

ORIGINAL ARTICLE

Applying new inter-individual approaches to assess fine-scale population genetic diversity in a neotropical frog, *Eleutherodactylus ockendeni*

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We assess patterns of genetic diversity of a neotropical leaf-litter frog, *Eleutherodactylus ockendeni*, in the upper Amazon of Ecuador without *a priori* delineation of biological populations and with sufficiently intensive sampling to assess inter-individual patterns. We mapped the location of each collected frog across a 5.4 × 1 km landscape at the Jatun Sacha Biological Station, genotyped 185 individuals using five species-specific DNA microsatellite loci, and sequenced a fragment of mitochondrial cytochrome *b* for a subset of 51 individuals. The microsatellites were characterized by high allelic diversity and homozygote excess across all loci, suggesting that when pooled the sample is not a panmictic population. We conclude that the lack of panmixia is not attributable to the influence of null alleles or biased sampling of consanguineous family groups. Multiple methods of population cluster analysis, using both Bayesian and max-

imum likelihood approaches, failed to identify discrete genetic clusters across the sampled area. Using multivariate spatial autocorrelation, kinship coefficients and relatedness coefficients, we identify a continuous isolation by distance population structure, with a first patch size of ca. 260 m and apparently large population sizes. Analysis of mtDNA corroborates the observation of high genetic diversity at fine scales: there are multiple haplotypes, they are non-randomly distributed and a binary haplotype correlogram shows significant spatial genetic autocorrelation. We demonstrate the utility of inter-individual genetic methods and caution against making *a priori* assumptions about population genetic structure based simply on arbitrary or convenient patterns of sampling.

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Introduction

The upper Amazon of Ecuador has exceptional amphibian species diversity and endemism, much of which is represented by terrestrial direct-developing frogs of the genus *Eleutherodactylus* (Duellman, 1999). The independence from water for reproduction is apparently very successful, as evidenced by its repeated and independent evolution (Hanken, 1999) and the observation that we currently recognize more than 600 direct-developing *Eleutherodactylus* species (AmphibiaWeb, 2006). From a population genetics perspective, *Eleutherodactylus* species are excellent study organisms because they facilitate analysis of inter-individual gene flow, rather than requiring a focus on pond-based (and therefore sampling biased) meta-population dynamics more common in amphibians (Marsh and Trenham, 2001).

Fine-scale population genetic structure is the first step in the continuum from population polymorphism, through

population differentiation, and ultimately to species diversity and richness (Templeton, 1981), so patterns among individuals may provide important insights into gene flow at higher hierarchical levels (Manel *et al.*, 2003). Though neotropical frogs are known to be particularly genetically diverse (Gascon *et al.*, 1998; Loughheed *et al.*, 1999; Crawford, 2003) and species rich (Duellman, 1999), there has been little research to date on fine-scale gene flow in neotropical amphibian populations.

Here we use new analytical approaches to quantify genetic diversity and gene flow in a neotropical leaf-litter frog, *Eleutherodactylus ockendeni*, within a single site in Ecuador using both nuclear DNA microsatellite and mitochondrial DNA markers. We employ both model- and distance-based statistical approaches without *a priori* delineating 'populations'. Our findings on the scale of gene flow in *E. ockendeni* may also provide insight into the population genetic processes that shape genetic diversity in other tropical species, especially small, direct-developing rainforest-restricted amphibians in this biodiverse but under-studied biogeographic region.

Materials and methods

Taxon sampling

Eleutherodactylus ockendeni (Leptodactylidae) frogs were collected opportunistically in primary and secondary

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rainforest in the upper Napo river basin in eastern Ecuador at the Jatun Sacha Biological Station and adjacent areas (W 77° 36', S 01° 04', 420 m above sea level with approximately 100 m local elevation variation). Trails for sampling were entered from the north access point (the biological station near the Napo River) and from the south (Arajuno River). Access to the central approximately 1 km of the reserve for sampling is not permitted. The locality is characteristic upper Amazonian habitat, with numerous small streams, moderate ridges and *terre firme* forest outside a narrow floodplain along the Napo River (see Pearman, 1997 for a complete description of local habitat). Geographic coordinates of each collected frog were taken with a handheld Global Positioning System (Garmin ETrex Vista) and mapped onto local topography with a Geographic Information System database developed by Sandler *et al.* (1997) using ArcGIS version 9 (Environmental Systems Research Institute, 2005) (Figure 1).

Frogs were killed with MS222, and then fixed with 10% formalin per standard protocols and stored in 70% ethanol. Liver and muscle samples were taken from each individual and stored in pure ethanol. Voucher specimens are deposited and catalogued at the Museo de Zoología, Pontificia Universidad Católica del Ecuador (QCAZ). DNA was extracted using standard phenol-

chloroform methods (Sambrook *et al.*, 1989) or Qiagen DNEasy Tissue Extraction kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and stored until use at -20°C.

