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Research Article

Assessing the molecular phylogeny of a near extinct group of vertebrates: the Neotropical harlequin frogs (Bufonidae; *Atelopus*)

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Neotropical harlequin frogs, *Atelopus*, are a species-rich bufonid group. *Atelopus* monophyly has been suggested but intergeneric, interspecific and intraspecific relationships are poorly understood. One reason is that morphological characters of harlequin frogs are often difficult to interpret, making species delimitations difficult. Molecular analyses (DNA barcoding, phylogeny) may be helpful but sampling is hampered as most of the more than 100 *Atelopus* species have undergone severe population declines and many are possibly extinct. We processed mitochondrial DNA (12S and 16S rRNA) of 28 available ingroup samples from a large portion of the genus' geographic range (Bayesian Inference, Maximum Likelihood). Our samples constitute a monophyletic unit, which is sister to other bufonid genera studied including the Andean genus *Osornophryne.* In contrast to previous morphological studies, our results suggest that *Osornophryne* is neither sister to *Atelopus* nor nested within it. Within *Atelopus*, we note two major clades with well supported subclades, one Amazonian–Guianan Clade (*Flavescens-spumarius* Clade plus *Tricolor* Clade) and an Andean–Chocó–Central American Clade (*Varius* Clade plus all other *Atelopus*). The first mentioned includes all species that possess a middle ear (i.e. stapes) except for *A. seminiferus* lacking it (like all remaining *Atelopus*). Previously proposed species groups based on frog-like versus toad-like overall appearance (i.e. *Longirostris* and *Ignescens* Groups) or phalangeal reduction in the thumb (i.e. *Flavescens* Group) are not monophyletic in our phylogeny, thus characters used to define them are not considered synapomorphies. We show that genetic divergence can be high between species belonging to different clades, in spite of their phenetic similarity (e.g. *A. pulcher*, *Atelopus* sp. 2). On the other hand, within the same clade, colour can vary tremendously, while genetic divergence is low (e.g. *A. flavescens* and allies). These observations demonstrate that *Atelopus* taxonomy is complicated and that an integrative approach is required before 'splitting' or 'lumping' nominal species.

Key words: Amphibia, *Atelopus*, Bufonidae, DNA barcoding, *Osornophryne*, species groups, systematics

Introduction

For many years, the large, nearly cosmopolitan toad family (Bufonidae) has been in the focus of evolutionary biologists, systematists and biogeographers (e.g. Blair, 1972; Graybeal, 1997; Pramuk, 2006; Pramuk *et al.*, 2008). However, little attention has been given to one of its oldest and mega-diverse lineages, the Neotropical harlequin frogs of the genus *Atelopus*, which is of Upper Cretaceous origin (Pramuk *et al.*, 2008). La Marca *et al.* (2005) recognized 113 species from Costa Rica south to Bolivia and eastwards via the Amazon basin onto the eastern Guiana Shield (Fig. 1), with the majority of species occurring in the Andes at elevations above 1500 m above sea level. Although, they belong to the family Bufonidae (toads), many *Atelopus* species are remarkably slender and have a frog-like appearance (Figs 2–7). Other remarkable features include brilliant colours in numerous species (Figs 2–5), visual communication, stream-adapted tadpoles developing a large belly sucker and the presence of tetrodotoxin in the skin (e.g. Lötters, 1996). Trends towards reduction of phalangeal and middle ear structures do occur (Figs 8, 9). Approximately half of the members in the genus lack the terminal phalange in the thumb (Lynch, 1993) and most, except a few Amazonian–Guianan *Atelopus*

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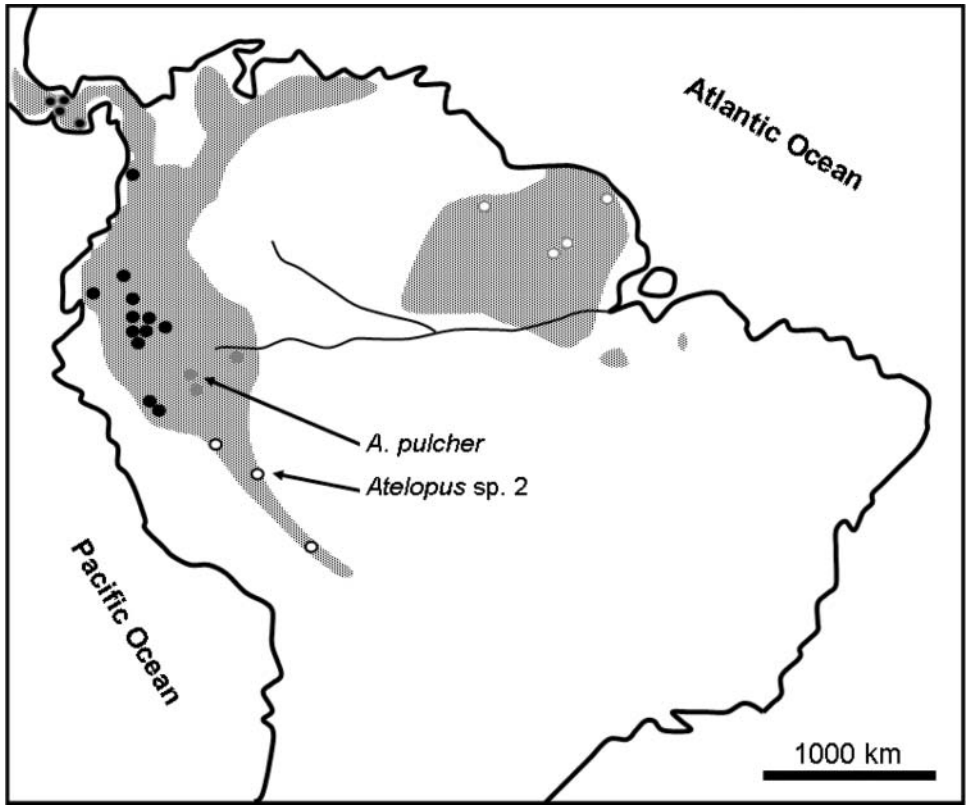


Fig. 1. Part of South and Central America with known distribution of the genus *Atelopus* (after Lötters, 1996; La Marca *et al.*, 2005; authors' unpubl. data). Dots give sample sites (see Table 1) with grey-scales indicating allocation to clades according to Fig. 10. Localities for two species are indicated by arrows (see text).

species lack stapes (e.g. McDiarmid, 1971; Coloma, 1997).

