Ridges and rivers: a test of competing hypotheses of Amazonian diversification using a dart-poison frog (*Epipedobates femoralis*)

S. C. Lougheed[1]*, **C. Gascon**[2]†, **D. A. Jones**[1], **J. P. Bogart**[3] and **P. T. Boag**[1]

[1]*Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6*
[2]*Biological Dynamics of Forest Fragments Project, Instituto Nacional de Pesquisas de Amazonica, CP 478, 69011-970 Manaus, Amazonas, Brazil*
[3]*Department of Zoology, University of Guelph, Guelph, Ontario, Canada N1G 2W1*

Mitochondrial DNA cytochrome *b* sequence data from a dart-poison frog, *Epipedobates femoralis*, were used to test two hypotheses of Amazonian diversification: the riverine barrier and the ridge hypotheses. Samples were derived from sites located on both banks of the Rio Juruá and on both sides of the Iquitos Arch in western Amazonia. The phylogeographic structure was inconsistent with predictions of the riverine barrier hypothesis. Haplotypes from opposite river banks did not form monophyletic clades in any of our phylogenetic analyses, nor was the topology within major clades consistent with the riverine hypothesis. Further, the greatest differentiation between paired sites on opposite banks was not at the river mouth where the strongest barrier to gene flow was predicted to occur. The results instead were consistent with the hypothesis that ancient ridges (arches), no longer evident on the landscape, have shaped the phylogeographic relationships of Amazonian taxa. Two robustly supported clades map onto opposite sides of the Iquitos Arch. The mean haplotypic divergence between the two clades, in excess of 12%, suggests that this cladogenic event dates to between five and 15 million years ago. These estimates span a period of major orogenesis in western South America and presumably the formation of these ancient ridges.

Keywords: vicariance hypotheses; speciation; Amazon; phylogeography; dart-poison frogs

1. INTRODUCTION

The lowland forests of the Amazon Basin contain a disproportionately large fraction of global species diversity (Gentry 1988; Wilson 1992). A number of vicariant speciation mechanisms have been presented to explain this high diversity (cf. Bush 1994; Haffer 1997). These hypotheses share the idea that historical and geographically pervasive barriers to gene flow have facilitated speciation in allopatry across much of Amazonia, but obviously differ with respect to the identity, location and duration of these barriers. The oldest of these, the riverine barrier hypothesis, derives from observations of animal distributions made by Wallace (1849, 1876). It posits a role for major Amazonian water courses in impeding gene flow between populations on opposite banks (Sick 1967; Salo *et al.* 1986; Capparella 1988, 1992; Ayres & Clutton-Brock 1992). The predictions for this hypothesis include that (i) many recently evolved sister taxa occupy opposite banks of large rivers, (ii) levels of genetic differentiation between populations on opposite river banks increases with increasing river width and flow rate and (iii) taxa of the upland terra firme forest show higher levels of differ-

entiation across rivers than taxa of the seasonally flooded várzea forests found adjacent to the river. This last prediction assumes that the strength of the barrier to gene flow is greater for exclusively terra firme species because it consists of both the river itself plus the várzea forests of both river banks (Capparella 1988, 1992; Patton *et al.* 1994; Gascon *et al.* 1996, 1998). A recently proposed and largely untested alternative hypothesis suggests an important role for Late Cenozoic palaeogeographical changes in topographical relief in defining and restricting distribution patterns of forest biota in western Amazonia (Räsänen *et al.* 1990). According to this hypothesis (hereafter the ridge hypothesis), historically prominent ridges may have facilitated allopatric differentiation of a variety of Amazonian taxa.

