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Genetic divergence is more tightly related to call variation than landscape features in the Amazonian frogs *Physalaemus petersi* and *P. freibergi*

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Abstract

Behavioural isolation from divergence in male advertisement calls and female preferences is hypothesized to cause genetic divergence and speciation in the Amazonian frogs *Physalaemus petersi* and *P. freibergi*, yet the importance of call variation and landscape features in genetic divergence is unresolved. We tested for correlations between genetic divergence at microsatellite loci and (1) call variables; and (2) landscape variables among 10 populations of these frogs. Genetic divergence was not correlated with geographical distance, rivers or elevation. There was a strong positive relationship, however, between genetic divergence and inter-population differences in one call variable, whine dominant frequency. Effective population sizes varied among sites (range = 15–846) and were often small, suggesting that genetic drift could influence call evolution. Evidence for fine-scale genetic structure within sites was also found. Our results support the hypothesis that behavioural isolation from divergence in male calls and female preferences causes genetic divergence and speciation.

Introduction

Numerous mechanisms have been hypothesized to cause population divergence and speciation, here defined as the evolution of reproductive isolation (Mayr, 1963). Potential speciation mechanisms include geographical barriers (Wallace, 1852; Mayr, 1963), divergent ecological selection (Endler, 1977; Moritz *et al.*, 2000) and sexual selection (Fisher, 1930; Lande, 1981, 1982; West-Eberhard, 1983), and genetic drift (Nei, 1976; Nei *et al.*, 1983). These mechanisms are often considered separately in the context of three ongoing debates about speciation: (1) the importance of geographical barriers; (2) the importance of divergent selection vs. genetic drift; and (3) the roles of ecological vs. behavioural isolation (Coyne & Orr, 2004). As these mechanisms may operate in concert in the same populations to cause speciation, or in different populations in different parts of a species' range, however, they should be considered together when evaluating speciation mechanisms. Furthermore, to demonstrate that an isolating mechanism such as behavioural isolation is leading to speciation, it is necessary to show that the isolating mechanism restricts gene flow. If an isolating mechanism does not restrict gene flow, then it will not cause continued population divergence and therefore will not complete the process of cladogenesis (speciation). Studying genetic divergence and speciation at this fine-scale, phylogeographical or landscape level is a particularly powerful approach for understanding speciation mechanisms (Panhuis *et al.*, 2001; Ritchie, 2007).

One isolating mechanism that continues to generate interest is behaviour (Blair, 1958; Alexander, 1962; Walker, 1974; Ryan & Wilczynski, 1988; Panhuis *et al.*, 2001; Coyne & Orr, 2004; Ritchie, 2007; Price, 2008). Behavioural isolation evolves when male mating signals and female preferences for these signals diverge among

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populations such that females prefer the local male signal (e.g. Coyne & Orr, 2004). The mechanisms causing divergence in male signals and female preferences may include divergent sexual selection, divergent ecological selection or genetic drift (Fisher, 1930; Lande, 1981, 1982; West-Eberhard, 1983; Coyne & Orr, 2004; Gavrilets, 2004; Ritchie, 2007). There are many examples of divergent male signals and female preferences among populations or incipient species (e.g. Ryan & Wilczynski, 1988; Seehausen *et al.*, 1997, 1999; Gray & Cade, 2000; Masta & Maddison, 2002; Coyne & Orr, 2004; Price, 2008). But only a handful of studies have shown that signal and preference divergence is correlated with restricted gene flow (Wilson *et al.*, 2000; Boul *et al.*, 2007; Ritchie *et al.*, 2007), suggesting that populations with different signals and preferences are diverging into distinct species. Showing this correlation between behavioural isolation and restricted gene flow is essential to demonstrate that behavioural isolation is leading to genetic divergence and speciation. Before prematurely concluding that behavioural isolation is the cause of genetic divergence, however, it is first necessary to assess the relative contribution of other factors such as geographical isolation, either by distance or by landscape features, to genetic divergence.

There is a long-standing interest in the importance of geographical barriers, such as rivers, and ecological gradients, such as elevation, in driving genetic divergence and speciation (Wallace, 1852; Mayr, 1963; Endler, 1977; Moritz *et al.*, 2000; Coyne & Orr, 2004). The field of landscape genetics focuses on understanding the effects of such landscape features on gene flow and genetic divergence (Manel *et al.*, 2003; Storfer *et al.*, 2007; Holderegger & Wagner, 2008). Geographical barriers may restrict gene flow, allowing populations to diverge (genetically or phenotypically) due to divergent selection or drift, whereas ecological gradients may cause population divergence and speciation via divergent selection in the presence of gene flow. Evidence that rivers act as barriers is mixed and varies depending on river width and species (da Silva & Patton, 1993; Gascon, 1996; Gascon *et al.*, 1996, 1998, 2000; Lougheed *et al.*, 1999; Hall & Harvey, 2002; Aleixo, 2004; Cheviron *et al.*, 2005). The importance of elevational gradients in genetic divergence has not been tested as extensively, but also varies among studies (Patton & Smith, 1992; da Silva & Patton, 1993; Graham *et al.*, 2004; Dingle *et al.*, 2006). Despite the importance of both landscape features and mating signal variation, relatively few studies have simultaneously tested their relative contributions to genetic divergence and speciation (Wilson *et al.*, 2000; Lampert *et al.*, 2003; Lovette, 2004; Ryan *et al.*, 2007).