Monophyly of *Atelopus* has been suggested on the basis of morphology, myology and osteology (McDiarmid, 1971; Graybeal & Cannatella, 1995). However, Coloma (1997), using a more comprehensive data set, argued that there is no compelling evidence for the historical reality of *Atelopus*, as suggested synapomorphies are found in other bufonid genera, as well. In addition, it remains to be resolved if the small Andean genus *Osornophryne* (about 10 species) is part of or sister to *Atelopus. Osornophryne* has been suggested to be closely related to *Atelopus* (e.g. Cannatella, 1986; Frost *et al.*, 2006), while the morphological (including osteology) study by Coloma (1997) revealed that *Osornophryne* may be nested within *Atelopus*, where it was placed originally by Peracca (1904).

Linked to the question of monophyly, only few attempts have been undertaken to assess harlequin frog phylogeny below the genus level, except for the yet unpublished study by Coloma (1997). So far, most species have been assigned to few species groups among each of which morphological similarity is high except in colour (Peters, 1973; Lynch, 1993). Paraphyly versus monophyly of these groups has been controversially discussed (e.g. Lynch, 1993; Coloma, 1997). Recently, promising molecular studies, using mitochondrial DNA, aiming at *Atelopus* species phylogeny have become available (Noonan & Gaucher, 2005; Zippel *et al.*, 2006; Richards & Knowles, 2007; Guayasamin *et al.*, 2010). However, the goal of each of these studies was to enlighten relationships of particular species within a restricted region (i.e. eastern Guiana Shield, Central America, Ecuadorian Andes) so that a molecular approach addressing the entire genus with its suggested species groups is pending.

As a result of little morphological variation within the genus *Atelopus*, some authors have doubted specific distinctness of certain populations and have 'lumped' nominal taxa (e.g. Savage, 1972; Peters, 1973; Lescure & Gasc, 1986). Such a view is, in part, corroborated by the few molecular approaches. Noonan & Gaucher (2005) found that despite remarkable variation in colour, genetic diversity was low among *Atelopus* on the eastern Guiana Shield. Similarly, Zippel *et al.* (2006), Richards & Knowles (2007) and Guayasamin *et al.* (2010) showed that intraspecific colour variation can be high among Central American and Andean *Atelopus*, respectively. However, the last three mentioned studies also demonstrated that there can be remarkable phenotypic overlap between

Figs 2–7. Colour variation is high among Guianan harlequin frogs, perhaps best considered as conspecifics: (2) *Atelopus spumarius barbotini* (photo S. Lötters), (3) *A. flavescens* (photo by B. Vilette). In contrast, (4) *A. pulcher* (photo by K.-H. Jungfer) and (5) *Atelopus* sp. 2 (photo by I. De la Riva) as well as (6) *A. bomolochos* complex 'Atillo' and (7) *A. bomolochos* complex 'Cutchil' (= *A. bomolochos sensu stricto*?) (photos by L.A. Coloma) each are similar in colour but belong to different phylogenetic lineages and may be considered cryptic species (see Fig. 10). Species shown in Figs 2–5 are representatives of frog-like while those in Figs 6–7 are representatives of toad-like appearance recognized in the genus *Atelopus* (i.e. *Longirostris* versus *Ignescens* Groups of Peters, 1973).

genetically clearly distinct lineages (species). These findings have indicated that both 'splitting' and 'lumping' can not be warranted in several nominal harlequin frog species. Further studies are necessary to better understand morphological and genetic variation in harlequin frogs.

A molecular phylogenetic analysis aiming at a larger set of samples from all over the genus' geographic range would help to better understand harlequin frog systematics. However, such a goal is hampered because field sampling has become extremely difficult. Over the last 20 years, the entire genus has become nearly extinct with only few species not

Table 1. *Atelopus* samples processed in this study, their voucher specimens and GenBank accession numbers (http://www.ncbi.nlm.nih.gov) for the mitochondrial 12S and 16S rRNA genes. Abbreviations: BPN, referring to field numbers by B.P. Noonan; KU, University of Kansas, Museum of Natural History, Lawrence, USA; MHNUC, Museo de Historia Natural de la Universidad del Cauca, Popayán, Colombia; MNCN, Museo Nacional de Ciencias Naturales, Madrid, Spain; MTD, Museum für Tierkunde, Dresden; MVZ, University of California, Museum of Vertebrate Zoology, Berkeley, USA; QCAZ, Museo de Zoología, Pontificia Universidad Católica del Ecuador, Quito, Ecuador; SMNS, Staatliches Museum für Naturkunde, Stuttgart.

Species, country, locality	Voucher	GenBank accession number 12S	GenBank accession number 16S
A. bomolochos complex, Ecuador, N Zhud	KU 201039	Missing	AF375508
A. bomolochos complex, Ecuador, Atillo A	QCAZ 2910	GU301887	GU252226
A. bomolochos complex, Ecuador, Atillo B	None	Missing	AF375509
A. bomolochos complex, Ecuador, Pachancho	KU 217428	GU301897	GU252232
A. bomolochos complex, Ecuador, Ingapirca	KU 217468	GU301893	GU252231
A. bomolochos complex (sensu stricto?), Ecuador, Cutchil	KU 217443	GU301886	GU252225
A. chiriquiensis, Panama	MVZ AG28	Missing	U52780
A. flavescens, French Guiana, Lac des Americains	None	GU301888	EU672970
A. halihelos, Ecuador, WSW Plan de Milagro	KU 201040	Missing	AF375510
A. hoogmoedi, French Guiana 1, Monts Bakra	None	GU301889	EU672972
A. hoogmoedi, French Guiana 2, Saül	BPN 754	DQ283260	DQ283260
A. hoogmoedi, Guayana, Mabura Hill region	SMNS 11970:1	Missing	EU672974
A. longirostris, Ecuador, Mindo region	KU 202268	Missing	AF375511
A. nanay, Ecuador, Las Tres Cruces	KU 217474	GU301891	GU252228
A. oxapampae, Peru, Oxapampa region	MTD 1276	GU301898	EU672979
A. peruensis, Peru, Abra Comulica	KU 211650	Missing	GU252230
A. peruensis, Peru, NNW Cajamarca	KU 211631	Missing	GU252229
A. pulcher, Peru, Tarapoto region	KU 211678	GU301895	EU672973
A. seminiferus, Peru, Alto Mayo	None	GU301896	EU672976
A. spumarius barbotini, French Guiana, Saül region	None	GU301892	EU672971
A. spumarius, Peru, Río Tahuayao	None	GU301894	EU672977
A. spurrelli, Colombia, Bahia Solano	MHNUC 273	Missing	EU672975
A. tricolor, Bolivia, Yungas de La Paz	MNCN 5885	GU301900	EU672978
A. varius, Costa Rica, near Las Alturas	None	Missing	AY325996
A. varius, Panama	MVZ AG29	Missing	U52779
A. zeteki, Panama, Las Filipinas	None	Missing	DQ283252
Atelopus sp. 1, Ecuador, Río Tililag	KU 217465	GU301890	GU252227
Atelopus sp. 2, Peru, near Puente Fortaleza	MNCN 5554	GU301899	EU672980