Laboratory methodology

Species-specific genomic library development, primer sequences, microsatellite motifs and amplification conditions are described in Elmer *et al.* (2006). We amplified seven tetranucleotide loci, Eloc-Bert&Ernie (hereafter ElocB&E), Eloc-Beauty&Beast (ElocB&B), Eloc-Jean-Paul&Simone (ElocJP&S), Eloc-Romeo&Juliet (ElocR&J), Eloc-Thelma&Louise (ElocT&L), Eloc-Laurel&Hardy (ElocL&H) and Eloc-Batman&Robin (ElocB&R). All amplifications included a negative control. PCR amplification was confirmed by agarose gel electrophoresis and ethidium bromide post-staining. Samples that amplified were subsequently genotyped on an ABI 3100 Automated Capillary DNA Sequencer and allele sizes determined according to GeneScan 500 LIZ Size Standard (Applied Biosystems, Foster City, CA, USA) and GeneScan Analysis Software version 3.7 (Applied Biosystems).

A fragment of the cytochrome *b* gene (approximately 800 bp) was amplified via PCR in 50 µl volumes that

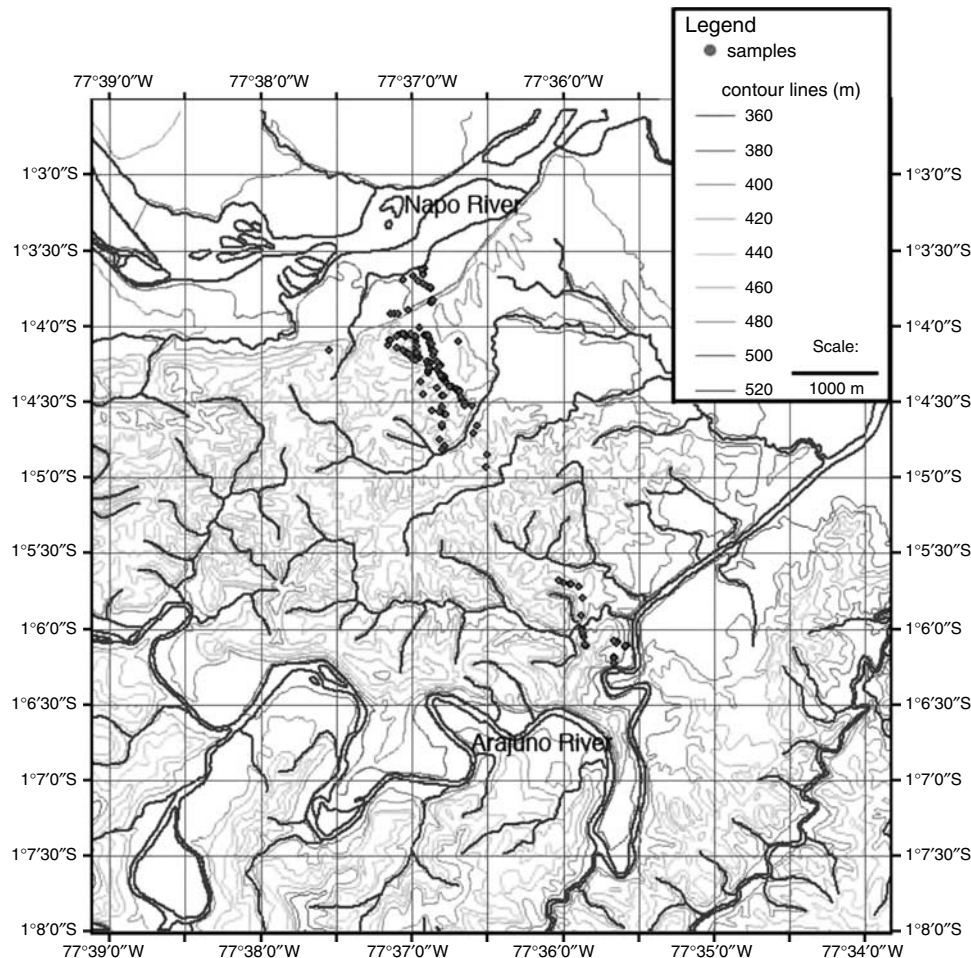


Figure 1 Map of collected individuals of *E. ockendeni* across the study area in the upper Amazon of Ecuador, the Jatun Sacha Biological Station.

contained 0.3 μM of the forward and reverse primer (MVZ15L and MVZ16H, Moritz *et al.*, 1992), PCR enhancing buffer (2.5 mM MgCl_2 , 10 mM Tris pH 8.4, 50 mM KCl, 0.02 mg bovine serum albumin and 0.01% gelatin), 0.3 mM dNTPs, 0.625 units *taq* DNA polymerase and approximately 1–5 ng DNA. All reaction sets included a negative control. Cycling parameters were: initial denaturing at 92°C for 3 min, 38 cycles denaturing 92°C for 1 min, annealing 51°C for 1 min and extension 72°C for 1 min, final extension of 72°C for 5 min, followed by extended cooling at 4 or 10°C.

PCR product was separated by electrophoresis on 1% 1 \times TBE (tris-borate-ethylenediamine tetraacetic acid) agarose gels and harvested bands cleaned with Qiaquick Gel Extraction kit as per manufacturer's protocol or remaining PCR product was purified using Pall Acro-Prep 96 Filter Plates according to the manufacturer's instructions. Samples were capillary sequenced using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) chemistry on an Applied Biosystems 3100 or 3730XL Gene Analyzer. Fifty-one samples were sequenced: 35 in both directions and the remainder only in the forward direction. Sequences are submitted to GenBank with accession numbers EF581013–EF581063.