Two sister species of Amazonian frogs, *Physalaemus petersi* and *P. freibergi*, provide an excellent opportunity to test the roles of signal variation and landscape features in genetic divergence. In both species, call divergence and behavioural isolation are implicated in speciation, with striking divergence in male advertisement calls observed over small spatial scales and short evolutionary time scales (Boul *et al.*, 2007; Guerra & Ron, 2008). One axis of call variation is call type. In some populations, males can only produce simple calls, consisting of two call components, a prefix and a whine (Fig. 1). In other populations, males also produce complex calls that include a third call component, the squawk (Fig. 1). Another axis of call variation is dominant frequency of simple calls. Phonotaxis experiments demonstrated that females strongly prefer the simple calls of local males over the calls of foreign males when the calls of local and foreign males differ in dominant frequency, demonstrating behavioural isolation (Boul *et al.*, 2007; Guerra & Ron, 2008). As dominant frequency is a salient feature in anuran call recognition (e.g. Ryan & Wilczynski, 1988), this leads to a simple prediction. If divergence in call dominant frequency and preferences for dominant frequency is causing genetic divergence and speciation, then there should be a positive correlation between genetic divergence and inter-population differences in dominant frequency.

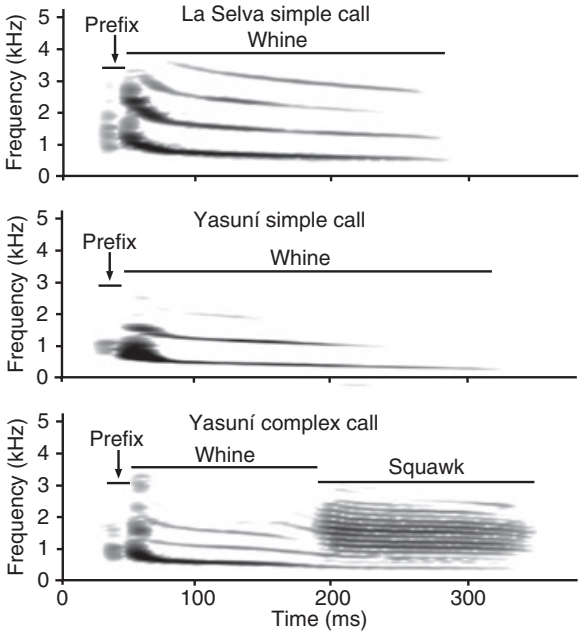

Fig. 1 Spectrograms of simple calls from La Selva and Yasuní and a complex call from Yasuní. Simple calls consist of two components, a prefix and a whine. Complex calls have an additional third component, the squawk.

Boul *et al.* (2007) demonstrated that divergence in call dominant frequency and female preferences in *P. petersi* is correlated with reduced gene flow between La Selva (with simple calls and high dominant frequencies on the north side of the Río Napo) vs. Yasuní and Tiputini (with complex calls and low dominant frequencies on the south side of the Río Napo; Figs 1 and 2). A large

