



# Neotropical diversification: the effects of a complex history on diversity within the poison frog genus *Dendrobates*

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## ABSTRACT

**Aim** We study the Neotropical poison frogs of the genus *Dendrobates* Wagler, 1830 in order to clarify their phylogenetic relationships and biogeographical history. The genus *Dendrobates* is an excellent taxon for examining patterns of Neotropical diversification as the four major species groups appear to correspond roughly to distinct geographical regions: (1) trans-Andean, (2) Andean foreland, (3) Brazilian Shield and (4) Guianan Shield/Central America. In order to test the agreement of five of the most prominent hypotheses of Amazonian diversification, phylogenetic patterns were examined for agreement with patterns predicted by these hypotheses.

**Location** Central and South America

**Methods** The phylogenetic relationships of the genus *Dendrobates* were examined from novel and existing (GenBank) sequences of four mitochondrial loci totalling c. 1400 bp from 40 specimens of 22 different species using maximum parsimony and Bayesian methods. Results were compared with traditional taxonomic arrangements by means of SH tests. Phylogenetic relationships and genetic distances were used to test the adequacy of various diversification hypotheses.

**Results** Phylogenetic analyses support the restructuring of two species groups of *Dendrobates* and the creation of a new species group. Statistical tests of the traditional taxonomic arrangement indicate a significantly bad fit to the molecular data. This restructuring has important implications for the understanding of the historical biogeography of *Dendrobates*. Biogeographical patterns within this genus suggest that a complex interaction of biotic and abiotic factors since the Eocene have produced the diversity observed today.

**Main conclusions** The current classification of *Dendrobates* into discrete species groups does not accurately reflect evolutionary history. Data presented here strongly support a monophyletic Brazilian Shield lineage whose members have previously been split among the *quinquevittatus* and *tinctorius* groups. Furthermore, previous attempts at elucidating the historical biogeography of this genus were compromised by incomplete sampling and conclusions drawn from a paraphyletic ingroup. Our findings demonstrate a role for numerous hypotheses of diversification (e.g. river, refuge, disturbance–vicariance) in the history of *Dendrobates*, supporting previous warnings about the dangers of oversimplification in the study of Neotropical diversification.

## Keywords

Amphibia, biogeography, Central America, *Dendrobates*, Dendrobatidae, disturbance–vicariance hypothesis, refuge hypothesis, South America.

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## INTRODUCTION

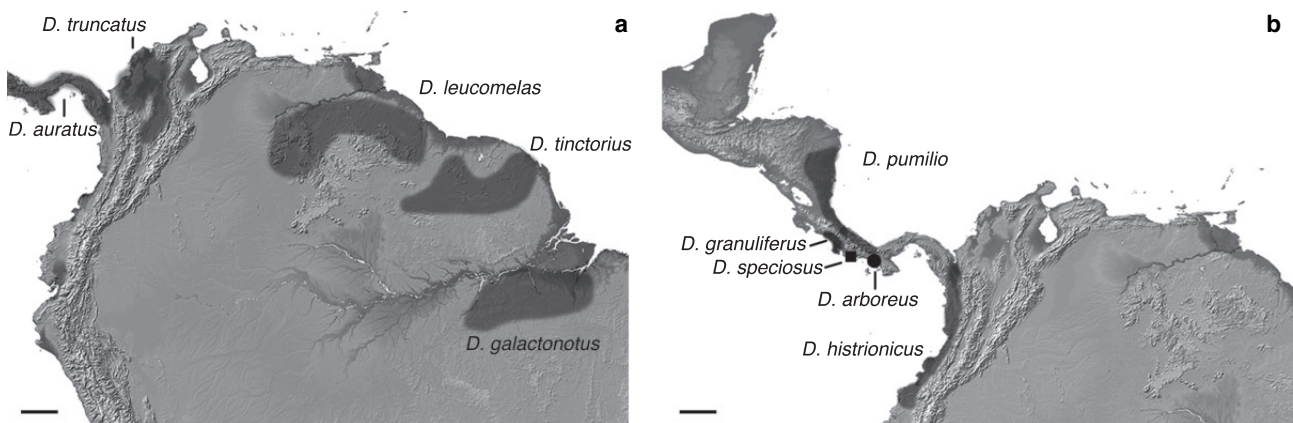
Few Neotropical organisms have sparked the interest of biologists like the poison frogs of the family Dendrobatidae. There have been numerous studies investigating their ecology (Wells, 1980; Toft, 1995; Caldwell, 1997; Summers, 2000), toxicology (Myers & Daly, 1980; Tokuyama *et al.*, 1992; Daly *et al.*, 1994) and systematic relationships (Clough & Summers, 2000; Vences *et al.*, 2000, 2003; Symula *et al.*, 2001; Santos *et al.*, 2003). With the exception of a single study (Symula *et al.*, 2003), the biogeography of this group has largely been ignored. Vences *et al.* (2003), however, did note that their results implied a significant concordance between evolutionary history and geographical distribution. This is surprising as the family is widely distributed throughout northern South America and attempts to understand the biotic history of this area are numerous. A number of hypotheses have been proposed to explain observed patterns of Amazonian diversity based primarily on the findings of studies of mammalian and avian groups. Many of these treatments address only cis-Andean taxa (those east of the Andes), and thereby overlook not only the direct effects of the Andean orogeny in the history of the South American biota, but also the potential information to be gleaned by examining groups whose representatives are found on both sides of this biogeographical barrier (but see Ribas *et al.*, 2005).

The benefit of examining a widespread Neotropical group such as Dendrobatidae is that inferences of historical evolutionary processes may incorporate our temporal knowledge of the formation of various mountain ranges, the dynamic history of Amazonian hydrology and the isthmian connection of the Americas. Herein, we examine biogeographical patterns within the genus *Dendrobates* in order to infer the role of geological change and climatic fluctuations on South American evolutionary diversification.