Of the handful of genetic studies assessing the potential importance of rivers in organismal diversification of the Amazon lowlands, the conclusions are varied. A few studies have indicated that large rivers can indeed play an important role in restricting gene flow in some vertebrate taxa (forest understory birds (Capparella 1988, 1992) and saddle-back tamarins (Peres *et al.* 1996)). Other studies of vertebrates (e.g. echimyid rodents and various frog species) show deep phylogeographical divisions that are unrelated to the presence of the river that bisects their ranges (Da Silva & Patton 1993; Patton *et al.* 1994, 1996; Gascon *et al.* 1996, 1998). Many of these empirical tests of
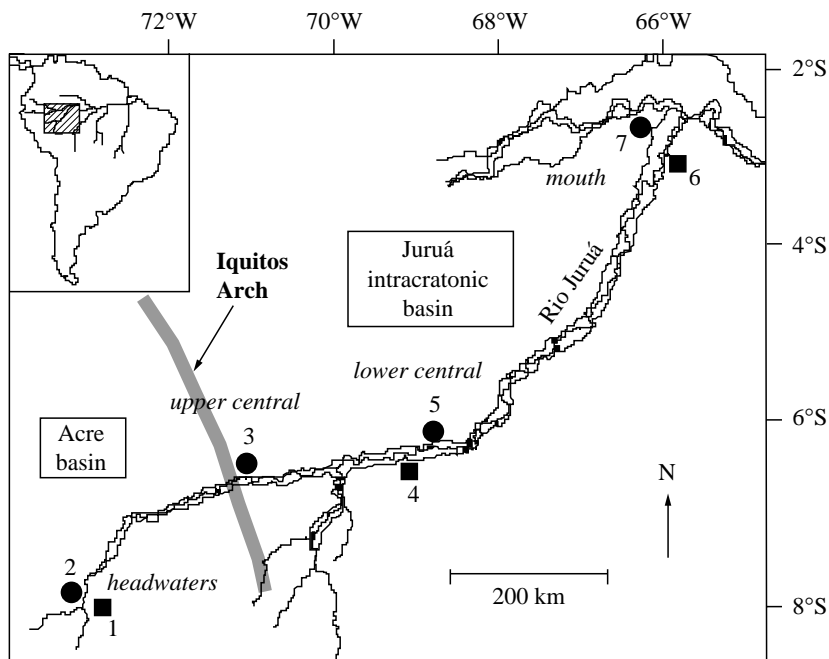
Figure 1. Map of the Juruá River indicating sampling localities. The box in the upper left corner indicates the location of the Juruá drainage basin relative to continental South America. Table 1 lists the locale names corresponding to the number codes. Four geographic sampling areas pertinent to phylogeographic analyses are shown (headwaters, upper central, lower central and mouth) falling into one of two basins separated by the Iquitos Arch. For consistency with other publications, the banks are designated as left (filled circles; sites 2, 3, 5 and 7) and right (filled squares; sites 1, 4 and 6).

the riverine barrier hypothesis have focused on the Rio Juruá, a 1000 km long, white-water tributary of the Amazon River (figure 1). For most echimyid rodents that have been studied, two roughly geographically coincident clades were apparent, corresponding to the upper and lower portions of the river (Da Silva & Patton 1993; Patton *et al.* 1994, 1996). Allozymic analyses of five anuran species suggested strong between-population differentiation that was inconsistent with the idea of a riverine barrier to gene flow, although the sample sizes precluded definitive statements regarding their phylogeographical structure (Gascon *et al.* 1996, 1998). Such patterns suggest the existence of historical impediments to gene flow other than major river systems.

Various authors have assembled evidence for the existence of major ridges that define different intraforeland (within a stable continental area adjacent to an orogenic belt) and intracratonic (within a stable continental region or craton) basins in western lowland Amazonia (Putzer 1984; Räsänen *et al.* 1987, 1990). These ridges or 'arches' originated during major orogenesis in western South America beginning some five to ten million years (Myr) before present (Räsänen *et al.* 1990). Gradual deposition of erosional sediments has largely erased evidence of these ridges on the modern landscape. One of these, the Iquitos Arch, runs perpendicularly to the Rio Juruá. It divides the Juruá drainage system into the Acre basin and a large intracratonic basin lying between the Guayanan and Brazilian Shields (figure 1). The major genetic disjunctions apparent in many echimyid rodents appear to map onto the Iquitos Arch, an observation that suggests that these ridges have acted as historical barriers to gene flow for these taxa. In the context of the Juruá drainage system, the riverine barrier and ridge hypotheses predict major axes of genetic differentiation that are orthogonal and, thus, clearly distinguishable (across-river versus between basins, respectively).