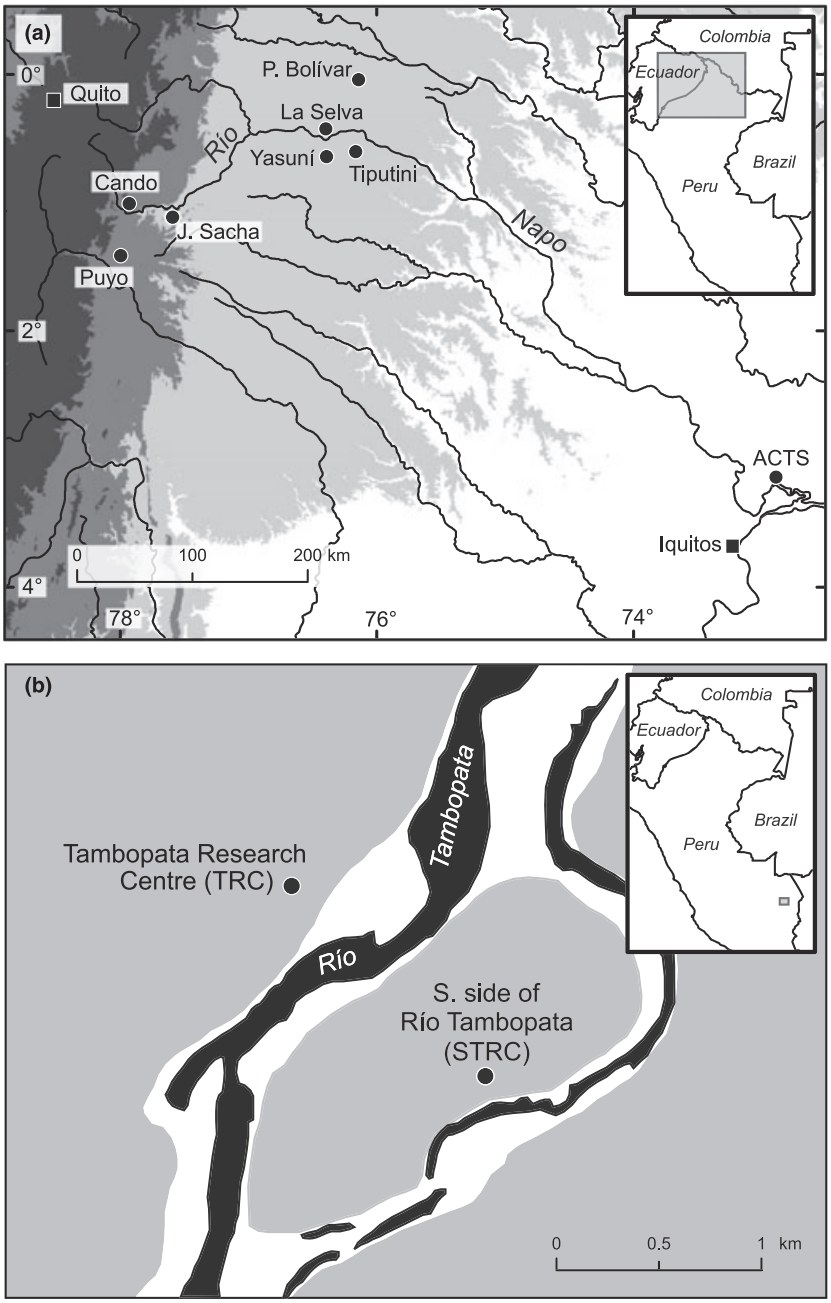

Fig. 2 Map of *Physalaemus petersi* (a) and *P. freibergi* (b) sites sampled and included in the analyses. In (a), white = 0–200 m elevation; light grey = 201–500 m; medium grey = 501–2000 m; and dark grey = 2000 + m. In (b), grey = forest; black = river; and white = beach. Insets in upper right of each panel show the areas detailed in maps (indicated with grey rectangles).

Amazonian tributary, the Río Napo, however, divides La Selva from Yasuní and Tiputini, confounding the potential contribution of behaviour and landscape features to genetic divergence. A phylogeographical analysis found no evidence that the Río Napo is a barrier (Funk *et al.*, 2007), but this study was based on a single locus, mtDNA. Thus in *P. petersi*, phonotaxis experiments have shown strong behavioural isolation between populations with different calls, but it is still unclear whether call divergence and behavioural isolation is resulting in genetic divergence and cladogenesis. To understand the mechanisms causing divergence in *P. petersi*, the effects of call variation and landscape features on genetic divergence need to be tested simultaneously using multiple nuclear loci.

We investigated patterns of genetic variation at microsatellite loci among populations of *P. petersi* and *P. freibergi* to test factors related to genetic divergence. Our specific objectives were to: (1) test whether genetic divergence among populations is related to landscape

features, including geographical distance, intervening rivers or elevational differences; and (2) test whether genetic divergence is related to call variation, specifically call type and dominant frequency. By simultaneously testing both landscape and call effects on genetic divergence, we were able to assess their relative importance in speciation.

Materials and methods

Study species

Physalaemus petersi (Jiménez de la Espada, 1872) and *P. freibergi* (Donoso-Barros, 1969) are pond-breeding, rainforest frog species in the family Leptodactylidae. *Physalaemus petersi* is found north of the Río Marañon and Río Amazonas in eastern Ecuador, north-eastern Peru, and south-eastern Colombia; *P. freibergi* is found south of these rivers in Amazonian Brazil, south-eastern Peru and Amazonian Bolivia (Funk *et al.*, 2008). *Physalaemus freibergi* was placed in the synonymy of *P. petersi* by Cannatella & Duellman (1984) based on morphology and was then resurrected based on limited molecular and call data (Cannatella *et al.*, 1998). Recently, differences between these species have been defined in more detail based on molecular, morphological and behavioural data (Funk *et al.*, 2007, 2008).

Together, *P. petersi* and *P. freibergi* form a clade that is the sister-group of *P. pustulosus*, the Túngara frog (Ron *et al.*, 2005, 2006). These three species form a well-supported clade (clade name Edentulus) that is the sister-group of a clade (clade name Duovox) containing all other species in the *P. pustulosus* species group. Although Nascimento *et al.* (2005) resurrected the genus *Engystomops* for the *P. pustulosus* species group, that action is not consistent with their own analysis of relationships. Ron *et al.* (2006) also followed the use of *Engystomops*. One of the authors of the latter paper (D.C.C.) agrees that resurrection of *Engystomops* as a genus was unjustified and has a larger manuscript in preparation on the molecular systematics of *Physalaemus*. Therefore the use of *Physalaemus* is continued here.

Sampling

We collected tissue samples (liver, muscle and toe-clips) from 10 sites and recorded calls from eight sites of *Physalaemus petersi* and *P. freibergi* from May to June 2004 and January to February 2005 (Table 1, Fig. 2). We refer to these as sites rather than populations because we did not want to assume *a priori* that sites were equivalent to randomly-mating populations. At Puyo and Tiputini, one toe-clip was taken from each frog that was then returned where it was found as these populations were part of ongoing mark–recapture and monitoring studies. Tissue samples were stored in 95% ethanol, tissue buffer or were frozen. This work was performed under Institutional Animal Care and Use Committee (IACUC) protocols 00010401 and 03021701.

Eight *P. petersi* sites and two *P. freibergi* sites were sampled. At each site, frogs were sampled from breeding ponds and along forest trails in an approximately 1–2 km diameter area. An exception was La Selva, Ecuador (Fig. 2a), where frogs were sampled from a single breeding aggregation spanning an approximately 50 m stretch of lake edge. To test the effects of landscape features such as rivers and elevational differences on genetic divergence, sites were sampled on opposite sides of major Amazonian rivers (the Río Napo for *P. petersi* and the Río Tambopata for *P. freibergi*; Fig. 2) and across

Table 1 Sites, coordinates in decimal degrees, elevation, mean call parameters, and genetic parameters of *Physalaemus petersi* and *P. freibergi* included in study.