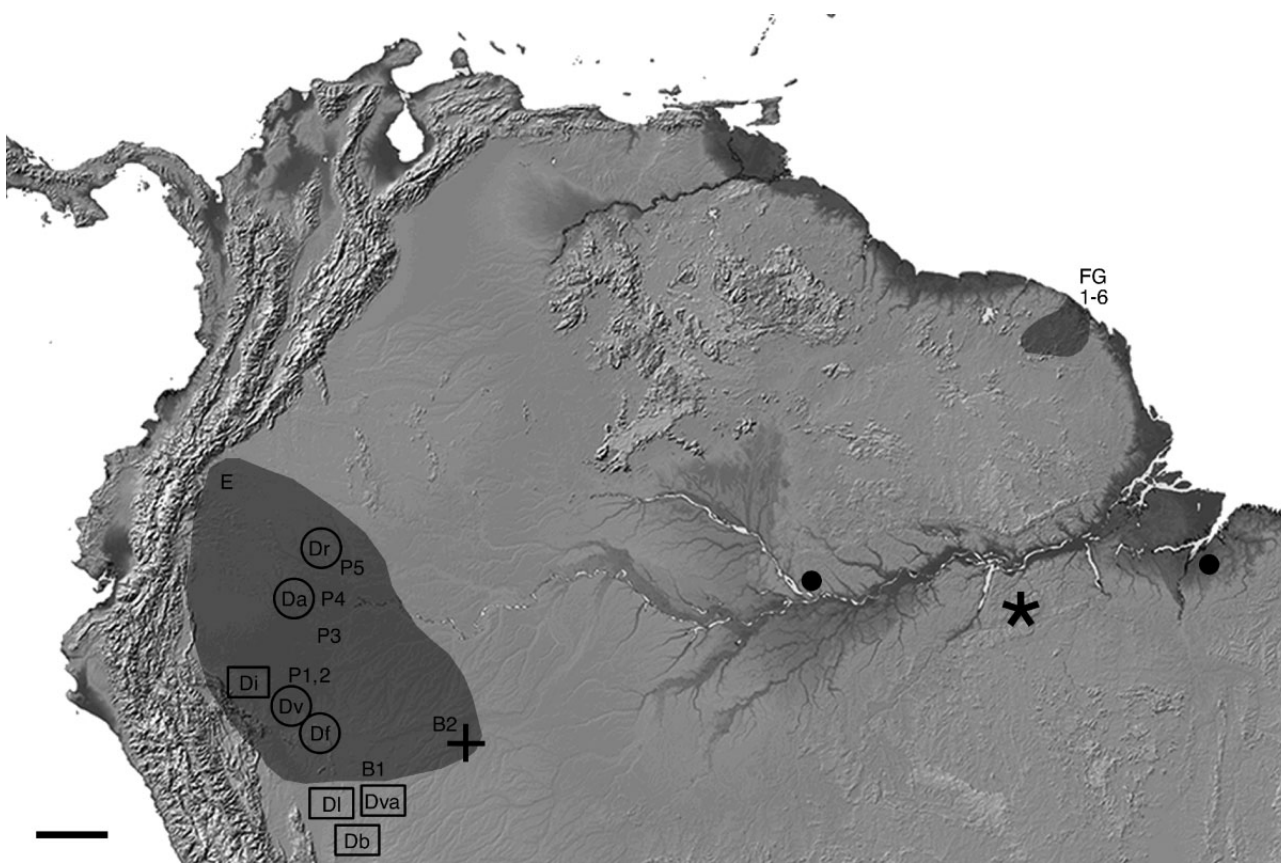
The members of the genus *Dendrobates* have long been considered to comprise at least three species groups that represent 'real' evolutionary entities. The *tinctorius* group (Silverstone, 1975 as modified by Myers & Daly, 1979) is a

poorly defined assemblage of five species that ranges from southern Nicaragua to north-eastern Colombia, the Guianan Shield and the north-eastern Brazilian Shield (Fig. 1a). The members of the *histrionicus* group (Myers *et al.*, 1984) range from the coastal lowlands of Colombia to southern Nicaragua (Fig. 1b) and are characterized by female parental care. The diminutive (< 3.0 cm) members of the *quinquevittatus* group (*sensu* Caldwell & Myers, 1990) are restricted primarily to the Amazonian lowlands of Ecuador, Peru, adjacent Brazil and isolated populations associated with the Amazon River [French Guiana and Brazil (Belem, Manaus, Santarem); Fig. 2]. An additional group, the *captivus* group, was proposed by Myers (1982), although it comprises two exceedingly uncommon (in scientific collections) species of unknown affinities that are known from intermediate altitudes (600–3000 m; *vide* Myers, 1982; Schulte, 1999) in eastern Peru.

Since the advent of molecular approaches to the investigation of biotic relationships, the family Dendrobatidae has been the focus of a great deal of attention. Initial studies were hampered by limited sampling, but generally provided support for current taxonomic arrangements (Clough & Summers, 2000; Vences *et al.*, 2000). Recent investigations into the evolutionary history of this family utilizing more comprehensive sampling indicate that neither the taxonomy nor the evolutionary history of the group are as simple as once thought (Santos *et al.*, 2003; Vences *et al.*, 2003). The general pattern illustrated by these studies indicates an early split in the history of *Dendrobates* between the small bodied Amazonian *quinquevittatus* group and all other species. The remaining taxa have frequently been demonstrated to sort into the classically recognized *tinctorius* and *histrionicus* groups. However, two recent investigations of relationships within the Dendrobatidae have tentatively suggested that this may not be the case (Symula *et al.*, 2003; Vences *et al.*, 2003). The results of these studies suggest that the *quinquevittatus* group is paraphyletic with respect to the (*tinctorius* + *histrionicus*) clade and indicate that the Amazonian *Dendrobates castaneoticus* and *Dendrobates quinquevittatus*, with *Dendrobates galactonotus* represent a monophyletic lineage more closely related to the *histrionicus*



**Figure 1** Geographical distribution of *tinctorius* (a) and *histrionicus* (b) species groups. Scale bar = 200 km.



**Figure 2** Geographical sampling of *quinquevittatus* (*sensu* Caldwell & Myers, 1990) group *Dendrobates* included in this study. The shaded area indicates the geographical extent of the distribution of *D. ventrimaculatus* s.l., unenclosed letter/number combinations correspond to localities of specific individuals included in this study (Fig. 4; Table 2). Enclosed two-letter codes correspond to localities of individuals of the following species included in this study (circles = Napo lineage, rectangles = Inambari lineage): Dr, *D. reticulatus*; Da, *D. amazonicus*; Dv, *D. variabilis*; Df, *D. fantasticus*; Di, *D. imitator*; Dl, *D. lamasi*; Db, *D. biolat*. Solid circles indicate reported (not sampled) localities for '*D. ventrimaculatus*', the cross and star indicates sampled localities for *D. quinquevittatus* and *D. castaneoticicus*, respectively. Scale bar = 200 km.

and *tinctorius* species groups. Although Symula *et al.* (2003) treat this result as preliminary (their study included only a single representative each of the *histrionicus* and *tinctorius* groups), it has subsequently been borne out by the work of Vences *et al.* (2003) who included broader sampling of the non-*quinquevittatus* *Dendrobates*. Both of these works noted the unusual pattern of relationships, but the lack of additional *Dendrobates* samples (Symula *et al.*, 2003) and the poor support of the relationships (Vences *et al.*, 2003) led the authors to urge cautious interpretation.

The work of Symula *et al.* (2003) was specifically aimed at interpreting the relationships within the *quinquevittatus* group in the context of historical Amazonian biogeography. While an examination of the biogeographical patterns within the Amazonian members of the genus *Dendrobates* should yield some very interesting insights into the history of the genus and the impact of biogeographical barriers on dendrobatid diversification, this study was limited by conclusions drawn from patterns observed within what is now known to be a paraphyletic group. High sequence divergence between members of the *ventrimaculatus* group (*sensu* this study) and

(*D. castaneoticicus* + *D. quinquevittatus*) was suggested to be the result of palaeogeographical barriers between the two. The conclusions drawn by Symula *et al.* (2003) were further hampered by lack of sampling of the widespread *Dendrobates ventrimaculatus* from the eastern portion of its range, as no samples of this species from any locality east of Acré, Brazil were examined. Vences *et al.* (2003) included two individuals of *D. ventrimaculatus* in their analysis, including one from French Guiana. Their findings suggest that the *D. ventrimaculatus* from French Guiana are closely related to conspecifics from Ecuador, although monophyly of this broadly distributed species and relationships to western Amazonian forms are unclear. Herein, we examine phylogenetic and biogeographical patterns within the genus *Dendrobates*, based on much broader sampling of species than that provided by Symula *et al.* (2003), and a more comprehensive sampling of species and populations of the *ventrimaculatus* group than provided by Vences *et al.* (2003). We specifically wish to address the apparent paraphyly of the *quinquevittatus* group and the applicability of the numerous hypotheses of Amazonian diversification to the genus *Dendrobates*.

## METHODS

### Taxon sampling and laboratory methods

Tissue samples of *D. ventrimaculatus* and *Dendrobates tinctorius* from French Guiana were obtained through the fieldwork of B.P.N. Samples from *D. galactonotus* were obtained from a captive individual of unknown origin. Samples of Brazilian *D. ventrimaculatus* collected by J. P. Caldwell were borrowed from the Louisiana State University Museum of Natural Sciences Collection of Genetic Resources. Localities of samples and voucher specimens are listed in Table 2. Sequence data used by Symula *et al.* (2003) and others were obtained from GenBank (see Table 2 for accession numbers).

