Available online at www.sciencedirect.com
ScienceDirect
Molecular Phylogenetics and Evolution xxx (2007) xxx–xxx
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Short Communication

Mitochondrial paraphyly in a polymorphic poison frog species (Dendrobatidae; *D. pumilio*)

Sabine Hagemann *, Heike Pröhl

Institut für Zoologie, Tierärztliche Hochschule Hannover, Bünteweg 17, D-30559 Hannover, Germany

Received 24 January 2007; revised 6 June 2007; accepted 11 June 2007

1. Introduction

Poison frogs (Dendrobatidae) have been the subject of scientific interest for a long time due to their bright colouration, astonishing phenotypic variability and complex breeding behaviour (Daly and Myers, 1967; Savage, 1968; Myers et al., 1983). In the past, species were diagnosed and their relationships evaluated mainly using morphological characteristics. However, the high phenotypic variation within some dendrobatid species as well as the occurrence of mimetic radiation (Symula et al., 2001; Darst and Cummings, 2006) complicated morphological diagnosis of species. Molecular markers offer an independent means to clarify species diagnoses and phylogenetic relationships in this group.

Molecular phylogenetic trees of dendrobatids frequently show discrepancies from the actual systematic grouping based on morphological characters. Conspecific populations often do not form monophyletic clades (Symula et al., 2003; Vences et al., 2003; Noonan and Wray, 2006) and low variation of many molecular markers precludes statistically reliable phylogenetic resolution (Summers et al., 1997; Clough and Summers, 2000; Summers and Clough, 2001; Vences et al., 2003). Most phylogenetic analyses include only a small number of conspecific individuals usually from the same population.

Genetic data that reveal intraspecific variability in dendrobatids sometimes show considerable divergence among populations (Summers et al., 1997; Lougheed et al., 1999). Lougheed et al. (1999) revealed that sequence divergences (genetic distances calculated using Kimura's two-parameter model) in a 282 bp fragment of the cytochrome *b* gene amounted up to 14.7% between haplotypes of different sampling areas of *Epipedobates femoralis* in western Amazonia, whereas Summers et al. (1997) found no more than 2% divergence in the same gene region between Panamanian *Dendrobates pumilio* populations. In yet another study, genetic distances among species of the *histrionicus* group (*D. pumilio*, *D. granuliferus*, *D. arboreus*, *D. speciosus*, and *D. histrionicus*; Myers et al., 1984) are also comparatively low (Summers et al., 1999). Examining the same fragment of the cytochrome b gene, genetic divergence was only 4.1% between *D. pumilio* and *D. arboreus*, and 9.3% between *D. histrionicus* and *D. granuliferus*. Speciation events of *D. pumilio* and related Central American species occurred after the establishment of the Pliocene land bridge (Silverstone, 1975) which therefore show lower average interspecific molecular divergences than do Amazonian species, whose cladogenetic history is much older (Lougheed et al., 1999; Clough and Summers, 2000; Noonan and Wray, 2006). Nevertheless, members of the *histrionicus* group are recognized as multiple species which is justified by differences in advertisement calls and distribution ranges (Myers et al., 1984) despite low genetic differences among them. Many species of this group show intraspecific phenotypic variation, which is remarkably high in *D. pumilio* and *D. histrionicus* (see http://www.dendrobase.de). Recently, Grant et al. (2006) resurrected the genus name *Oophaga* from synonymy for species assigned to the *histrionicus* group by Myers et al. (1984), taking into account genetic, ethological, and phenotypic data. Since the suggested taxonomy is not yet widely accepted, we will continue to use the traditional nomenclature in the present study.

The strawberry poison frog (*D. pumilio*) ranges from the Caribbean coast of Nicaragua to the Caribbean lowlands

* Corresponding author. Present address: Department Epigenetics, German Cancer Research Center, Im Neuenheimer Feld 580, D-69120 Heidelberg, Germany. Fax: +49 6221 423802.
E-mail address: s.hagemann@dkfz.de (S. Hagemann).

1055-7903/$ - see front matter
doi:10.1016/j.ympev.2007.06.010

of Costa Rica and Panama. Whereas individuals from Nicaragua and Costa Rica generally show red dorsal colouration and blue, black or red legs, frogs of the Bocas del Toro province in Panama exhibit extreme polymorphism in pattering and colouration. Red, orange, green, olive green, blue, or black coloured frogs occur on the archipelago Bocas del Toro and the adjacent mainland (Daly and Myers, 1967). The phenotypic variation is also apparent in varying body size and differences in advertisement calls (Pröhl et al., in press).

Our aim in this paper is to reconstruct the phylogeny of *D. pumilio* and related species by analysing three mitochondrial genes to reveal: (1) whether morphologically disparate populations of Panamanian strawberry poison frogs show greater molecular genetic divergence than do more monomorphic populations in Costa Rica, (2) whether the strawberry poison frogs form a monophyletic clade and (3) to compare within-species molecular variation to molecular variation among closely related species within the *histrionicus* group. The precisely defined geographic distribution as well as the accessibility of *D. pumilio* populations in Costa Rica and Panama allows an accurate evaluation of genetic variation within this polymorphic dendrobatid species.

