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Phylogeography and demography of Guianan harlequin toads (*Atelopus*): diversification within a refuge

BRICE P. NOONAN* and PHILIPPE GAUCHER†
*University of Texas at Arlington, Department of Biology, Box 19498, Arlington TX 76109, †Mission pour la creation du parc de la Guyane, BP 275, 97326 Cayenne cedex, France

Abstract

We investigated the genetic structure of populations of Guianan harlequin toads (genus *Atelopus*) and their evolutionary affinities to extra-Guianan congeners. Phylogenetic analysis of mitochondrial cytochrome *b* (cyt *b*) and NADH dehydrogenase subunit 2 (ND2) gene sequences produced well-supported clades largely corresponding to the four recognized taxa in the Guianas (*Atelopus spumarius hoogmoedi, Atelopus spumarius barbotini, Atelopus franciscus,* and *Atelopus flavescens*). Our findings suggest that the Guianan *A. spumarius* represent distinct evolutionary lineages that merit distinction from Amazonian conspecifics, and that the status of *A. flavescens* and *A. franciscus* is somewhat less clear. Approximately 69% of the observed genetic variation is accounted for by differences between these four recognized taxa. Coalescent-based estimates of gene flow between taxa suggest that these lineages are largely isolated from one another. Negligible rates of migration between populations and significant divergence within such close proximity suggests that although the region inhabited by these taxa is almost entirely undisturbed, significant habitat heterogeneity exists as to have produced a remarkable diversification of *Atelopus* within the eastern Guiana Shield. These results contradict the commonly held view of the Guiana Shield as a 'refuge' whose stability during late Tertiary and Quaternary climatic fluctuations served as a biotic reservoir. Instead, we provide evidence that climatic fluctuations during this time had a diversifying effect within the Guianan region.

Keywords: *Atelopus*, gene flow, phylogeography, Pleistocene refugia, speciation

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Introduction

Toads of the genus *Atelopus* have long attracted the attention of biologists working in the Neotropics. Their bright colouration and diurnal habit make them a conspicuous component of the vertebrate fauna. There are currently 72 recognized species of *Atelopus*. The great majority of these species are restricted to the highlands of Peru, Ecuador, Colombia and Venezuela. Scientific exploration of these highlands through the 1980s continued to produce new species, after which time the rate of discovery declined rapidly.

It is now clear *Atelopus* were particularly hard hit by the recent amphibian declines. There have been numerous reports of probable extinction of *Atelopus* with most affected species being restricted to highland regions (La Marca & Reinthaler 1991; Vial & Saylor 1993; Pounds & Crump 1994; Durant & Arellano 1995; La Marca 1995; Lötters 1996; La Marca & Lötters 1997; Ron *et al*. 2003). Of the 13 species for which there are published reports of significant declines or probable extinction, 10 of them live exclusively above 2000 m. As a rule, these highland species of *Atelopus* have very limited distributions. No single species has a distribution that spans more than 500 km straight-line distance, and most are known from single or a very few localities (Lötters 1996). Conversely the Amazonian *Atelopus spumarius* ranges from *cis*-Andean Ecuador and Peru, along the Amazon River, to the Guianas and the Brazilian state of Amapa (Fig. 1).

Although a great deal of effort has been aimed at determining exactly what these frogs ought to be named (confounded by loss of types and description of dubious

Correspondence: B. P. Noonan, Brigham Young University, Integrative Biology, 501 WIDB, Provo, UT 84602. Fax: 801-422-0090; E-mail: noonan@byu.edu

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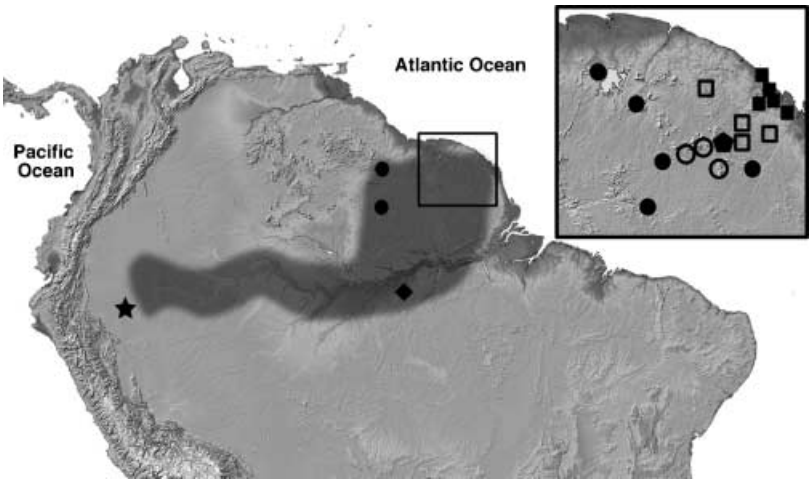

Fig. 1 Approximate geographical distribution of *Atelopus spumarius* (shaded area) with sampled localities: *Atelopus pulcher*, star; *Atelopus spumarius s.l.*, diamond; *Atelopus s. hoogmoedi*, solid circles; *Atelopus spumarius barbotini*, open circles; *Atelopus franciscus*, open squares; *Atelopus flavescens*, closed squares; Pic Matecho (*Atelopus vermiculatus*), closed pentagon.

subspecific forms), little effort has been directed at using quantitative methods in the delimitation of species of this genus and the clarification of relationships with presumed closely related species. Indeed, neither the entire genus nor any subset has ever been the subject of a serious phylogenetic investigation. This is likely due, at least in part, to the aforementioned recent declines and the lack of readily available material (especially tissues).

Three species of *Atelopus* are found in Amazonia (including the Guianas), namely the widespread *A. spumarius*, and two taxa that are restricted to NE French Guiana (*Atelopus flavescens* and *Atelopus franciscus*). Numerous subspecific names have been ascribed to certain populations of *A. spumarius* throughout its range. Recent work has seen various taxa placed in, and removed from synonymy with *A. spumarius* as well as the suggestion that some subspecies actually represent variants of a different species altogether (Kok 2000). *Atelopus spumarius* was originally described from the upper Amazon basin of Peru (Cope 1871), although the material has been lost (Rivero 1968; Peters 1973). When Lescure (1981) designated a neotype for this taxon, *Atelopus pulcher*, a species known from an area some 600 km to the southwest of the type locality, was synonymized with *A. spumarius*. Lötters *et al.* (2002) recently provided evidence that *A. pulcher* represented a distinct species and thus resurrected this name. Populations further south in the Peruvian states of San Martin and Loreto were described by Rivero (1968) as *Atelopus spumarius andinus*. Lötters & de la Riva (1998) provided evidence that this taxon too should be recognized as a full species. Lescure (1981) also described a subspecies (*Atelopus spumarius barbotini*) to represent forms known from central French Guiana. Examination of advertisement call data has been used to suggest that animals referable to this taxon are actually more closely related to the northeastern French Guianan *A. flavescens* (Kok 2000). Perhaps most confusing of all, Lescure [1973 (1974)] described *Atelopus spumarius hoogmoedi* from the southern region of French Guiana and applied this name to populations in Guyana and Suriname (Lescure 1973). Lescure & Gasc (1986) subsequently indicated that this taxon did not merit distinction from the nominate form of this species, yet Lescure later recognizes this taxon with no explanation (Lescure & Marty 2000). Lötters *et al.* (2002) includes these animals in '*A. spumarius sensu lato*' and suggest that they may represent a distinct species (*A. hoogmoedi*).

The distributions of *A. franciscus* and *A. flavescens* are much more localized, with each restricted to the coastal region of eastern French Guiana (Fig. 1). Phenotypic variation among populations of *A. franciscus* is presumably quite low, and they appear to lack any aposematic colouration whatsoever (Fig. 2). *Atelopus flavescens* on the other hand is very brightly coloured, and although most populations are very similar in appearance (Fig. 2) the synonymization of *Atelopus vermiculatus* (Fig. 2) introduces significant phenotypic differentiation from the 'typical'. Populations of *Atelopus* referable to *A. vermiculatus* [hereafter referred to by the lone locality (Pic Matecho) sampled for this study as this taxon is no longer recognized as valid] are geographically intermediate between *A. s. barbotini* and *A. franciscus* (Fig. 1).