Tissues were taken from liver and preserved in 95% ethanol. Genomic DNA was isolated using the Qiagen Inc. (Valencia, CA, USA) DNEasy Tissue Kit according to the standard protocol. Portions of the mitochondrial cytochrome *b* (*cyt b*), cytochrome oxidase subunit 1 (CO1), 12S rRNA and 16S rRNA genes were amplified using published primers (Symula *et al.*, 2003). Amplifications were performed in 20 µL reaction volumes using TaKaRa hotstart *Taq* DNA polymerase and 10× reaction buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl<sub>2</sub>). Amplification was performed in a PTC100 (MJ Research, Waltham, MA, USA) thermocycler under the following profile: 94 °C for 2 min, 25 cycles of denaturation at 94 °C for 20 s, annealing at 52 °C for 30 s, elongation at 72 °C for 1 min and a final elongation at 72 °C for 15 min. Polymerase chain reaction (PCR) products were then cut from 1% agarose gels stained with ethidium bromide and purified using the Qiagen QIAquick Gel Extraction Kit. The purified double-stranded products were used directly in quarter-volume dideoxy-termination sequencing reactions using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA). Unincorporated dye terminators were removed by precipitation with PelletPaint (Novagen, San Diego, CA, USA) and ethanol/sodium acetate.

Sequences were edited and aligned with Sequencher v. 4.1 (Gene Codes Corp., Ann Arbor, MI, USA) and checked by eye. Alignments of protein-coding genes (*cyt b*, 269 bp and CO1, 437 bp) were unambiguous with no apparent insertions or deletions. The 12S and 16S rRNA genes contained highly variable regions for which alignment was ambiguous and, therefore, these regions were excluded from analyses, yielding alignments of 287 bp and 435 bp respectively. The combined data set consisted of 1429 aligned sites, of which 799 were constant and 440 of 630 variable sites were parsimony-informative. Sequences were deposited in GenBank (DQ163059–DQ163090).

### Sequence analysis

Sequences were analysed using PAUP\* version 4.0b10 (Swofford, 2001) employing maximum parsimony (MP) as the optimality criterion. In order to determine support for proposed clades we performed 2000 nonparametric bootstrap

pseudoreplicates with the heuristic search option, tree bisection–reconnection branch swapping (TBR) and 10 random taxon addition replicates. Bayesian analysis of the data employed MrBayes version 3.0b4 with four chains (one cold) for 50 million generations and a mixed model approach (Nylander *et al.*, 2004). Models of sequence evolution for each of the four genes were determined using Modeltest version 3.6 (Posada & Crandall, 1998). Adequate burn-in was determined by examining a plot of the likelihood scores of the heated chain for convergence on stationarity (Leache & Reeder, 2001). Topological conformity with previous systematic hypotheses was tested using SH tests (Shimodaira & Hasegawa, 1999) as implemented in PAUP\*.

### Evaluation of biogeographical hypotheses and previous findings

We suspected that the attempts by Symula *et al.* (2003) to interpret evolutionary history in terms of historical biogeographical processes might have been confounded by incomplete taxon sampling. We therefore re-evaluate the adequacy of the biogeographical hypotheses tested by these authors. We have designed our analyses to test five of the models of speciation that are commonly ascribed to the Amazonian biota (*sensu* Haffer, 1997 and Moritz *et al.*, 2000). Two models (gradient and vanishing refuge) were not considered as they implicate adaptive divergence across ecological gradients (e.g. invasion of dry forest during periods of reduced rainfall) and do not appear to be applicable or testable based on the ecology and geographical distributions of extant *Dendrobates*. Additionally, with the increased sampling of the genus *Dendrobates* presented here, we were able to test earlier instances of evolutionary divergence within the genus for consistency with these historical hypotheses. While a detailed review of these hypotheses is beyond the scope of this paper, we summarize their foundations and predicted effects on the evolution of *Dendrobates* in Table 1 and Fig. 3. For the purposes of testing these hypotheses we consider the following phylogenetic groups within the genus *Dendrobates*: the *histrionicus* group (*sensu* Myers *et al.*, 1984); a revised *quinquevittatus* group including *D. galactonotus*, *D. castaneoticus* and *D. quinquevittatus*; the *ventrimaculatus* group [which here includes all members of the *quinquevittatus* group of Caldwell & Myers (1990) except *D. castaneoticus* and *D. quinquevittatus*]; and the *tinctorius* group (*sensu* Silverstone, 1975; Myers & Daly, 1979). These *a priori* assumptions are based on the findings of Santos *et al.* (2003) and Vences *et al.* (2003) and are borne out by our analyses.

It should be noted that the possibility of determination of the exact timing of divergence events within this group using modern Bayesian or maximum likelihood techniques is impractical due to the paucity of the neobatrachian fossil record. For the purposes of this project we will not be conducting rigorous tests of the accuracy of the following hypotheses, rather we will be qualitatively gauging the adequacy of these hypotheses to explain the diversity of

**Table 1** Hypotheses of Amazonian diversification and their predicted effects on the evolutionary history of *Dendrobates*

Hypothesis	Timing	Factors	Effects	Predictions for <i>Dendrobates</i>
Palaeogeographical*	Tertiary	Andean orogeny (and associated uplift in Andean foreland), marine incursions, formation of Panamanian isthmus	Vicariant differentiation of Neotropical lineages associated with the formation of barriers to dispersal	Distinct cis- and trans-Andean lineages with the former divided into two groups corresponding with the Guianan and Brazilian shields; Panamanian invasion by trans-Andean lineages
River†	From the Late Miocene	Amazonian tributaries	Large tributary rivers are expected to fragment faunal distributions into intra-riverine corridors	Sister species or interspecific populations separated by lower reaches of Amazonian tributaries with less structure apparent in headwater regions. Most applicable to <i>D. ventrimaculatus</i> group in Napo region
Refuge‡	Cenozoic	Repeated cycles of drastic fluctuations in precipitation	Episodic fragmentation of Amazonian forest (and associated fauna) into discrete patches of habitat capable of supporting wet forest despite reduction in precipitation	Evolutionary lineages associated with proposed refugia (Napo, Marañon, Guiana, etc.) whose inhabitants are paraphyletic with respect to those of nearby refugia
River refuges§	Post-Miocene	Reduced precipitation and Amazonian tributaries	Similar to river with latitudinal restriction of distributions into intrariverine corridors associated with increased aridity adversely affecting habitat quality at headwater regions	Sister species occurring in adjacent intrariverine corridors ( <i>D. galactonotus</i> , <i>D. castaneoticus</i> , <i>D. quinquevittatus</i> )
Disturbance–vicariance¶	Quaternary	Decreased temperatures, reduced atmospheric CO <sub>2</sub> , moderate reduction in precipitation	Decreased temperatures facilitate eastward spread of Andean (cool-adapted) organisms. Cyclic fluctuations in temperature and associated factors fragmented habitat according to altitude (rather than rainfall as in refuge)	Diversity within monophyletic lineages associated with a single region (refuge). This would predict that the high species diversity within the Napo and Marañon regions are monophyletic with respect to each other

\*Räsänen *et al.* (1990; 1995); Coates & Obando (1996); Haffer (1997).

†Wallace (1853); Capparella (1991); Patton *et al.* (1994); Haffer (1997); Peres (1997); Lougheed *et al.* (1999); Gascon *et al.* (2000); Patton *et al.* (2000).

‡Haffer (1969, 1990, 1997); Endler (1982); Brown (1987); Nores (1999); Gascon *et al.* (2000).