Population genetics analyses

Summary statistics: Problems with single allele amplification and genotyping errors were inferred using the program MICRO-CHECKER version 2.2.3 (van Oosterhout *et al.*, 2004). Departures from Hardy–Weinberg equilibrium (HWE) and linkage equilibrium across all loci and samples pooled were tested in GENEPOP web version 3.4 (Raymond and Rousset, 1995) using Monte Carlo approximation of exact tests. Sequential Bonferroni correction was applied (Rice, 1989). We calculated F_{IS} values with the bias-corrected (Robertson and Hill, 1984) estimator, which is most appropriate when genetic differentiation is low (Raufaste and Bonhomme, 2000; Waples and Gaggiotti, 2006). Gene diversity (H_e) for each locus was calculated in SPAGEDi version 1.2 (Hardy and Xavier, 2002).

Inbreeding: With all samples pooled, we ran 1000 genotype permutations in IDENTIX version 1.1 (Belkhir *et al.*, 2002) using the $rx_{y_{Identity}}$ identity (Belkhir *et al.*, 2002), $rx_{y_{QG}}$ (Queller and Goodnight, 1989) and $rx_{y_{LR}}$ (Lynch and Ritland, 1999) estimators of relatedness. Significant deviations of the mean from the null would suggest that individuals are more consanguineous than expected under random mating. If multiple consanguineous groups had been sampled, overall mean identity values of the relatedness distribution may not differ significantly from random though variance would (Belkhir *et al.*, 2002). Therefore, we tested for deviations of both mean and variance from the null expectation.

Demographic history: We tested for genetic signatures of a recent population bottleneck using the method of Garza and Williamson (2001), which compares richness versus size distribution of alleles.

Population clustering: The program PARTITIONML (Castric *et al.*, 2002) uses maximum likelihood criteria to search for the best clustering strategy and place

individuals into their most likely population of origin, assuming random mating within clusters. We successively increased k until there was no improvement in fit ($P < 0.1$) after penalizing the addition of extra model parameters with a likelihood ratio test.

We tried four Bayesian approaches to identifying population structure. In STRUCTURE version 2.1 (Pritchard *et al.*, 2000; Falush *et al.*, 2003), we used the admixture model, a separate α -inferred for each population and correlated allele frequencies (λ) estimated for each population, iterating $k=1$ to 10 five times, with and without prior population information (that is, all samples as one population or northern and southern samples as two populations; Figure 1). Analyses were run for 500 000 generations of which the first 50 000 were discarded as burn-in. We used Bayesian analysis of population structure (BAPS) version 4.14 (Corander *et al.*, 2003, 2004) without spatial reference with 5, 10, 15, 20 or 25 iterations of maximum $k=55$ to cluster individuals, after experimenting to find suitable upper bounds of k . With BAPS version 2.2 (Corander *et al.*, 2003, 2004) in multiple Markov chain Monte Carlo clustering mode with maximum $k=55$ and no initial deterministic partition, we tried to identify population clusters five times with ten parallel chains of 50 000 iterations, 10 000 burn-in and thinning = 5. Convergence was estimated by the apparent stability of chains in the $\ln L$ plots after burn-in.

Multivariate spatial autocorrelation: Using GENALEX version 6 (Peakall and Smouse, 2006), we assessed spatial structure of all samples at five unlinked microsatellite loci using graphs of increasing cumulative distance class sizes and correlograms with equal sample sizes (that is, pairwise contrasts) per distance class. Genetic distances were calculated using the 'multilocus' and 'interpolate missing' options. Statistical significance of r was tested using 999 random permutations and 1000 bootstrap replicates. To test whether the correlogram pattern could be an artefact of the genetic distances in our sample, we also generated a correlogram using a random genetic distance matrix bounded by the genetic distance values found in the real data (0–15).

Multivariable inter-individual estimators: We calculated the inter-individual estimator Rousset's a (Rousset, 1997, 2000) and the similarity measures F_L (Loiselle *et al.*, 1995), F_R (Ritland, 1996) and R_M Moran's- I (per Epperson and Li, 1996) with inter-individual geographic distances in the software SPAGeDi. Genes and locations were permuted 1000 times to create a null distribution. The coefficients were regressed (r) against the logarithm of geographic distance.

MtDNA diversity and gene genealogy analyses: Sequences were assembled in MACCLADE version 4.07 (Maddison and Maddison, 2003). Alignment was not problematic and included no internal gaps. To help verify that we had true mitochondrial sequences and not nuclear homologues, we translated DNA into amino-acid corresponding sequences to confirm the absence of stop codons (Zhang and Hewitt, 1996). For analyses, sequence ends were

trimmed at the 5' and 3' ends to a total fragment size of 709 base pairs.