2. Materials and methods

2.1. Sample collection and selection of gene fragments

We collected tissue samples (toe clips) of Costa Rican and Panamanian frogs from 20 different populations (Fig. 1). Samples were preserved in 100% ethanol for genetic analyses. Frog colouration was documented using a digital camera (Nikon Coolpix 4500) and longitude and latitude of all sample sites were measured with a Garmin eTrex Legend GPS. Localities of the populations and colour morphs are presented in Table 1.

Fig. 1. Distribution of sample localities in Costa Rica and Panama. 1, Upala; 2, Caño Negro; 3, La Selva; 4, Tortuguero; 5, Guápiles; 6, Pueblo Nuevo; 7, Siquirres; 8, Bribri; 9, Puerto Viejo; 10, Almirante; 11, Tierra Oscura; 12, Colón; 13, Solarte; 14, Bastimentos; 15, San Cristóbal; 16, Pastores; 17, Popa; 18, Cayo de Agua; 19, Loma Partida; 20, Escudo de Veraguas. Populations 1 to 6 belong to the Northern geographic/genetic group, populations 7 to 19 are among the Southern geographic/genetic group, and population 20 was assigned to the Escudo group.

To estimate phylogenetic relationships among populations of *D. pumilio*, we selected three fragments of mitochondrial genes (cytochrome *b*, cytochrome oxidase I and 16S rRNA), which have been used in previous molecular studies of dendrobatids (e.g. Summers et al., 1997, 1999; Clough and Summers, 2000; Vences et al., 2000).

2.2. DNA extraction, amplification and sequencing

DNA was extracted using Qiagen DNeasy Tissue Kit. Mitochondrial DNA (mtDNA) sequence fragments of 16S rRNA, cytochrome *b* (Cyt *b*), and cytochrome oxidase I (COI) gene regions were amplified using PCR. We used primers 16SA (5′-CGC CTG TTTATC AAAAAC AT-3′) and 16SB (5′-CCG GTC TGAACT CAG ATC ACG T-3′) of Palumbi et al. (1991) to amplify a portion of 16S ribosomal RNA gene. Primers L14841 (5′-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA-3′) and H15149 (5′-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3′) of Kocher et al. (1989) were utilised to amplify a section of mitochondrial Cyt *b* gene and primer set COIA (5′-ATC CTC CCA GGC TTC GGA ATC ATC T-3′) and COIB (5′-CCG GCA AAT ATT ACA CCA AAG TGG G-3′) amplified the mitochondrial COI region. The latter primers were established in our laboratory based on the *D. pumilio* COI sequence of Summers et al. (1999). Each PCR reaction (20 μl) contained 5–10 ng of genomic DNA, 1 U Taq-Polymerase (Eppendorf), 1× Advanced Buffer containing self-adjusting $MgCl_2$ (Eppendorf), 0.8 mM dNTPs and 0.25 mM of each primer for 16S and COI amplifications and 0.5 mM of each primer for Cyt*b* reactions, respectively. Following cycle sequencing, samples were purified via ethanol precipitation, resuspended in 10 μl MegaBACE Loading Solution (GE Healthcare) and electrophoresed on the MegaBACE 1000 Automated DNA Sequencer. Resulting sequences were deposited at GenBank (see Table 1 for accession numbers).

2.3. Sequence and phylogenetic analysis

Chromatograms of mtDNA sequences were edited and base calls checked using SeqMan module of the Lasergene program (DNASTAR Inc., Madison, Wis.). Complementary sequences were aligned with the same program. Homologous regions of consensus sequences were aligned using ClustalX (Thompson et al., 1997) and adjusted manually in SEAVIEW (Galtier et al., 1996). Further DNA sequences of closely related species available from GenBank were added to the dataset (Table 1).

Distance analysis was performed using MEGA, version 4.0 (Kumar et al., 1993). Genetic distances between populations of *D. pumilio* and related species (*D. histrionicus*, *D. speciosus*, *D. arboreus*, and *D. granuliferus*) and between groups of populations were calculated using Kimura's

Please cite this article in press as: Hagemann, S., Pröhl, H., Mitochondrial paraphyly in a polymorphic poison frog ..., Mol. Phylogenet. Evol. (2007), doi:10.1016/j.ympev.2007.06.010

Table 1
Localities of the investigated *D. pumilio* populations and related species, characteristic colouration and pattern, and accession numbers of gene fragments