§Mossman *et al.* (1986); Haffer (1993a,b, 1997).

¶Colinvaux (1993); Bush (1994); Haffer (1997).

*Dendrobates* based on generally accepted rates of molecular evolution of the anuran mitochondrion.

## RESULTS AND DISCUSSION

### Phylogenetic analysis

The phylogram obtained from the combined Bayesian analysis is shown in Fig. 4. This topology does not disagree with that obtained by a strict consensus of 48 equally parsimonious trees produced by MP analysis. All analyses strongly supported (1.0 Bayesian posterior probability (pp)/100 MP bootstrap) a monophyletic *ventrimaculatus* group that includes all members of Myers' (1982) *quinquevittatus* group except *D. quinquevitt-*

*atus* and *D. castaneoticus*. This result also supports the paraphyly of the *quinquevittatus* group and brings into question the biogeographical conclusions of Symula *et al.* (2003). Support was also strong (1.0/100) for a monophyletic *D. histrionicus* group, which includes all species with maternal care of the young. Two additional lineages, a *tinctorius* group (minus *D. galactonotus*) and a new *quinquevittatus* group (*D. galactonotus*, *D. castaneoticus*, *D. quinquevittatus*), which were hinted at in previous analyses (Symula *et al.*, 2003; Vences *et al.*, 2003), received strong support (1.0/82 and 1.0/76, respectively). Support for the placement of *D. quinquevittatus* and *D. castaneoticus* with *D. galactonotus* and outside the *ventrimaculatus* group is quite strong, thus rendering Myers' (1982) *quinquevittatus* group paraphyletic. SH tests constraining

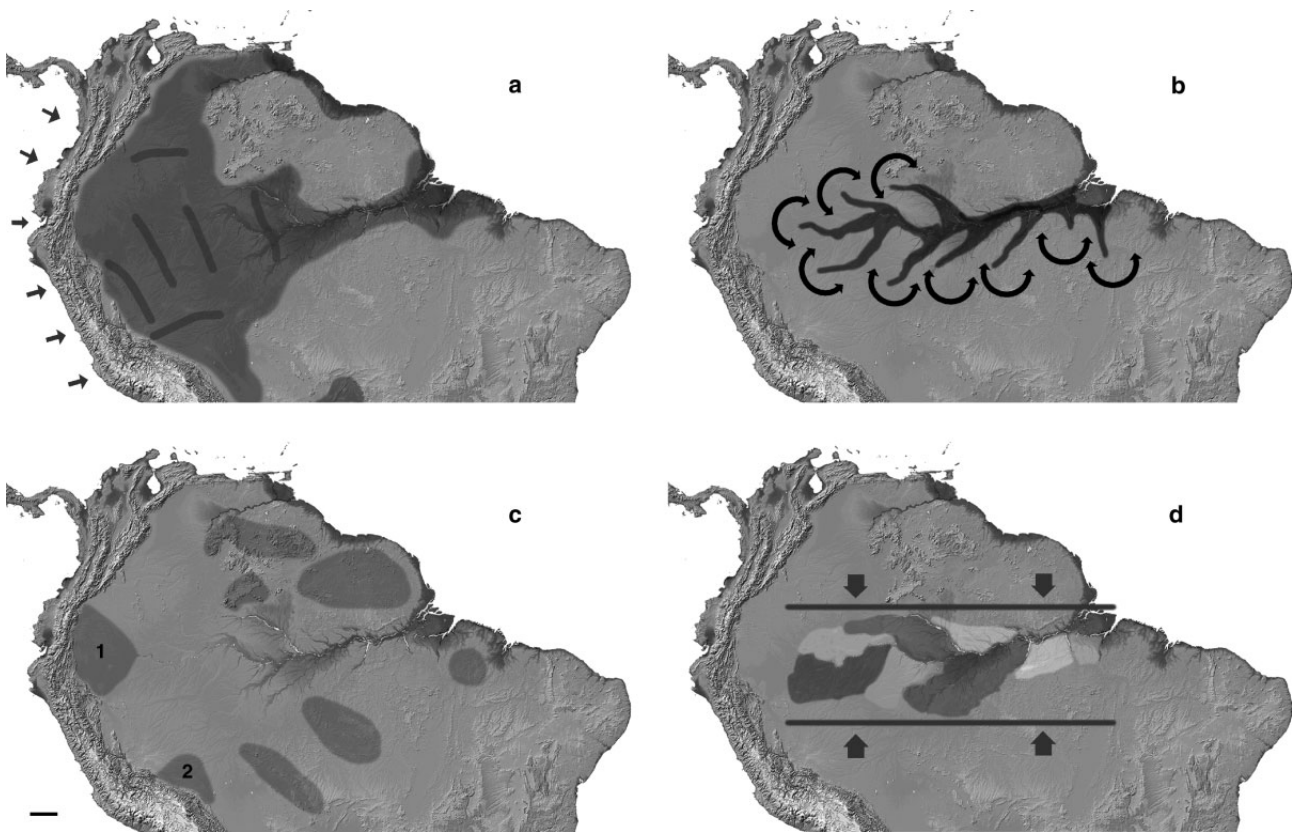
**Table 2** Species, localities and GenBank accession numbers for samples