Mitochondrial sequence divergence was estimated in PAUP* version 4.0b10 (Swofford, 2003). Haplotype and nucleotide diversity were calculated in DNASP version 4.10.7 (Rozas *et al.*, 2003). Haplotypes were collapsed and a maximum parsimony network with 95% connection limit was built in TCS version 1.21 (Clement *et al.*, 2000) using the method of Templeton *et al.* (1992).

A binary (0 when identical, 1 when different) inter-individual haplotype distance matrix was calculated in GENALEX for 50 individuals. A graph of increasing cumulative distance class size and a correlogram were calculated as with the microsatellite data.

Results

Microsatellite population genetics

Summary statistics: Three of the 21 contrasts among loci showed significant linkage disequilibrium ($P < 0.001$; Eloc-B&B and Eloc-JP&S, Eloc-B&B and Eloc-R&J, and Eloc-JP&S and Eloc-R&J). Therefore, the loci Eloc-R&J and Eloc-B&B were eliminated from all subsequent analyses, leaving a data set of five loci that showed no significant linkage disequilibrium.

Using the Brookfield equation (Brookfield, 1996) and based on the distribution of homozygotes across allele sizes, two loci (Eloc-JP&S and Eloc-T&L) showed evidence of null alleles being rare ($P \leq 0.02$), two loci (Eloc-B&E and Eloc-B&R) showed slight evidence for the presence of null alleles ($P < 0.10$) and one locus (Eloc-L&H) showed moderate levels of null alleles ($P = 0.30$) (based on Dakin and Avise, 2004).

With all samples pooled, three loci (Eloc-B&E, Eloc-L&H and Eloc-B&R) departed significantly from HWE expectations ($P \leq 0.01$); there was no significant deviation for Eloc-JP&S and Eloc-T&L. When HWE was tested among samples divided by geographic cluster, northern samples ($n = 147$) showed the same pattern as all samples pooled. Southern samples ($n = 38$) showed significant deviation from HWE at Eloc-L&H and Eloc-B&R. A heterozygote deficiency ranging from weak to strong was found across all loci (Table 1). Average F_{IS} value was +0.154 for all loci combined. All loci were highly variable, with between 14 and 29 alleles per locus and a total of 110 alleles across all five loci. Average gene diversity (H_e) was 0.902.

Inbreeding: Neither the mean nor the variance of any of the three inbreeding estimators differed significantly from the null distribution of genotypes ($rx_{yIdentity}$ mean $P = 0.069$, variance $P = 0.269$; rx_{yQG} mean $P = 0.39$,

variance $P = 0.298$; rx_{yLR} mean $P = 0.880$, variance $P = 0.611$). Because mean $rx_{yIdentity}$ has a P -value close to the 5% significance level, we reanalysed the distribution using 10 000 permutations to increase accuracy; the estimator remained not significantly different from the null distribution ($P = 0.080$).

Demographic history: The *E. ockendeni* at this locality do not show a signature of having experienced a recent bottleneck in effective population size (Garza and Williamson, 2001): $M = 0.836$ averaged across five loci.

Population clustering: PARTITIONML analyses did not support the existence of more than one cluster ($P < 0.001$ for $k = 1$; $P = 0.074$ for $k = 2$; $P = 0.975$ for $k = 3$). Because the P -value for $k = 2$ clusters is only slightly below significance, we tested the content of each of those two clusters as separate genetic populations in GENEPOP and neither was in HWE (Fisher's method χ^2 was highly significantly different than expected for all loci combined within each cluster).

Clustering analyses in STRUCTURE showed that no value of k was strongly supported. When no prior population information was included in the model, the greatest increase in likelihood was between 1 and 2 clusters but higher k values did not plateau; beyond $k = 3$ clusters there was a lack of convergence (data not shown). When the model included whether the sampled frog came from the north or south group, there was poor convergence on $k = 1$ and a lack of convergence at all higher values of k (data not shown).

In repeated runs of BAPS 4 using a maximum $k = 55$, the number of determined clusters varied from 36 to 41 depending on whether we used 5, 10, 15, 20 or 25 iterations. Changing the upper bound of k to 110 did not change the range of clusters identified. Overall, this strongly suggests that the program was not converging repeatedly on the same likelihood space and could not identify clusters with confidence. The authors suggest that BAPS 2 be used if BAPS 4 has difficulty in ascertaining a number of clusters. However, BAPS 2 also failed to converge on a consistent number of population clusters. In five identical runs, four different numbers of clusters were found (40, 41 twice, 49 and 51).

Multivariate spatial autocorrelation: Spatial genetic structure was found with cumulative distances class sizes ranging from 100 to 5400 m (Figure 2 and Supplementary Table 1). Permutation tests identified significant structure from 200 m ($r = 0.010$, $P = 0.003$) up to almost the entire study area (4000 m; $r = 0.001$, $P = 0.014$). Bootstrap error bars (less powerful than permutations; see Peakall *et al.*, 2003) find positive spatial genetic structure at distances of 0–200 m, and from 0 to 600 m through 0 to 1200 m.