The present study examines the phylogeography of a largely terrestrial dart-poison frog species, *Epipedobates femoralis*, using DNA sequence data. Preliminary allozymic

evidence indicates that movement is uncommon, even among populations that are in close proximity (Gascon *et al.* 1998). Such low vagility implies that historical barriers to gene flow may have played a prominent role in shaping the present-day patterns of genetic diversity in *E. femoralis*. The ecology of this species suggests that it may be particularly impacted on by a riverine barrier to gene flow. *E. femoralis* is an inhabitant of upland terra firme forests and breeding and development to the tadpole stage are largely independent of aquatic habitat (Zimmerman & Simberloff 1996). The specific objectives of the present study were to (i) examine the apportionment of genetic variation between and within populations in an Amazonian upland forest frog and (ii) construct phylogenetic hypotheses to test whether the Rio Juruá, the Iquitos Arch or neither have influenced the present-day patterns of genetic differentiation.

2. MATERIAL AND METHODS

(a) *Sample collection and DNA extraction*

The samples used in this study were derived from the seven sites along the Rio Juruá shown in figure 1. Individuals were pithed and liver, heart, kidney and leg muscle tissues were removed and either immediately frozen in liquid nitrogen or placed in 70% ethanol. The animals were fixed in 10% formalin and preserved in 70% ethanol. All specimens were deposited in the Instituto Nacional de Pesquisas da Amazonia (INPA) collection.

The tissues were transported to Queen's University where total genomic DNA was extracted from small plugs of liver tissue using iminodiacetic acid chelating resin suspension (5 g Chelex in 95 ml H$_2$O; Sigma). The samples were put in 450 ml Chelex solution, incubated overnight in a 56 °C water bath, vortexed for 15 s, incubated at 95 °C for 15 min, vortexed again for 15 s and then spun at 12 000 rpm for 5 min. Extracted samples were stored at −20 °C until amplification.

(b) *Amplification and sequencing*

All PCR amplifications were performed in a Perkin Elmer 9600 thermocycler. A negative control was included for all

Table 1. *Summary statistics of cytochrome* b *sequence variation within population samples of* E. femoralis *from the Rio Juruá*

(Genotypic diversity (h) and nucleotide diversity (π) are calculated according to the methods of Nei (1987). The sample names and numbers and bank designations correspond to those shown in figure 1.)

sample	bank	n	basin	number of haplotypes	mean haplotypic divergence (%)	h	π
headwaters							
1. Porongaba	R	5	Acre	3	2.40	0.80	0.018
2. Porongaba	L	1	Acre	1	—	—	—
upper central							
3. Condor	L	4	intracratonic	3	0.44	0.83	0.0038
lower central							
4. Altamira	R	5	intracratonic	4	0.66	0.90	0.0059
5. Jainu	L	6	intracratonic	5	0.98	0.93	0.0090
mouth							
6. Vai-Quem-Quer	R	3	intracratonic	2	0.33	0.67	0.0022
7. Vira-Volta	L	1	intracratonic	1	—	—	—