Taxon	Locality	12S	16S	COI	cyt <i>b</i>
<i>Colostethus talamancae</i>	Unknown	AF128587	AF098740	AF097496	U70140
<i>tinctorius</i> group					
<i>D. auratus</i>	Unknown	AY263212	AY364370	AF097501	AF128603
<i>D. galactonotus</i>	Unknown	DQ163082	NA	NA	DQ163071
<i>D. leucomelas</i>	Unknown	AF128593	AF124119	AF097499	AF128594
<i>D. tinctorius</i>	Saul, French Guiana	DQ163090	NA	NA	DQ163072
<i>quinquevittatus</i> group					
<i>D. amazonicus</i>	Almendras, Loreto, Peru	AF482770	AF482785	AF482815	AF482800
<i>D. biolat</i>	Tambopata, Madre de Dios, Peru	AF482779	AF482794	AF482823	AF482809
<i>D. castaneoticus</i> (1)	102 km SE Santarem, Para, Brazil	AF482774	AF482789	AF482818	AF482804
<i>D. castaneoticus</i> (2)	102 km SE Santarem, Para, Brazil	AF482775	AF482790	AF482819	AF482805
<i>D. fantasticus</i> (1)	Sauce, San Martin, Peru	AF412444	AF412472	AF412416	AF412500
<i>D. fantasticus</i> (2)	Huallaga Canyon, San Martin, Peru	AF412440	AF412468	AF412412	AF412496
<i>D. imitator</i> (1)	Shapaja, San Martin, Peru	AF412460	AF412488	AF412432	AF412516
<i>D. imitator</i> (2)	Huallaga Canyon, San Martin, Peru	AF412448	AF412476	AF412420	AF412504
<i>D. imitator</i> (3)	Achinamisa, San Martin, Peru	AF412459	AF412487	AF412431	AF412515
<i>D. lamasi</i>	Tingo Maria, Huanuco, Peru	AF482778	AF482793	AF482822	AF482808
<i>D. quinquevittatus</i>	Rio Ituxi, Amazonas, Brazil	AF482773	AY263253	NA	AF482803
<i>D. reticulatus</i>	Loreto, Peru	AF482771	AF482786	AF482816	AF482801
<i>D. vanzolinii</i>	Porto Walter, Acre, Brazil	AF128599	AF128598	NA	AF128600
<i>D. variabilis</i> (1)	26 km NE Tarapoto, San Martin, Peru	AF412463	AF412491	AF412435	AF412519
<i>D. variabilis</i> (2)	26 km NE Tarapoto, San Martin, Peru	AF412464	AF412492	AF412436	AF412520
<i>D. ventrimaculatus</i> (B1)	Porto Walter, Acre, Brazil	AF485782	AF482797	AF482826	AF482812
<i>D. ventrimaculatus</i> (B2)	Rio Ituxi, Amazonas, Brazil	DQ163089	DQ163079	DQ163063	DQ163074
<i>D. ventrimaculatus</i> (E)	Pompeya, Sucumbios, Ecuador	AF482780	AF482795	AF482824	AF482810
<i>D. ventrimaculatus</i> (FG1)	Saul, French Guiana	DQ163084	DQ163081	DQ163065	DQ163070
<i>D. ventrimaculatus</i> (FG2)	Pic Matecho, French Guiana	DQ163083	DQ163075	DQ163059	DQ163066
<i>D. ventrimaculatus</i> (FG3)	Maripa, French Guiana	DQ163085	DQ163077	DQ163061	DQ163067
<i>D. ventrimaculatus</i> (FG4)	Maripa, French Guiana	DQ163086	DQ163078	DQ163062	DQ163068
<i>D. ventrimaculatus</i> (FG5)	Maripa, French Guiana	DQ163088	DQ163080	DQ163064	DQ163069
<i>D. ventrimaculatus</i> (FG6)	Kaw, French Guiana	DQ163087	DQ163076	DQ163060	DQ163073
<i>D. ventrimaculatus</i> (P1)	Bonilla, San Martin, Peru	AF412466	AF412494	AF412438	AF412522
<i>D. ventrimaculatus</i> (P2)	Bonilla, San Martin, Peru	AF412465	AF412493	AF412437	AF412521
<i>D. ventrimaculatus</i> (P3)	Nauta, Loreto, Peru	AF482784	AF482799	AF482828	AF482814
<i>D. ventrimaculatus</i> (P4)	Allpahuayo, Loreto, Peru	AF482776	AF482791	AF482820	AF482806
<i>D. ventrimaculatus</i> (P5)	N. Bank Napo R., Loreto, Peru	AF482781	AF482796	AF482825	AF482811
<i>histrionicus</i> group					
<i>D. arboreus</i>	Unknown	AF128611	AF098748	AF097504	AF128612
<i>D. granuliferus</i>	Unknown	AF128608	AF098749	AF097505	AF128609
<i>D. histrionicus</i>	Unknown	AF128617	AF098742	AF097498	U70154
<i>D. lamasi</i>	Unknown	AF482778	AF482793	AF482822	AF482808
<i>D. pumilio</i>	Unknown	AF128614	AF128613	AF097500	U70147
<i>D. speciosus</i>	Unknown	AF128596	AF098747	AF097503	AF128597

Codes in parentheses match those of Figs 2 and 4.

Myers' (1982) *quinquevittatus* group to be monophyletic were significantly less likely than the unconstrained topology ( $P < 0.002$ ). Similar tests of the monophyly of the *tinctorius* group (including *D. galactonotus*) suggest that there is strong support in this data set for the rejection of this group as a clade ( $P < 0.02$ ). Comparisons were also made of two trees with unresolved relationships within and among species groups to test the fit of the data to both the classical three species group hypothesis (*tinctorius*, *histrionicus* and *quinquevittatus*) and the novel four-group hypothesis (with the new *quinquevittatus* and *ventrimaculatus* groups). SH tests strongly rejected the three

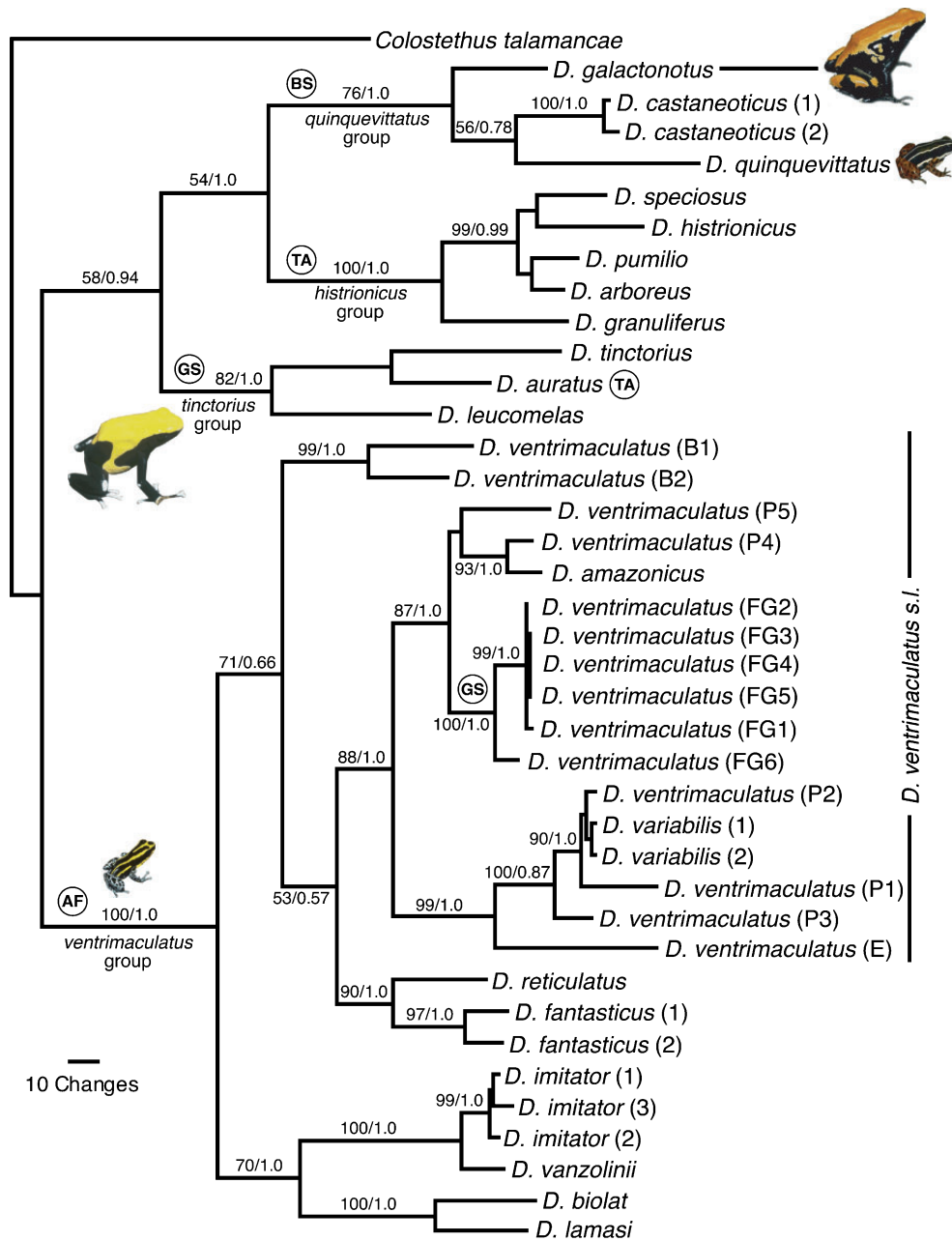
species group hypothesis ( $P < 0.001$ ;  $-\ln L = 15,729.5$ ) in favour of the new hypothesis ( $-\ln L = 14,890.8$ ). While relationships within these four lineages (*tinctorius*, *quinquevittatus*, *ventrimaculatus*, *histrionicus*) appear to be strongly supported in the Bayesian analysis, there is not significant support from MP analyses to resolve relationships within the *histrionicus*, and newly defined *quinquevittatus*, and *histrionicus* groups. Relationships among the four lineages are weakly supported by MP analysis, though Bayesian analyses support the *quinquevittatus* and *histrionicus* groups as sister, which are in turn sister to the *tinctorius* group.



