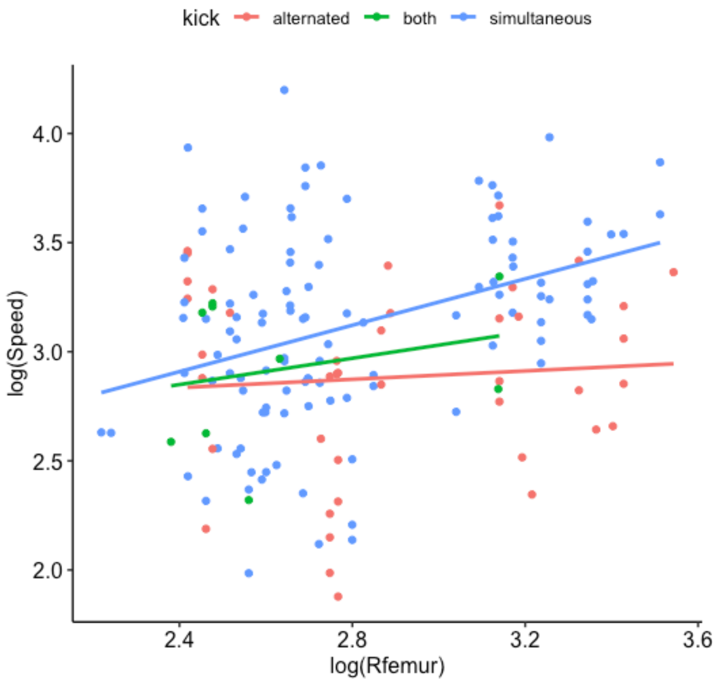
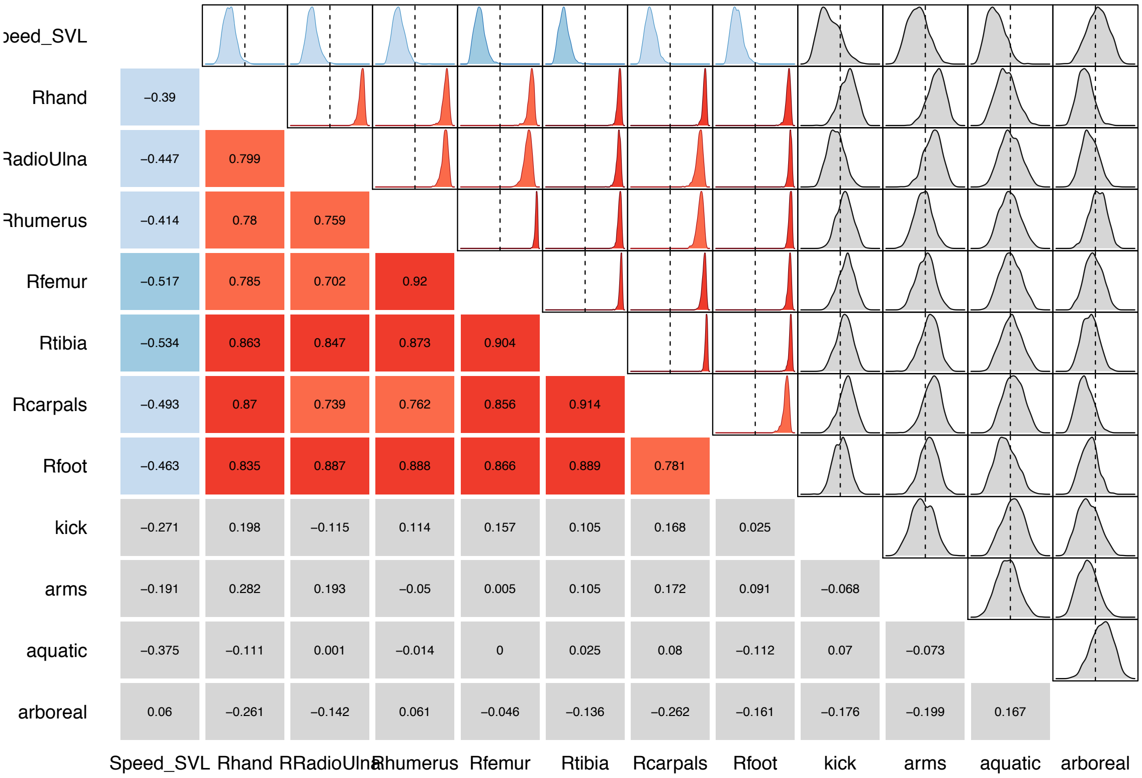