categorized as Critically Endangered when applying IUCN Red List criteria. Many of the known *Atelopus* species, despite increased efforts to trace them, have not been found in their habitat for years and may have become extinct (La Marca *et al.*, 2005; Lötters *et al.* 2005*b*; Lötters, 2007).

We have been able to collect 28 *Atelopus* samples. Although this number is only about one quarter of the number of known taxa, our samples belong to species in previously suggested groups and cover a large portion of the geographic range of the genus (Fig. 1). We used them to reconstruct a phylogeny using mitochondrial DNA. Our purposes are (1) to study if *Osornophryne* clusters within *Atelopus* or as an outgroup; (2) to test the monophyly of proposed species groups and see if evolutionary trends in skeletal morphology (i.e. thumb and middle ear reduction) can be linked to these groups; (3) to further study if phenotypic overlap among distinct lineages does occur (i.e. suggesting 'splitting' into cryptic species) or if high morphological similarity within the genus can be more generally linked to low genetic diversity, thus making 'lumping' of nominal species more warranted.

Materials and methods

Sampling

DNA was extracted from tissue samples (toe, muscle) preserved in 99% ethanol using a DNeasy® blood and tissue kit (Qiagen). Primers for fragments of the 12S rRNA and the 16S rRNA genes were 12SA-L and 12SB-H and 16SA-L and 16SB-H (used as in Palumbi *et al.*, 1991). For several taxa, PCR amplifications repeatedly failed for one of the genes, possibly due to poor preservation of the samples. PCR was performed in 25 μl reactions using REDTaq Polymerase Readymix (Sigma). PCR products were purified via spin columns (Qiagen). Sequencing was performed directly using the corresponding PCR primers on an ABI3730XL sequencer.

New sequences obtained in the described way were deposited in GenBank (Benson *et al.*, 2004) and combined with existing ones available from this source in the final data set. For a complete list of *Atelopus* species, samples, their vouchers and GenBank accession numbers see Table 1. We included, as outgroups, samples of the

Bufonidae genera (GenBank accession number 12S, 16S): *Dendrophryniscus brevipollicatus* (AF375490, AF375515), *Melanophryniscus stelzneri* (GU301901, GU252233), *Oreophrynella* sp. (GU301902, GU252234), *Osornophryne antisana* (AF375496, EU672983), *O. puruanta* (missing, EU672982), *Osornophryne* sp. 1 (GU301903, EU672984), *Osornophryne* sp. 2 (missing, EU672981), *Peltophryne lemur* (DQ283273, DQ283273), *Rhamphophryne rostrata* (AF375533, AF375506), *Rhinella marina* (AY028485, DQ283062).

Phylogenetic analysis

Chromatograms were checked by eye using FinchTV 1.4 (http://www.geospiza.com) and the sequences were subsequently aligned using the Muscle alignment program, version 3.6 (Edgar, 2004) using the default settings (best accuracy). The resulting alignments were checked by eye and were not found to require additional editing. Missing genes were coded as 'missing' in the concatenated data set (see Table 1).

A homogeneity partition test (Farris *et al.*, 1994), as implemented in PAUP*, version 4.0b10 (Swofford, 2002), did not reject homogeneity of the two markers ($P = 0.35$). Besides an analysis of the combined data set we also performed separate analyses for each gene. Phylogeny reconstruction based on the separate and combined data sets was performed using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. The best fitting models of sequence evolution were determined by the Akaike Information Criterion in Modeltest 3.7 (Posada & Crandall, 1998). ML tree searches were performed using PhyML, version 2.4.4 (Guindon & Gascuel, 2003). Bootstrap branch support values were calculated with 500 replicates. The BI analysis was conducted with MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001), using models estimated with Modeltest under the AIC criterion, with 2 500 000 generations, sampling trees every 100th generation (and calculating a consensus tree after omitting the first 6250 trees). Log likelihood scores for the remaining trees were graphed in Tracer 1.4 (http://beast.bio.ed.ac.uk/Tracer) and checked for appropriateness of the burn-in-period.