**Figure 3** Predictions of habitat continuity. (a) Based on the palaeogeography hypothesis. The shaded region illustrates the potential extent (there is no clear consensus on the actual extent) of the proposed Miocene marine incursion, the dark lines in the Amazonian region indicate palaeoridges and arrows illustrate crustal movement causing Andean and palaeoridge orogeny. (b) Simplified representation of the riverine barrier hypothesis; the breadth of the shaded river courses illustrates the putative strength of the barrier, arrows indicate potential gene flow. (c) The refuge hypothesis; shaded areas represent consensus refugia from previous studies (see text): 1 = Napo refuge, 2 = Inambari refuge. (d) Simplified representation of the river refuge hypothesis; horizontal bars and arrows illustrate latitudinal constriction of humid forest and shaded regions represent interriverine corridors proposed to have retained forest cover. Scale bar = 200 km.

Several interesting observations can be made within the *ventrimaculatus* group. *Dendrobates ventrimaculatus* appears to represent a complex of species within the Andean foreland area, two of which have been described (*Dendrobates variabilis* and *Dendrobates amazonicus*). The genetic diversity within this 'super species' is as great as or greater than that between any randomly selected pair of *ventrimaculatus* group species. It should be noted that the significant differentiation observed by Symula *et al.* (2003) of *D. ventrimaculatus* from Acré Brazil is substantiated herein by the addition of a sample from the nearby state of Amazonas. It appears that the genetic diversity within *D. ventrimaculatus* merits the recognition of additional species, though this process appears to be hindered by the relatively conservative nature of phenotypic variation within this species complex. The validity of *D. variabilis* (Zimmermann & Zimmermann, 1988) has long been a subject of debate, due in no small part to the inadequacy of the original description (see Caldwell & Myers, 1990), although it has received support on the basis of differences in advertisement call (Symula *et al.*, 2003) and elevational distribution (Schulte, 1999). It is interesting to note that *D. variabilis* appears to be nested well

within a noticeably western group of *D. ventrimaculatus* and exhibits only minimal sequence divergence from a nearby population of *D. ventrimaculatus* (P1 & P2, Fig. 2). Within *D. ventrimaculatus sensu lato (s.l.)*, populations from French Guiana seem to be most closely related to those populations situated directly at the confluence of the major northern Peruvian rivers (the Napo and Marañon), which combine to form the beginning of the Amazon River (Fig. 5). Schulte (1999) suggested in his description of *D. amazonicus* that this taxon was closely related to *D. ventrimaculatus* from French Guiana. This observation is supported by our findings. *Dendrobates amazonicus* and *D. ventrimaculatus* from the Iquitos area along the Amazon River are closely related and, surprisingly, are more closely related to populations in French Guiana (c. 2400 km away) than they are to populations in western Peru (100–600 km away). It appears that genetic divergence in the eastern portion of the Andean forelands is latitudinally structured, corresponding with the courses of the Amazonian tributaries, and this eastern group is then significantly divergent from an Andean slope clade that extends from northern Ecuador to Tarapoto, Peru. The populations of *D. ventrimaculatus*



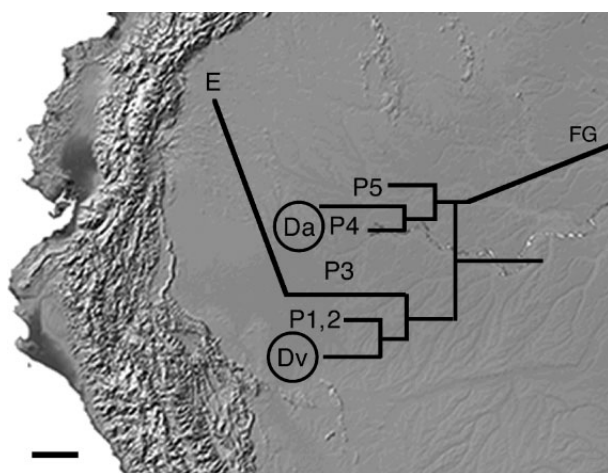
**Figure 4** Phylogenetic relationships within *Dendrobates* as inferred from Bayesian analysis of combined data set (CO1, 12S, 16S, cyt *b*). Bayesian posterior probabilities and MP bootstrap support values (> 50%) are illustrated above the branches. Species groups (*sensu* this study) discussed in the text are indicated below the branches. The generalized geographical distribution of species groups is indicated at the root node of each group with any variation from this generalization being indicated on branches within these groups: Brazilian Shield (BS), trans-Andean (TA), Guiana Shield (GS), Andean foreland (AF). Photographs illustrate representatives (roughly to scale) of the *ventrimaculatus* and *tinctorius* groups as well as members of the *quinquevittatus* group, demonstrating the variation in both colour pattern and body size present in the *quinquevittatus* group that has confounded previous hypotheses of phylogenetic affinities.

from western Brazil are significantly differentiated from the remainder of the species complex, and their isolation on the eastern side of the Ucayali River appears to have occurred early in the diversification of *D. ventrimaculatus* s.l. Within the *ventrimaculatus* group, we obtained strong support for the northern (*D. ventrimaculatus*, *Dendrobates fantasticus*, *D. reticulatus*, *D. variabilis*, *D. amazonicus*) and southern (*Dendrobates*

*imitator*, *Dendrobates vanzolinii*, *Dendrobates biolat*, *Dendrobates lamasi*) groups observed by Symula *et al.* (2003).

### Historical biogeography

The phylogenetic pattern presented here, in conjunction with estimates of divergence time, provide a backdrop upon which



**Figure 5** Phylogeographical relationships within *D. ventrimaculatus* s.l. (excluding B1 and B2, see Fig. 4). Refer to Fig. 4 and Table 2 for identification of individual samples: Da = *D. amazonicus*, Dv = *D. variabilis*. Scale bar = 100 km.

the predictions of the various hypotheses of Amazonian diversification (Table 1) can be examined. We now examine the fit, or lack thereof, of the observed evolutionary patterns within *Dendrobates* to these models.

#### Palaeogeography hypothesis

Symula *et al.* (2003) estimated the divergence time between *D. castaneoticus*/*D. quinquevittatus* and the *ventrimaculatus* group to be between 10 and 30 Myr. This estimate is now shown to represent the age of the first major split within the genus, that between the *ventrimaculatus* group and all other *Dendrobates*. While the biogeographical pattern appears to resemble closely the palaeogeographical projection (Table 1; Fig. 3a), the Andes are estimated to have reached 5000 m in elevation by *c.* 45 Ma (Kellogg, 1984). Although this Andean uplift would be expected to act as a biogeographical barrier to *Dendrobates* at substantially lower altitudes, the age of these mountains significantly exceeds estimated divergence times based on even the most conservative estimates of substitution rates. It appears that the current pan-Andean distribution of *Dendrobates* is largely the result of the Chocoan–Andean corridor in northern South America (as suggested by Haffer (1997), which was unaffected by the Andean orogeny until the Neogene uplift of the northern Andes.