Figure 4. Relationship between femur length (log) and swimming speed (cm/sec) color coded by hindlimb gait behavior.



	Speed_SVL	Rhand	RRadioUlna	Rhumerus	Rfemur	Rtibia	Rcarpals	Rfoot	kick	arms	aquatic	arboreal
Speed_SVL												
Rhand	−0.39											
RadioUlna	−0.447	0.799										
Rhumerus	−0.414	0.78	0.759									
Rfemur	−0.517	0.785	0.702	0.92								
Rtibia	−0.534	0.863	0.847	0.873	0.904							
Rcarpals	−0.493	0.87	0.739	0.762	0.856	0.914						
Rfoot	−0.463	0.835	0.887	0.888	0.866	0.889	0.781					
kick	−0.271	0.198	−0.115	0.114	0.157	0.105	0.168	0.025				
arms	−0.191	0.282	0.193	−0.05	0.005	0.105	0.172	0.091	−0.068			
aquatic	−0.375	−0.111	0.001	−0.014	0	0.025	0.08	−0.112	0.07	−0.073		
arboreal	0.06	−0.261	−0.142	0.061	−0.046	−0.136	−0.262	−0.161	−0.176	−0.199	0.167	

Figure 5. Evolutionary correlation values estimated based on the threshold model (Felsenstein 2005, 2012). Continuous traits include linear measurements and swimming speed. Discrete traits include forelimb behavior, hindlimb gait, and microhabitat. Grey corresponds to correlations that are not significant, blue corresponds to negatively correlated traits that are significant, and red corresponds to positively correlated traits that are significant.

Table 1. Summary of the morphological data and the swimming performance, and the binary states for the behavior and the microhabitat