At inter-individual distances less than approximately 800 m, the pattern of spatial autocorrelation is positive at most distance classes; r -values are low and range from 0.013 to -0.004 with a y -intercept of 0.012 (Supplementary Figure 1 and Supplementary Table 2). Beyond approximately 800 m there is no positive spatial autocorrelation. The x -intercept approximately represents patch size (roughly akin to neighbourhood area; Sokal and Wartenberg, 1983); here the furthest extent of

Table 1 Number of alleles (N_A), ratio of observed over 'expected' heterozygosity (H), Robertson and Hill (1984) F_{IS} and unbiased estimated of gene diversity (H_e), for each locus

Locus	N_A	H_{obs}/H_{exp}	F_{IS}	H_e
Eloc-Bert&Ernie	22	0.872	+0.099	0.921
Eloc-JeanPaul&Simone	17	0.956	+0.021	0.911
Eloc-Thelma&Louise	14	0.960	+0.007	0.805
Eloc-Laurel&Hardy	28	0.362	+0.497	0.934
Eloc-Batman&Robin	29	0.802	+0.156	0.940

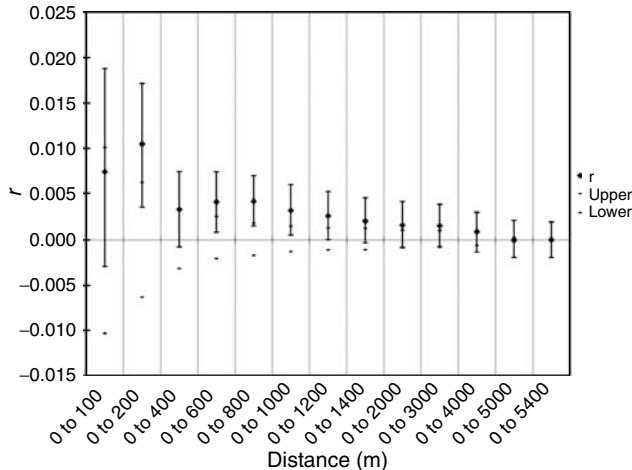


Figure 2 A graph of cumulative increasing distance classes from 100 to 5400 m and the spatial autocorrelation (r) of microsatellite genotypes with distance. Error bars from bootstrapping and upper and lower bounds of a null distribution are included.

positive autocorrelation is ca. 800 m and the first x -intercept (patch size) is 263 m.

The correlogram of random genetic distances fluctuates around zero ($r=0.053$ to -0.032) but shows no significant r -values at any distance class (data not shown).

Multivariable inter-individual estimators: The regression of Rousset's a -distance estimator by the logarithm of geographic distance had a y -intercept of 0.214 (not standardized to zero) and a weakly positive regression slope of 0.002, not significantly different than random spatial genetic structure ($P=0.327$; data not known). The similarity measures F_L , F_R and R_M showed a consistent statistically significant pattern of decreasing kinship or relatedness with increasing geographic distance. The y -intercept of r_L was 0.014 and the regression slope was very slightly negative at -0.002 ($P<0.001$; data not shown). The regression r_R had a y -intercept of 0.009, a slope of -0.002 ($P<0.001$), almost identical to r_L (Supplementary Figure 2). The regression of Moran's- I (r_M) had a y -intercept of 0.025, slope of -0.004 ($P<0.001$; Supplementary Figure 3). To illustrate the relationship between Moran's- I and natural geographic distance, we plotted a logarithmic trend line following R_M (Figure 3), which shows a gradual decrease in Moran's- I with distance although there is notable variation in R_M between distance classes. The first distance class Moran's- I value is 0.016.

MtDNA diversity and gene genealogy analyses

The maximum parsimony network of 51 sequences resulted in five nodes each separated by one step (Figure 4b). Thirty-four individuals resolved into haplotype II, five in haplotype I (putatively ancestral to the other haplotypes in this sample based on outgroup weight), nine in haplotype IV, two in haplotype III and one into haplotype V. Haplotypes do not appear to be randomly geographically distributed: for example, haplotypes V and IV are more prevalent in the southern portion of the reserve, while haplotype I is more common in the north (Figure 4a).

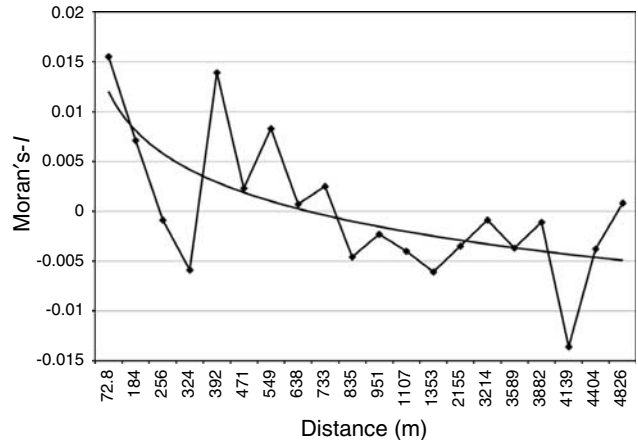


Figure 3 Multivariable Moran's- I plotted against average geographic distance classes (in metres), with a logarithmic trend line.

The maximum uncorrected p sequence distance is 0.0063 between haplotypes III and V. Haplotype diversity (h) is 0.470 ± 0.073 and per site nucleotide diversity (π) is 0.00122 ± 0.00020 . DNASP accommodates missing data differently than Templeton, Crandall, Sing and identified four haplotypes.