Interestingly, the distribution of these animals throughout the Guianas, as it is currently understood, is highly fragmented. Although commonly found at very low elevations (*c.* 10 m) they seem to be restricted to forests associated with 'significant' topographical relief. Indeed, their absence from lowland forest is striking when one considers that the highlands that appear to be prerequisite are commonly less than 300 m. This pattern has also been observed for the broadly sympatric poison frog *Dendrobates tinctorius* (BPN, personal observation), which is also a diurnal, aposematic species. Our knowledge of the distribution of

A. spumarius in Amazonian Brazil is very poor, and it is unknown if their distribution exhibits a similar altitudinal requirement. The fragmented nature of the distribution of these animals in the Guianas has undoubtedly contributed to the phenotypic diversification that has occurred within and among what are assumed to be three closely related species.

The apparent extension of the primarily Andean *Atelopus* lineage into Amazonia and the Guianas (Lynch 1993), coupled with distributional fidelity to the Guianan uplands follow the patterns predicted by Bush's (1994) Disturbance-Vicariance hypothesis (DV). Bush argues that a complex combination of factors, including fluctuating temperatures, a reduction in atmospheric CO_2, and localized, moderate reduction of precipitation [rather than an Amazonia-wide, significant increase in aridity as suggested by Haffer (1969)] associated with Pleistocene glacial events, of which there were at least 10 (Hooghiemstra 1984, 1989), were primarily responsible for Amazonian diversification. These temperature fluctuations (± 7.5°) are presumed to have facilitated the eastward spread of cool-adapted Andean taxa into Amazonia (as temperatures cooled, the Amazon basin became more suitable for taxa that were previously restricted to higher altitudes). This is in direct contrast to the predictions of the Refuge hypothesis (Haffer 1969) which suggests that reduced precipitation in the Tertiary resulted in the fragmentation of the Amazonian forest into numerous isolated refugia. The DV hypothesis also suggests that precipitation may have periodically been reduced by as much as 20% during this time. However, unlike the Refuge hypothesis which divides the Amazonian forest into numerous forest islands, this reduction in precipitation is predicted to have fragmented the South American rainforest cover into two large fragments (western Amazonian/Andean and Guianan coastal zones, Fig. 3 of Bush 1994). Furthermore, significant fragmentation of populations (not forest cover in general) of cooler adapted species within these forested regions is expected to have resulted from these glacial cycles and associated temperature fluctuations. As temperatures increased, the distributions of these Andean, cool-adapted species would retract to nearby highlands. The few members of the primarily Andean genus *Atelopus* inhabiting the patchily distributed mountains of the eastern Guiana Shield provide an excellent opportunity to test the predictions of the DV hypothesis. These predictions would lead us to expect genetic lineages of *Atelopus* to correspond with generally contiguous or at least proximate uplands and date to the Quaternary. We would also expect little gene flow among those upland regions due to the inhospitably warm temperatures of the intervening lowland forest.

The phenotypic diversity among populations of Guianan *Atelopus* provides an interesting backdrop upon which to test for Pleistocene differentiation. Such phenotypic diversity between geographically proximate populations of the same or even closely related species is surprising, especially so given the bright colours that characterize these frogs are presumed to act as a warning to potential predators of the unpalatability of the animal. The assumption that the bright colouration serves as a warning is supported by the observation of unken reflex (arching of the back to reveal ventral flash colouration) behaviour (Rodríguez & Duellman 1994; BPN, personal observation) and the presence of a significant amount of tetrodotoxin in *A. spumarius* (Daly *et al*. 1994).

Previous studies of *Atelopus* have focused on taxonomy, toxicity, and ecology (including population declines) while little effort has been put towards using molecular methods to make inferences about the evolutionary history of these animals. Herein we examine mitochondrial gene sequences from numerous populations throughout Amazonia, with particularly intensive sampling in the Guianas to examine (i) phylogenetic relationships and their bearing on taxonomy, (ii) evolutionary history (demography, age and origins) of Guianan (Guyana, Suriname, French Guiana) animals, and (iii) phylogeographical patterns within the Guianas, with particular reference to the effect of potential barriers to dispersal and the implications in terms of conservation priorities.

Materials and methods

Sampling and amplification

Samples of *Atelopus* 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, M. Donnelly, P. Kok, M. Blanc, Smithsonian Institution, Louisiana Museum of Natural History, and University of Kansas Museum of Natural History. Localities of samples are listed in Tables 1 (Guianan) and S3 (Supplementary material, all others). A total of 85 individuals representing four outgroup species ($n = 6$) and all described Guianan forms were sampled. Tissues were taken from toe clips, liver or muscle and preserved in 95% ethanol and then stored at –80 °C prior to DNA extraction. The published mitochondrial genome of *Bufo melanostictus* (GenBank Accession no. NC005794) was used as an outgroup for phylogenetic analysis.

Genomic DNA was isolated using the QIAGEN DNeasy Tissue Kit according to the standard protocol, or using standard PCI/CI extraction (Sambrook *et al*. 1989) for problematic samples. Amplification of a *c*. 1400 bp fragment including ND2 and flanking t-RNAs was carried out using the primers H5934–L4437 of Macey *et al*. (1997). Additional primers were developed to obtain completely overlapping sequence (Table 2). Amplification of this region was problematic and for the purposes of this study,

Table 1 Summary of within-population diversity of cytochrome *b* sequences from Guianan *Atelopus* populations. Geographic location, number of individuals sampled, number of haplotypes, number of variable sites, gene and nucleotide (Π) diversity with 95% confidence intervals, Tajima's *D* and Fu's F_S are reported

Species/ population	Latitude and longitude	N_{ind}	N_{hap}	# Polymorphic sites	Gene diversity	Π (100×)	*D*	F_S
A. s. hoogmoedi								
Brownsberg	04°52′N, 55°13′W	6	2	1	0.53 ± 0.17	0.14 ± 0.16	0.851*	0.6254*
Iwokrama	04°23′N, 58°46′W	4	3	1	0.83 ± 0.73	0.22 ± 0.23	0.592*	–0.658*
Lely	04°16′N, 54°44′W	2	2	1	1.0 ± 0.5	0.33 ± 0.46	0*	0*
Mitaraka	02°16′N, 54°32′W	5	2	3	1.8 ± 1.24	0.48 ± 0.38	1.572*	2.429*
Lac Toponowini	03°02′N, 52°42′W	13	4	17	2.55 ± 1.46	0.01 ± 0.01	–0.382*	–0.362*
Acarai Mountains	01°18′N, 58°45′W	1	1	0	0.00	0.00	NA	NA
A. s. barbotini								
Piton Baron	03°15′N, 53°04′W	5	3	2	1.0 ± 0.18	0.31 ± 0.31	–0.710*	–0.887*
Roche Dachine	03°28′N, 53°13′W	1	1	0	0.00	0.00	NA	NA
Saul	03°37′N, 53°12′W	6	3	5	0.73 ± 0.16	0.73 ± 0.52	1.390*	1.624*
A. vermiculatus								
Pic Matecho	03°45′N, 53°02′W	6	5	10	0.93 ± 0.12	15.85 ± 1.01	0.759*	–0.373*
A. franciscus								
Mount Chauve	03°49′N, 52°45′W	5	5	6	1.0 ± 0.13	0.80 ± 0.58	–0.191*	–2.371†
Nouragues	04°07′N, 52°40′W	5	2	2	0.67 ± 0.31	0.37 ± 0.32	1.893*	1.530*
Mount Trinité	04°36′N, 53°22′W	2	2	2	1.0 ± 0.5	0.56 ± 0.68	0*	0*
Armontabo	03°46′N, 52°20′W	2	1	0	0.00	0.00	NA	NA
A. flavescens								
Mount Kaw	04°29′N, 52°02′W	11	2	1	0.44 ± 0.13	0.12 ± 0.13	0.671*	0.779*
Mount Favard	04°30′N, 52°02′W	4	3	2	1.0 ± 0.27	0.37 ± 0.38	0.00*	–1.216*
Mount Grand Matoury	04°52′N, 52°20′W	2	1	0	0.00	0.00	NA	NA
Trois Pitons	04°13′N, 51°51′W	2	1	0	0.00	0.00	NA	NA

*$P > 0.05$; †$P \leq 0.02$.