reaction cocktails. A 308 bp fragment of mitochondrial DNA (mtDNA) cytochrome *b* was amplified using shortened versions of L14841 (5′-CCATCCAACATCTCAGCATGATGAAA-3′) and H15149 (5′-CCCTCAGAATGATATTTGTCCTCA-3′) (Kocher *et al*. 1989). To generate a light single-stranded product we used a biotinylated version of L14841 paired with non-modified H15149 and for a heavy single-stranded product the reverse. For some samples we used internal primers of our own design to amplify shorter fragments (Epip-Int H, 5′-ATGAAGAAGAATGAAGCGCC-3′ and Epip-Int L, 5′-TCCATTGCTCATATTTGCCG-3′). Each PCR reaction contained 2 µl of sample and 48 µl of reaction cocktail containing 2 µM MgCl$_2$, 200 µM dNTPs, 1 µM of each primer, 1X Gibco BRL react buffer and 0.25 units of Gibco BRL taq polymerase. The amplification profile was as follows: 3 min at 95 °C, 35 cycles of 10 s at 95 °C, 15 s at 50 °C and 15 s at 72 °C, with a final extension of 1 min at 72 °C. Amplified products were cleaned and single-stranded template isolated using supermagnetic polystyrene beads (Dynabeads M-280 Streptavidin; Dynal) according to the manufacturer's instructions.

Each single-stranded product was sequenced with the non-biotinylated member of the primer pair used in amplification. Sequencing was performed using a Thermo-Sequenase cycle-sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) following the manufacturer's protocols. The samples were denatured at 90 °C for a minimum of 3 min and subjected to PAGE (6% gels) for durations of 2 and 4 h. Sequences were visualized using autoradiography and scored by hand.

(c) *Statistical and phylogenetic analyses*

Sequences were aligned using GeneWorks (IntelliGenetics, Inc., Mountain View, CA, USA). Sequence divergence and the number of transitions and transversions between haplotypes were calculated in MEGA (v. 1.01, Kumar *et al*. 1993). For samples containing more than a single individual, haplotypic diversity (h) and nucleotide diversity (π) were calculated in Arlequin (v. 1.1, Schneider *et al*. 1997) according to the methods outlined in Nei (1987). To examine the relationships between haplotypes and facilitate direct comparison with previous studies in the Juruá basin (Patton *et al*. 1996), a minimum spanning network was generated using NTSYS-pc (v. 1.80, Rohlf 1994) based on a matrix of substitutional differences between haplotypes.

For the phylogenetic reconstructions, we employed distance-based neighbour-joining (Saitou & Nei 1987) and maximum-

parsimony methods using PAUP* (v. 4.0 beta, Swofford 1998). For the former we used Kimura two-parameter distances. Support for nodes within the neighbour-joining tree was estimated using bootstrap analysis with 500 replicates. For maximum-parsimony analyses we used the heuristic search option in PAUP* with TBR branch swapping. Support for resolved nodes was generated again using bootstrap analysis with 500 replicates and ten random additions per replicate. In addition, Bremer (1994) decay indices were generated for each node using AUTODECAY 4.0 (Erikkson 1998). Parsimony analyses were performed (i) with all characters weighted equally, (ii) with transversions-weighted, five-times transitions (based on the empirical ratio) and (iii) using six-parameter parsimony (Williams & Fitch 1989, 1990) according to the methods outlined in Cunningham (1997). All analyses were performed with and without each of four outgroup taxa considered singly and combined: *Epipedobates trivittatus* (this study), *Dendrobates histrionicus* (GenBank accession U70154), *Minyobates minutus* (GenBank accession U70163) and *Phyllobates lugubris* (GenBank accession U70164).

To examine the riverine and ridge hypotheses outlined in § 1 explicitly, we performed additional maximum-parsimony analyses in which resulting trees were constrained such that (i) haplotypes on opposite river banks were monophyletic and (ii) haplotypes within each basin were monophyletic. These constraint trees are obviously the most stringent versions of the two hypotheses and assume no gene flow across the respective putative barriers. Topology-dependent permutation tail probability (PTP) tests (Faith & Cranston 1991) were used to compare the constrained to the shortest unconstrained tree(s) derived from parsimony analyses.

