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Refugial isolation and secondary contact in the dyeing poison frog *Dendrobates tinctorius*

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Abstract

Recent palaeoclimactic research suggests that fluctuating environmental conditions throughout the Pleistocene of Amazonia occurred with previously unrecognized frequency. This has resulted in a theoretical shift from glacially influenced fluctuations to those driven by precessional rhythms. This theoretical revolution has a profound impact on expectations of biotic diversity within biogeographical regions that have long been based on the idea of large-scale landscape fragmentation associated with increased aridity and glacial cycles. Generally speaking, this shifts phylogeographical expectations from that of (i) large areas of sympatry of closely related (but not sister) species whose origins lie in separate refugia, and current distributions are the results of cyclic connectivity of those two refugia (refuge hypothesis), to that of (ii) fine scale genetic structure, often associated with elevation, and divergence well below expected speciation levels [disturbance–vicariance (DV) hypothesis]. To date there have been few tests of the expectations of the DV hypothesis based on empirical studies of Neotropical floral and faunal communities. Herein we examine phylogeographical structure of *Dendrobates tinctorius*, an amphibian species endemic to the uplands of the eastern Guiana Shield, based on sampling of 114 individuals from 24 localities. Phylogenetic, nested clade, and dispersal–vicariance (DIVA) analyses of cytochrome *b* sequence data reveal the presence of two mitochondrial lineages that are associated with previously identified western and eastern uplands of this area. The geographical distribution of mitochondrial haplotypes and the results of DIVA and coalescent analyses suggest that there has been extensive secondary contact between these lineages indicating a complex history of connectivity between these western and eastern highlands, supporting the predictions of the DV hypothesis.

Keywords: coalescence, *Dendrobates*, gene flow, phylogeography, population genetics

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Introduction

The eroded highlands of the Precambrian Guiana Shield represents the oldest geological formation in South America (Huber 1995). This region is located on the NE coast of South America and is bound on the west by the Rio Orinoco and Rio Negro, and in the south by the lower reaches of the Amazon (Fig. 1, inset). The Guiana Shield is inhabited by more than 272 recognized species of amphibian, of which no less than 47% are endemic (Huber & Foster 2003). While the Guiana Shield has been identified as a distinct geological and zoogeographical unit with an important role in the evolutionary history of numerous Neotropical groups, it has received little attention in recent evaluations of biogeographical patterns of South American taxa (but see Voss *et al*. 2001; Steiner & Catzeflis 2004; Noonan & Gaucher 2005).

Dendrobates tinctorius is the nominate species of the Neotropical family of poison frogs, the Dendrobatidae. Although this family has been the focus of much ecological and phylogenetic research there has yet to be a single study examining intraspecific phylogeography that covers a species entire distribution or any significant portion thereof.

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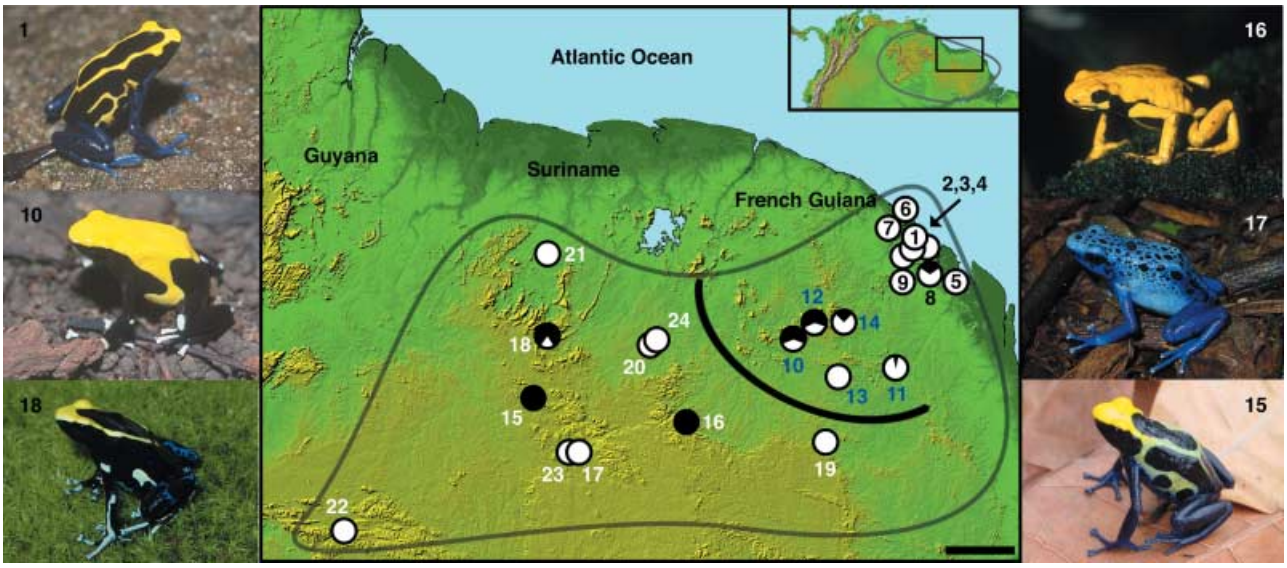

Fig. 1 Geographical sampling of *Dendrobates tinctorius* localities sampled for this study. Fill colour of circles indicates proportion of representation of W (black) and E (white) haplotype groups in each population. Numbers correspond to localities listed in Table 1. Color of numbers on map indicate populations that were assigned to W (white), C (blue) and E (black) for DIVA. Photographs illustrate interpopulation variation in phenotype with numbers corresponding to locality and represent populations with single or mixed haplotypic lineage representation. Solid black line on map indicates generalized demarcation between western and eastern regions discussed in text. Grey circles indicate distribution of Guiana Shield (inset) and *D. tinctorius*, respectively. Number 17 illustrates the population previously assigned to *Dendrobates azureus*. Scale bar = 70 km.