Table 2 Primers used for the amplification and sequencing of the cytochrome oxidase *b*, and NADH dehydrogenase subunit 2 (ND2) genes

Primer	Source	Sequence (5′ to 3′)
ND2		
Lint	this study	GGMATYGCCCCVTTYCACTTCTG
Hint	this study	CTATTAAGGGCTTTGAAGGCTC
L1353	this study	GCTTCAATTGCTCGTGGGTG
Atel-HintR	this study	CAGGACATAATGATATTCACC
Atel-LintR	this study	GCAATTGAGGAGAAAGCTAA
H5934	Macey *et al*. (1997)	AGRGTGCCAATGTCTTTGTGRTT
L4437	Macey *et al*. (1997)	AAGCTTTCGGGCCCATACC
cyt *b*		
Atel-cbF	this study	GAGTAATAGGGGTGAAATGG
Atel-cbIF	this study	ACYTCCACATCGGACGAGG

sequences were available from only 32 individuals from the Guianas. Additionally, two *Atelopus* specific primers were developed to amplify a 375 bp fragment of cyt *b* (Table 2), which was obtained for 79 individuals. 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 $MgCl_2$). Amplification was performed in a PTC100 (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. 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 of sequences was unambiguous with no apparent insertions or deletions.

Phylogenetic analysis

Phylogenetic relationships among representative mtDNA haplotypes were estimated using `paup*` version 4.0b10 (Swofford 2001) employing maximum-parsimony (MP) and

maximum-likelihood (ML) methods. Support for proposed clades was assessed via 2000 nonparametric bootstrap pseudoreplicates with the heuristic search option, tree-bisection–reconnection (TBR) branch swapping and 10 random taxon addition replicates for MP and ML analysis. The model of sequence evolution implemented in ML analysis was determined using hierarchical likelihood ratio testing with modeltest version 3.6 (Posada & Crandall 1998) and substitution patterns for both genes suggested a best fit to the Hasegawa *et al*. (1985) model with rate heterogeneity (HKY + Γ). This model was also implemented in a Bayesian phylogenetic analysis using mrbayes 3.0 (Huelsenbeck & Ronquist 2001) to determine topological support from a posterior probability distribution. Bayesian analysis produced 100 001 trees (obtained from a run of 50.0×10^6 generations) of which 5000 were discarded as burn-in. Adequate burn-in was determined by examining a plot of the likelihood scores of the heated chain for convergence on stationarity (Leaché & Reeder 2001). Topological conformity with previous taxonomic hypotheses was tested using SH-tests (Shimodaira & Hasegawa 1999) as implemented in paup*.

A minimum-spanning network with connections exceeding 95% parsimonious probability was obtained from 79 Guianan individuals for cyt *b* using the program tcs (Clement *et al*. 2000), and thereby implementing the statistical approach of Templeton *et al*. (1992). To determine whether there is a geographical association between haplotypes/clusters, a nested clade analysis (NCA; Templeton *et al*. 1995; Templeton 1998) was performed. Clade and nested clade distances (D_c and D_n, respectively) were measured as straight-line distances between localities and were computed using the program geodis 2.2 (Posada *et al*. 2000). As sampling of ND2 was incomplete for all populations and representative sample sizes were low, these data were excluded from analysis of haplotype structure and demographic history.

Genetic structure of cytochrome b *haplotypes*

Measures of gene, nucleotide, and haplotypic diversity were estimated for the cyt *b* data using arlequin (Schneider *et al*. 2000) with Kimura 2-parameter (K2P) distances with gamma correction. In order to test the sequences for deviation from the expectations based on neutral theory, Tajima's (1989) *D* was calculated and significance determined by comparison to a beta distribution. The hierarchical structure of cyt *b* variation within Guianan *Atelopus* was examined via an analysis of molecular variance (amova, Excoffier *et al*. 1992) utilizing the program arlequin. This analysis was performed with with a K2P distance with the aforementioned gamma correction, 20 000 random permutations and the populations structured as follows: *A. s. hoogmoedi*, *A. flavescens*, *A. franciscus*, *A. s. barbotini*, and the locality of Pic Matecho. This analysis provides insight into the amount of genetic variation observed in the sample attributable to within-population (Φ_{ST}), within-group (Φ_{SC}), and among-group variation (Φ_{CT}). The population of Pic Matecho was treated separately as this population includes haplotypes from both the *A. flavescens*/*A. franciscus* and the well-supported *A. s. barbotini* groups.

In order to test for evidence of population expansion within lineages, arlequin was used to examine mismatch distributions. This analysis examines the distribution of paired haplotype differences and infers 95% confidence intervals for time in generations since last population expansion ($\tau = 2\,\mu t$, where μ is the mutation rate and t is the generation time in years), initial population size ($\theta_0 = 2\,\mu N_0$), and final population size ($\theta_1 = 2\,\mu N_1$) according to the method of Schneider & Excoffier (1999). Parametric bootstrapping is used to obtain these 95% confidence intervals, which, it should be noted, for θ_0 and θ_1 have been shown to be excessively large (Schneider & Excoffier 1999). This analysis also produces the index of raggedness (Harpending 1994) and an estimate of fit between the observed and expected mismatch (sum of squares deviations, SSD). The raggedness index is generally expected to assume a large value in relatively stable populations which themselves generally exhibit a multimodal, or non-Poisson, distribution of haplotypic differences. The significance of both of these statistics is assessed by P_{HARP}, and P_{SSD}, which when < 0.05 support a model of recent population expansion (by rejecting the null hypothesis of no recent expansion). Additionally, the raggedness index has been shown to be more powerful in quantifying population growth with limited sample sizes (Ramos-Onsins & Rozas 2002). Fu's F_S (Fu 1997) was also employed to detect population growth within individual localities. To estimate t (time in years since population expansion), we assumed a mitochondrial mutation rate of 2.5% per Myr (Lougheed *et al*. 1999) and a generation time of 3 years (K. Zippel & R. Gagliardo, personal communication).

Coalescent-based estimates of demographic parameters

In order to overcome the limitations of conventional estimates of migration rates based on F_{ST} values (e.g. estimation of divergence time assuming isolation without gene flow, Whitlock & McCauley 1999; Nielsen & Wakeley 2001; Beerli 2004), we employed a coalescent-based approach. Nielsen & Wakeley (2001) and Hey & Nielsen (2004) have developed a Markov chain Monte Carlo (MCMC) method that includes both isolation and migration in a single model and simultaneously estimates demographic parameters of interest (migration, population size, divergence time) scaled by the neutral mutation rate (u). Joint maximum-likelihood estimates of the population mutation rate ($\theta = 2N_{ef}u$), the rate of migration ($m = m/u$, where m is the

rate of migration for each gene copy [this migration rate is not to be confused with the more common M (= 2Nm) which is equivalent to $\theta m/2$]), divergence time t (= tu, with t being time in generations since divergence), and time to most recent common ancestor (TMRCA) were obtained from the Bayesian posterior distributions generated by the program IM (Hey & Nielsen 2004). The joint estimates of demographic parameters obtainable with the use of a full isolation with migration model allow the differentiation of historical processes whose signature would have been unclear with traditional methods. For example, joint estimates of parameters allow one to determine whether two populations that do not represent reciprocally monophyletic lineages are the result of a recent separation with little to no gene flow (incomplete lineage sorting) or an older separation with measurable gene flow (hybridization).