Species	SVL	Hadnd	Radio-Ulna	Humerus	Femur	Tibia	Carpals	Foot	Hind limb	Fore limb	Speed	Gait	Forearm	arboreal	aquatic
Anotheca_spinosa	7.497	2.243	1.55	1.022	3.005	3.188	1.377	3.244	10.814	4.815	2.804	0	1	2	0
Charadrahyla_altipotens	7.253	2.134	1.366	1.107	3.081	3.745	1.951	3.152	11.929	4.607	3.374	1	0	1	1
Charadrahyla_nephila	5.506	1.757	1.289	1.048	2.383	2.783	1.273	2.96	9.399	4.094	4.958	0	0	2	2
Charadrahyla_taeniopus	5.916	1.861	1.18	1.087	2.626	3.122	1.596	2.654	9.998	4.128	3.22	1	0	2	1
Diaglena_spatulata	6.496	0.985	1.225	0.767	2.274	2.283	1.064	2.445	8.066	2.977	6.168	0	0	2	1
Dryophytes_eximia	3.19	0.846	0.646	0.404	1.417	1.598	0.885	1.5	5.4	1.896	6.909	0	0	1	0
Duellmanohyla_chamulae	2.847	0.818	0.554	0.443	1.189	1.329	0.741	1.129	4.388	1.815	6.175	0	1	2	1
Duellmanohyla_ignicolor	3.69	1.019	0.645	0.619	1.634	1.909	1.071	1.425	6.039	2.283	6.593	0	0	2	1
Duellmanohyla_schmidtorum	3.072	0.851	0.557	0.492	1.312	1.478	0.837	1.17	4.797	1.9	8.913	0	0	2	2
Ecnomiohyla_miotympanum	2.934	0.871	0.585	0.539	1.341	1.526	0.807	1.28	4.954	1.995	6.895	0	1	2	2
Exerodonta_abdivita	3.492	0.681	0.789	0.531	1.294	1.635	0.747	1.225	4.901	2.001	2.686	0	1	2	1
Charadrahyla_juanitae	3.432	1.061	0.727	0.618	1.644	2.013	1.159	1.516	6.332	2.406	4.58	0	0	2	2
Exerodonta_sumichrasti	2.872	0.804	0.561	0.555	1.202	1.378	0.712	1.147	4.439	1.92	7.476	0	1	2	3
Megastomatohyla_mixe	3.668	1.121	0.696	0.603	1.567	1.753	0.938	1.659	5.917	2.42	4.119	1	0	1	2
Megastomatohyla_pellita	2.688	0.828	0.609	0.492	1.123	1.324	0.654	1.311	4.412	1.929	10.859	1	0	1	0
Plectrohyla_matudai	3.839	1.3	0.774	0.667	1.712	1.893	1.019	1.686	6.31	2.741	4.441	1	1	1	1
Ptychohyla_achrocorda	2.993	0.845	0.556	0.451	1.21	1.423	0.806	1.197	4.636	1.852	6.355	1	0	1	1
Ptychohyla_euthysanota	3.531	0.998	0.683	0.562	1.53	1.774	0.969	1.484	5.757	2.243	6.494	0	1	2	2
Ptychohyla_leonhardschultzei	3.346	0.904	0.658	0.547	1.427	1.653	0.904	1.332	5.316	2.109	10.406	0	0	2	3
Ptychohyla_zophodes	3.271	0.946	0.61	0.526	1.442	1.624	0.896	1.357	5.319	2.082	8.591	0	0	2	2
Sarcohyla_bistincta	6.292	1.914	1.434	1.097	2.311	2.289	1.302	3.143	9.045	4.445	3.991	0	1	1	2
Sarcohyla_cyclada	4.901	0.972	0.519	0.975	2.416	1.66	0.928	1.656	6.66	2.466	4.813	1	0	2	2
Sarcohyla_hazelae	3.424	1.02	0.706	0.701	1.39	1.624	0.865	1.221	5.1	2.427	5.19	1	0	2	1

Sarcohyla_thorectes	3.034	0.902	0.605	0.634	1.19	1.459	0.713	1.331	4.693	2.141	7.367	1	0	2	1
Smilisca_baudinii	5.659	1.564	1.115	0.92	2.53	2.786	1.402	2.317	9.035	3.599	5.328	0	0	1	1
Smilisca_cyanosticta	5.737	1.806	1	0.916	2.895	3.224	1.69	2.458	10.267	3.722	6.045	0	1	1	0
Tlalocohyla_picta	2.324	0.528	0.362	0.309	1.027	1.122	0.65	0.879	3.678	1.199	13.597	0	1	1	0
Tlalocohyla_smithii	2.621	0.376	0.453	0.391	1.16	1.3	0.672	1.146	4.278	1.22	6.082	0	0	1	1
Triprion_petasatus	7.836	0.998	1.172	1.137	2.86	2.747	0.896	3.228	9.731	3.307	2.975	0	0	2	0