Subsequent to this apparent complete separation of cis- and trans-Andean regions, fluctuating sea levels in the Quaternary make the identification of a specific time of complete isolation of lowland floral and faunal communities on either side impossible as this may have periodically created a corridor for dispersal along the Colombian coast. However, the comparative sequence divergence within the *histrionicus* group, which spans the Quaternary Isthmus of Panama, is roughly half that of the cis/trans Andean representatives of the *tinctorius* group. Divergence between sister species of the *tinctorius* group

suggests relatively ancient [in relation to the age of the genus (10–30 Myr)] speciation events, compared with the relatively recent diversification within the *histrionicus* group. Thus, it appears that the trans-Andean distribution of the *tinctorius* group is not the result of periodic migration around the northern Andes during fluctuations in Quaternary sea levels. Estimates of divergence times between trans-Andean *Dendrobates auratus* and cis-Andean *Dendrobates leucomelas* based on the approximate rate of substitution for *cyt b* (0.8–2.5% divergence Myr<sup>-1</sup>) reported by Loughheed *et al.* (1999) and used by Symula *et al.* (2003) suggest that these events took place during the Miocene (6–17 Ma). This estimate coincides with the proposed South American marine introgression (Räsänen *et al.*, 1995) that may have (depending on the extent) resulted in the fragmentation of cis-Andean South America into large islands that coincide with the distribution of these taxa: western Guiana (*D. leucomelas*), eastern Guiana (*D. tinctorius*) (potentially separated by Branco River/Rupununi lowlands; see Nores, 1999) and the Brazilian Shield (*D. galactonotus*, *D. quinquevittatus*, and *D. castaneoticus*).

A number of recent studies have suggested that the western Amazonian ridges, primarily the Iquitos arch, had a significant impact on faunal diversification (Patton & da Silva, 1998; Loughheed *et al.*, 1999; Symula *et al.*, 2003) between eastern and western Amazonia. These studies estimated divergence times between clades, presumably separated by these barriers, which supported late Tertiary (5–31 Ma) isolation. Although these authors attribute this divergence to the presence of these ridges bisecting the Amazonian tributaries, they overlook the fact that the timing of origin for these ridges (particularly the Iquitos ridge that is the focus of these studies) coincides with the estimated timing of a north–south sub-Andean seaway (Räsänen *et al.*, 1995) that may have submerged the Iquitos arch, superseding it as a biogeographical barrier. Haffer (1997) notes that ‘no (or very little) effect is seen of the Iquitos Arch’ and that it is unclear if this formation was ever even above sea level during the Tertiary. Thus, it would seem that a more parsimonious interpretation of the results presented by previous authors (e.g. Patton & da Silva, 1998; Loughheed *et al.*, 1999; Symula *et al.*, 2003) might be that an east–west divergence occurring in the late Tertiary was due to eustatic sea-level fluctuations rather than an arch that was probably submerged at this time. This might explain why a number of pan-Amazonian species exhibit east–west diversification that appears to occur somewhere in the Andean foreland, but does not coincide with the position of one of the palaeogeographical ridges. Many studies that have proposed a significant historical influence of these ridges on Amazonian diversification have limited sampling to populations along both banks of major rivers in this area (e.g. the Juruá), and have no sampling of additional populations to the north or south in the headwater region (Gascon *et al.*, 1998; Loughheed *et al.*, 1999; but see Patton *et al.*, 1994, 2000). Our results suggest that latitudinal divergence in the intracratonic basins between populations is significant (as great as that observed across these palaeoridges).

### Riverine barrier hypothesis

Despite the lingering questions as to the applicability of this hypothesis to diversification within Amazonia (Haffer, 1997), it has frequently been incorporated into recent studies. Moreover, this hypothesis has been utilized essentially as a 'straw man' in biogeographical studies of Amazonian taxa (e.g. Loughheed *et al.*, 1999). Although rivers unquestionably act as barriers to dispersal in a number of groups (e.g. birds, primates; see Ayres & Clutton-Brock, 1992), the dynamic nature of their course and the potential for genetic continuity in headwater regions make them a poor choice for 'either/or' hypothesis testing. Additionally, rivers are expected to affect the distributions of species with different habits in different ways, resulting in differing genetic signatures among co-distributed taxa.

Within the Amazonian *Dendrobates* there is only one taxon for which we have reasonable sampling and that has a distribution for which the riverine barrier hypothesis can be tested. *Dendrobates ventrimaculatus* occurs throughout the Amazon basin, and on both sides of the Amazon River in both the headwater and terminal regions. The distribution of this species in the headwater region is broad, yet the known distribution near the terminus of the Amazon is limited and our sampling is restricted to a number of localities in French Guiana. Samples from Belém were unavailable for this study, but we believe that it is informative to examine patterns within the Andean foreland basin, as the major tributaries of the Amazon that drain this region may provide similar insight into the role of rivers in faunal diversity.

Within *D. ventrimaculatus*, phylogenetic relationships reveal a biogeographical pattern not entirely inconsistent with the riverine hypothesis (Table 1). This pattern is particularly clear in populations near the confluence of the major Amazonian tributaries (Napo, Marañon etc.) in eastern Peru. Individuals generally appear to be more closely related to individuals (populations) within the same intrariverine corridor (east-west) than more geographically proximate populations that are on the opposite side of one of the region's major rivers (north-south). The relationship of the individual from Ecuador to the southernmost populations (both occurring at the base of the Andean slopes) suggests that the headwaters of the region's rivers are ineffective barriers to gene flow. An aspect of the role of rivers in the evolution of species that is often ignored is that of a corridor for dispersal. The sequence divergence between populations from French Guiana (corrected for variation within this lineage) and near Iquitos, Peru is less than that between the latter and a population < 100 km to the north. As the sequence divergence between these Peruvian and French Guianan populations is roughly equivalent to that observed between other populations (and species) within this complex, it would seem that the Guianan population is a dispersal relict and continued gene flow between these two regions is unlikely. This in turn suggests that gene flow between these eastern Peruvian populations is equally unlikely, because intra-Peruvian differentiation is even greater despite the proximity.

The data presented here suggest that the *Dendrobates* of the Napo region merit more detailed examination in order to determine the potential for a 'river effect' on the distribution of genetic diversity within this region.

### Refuge hypothesis

The distribution of *Dendrobates* appears to be closely associated with the refugia suggested by Brown (1987) (Fig. 2c). Although patterns of relationships between taxa originating as a result of refugial isolation cannot be predicted, one expectation is that climatic cycles would have affected these refugia at similar times in similar ways. Taxa originating as a result of these climatic fluctuations are expected to have diverged from congeners at roughly the same time. Average estimates of divergence time within the four species groups based on the standard range of evolutionary rates suggested for mitochondrial sequences (see above) suggest a Miocene (mid to late) age. This timing would support Haffer's (1997) assertion that climatic fluctuations throughout the Cenozoic have affected the distribution of Amazonian forest and have played a role in modern biogeographical patterns and the origin of extant species. Within the *ventrimaculatus* group, our findings corroborate the conclusion of Symula *et al.* (2003) that the basal split within this group appears to be associated with taxa whose distributions are allied with the Napo and Inambari refugia.

The refuge hypothesis predicts repeated habitat isolation throughout the Cenozoic, and therefore we would expect to see multiple species in each of the hypothesized refugia as a result of multiple cycles of refuge connectivity and dispersal. With the exception of the *ventrimaculatus* group we see only one species per refuge (*D. tinctorius*, *D. leucomelas*, *D. galactonotus*, *D. quinquevittatus*, *D. castaneoticus*, *D. auratus*). This may indicate that a particularly drastic cycle of climate change occurred during this period, early in the history of *Dendrobates*. Subsequent refugial connectivity may not have occurred to an extent that favoured dispersal from one refuge to another, or ecological divergence between species may not have been significant enough to permit sympatry. However, the observed hybrid viability of members of this group (*D. tinctorius*, *D. leucomelas*, *D. auratus*; B.P.N., pers. obs.) combined with the extent of genetic differentiation suggest that these have remained spatially isolated since their Miocene origin. Within the *ventrimaculatus* group, the high number of species associated with each refuge suggests a more complex history. However, these data illustrate only one instance of mixed history/distribution (*D. imitator*, a member of the Inambari clade currently found in the Napo region) as would be expected from periodically reconnected refugia. Phylogeographical patterns within *D. ventrimaculatus* indicate that the presence of this species in French Guiana (and presumably in the Belem area) is the result of eastward dispersal, rather than a zone of secondary contact between Napo and Guianan/Belem refugia. These findings suggest that Tertiary climatic cycles may have initiated varying levels of habitat change associated

with differential degrees of climatic change and longevity. Therefore, minor climatic cycles may have affected the genetic continuity within refugia relatively frequently, while cycles creating drastic changes in climate and forest cover between refugia were less common.