		Coordinates			Call parameters				Genetic parameters		
Species	Site	Latitude	Longitude	Elev (m)	*n*	Call type	Call dom freq (Hz)	Whine dom freq (Hz)	*n*	H_E	R_S
P. petersi	Puerto Bolívar	−0.0886	−76.1419	240	0	NA	NA	NA	15 (NA)	0.79	7.2
	Puyo	−1.4431	−77.9967	954	6*	Simple	628	517	38 (0)	0.64	7.8
	Cando	−1.0669	−77.9331	702	15	Simple	578	519	25 (0)	0.55	4.9
	Jatun Sacha Biological Station	−1.0667	−77.6000	450	8	Simple	620	603	11 (8)	0.64	6.1
	La Selva Lodge	−0.4889	−76.3747	226	24	Simple	743	700	30 (10)	0.32	2.4
	Tiputini Biodiversity Station	−0.6372	−76.165	208	10	Complex	445	438	30 (10)	0.70	7.2
	Estación Cientifica Yasuní	−0.6781	−76.3967	250	15	Complex	436	474	30 (0)	0.75	7.3
	Amazon Conservancy for Tropical Studies (ACTS)	−3.2594	−72.9028	102	0	NA	NA	NA	30 (NA)	0.66	7.2
P. freibergi	Tambopata Research Centre (TRC)	−13.1351	−69.6064	167	7	Simple	541	404	30 (7)	0.82	9.2
	South side of Río Tambopata across from TRC (STRC)	−13.1434	−69.5975	201	7	Simple	575	472	30 (7)	0.83	9.5

*Call parameters for Puyo are from Guerra & Ron (2008). *N* is the number of individuals for call or genetic analysis. The number of recorded males that were also used for genetic analysis is shown in parentheses by the genetic sample size for each site. H_E, expected Hardy–Weinberg heterozygosity; R_S, allelic richness; NA, not applicable.

most of the elevational range of *P. petersi* (from 102 m at the Amazon Conservancy for Tropical Studies, Peru to 954 m at Puyo, Ecuador, close to the species' maximum elevation of 1069 m; Funk *et al.*, 2008). Although we were only able to sample two *P. freibergi* sites, they were located on opposite sides of the Río Tambopata, providing an independent test of the importance of rivers in restricting gene flow. We collected tissues from a total of 269 individuals from 10 sites (a mean of 26.9 individuals per site) and recorded calls from a total of 86 males from seven sites (a mean of 12.3 males per site; Table 1). At some sites, the genetic and call data were collected from the same individuals (Table 1).

Call analysis

Advertisement calls were recorded with a Sennheiser SE66 microphone (frequency response 40–20 000 Hz) or a Sony ECM-MS 907 microphone (frequency response 100–15 000 Hz), a Sony WM-D6C professional analogue tape recorder (frequency response 40–15 000 Hz) (Sony Electronics Inc., San Diego, CA, USA) and metal cassette tapes. Calls were digitized and analysed using batch processing in SIGNAL (Engineering Design, Belmont, MA, USA). Batch processing enforces a degree of standardization that is sometimes lost when calls are analysed individually. Only one randomly chosen call from each male was analysed to avoid pseudo-replication. Calls were examined prior to analysis to make sure they had a high signal to noise ratio (i.e. no interference from calls from other males). As preference based on spectral aspects of the whine is well known in *P. pustulosus* (Ryan, 1980; Wilczynski *et al.*, 1995; Bosch *et al.*, 2000), we focused on call dominant frequency (dominant frequency of the prefix and whine) and whine dominant frequency (dominant frequency of the whine only). The significance of differences in the dominant frequency of calls and whines among sites was tested using ANOVA. We also tested the correlation between call and whine dominant frequency among individuals using a Pearson correlation.

Microsatellite genotyping

We analysed genetic variation at nine variable microsatellite loci (Table 2). DNA extraction, PCR, and fragment analysis were performed as described previously (Boul *et al.*, 2007). Microsatellite primers were developed from pooled *P. petersi* and *P. freibergi* genomic DNA by Genetic Identification Services (Chatsworth, CA, USA). Primer sequences, PCR annealing temperatures, and GenBank accession numbers are shown in Table 2. PCR fragments were analysed on an ABI 3100 capillary DNA sequencer (Applied Biosystems Inc., Carlsbad, CA, USA) and fragment data were scored using GENEMARKER vs. 1.3 (Soft Genetics, LLC, State College, PA, USA). Previously genotyped individuals were included on all plates as size standards to make sure that loci were scored consistently among experiments.

Genetic analysis

Standard population genetic analyses

We first used standard population genetic analyses to test the quality of our data and estimate within-population genetic variation. Exact probabilities for Hardy–Weinberg

Table 2 Primer sequences of nine variable microsatellite loci that were used to examine genetic variation in *Physalaemus petersi* and *P. freibergi*.

Locus	Repeat motif	Primer sequences (5′–3′)	Allele size	No. alleles (mean per site)	T_a (°C)	GenBank accession no.
Ppet1	$(TAGA)_9$	F-GAGGCACTTCATCTACACAGTC R-CCGCCACATACACTTGTC	254	30 (5.4)	57	DQ995212
Ppet4	$(CA)_{12}$	F-ATCCAACCGTAAATCACAA R-GCAAGTCTCCTCACACTATTG	157	18 (4.2)	55	DQ995213
Ppet7	$(TAGA)_{18}TACAA(TAGA)_{15}$	F-CCTTGGAGTCTTTGTCATTG R-CACCACTTTCGTTTTTGAAC	235	51 (14.3)	57	DQ995214
Ppet11	$(TG)_{21}$	F-ACCATTAAAGAACATCCACCAC R-AAGAGCAGATCCTGCAAGAG	128	21 (8.9)	56	DQ995215
Ppet114	$(TG)_{13}$	F-TTGGTCCTGTGATGTCAGTG R-GACTCCGATTGGTTTGTCTC	280	24 (5.5)	58	DQ995216
Ppet118	$(TAGA)_6TTAGATAA(TAGA)_{10}$	F-GAACTGGGATGGATGATAGAC R-GAGGCTGCATATAATGGAATT	196	43 (12.4)	57	DQ995217
Ppet123	$(TCTA)_{25}$	F-CATTTTTTTATCCACGCTGAAC R- GGGTGCTCAGAAGCAATACTAG	264	36 (7.6)	58	GQ281739
Ppet125	$(TCTA)_{27}TCAA(TCTA)_{28}$	F-CCTTGAAGTATTGATTGAGGAT R-TAGGCAATGAGCATAAGACAG	389	86 (14.6)	58	DQ995218
Ppet131	$(TAGA)_{24}$	F-GGAACAACAAGTACACATCAAA R-TGGGTTACAATGAGCAGTG	287	60 (14.6)	57	DQ995219

