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The *Phyllomedusa perinesos* group (Anura: Hylidae) is derived from a Miocene Amazonian Lineage

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Abstract

The *Phyllomedusa perinesos* group is composed of four species that inhabit cloud forests in the eastern Andean slopes. We estimated the phylogenetic relationships among them and their closest relatives using mitochondrial DNA sequences. Our results confirm the monophyly of the group and a close relationship with the Amazonian species *Phyllomedusa atelopoides* and *Phyllomedusa tomopterna*. A chronogram indicates that the group originated during the Miocene and the contemporary species diverged from their closest relatives during the Miocene and early Pliocene. The timing of the group's origin suggests that its evolution was linked to the rise of the eastern Andes. Based on the phylogeny we expand the species content of the group to include *P. atelopoides* and *P. tomopterna.*

Key words: Andes, Biogeography, Ecuador, Hylidae, Peru, Speciation

Introduction

The *Phyllomedusa perinesos* group was first defined by Cannatella (1982) to accommodate four species of leaf-frogs from cloud forests from the eastern Andean slopes from Ecuador and Peru: *P. baltea* Duellman & Toft 1979, *P. duellmani* Cannatella 1982, *P. ecuatoriana* Cannatella 1982, and *P. perinesos* Duellman 1973. The group was principally diagnosed by having purple coloration in the hands, feet, belly, flanks and concealed surfaces of the limbs (Cannatella 1982).

Two phylogenetic analyses of Phyllomedusinae each included two species of the *P. perinesos* group (*P. baltea* and *P. duellmani* in Faivovich et al. [2010] and *P. perinesos* and *P. duellmani* in Wiens et al. [2010]) and found that the two species were phylogenetically closest to each other. More recently, Pyron & Wiens (2011) re-analyzed the same sequences (originally published by Faivovich *et al.* 2010 and Wiens *et al.* 2005, 2010) and found moderate support (bootstrap = 59) for a clade composed of the three species, as the sister-group of *P. atelopoides*. Faivovich et al. (2010) and Pyron & Wiens (2011) found that the *P. perinesos* group + *P. atelopoides* are sister to *P. tomopterna*. Until know, *P. ecuatoriana* has not been included in a phylogenetic analysis and thus its evolutionary affinities have not been rigorously tested.

Herein we add new sequence data of *P. ecuatoriana* to determine its phylogenetic position and to resolve the relationships within the *P. perinesos* species group. The genetic data confirms the monophyly of the group and suggest that its origin was associated with the uplift of the Andean Eastern Cordillera.

Methodology

We estimated the phylogenetic position of *P. ecuatoriana* within the *P. perinesos* group based on new sequence

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data for 12S, 16S (partial sequence), tRNA ^{Leu}, NADH dehydrogenase subunit 1 (ND1), tRNA ^{Ile}, and tRNA ^{Gln} for a total of 2209 bp. We included sequences of the *P. perinesos* species group and the closely related *P. atelopoides* and *P. tomopterna* from GenBank. Table 1 includes the specimen data and their Genbank accession numbers. For the outgroup we added two samples of *P. rohdei,* which belongs to the sister clade of the *P. perinesos* group + *P. atelopoides* + *P. tomopterna* (Faivovich *et al*. 2010).

TABLE 1. Specimens and Genbank accession numbers used in the phylogenetic analysis.