3. RESULTS

(a) *Sequence variability*

A total of 305 base pairs of the cytochrome *b* gene (101 complete codons plus an additional first and second position) were sequenced for each of 25 *E. femoralis* and two *E. trivittatus* (GenBank accession AF163914–AF163940). No stop codons were detected in any haplotypes. The base composition of the amplified fragment was significantly biased, with a deficit of guanine (G, mean = 15.6%), an excess of thymine (T, mean = 37.9%) and with adenine
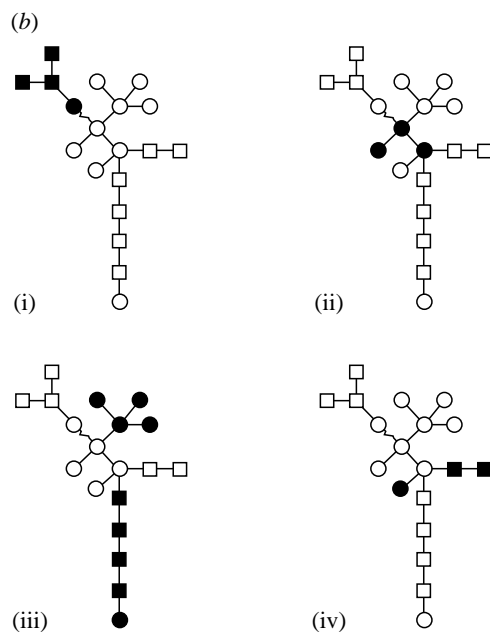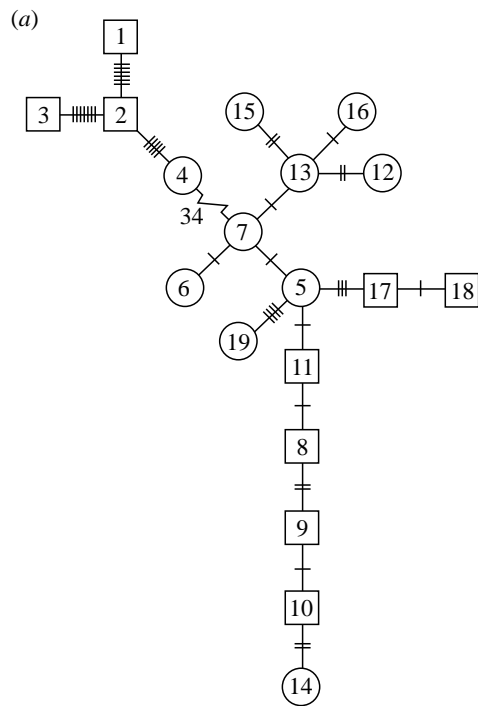
(a)

Table 2. *Mean uncorrected sequence divergence between haplotypes found in sites situated on adjacent banks within sampling areas, between sampling areas and between basins separated by the Iquitos Arch*

comparison	mean divergence between haplotypes (%)
within areas across river	
headwaters (site 1 versus site 2)	2.08
lower central (site 4 versus site 5)	6.13
mouth (site 6 versus site 7)	1.80
between areas	
headwaters versus upper central	12.04
headwaters versus lower central	12.17
headwaters versus mouth	12.36
upper central versus lower central	4.00
upper central versus mouth	1.53
lower central versus mouth	4.93
between basins	
Acre versus Juruá Intracratonic	12.16

(b)

Figure 2. (*a*) Minimum spanning tree for 19 cytochrome *b* haplotypes found among 25 individuals and seven populations of *E. femoralis*. Haplotype number is used for reference purposes in the text. Each bar indicates a base substitution between adjacent haplotypes. Squares indicate haplotypes found in right bank samples and circles those originating from the left bank. (*b*) The same tree as in (*a*) but with haplotypes for each of the four sampling areas listed in figure 1 shown by solid symbols. (i) Headwater/Acre basin, (ii) upper central/intracratonic basin, (iii) lower central/intracratonic basin and (iv) mouth/intracratonic basin.

and cytosine found in approximately equal proportions (A, mean = 24.0% and C, mean = 22.5%). These percentages are typical for cytochrome *b* in anurans (e.g. Summers *et al.* 1997). For the ingroup, 19 distinct haplotypes were detected (table 1) with a total of 61 variable sites: four

in the first codon position, two in the second position and 55 in the third position. The mean pairwise transition/transversion ratio was 4.62 (s.d. 3.22); this is an underestimate because comparisons where only transitional differences existed were excluded from the calculation.