The graph of increasing cumulative distance classes (Supplementary Figure 4 and Supplementary Table 3) and the correlogram (Supplementary Figure 5 and Supplementary Table 4) indicates significant positive spatial autocorrelation of a binary-distance haplotype matrix against geographic distance. The pattern of autocorrelation is similar to the microsatellite correlograms: the first distance class has positive autocorrelation, which then decreases before increasing again at ca. 759–1215 m, and then decreases to no or negative autocorrelation for the remaining distance classes. Patch size, or the x -intercept, is 506 m. Significant cumulative inter-individual spatial autocorrelation can be detected up to 4800 m.

Discussion

Quantifying spatial genetic structure

We found no evidence that the population as a whole nor the two sampling clusters is in HWE. Our thorough testing to identify reasons for the lack of HWE found neither evidence for discrete subpopulation clusters nor support for panmixia. Lack of HWE can be attributed to: (i) null alleles; (ii) consanguineous mating or sampling individuals from a few families; or (iii) a Wahlund effect (adapted from Castric *et al.*, 2002) and we specifically addressed each of these possibilities.

Neither null alleles nor inbreeding underlie the homozygote excess in our data. Combined probability tests based on expected frequencies of homozygotes and heterozygotes across allele size classes (van Oosterhout *et al.*, 2004) did not detect a significant influence of null alleles or other genotyping errors. Therefore, the homozygote excess across loci is apparently not an artefact of null alleles and instead can be considered an actual pattern in the distribution of genotypes.

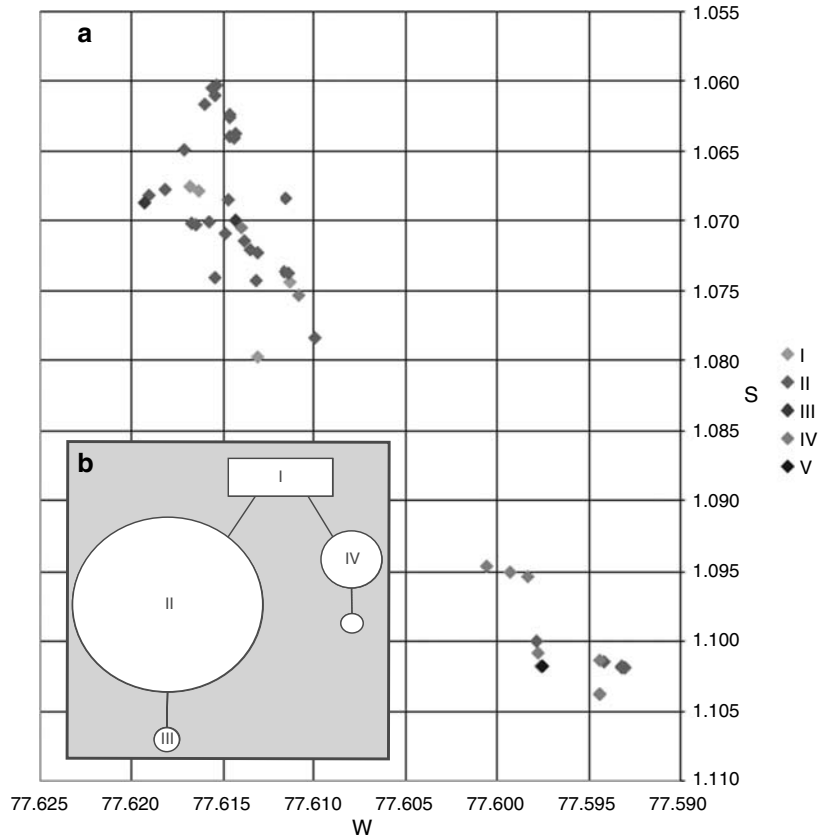


Figure 4 A plot of (a) haplotypes across the sample area and (b) a maximum parsimony haplotype network representing haplotype frequencies. Spatial referencing of the distribution of individuals is a simplification of Figure 1 (map).

Estimators of inbreeding did not identify significant consanguinity in the data using two different approaches: Queller and Goodnight (1989) and Lynch and Ritland (1999) test the probability of identity by descent of genes among individuals at random, while the Belkhir *et al.* (2002) method assesses the expected proportion of loci that are homozygous in the offspring of a chosen pair (Belkhir *et al.*, 2002). Therefore, we consider it very unlikely that the homozygote excess in this *E. ockendeni* population is a result of our having sampled a few closely related kin groups.