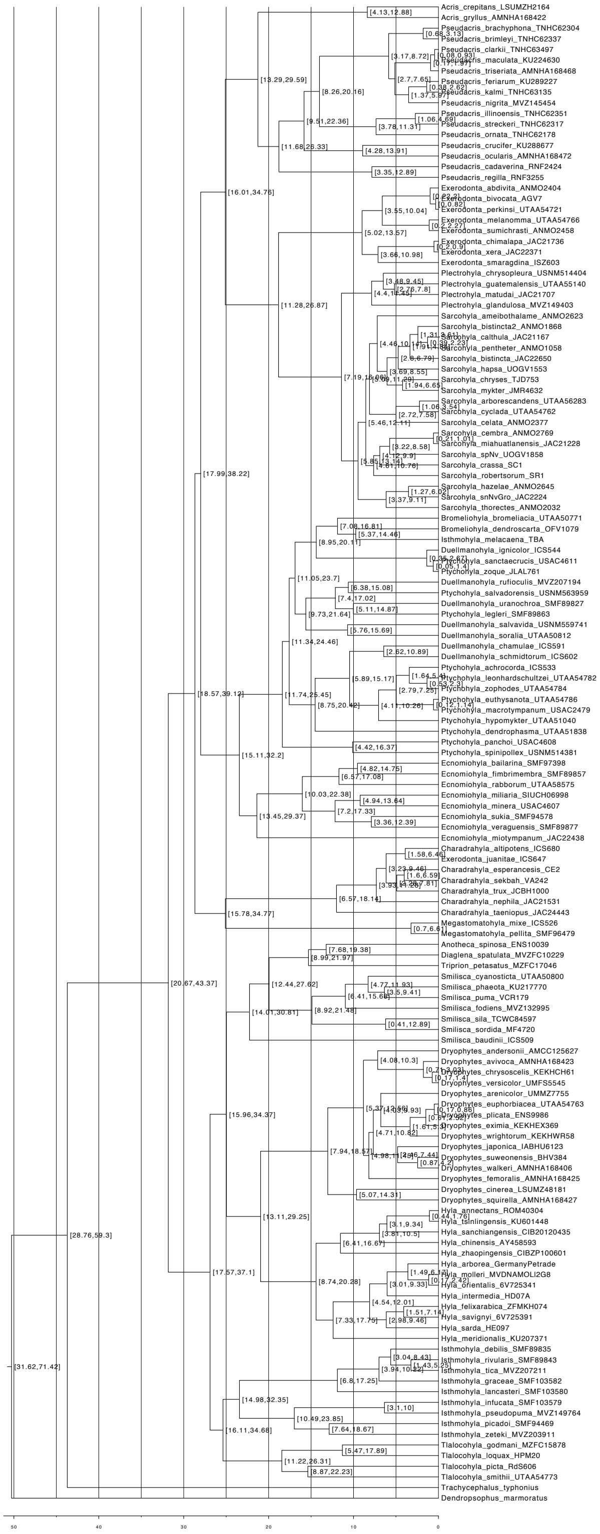
Table 2. Akaike Information Criteria (AIC) values and their corresponding weights for each model of character evolution for swimming speed.

	Brownian Motion (BM)	Ornstein-Uhlenbeck (OU)	Early-Burst (EB)
AIC	44.656	**35.224**	47.154
AIC weight	0.008	0.988	0.002

Table 3. PGLS p-values for the association between swimming speed and each of the characters for behavior, microhabitat, and morphology (femur length).

		p-value
Swimming behavior	Forelimb behavior	0.744
	Hindlimb gait	0.4299
Microhabitat	Aquatic	0.498
	Arboreal	0.6172
Morphology	Femur length	1-e-04

APPENDIX 1



Appendix A-1. Phylogenetic inferences for the 139 species of the subfamily hylinae. Node ages and corresponding HD 95%

VITA

Itzue Caviedes-Solis is a frog lover and a travel enthusiast. She was born and raised in the Mexico City. Her career is dedicated to study tree frog’s natural history and evolution using multidisciplinary approaches. She earned her bachelor and master’s degree degree at the Universidad Nacional Autónoma de México (UNAM). For her undergraduate thesis, she worked on a herpetological survey of the municipality of Pluma Hidalgo, Oaxaca. For her master’s thesis she focused on the molecular phylogeny of the *Sarcohyla* genus (Anura: Hylidae). She earned her Doctoral degree at the Leaché lab at the University of Washington. Her dissertation research focuses on the systematics and evolution of the subfamily Hylidae, composed by tree frogs from Middle America. She loves the mountains, biking, crafting and learning about nature.