Skeletal characters

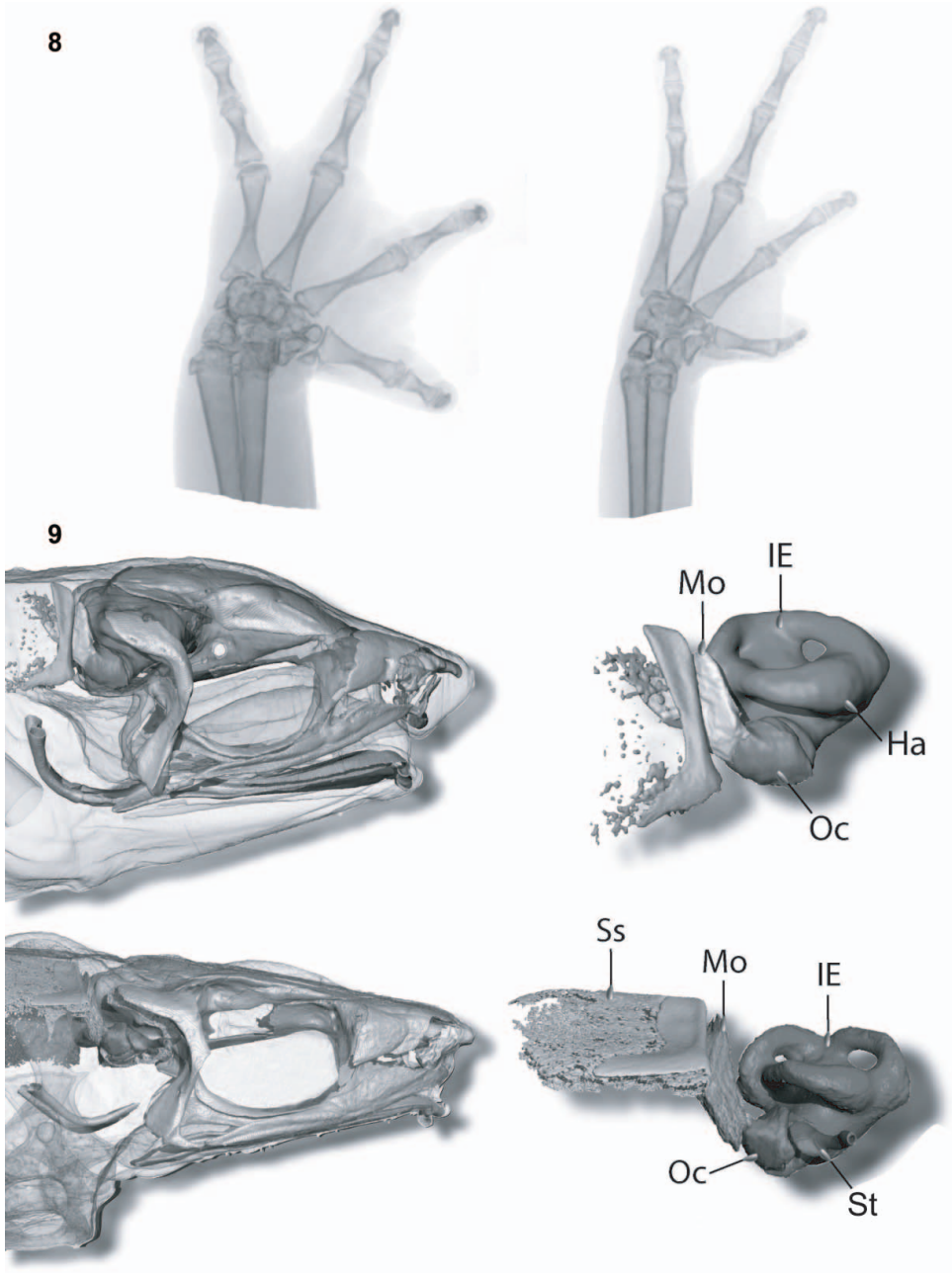
Information on phalangeal reduction in the thumb and presence or absence of stapes was obtained from published references (see below). Of those species in which middle ear information was lacking, adults of *Atelopus pulcher* and *A. seminiferus* were available to us (Figs 8, 9). Conditions were studied in the manner described by Guigay *et al.* (2007) and Van der Meijden *et al.* (2007) with X-ray synchrotron propagation phase contrast microtomography and holotomography at the ID19 beam-line of the European Synchrotron Radiation Facility (ESRF, Grenoble). For this purpose, a specimen was placed in a small polypropylene tube for imaging. For *A. pulcher* we acquired tomographic data in phase contrast mode (energy: 35 Kev, propagation distance: 300 mm, voxel size: 8.06 μm). Holotomographic images of *A. seminiferus* were taken at a pixel size of 4.91 and 8.06 μm at three sample-detector distances (40/500/900 mm at 35 KeV and 25/300/995 mm at 35 Kev). Fifteen, twelve, ten and nine hundred radiographic images with a size of 2048 $\times$ 2048 and 1024 $\times$ 1024 pixels were acquired respectively using a FReLoN CCD camera at different angles ranging 0–180 degrees. Dark current and reference images without sample were recorded to perform flat field corrections on the projections. Phase retrieval was performed using the mixed approach. After phase retrieval, tomographic reconstruction was performed using a 3D version of the filtered back projection algorithm to reconstruct the 3D refractive index distribution. From this the 3D skull structure and soft tissue details were extracted. Three-dimensional renderings were obtained after semi-automatic segmentation of the skeleton, using Avizo 6.1 (Mercury Computer Systems, Chelmsford, MA, USA).

Results

In our ML and BI reconstructions, all *Atelopus* constitute a well supported monophyletic group with all *Osornophryne* representing a related monophyletic clade which is not sister to *Atelopus* (Fig. 10).

Within *Atelopus*, our analysis resolved two major clades, each exhibiting moderate to high support in both ML and BI approaches (Fig. 10). One exclusively incorporates all samples available from Amazonia and the eastern Guiana Shield (see Fig. 1), hereafter termed Amazonian–Guianan Clade. All species included have a frog-like appearance (cf. Figs 2–7) and show phalangeal reduction in the thumb as illustrated in Figure 8 (information taken from McDiarmid, 1971, 1973; Lynch, 1993; Coloma, 1997; authors' unpubl. data). The second major clade contains all Andean (i.e. samples from higher than 2000 m above sea level) *Atelopus* and those from west of the Andes including the Chocó Region and Central America (see Fig. 1), hereafter termed Andean-Chocó-Central American Clade. As shown in Fig. 10, Central American *Atelopus* plus *A. spurrelli* from the adjacent South American Chocó region of Colombia constitute in both ML and BI reconstructions a distinct, well supported lineage within this arrangement (*Varius* Clade). Species in the Andean–Chocó–Central American Clade all lack a middle ear (information taken from McDiarmid, 1971; Coloma, 1997; authors' unpubl. data). They have both frog- and toad-like appearances (cf. Figs 2–7). Phalangeal reduction in the thumb is found throughout the entire clade but not in all taxa (information taken from McDiarmid, 1971; Lynch, 1993; Coloma, 1997; authors' unpubl. data).