Dendrobates tinctorius occurs throughout the eastern portion of the Guiana Shield, including southeastern Guyana, Suriname, French Guiana, and extreme northeastern Brazil, where its occurrence appears to be strongly correlated with that of the patchily distributed uplands (200 m +) of this area. Although this species may be observed at elevations as low as 0 m, these instances are always in association with the slopes of nearby uplands. Much of this eastern portion of the Guiana Shield, particularly the coastal region, is comprised of lowland rainforest that experiences some level of seasonal inundation, and these areas appear to represent unsuitable habitat for *D. tinctorius*. The apparently patchy distribution of suitable habitat for *D. tinctorius* throughout its range is substantiated by extreme interpopulational phenotypic variation (Fig. 1) that has led to the description of some populations as distinct species (e.g. *Dendrobates azureus*, synonymized by Wollenberg *et al*., 2006). This drastic phenotypic variation observed within what is often considered a single biotic region provides an excellent backdrop for investigations into fine-scale population structure.

This examination of population structure within the Guianan region lends itself to interpretation in terms of phenomena predicted by the disturbance–vicariance hypothesis (DV). This hypothesis attempts to relate observed biogeographical patterns to historical climactic fluctuations. The DV hypothesis as first proposed by Colinvaux (1993), expanded by Bush (1994; Bush *et al*. 2002, 2005) and Mayle *et al*. (2004) and applied specifically to the Guiana Shield by Rull (2004a, 2004b, 2004c, 2005a, 2005b) invokes changes in temperature and atmospheric CO_2 levels associated with precessional cycles as the dominant factor influencing the historical continuity of Amazonian communities. This theory is founded on the observed palynological and palaeoclimactic record which indicates cyclic, altitudinal migration of floral communities throughout the Pleistocene. Presumably, this would have affected *D. tinctorius* by fragmenting the distribution in warmer periods when appropriate habitat migrates to higher altitudes, and reconnecting populations following migrations to lower elevations associated with subsequent cooling.

Within the eastern portion of the Guiana Shield, only a single phylogeographical study has been published that examines patterns at a scale similar to that examined here (Noonan & Gaucher 2005). Those results indicated that the largely contiguous uplands of the Guianan interior (south) harbour a lineage distinct from that inhabiting the fragmented uplands of north-central French Guiana (Fig. 1). Based on this evidence and the observed interpopulation phenotypic variation we considered and tested, via coalescent simulation and dispersal–vicariance analysis (DIVA), three alternative hypotheses of population history: (1) fragmentation of a widespread ancestor; (2) panmixia; and

(3) vicariance associated with refugial isolation in southern and northeastern regions. Support for (1) and (2) would be provided by an observed genealogy whose fit within an unresolved population tree (differing only in population divergence times) is consistent with the fit of trees simulated under a coalescent model within such a population tree and a poorly resolved DIVA (e.g. no unambiguous ancestral area[s]). A history of refugial isolation (3) would be supported by coalescent simulations in which the observed genealogy is a better fit to a population tree reflecting refugial groupings and a well-resolved DIVA (e.g. unambiguous ancestral areas consistent with proposed refugia). We would like to point out that our third hypothesis is not to be allied to the long-favoured Refuge hypothesis of Amazonian diversification first put forward by Haffer (1969, 1990, 1997), as the climactic factors implicated in this hypothesis have been demonstrated to be untenable (Mayle *et al*. 2004). We consider, rather, the possibility that *D. tinctorius* was subject to the same climactic factors that affected the observed genetic structure of the codistributed anuran *Atelopus* (Noonan & Gaucher 2005) that reflect habitat fragmentation within the Guianas due to temperature fluctuations (following the altitudinal migrations documented for floral communities) throughout the Pleistocene.

Materials and methods

Sampling and amplification

Samples of *Dendrobates tinctorius* from the Guianas were obtained directly through the fieldwork of the authors, or from borrowed/collaboratively collected material. Tissue grants were obtained from F. Catzeflis, P. Kok, B. Villette, and H. Claessen. Localities of samples are listed in Table 1 and illustrated in Fig. 1. Due to collection restrictions, some individuals are not represented by voucher specimens and were sampled by toe clipping. A total of 116 samples representing two outgroup species [one each of *Dendrobates leucomelas* and *Dendrobates galactonotus*; chosen based on results of recent analyses of *Dendrobates* phylogeny (Noonan & Wray 2006)] and 24 populations of *D. tinctorius* were

Table 1 Summary of within-population diversity of cytochrome *b* sequences from *Dendrobates tinctorius*. # column corresponds to population numbers in Fig. 1

Locality	#	Latitude and longitude	N_{ind}	N_{hap}	# Polymorphic sites	Gene diversity	π (100×)
D. tinctorius							
Roura	1	04 34′N, 52 12′W	3	3	4	1.0 ± 0.27	0.38 ± 0.34
Regina	2	04 20′N, 52 10′W	1	1	0	0	0
Mt. Favard	3	04 30′N, 52 02′W	2	2	2	1.0 ± 0.50	0.28 ± 0.34
Mt. Baugé	4	04 14′N, 52 13′W	2	1	0	0	0
Ouanary	5	04 12′N, 51 40′W	8	3	2	0.46 ± 0.20	0.07 ± 0.07
Petit Matoury	6	04 54′N, 52 21′W	6	2	1	0.53 ± 0.17	0.08 ± 0.07
Grand Matoury	7	04 52′N, 52 20′W	3	1	0	0	0
Mt. Kaw	8	04 29′N, 52 02′W	13	5	20	0.83 ± 0.06	1.25 ± 0.69
Nouragues	9	04 07′N, 52 40′W	1	1	0	0	0
Saul	10	03 37′N, 53 12′W	5	2	17	0.60 ± 0.17	1.44 ± 0.93
Lac Toponowini	11	03 02′N, 52 42′W	18	6	22	0.63 ± 0.12	0.53 ± 0.32
Pic Matecho	12	03 45′N, 53 02′W	6	3	22	0.73 ± 0.16	1.81 ± 1.10
Mt. Bakra	13	03 17′N, 52 56′W	2	2	1	1.0 ± 0.50	0.14 ± 0.20
Mt. Chauve	14	03 49′N, 52 45′W	4	2	22	0.50 ± 0.26	1.68 ± 1.16
Ellerts de Haan	15	03 05′N, 56 28′W	8	4	3	0.75 ± 0.14	0.14 ± 0.12
Mitaraka	16	02 16′N, 54 32′W	2	1	0	0	0
Tafelberg	18	03 47′N, 56 09′W	5	3	20	0.70 ± 0.22	1.12 ± 0.78
Mt. St. Marcel	19	02 23′N, 53 00′W	5	2	2	0.40 ± 0.24	0.12 ± 0.12
Pimba Creek	20	03 32′N, 54 59′W	1	1	0	0	0
Ralleighvallen	21	04 43′N, 56 12′W	4	2	1	0.50 ± 0.26	0.07 ± 0.09
Acarai	22	01 18′N, 58 45′W	1	1	0	0	0
Sipaliwini	23	02 01′N, 56 07′W	3	1	0	0	0
Tapanahony R.	24	03 33′N, 54 59′W	1	1	0	0	0
D. azureus							
Vier Gebroeders	17	02 00′N, 55 59′W	10	1	0	0	0