Population/species	Country	Colour and pattern	GPS coordinates or localities	GenBank accession numbers		
				16S	Cyt *b*	COI
Upala	Costa Rica	Red dorsum and venter, blue legs	N10° 54.448′ W85° 02.494′	EF597169	EF597209	EF597189
Caño Negro	Costa Rica	Red dorsum and venter, blue legs	N10° 51.543′ W84° 46.461′	EF597162	EF597202	EF597182
La Selva	Costa Rica	Spotted, with red dorsum and venter, blue legs	N10° 26.499′ W84° 46.461′	EF597164	EF597204	EF597184
Tortuguero	Costa Rica	Spotted, with red dorsum and venter, blackish-blue legs	N10° 36.773′ W83° 31.868′	EF597168	EF597208	EF597188
Guápiles	Costa Rica	Red dorsum and venter, blue legs	N10° 11.297′ W83° 49.274′	EF597163	EF597203	EF597183
Pueblo Nuevo	Costa Rica	Red dorsum and venter, blue legs	N10° 19.305′ W83° 34.440′	EF597165	EF597205	EF597185
Siquirres	Costa Rica	Spotted, with red dorsum and venter, blackish-blue legs	N10° 05.546′ W83° 31.121′	EF597167	EF597207	EF597187
Puerto Viejo	Costa Rica	Red dorsum and venter with brownish pattern, red legs	N09° 38.512′ W82° 45.223′	EF597166	EF597206	EF597186
Bribri	Costa Rica	Spotted, with red dorsum and venter, red legs	N09° 38.437′ W82° 52.573′	EF597161	EF597201	EF597181
Almirante	Panama	Red dorsum and venter, grey legs	N9° 17.166′ W82° 23.439′	EF597170	EF597210	EF597190
Tierra Oscura	Panama	Black/blue dorsum and legs, blue venter	N9° 10.736′ W82° 15.527′	EF597180	EF597220	EF597200
Isla Colón	Panama	Spotted, with green dorsum, yellow venter, brown legs	N9° 23.418′ W82° 14.182′	EF597172	EF597212	EF597192
Isla Bastimentos	Panama	Red dorsum, white venter, grey legs	N9°17.420 ′ W82° 04.949′	EF597171	EF597211	EF597191
Isla Solarte	Panama	Orange dorsum and venter, orange legs	N9°20.096 ′ W82° 13.140′	EF597179	EF597219	EF597199
Isla San Cristóbal	Panama	Red dorsum and venter, grey legs	N9° 16.291′ W82° 17.403′	EF597178	EF597218	EF597198
Isla Pastores	Panama	Yellow/brown dorsum and legs, yellow venter	N9° 14.395′ W82° 21.033′	EF597176	EF597216	EF597196
Isla Popa	Panama	Dark green dorsum, blue venter and legs	N9°13.109 ′ W82° 08.468′	EF597177	EF597217	EF597197
Isla Cayo de Agua	Panama	Green dorsum, yellow venter, blue legs	N9° 09.187′ W82° 03.305′	EF597173	EF597213	EF597193
Isla Loma Partida	Panama	Spotted, with green/blue dorsum, blue venter, brown legs	N9° 08.202′ W82° 09.955′	EF597175	EF597215	EF597195
Isla Escudo de Veraguas	Panama	Red dorsum, light blue flanks, venter, and legs	N9° 06.106′ W81° 32.959′	EF597174	EF597214	EF597194
D. arboreus	Panama	Yellow spots, brown or black dorsum and venter	Fortuna	AF098748	AF120015	AF097504
D. speciosus	Panama	Irregular black spots, bright red dorsum and venter	Fortuna	AF098747	AF120014	AF097503
D. histrionicus	Ecuador	Black pattern on orange, yellow, red, white or blue base colour	Santo Domingo	AF098742	AF120009	AF097498
D. granuliferus	Costa Rica	Red or orange dorsum, green to blue-green legs	Corcovado national park	AF098749	AF120016	AF097505

D. pumilio populations are arranged according to geographic distribution from N-W to S-E, and from mainland to islands.

Please cite this article in press as: Hagemann, S., Pröhl, H., Mitochondrial paraphyly in a polymorphic poison frog ..., Mol. Phylogenet. Evol. (2007), doi:10.1016/j.ympev.2007.06.010

(1980) two-parameter model. To evaluate genetic variation within populations we examined the COI sequence fragment (which was the most variable marker in this study) of eight individuals from populations San Cristóbal, Bastimentos, and Puerto Viejo, respectively.

Phylogenetic maximum parsimony (MP), maximum likelihood (ML), and Bayesian analyses were performed with PAUP*, version 4.0b10 (Swofford, 2002) and MrBayes, version 3.1.1 (Ronquist and Huelsenbeck, 2003), respectively. After analysing all fragments separately we conducted a combined analysis of the complete dataset concatenating all three gene fragments. In addition to *D. pumilio* sequences, homologous DNA-regions of two closely related Panamanian species (*D. arboreus* and *D. speciosus*) were included in the analysis. The South American species *D. histrionicus* was denoted as outgroup. We calculated maximum-parsimony trees using a heuristic search with tree-bisection-reconnection (TBR) branch swapping. Support for resolved nodes was generated by bootstrap analysis with 2000 replicates and 10 random additions per replicate. For ML analysis, models of sequence evolution were compared by likelihood-ratio tests using MODELTEST 3.7 (Posada and Crandall, 1998). The maximum-likelihood tree was searched with PAUP*, based on the best-fit model (HKY85+G) and parameter estimates given by MODELTEST. Analyses were conducted using a heuristic search with stepwise addition with 10 random additions per replicate and TBR branch swapping. Confidence in each node was assessed by 500 bootstrap replicates. Additionally, Bayesian posterior probabilities were calculated using MrBayes, with parameters estimated from the data. As many as 1,000,000 generations were run, every 100th tree collected, and 10% of the initial trees discarded as burn-in.