The Amazonian–Guianan Clade can be divided into two subclades, each supported by high ML and BI values. One



Figs 8, 9. (8) Phalangeal characteristic without (left) and with reduction in the thumb: *Atelopus pastuso* (QCAZ 15013, Carchi province, Ecuador) and *A. spumarius* (QCAZ 32316, Pastaza province, Ecuador), each the ventral aspect of right hand (not to scale). (9) Absence in *A. seminiferus* (SL, unnumbered) versus presence in *A. pulcher* (SL, unnumbered) of middle ear (stapes = St) in harlequin frogs: volume rendering of lateral view of skull with the skin transparent with position of the inner and middle ear in situ (left) and 3D visualization of lateral view of the inner and middle ear (opercular muscle = Mo, horizontal ampulla = Ha, inner ear = IE, opercular cartilage = Oc, suprascapula = Ss).

unites species from southern pre-Andean Peru and adjacent Bolivia (*Tricolor* Clade) and the other one contains upper Amazonian and Guianan *Atelopus* (*Flavescens-spumarius* Clade). Within the last mentioned, all Guianan harlequin frogs constitute a monophyletic group (Guianan Clade in Fig. 10). Further, the *Flavescens-spumarius* Clade contains all the *Atelopus* species, including *A. pulcher* (Fig. 9), in which a middle ear is present (information taken from McDiarmid, 1971, 1973; Coloma, 1997). However, this is not a character present throughout the entire clade, since *A. seminiferus*, which is well nested within it (cf. Fig. 10), lacks stapes (Fig. 9). As far as is known, the members of closely

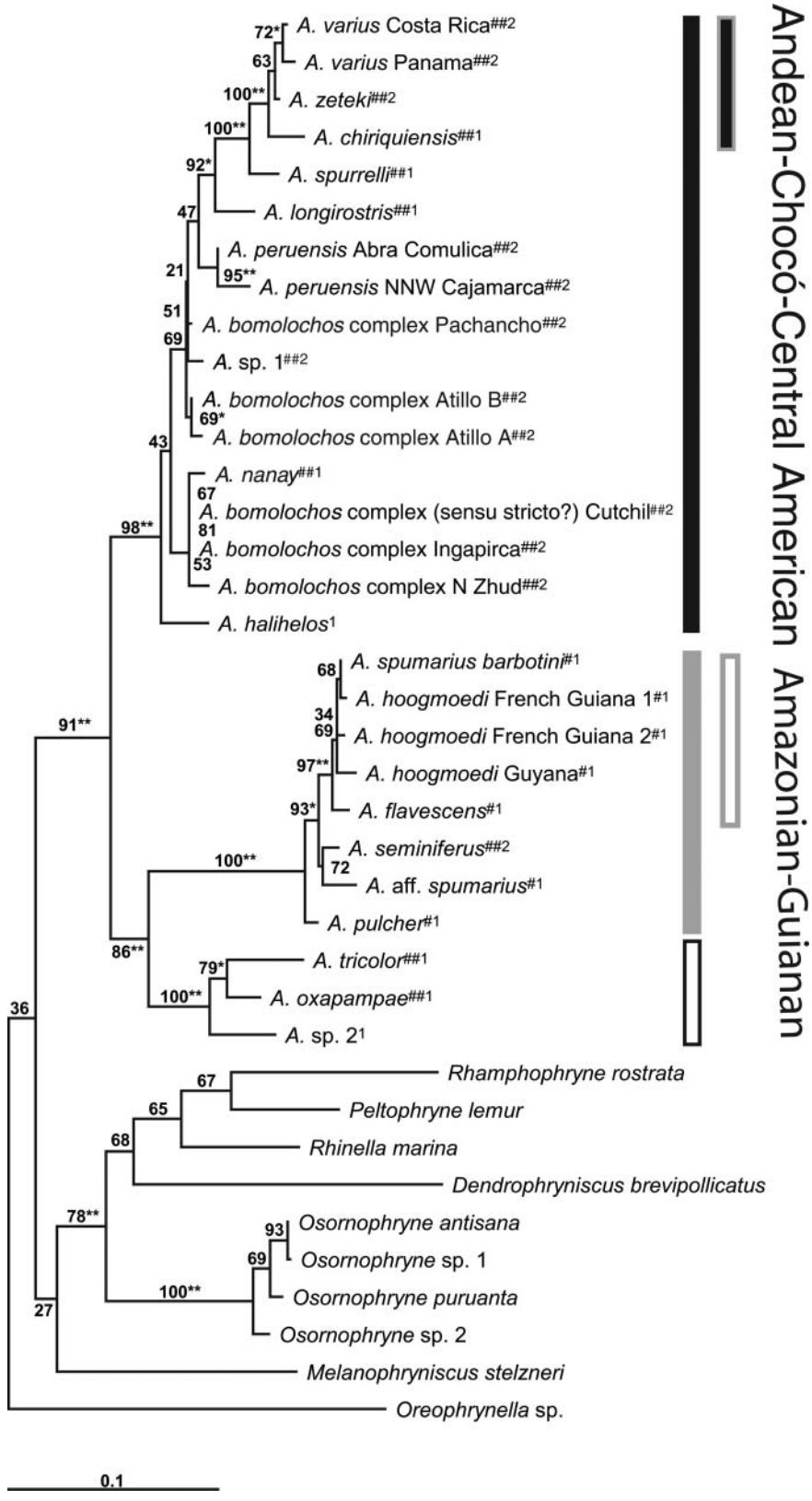


Fig. 10. Maximum Likelihood (ML) tree of *Atelopus* species and additional Neotropical bufonid genera with species clades referred to in this paper indicated by text and grey-scale bars (Andean-Chocó-Central American Clade including *Varius* Clade = black with grey frame; Amazonian-Guianan Clade with *Flavescens-spumarius* Clade = grey; *Tricolor* Clade = white with black frame; Guianan Clade = white with grey frame). ML bootstrap values are given, branches with 100% Bayesian support have ** and those with > 95% have *; middle ear present = #, absent = ##; phalangeal reduction present = [1], absent = [2].

related *Tricolor* Clade, like all other *Atelopus*, also lack a middle ear (McDiarmid, 1971; Coloma, 1997; SL unpubl. data).