Addressing the possibility that the lack of HWE is due to discrete genetic subpopulations within the sampled area (Wahlund effect; Sinnock, 1975) is more complicated: neither the maximum likelihood nor the Bayesian model-based tests identified any notable structure or population clustering. It has been demonstrated in other studies that the algorithms of STRUCTURE and BAPS successfully assign clusters when they exist (Castric *et al.*, 2002; Berry *et al.*, 2004; Corander *et al.* 2004; Funk *et al.*, 2005; Frantz *et al.*, 2006; Waples and Gaggiotti, 2006; Cabe *et al.*, 2007). STRUCTURE is not well suited to the continuous fine-scale sampling scheme used here (Pritchard *et al.*, 2000), it may perform better with more loci than we have (Evanno *et al.*, 2005; Waples and Gaggiotti, 2006), and performs poorly when divergence among clusters, if they do exist, is low (Castric *et al.*, 2002; Waples and Gaggiotti, 2006) or there is isolation by distance (Pritchard and Wen, 2003). PARTITIONML and BAPS are to date less tested in the literature than

STRUCTURE, but presumably are hindered by similar issues. Given their frequent successes at assigning individuals to genetic groups in other biological systems, we do not suggest that these four model-based programs have failed here. Rather, we suggest that there are no significant genetic clusters to be found. Thus, in our study the lack of obvious clustering in the data is an important result about spatial genetic population structure because it suggests there is no discrete subpopulation structuring at this level.

We conclude that the *E. ockendeni* from this locality comprise a continuous non-panmictic population of inter-individual isolation by distance (hereafter IBD). Here, IBD is evidenced by kinship and relatedness measures that decrease with the logarithm of geographic distance (Fenster *et al.*, 2003) (Figure 3 and Supplementary Figures 2 and 3), a homozygote excess with microgeographic differentiation in gene frequencies (Turner *et al.*, 1982; Epperson, 1995; Vekemans and Hardy, 2004) and spatial genetic autocorrelation that mirrors the signature expected under IBD (Barbujani, 2000; Peakall *et al.*, 2003). It is clear from the pattern in all correlograms that there is spatial genetic structure of IBD in this data set, both for microsatellite genotypes and mtDNA binary haplotypes. Spatial autocorrelation of genotypes and spatial distance is found at the scale of minimally approximately 200 m and, including the second periodic increase in the correlogram, approximately 450 m; no positive spatial autocorrelation is found after approximately 800 m and at most distance classes

the association is weak (low r -value) (Figure 3; Supplementary Figure 1 and Supplementary Table 2). The increasing cumulative distance measures identified positive genetic structure at distances of up to 4 km (Figure 2 and Supplementary Table 1), suggesting that genetic neighbourhoods or patches are present but not highly restricted or differentiated.

It is difficult for us to compare the *E. ockendeni* r -values with other amphibian studies using spatial autocorrelation correlograms: there are to date relatively few studies using inter-individuals spatial autocorrelation on animals and they are more common on plants (Vekemans and Hardy, 2004). In a study of Australian bush rats, *Rattus fuscipes*, trapped along a transect, r -values were proposed to be four times higher than what had previously been found in plants and ranged from 0.110 at 50 m to 0.004 at 600 m (Peakall *et al.*, 2003). An inter-individual microsatellite spatial autocorrelation study of the marsupial carnivore, *Antechinus agilis*, r -values in the first distance class ranged from ca. 0.03 to 0.07 (Banks *et al.*, 2005), while in Pacific jumping mice, *Zapus trinotatus*, the r -value was similar to that lower bound at ca. 0.025 (Vignieri, 2005). Spatial autocorrelation studies of amphibians tend to pool individuals by sample population and examine broader geographic scales than we have here, making direct comparisons difficult. Nonetheless, the first distance class values tend to be higher than those that we found. With microsatellites, r -values of 0.015–0.06 in *Rana temporaria* (Johansson *et al.*, 2005) and ca. 0.06 in spotted salamanders, *Ambystoma maculatum* (Zamudio and Wieczorek, 2007), were reported. Moran's- I of single minisatellite alleles in *Bufo bufo* in Great Britain ranged widely, from –0.03 to 0.07 (Scribner *et al.*, 2001).

The mitochondrial haplotype correlogram shows a very similar pattern to that of the microsatellite genotypes, though at larger geographic distances (that is, mtDNA patch size is 505 m; Supplementary Figures 4 and 5, and Supplementary Tables 3 and 4). While the information in haplotype correlograms is simplified because the genetic distance matrix is developed from binary difference (Peakall and Smouse, 2006), earlier studies found such correlograms to reflect biologically real genetic diversity (Cassens *et al.*, 2000) and even corroborate nuclear patterns (Ehrich and Stenseth, 2001; Banks *et al.*, 2005) as we see here in *E. ockendeni*.

Spatial autocorrelation can reveal the scale and pattern of correlation but cannot identify the specific location of discontinuities (Manel *et al.*, 2003), so ideas of 'patch size' should be interpreted cautiously (Fortin, 1999; Radeloff *et al.*, 2000; Rousset, 2001). The oscillating pattern of r found in early distance classes of the correlograms is similar to the periodicity patterns expected with spatial patchiness (Fortin, 1999; Radeloff *et al.*, 2000) and IBD (Barbujani, 2000; Peakall *et al.*, 2003). The pattern of the correlogram is also possibly an exaggeration of the stochastic oscillations expected under IBD (Vekemans and Hardy, 2004), a function of inconsistent signal among loci (Sokal and Oden, 1991), or an artefact of our uneven sampling design (Fortin *et al.*, 1989). We think it unlikely that the periodicity is a result of the gap in sampling at the centre of the study area because the gap in pairwise inter-individual geographic distances (most notably at approximately 1.5–3 km) does not coincide with the

fluctuation in r at short inter-individual geographic distances (that is, approximately 300 m).