Voucher No.	Species	12S	16S-ND1	Reference
QCAZ 23030	*Dendropsophus sarayacuensis*	KF756941	--	this publication
QCAZ 23680	*Hypsiboas pellucens*	JN970516	--	Funk et al. 2012
SAMA R53945	*Litoria inermis*	DQ283211	--	Frost *et al.* 2006
QCAZ 28646	*Osteocephalus mutabor*	HQ600641	--	Ron *et al.* 2010
KU 215381	*Phyllomedusa atelopoides*	AY819413	--	Wiens *et al.* 2005
SMNS ??	*Phyllomedusa baltea*	GQ366252	GQ366321	Faivovich *et al.* 2010
KU 212206	*Phyllomedusa duellmani*	AY819414	--	Wiens *et al.* 2005
QCAZ 47115	*Phyllomedusa ecuatoriana*	KF756940	KF756942	this publication
KU178854	*Phyllomedusa perinesos*	GQ896278	--	Wiens *et al.* 2010
CFBHT 93	*Phyllomedusa rohdei*	GQ366237	GQ366315	Faivovich *et al.* 2010
CFBH 7196	*Phyllomedusa rohdei*	GQ366238	GQ366316	Faivovich *et al.* 2010
KU 221949	*Phyllomedusa tomopterna*	AY819404	AY819535	Wiens *et al.* 2005
MJH 7076	*Phyllomedusa tomopterna*	AY843728	GQ366337	Faivovich *et al.* 2005; Faivovich *et al.* 2010
CFBH 2451	*Phyllomedusa tomopterna*	GQ366286	GQ366336	Faivovich *et al.* 2010

Preliminary sequence alignment was done with Geneious 5.4.4 software (GeneMatters Corp.) using the Geneious alignment algorithm. The matrix was imported to Mesquite (version 2.72; Maddison & Maddison 2009) and the ambiguously aligned regions were adjusted manually to produce a parsimonious alignment (i.e., informative sites minimized). Phylogenetic trees were obtained using Bayesian inference and maximum likelihood. Because our dataset includes several genes, it is unlikely that it fits a single model of nucleotide substitution. Thus, we partitioned the data to analyze each partition under a separate model chosen with JModelTest version 0.1.1 (Posada 2008) using the Akaike Information Criterion with sample size correction as optimality measure. Four partitions were defined: 12S, 16S, ND1, and combined tRNAs. To test the effect of the partition strategy on the phylogeny, we also analyzed the data under a single partition and chose between both partition strategies using Bayes factors under a threshold of 10 or higher as evidence in favor of the more complex partition (Brandley *et al.* 2005).

Each Bayesian analysis consisted of two parallel runs of the Metropolis-coupled Monte Carlo Markov chain for 2 x 10^6 generations. Each run had four chains. The prior for the rate matrix was a uniform dirichlet distribution and all topologies were equally probable a priori. Convergence into a stationary distribution was determined by reaching average standard deviation split frequencies < 0.05 between runs. We also used Tracer ver. 1.5 (Rambaut & Drummond 2007) to visually inspect convergence and stationarity of the runs and to obtain effective sample sizes (ESS) for all model parameters. The analysis was stopped when all ESS were > 100. Each run was sampled every 1000 generations. The first 50% of the samples were discarded as burn-in and the remaining were used to estimate the Bayesian tree, posterior probabilities and other model parameters. Bayesian phylogenetic analyses were carried out in MrBayes 3.2.1 (Ronquist *et al.* 2012).

The phylogeny was also estimated under maximum likelihood with a genetic algorithm using GARLI version 2.0 (Zwickl 2006). Ten stochastic likelihood searches from different starting trees were conducted to ensure recovery of the best tree. Nodal support was assessed with non-parametric bootstrapping from 1000 maximum likelihood searches. Search settings for each replicate were the same as those of the full search except for the number of stochastic searches (two instead of ten).

We estimated the time to most recent common ancestor (TMRCA) for the *P. perinesos* species group using 12S sequences with BEAST v. 1.7.1 (Drummond & Rambaut 2007). Following Wiens et al. (2011) estimate, we used as

a temporal constraint the crown-group age of the clade of Pelodryadinae + Phyllomedusinae (71.91 My; SD = 1.5). To apply the time calibration, we added outgroup sequences of *Litoria inermis*, *Osteocephalus mutabor*, *Dendropsophus sarayacuensis,* and *Hypsiboas pellucens*. Because the analysis was based on sequences from a single gene, we constrained the tree topology to mirror the Bayesian tree, which was based on a larger number of loci and thus is more likely to represent the true phylogeny.