Repeat motif and allele size (number of bp) refer to alleles of the sequenced clones. The total number of observed alleles and the mean number of alleles per site were calculated from all 209 *P. petersi* and all 60 *P. freibergi* individuals included in the study. T_a, annealing temperature.

proportions and linkage disequilibrium were calculated using GENEPOP 3.4 (Raymond & Rousset, 1995). MICROCHECKER was used to test for null alleles (van Oosterhout *et al.*, 2004). Expected heterozygosities (H_E), the number of alleles per locus and allelic richness (R_S) were calculated with MICROSATELLITE ANALYZER 4.05 (Dieringer & Schlötterer, 2003). Allelic richness is the number of alleles per locus corrected for differences in sample sizes among sites (El Mousadik & Petit, 1996).

Tests for genetic structure within sites

Recent research on Amazonian frogs demonstrated genetic substructure over small distances of 200–4000 m (Elmer *et al.*, 2007). Therefore, we tested for genetic substructure within each of our sampling sites using a Bayesian clustering approach implemented in STRUCTURE 2.2 (Pritchard *et al.*, 2000). We did not have coordinates for each individual, so we did not use analyses that require this information. STRUCTURE estimates the number of populations (K) in a sample by minimizing deviations from Hardy–Weinberg proportions and linkage equilibrium within populations and then assigns individuals to one or more of these populations (k). The estimation procedure consists of iterations for different values of K and then comparing the estimated log probability of the data under each K, ln [Pr($X|K$)], called ln $P(D)$ in STRUCTURE. We used the admixture model that assumes gene flow among populations and correlated allele frequencies. We performed 20 runs for each K, from K = 1–3, and calculated the mean ln $P(D)$ across runs for each K (e.g. Waples & Gaggiotti, 2006). For each run, we used a burn-in (the number of steps to run the simulation before collecting data) of 30 000 and a total run length of 100 000 which gave consistent results across runs. We ran this analysis separately for each site with a sample size of 30 or more (Puyo, La Selva, Tiputini, Yasuní, ACTS, TRC, STRC; Table 1, Fig. 2).

Tests of population bottlenecks and estimation of effective population sizes

Because we observed low levels of within-population genetic variation at some sites (see Results), we used bottleneck tests and estimated effective population sizes (N_e) to test whether bottlenecks or small effective population sizes were potentially responsible for low genetic variation. Estimation of N_e is also relevant for understanding divergence in male calls and female preferences as drift in small populations is expected to increase phenotypic divergence. These analyses were conducted for all sites except Jatun Sacha in which the sample size was small (Table 1). We tested for recent population bottlenecks following Cornuet & Luikart (1996). This method, implemented in program BOTTLENECK 1.2.02 (Piry *et al.*, 1999), is based on the predicted loss of rare alleles in recently bottlenecked populations. It uses a single population sample to test whether there has been a recent reduction in allelic variation. Simulations (Cornuet & Luikart, 1996; Williamson-Natesan, 2005), theory (Garza & Williamson, 2001) and case studies (Cornuet & Luikart, 1996; Beebee & Rowe, 2001; Goossens *et al.*, 2006; Spear *et al.*, 2006) all show that this is the best method for detecting recent, low-magnitude declines in N_e. The stepwise mutation model (SMM) and two-phase mutation model with 12% multi-step mutations were used to generate null distributions under mutation-drift equilibrium, as these models span the range of mutation models considered reasonable for microsatellites (Shriver *et al.*, 1993; Di Rienzo *et al.*, 1994; Garza & Williamson, 2001). We tested the sensitivity of bottleneck tests to loci with possible null alleles by repeating these tests without loci identified by MICROCHECKER as potentially having null alleles at the given site.

We estimated N_e for each site using approximate Bayesian computation with the program ONESAMP (Tallmon *et al.*, 2004, 2008). This program uses eight summary statistics with a known relationship with N_e and approximate Bayesian computation to estimate N_e from a single sample of microsatellite data. It has been shown to be robust under a wide range of population parameters (Tallmon *et al.*, 2004, 2008). We used a liberal prior of 2–1000 for the upper and lower bounds for N_e. A conservative prior of 4–500 was also used for one randomly chosen site, Puerto Bolívar, to test the sensitivity of the results to the prior. We also tested the sensitivity of N_e estimates to loci with potential null alleles by repeating ONESAMP without loci identified by MICROCHECKER as possibly having null alleles at the given site.

Tests of factors related to genetic divergence

Weir & Cockerham's (1984) pairwise F_{ST} values and the significance of allelic differentiation among sites were calculated in GENEPOP. Critical α values for pairwise tests of allelic differentiation were determined using a sequential Bonferroni adjustment (Rice, 1989).