Parametric bootstrapping (Huelsenbeck et al., 1996) was conducted to test for monophyly of investigated populations. The ML tree was determined using PAUP* (Swofford, 2004), under the constraint that *D. pumilio* populations form a monophyletic group. Hundred data matrices were simulated on the constraint tree using MESQUITE, version 1.12 (Maddison and Maddison, 2006) implementing substitution model parameters estimated using MODELTEST in conjunction with PAUP*. For each simulated dataset, two ML trees were created in PAUP*one under the constraint that populations form a monophyletic group and one without the constraint. Differences in likelihood values between constraint and unconstraint trees were calculated using PAUP* and the distribution of these values was subsequently calculated using MESQUITE.

3. Results

3.1. Sequence characteristics

The aligned dataset, combining three mitochondrial gene fragments of 20 *D. pumilio* populations and homologous sequence information of *D. histrionicus*, *D. arboreus*, and *D. speciosus*, consisted of 1215 characters representing 505 bp of 16S rDNA, 281 bp of Cyt *b* and 429 bp of COI. A total of 137 variable sites were detected, where 84 of them were parsimony-informative. The 20 individuals analysed represented 19 haplotypes; only the frogs from Caño Negro and Pueblo Nuevo in Costa Rica showed no differences in the examined markers.

3.2. Distance analyses

Intraspecific genetic K2P (Kimura 2-parameter) distances between populations varied from 0 to 4.5% of divergence (see Supplementary S1). In general, genetic distances between monomorphic populations from Costa Rica were greater than distances between polymorphic Panamanian populations. The overall mean genetic K2P distance of *D. pumilio* populations was 2.5%. Mean genetic K2P distances within populations using COI sequence information were 0% (San Cristóbal), 0.2% (Bastimentos), and 0.3% (Puerto Viejo), whereas the mean genetic distance between populations was 4.2% in the same gene region (data not shown).

3.3. Phylogenetic analyses

Phylogenetic MP, ML, and Bayesian analyses favoured identical tree topologies. All analyses based on single or combined markers clearly divided populations of *D. pumilio* into three monophyletic genetic groups (Fig. 2). The first group comprised of the northern Costa Rican populations (Northern group). The second group consisted of a single Panamanian population from the island Escudo de Veraguas (Escudo group) and the third group included populations from southern Costa Rica and Panama (Southern group). Mean genetic distances within and between groups are shown in Table 2.

3.4. Genetic variation and phylogenetic relationships among species of the histrionicus group

The comparison of homologous gene regions of related species demonstrated that some genetic distances were higher between intraspecific populations of *D. pumilio* than among different species. For example 4.5% sequence divergence was calculated between two *D. pumilio* populations (Colón and Guápiles) but the divergence between *D. pumilio* (Popa) and *D. arboreus* was only 1.6%. DNA sequences of the Escudo de Veraguas population are more similar to a distinct species, *D. speciosus*, than to individuals of its own species. Highest genetic divergence (6.5–8.2%) was calculated between the sampled populations and *D. granuliferus*. Phylogenetic MP, ML and Bayesian analyses including *D. speciosus* and *D. arboreus* strongly support the distance analysis, demonstrating that the populations of *D. pumilio* do not form a monophyletic group (Fig. 2). On comparing the difference in likelihood values of constraint and unconstraint trees of real data to the distribution of differences from simulated data, we found significant support for rejection of monophyly in *D. pumilio* populations ($P < 0.001$).

Please cite this article in press as: Hagemann, S., Pröhl, H., Mitochondrial paraphyly in a polymorphic poison frog ..., Mol. Phylogenet. Evol. (2007), doi:10.1016/j.ympev.2007.06.010