Coalescent parameters are subject only to relative interpretation due to their scaling to u. However, incorporating published calibrations of rates of molecular divergence and observed generation times (see above) allows the conversion of divergence time to years, thereby enabling a more useful biogeographical interpretation of results. Inferred rates of sequence divergence for cyt *b* of amphibians are summarized by Lougheed *et al*. (1999), and range from 0.8% to 2.5% per Myr. For the fragment examined here (cyt *b*) this translates to a mutation rate (u) between 3.0×10^{-6} and 9.4×10^{-6} mutations per sequence per year. These values were used as an upper and lower bound for subsequent coalescent estimates of divergence times. While the assumption of a constant rate of evolution over time is generally considered to be unrealistic, alternative methods which do not make such an assumption require calibrations in the form of fossil material or prior knowledge of the age of at least some of the included lineages. As such information is lacking for this group, we feel that the use of a molecular clock is justified, although the results should be interpreted with these limitations in mind.

Results

Phylogenetic analysis

The results of phylogenetic analyses of both mitochondrial gene regions suggest that the *Atelopus* of the Guiana Shield represents an evolutionary lineage distinct from Amazonian congeners. Among the Guianan samples of *Atelopus*, the 32 and 15 unique haplotypes for cyt *b* and ND2, respectively, are listed numerically with their corresponding localities in Fig. 2a. Parsimony-informative characters represented 94 (25 within Guianan samples) of the 375 cyt *b* characters and 169 (87 within Guianan samples) of the 1476 ND2 characters. Parsimony analysis resulted in 32 and 28 equally parsimonius trees of 208 and 495 steps (consistency indices = 0.760 and 0.917) for cyt *b* and ND2, respectively (not shown). Corrected (HKY + Γ), pairwise sequence divergence between Guianan haplotypes ranged from 0.27% to 7.67% for cyt *b* and 0.14% to 5.67% for ND2. Phylogenetic analysis of both cyt *b* and ND2 sequence data reveal few well-supported patterns of relationship within the sampled Guianan populations (Fig. 2a). Importantly these analyses do reveal significant genetic differentiation from Peruvian populations of the closely related *Atelopus pulcher*, as well as the more proximate population from Santarem, Brazil, which is currently recognized as a member of *Atelopus spumarius s.l.* (Fig. 2a). Monophyly of the Guianan *Atelopus* with respect to these conspecifics and closely related congeners is strongly supported by both ML and BI analysis of both gene regions (0.92/84; 1.0/95, BI/ML, cyt *b* and ND2, respectively; subsequent reports of nodal support will follow this format).

Within the Guianan samples, populations (and all but one individual) referred to *Atelopus s. hoogmoedi* are strongly supported as a monophyletic lineage by analyses of both genes (1.0/94; 1.0/96). This lineage appears to be sister to an assemblage containing all other Guianan *Atelopus* (*Atelopus s. barbotini*, *Atelopus flavescens*, *Atelopus franciscus*, Pic Matecho) that, although greatly differentiated, is itself well supported only in the ND2 analysis (1.0/98). This clade is restricted in geographical distribution to the northern half of French Guiana and includes four previously described forms (three of which are currently recognized). Within this French Guianan clade, there is strong support from all analyses (0.96/68; 1.0/100) for a group restricted to the central mountain region (and one individual of *A.* s. *hoogmoedi* from the nearby locality of Toponowini) largely corresponding to the recognized taxon *A. s. barbotini*. Within the remainder of the French Guianan assemblage, the data provide poor support for the observed structure. It should be noted that ND2 data are lacking for Mount Chauve, a population referred to *A. franciscus* which appears to be quite distinct from other French Guianan *Atelopus*.

One population, Pic Matecho, included a diverse array of mitochondrial haplotypes and members of this population were nested well within both the strongly supported central mountain clade (*A. s. barbotini*) and the group of coastal populations referable to *A. flavescens* and *A. franciscus*.

Population structure

Analysis of haplotypic diversity reveals variable levels of interpopulational diversity and indicates that the majority of observed genetic variation is accounted for by differences between recognized taxa. The haplotypic diversity observed within populations of Guianan *Atelopus* is summarized in Table 1 (all haplotypes and their distributions are listed in Tables S1 and S2; sequences of each individual and all outgroup samples were deposited in GenBank under Accession nos AY995949–AY996036 and DQ068410–DQ068448).

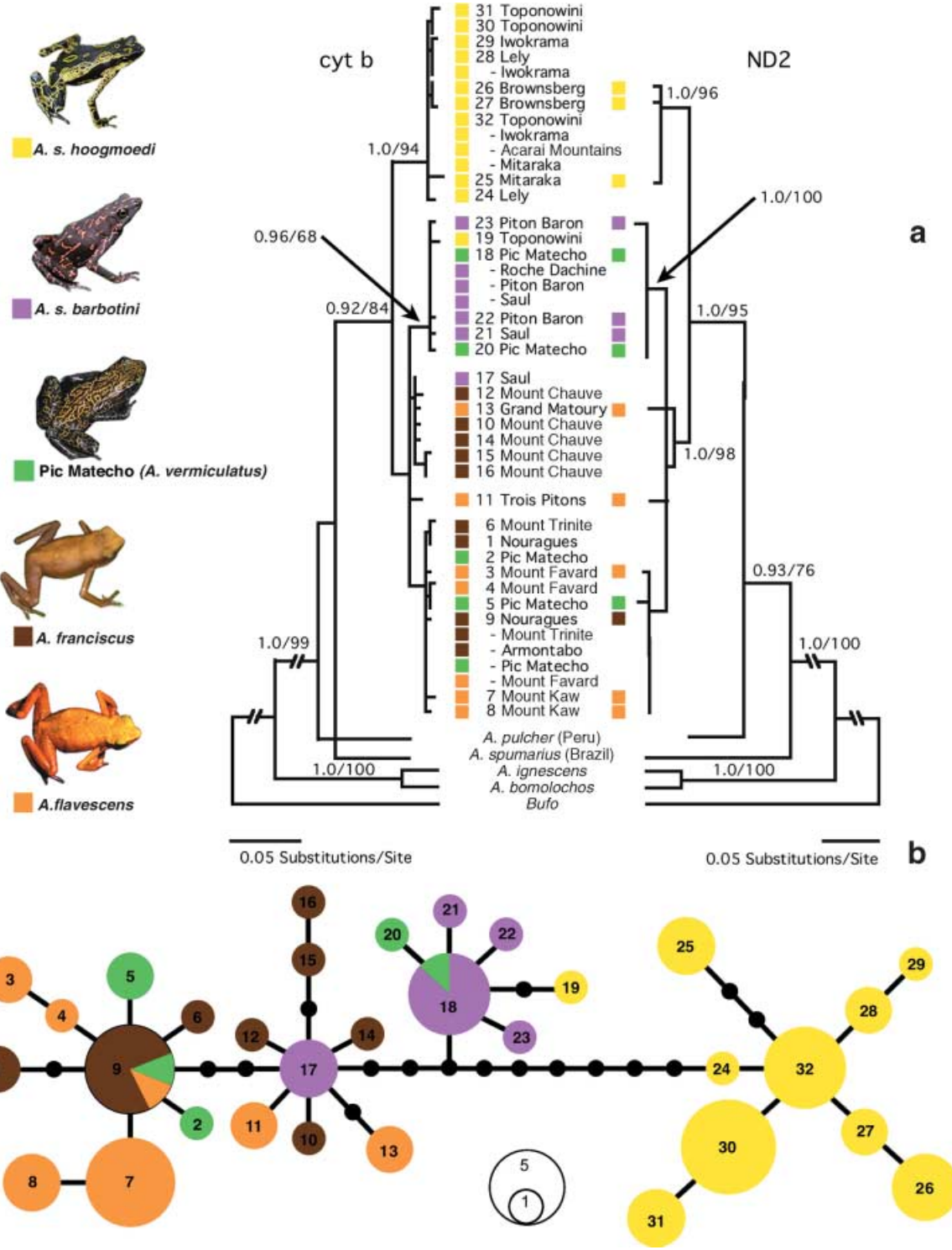

Fig. 2 Relationships among mitochondrial haplotypes: (a) Phylogenetic relationships among unique Guianan *Atelopus* cyt *b* (left) and ND2 (right) haplotypes. Trees shown are phylograms based on heuristic maximum-likelihood (ML) analyses. Support values on cladogram represent Bayesian posterior probabilities and ML nonparametric bootstrap values, respectively. Haplotype numbers at tips of cladogram follow scheme of Fig. 2b; localities listed for haplotypes are illustrated in Figs 1 and 3 and all are listed in Table 1. Localities preceded by '-' represent instances of haplotype sharing among populations/species (number above '-' indicates shared haplotype). (b) Minimum-spanning networks of 32 unique haplotypes cyt *b* (375 bp) of Guianan *Atelopus*. Shared haplotypes are indicated by circles with area being proportional to the number of individuals sharing that haplotype. Inferred intermediate haplotypes between observed haplotypes are indicated by small circles. Colours correspond to phenotype presented on left side of figure.