Mantel tests (Mantel, 1967) and partial Mantel tests (Smouse *et al.*, 1986) were used to test the relationship between genetic divergence [$F_{ST}/(1 - F_{ST})$; Rousset, 1997] and straight-line geographical distance, intervening rivers, elevational differences, call type, differences in call dominant frequency and differences in whine dominant frequency among *P. petersi* sites using FSTAT vs. 2.9.3.2 (Goudet, 2002). Mantel tests were not used for *P. freibergi* because several sites are required for this analysis. If divergence in call dominant frequency and preferences for dominant frequency is causing genetic divergence, then there should be a positive relationship between genetic divergence and differences in whine and/or call dominant frequency among populations. This prediction assumes that populations were originally connected by some level of gene flow and that the evolution of behavioural isolation subsequently restricted gene flow in proportion to the level of call and preference divergence. Because call recordings were

not available for two *P. petersi* sites (Puerto Bolívar and ACTS), separate analyses were conducted to test the effects of landscape variables (geographical distance, rivers and elevation) and call variables (call type, call dominant frequency and whine dominant frequency) on genetic divergence. The landscape analysis included all eight *P. petersi* sites and the call analysis included the six *P. petersi* sites with call data. As ACTS was a geographical outlier (i.e. it was distant from the other seven sites), the landscape analyses were also conducted without ACTS to test the sensitivity of the results to inclusion of this site. Call data for Puyo were taken from Guerra & Ron (2008). Prior to Mantel tests, we tested whether natural-log-transformation of predictor variables improved the linear fit between genetic divergence and these variables by calculating correlation coefficients with and without transformation. Log-transformation improved the fit for two variables, elevational differences and differences in call dominant frequency. Thus the transformed values for these variables were used in Mantel tests.

Partial Mantel tests were used to test the effects of intervening rivers, elevational differences, call type, differences in call dominant frequency and differences in whine dominant frequency after removing the effects of geographical distance. Although there is an unresolved debate regarding the statistical validity of partial Mantel tests (Raufaste & Rousset, 2001; Castellano & Balleto, 2002; Rousset, 2002), the validity of simple Mantel tests is not in question (Raufaste & Rousset, 2001). Our conclusions do not hinge on the results of the partial Mantel tests (see Results and Discussion), but we still include these results as partial Mantel tests remain a standard analysis. We corrected critical α values for multiple Mantel and partial Mantel tests using a sequential Bonferroni adjustment (Rice, 1989).

We tested the sensitivity of Mantel and partial Mantel tests to loci with possible null alleles in two ways. First, Mantel and partial Mantel tests were repeated using genetic divergence estimates $[F_{ST}/(1 - F_{ST})]$ calculated without the two loci (Ppet118 and Ppet125) identified by Microchecker as potentially having null alleles in multiple sites. Second, for each locus identified by Microchecker as potentially having null alleles in any site (Ppet1, Ppet7, Ppet11, Ppet118, Ppet123, Ppet125 and Ppet131), Mantel and partial Mantel tests were repeated using genetic divergence estimates calculated without the given locus.

Results

Call variation among populations

All frogs at four *P. petersi* sites made simple calls (Puyo, Cando, Jatun Sacha and La Selva) and at two sites complex calls were also heard (Yasuní and Tiputini; Table 1). Frogs at both *P. freibergi* sites made only simple calls. There was also significant variation among *P. petersi* and *P. freibergi* sites (all eight sites with recordings) in the dominant frequency of calls ($F_{6,79} = 9.85$, $P < 0.001$) and whines ($F_{6,79} = 7.39$, $P < 0.001$; Table 1). Mean call dominant frequencies ranged from 436 Hz at Yasuní to 743 Hz at La Selva; mean whine dominant frequencies ranged from 404 Hz at TRC to 700 Hz at La Selva. Call and whine dominant frequencies were significantly correlated ($r = 0.851$, $P < 0.001$).

Genetic analysis

Standard population genetic analyses

Only one out of 33 possible tests for departure from linkage equilibrium was significant, less than the value ($1.65 = 0.05 \times 33$) expected to be significant by chance. Thus loci were inferred to be independent. In Hardy–Weinberg tests, there was significant homozygote excess at all loci except Ppet11 and Ppet114 and at all sites except La Selva, the one site in which only a single breeding aggregation was sampled. Microchecker identified potential null alleles at all loci except Ppet4 and Ppet114 and at all sites except La Selva. For loci with potential null alleles, the number of sites identified as having null alleles at that locus was one (Ppet7 and Ppet11), two (Ppet123), three (Ppet1 and Ppet131), five (Ppet125) or six (Ppet118). For sites with potential null alleles, the number of loci identified as having null alleles at that site was one (TRC and STRC), two (Puerto Bolívar, Yasuní, and Tiputini), three (Cando, Jatun Sacha, and ACTS) or four (Puyo).

Within-population genetic variation varied substantially among populations (Table 1). Expected heterozygosity (H_E) ranged from 0.32 at La Selva to 0.83 at STRC. Allelic richness (R_S) was lowest at La Selva (2.4) and highest at STRC (9.5).

Genetic structure within sites

In the Structure analysis, each sampling site was inferred (by posterior probability) to consist of one population ($K = 1$) except for TRC where $K = 2$ had a slightly higher posterior probability (0.53). But even at TRC, all individuals had approximately 50% membership in both clusters, indicating lack of structure (Pritchard *et al.*, 2000). Thus Structure did not find genetic substructure within sites.

Bottlenecks and effective population sizes

No evidence was found for heterozygosity excess (indicative of population bottlenecks) in any site regardless of the mutation model. Under the SMM, heterozygosity deficiency (indicative of population expansion) was significant for Tiputini, ACTS and TRC ($P = 0.027$, 0.027 and 0.020 respectively). After removing loci with potential null alleles, heterozygosity deficiency was no longer significant at Tiputini ($P = 0.078$), but remained significant at ACTS and TRC ($P = 0.047$ and 0.023).