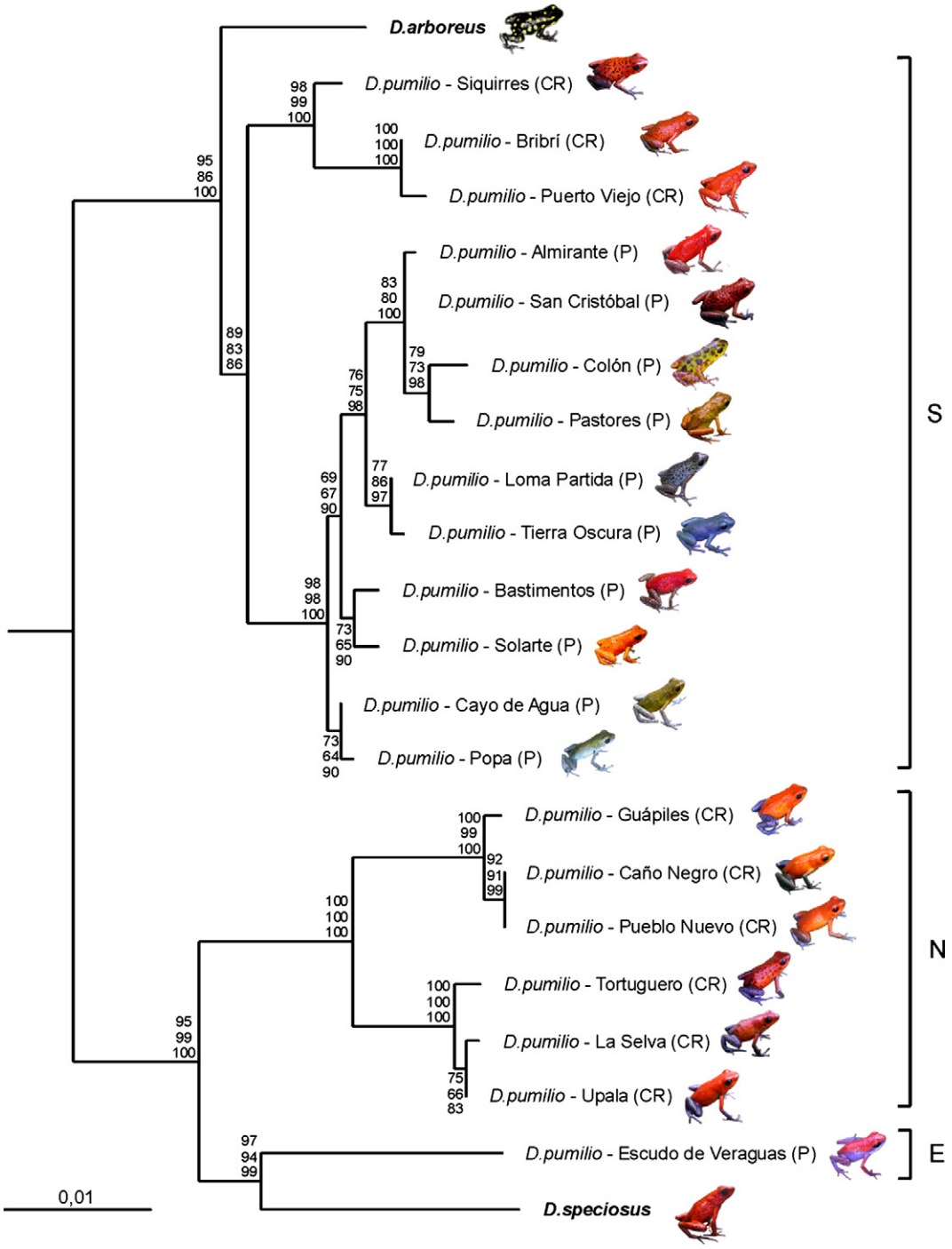

Fig. 2. MP, ML and Bayesian phylogeny of *D. pumilio* populations and related species based on combined mitochondrial 16S, Cyt *b* and COI sequences. Values (top down) are (1) parsimony and (2) maximum likelihood bootstrap supports (in percentage, MP: 2000 bootstraps, ML: 500 bootstraps), and (3) approximated bayesian posterior probabilities in percentage (1,000,000 replicates) for the equivalent node. Branch lengths were estimated using ML. CR, Costa Rica; P, Panama; S, Southern group; N, Northern group; E, Escudo group. The tree was rooted using *D. histrionicus* as outgroup (not shown).

4. Discussion

4.1. Phentoypic and genetic variation in D. pumilio

Phenotypic and genetic divergences among populations do not correlate in *D. pumilio*. Genetic distances between frogs of Panamanian polymorphic populations are not greater than distances between similarly coloured frog populations from Costa Rica. Thus, the extreme colour polymorphism of the island populations cannot be explained by longer genetic fragmentation in comparison with monomorphic Costa Rican populations. The low mitochondrial genetic distances between polymorphic frogs might result from repeated connection of Panamanian islands among

Please cite this article in press as: Hagemann, S., Pröhl, H., Mitochondrial paraphyly in a polymorphic poison frog ..., Mol. Phylogenet. Evol. (2007), doi:10.1016/j.ympev.2007.06.010

Table 2
Mean genetic distances (K2P) within and between monophyletic groups of investigated populations and *D. histrionicus*