The time calibrations of Wiens et al. (2011) are based on fossil and presumptive dates of geologic events. The use of fossils and geologic events to calibrate phylogenies could be biased by the uncertainties of fossil identifications and the difficulties to rule out non-vicariant divergence scenarios for groups inhabiting vicariant land masses (Bell *et al*. 2010; Kodandaramaiah 2011). To evaluate the reliability of the chronogram obtained from calibration of the Pelodryadinae + Phyllomedusinae node, we compared it to chronograms derived from calibrations based on two estimates of the rate of evolution of the 12S and 16S mitochondrial gene. The first calibration assumes a rate of evolution of 0.00249 substitutions per site per lineage (based on Pipidae; Evans *et al.* 2004) while the second a rate of 0.00277 (based on *Pseudacris*; Lemmon *et al*. 2007). The estimate of the second rate is partly based on calibration points used by Wiens et al. (2011) and thus is not completely independent from our node-based calibration.

Divergence times were estimated under an uncorrelated relaxed-clock tree model with a Yule process as tree prior. The analyses run between 0.5 and 1.5 billion, until the searches attained Effective Sample Sizes (ESS) > 150 for all parameters. In all searches we removed 10% of the samples as burn-in.

Results

The Bayesian analysis of 2209 sites resulted in a majority-rule consensus tree showing strong support for the *P. perinesos* group and sister relationships between the northern species (*P. perinesos* + *P. ecuatoriana*) and the southern ones (*P. baltea* + *P. duellmani*). The *P. perinesos* group + *P. atelopoides* form a strongly supported clade that is the sister-group of *P. tomopterna* (Fig. 1). The topology of the maximum likelihood tree was identical to that of the Bayesian tree.

The two estimates of TMRCAs of the *P. perinesos* group based on the rates of molecular evolution are consistently lower than the TMRCA based on the crown-group age of Pelodryadinae + Phyllomedusinae (Table 2). However, the former fall within the 95% highest posterior densities of the crown-group age estimate. Regardless of the calibration procedure, all three estimates show that the earliest divergence within the *P. perinesos* group took place in the Miocene (12.9–17.5 My) and the speciation events of the four contemporary species took place between the late Miocene and early Pliocene (7.5–10.0 My for *P. perinesos* + *P. ecuatoriana*; 4.3–6.1 My for *P. baltea* + *P. duellmani*).

TABLE 2. Times to most recent common ancestor (TMRCA; in My) and 95% posterior density interval (in parentheses) for various clades in the *P. perinesos* species group. The node calibration assumed a crown age of 71.9 My for the clade Pelodryadinae + Phyllomedusinae.

Clade	Node calibration	*Pseudacris* rate calibration	*Xenopus* rate calibration
P. perinesos group	17.5 (10.9–24.9)	12.9 (10.0–16.2)	14.4 (11.1–18.0)
P. perinesos + *P. ecuatoriana*	10.0 (4.7–15.7)	7.5 (5.0–10.2)	8.3 (5.6–11.4)
P. baltea + *P. duellmani*	6.1 (2.46–10.32)	4.3 (2.60-6.40)	4.8 (2.90–7.15)
P. perinesos group + *P. atelopoides*	24.0 (15.6–33.0)	17.8 (14.23–21.9)	19.7 (15.7–24.3)

Discussion

The phylogeny is consistent with previous analyses (Faivovich *et al.* 2010, Pyron & Wiens 2011) that used fewer species in that the *P. perinesos* species group has strong support (PP = 0.99) and is closely related to two lowland species from the Amazon Basin: *P. atelopoides* and *P. tomopterna*. Given that the *P. perinesos* species group is

distributed in the eastern Andean slopes (1000–2000 m), and that its two closest relatives inhabit the Amazonian lowlands, the origin of the group was likely associated with a single dispersal or vicariance event of a lowland ancestor. Regardless of the set of calibrations used, our estimate about times of divergence is consistent with geologic data.