#, number corresponding to locality in Fig. 1; N_{ind}, number of individuals sampled for locality; N_{hap}, number of haplotypes observed within locality; π, nucleotide diversity (×100) ± 1 SD.

sampled (Fig. 1). Tissues were taken from toe clips, liver or muscle and preserved in 95% ethanol and then stored at –80 C prior to DNA extraction.

Genomic DNA was isolated using the QIAGEN DNeasy Tissue Kit according to the standard protocol. Problematic samples were extracted using the standard PCI/CI extraction method of Sambrook *et al*. (1989). Amplification of a 705-bp fragment of the mitochondrial cytochrome oxidase subunit *b* (cyt *b*) was carried out using the primers Dend-cbIF (5′ GCTTTCTCATCTGTAGCCCA 3′) — CytbAR-H (5′ TAWAAGGGTCTTCTACTGGTTG 3′). Amplifications were performed in 20- L reaction volumes using TaKaRa hotstart *Taq* DNA polymerase and 10 × reaction buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$]. Amplification was performed in a PTC-100 (MJ Research) 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 ethidium bromide stained, 1% agarose gels and purified using the QIAGEN QIAquick Gel Extraction Kit. The purified double-stranded products were used directly in 1/4 volume dideoxy-termination sequencing reactions using BigDye Terminator version 3.1 (Applied Biosystems). Unincorporated dye terminators were removed by precipitation with PelletPaint (Novagen) and EtOH/NaAcetate. Sequences were edited and aligned with SEQUENCHER version 4.1 (Gene Codes Corp) and checked by eye. Alignment was unambiguous with no apparent insertions or deletions.

Sequence analysis

Phylogenetic relationships among unique mtDNA haplotypes were estimated using PAUP* version 4.0b10 (Swofford 2001) employing maximum parsimony (MP) and maximum likelihood (ML) methods. An appropriate nucleotide substitution model for ML analysis was selected using the Akaike Information Criterion implemented in MODELTEST version 3.7 (Posada & Crandall 1998). Support for proposed clades was assessed via 2000 nonparametric bootstrap pseudoreplicates (MP and ML analyses) with the heuristic search option, tree-bisection–reconnection branch swapping (TBR) and 10 random taxon addition replicates for both analyses. DNASP version 4.10.4 (Rozas & Rozas 1995) was used to test for violations within the data set of the assumption of selective neutrality by implementing the McDonald–Kreitman test (McDonald & Kreitman 1991).

Genetic structure of cyt b *haplotypes*

Measures of gene, nucleotide, and haplotypic diversity were estimated for the cyt *b* data using the program ARLEQUIN 2.000 (Schneider *et al*. 2000) with Tamura & Nei (TrN) distances and gamma (= 0.452) correction (model selection from MODELTEST). The hierarchical structure of cyt *b* variation within *D. tinctorius* was examined via an analysis of molecular variance (AMOVA, Excoffier *et al*. 1992) utilizing the program ARLEQUIN 2.000 (Schneider *et al*. 2000) also with TrN distance and the aforementioned gamma correction in order to determine the amount of genetic variation observed in the sample attributable to within (Φ_{ST}) and among population variation (Φ_{CT}). Significance of results were assessed by running 20 000 random permutations.

We examined the relationship between haplotypes and geography using Nested Clade Analysis (NCA) by first using TCS version 1.13 (Clement *et al*. 2001) to compute a parsimony network that was subsequently nested according to the criteria of Templeton (1998). In order to infer geographical associations among haplotype clusters, clade (D_c) and nested clade (D_n) distances were measured as straight-line distances by GEODIS 2.2 (Posada *et al*. 2000). Historical factors influencing the evolutionary history of nested clades that demonstrated a significant association for haplotype and geography were inferred using the key of Templeton (2004; 14 July 2004 version).

Ancestral area reconstruction

In order to infer the ancestral distributions for each node of the haplotype phylogeny, DIVA was conducted using DIVA version 1.1 (Ronquist 1996). This event-based method reconstructs ancestral distributions without the need to specify, a priori, relationships among geographical areas utilizing a cost matrix in which speciation (presumably via vicariance) incurs no cost, and extinction and dispersal each incur cost penalties. Thus, the method assigns hypothetical distributions to the internal nodes of the specified topology in a manner that most effectively minimizes cost (dispersal and extinction events). This method has been widely applied to large-scale, higher-level analyses of biogeographical patterns, but has seen little use in studies of phylogeographical patterns within species. The ability of DIVA to account for the occurrence of haplotypes in multiple areas while recovering ancestral areas should prove most useful in such studies. Based on the topography of the Guianas, the observed distribution of mitochondrial haplotypes, and the results of previous study of a codistributed amphibian group (Noonan & Gaucher 2005), we chose to be conservative in our definition of regions (by over rather than underestimating the number of units) and designated three unit areas: western (W), central (C), and eastern (E) (Fig. 1). Increasing the number of regions considered leads to a greater chance of inconclusive or nonexclusive hypotheses of ancestral areas for the phylogenetic lineages of interest. Each of the 24 observed haplotypes was scored according to its observed