Mean genetic distance within groups (%)	
Northern group	1.8
Southern group	0.9
Mean genetic distance between groups (%)	
Northern group–Southern group	3.5
Northern group–Escudo group	3.5
Southern group–Escudo group	4.1
Northern group–*D. histrionicus*	4.6
Southern group–*D. histrionicus*	4.3
Escudo group–*D. histrionicus*	4.9

one another and the adjacent mainland due to cyclical sea level changes during the Pleistocene period. This may have caused gene flow between populations and mitochondrial introgression. Genetic distances among populations from the Bocas del Toro province reflect geological data regarding the sequence of island formation (Anderson and Handley, 2002). Individuals from islands that were connected for a longer period of time (e.g. Isla Solarte and Isla Bastimentos; Isla Popa and Isla Cayo de Agua) show higher genetic and phenotypic similarity to each other, and populations from islands located close to the mainland show higher genetic and phenotypic similarity to adjacent mainland populations (e.g. Isla Loma Partida and Tierra Oscura; Isla Colón and Pastores; Isla San Cristóbal and Almirante; Anderson and Handley, 2002). Moreover, frogs from the geographically disconnected island Escudo de Veraguas are genetically distinct from all other populations. The observed polymorphism may be a result of sexual selection (Summers et al., 1997) or more likely in our opinion, different selection pressures favouring distinct morphs in diverse habitats.

4.2. Genetic variation among species of the histrionicus group and biogeographic implications

Distance analyses revealed high genetic divergences between *D. granuliferus* and all individuals investigated in this study. The genetic distance between *D. granuliferus*, which is allopatrically distributed at the Pacific coast of Costa Rica, and Costa Rican *D. pumilio* populations even exceeds the distance between *D. pumilio* and *D. histrionicus* from Ecuador (see Supplementary S1). These findings support the hypothesis that *D. granuliferus* had diverged from a more ancestral lineage that later diverged to form *D. histrionicus*, *D. pumilio*, *D. speciosus*, and *D. arboreus* (Clough and Summers, 2000). Thus, *D. granuliferus* was excluded from our phylogenetic analyses and trees were rooted with *D. histrionicus*, probably representing the ancestral lineage of investigated species. Dendrobatids of the *histrionicus* group may have colonised Central America in two migration events. The first, phenotypically more monomorphic lineage diverged to form the Northern genetic group, the Escudo genetic group and *D. speciosus*. This was followed by a phenotypically polymorphic lineage with higher genetic similarity to *D. histrionicus* that diverged to form the Southern genetic group and *D. arboreus*.

4.3. Mitochondrial paraphyly in D. pumilio

The haploptypes of *D. speciosus* and *D. arboreus* are phylogenetically nested within the haplotypes of the investigated *D. pumilio* populations, indicating that *D. pumilio* was found to be paraphyletic. Paraphyly is observed in many species (Funk and Omland, 2003) and has multiple potential causes, including: (a) introgressive hybridization through interspecific mating followed by backcrossing of hybrids into parental populations, (b) incomplete lineage sorting due to recent speciation events and (c) imperfect taxonomy caused by misidentification of inter- or intraspecific variation.

Introgressive hybridisation seems to be an unlikely explanation for the observed paraphyly as *D. arboreus* and *D. speciosus* generally do not occur in sympatry with *D. pumilio*, and *D. arboreus* additionally shows ecological isolation (Myers et al., 1984). Both aspects will be discussed in detail below.

Assuming recent speciation within the *histrionicus* group as supported by geographical data and low genetic distances between species, incomplete lineage sorting may explain species paraphyly in the haplotype tree. *D. pumilio* shows a comparatively wide distribution and polymorphic allelic lineages. Hence, it may represent the ancestral species of *D. speciosus* and *D. arboreus* whereby all three species retain common alleles.

However, the most likely explanation for the observed paraphyletic pattern is, in our opinion, inaccurate taxonomy caused by two possible misinterpretations: Ample intraspecific variation within the *histrionicus* group may have been misidentified as species-level variation where *D. pumilio*, *D. speciosus* and *D. arboreus* might represent polymorphic forms of a single species. However, both nested species are clearly differentiated from *D. pumilio* in biologically relevant characters and show obvious divergences in several advertisement-call parameters (Myers et al., 1984; Jungfer et al., 1996). *D. speciosus* is distinguished by its distribution exclusively in the mountains of western Panama and by its larger body size (27.5–30 mm versus 17.5–24 mm in *D. pumilio*; Schmidt, 1857). *D. arboreus* is ecologically isolated from *D. pumilio* where *D. pumilio* occupies the forest ground and low vegetation and *D. arboreus* inhabits trees in the western Panamanian low- and high-lands (Myers et al., 1984).