Table 3 Effective population size (N_e) estimates from approximate Bayesian computation in program ONESAMP.

Species	Site	Mean	Median	95% credible limits
Physalaemus petersi	Puerto Bolívar	34	34	20–84
	Puyo	389	385	161–1864
	Cando	41	41	25–125
	La Selva Lodge	15	15	8–36
	Tiputini Biodiversity Station	247	243	120–893
	Estación Cientifica Yasuní	256	263	134–829
	Amazon Conservancy for Tropical Studies	406	416	192–2005
Physalaemus freibergi	Tambopata Research Centre (TRC)	755	723	305–3171
	South side of Río Tambopata across from TRC	846	825	366–4034

Effective population sizes estimated with ONESAMP varied from a mean of 15 at La Selva to 846 at STRC (Table 3). Changing the prior for the low and high values of N_e or removing loci with potential null alleles did not change the results. The 95% credible limits for N_e overlapped between analyses with different priors and with or without possible null alleles, demonstrating relative insensitivity to the prior and to inclusion of loci with possible null alleles.

Factors related to genetic divergence

Pairwise F_{ST} values among *P. petersi* sites ranged from 0.046 between Tiputini and Yasuní to 0.514 between La Selva and Cando (Table 4). Mean pairwise F_{ST} among *P. petersi* sites was 0.280. Pairwise F_{ST} between the two *P. freibergi* sites, TRC and STRC, was 0.020. Allelic differentiation between all pairs of sites was significant ($P < 0.001$) after correcting for multiple tests.

Mantel and partial Mantel tests demonstrated that the only predictor variable significantly related to genetic divergence in *P. petersi* was inter-population differences in whine dominant frequencies (Table 5, Fig. 3). In tests focusing on landscape variables (geographical distance, the Río Napo and elevational differences), none of the five tests was significant (regardless of whether or not ACTS was included). In tests focusing on call variables (call type, call dominant frequency and whine dominant frequency) and geographical distance, only the Mantel test of genetic divergence vs. differences in whine dominant frequency and the partial Mantel test of the same relationship were significant after correcting for multiple tests. The Mantel and partial Mantel tests of genetic divergence vs. differences in call dominant frequency were marginally significant. Removing loci with potential null alleles did not change which Mantel and partial Mantel tests were significant.

Discussion

Genetic divergence is more tightly related to call variation than to landscape features

We found a strong, positive relationship between genetic divergence and differences among sites in mean whine dominant frequency in *P. petersi*. In both Mantel and partial Mantel tests, differences in whine dominant frequency explained over half the variation in genetic divergence (53% and 56% of the variation respectively; Table 5, Fig. 3). Moreover, as genetic divergence was not significantly related to geographical distance, this result does not depend on the partial Mantel test used to control for geographical distance. Thus the debate regarding the statistical soundness of partial Mantel tests does not affect our conclusions. In addition, removing loci with potential null alleles did not change the significance of the relationship between genetic divergence and differences in whine dominant frequency.

The positive relationship between genetic divergence and differences in whine dominant frequency was predicted based on female preferences tested with phonotaxis experiments (Boul *et al.*, 2007; Guerra & Ron, 2008). In these experiments, females strongly

Table 4 F_{ST} estimates between *Physalaemus petersi* sites.

	Puerto Bolívar	Puyo	Cando	J. Sacha	La Selva	Tiputini	Yasuí
Puyo	0.185						
Cando	0.254	0.107					
J. Sacha	0.206	0.108	0.177				
La Selva	0.397	0.435	0.514	0.479			
Tiputini	0.155	0.250	0.286	0.239	0.465		
Yasuní	0.131	0.246	0.289	0.236	0.449	0.046	
ACTS	0.116	0.322	0.373	0.302	0.533	0.240	0.300

J. Sacha, Jatun Sacha; ACTS, Amazon Conservancy for Tropical Studies. Allelic differentiation was significant between all population pairs.

Table 5 Results of simple and partial Mantel tests (with geo dist in parentheses) to investigate the relationship between genetic distance, landscape variables and call variables in *Physalaemus petersi*.

Predictor variables	No. sites	Mantel test predictor variables	*r*	*P*
Landscape	8	geo dist	0.138	0.485
		river	−0.019	0.925
		ln elev diff	−0.049	0.803
		river (geo dist)	−0.013	0.952
		ln elev diff (geo dist)	−0.114	0.560
Call	6	geo dist	0.200	0.474
		call type	0.012	0.965
		ln diff call dom Hz	0.620	0.012
		diff whine dom Hz	0.727	**0.002**
		call type (geo dist)	−0.047	0.870
		ln diff call dom Hz (geo dist)	0.592	0.019
		diff whine dom Hz (geo dist)	0.748	**0.001**

$F_{ST}/(1 - F_{ST})$ was used as genetic distance (Rousset, 1997).
Geo dist, geographical distance (km) between sites; river, same vs. opposite side of Río Napo; elev diff, difference in elevation (m); call type, same vs. different call type; diff call dom Hz, difference in mean call (prefix + whine) dominant frequency; diff whine dom Hz, difference in mean whine dominant frequency; *r*, standardized Mantel test statistic which is equivalent to a Pearson product-moment correlation coefficient.
P was estimated from 10 000 randomizations.
Elev diff and diff call dom Hz were natural-log-transformed to improve the linear fit between $F_{ST}/(1 - F_{ST})$ and these variables.
Controlled variable (geographical distance) in partial Mantel tests in parentheses.
P values significant after correcting for multiple tests are shown in bold.