Table 3 Summary of Φ-statistics produced by amova with populations grouped by species

	F	%	*P*
Within populations (Φ_{ST})	0.87697	12.30	<< 0.001
Among populations (Φ_{SC})	0.59913	18.39	<< 0.001
Among species (Φ_{CT})	0.69309	69.31	<< 0.001

Each GenBank record is annotated with the individual field number, locality (from Table 1) and haplotype numbers (from Fig. 2). With the exception of Pic Matecho, nucleotide diversity ranged from 0.14% to 0.8% (mean 0.39%). The value for Pic Matecho is extremely high (15.85%) due to the presence of two, evolutionarily divergent haplotype lineages. Haplotypic diversity within populations was variable, although it appears that significant variation exists. All populations sampled for three or more individuals had at least two haplotypes present. The amova revealed significant differentiation at all hierarchical levels, and the great majority of this variance (69.3%) was explained by differences between designated groups (*A. s. hoogmoedi*, *A. s. barbotini*, *A. flavescens*, *A. franciscus*, and Pic Matecho), while relatively little was due to inter (18.4%) or intragroup (12.3%) variance (Table 3).

Analysis of haplotypic relationships revealed significant structure within the Guianas. A single haplotype network was recovered by tcs based on cyt *b* sequences of 79 Guianan individuals from 18 populations (Fig. 2b). The network presents a pattern similar to the results of phylogenetic analysis in recovering four major haplotypic groups that can be generally characterized as (I) *A. s. hoogmoedi*, haplotypes 24–32, (II) *A. s. barbotini*, haplotypes 18–23, (III) a mix of *A. flavescens*/*A. franciscus*/*A. s. barbotini*, haplotypes 10–17, (iv) *A. flavescens*/*A. franciscus*, haplotypes 1–9. The haplotype network also clearly shows Pic Matecho haplotypes to be nested well within groups II and III. The NCA (Fig. S1, Table S3) resulted in 'inconclusive outcome' for most 1-step clades, yet provided support for patterns of fragmentation and restricted gene flow with isolation by distance for higher nesting levels corresponding with clades I, III.

All individuals a priori referred to *A. s. hoogmoedi* (with the exception of one sample from Lac Toponowini, haplotype 19) formed a distinct haplotypic group differentiated by no less than eight steps from any other Guianan *Atelopus*. This group included 30 individuals from five populations in which 10 haplotypes were observed and as many as three haplotypes per population. Genetic differentiation between haplotypes was as great as five steps. No geographical structure is apparent from the current sampling of this taxon, although haplotype 32 spans the entire sampled distribution (Acarai, Iwokrama, Mitaraka, Toponowini). NCA suggests long-distance colonization for this taxon, particularly with respect to populations at the northeastern periphery of the distribution (Brownsberg and Lac Toponowini).

A second distinct haplotypic group was formed in both analyses that included nearly all individuals from the mountainous region of central French Guiana referable to *A. s. barbotini*. This group also included individuals from Pic Matecho that are phenotypically referable to *A. vermiculatus* (syn. *A. flavescens*) and one *A. s. hoogmoedi* from Lac Toponowini (roughly 30 km SE of the *A. s. barbotini* locality of Piton Baron). This group is separated by ≥ 8 and ≥ 5 steps from the *A. s. hoogmoedi* and *A. flavescens*/*A. franciscus* groups, respectively. The data included 15 individuals from five populations from which six haplotypes were observed. Haplotype sharing was observed between four populations (no. 18; Pic Matecho, Savane Roche Dachine, Saül and Piton Baron), as many as three haplotypes were observed in a single population (Piton Baron). No two haplotypes differed by more than three steps.

The remaining haplotypes, which appear to cluster into two groups, included all members a priori referred to *A. flavescens* and *A. franciscus* as well as four individuals from Pic Matecho referable to *A. vermiculatus* (syn. *A. flavescens*) and three *A. s. barbotini* from Saül. Within this group, 39 individuals were sampled from 10 localities (including Pic Matecho). Only one instance of haplotype sharing (no. 9) across populations was observed. This haplotype was observed in populations of *A. flavescens* (Mount Favard) and *A. franciscus* (Nouragues, Mount Trinitie, Aromontabo) as well as in one individual from Pic Matecho. Within *A. flavescens*, seven haplotypes were observed across four populations. Two populations (Trois Pitons, no. 11 and Mount Grand Matoury, no. 13) were very divergent (separated by ≥ 4 steps) from their conspecifics. Within *A. franciscus*, eight haplotypes were observed across four populations with six of those haplotypes being represented by only a single individual. Divergence within *A. franciscus* was as great as seven steps. Geographic analysis of haplotypic structure (NCA) suggests that a complex array of factors (past fragmentation and restricted gene flow with isolation by distance) have shaped the genetic structure of the northeastern French Guianan *Atelopus*.

Analysis of mismatch distributions revealed a general lack of support for recent expansion in all groups. The largest observed value of τ (time since last divergence) was in *A. s. barbotini* (τ = 10.6) (Table 4). Generally the group with the largest value of τ is assumed to represent the oldest lineage. Models of population expansion were found to be a significantly bad fit for all populations ($P_{(HARP)}$ and $P_{(SSD)} > 0.05$). The low ragged indices obtained from these analyses suggest a pattern of relative stability (e.g. *A. s. hoogmoedi*, $P_{(HARP)} = 0.837$, $P_{(SSD)} = 0.619$). This is further substantiated by observation of mismatch distribution plots for all groups considered, which reveal clearly multimodal

Table 4 Results of mismatch analysis of the cyt *b* data

Clade	τ	Obs. mean	θ_0	θ_1	Ragged	$P_{(HARP)}$	$P_{(SSD)}$
A. s. barbotini	10.621 (4.58–25.50)	5.745	0.0 (0.0–14.9)	13.16 (4.7–1653.8)	0.150	0.187	0.088
A. s. hoogmoedi	2.215 (0.80–4.72)	2.93	0.35 (0.0–1.6)	9.97 (2.8–6651.2)	0.028	0.837	0.619
A. flavescens	7.093 (2.53–15.97)	2.974	0.002 (0.0–7.8)	4.26 (1.0–71.4)	0.083	0.537	0.394
A. franciscus	6.332 (2.53–11.01)	4.4	0.0 (0.0–3.4)	11.75 (4.5–4754.2)	0.053	0.651	0.612
Pic Matecho (*A. vermiculatus*)	9.680 (4.1–29.7)	4.933	0.0 (0.0–19.0)	12.4 (2.7–4679.8)	0.164	0.599	0.138

Table 5 Maximum-likelihood estimates of mutation parameter θ, migration rate M = 2Nm, divergence time Tm, and time to most recent common ancestor (TMRCA) inferred from the mode of the marginal densities reported by im (Hey & Nielsen 2004). Values of θ listed are those of taxa listed in first column in comparisons with taxa from columns 2–6. Migration rate estimates (M) are reported as rates from taxon in first column to taxa from columns 2–6. NM, not measured