Considering the relatively small sample sizes, the intrasite genetic diversity detected was surprisingly high. For all sites where more than a single individual was sampled, multiple haplotypes were detected (table 1). The mean haplotypic divergence within sites ranged from 0.33 to 2.40% while the nucleotide diversity (π) ranged from 0.0022 to 0.018, with the highest values for both measures recorded for site 1 in the headwaters sampling area (table 1). No haplotype was shared between localities.

(b) *Phylogeographic perspectives*

Two major, robustly supported clades were apparent in all analyses (figures 2 and 3) and separated the four headwater haplotypes from all others. These two clades correspond to the Acre and Juruá intracratonic basins. This same division was obtained using both the neighbour-joining and maximum-parsimony approaches. The tree topology was similar with or without the use of the various outgroup taxa considered in combination or singly. Further, the tree topologies resulting from the parsimony analysis were similar under the three different weighting schemes with the well-supported clades shown in figure 3 (bootstrap support ⩾ 80%) apparent in all. Within the clade corresponding to the intracratonic basin, geographical proximity was not a good predictor of evolutionary affinity; the upper central haplotypes (numbers 5–7) were consistently grouped with the mouth haplotypes (numbers 17–19; see figure 3).

The mean sequence divergence between across-river sites within the sampling areas ranged from 1.80 to 6.13% with the highest value being for the lower central area (table 2). The major genetic disjunction evident in the minimum spanning tree (34 steps separating haplotypes 1–4 from the rest; see figure 2) is reflected in the mean haplotypic sequence divergence between sampling areas and between basins
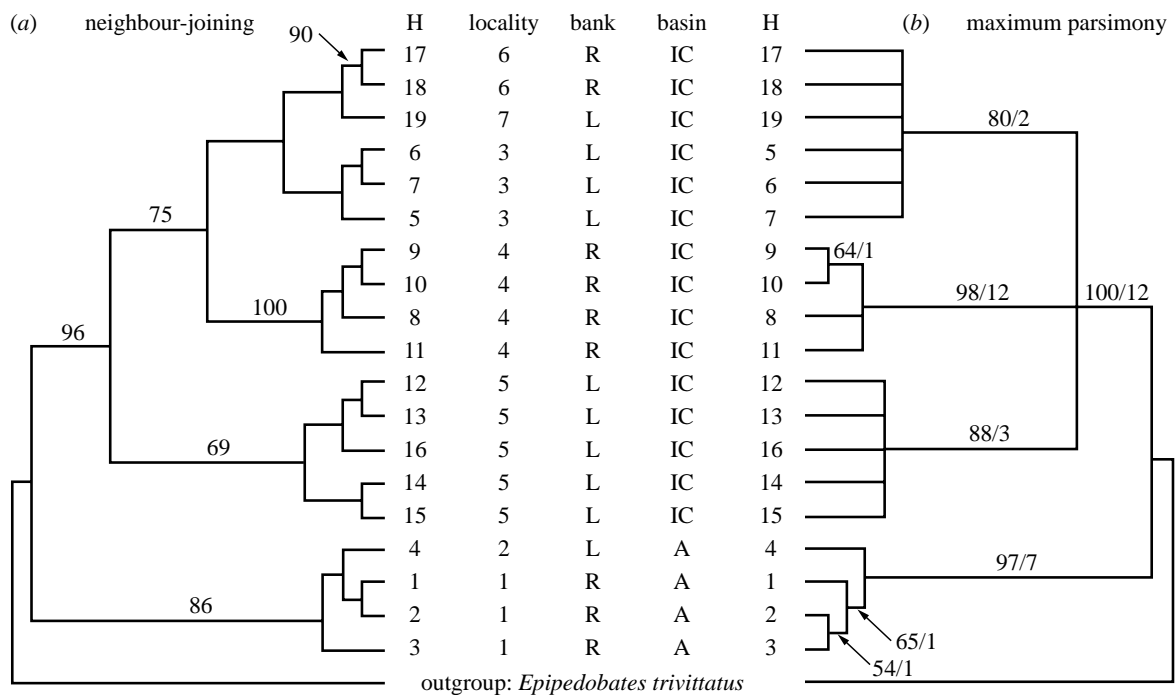
Figure 3. Rooted phylogenetic trees for 19 *E. femoralis* cytochrome *b* haplotypes from samples originating from the Rio Juruá using one of the four outgroup taxa considered in the analyses. (*a*) Neighbour-joining tree using Kimura two-parameter distances with percentage bootstrap support indicated for each branch. (*b*) Strict consensus of 28 equally parsimonious trees (131 steps) from a heuristic search with transversions and transitions weighted equally. Percentage bootstrap support and Bremer decay indices (after the forward slash) are indicated for non-polytomous nodes. The haplotype codes (H) correspond with those listed in figure 2, the banks codes correspond to right (R) and left (L) and the basin designation indicates whether the haplotype derives from west (Acre basin, A) or east (intracratonic basin, IC) of the Iquitos Arch. See the text for details.