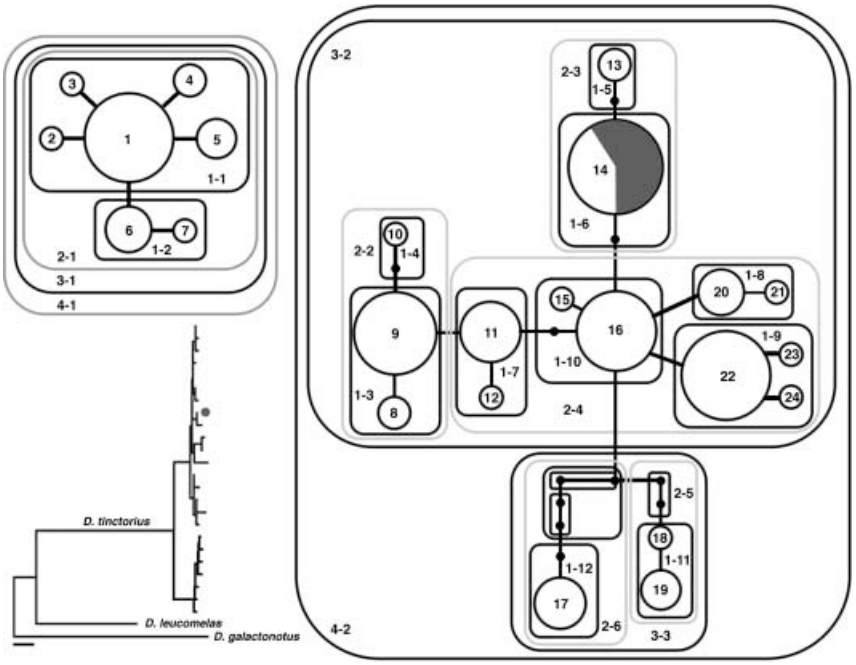

Fig. 2 Nested clade diagram for 24 unique haplotypes recovered from analysis of 114 *Dendrobates tinctorius* illustrating W (4-1) and E (4-2) groups discussed in text and phylogenetic hypothesis (ML) of relationships among *D. tinctorius* haplotypes. Shaded portion of haplotype 14 circle and shaded circle on tree correspond to individuals previously recognized as *Dendrobates azureus*. Scale bar = 0.01 substitutions/site.

presence or absence in each of these three geographical regions. Unconstrained optimization was carried out on the ML topology illustrated in Fig. 2.

Coalescent-based analysis of demography

In order to infer current and historical demographic parameters of *Dendrobates tinctorius*, we implemented Hey & Nielsen's (2004) (see also Nielsen & Wakeley 2001) isolation with migration model using the application IM. Using this method we were able to simultaneously estimate the effective female population size ($N_{ex} = \theta_x/2\ _G$; where x = W or E for the western and eastern regions, respectively, and $_G$ is the per-generation mutation rate) for each region (θ_W and θ_E defined by the grouping of populations west and east of the demarcation illustrated in Fig. 1), the ancestral population size (θ_A) before population splitting, the number of migrant females per generation (e.g. $M_{W\rightarrow E} = \theta_W m_W/2$), and the time, in years, since population divergence ($t = t/\ _L$; where t is the time parameter in terms of mutations), and $_L$ is mutation rate per locus per year. For these calculations we assumed a per-generation mutation rate ($_G$) of 5.28×10^{-5} (from Crawford 2003; generation time = 3 years; B.P.N., personal observation).

Five initial runs were used to obtain broad prior estimates of maximum parameter values that were specified in subsequent runs. All runs implemented Metropolis coupling over three chains with a single genealogy update per step and included a burn-in period of 500 000 steps. We ran four separate analyses with identical conditions but different random number seeds. Runs were considered complete when effective sample sizes (ESS) for each parameter exceeded 500.

Results

Phylogenetic relationships

More than 95% of the analysed 705-bp fragment of cyt *b* was obtained for all but eight of the 114 *Dendrobates tinctorius* individuals included in this analysis as well as the two outgroup taxa. Of these characters, 194 sites were variable with 77 being parsimony-informative. The McDonald–Kreitman test of selective neutrality revealed no support for selection on this locus. Within *D. tinctorius* 24 distinct haplotypes were observed that differed by as much as 3.4% (uncorrected distance, haplotypes 7 and 17). Maximum parsimony and ML analyses of the 24 *D. tinctorius* haplotypes and two outgroup species produced largely concordant topologies with relatively little support for patterns of relationship within haplotype groups. These analyses reveal a level of differentiation from the sister taxon, *Dendrobates leucomelas* (~14.5% uncorrected distance) that is in general accordance with observed levels of cyt *b* divergence between closely related species within this genus (Noonan & Wray 2006). The single haplotype observed in all sampled individuals of *Dendrobates azureus* (14, Fig. 2; population 17, Fig. 1) was also observed in the nearby populations of Sipaliwini and Ellerts de Haan (populations 23 and 3, respectively, Fig. 1) making the recovery of a distinct *D. azureus* impossible with this data set. This is consistent with the recent synonymy of *D.*

Table 2 Geographic distribution of 24 cytochrome *b* haplotypes observed in *Dendrobates tinctorius*. Vertical line indicates division between W and E group haplotypes, horizontal line indicates geographical division between E and W populations

#	Locality	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	Roura											**1**									**1**		**1**		
2	Regina														**1**										
3	Mt. Favard														**1**		**1**								
4	Mt. Bagué			**2**																					
5	Ounary																						**6**	**1**	**1**
6	Petit Matoury																**4**						**2**		
7	Grand Matoury																**3**								
8	Mt. Kaw	**4**															**2**				**3**	**1**	**3**		
9	Nouragues	WEST in East														EAST							**1**		
10	Saul	**3**										**2**													
11	Lac Toponowini	**1**							**2**	**11**	**1**					**1**	**2**								
12	Pic Matecho						**3**	**1**										**2**							
13	Mt. Bakra																**1**						**1**		
14	Mt. Chauve						**1**											**3**							
15	Ellerts de Haan	**4**	**1**		**2**	**1**																			
16	Mitaraka					**2**																			
17	Vier Gebroders														**10**										
18	Tafelberg	**3**		**1**											**1**										
19	Mt. St. Marcel	WEST										**4**	**1**			EAST in West									
20	Pimba Creek													**1**											
21	Ralleighvallen																		**1**	**3**					
22	Acarai														**1**										
23	Sipaliwini														**3**										
24	Taponahony R.													**1**											