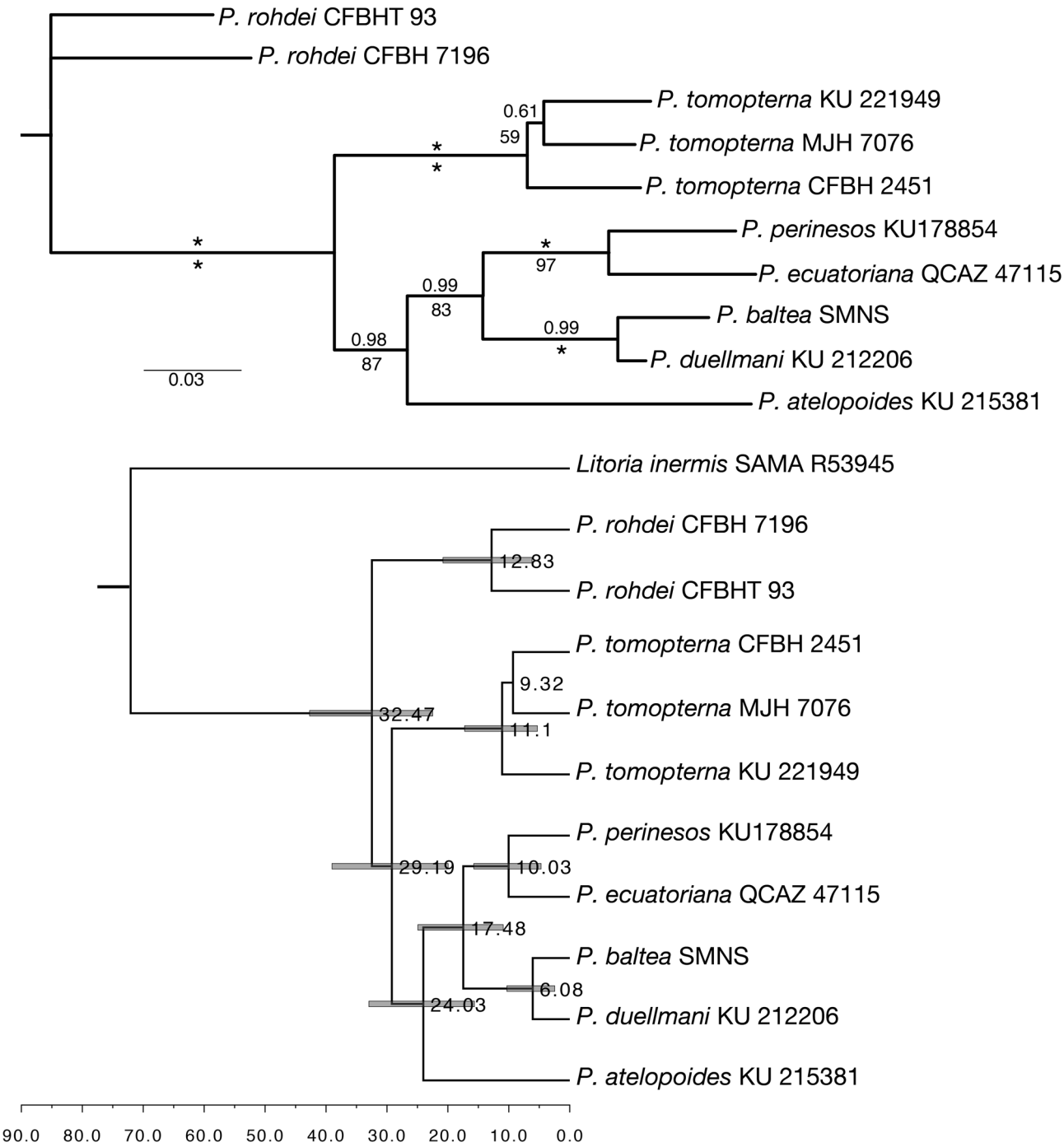

FIGURE 1. Above: Bayesian consensus phylogram depicting relationships among members of the *Phyllomedusa perinesos* species group. The phylogram was obtained from analysis of 2209 bp of mitochondrial DNA genes 12S, 16S (partial sequence), tRNA ^{Leu}, ND1, tRNA ^{Ile}, and tRNA ^{Gln}. Museum catalog no. is shown for each sample. Posterior probabilities resulting from Bayesian Markov chain Monte Carlo searches appear above branches; an asterisk represents a value of 1. Non-parametric bootstrap support values from maximum likelihood searches (1000 pseudoreplicates) appear below branches; an asterisk represents a value of 100. Below: Chronogram obtained from a relaxed-clock Bayesian analysis assuming a crown-group age for the clade Pelodryadinae + Phyllomedusinae of 71.91 My. The scale below represents time in My. Gray horizontal bars are 95% highest posterior density intervals for each divergence time. Outgroup taxa are not shown on either tree.

By the early to middle Miocene, the Eastern Andean Cordillera had elevations below 700 m and by 4 Mya its elevations were below 40% of their current values (Gregory-Wodzicki 2000). Thus, by the time when the MRCA of the *P. perinesos* group originated (i.e., when its MRCA diverged from *P. atelopoides*, 18–24 My) the montane habitats characteristic of the group did not exist. The origin of the extant species was likely associated with the uplift of the Eastern Cordillera and a switch of ecological niche to inhabit montane forests with cooler temperatures. This scenario will suggest a parapatric mode of speciation along an environmental gradient. Similar speciation scenarios have been proposed for *Osteocephalus festae* and *Osteocephalus verruciger* (Ron et al. 2012).