Moran's- I calculated from genotypes has a well-characterized distribution under IBD and offers valuable information on dispersal, neighbourhoods and effective population sizes (N_e) (Epperson, 2005). The relatively low Moran's- I value in the first distance class of the microsatellite correlogram (Figure 3) implies N_e values of approximately 250 or 300 individuals (Figure 2 and Table 1 in Epperson, 2005). The gradual decrease in the logarithmic trend line of geographic distance and Moran's- I seen in our data also suggests that effective population sizes are large (Figure 1 in Epperson, 2005). Crawford (2003) proposed that populations of terrestrial *Eleutherodactylus* in neotropical rainforests are huge and that this may in part be responsible for their high levels of genetic diversity. Data presented here and the high numbers of *E. ockendeni* found in the Jatun Sacha area (KR Elmer, unpublished data; Pearman, 1997) certainly would corroborate that hypothesis.

Assessing genetic diversity

We found high polymorphism in both microsatellites and mtDNA in *E. ockendeni* at this upper Amazonian locality. Inter-specific comparisons of levels of genetic diversity can be problematic because of differences in ecology and taxonomy of compared species, geographic regions and/or molecular markers, especially non-homologous microsatellite loci. Nonetheless, such comparative inferences are valuable for placing our findings in context, and are a fundamental element of comparative biogeography because patterns independently identified across taxa together can be used to test regional or taxon-specific hypotheses of evolution. The heterozygosity across microsatellite loci in this study (average H_e 0.902) is much higher than anuran populations' microsatellite heterozygosities in the temperate Americas: for example, 0.427–0.731 among populations of *Rana catesbeiana* in Ontario, Canada (Austin *et al.*, 2004); 0.23–0.70 in *Rana luteiventris* populations in the western USA (Funk *et al.*, 2005); 0.25–0.87 in north western USA populations of *Rana cascadae* (Monsen and Blouin, 2003); and 0.47–0.66 in *Rana temporaria* in Sweden (Johansson *et al.*, 2005). It is difficult to make biogeographically more appropriate comparisons because almost no studies of amphibian microsatellite population genetics have been published for the Neotropics. An exception, a study of túngara frog population genetics in Panamá, found average heterozygosity of 0.820 across 17 populations and 7 loci (Lampert *et al.*, 2003, our calculation based on authors' tables), a value which is more comparable to our findings here.

Spatial genetic structure, conservation and species diversity

Understanding patterns of inter-individual gene flow will prove critical in allowing biologists to quantify the impact that increasing anthropogenic disturbance in the upper Amazon, such as road construction, farm colonization and oil pipeline construction (McLearn, 2003; Bilsborrow *et al.*, 2004), has on amphibians. Amazonian *Eleutherodactylus* are almost exclusively primary and secondary forest species (Duellman, 1978) and therefore are particularly sensitive to environmental disturbance

and deforestation; for example, species richness at Jatun Sacha has been shown to decrease with proximity to deforested pasture (Pearman, 1997). Our findings that the Jatun Sacha population has not experienced a recent demographic bottleneck are positive news that the population was probably not greatly affected by historical selective logging in some parts of the Jatun Sacha forest. Neotropical terrestrial direct-developing frogs, in the scant ecological evidence to date, do not disperse very far. Caribbean terrestrial direct-developing frogs are thought to have extremely small home ranges and move little per night, such as 3–4.5 m in *Eleutherodactylus coqui* (Woolbright, 1985) and 2.8–4.0 m in *Eleutherodactylus johnstonei* (Ovaska, 1992). There is evidence that the Central American frog *Eleutherodactylus fitzingeri* may have a somewhat larger home range, averaging 7 m between sightings (Höbel, 1999). The small genetic patch sizes implied here in *E. ockendeni* corroborate restricted dispersal and, coupled with amphibians' sensitivity to deforested edge effects (Pearman, 1997), suggests that anthropogenic deforestation, especially linear habitat degradation such as roads and pipelines, may have a strong negative impact on terrestrial amphibian, inter-individual population dynamics and gene flow.

Here we present a neotropical amphibian genetic study with sufficiently intensive sampling within a geographic area to characterize population structure and gene flow dynamics at inter-individual scales. By focussing on a direct-developing terrestrial species, *E. ockendeni*, we uncover the fine-scale population genetics of an amphibian that is not tied to water for reproduction and is therefore distributed throughout the forest and presumably less subject to classical amphibian metapopulation dynamics (discussed in Smith and Green, 2005). Because the ecology of *E. ockendeni* is likely similar to numerous upper Amazonian terrestrial, direct-developing *Eleutherodactylus* frogs, the pattern we identified here of continuous population IBD and high genetic polymorphism may be a general pattern of population structure at these small scales. If this restricted gene flow is a product of the limited vagility of neotropical terrestrial amphibians, generally, then this may be a factor of natural history that promotes allopatric or parapatric speciation in these taxa (also suggested by, for example, Garcia-Paris et al., 2000; Graham et al., 2004; Wake, 2005) and may ultimately underlie the high biodiversity in the area.

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