Clade	χ^2	Probability	Inference chain	Inferred pattern
2-1	27.0	0.00	1-2-3-4-NO	RGF w/IBD
2-4	78.07	0.00	1-3-4-NO	RGF w/IBD
3-2	136.51	0.00	1-2-11-Yes	Contiguous Range Expansion
3-3	9.00	0.00	1-19-20-NO	Inadequate Sampling
4-2	1.00	0.00	1-2-11-17-NO	Inconclusive
Total	66.42	0.00	1-2-11-17-NO	Inconclusive

Table 3 Nested contingency results based on 10 000 permutations in GEODIS of clades exhibiting a significant association for haplotype and geography. Inferences were made with the 14 July 2004 key available from David Posada's website (http://darwin.uvigo.es)

azureus with *D. tinctorius* (Wollenberg *et al*. 2006). Hereafter, references to *D. tinctorius* will refer to *D. tinctorius* and *D. azureus*. Within *D. tinctorius*, resolution of phylogenetic relationships among haplotypes was poor due to the generally low level of intraspecific differentiation. However, all analyses strongly support a distinct split between two well-supported clades (MP bootstrap = 92% for both) comprised of haplotypes 1–7 (ML = 93%) and 8–24 (ML = 89%), respectively (Fig. 2). Uncorrected levels of sequence divergence between these two groups range from 2.3% to 3.4% and are within the levels commonly observed among conspecifics. Within haplotype groups, pairwise sequence divergence was much greater in the 8–24 lineages (0.14–1.9%) than within the 1–7 lineages (0.1–0.6%). Interestingly, the distributions of these two haplotype lineages overlap extensively, and in seven instances occur in the same population (Fig. 1).

Population structure

Two separate haplotype networks were recovered by TCS based on cyt *b* sequences of 114 individuals of *D. tinctorius* from 24 populations (Fig. 2, Table 2). These two networks correspond to the well-supported clades recovered by phylogenetic analysis (Fig. 2). Haplotype group 1–7 [hereafter referred to as the western (W) group] appears to be less common in that it was observed in fewer populations, yet its geographical distribution spans nearly the entire sampled area. Haplotype group W does appear to be quite uncommon in the extreme eastern portion of the range of *D. tinctorius* with only 5 of 55 (9%) individuals east of the Mount Chauve population (# 14, Fig. 1) possessing a W group haplotype, compared to 22 of 59 (37%) west of (and including) this locality. The low levels of genetic divergence within the W group observed in the phylo-

genetic analysis are reflected by the low haplotypic differentiation as no two haplotypes are separated by more than three steps. Haplotype group 8–24 [hereafter referred to as the eastern (E) group] is also widely distributed, yet harbours a great deal more haplotypic diversity with a greater amount of differentiation than group W. Within this group, haplotypes are separated by as many as 11 steps (haplotypes 17 and 10, Fig. 2). Within populations, as many as six haplotypes were observed (Lac Toponowini; Table 2), and single haplotypes were distributed across as many as six populations and > 500 km (haplotypes 14, 16, 22). Nucleotide diversity for all 24 populations ranged from 0.0007 to 0.018 (mean = 0.007; Table 1) and was highest within the populations in which both W and E haplotype groups were represented. Results of AMOVA indicate that the observed genetic variation is due to significant variation within (55.8%) and among (44.2%) populations. Nested clade analysis revealed significant support for restricted gene flow with isolation by distance for clades 2-1 and 2-4, and contiguous range expansion for clade 3-2 (Table 3, Fig. 2).

Ancestral area reconstruction

Our phylogenetic hypothesis of haplotype relationships suggests two distinct, historic lineages, although their current overlapping distribution complicates phylogeographical interpretation to some extent. However, despite the tendency for DIVA to be characterized by reduced precision in assignment of historical distributions near the root node, the two haplotype lineages recovered in our analysis were unequivocally recovered as originating in the W and E regions of the Guianas (Fig. 3). It should be noted that although Fig. 3 represents the singlemost parsimonious tree, the support for the interrelationships of the three main lineages of the H8–H24 group were not strongly supported. Placement of the western lineage of this group (corresponding to haplotypes 13,14,17–19) as sister to the other two lineages would result in an ambiguous E/W inferred ancestral area with a W origin of clades 2-3 and 3-3 (Fig. 2) and an E origin of 2-2 and 2-4. This alternative pattern would still support an eastern origin of the majority of haplotypes in clade 4-2 (Fig. 2) including haplotype 16, the central and inferred ancestral haplotype of this clade.

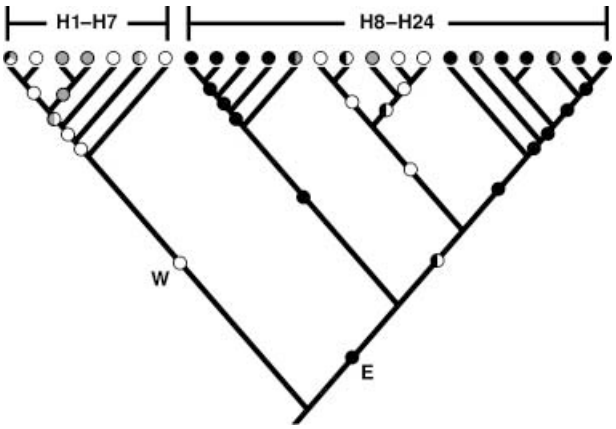

Fig. 3 Reconstructed ancestral areas for each internal node of the ML phylogeny using DIVA. The three regions specified are indicated by the coloured fill of each circle as follows: white = West; grey = central French Guiana mountains (populations 2, 17, 17, 18); black = East. Haplotype group 4-1 (Fig. 2) is unambiguously recovered as a western lineage, and Haplotype group 4-2 (Fig. 2) as associated with the eastern region.