θ	*A. flavescens*	*A. franciscus*	Pic Matecho	*A. s. barbotini*	*A. s. hoogmoedi*
A. flavescens	—	2.27	NM	NM	NM
A. franciscus	48.43	—	19.37	NM	NM
Pic Matecho	NM	8.55	—	5.58	NM
A. s. barbotini	NM	NM	7.10	—	7.82
A. s. hoogmoedi	NM	NM	NM	10.60	—
M	*A. flavescens*	*A. franciscus*	Pic Matecho	*A. s. barbotini*	*A. s. hoogmoedi*
A. flavescens	—	0.09	NM	NM	NM
A. franciscus	0.1 (43.6*)	—	0.1 (18.4*)	NM	NM
Pic Matecho	NM	0.08	—	1.0	NM
A. s. barbotini	NM	NM	0.03	—	0.01
A. s. hoogmoedi	NM	NM	NM	0.61	—
Tm/TMRCA†	*A. flavescens*	*A. franciscus*	Pic Matecho	*A. s. barbotini*	*A. s. hoogmoedi*
A. flavescens	—	229–715	NM	NM	NM
A. franciscus	708.4–2215.1	—	159–499	NM	NM
Pic Matecho	NM	1052.6–3291.1	—	89.9–281	NM
A. s. barbotini	NM	NM	955.5–2987.5	—	1489.8–4658.1
A. s. hoogmoedi	NM	NM	NM	1479.2–4624.9	—

*Marginal densities for migration parameter (*m*) of *A. franciscus* (to Pic Matecho and to *A. flavescens*) exhibited a sharp peak at $m = 0$ yet a second peak occurred at $m = 1.9$ and 1.8, respectively. See Discussion for justification of use of nonzero *m*.
†Tm = $t \times 3$ (in Kyr above diagonal) and time to most recent common ancestor (in Kyr below diagonal).
Range presented represents values calculated with rates of cyt *b* discussed in text.

distributions (with the potential exception of *A. flavescens*) indicative of stable populations (Fig. 3).

Coalescent analyses

Coalescent-based maximum-likelihood estimates of population divergence times (Tm), migration rates (M), and population mutation rates (θ) are presented in Table 5. Results presented represent the average of three independent Markov chains initiated with identical parameters and different random number seeds that were run until the lowest effective sample size (ESS) for any of the six parameters was at least 1000. Results were also plotted in order to verify congruence of the likelihood surfaces (not shown).

The results of these analyses suggest the possibility of a hybrid origin of the Pic Matecho population as indicated by phylogenetic analysis and population structure. Initially, it seems that Pic Matecho (as well as the coastal *A. flavescens*) are receiving an immeasurably low number immigrants from *A. franciscus* (Fig. 3, Table 5). Although preliminary estimates of migration rates for *A. franciscus* (with *A. flavescens* and Pic Matecho) were small ($m < 0.1$), examination of the posterior probability distributions revealed secondary peaks near $m = 2$ (e.g. Fig. 4). Parenthetical values of M in Table 5 represent the use of *m*-values from second peaks in the posterior distributions. These values are undoubtedly artificial, and illustrate the need for additional sampling in this geographical region, yet suggest that there may be migration taking place in NE French Guiana. Aside from

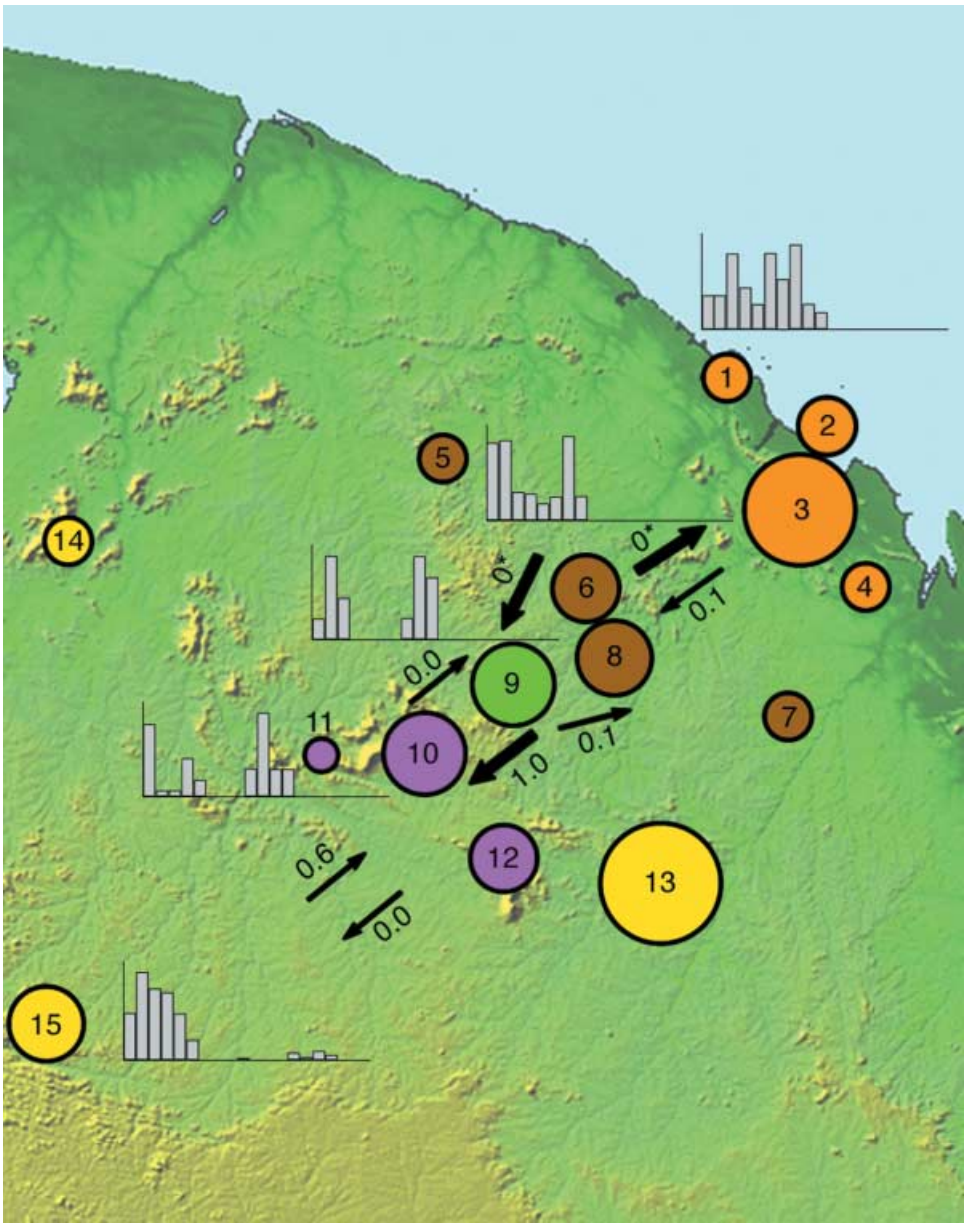

Fig. 3 Estimates of gene flow and demography for French Guianan *Atelopus* populations. Area of circle for each population is proportional to sample size, colour indicates phenotype present and follows colouring scheme of Fig. 2. Arrows indicate directional gene flow (in units of $2N_m$) per generation between adjacent groups based on coalescent estimates. Large arrows indicate M equal to or greater than 1; large arrows with $M = 0^*$ are discussed in text. Mismatch distributions for designated groups (see text) are also presented. Localities are numbered as follows: (1) Mount Grand Matoury, (2) Mount Favard, (3) Mount Kaw, (4) Trois Pitons, (5) Mount Trinitie, (6) Mount Chauve, (7) Aromontabo, (8) Nouragues, (9) Pic Matecho, (10) Saul, (11) Savane Roche Dachine, (12) Piton Baron, (13) Toponowini, (14) Lely Mountains, (15) Mitaraka. The localities of Brownsberg, Iwokrama, and Acarai Mountains are west of the region illustrated (see Fig. 1 for these localities).

the complicated situation surrounding *A. franciscus*/Pic Matecho, it appears that little migration is taking place among Guianan *Atelopus*. Indeed, the only other measurable estimate of migration was from *A. s. hoogmoedi* to *A. s. barbotini*; yet that estimate was less than one ($2N_m = 0.61$, Fig. 3).