A second possible misinterpretation is that species-level variation has been considered as intraspecific variation. It was assumed that red and blue coloured frogs from northern Costa Rica and the Panamanian island Escudo de Veraguas represent phenotypic variations of polymorphic frogs found in Panama (Savage, 1968). However, distance

Please cite this article in press as: Hagemann, S., Pröhl, H., Mitochondrial paraphyly in a polymorphic poison frog ..., Mol. Phylogenet. Evol. (2007), doi:10.1016/j.ympev.2007.06.010

analyses strongly support the division into three genetic groups. Phylogenetic analyses revealed that (a) the Costa Rican red and blue morph (Northern group), (b) the polymorphic Panamanian frogs including entirely red individuals and red individuals with black legs from southern Costa Rica (Southern group), and (c) the Escudo de Veraguas population (Escudo group) are distinct genetic lineages. Genetic distances between different groups even exceed distances between clearly differentiated species within the *histrionicus* group. Besides a distinct colour pattern, strawberry poison frogs show differences in body size and call parameters between genetic groups (Pröhl et al., in press; S. Hagemann, unpublished data). Genetic grouping is in accordance with geographic distribution of investigated populations. Red morphs with blue legs from the Northern group are geographically separated by the river Rio Reventazón from red morphs with black or red legs, which were genetically assigned to the Southern group. Further studies should investigate the importance of this river as geographic barrier.

Interestingly, the Escudo de Veraguas population and *D. speciosus* are genetically similar. Nonetheless, morphological differences argue against their consolidation into a single species: while the 15–16 mm small frogs (snout to vent length) from the Escudo de Veraguas island show a red dorsal colouration and light blue coloured flanks, hind legs and venter (S. Hagemann, pers. observ.), *D. speciosus* individuals are almost twice as large and present a bright red colouration with irregular black spots on the back (Jungfer, 1985). Additionally, frogs show differences in advertisement calls (http://www.dendrobase.de).

Provided that morphological and genetic divergences are sufficient to place *D. speciosus* and *D. arboreus* into distinct species, comparable degrees of divergence in the same parameters should justify dividing *D. pumilio* populations into three distinct species. *D. pumilio* populations would be split to form one species represented by the Northern group for which the synonymy *D. typographus* (type species from an unknown locality in Costa Rica, Keferstein, 1867) or *D. ignitus* (type species from Nicaragua, Cope, 1874) may be resurrected. The second polymorphic species would include all individuals assigned to the Southern group (described as *D. pumilio* by Schmidt, 1857) and a new third species represented by the Panamanian Escudo de Veraguas population.

This study shows that morphological characteristics alone do not allow a discrimination between species within the *histrionicus* group. Genetic markers offer an additional, more objective means of diagnosing dendrobatid species. Nuclear genomic information and phenotypic characters should be used to confirm the results of mtDNA analyses and to establish a consistent and generally accepted species definition within the *histrionicus* group. Additionally, behavioural experiments and bioacoustic analyses might prove useful to support the division into different species, especially by examining whether different genetic groups are reproductively isolated.

Acknowledgments

We thank Costa Rican and Panamanian authorities for collecting and exporting permits. We are grateful to Jan Karsch for supplying additional tissue samples and to Edilberto Morales Mendez for help in the field. We also thank Thomas Ostrowski for helpful discussions and support of this study and Miguel Vences for helpful comments on this manuscript. Financial support was provided by the DAAD (D/04/33799).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2007.06.010.

References

Anderson, R.P., Handley, C.O., 2002. Dwarfism in insular sloths: biogeography, selection, and evolutionary rate. Evolution 56 (5), 1045–1058.

Clough, M., Summers, K., 2000. Phylogenetic systematics and biogeography of the poison frogs: evidence from mitochondrial DNA sequences. Biol. J. Linn. Soc. Lond. 70 (3), 515–540.

Cope, 1874. *Dendrobates ignitus*. Proc. Acad. Nat. Sci. Philadelphia 26, 68.

Daly, J.W., Myers, C.W., 1967. Toxicity of Panamanian poison frogs (Dendrobatidae). J. Chem. Ecol. 20, 943–955.

Darst, C.R., Cummings, M.E., 2006. Predator learning favours mimicry of a less-toxic model in poison frogs. Nature 440, 208–211.

Funk, D.J., Omland, K.E., 2003. The frequency, causes and consequences of species level paraphyly and polyphyly: insights from animal mitochondrial DNA. Annu. Rev. Ecol. Evol. Syst. 34, 397–423.

Galtier, N., Gouy, M., Gautier, C., 1996. SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput. Appl. Biosci. 12, 543–548.

Grant, T., Frost, D.R., Caldwell, J.P., Gagliardo, R., Haddad, C.F.B., Kok, P.J.R., Means, D.B., Noonan, B.P., Schargel, W.E., Wheeler, W., 2006. Phylogenetic systematics of dart-poison frogs and their relatives (Amphibia, Athesphatanura, Dendrobatidae). B. Am. Mus. Nat. Hist., 299.

Huelsenbeck, J.P., Hillis, D.M., Jones, R., 1996. Parametric bootstrapping in molecular phylogenetics: applications and performance. In: Ferraris, J.D., Palumbi, S.R. (Eds.), Molecular Zoology: Advances, Strategies and Protocols. John Wiley & Sons, Inc, New York, pp. 19–45.