Coalescent analysis of demography

Demographic parameters estimated for W and E geographical groups of populations indicate that the Eastern region has a much larger female effective population size [$N_{e\text{East}}$ = 204 000; 90% highest posterior density interval (HPD) 115 000–335 000] than the Western region ($N_{e\text{West}}$ = 81 000; 90% HPD 28 000–175 000). Divergence time isolation between these two regions indicates an early Pleistocene [t = 0.6 – 1.3 million years ago (Ma); results from multiple, independent runs converged on values within this range although HPD intervals were large and the distributions did not approach 0], temporally congruent with the findings of Noonan & Gaucher (2005) for the codistributed *Atelopus*, as well as the timing of isolation of Guianan rattlesnakes (Wüster *et al*. 2005). Migration rates suggest that gene flow is unidirectional ($M_{W\rightarrow E}$ = 3.87; $M_{E\rightarrow W}$ = 0.05), as is the case in *Atelopus* (Noonan & Gaucher 2005).

Tests of lineage sorting

The observation of broad sympatry of divergent lineages has been categorized by Avise *et al*. (1987) and Avise (2000) as a category II pattern. Avise suggests that this pattern is the result of either a species with a large effective population size and high levels of gene flow or, more commonly, secondary contact between divergent lineages. Based on the observed phylogeographical structure of genetic variation within *D. tinctorius* we tested for a genetic signature consistent with incomplete lineage sorting resulting from fragmentation of a widespread ancestor or panmixia against a null hypothesis of historical fragmentation and recent secondary contact. This possibility of the observed pattern resulting from fragmentation of a widespread ancestor is supported by the potential for retained ancestral polymorphism given the observed divergence time of ~500 000 generations is far less than the predicted time to monophyly ($4N_e$ generations; Rosenberg 2003 and references therein) of isolated populations. We therefore implemented the gene-tree population-tree method of Knowles (2001) and Knowles & Maddison (2002) with

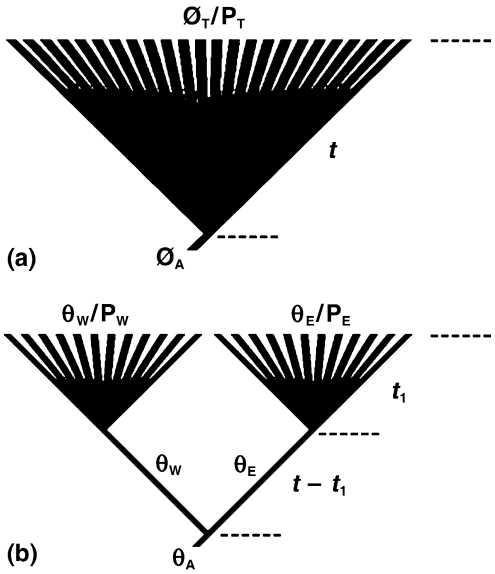

Fig. 4 Population trees used in coalescent simulation tests of incomplete lineage sorting using MESQUITE. Branch widths are indicated by specific θ values that are discussed in the text. Terminal branch widths sum to their representative θs (e.g. θ_W/P_W indicates that the width of each branch is the total θ for the W group divided by the number of W populations; θ_A is the ancestral population size), and branch lengths sum to *t* (in generations).

coalescent simulations to test the fit of the data to models of panmixia and incomplete lineage sorting. In order to do this we specified a population tree reflecting the fragmentation of a widespread ancestor (Fig. 4a) in which *t* is specified as either ~0 (panmixia) or as equal to the empirical estimate of divergence time obtained from isolation with migration (IM) analysis. Gene trees were simulated by neutral coalescence within this population tree with an empirical estimate of divergence times (as measured in generations) and population sizes (N_e; both obtained from IM) using the program MESQUITE version 1.06 (Maddison & Maddison 2004). Simulated gene trees were then fit to the alternative population model of refugial isolation (Fig. 4b). The discord between the simulated gene trees and the specified population model was measured as the failure of the gene trees to coalesce within population lineages or the deep coalescence of gene lineages using the *s* statistic of Slatkin & Maddison (1989). We then compared the discord (*s*) obtained by containing the observed gene tree within the fragmentation model (Fig. 4b) to the distribution of *s* values from simulated trees.

All gene trees were simulated within a population tree with an ancestral population size of N_e = 248 000 (θ_A = 26.2) applied to the root node, and E and W specific population sizes assigned based on empirical estimates obtained from IM analysis ($N_{e\text{East}}$ = 204 000, θ_{East} = 21.6; $N_{e\text{West}}$ = 81 000, θ_{East} = 8.6) with those totals divided equally among the respective terminals. For the recovered gene tree we computed Slatkin & Maddison's *s* = 36. The discord predicted by coalescent simulation was entirely congruent with the population model of refugial isolation (average *s* = 35.71) and we were able to reject hypotheses of fragmentation of a widespread ancestor and panmixia at α = 0.05.

Discussion

Phylogeographical structure and demographic history

Given the assumption that these two lineages are now freely introgressing, the focus turns to the demographic history of each lineage. The results of NCA suggest that clade 3-2 (Fig. 2) has undergone contiguous range expansion (Table 3). Further support for expansion within the E lineage is provided by DIVA that indicates the lineage comprised of haplotype groups 2–3 and 2–5 are unambiguously associated with the western region of the Guianas (Fig. 3). Indeed, outside these two clades, no other E haplotype is found outside the central-northern French Guianan region. This suggests that the current phylogeographical pattern reflects an older, Pleistocene, westward expansion of the E group rather than current expansion/connectivity. This conclusion is supported by the near absence of westward gene flow recovered by coalescent analysis ($M_{E\rightarrow W}$ = 0.05).