Separate analyses were conducted partitioning the *A. franciscus* haplotypes into those originating from Mount Chauve and all others. The reason behind examining Mount Chauve separately is that these haplotypes were shown to be quite unique and divergent from all conspecifics (and the closely related *A. flavescens*) in both the phylogenetic and network analyses. The results of these analyses (not shown) suggest that the genetic structure of the population of Mount Chauve may be complicating the interpretation of

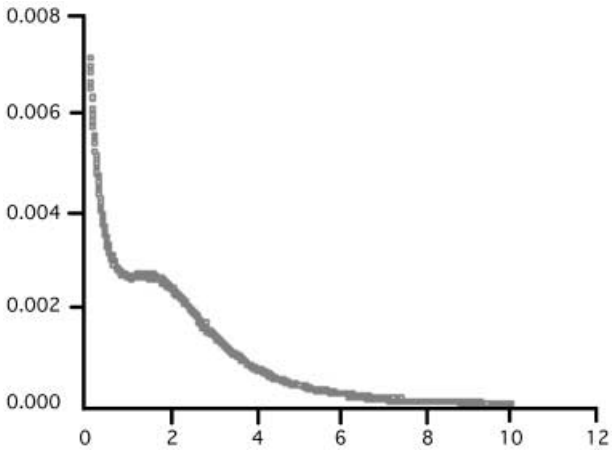

Fig. 4 The marginal posterior probability distribution for the migration parameter of *Atelopus franciscus* (scaled by the neutral mutation rate) from the analysis of *Atelopus franciscus* and *Atelopus flavescens*.

patterns of gene flow within this region. As our sampling from Mount Chauve makes up more than one-third of our samples of *A. franciscus*, it is likely that this is the reason we are observing a bimodal posterior distribution of migration rates for this species.

Estimates of divergence time between lineages of Guianan *Atelopus*, based upon coalescent estimates of t (given $u = 3.0–9.4 \times 10^{-6}$), reveal a relatively old (1.5–4.7 Myr) split between *A. s. hoogmoedi* and the French Guianan taxa. Divergence estimates between lineages within French Guiana indicate that these lineages are the result of Quaternary differentiation (90–499 Kyr; Table 5).

Discussion

Phylogenetic relationships of Guianan Atelopus *and taxonomic implications*

Phylogenetic analysis of all Guianan haplotypes and extra-Guianan species reveals extensive sequence divergence (*c*. 20% uncorrected in both cyt *b* and ND2) between the members of the *Atelopus ignescens* (*A. ignescens* and *A. bomolochos*) and *Atelopus flavescens* species groups. Within the *A. flavescens* group, we have very few samples originating outside the Guianan portion of the distribution. However, sequence divergence between the individual from Santarem (*Atelopus spumarius s.l.)*, all individuals in the Guianas (including the putative conspecific *Atelopus spumarius hoogmoedi* and *Atelopus spumarius barbotini*), and the closely related *Atelopus pulcher* in Peru is roughly equivalent at 7.2–8.9%. This suggests significant evolutionary differentiation of the Guianan *Atelopus* and highlights the need for additional investigation into the distribution and relationships of populations of *Atelopus* in the Amazon basin.

Perhaps the most telling example of the distinctiveness of the Guianan populations lies in the observed divergence between the Santarem specimen and the sampled individuals of *A. s. hoogmoedi*, a presumed conspecific. This taxon is here demonstrated to represent a well-supported (1.0/94, 1.0/96) apparently genetically contiguous, distinct evolutionary unit (the first such demonstration for any member of *A. spumarius s.l.*). This assertion is supported by observed low level of interpopulation differentiation and the distribution of cyt *b* haplotype 32 which spans the entire sampled range for this taxon. This genetic continuity over such a distance is in stark contrast to the divergence observed between *A. s. hoogmoedi* and the individual from Santarem (*c*. 8%, corrected) which is roughly equidistant from Mount Mitaraka as is Iwokrama (among which cyt *b* haplotype 32 is shared).

Within the Guianas it is clear that there are three major lineages. Phylogenetic analysis reveals a basal split between the comparatively widespread *A. s. hoogmoedi* and those populations found in northern French Guiana. The *A.* s. *hoogmoedi* lineage includes all Surinamese and Guyanan populations as well as those on the southern and eastern borders of French Guiana. These animals are differentiated from all other Guianan populations by eight unique mutational steps for cyt *b* (with the exception of haplotype 19). Within this group there appears to be little phylogeographical structure with widespread haplotype sharing (no. 32), although no more than two haplotypes were observed in any one population. Although a taxonomic revision is beyond the scope of this paper, it is clear that *A. s. hoogmoedi*, as described by Lescure [1973 (1974)], merits consideration as a full species.

Among the populations of northern French Guiana, our findings lend support to the assertion of Kok (2000) that the populations of central French Guiana, referred to *A. s. barbotini*, are indeed more closely related to *A. flavescens* and *A. franciscus* than to the presumed conspecific *A. s. hoogmoedi*. However, the suggestion that this taxon may be synonymous with *A. flavescens* (Kok 2000) is not supported. Populations associated with the central mountain range of French Guiana, most of which are phenotypically representative of *A. s. barbotini*, represent a well-supported monophyletic unit (0.96/68, 1.0/100) with the exception of haplotype 17. Here, NCA suggests restricted gene flow with isolation by distance between *A. s. barbotini* (haplotype group 18–23) and the coastal populations. Although this taxon can no longer be considered a form of *A. spumarius*, further sampling is needed to support taxonomic assignation. This effort will undoubtedly be complicated by potential hybridization with congeners in the area around Pic Matecho.

The status of populations in the northeastern portion of French Guiana is less clear. It appears that none of the taxa from this region represents mutually exclusive genetic lineages. Joint estimates of divergence time and migration rates (with populations grouped by taxonomic assignment)

indicate that this is likely the result of Quaternary divergence with incomplete lineage sorting (recent divergence + low migration). NCA suggests that haplotype groups 1–9 and 10–17 are both the result of restricted gene flow with isolation by distance. While our results indicate that the support for the differentiation of the three recognized species from this region is somewhat questionable, it is clear that an assessment of the validity of these taxa necessitates further investigation.

Demographic history and differentiation within Guianan Atelopus

Analysis of haplotype structure of Guianan *Atelopus* reveals significant structure that is generally concordant with previously named forms. Indeed, amova reveals that a very high proportion of the observed genetic variation (69%) is explained by differences between the taxonomic groupings of *A. s. hoogmoedi*, *A. s. barbotini*, *A. flavescens*, *A. franciscus* and Pic Matecho. Nucleotide diversity within populations (except Pic Matecho) is in accordance with, although slightly lower than, reported levels of diversity for other anurans (e.g. Vences *et al*. 2004). The low level of variance attributable to within population (12%, reduced to 9% when Pic Matecho is excluded) and within group (18%) differentiation suggests that overall genetic diversity is primarily attributable to differences between designated groups.

Our results strongly suggest *A. s. hoogmoedi* to be a genetically conserved lineage with broad-scale haplotype sharing over the entire distribution. Although our geographical sampling of *A. s. hoogmoedi* was broad, spanning the entirety of the known distribution in the Guianas, too few samples were included to adequately assess the distribution of haplotypes of this taxon. This apparent lack of geographical structure to the distribution of individual haplotypes is consistent with the high raggedness index (= 0.028) and the distribution of haplotypic mismatch (Fig. 3, Table 4). The mismatch distribution for *A. s. hoogmoedi* (Fig. 3) is strongly bimodal, and was not significantly smoother than the mismatch distribution simulated from stationary populations ($P_{(HARP)} = 0.837$, $P_{(SSD)} = 0.619$). All of these observations point to a widespread, genetically contiguous, stable population. However, the results of NCA suggest that the eastern periphery of the distribution of this lineage may represent an advancing front of colonization. This is supported by inferences of long distance dispersal, particularly those populations at the northern (Brownsberg) and eastern (Lac Toponowini) edge of the distribution. This may explain the lone instance of haplotypic introgression involving an *A. s. hoogmoedi* (Lac Toponowini) individual with a *A. s. barbotini*-like haplotype (19).