Regarding relationships within the Amazonian–Guianan Clade, it can be noted that nominal species in the Guianan Clade, remarkably different in colour (e.g. Figs 2–3), show rather limited genetic diversity in the mitochondrial 12S and 16S rRNA genes, especially when compared with other species in the genus (Table 2). In contrast, more similarly coloured species, like *A. spumarius*, *A. pulcher* and *Atelopus* sp. 2 (Figs 4–5), show higher divergence (Table 2), are clearly paraphyletic and differ in middle ear condition (Fig. 10). Within the Andean–Chocó–Central American Clade, ML and BI support is limited for the position of many samples (Fig. 10). So far, it can be observed that both similarly coloured (Figs 6–7) and different looking populations under the name *A. bomolochos* complex are paraphyletic; divergence between them is limited when compared with congenerics (Table 2).

Discussion

Osornophryne-Atelopus relationship

The position of *Osornophryne* in our gene tree suggests that neither it is an ingroup (Coloma, 1997), nor the sister group to *Atelopus* (Cannatella, 1986). According to our results, *Osornophryne* is not even closely related to *Atelopus*, but the sister taxon of a clade including other Neotropical bufonids, here represented by *Dendrophryniscus*, *Peltophryne*, *Rhamphophryne* and *Rhinella*. The resulting conflict between morphology including osteology (Cannatella, 1986; Coloma, 1997; Frost *et al.*, 2006) versus molecular data requires further studies, as it may reflect apparent homoplasy in the form of parallel evolution, convergence or plesiomorphic conditions. However, it has to be noted that at the current stage, we are far away from a comprehensive understanding of Neotropical Bufonidae, especially as molecular data for critical genera and species are not available, e.g. *Andinophryne*, *Frostius*, *Truebella*, *Atelopus mucubajiensis* or *A. pinangoi* (e.g. Frost *et al.*, 2006; Pramuk *et al.*, 2008); the two latter are hypothetically closely related to *Osornophryne* (Coloma, 1997).

Intra-generic grouping

Peters (1973) defined the *Longirostris* and *Ignescens* Groups for *Atelopus* with frog- and toad-like appearance, respectively (cf. Figs 2–7). This author already claimed that the groups were phenetic and represented ecologically functional rather than monophyletic units. In concordance with this, none of the two groups is mirrored by our phylogeny, as, for instance, *A. longirostris* itself is member of the Andean–Chocó–Central American Clade like *A. bomolochos* and *A. halihelos*, which Peters (1973) placed with his *Ignescens* Group.

A hypothetically monophyletic *Flavescens* Group that included 24 species was defined by Lynch (1993), who proposed that phalangeal reduction in the thumb (Fig. 8) was a synapomorphy. In contrast, our study indicates that this character has likely evolved independently in different *Atelopus* lineages (Fig. 10). Rejection of phylogenetic signals in the proposed species groups mentioned is in concert with Coloma (1997) who studied *Atelopus* morphology and osteology.

McDiarmid (1973) proposed that the *Atelopus* species with a middle ear (i.e. stapes) were a natural group that retained the 'internal tympani', which was considered as a primitive condition (McDiarmid, 1971). Our results show that all species known to possess a middle ear constitute a monophyletic lineage (*Flavescens-spumarius* Clade) with one species part of this assemblage lacking this character, *A. seminiferus* (Fig. 10). The *Flavescens-spumarius* Clade is a derived group of *Atelopus* in our analysis and it remains to be studied if the middle ear disappeared in an ancestral *Atelopus* but was secondary gained by species in this clade, except *A. seminiferus.* Alternatively, multiple middle ear loss may have occurred, at least three times, i.e. in all *Atelopus* outside the Amazonian–Guianan Clade, in *A. seminiferus* and in the *Tricolor* Clade (Fig. 10). The four Amazonian species possessing stapes examined by Coloma (1997), based mainly on external morphological and skeletal characters, formed a monophyletic unit including the earless *A. spurrelli* from west of the Andes. In contrast, *A. spurrelli* in our molecular study is well nested within the Andean–Chocó–Central American Clade (Fig. 10). This conflict between the morphological versus molecular data sets remains to be assessed with additional data.

'Splitting' versus 'lumping'

Noonan & Gaucher (2005) demonstrated that Guianan *Atelopus* exhibit little mitochondrial gene variation (cyt*b*, NADH2) and that even introgression may have occurred among the nominal species. Their findings can be well explained with the predictions of the disturbance-vicariance hypothesis in which speciation remains incomplete due to repeated contact of developing lineages (Noonan & Gaucher, 2005; Lötters *et al.*, 2010). It was not the purpose of these authors to draw taxonomic conclusions, but their data suggest that the available names *A. flavescens*, *A. franciscus*, *A. spumarius barbotini* and *A. vermiculatus* may best be referable to a single polymorphic taxon, *A. flavescens*. In life, it can be ventrally pink to purple and dorsally brown, orange or yellow with or without purple or brownish pattern (Figs 2–3; Boistel *et al.*, 2005*b*). The Guianan *A. hoogmoedi*, in life ventrally cream to yellow and dorsally brown to black with orange to yellow pattern (Lötters *et al.*, 2005*a*), may either represent a second Guianan taxon (apparently ranging into the adjacent

Table 2. Uncorrected p-distances for the mitochondrial 16S rRNA gene data set of 28 *Atelopus* samples. For GenBank accession numbers and vouchers see Table 1.

	Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	*A. bomolochos* complex Cutchil																											
2	*A. bomolochos* complex Atillo A	0.03																										
3	*A. flavescens*	0.107	0.112																									
4	*A. hoogmoedi* French Guiana 1	0.119	0.117	0.01																								
5	*A. hoogmoedi* Guayana	0.103	0.123	0.02	0.01																							
6	*A. bomolochos* complex N Zhud	0.01	0.03	0.084	0.092	0.09																						
7	*Atelopus* sp. 1	0.03	0.02	0.112	0.121	0.113	0.03																					
8	*A. chiriquiensis*	0.063	0.06	0.143	0.159	0.146	0.061	0.055																				
9	*A. bomolochos* complex Atillo B	0.02	0	0.09	0.099	0.097	0.02	0.01	0.052																			
10	*A. halihelos*	0.03	0.04	0.093	0.099	0.092	0.02	0.04	0.072	0.04																		
11	*A. longirostris*	0.03	0.04	0.102	0.114	0.102	0.04	0.04	0.056	0.03	0.05																	
12	*A. nanay*	0.01	0.04	0.108	0.117	0.113	0.02	0.03	0.067	0.02	0.04	0.04																
13	*A. peruensis* NNW Cajamarca	0.046	0.03	0.122	0.118	0.126	0.04	0.03	0.068	0.02	0.052	0.052	0.04															
14	*A. peruensis* Abra Comulica	0.03	0.03	0.107	0.118	0.109	0.03	0.02	0.059	0.02	0.04	0.03	0.04	0.02														
15	*A. spumarius barbotini*	0.102	0.114	0	0	0.01	0.086	0.107	0.139	0.092	0.09	0.098	0.108	0.109	0.104													
16	*A. hoogmoedi* French Guiana 2	0.095	0.113	0.01	0	0.01	0.081	0.104	0.136	0.088	0.084	0.094	0.105	0.116	0.099	0												
17	*A. spurrelli*	0.049	0.055	0.126	0.142	0.124	0.05	0.05	0.04	0.04	0.052	0.04	0.057	0.061	0.044	0.122	0.114											
18	*A. varius* Panama	0.059	0.067	0.135	0.153	0.135	0.057	0.059	0.02	0.056	0.066	0.051	0.069	0.073	0.055	0.131	0.125	0.04										
19	*A. varius* Costa Rica	0.057	0.066	0.137	0.157	0.132	0.057	0.055	0.02	0.054	0.066	0.05	0.07	0.071	0.052	0.135	0.125	0.03	0.01									
20	*A. zeteki*	0.05	0.055	0.121	0.137	0.12	0.05	0.05	0.02	0.05	0.06	0.04	0.059	0.061	0.044	0.118	0.11	0.03	0.01	0.01								
21	*A. bomolochos* complex Ingapirca	0	0.03	0.099	0.112	0.099	0.01	0.024	0.063	0.02	0.03	0.03	0.01	0.04	0.03	0.096	0.092	0.05	0.06	0.058	0.05							
22	*A. spumarius*	0.099	0.104	0.05	0.03	0.05	0.078	0.105	0.137	0.084	0.094	0.102	0.096	0.11	0.103	0.03	0.04	0.116	0.132	0.13	0.116	0.095						
23	*A. pulcher*	0.09	0.106	0.03	0.02	0.04	0.08	0.096	0.126	0.083	0.081	0.094	0.102	0.116	0.1	0.03	0.03	0.112	0.12	0.12	0.108	0.088	0.04					
24	*A. seminiferus*	0.097	0.107	0.03	0.03	0.03	0.08	0.099	0.131	0.085	0.086	0.095	0.103	0.106	0.1	0.02	0.02	0.116	0.126	0.126	0.11	0.093	0.03	0.03				
25	*A. bomolochos* Pachancho	0.02	0.01	0.101	0.115	0.108	0.02	0.01	0.051	0	0.04	0.03	0.03	0.03	0.02	0.1	0.098	0.04	0.053	0.051	0.04	0.02	0.099	0.091	0.093			
26	*A. oxapampae*	0.086	0.103	0.11	0.12	0.112	0.086	0.097	0.12	0.095	0.09	0.088	0.099	0.113	0.097	0.106	0.106	0.106	0.114	0.112	0.1	0.084	0.115	0.099	0.107	0.089		
27	*Atelopus* sp. 2	0.094	0.103	0.113	0.12	0.114	0.082	0.098	0.112	0.086	0.081	0.084	0.105	0.115	0.101	0.11	0.108	0.097	0.107	0.104	0.093	0.09	0.126	0.099	0.107	0.089	0.058	
28	*A. tricolor*	0.105	0.11	0.115	0.126	0.122	0.095	0.108	0.125	0.101	0.103	0.099	0.109	0.114	0.111	0.117	0.12	0.121	0.129	0.128	0.113	0.103	0.113	0.111	0.116	0.103	0.055	0.065

Amazon basin; e.g. Lescure, 1981) or may also be part of the polymorphic *A. flavescens*.

Our analysis, along with Noonan & Gaucher (2005) and Lötters *et al.* (2010), reveals that Guianan harlequin frogs are a monophylum (Guianan Clade in Fig. 9) displaying limited genetic diversity in the mitochondrial 12S and 16S rRNA genes (Table 2). In anuran amphibians, the mitochondrial 16S rRNA gene, because of its highly conserved priming sites, has been suggested as a standard marker for species delimitation via a DNA barcoding approach (e.g. Vences *et al.*, 2005). Divergence of 3% has been suggested an operable threshold for the consideration of possible species, to be confirmed by an integrative approach (Fouquet *et al.*, 2007; Vieites *et al.*, 2009). Divergence among the Guianan harlequin frogs included in this study in the uncorrected p-distances in the mitochondrial 16S rRNA gene at maximum is 2% (Table 2). This low genetic variation supports a view in which previous workers have taxonomically 'oversplit' Guianan *Atelopus* and that high colour polymorphism is the result of within-species variation. The integration of aspects of life history and tadpole morphology also suggest that Guianan harlequin frogs rather are conspecifics (Boistel *et al.*, 2005*a*; Luger *et al.*, 2009; senior author's unpubl. data). We here refrain from any formal taxonomic action, because the available information so far relies on mitochondrial gene trees only. We suggest that, for instance, nuclear markers may be useful for more sound taxonomic conclusions.

Populations previously described as *A. spumarius barbotini* and *A. spumarius hoogmoedi* (here referred to as *A. hoogmoedi* following Lötters *et al.*, 2005*a*) were treated as conspecific with the western Amazonian *A. andinus*, *A. pulcher* and *A. spumarius* by Lescure (1981) and Lescure & Gasc (1986) based on similarity in colour. In life, these harlequin frogs are ventrally cream or yellow to red and dorsally brown to black with yellow to green pattern (Figs 4–5). Genetic information for *A. andinus* is not available, so that this name cannot be discussed here. We do not consider *A. pulcher* and *A. spumarius* as conspecifics (divergence 4%; Table 2), because in the phylogeny presented in Fig. 10, *A. pulcher* is sister to all other samples in the Amazonian–Guianan Clade including *A. spumarius*. It may be considered that paraphyly in mitochondrial gene trees may result from an underestimation of the breadth of species limits because occasionally allele variation can be higher within than between species (Funk & Omland, 2003). However, this may not apply here, as treating these *A. pulcher* and *A. spumarius* as separate taxa is supported by differences in adult morphology and vocalizations (Cocroft *et al.*, 1990; Lötters *et al.*, 2002). Based on the position in our phylogeny (Fig. 10), we also reject conspecifity of *A. spumarius barbotini* or *A. hoogmoedi* with *A. pulcher*, although divergence is $\geq$ 2% only (Table 2). *Atelopus pulcher* is well distinguished from *A. spumarius barbotini* and *A. hoogmoedi* on the basis of vocalizations and tadpole morphology (Lescure, 1981; Cocroft *et al.*, 1990; Lötters *et al.*, 2002; Boistel *et al.*, 2005*a*). We cannot rule out that *A. spumarius* is conspecific with *A. spumarius barbotini* or *A. hoogmoedi* (divergence $\geq$ 3%; Table 2) as data for a more integrative approach are lacking. However, it may be noted that in the 16S mitochondrial rRNA gene divergence of confirmed species can even be lower that the rule-of-thumb 3%-threshold (e.g. Schick *et al.*, 2010; Zimkus & Schick, 2010).