Jungfer, K.H., Weygoldt, P., Juraske, N., 1996. *Dendrobates vicentei*, ein neuer Pfeilgiftfrosch aus Zentral-Panama. Herpetofauna 18 (103), 17–26.

Keferstein, W. 1867. Ueber einige neue oder seltene Batrachier aus Australien und dem tropischen Amerika. – Nachrichten von der Georg-Augusts Universität und der Königlichen Gesellschaft der Wissenschaften zu Göttingen 18, 341–361.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Paabo, S., Villablanca, F.X., Wilson, A.C., 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA 86, 6196–6200.

Kumar, S., Tamura, K., Nei, M., 1993. MEGA: Molecular Evolutionary Genetics Analysis. Pennsylvania State University, University Park, PA.

Lougheed, S.C., Gascon, C., Jones, D.A., Bogart, J.P., Boag, P.T., 1999. Ridges and rivers: a test of competing hypotheses of Amazonian diversification using a dart-poison frog, *Epipedobates femoralis*. Proc. R. Soc. Series B 266, 1829–1835.

Maddison, W.P. Maddison, D.R. 2006. Mesquite: a modular system for evolutionary analysis. Version 1.12. Available from <http://mesquiteproject.org/>.

Myers, C.W., Daly, J.W., Martinez, V., 1983. Dart poison frogs. Sci. Am. 248, 120–133.

Myers, C.W., Daly, J.W., Martinez, V., 1984. An arboreal poison frog (Dendrobates) from western Panama. Amer. Mus. Novit. 2783, 1–20, New York.

Noonan, B.P., Wray, K.P., 2006. Neotropical diversification: the effects of a complex history on diversity within the poison frog genus Dendrobates. J. Biogeogr. 33 (6), 1007–1022.

Palumbi, S.R., Martin, A., Romano, S., McMillan, W.O., Stice, L., Grabowski, G. 1991. A simple fool's guide to PCR, Version 2.0., Honolulu, Hawaii.

Posada, D., Crandall, K., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14 (9), 817–818.

Pröhl, H., Hagemann, S., Karsch, J., Höbel, G. in press. Geographic advertisement call variation in the strawberry poison frog *Dendrobates pumilio*. Ethology.

Ronquist, F., Huelsenbeck, J.P., 2003. MRBAYES 3: bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Savage, J.M., 1968. The dendrobatid frogs of Central America. Copeia, 745–776.

Schmidt, O. 1857. Sitzungsber. Akad. Wiss. Wien, Math. Naturwiss. Kl. 24, 12.

Silverstone, P.A., 1975. A revision of the poison-arrow frogs of the genus *Dendrobates wagler*. Nat. Hist. Mus. LA Co. Sci. Bull. 21, 1–55.

Summers, K., Clough, M.E., 2001. The evolution of coloration and toxicity in the poison frog family (Dendrobatidae). Proc. Natl. Acad. Sci. USA 98, 6227–6232.

Summers, K., Bermingham, E., Weight, L., Mc Cafferty, S., Dahlstrom, L., 1997. Phenotypic and genetic divergence in three species of dart-poison frogs with contrasting parental behaviour. J. Hered. 88, 1.

Summers, K., Weigt, L.A., Boag, P., Bermingham, E., 1999. The evolution of female parental care in poison frogs of the genus Dendrobates: evidence from mitochondrial DNA sequences. Herpetologica 55 (2), 254–270.

Swofford, D.L. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (* and other methods). Version 4b10. Sinauer Associates, Sunderland, MA.

Symula, R., Schulte, R., Summers, K., 2001. Molecular phylogenetic evidence for a mimetic radiation in Peruvian poison frogs supports a Müllerian mimicry hypotesis. Proc. R. Soc. Lond. B. 268, 2415–2421.

Symula, R., Schulte, R., Summers, K., 2003. Molecular systematics and phylogeography of Amazonian poison frogs of the genus Dendrobates. Mol. Phylogenet. Evol. 26 (3), 452–475.

Thompson, J.D., Gebson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTALX window interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acid. Res. 30, 109–112.

Vences, M., Kosuch, J., Boistel, R., Haddad, C.F.B., La Marca, E., Lötters, S., Veith, M., 2003. Convergent evolution of aposematic coloration in Neotropical poison frogs: a molecular phylogenetic perspective. Org. Divers. Evol. 3, 215–226.

Vences, M., Kosuch, J., Lötters, S., Widmer, A., Jungfer, K.H., Köhler, J., Veith, M., 2000. Phylogeny and classification of poison frogs (Amphibia: Dendrobatidae), based on mitochondrial 16S and 12S ribosomal RNA gene sequences. Mol. Phylogenet. Evol. 15 (1), 34–40.

Please cite this article in press as: Hagemann, S., Pröhl, H., Mitochondrial paraphyly in a polymorphic poison frog ..., Mol. Phylogenet. Evol. (2007), doi:10.1016/j.ympev.2007.06.010