The general stability of *A. s. hoogmoedi* is likely due in part to the relatively great age of this taxon ($t = 5.2$), which is more than five times greater than any other Guianan lineage. Estimates place the divergence between this taxon and French Guianan taxa at greater than 1.5 Myr implying a Pliocene origin (Table 5). Throughout this extensive period of isolation, it appears that there has been little genetic contribution of this lineage to those of central and northern French Guiana with our results indicating low levels of emigration ($2N_m = 0.61$) and only one instance of haplotype sharing.

Analysis of haplotypic structure supports the phylogenetic recovery of a monophyletic group corresponding largely to *A. s. barbotini*. Although the validity of this taxon has recently been brought into question, there is little doubt of its distinctiveness based on our data. It is interesting to note that mismatch analyses suggest this to be the oldest lineage within the Guianan *Atelopus* ($\tau = 10.621$), yet coalescent estimates of divergence times as well as time to most recent common ancestor (TMRCA) suggest that this lineage is relatively young (< 300 Kyr), especially compared to the age of *A. s. hoogmoedi*. Haplotypic mismatch distribution also reveals a bimodal distribution inconsistent with a model of population expansion (Fig. 3). Estimates of ($P_{(SSD)} = 0.088$) suggest that we can reject the hypothesis of no population expansion at $\alpha = 0.1$, although ($P_{(HARP)} = 0.187$) is not significant at any acceptable level. Estimates of migration rates suggest that this taxon is exchanging very few migrants with its Guianan congeners and are in concordance with the NCA results. Estimates of *A. s. hoogmoedi* immigrants are less than 1 ($2N_m = 0.61$) and no emigration was discernable. However, individuals from Pic Matecho, a mountain located at both the northeastern extreme of the central mountain range (range of *A. s. barbotini*) and the headwaters of the Aratai River (which flows northward to the area inhabited by *A. franciscus* and *A. flavescens*), were unusual in that they contained both *A. s. barbotini* and *A. flavescens/franciscus* haplotypes. This population also produced the only estimate of migration ($2N_m$) greater than 1, with migrants apparently emigrating to the area inhabited by *A. s. barbotini*. Mismatch distributions of Pic Matecho were strongly bimodal (Fig. 3) and are a very poor fit to a model of population expansion ($P_{(HARP)} = 0.599$; $P_{(SSD)} = 0.138$) although this is potentially a result of hybridization rather than demographic equilibrium.

Our results suggest that the problematic taxonomic assignation of animals from the area of Pic Matecho, which has been complicated by intermediacy of characters, may also be a result of introgression by *A. franciscus*. It has been noted that one of the most important reproductive isolation methods in anurans is the advertisement call, and the call similarity between *A. s. barbotini* and *A. flavescens* noted by Kok (2000) provides additional support for a zone of secondary contact in this area. Further evidence to this effect was provided by Kok (2000), who photographed amplectant pairs of mixed identity as well as phenotypically intermediate individuals. This apparent mixing of phenotypic forms is not known from other populations in the

Guianas where phenotypic variation within populations is extremely low.

Atelopus franciscus and *A. flavescens*, while apparently forming distinct geographical entities, do not appear to represent mutually exclusive evolutionary lineages. Mismatch analyses (Fig. 3) for neither taxon provide any indication of expansion ($P_{(HARP)} = 0.651$, $P_{(SSD)} = 0.612$ and $P_{(HARP)} = 0.537$, $P_{(SSD)} = 0.394$). Joint estimates of both migration and divergence time suggest a recent divergence (< 715 kyr) and insignificant levels of genetic exchange ($2N_m \leq 0.1$), implicating incomplete lineage sorting of two young, distinct species. This conclusion is supported by the NCA results suggesting past fragmentation (haplotype group 1–9) and restricted gene flow with isolation by distance (haplotype group 10–18). However, the apparent presence of two mitochondrial lineages within *A. franciscus* (see above) complicate these interpretations.

Conclusions

The Guiana Shield is the most intact (80–90%), least inhabited (0.6–0.8 people/km^2) tropical rainforest region in the world (Huber & Foster 2003). As such it is not surprising that the widespread amphibian declines reported from tropical regions around the world have yet to be documented in this region. Within the Guianas themselves, conservation has been made an extremely high priority with protected areas approaching 25% of the total area of Suriname and 40% of French Guiana. Our findings suggest that the relatively narrowly distributed *Atelopus* of northern French Guiana represents, a complex assemblage of populations including very distinct, presumably isolated lineages (Grand Matoury, Trois Pitons) within species. It appears that efforts underway to provide strict protection for this region would preserve an area of high genetic diversity and complex evolutionary history unlike any other observed throughout the distribution of Guianan *Atelopus*. Further investigations of the population structure of the widespread *Atelopus hoogmoedi* utilizing additional markers will elucidate patterns of diversity across this vast area and provide insight into regions of particular importance to the genetic continuity of the eastern portion of the Guiana Shield.

Our findings suggest that the *Atelopus* of the Guianas represent an evolutionary unit distinct from Andean foreland conspecifics and closely related congeners (concordant with the fragmentation of Amazonia into Amazonian/Andean-Foreland and Guianan regions predicted by the DV hypothesis), and exhibit significant population structure among what appear to be three distinct lineages. These data support a late Miocene or early Pleistocene origin for this group with diversification within the region occurring in the Quaternary. This too supports the predictions of the DV hypothesis. The apparent association of some of the lineages with particular mountain formations (*A. s. hoogmoedi*: Tumock-Homoeck Mountains spanning the southern borders of all three Guianas; *A. s. barbotini*: central highlands of French Guiana; *Atelopus flavescens*: coastal French Guianan ranges) and the general genetic discontinuity of these geographically proximate lineages all support the presence of an Andean cool-adapted invader now restricted to suitable upland habitat and isolated by surrounding lowlands. This pattern is even evident within the coastal *A. flavescens* in which populations at the western and easternmost extent of the distribution appear to have been isolated for a significant period of time on mountains separated from the main of the distribution by large rivers (potentially less of a barrier with reduced sea levels and precipitation predicted by DV). The patchy distribution of *Atelopus* along the Amazon itself, as evidenced by the specimen from Santarem, Brazil, only strengthens the argument for the DV climactic factors effect on the distribution and diversity of this taxon. That there is such diversity (both genetic and phenotypic) within this lineage in the Guianan refuge, follows Bush's (1994) assertion that these refugia were areas of 'maximal disturbance' rather than 'maximal stability'. This is not to imply that the late Tertiary aridity that forms the foundation of the Refuge Hypothesis had no effect on the distribution of species. However, our results suggest that the temperature fluctuations forming the basis of the DV Hypothesis resulted in the diversification of organisms within what are assumed by the Refuge Hypothesis to be geographically homogeneous reservoirs of biodiversity. These findings argue for the recognition of the complexity of factors that have contributed to Amazonian diversification (many of which are integrated into the DV Hypothesis, including to some degree increased aridity) in the study of Neotropical biogeography.

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2624/MEC2624sm.htm

Fig. S1. Nested clade diagram of 32 unique cyt *b* haplotypes of Guianan *Atelopus*.

Table S1. Variable sites of 32 unique cyt *b* haplotypes of Guianan *Atelopus*.

Table S2. Frequencies of unique cyt *b* haplotypes in 17 sampled populations of Guianan *Atelopus*.

Table S3. Locality data for extra-Guianan specimens.

Table S4. Nested Contingency results based on 5000 permutations in GeoDIS. Significance at the $p < 0.05$ level is indicated by bold font. Inferences were made with the 14 July 2004 key available from David Posada's website (http://darwin.uvigo.es).

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Brice Noonan's research currently focuses on the geographic distribution of genetic variation of the Neotropical biota (with an emphasis on the Guiana Shield) and the diversification of the fauna of Gondwana. Philippe Gaucher is the scientific director of the French Guianan national park task force, charged with delimiting new protected areas. He is primarily interested in incorporating data on the genetic structure of Guianan taxa in the process of creating protected areas.