In summary, we note that in the Amazonian–Guianan Clade: (1) colour is remarkably variable within the Guianan Clade of *Atelopus* which exhibits low genetic diversity (i.e. suggesting 'lumping' of nominal species); (2) genetically more distant *Atelopus* show high phenotypic overlap (i.e. suggesting 'splitting' into cryptic species). The second observation is further corroborated by *Atelopus* sp. 2 (Fig. 5). It is morphologically similar to *A. pulcher* (Fig. 4), but lacks a tympanum and is nested in another, well supported subclade (Fig. 10); divergence is 9.9% (Table 2).

Both high colour variation within species as well as low such variation between different phylogenetic lineages does also occur in the Andean–Chocó–Central American Clade, i.e. the Panamanian *A. zeteki* and allies (Richards & Knowles, 2007) and the Ecuadorian high-Andean harlequin frogs related to *A. bomolochos* and *A. ignescens*, also known as 'jambatos' (Guayasamin *et al.*, 2010). Our sampling for Mesoamerican harlequin frogs is limited and they are not discussed here. With regard to the *Atelopus* from the Andes of southern Ecuador, Peters (1973) and Gray (1983) proposed *A. bomolochos* to represent a wide-spread polymorphic harlequin frog, justified by external morphology including colour. This concept was disputed by Coloma (2002) and Coloma *et al.* (2000, 2007) through the redescription of *A. exiguus* and the descriptions of *A. nanay* and *A. onorei* from Andean Ecuador after comprehensive analyses of adult external morphology, osteology and tadpoles. Despite this, *A. bomolochos* has been tentatively considered a highly variable taxon with some populations phenotypically more similar to other species than to conspecifics (Coloma *et al.*, 2000, 2007; Coloma, 2002). Our phylogeny suggests that intraspecific variation of *A. bomolochos* may not be as high as expected, since samples of distinctly coloured populations allocated to this nominal species are paraphyletic in our gene tree (Fig. 10). On the other hand, similar looking populations in this apparent species complex may represent distinct taxa, like the Atillo and Cutchil populations which are dorsally dull green in life (Figs 6–7). Although there is limited support for the positions of these populations in our gene tree, it may be noted that in the molecular study by Guayasamin *et al.* (2010), performed with mitochondrial DNA (16S, tRNA-Leu, ND1, tRNA-Ile), likewise *A. bomolochos*, including the Atillo and Cutchil (= *A. bomolochos* sensu stricto?) populations, was shown to be

paraphyletic. When comparing uncorrected p-distances in the mitochondrial 16S rRNA gene of these two populations, they differ at 3.2% (Table 2), which may support the existence of cryptic species diversity (see Fouquet *et al.*, 2007; Vieites *et al.*, 2009). However, an integrative approach is pending due to the lack of data other than molecular. Also, as mentioned above, paraphyly in mitochondrial gene trees may occur when variation of some alleles is higher within than between species. Other possibilities are incomplete lineage sorting, introgression and hybrid speciation (Funk & Omland, 2003). These aspects have been little studied in the genus *Atelopus* but may play a role (e.g. Coloma *et al.*, 2000, 2010). On the other hand, McKay & Zink (2010) recently uncovered that paraphyly, at least in birds, is more often the result of incorrect taxonomy and concluded that mitochondrial gene trees are rarely misleading with regard to species delimitation.

Our findings point out the difficulty of defining *Atelopus* species and emphasize that a simple explanation of 'splitting' versus 'lumping' is inadequate. They also raise questions regarding mechanisms leading to high intraspecific and low interspecific colour variation. Within-species colour variation is also high in the aposematic dendrobatid frogs from the Neotropics, e.g. *Dendrobates tinctorius* from the same general area as *A. flavescens* and relatives (Noonan & Gaucher, 2005, 2006). It has been demonstrated that polymorphism in *D. tinctorius* is phylogenetically relatively uninformative (Wollenberg *et al.*, 2008). Instead, it has been suggested that visual (avian) predators possess an inherent avoidance of brightly coloured diurnal anurans evoking a strong selective pressure to local colours. In field experiments with painted clay frogs brightly coloured, novel forms were more likely to suffer an attack (Noonan & Comeault, 2009). Like dendrobatids, *Atelopus* species are diurnal and often colourful and all species studied so far contain potent skin toxins (Lötters, 1996). It may be worth studying if mechanisms of polymorphism as in *Dendrobates* may be involved in *Atelopus.*

There are other examples from the Dendrobatidae as well as aposematic diurnal Malagasy poison frogs (Mantellidae), in which species from different clades exhibit strikingly similar colours. It has been hypothesized that this is the result of Müllerian mimicry, as similar looking species occur syntopically (Symula *et al.*, 2001; Schaefer *et al.*, 2002). Although syntopic occurrence in *Atelopus* species is rare and their geographic ranges often are remarkably limited (Lötters, 1996), Müllerian mimicry may help to explain similarity in *Atelopus* taxa which occur in relatively close geographic proximity, like the Mesoamerican *A. varius* and *A. zeteki* (Richards & Knowles, 2007) or *A. bomolochos* and similar species (Coloma *et al.*, 2000, 2007; Coloma, 2002). However, Müllerian mimicry is less plausible in the case of *A. pulcher* and *Atelopus* sp. 2 which occur in entirely different drainage systems almost 1000 km by air apart from each other (Fig. 1).

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