The low level of genetic diversity within the W haplotype group and the NCA inference of restricted gene flow with isolation by distance suggest a very different demographic history for this haplotype lineage. The single-nested haplotype (1) is widely distributed, occurring in central Suriname and central and coastal French Guiana (localities 8,9,10,15,18; > 500 km). DIVA suggests this lineage arose in the western portion of the Guianas and is thus associated with the generally contiguous highlands of the Guianan interior (Fig. 1). Combined with the high eastward migration rate recovered ($M_{W\rightarrow E}$ = 3.87), this suggests the presence of this lineage in the central and coastal mountain regions of French Guiana is due to recent dispersal rather than retained ancestral polymorphism.

Coalescent simulations are entirely consistent with isolation in western and eastern refugia. Based on these findings and the observed distribution of the two haplotype groups and estimates of gene flow, it appears that secondary contact between two previously isolated lineages has occurred. This mixture of lineages is also reflected in the AMOVA results, which indicate that the majority of genetic variation observed (55%) is due to within population variation. Clearly demonstrating secondary contact in intraspecific phylogeography using mitochondrial data is difficult, yet we feel that the combined results of DIVA,

NCA, and coalescent analyses provide a clear picture of two historically separated lineages that have recently or currently are expanding and are now broadly codistributed.

Guianan biogeography

While there have only been two other published studies of phylogeographical structure within the Guianas (Steiner & Catzeflis 2004; Noonan & Gaucher 2005), the results presented here do illustrate a common pattern. Both amphibian taxa studied to date (*Dendrobates*, this study; *Atelopus*, Noonan & Gaucher 2005) demonstrate a biotic break between the southern upland block and the fragmented mountains of central and northeastern French Guiana (Fig. 1). Interestingly, both of these species show evidence of current/recent east to west gene flow across this divide, although this mixing appears to be far greater in *Dendrobates*. The phylogeographical patterns observed within *D. tinctorius* support the conclusions of Noonan & Gaucher (2005) and Mayle *et al*. (2004) that the Guianan Shield (particularly this Eastern portion) did not serve as a single, contiguous refuge surrounded by inhospitable grassland/savannah throughout the Quaternary. Furthermore, factors affecting the observed split between W and E groups predate the Holocene and the Last Glacial Maximum and are congruent with the estimated timing of divergence across this divide in the similarly distributed Guianan *Atelopus* (Noonan & Gaucher 2005). Secondary contact between these refugia appears to have occurred more than once (older westward expansion of E group and recent eastward expansion of W group) and may have been driven by precessional cycles that have occurred at regular intervals for relatively short periods of time (~11 000 years) and appear to have superceded the effects of Pleistocene temperate glaciation within Amazonia (Bush *et al*. 2002; Bush 2005) and the Guianan region specifically (Rull 2004a, 2004b, 2004c, 2005a, 2005b). Indeed, increased aridity does appear to have affected biotic distributions but was not associated with glacial cycles but rather with these precessional variations in insolation, nor was it the sole factor as fluctuations in temperature and atmospheric concentration of CO_2 were also associated with these cycles and presumably affected habitat continuity. This combination of factors, with aridity merely a contributor rather than the driving force behind habitat fragmentation is the main distinction between the Refuge and DV hypothesis. Indeed, were increased aridity the primary contributor to the fragmentation of suitable forest habitat, one would predict these forests would concentrate around lowland sources of water such as the numerous rivers throughout the region. However, if instead temperature were the main contributing factor, one would expect upward altitudinal migration with increasing temperature (congruent with the observed current distributional fidelity of *D. tinctorius* and *Atelopus* to the Guianan uplands).

Conservation implications

The findings presented here, in conjunction with those of Noonan & Gaucher (2005) strongly argue for the recognition of north-central French Guiana as a biotic region distinct from the remainder of the eastern block of the Guiana Shield. Current conservation efforts focus on the formation of an enormous national park including nearly the entire southern half of this country. Our findings suggest that such a park, including the central mountain region, would include a great deal of genetic diversity by covering both western and eastern biotic regions. However, it is increasingly apparent that the coastal ranges of French Guiana are likely to represent "the 'cradle' " of diversity for this eastern region. This is alarming as this region is essentially the only place in which *D. tinctorius* and human distributions overlap. Recent completion of a highway between the capital of French Guiana (Cayenne) and Oiapoque (Brazil) bisects this region and highlights the need for foresight in the designation of protected areas in this coastal region in order to mitigate the adverse effects of a burgeoning human population.

Conclusions

Phylogenetic analysis of *Dendrobates tinctorius* recovered two distinct haplotype lineages that overlap extensively in their geographical distribution. DIVA suggests an allopatric origin for these lineages in separate western and eastern areas consistent with the distributional pattern of the codistributed *Atelopus* (Noonan & Gaucher 2005). Genetic evidence suggests a relatively old separation of these W and E lineages concordant with the observed divergence of *Atelopus* (0.5–1.5 Ma) and is consistent with the isolation of Guianan snakes (~1 Ma; Wüster *et al*. 2005). These findings demonstrate the complexity of the Guianan fauna and the dangers of oversimplifying Neotropical biogeography into general biotic realms (e.g. Guiana Shield) which misrepresent the effects of a complex climactic history on the diversity within these areas.

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References

Avise JC (2000) *Phylogeography: the History and Formation of Species*. Harvard University Press, Cambridge, Massachusetts.

Avise JC, Arnold J, Ball Jr RM *et al.* (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecological Systematics*, **18**, 489–522.

Bush M (1994) Amazonian speciation: a necessarily complex model. *Journal of Biogeography*, **21**, 5–17.

Bush M (2005) Of orogeny, precipitation, precession and parrots. *Journal of Biogeography*, **32**, 1301–1302.

Bush MB, Miller MC, De Oliveira PE, Colinvaux PA (2002) Orbital forcing signal in sediments of two Amazonian lakes. *Journal of Paleolimnology*, **27**, 341–352.

Clement X, Posada D, Crandall K (2001) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1659.

Colinvaux P (1993) Pleistocene biogeography and diversity in tropical forests of South America. In: *Biological Relationships Between Africa and South America*, **16**, 473–499. (ed. P Goldblatt). Yale University Press, New Haven, Connecticut.