(table 2). The mean divergence between the headwater haplotypes and each of the other three sampling areas ranged from 12.04 to 12.36% and that between haplotypes situated on opposite sides of the Iquitos Arch was 12.16%. The results of our *a priori*, topology-dependent permutation tests support the ridge hypothesis (T-PTP = 0.001) but not the riverine barrier hypothesis (T-PTP = 1.000), at least in its most stringent form assuming monophyly of the haplotypes on opposite banks.

4. DISCUSSION

Each of the 19 haplotypes detected was unique to a single locality. Further, the results from most phylogenetic analyses are consistent with the idea that haplotypes within localities comprise monophyletic groups (figures 2 and 3). Although a somewhat provisional conclusion because of small sample sizes, these observations suggest a high degree of compartmentalization (*sensu* Neigel & Avise 1986; Slatkin 1989). The finding of high viscosity of gene flow is congruent with an earlier allozyme study of *E. femoralis* (Gascon *et al.* 1998). The high apparent nucleotide diversity, particularly within headwater sites, suggests two possibilities. First, the effective population sizes are large because we would otherwise expect genetic drift to have eliminated much of the intrapopulation genetic diversity (Wright 1969). Second, gene flow may be restricted between regions but high between local populations within regions, thus maintaining high intrapopulational diversity. Our sampling is of an insufficiently fine geographical scale to distinguish between these possibilities.

(a) *Riverine barrier hypothesis*

The phylogeographic pattern revealed by our analyses appears inconsistent with the riverine hypothesis, at least insofar as the Rio Juruá was posited to be the most important historical barrier to gene flow in this region. The topology-dependent permutation test confirmed that haplotypes derived from opposite river banks do not form monophyletic clades. One could argue that our test of the riverine barrier hypothesis is too stringent and that perhaps the river is still important in restricting gene flow within basins. If we consider the intracratonic basin clade only, a topology-dependent permutation test for intrabank monophyly still suggests that the river has had little impact in shaping current phylogeographic patterns (T-PTP = 0.220). While we recognize the criticisms that have been directed at some applications of these topology-dependent permutation tests (e.g. Swofford *et al.* 1996), these tests merely formalize what is evident from figures 2 and 3. In sum, right- and left-bank haplotypes do not group together even within the two major clades evident in figure 3. Further, the greatest differentiation between spatially proximate sites on opposite banks does not occur at the junction of the Juruá and Amazon Rivers where the putative barrier was predicted to be strongest, but in the lower central sampling area some 500 km upriver from the mouth. Within the headwaters sampling area there is actually a greater mean sequence divergence between haplotypes within site 1 than between the haplotypes found on opposite banks of the river.