#### River refuge hypothesis

The river refuge theory is rarely considered when exploring patterns of Amazonian diversification despite its potential influence on biogeographical history within the last 10 Myr. There are three species of *Dendrobates* (*D. quinquevittatus*, *D. castaneoticus*, *D. galactonotus*) whose distributions are closely associated with the Amazon River and have potentially been influenced by historical latitudinal habitat constriction. Unfortunately, the temporal overlap with the predictions of the refuge theory prevents the differentiation of late Miocene–Quaternary divergence when distribution alone does not necessarily exclude one of these two hypotheses. For the purposes of this study, however, it appears that we can discount an intrariverine origin for these taxa due to the overlapping distributions of *D. galactonotus* and *D. castaneoticus*, and the estimated time of origin of these three taxa (6–23 Ma), which largely pre-dates the proposed origin of the Amazon and its tributaries as they are known today.

#### Disturbance–vicariance hypothesis

The biogeographical evidence presented here provides little material for a thorough examination of the merits of this hypothesis, particularly for members of the *tinctorius* group. However, sampling within the *ventrimaculatus* group is sufficiently adequate to enable us to make some observations on the potential influence of Quaternary temperature fluctuations. Based on rates of sequence divergence for mitochondrial genes, divergence attributable to Quaternary events should not exceed 5% (assuming a maximum rate of 2.5% Myr<sup>-1</sup>). While the divergence among members of some groups (e.g. populations of *D. imitator*) falls within this range, diversity within the broadly distributed, phenotypically and genetically divergent clade of *D. ventrimaculatus* s.l. exceeds this measure in all but a few instances. Furthermore, divergence within this group appears to have occurred at numerous, non-synonymous times throughout the Neogene. Additionally, the monophyly of the representatives of the *ventrimaculatus* group in both the Napo and Marañon regions (discussed by Symula *et al.*, 2003; see discussion above for the single exception) suggests that the high diversity within these areas is not the result of serial connectivity with interrefugial migration, but diversification within each refuge. Such intrarefugial diversification has been documented for Neotropical amphibians based on more comprehensive sampling (Noonan & Gaucher, 2005), so these factors cannot be ignored.

The disturbance–vicariance hypothesis requires that eastward-dispersing Andean lineages should have geographical distributions coincident with intermediate (100–300 m) Ama-

zonian altitudes. Interestingly, this pattern appears to conform to the observed distribution of several cis-Andean *Dendrobates*, which seem to be generally absent from broad areas of Amazonian bottomland and restricted to marginally elevated areas and their immediately adjacent lowlands. It is difficult to make such distributional assertions for most species of *Dendrobates* as sampling of most taxa is not representative of their entire distribution. However, this pattern is apparent in the Guianan *D. tinctorius*, whose distribution is extremely patchy, and is closely associated with the eroded remnants of the eastern Guianan Shield, whose maximum elevation ranges from 150 to 1000 m (B.P.N., pers. obs.). The disturbance–vicariance hypothesis seems to be applicable to the evolution of the genus at a much finer scale than that addressed by this study. Further work employing intensive sampling of species throughout their distribution will prove a better test of the intraspecific effects predicted by the palynological record.

## CONCLUSIONS

Despite the amount of effort put into the study of the genus *Dendrobates* it is clear that our understanding of its evolutionary history is still inadequate. The diversification of *Dendrobates*, as Vences *et al.* (2003) suggested, does appear to 'have a strong biogeographic component'. As has been supposed for some time, there appear to be distinct, divergent groups of species within this genus, although the estimation of their composition seems to have been slightly erroneous. The widespread *tinctorius* group appears to have undergone comparatively little diversification throughout its distribution and to have been little affected by the climatic fluctuations associated with serial post-Miocene ice ages. Sequence divergence between sister species within this group suggests a Miocene origin. This temporal estimate, combined with current distributions, leads to the conclusion that this diversity may be the result of isolation associated with rising sea levels. Although the distributions of these taxa are clearly associated with putative refugia, our knowledge of times for climatic phenomena that would have restricted faunal distributions to these refugia is scant. It is possible that a combination of climatic fluctuations and changes in sea level have contributed to diversification within these areas. This may explain why subsequent climatic cycles, which have been suggested to have augmented biotic diversity (e.g. Quaternary glacial cycles), had little effect on the diversity within these isolated lineages.

A well-resolved group within this genus (the *histrionicus* group) appears to have recently undergone relatively rapid diversification that may be associated with a shift to female-dominated and more involved parental care, including larval feeding (Summers *et al.*, 1999). The diversification may be attributable to this ecological adaptation as a similar pattern of radiation is not observed in the sympatric *tinctorius* group species. It is unknown whether this diversification is also associated with one of the aforementioned historical hypotheses of abiotic changes. The diminutive members of the *ventrimaculatus* group pose a far more complex system

biogeographically. With the exception of the recently dispersed populations of *D. ventrimaculatus*, the estimated time of origin and its distribution suggest that events in the Miocene (potentially marine introgression or the formation of the Lago Amazonas) and not the Andean orogeny were the driving factors for the origin of this lineage. Subsequent to this, the incredible diversity within this group and significant structure within species appears to be the result of multiple factors, including the influences suggested by the riverine barrier, refuge, disturbance–vicariance, and gradient/vanishing refuge hypotheses. This is not to suggest that these data are unable to distinguish a single hypothesis that best explains the observed diversity. Rather, these events appear to have affected the diversification at different levels: divergence between the *ventrimaculatus* group and the rest of *Dendrobates* (palaeogeographical); divergence among major lineages of the group (refuge, palaeogeographical); interspecific divergence, including among *D. ventrimaculatus* s.l. (riverine barrier, disturbance–vicariance). It should be noted that recent work suggests a relationship between this *ventrimaculatus* group and the Choco-Panamanian *Minyobates* (Santos *et al.*, 2003), indicating the possibility of a cis-Andean slope/north-west Colombian corridor, the continuity of which was disrupted by the aforementioned uplift of the northern Andes.

Our findings support Bush's (1994, 2005) assertion that Amazonian speciation is very complex. Although this conclusion is not novel, it strengthens the argument against the all too common approach of 'either/or' in studies of historical biogeography. Furthermore, the apparent desire to attribute observed patterns of biotic diversification to one particular hypothesis will necessarily oversimplify. Although the consideration of the likely non-independence of these proposed historical phenomena has been widely noted (Haffer, 1997; Moritz *et al.*, 2000), this has rarely translated into empirical studies (but see Aleixo, 2004). It is clear that the conceptual basis of each of these hypotheses has some measure of support (be it biological or geological) and that novel evidence for additional historical factors does not entirely discount the possibility of any particular hypothesis. For example, the Quaternary climatic fluctuations described by the disturbance–vicariance hypothesis do not negate the potential effects of the change in precipitation proposed by the refuge hypothesis, rather research into these fluctuations adds to our comprehension of an intricate history of which we know very little.

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