Crawford AJ (2003) Huge populations and old species of Costa Rican and Panamanian dirt frogs inferred from mitochondrial and nuclear gene sequences. *Molecular Ecology*, **12**, 2525–2540.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial restriction data. *Genetics*, **131**, 479–491.

Haffer J (1969) Speciation in Amazonian forest birds. *Science*, **165**, 131–137.

Haffer J (1990) Geoscientific aspects of allopatric speciation. In: *Vertebrates in the Tropics* (eds Peters G, Hutterer R), pp. 45–60. Museum A. Koenig, Bonn, Germany.

Haffer J (1997) Alternative models of vertebrate speciation in Amazonia: an overview. *Biodiversity and Conservation*, **6**, 451–476.

Hey J, Nielsen R (2004) Multilocus methods for estimating population sizes, migration rates and divergence time, with applications to the divergence of *Drosophila pseudoobscura* and *D. persimilis*. *Genetics*, **167**, 747–760.

Huber O (1995) Geographical and physical features. In: *Flora of the Venezuelan Guayana, Volume 1: Introduction* (eds Berry P, Holst BK, Yatskievych K), pp. 1–51. Missouri Botanical Garden Press, St. Louis, Missouri.

Huber O, Foster MN (eds) (2003) *Conservation priorities for the Guayana Shield*. Conservation International, Washington.

Knowles LL (2001) Did the Pleistocene glaciations promote divergence? Test of explicit refugial models in Montane grasshoppers. *Molecular Ecology*, **10**, 2623–2635.

Knowles LL, Maddison WP (2002) Statistical phylogeography. *Molecular Ecology*, **10**, 2623–2635.

Maddison WP, Maddison DR (2004) *MESQUITE: a Modular System for Evolutionary Analysis*, version 1.06b1. available at http://mesquiteproject.org.

Mayle FE, Beerling DJ, Gosling WD, Bush MB (2004) Responses of Amazonian ecosystems to climactic and atmospheric carbon dioxide changes since the Last Glacial Maximum. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, **359**, 499–514.

McDonald JH, Kreitman M (1991) Adaptive protein evolution at the *Adh* locus in *Drosophila*. *Nature*, **352**, 652–654.

Nielsen R, Wakeley J (2001) Distinguishing migration from isolation. A Markov chain Monte Carlo approach. *Genetics*, **158**, 885–896.

Noonan BP, Gaucher P (2005) Phylogeography and demography of Guianan harlequin toads (*Atelopus*): diversification within a refuge. *Molecular Ecology*, **14**, 3017–3031.

Noonan BP, Wray KP (2006) Neotropical diversification: the effects of a complex history on diversity within the poison frog genus *Dendrobates*. *Journal of Biogeography*, **33**, 1007–1020.

Posada D, Crandall KA (1988) MIDELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Posada D, Crandall KA, Templeton AR (2000) GEODIS: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, **9**, 487–488.

Ronquist F (1996) *DIVA version 1.1. Computer program and manual available by anonymous FTP from Uppsala University* (ftp.uu.se or ftp.systbot.uu.se).

Rosenberg NA (2003) The shapes of neutral gene genealogies in two species: probabilities of monophyly, paraphyly, and polyphyly in a coalescent model. *Evolution*, **57**, 1465–1477.

Rozas J, Rozas R (1995) DNASP, DNA sequence polymorphism: an interactive program for estimating population genetics parameters from DNA sequence data. *Computer Applications in Bioscience*, **11**, 621–625.

Rull V (2004a) Biogeography of the'Lost World': a palaeoecological perspective. *Earth-Science Reviews*, **67**, 125–137.

Rull V (2004b) An evaluation of the Lost World and Vertical Displacement hypotheses in the Chimant· Massif, Venezuelan Guayana. *Global Ecology and Biogeography*, **13**, 141–148.

Rull V (2004c) Is the 'Lost World' really lost? Palaeoecological insights into the origin of the peculiar flora of the Guayana Highlands. *Naturwissenschaften*, **91**, 139–142.

Rull V (2005a) Palaeovegetational and palaeoenvironmental trends in the summit of the Guaquinima massif (Venezuelan Guayana) during the Holocene. *Journal of Quaternary Science*, **20**, 135–145.

Rull V (2005b) Biotic diversification in the Guayana Highlands: a proposal. *Journal of Biogeography*, **32**, 921–927.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schneider S, Roessli D, Excoffier L (2000) *ARLEQUIN version 2.000: A Software for Population Genetics Data Analysis*. Genetics and Biometry Laboratory. University of Geneva, Geneva, Switzerland.

Slatkin M, Maddison WP (1989) A cladistic measure of gene flow inferred from phylogenies of alleles. *Genetics*, **123**, 603–613.

Steiner C, Catzeflis FM (2004) Genetic variation and geographical structure of five mouse-sized opossums (Marsupialia, Didelphidae) throughout the Guiana Region. *Journal of Biogeography*, **31**, 959–973.

Swofford DL (2001) *PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods)*, version 4b10. Sinauer, Sunderland, Massachussets.

Templeton AR (1998) Nested clade analysis of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology*, **7**, 381–397.

Templeton AR (2004) Statistical phylogeography: methods of evaluating and minimizing inference errors. *Molecular Ecology*, **4**, 789–809.

Voss RS, Lunde DP, Simmons NB (2001) The mammals of Paracou, French Guiana: a Neotropical lowland rainforest fauna. Part 2. Nonvolant species. *Bulletin of the American Museum of Natural History*, **263**, 1–236.

Wollenberg K, Noonan BP, Lötters S (2006) Polymorphism versus species richness. Systematics of large *Dendrobates* from the eastern Guiana Shield (Amphibia: Dendrobatidae). *Copeia*, in press.

Wüster W, Ferguson JE, Quijada-Mascareñas A, Pook CE, Salomao Mda G, Thorpe RS (2005) Tracing an invasion: landbridges, refugia, and the phylogeography of the Neotropical rattlesnake (Serpentes: Viperidae: *Crotalus durissus*). *Molecular Ecology*, **14**, 1095–1108.

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