A caveat to the preceding discussion is the notion that we are dealing with a complex of morphologically similar

phylogenetic species, even within the intracratonic basin. If the defining cladogenic events predate the formation of the present-day Juruá drainage system, then it is the pattern within major clades that may be more appropriate for testing the riverine barrier hypothesis. Our ability to test the hypothesis at this level in the phylogenetic hierarchy is somewhat limited but the patterns shown in figure 2 again seem to run against the idea that the Rio Juruá has been an important impediment to gene flow.

(b) Ridge hypothesis

Clearly, the major genetic disjunction between the headwaters and all remaining sampled areas is geographically coincident with the Iquitos Arch and, thus, consistent with the ridge hypothesis. This result is also consistent with phylograms generated using allozyme-based distances that showed the headwaters as the most divergent among the samples compared (Gascon *et al.* 1998). However, to decide whether this major genetic disjunction is temporally coincident with the estimated formation of the Iquitos Arch, we must attempt to date the origin of the two major clades shown in figure 3. Assuming rates of change in cytochrome *b* of between 0.8 and 2.5% per Myr (Brown *et al.* 1982; Shields & Wilson 1987; Irwin *et al.* 1991; Martin *et al.* 1992; Tan & Wake 1995), separation of the two mitochondrial clades occurred some 5–15 Myr ago. These dates bracket the period of major orogenic events in western South America and presumably the formation of the Iquitos Arch (Räsänen *et al.* 1990).

Our results are mirrored by the patterns of genetic structure described from some echimyid rodents (e.g. *Mesomys hispidus*; Patton *et al.* 1994). Although the amount of haplotype sharing varies across taxa, the major axis of differentiation is clearly orientated east–west corresponding approximately to the headwater and mouth regions of the Rio Juruá. Such commonality of phylogeographic pattern suggests some historically pervasive barrier to gene flow that has impacted on a variety of taxa. As we have argued above, the Iquitos Arch is a probable candidate.

(c) Depth of divergence

Exceptional levels of intraspecific genetic divergence, as we found between the headwaters and intracratonic basin haplotypes (table 1), may not be atypical of Amazonian anurans (Heyer & Maxson 1982; Hass *et al.* 1995) or indeed of neotropical vertebrates in general when compared to their Nearctic counterparts (e.g. birds (Gerwin & Zink 1989; Seutin *et al.* 1993, 1994) and rodents (Da Silva & Patton 1993)). Possible explanations include lower vagility, a smaller effective population size or simply longer independent evolutionary histories of neotropical versus Nearctic organisms (e.g. Hackett & Rosenberg 1990). The only available comparative data for the exclusively neotropical dendrobatids suggest that *E. femoralis* populations may show greater population structure as assayed by mtDNA cytochrome *b* than even other confamilial species (Summers *et al.* 1997). This difference may be due to the relatively recent age of much of the Central American isthmus (*ca.* 3 Myr; see Coates & Obando 1996) compared to lowland Amazonia. Definitive statements on the generality of a pattern of differences in anuran genetic population structure between the neotropics and Nearctic await analyses of additional data.

5. CONCLUSION

Various hypotheses of diversification of Amazonian biota have proved generally difficult to evaluate because some predictions are difficult to test or are non-exclusive to individual hypotheses (e.g. Bush 1994). In contrast, the riverine barrier and ridge hypotheses as applied to the Rio Juruá basin make clear and distinguishable predictions regarding major axes of phylogeographic differentiation. Our present results and those of a few other studies (Da Silva & Patton 1993; Patton *et al.* 1994, 1996; Gascon *et al.* 1996, 1998), indicate that the notion of riverine barriers as important and pervasive 'engines' of biodiversity generation is problematic. Our study instead suggests that the present-day patterns in genetic population structure in the western Amazon may be the result of barriers that are no longer evident on the landscape. While our evidence is, by necessity, correlational, we can definitively reject the idea that the Rio Juruá has been important in shaping the patterns of mtDNA diversity in *E. femoralis*. Our results also add to an emerging pattern of surprising deep genetic divisions among many neotropical taxa including anurans. Further work with other taxa and both mitochondrial and nuclear markers will add to our understanding of vicariance mechanisms and the generation of biological diversity in lowland Amazonia.

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