In his review of the *P. perinesos* group, Cannatella (1982) hypothesized that speciation of the common ancestor of the group, was associated with vicariance associated with the Huancabamba Depression, a well-known biogeographic barrier in northern Peru (Duellman 1979; Parker *et al.* 1985). Our phylogeny supports that hypothesis because it shows that species on both sides of the depression are each other's closest relatives; however, alternative speciation scenarios are also possible. The importance of the Huancabamba Depression as a barrier promoting speciation in amphibians has yet to be confirmed. In frogs of the *Osteocephalus buckleyi* species group, the Huancabamba depression does not appear to represent a significant barrier (Ron *et al.* 2012).

It is worth reconsidering the definition and content of the *P. perinesos* group. Cannatella (1982) diagnosed the group as having purple coloration in the hands, feet, belly, flanks, and concealed surfaces of the limbs, which he postulated was a synapomorphy (Cannatella 1982). However, *P. atelopoides* has similar coloration, although with a lesser intensity of purple (Duellman *et al.* 1988:92).

We also note that several species in the *P. hypochondrialis* group (e.g., *P. palliata*, *P. nordestina*), which is the sister-group of the *perinesos* group + *P. atelopoides* + *P tomopterna*. (*sensu* Faivovich et al. [2010]) have purplish color on the flanks associated with orange regions. Thus, it is likely that the purple-orange coloration in the flanks is a synapomorphy for a clade that joins the *P. perinesos* group + *P. atelopoides* + *P. tomopterna* + *P. hypochondrialis* group (*sensu* Faivovich et al. 2010).

Duellman et al. (1988) did not make any direct comparisons with the species of the *P. perinesos* group. This molecular phylogenetic analysis then, is an example in which molecular phylogenies can illuminate the interpretation of the evolution of phenotypic characters.

Our phylogeny indicates that *P. atelopoides* and *P. tomopterna* are sequential sister-species to the *perinesos* group. Neither is assigned to a species group. For this reason we expand the content of the perinesos group to include *P. tomoptera* and *P. atelopoides*. As currently defined, the group lacks known morphological synapomorphies.

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