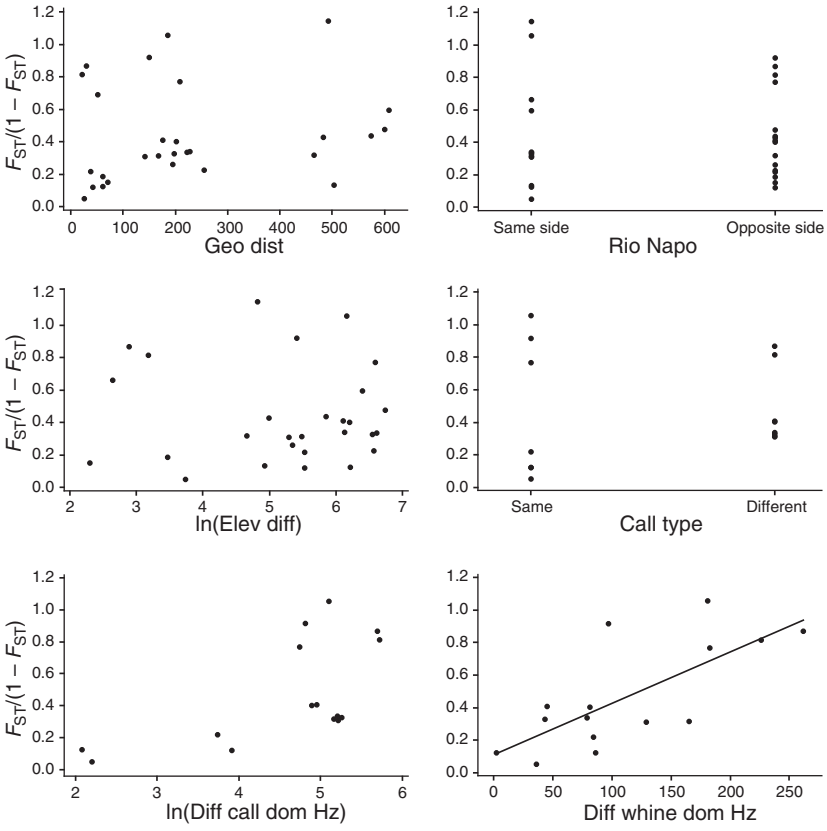

Fig. 3 Plot of $F_{ST}/(1 - F_{ST})$ against different predictor variables in *Physalaemus petersi*: geo dist = geographical distance (km) between sites; Río Napo = same vs. opposite side of the Río Napo; elev diff = difference in elevation (m); call type = same vs. different call type; diff call dom Hz = difference in mean dominant frequency of calls (prefix + whine); and diff whine dom Hz = difference in mean whine dominant frequency. Elev diff and diff call dom Hz were natural-log-transformed to improve the linear fit between $F_{ST}/(1 - F_{ST})$ and these variables. Only the relationship between $F_{ST}/(1 - F_{ST})$ and diff whine dom Hz was significant in Mantel tests (indicated with regression line).

discriminated against the calls of foreign males and the most obvious difference between the local and foreign calls was the dominant frequency. This local mate preference should restrict gene flow and increase genetic divergence, leading to the observed positive relationship between genetic divergence and differences in whine

dominant frequency. In contrast, there was not a significant relationship between genetic divergence and call type. This is also consistent with preference tests showing no significant difference in preference for complex calls between populations with different call types (Boul *et al.*, 2007).

As our results are correlative, we have not directly demonstrated the causal relationship between genetic and call divergence. Another possibility is that restricted gene flow among sites caused by geographical barriers has allowed call frequencies to diverge. The observation that genetic divergence was not related to geographical distance, intervening rivers or elevational differences indicates, however, that these landscape features do not restrict gene flow. This suggests that divergence in male calls and female preferences may be the causative mechanism driving genetic divergence. Additional phonotaxis experiments could be used to test whether the positive relationship between genetic divergence and differences in whine dominant frequency is caused by divergence in female preferences and male calls. If this hypothesis is correct, then the strength of female preferences for local calls should be proportional to the difference in whine dominant frequency between local males and males from the given foreign population. This could be tested using synthesized calls based on the mean call parameters of different populations throughout the species' range as done for *P. pustulosus* (Ryan *et al.*, 2007).

The lack of landscape effects on genetic divergence is consistent with a previous mtDNA phylogeographical study of many of the same populations of *P. petersi* and *P. freibergi* (Funk *et al.*, 2007), but is in contrast to other studies that have shown strong landscape effects on population structure in some amphibians (e.g. Funk *et al.*, 2005a; Spear *et al.*, 2005; Giordano *et al.*, 2007). Funk *et al.* (2007) found no significant relationship between genetic divergence (measured as sequence divergence) and the Río Napo or the Río Tambopata or between genetic divergence and elevational differences. Some studies of birds and butterflies support a barrier effect of some Amazonian rivers (Hall & Harvey, 2002; Aleixo, 2004; Hayes & Sewlal, 2004; Höglund & Shorey, 2004; Cheviron *et al.*, 2005), but other studies of birds, mammals and amphibians found little or no such effect (da Silva & Patton, 1993; Gascon, 1996; Gascon *et al.*, 1996, 1998, 2000; Lougheed *et al.*, 1999; Symula *et al.*, 2003; Aleixo, 2004). The Río Napo is wide (approximately 1 km wide between La Selva and Yasuní) and likely acts as a significant current barrier to gene flow for *P. petersi*. The Río Tambopata is narrower (approximately 300 m wide between TRC and STRC), but could be a current barrier to movement for *P. freibergi*. The lack of an effect on genetic divergence may therefore be partly due to historic gene flow among populations prior to becoming divided by rivers. For example, lateral channel migration is common in the western Amazon basin and can cause across-river transfers of large pieces of land such that populations that are currently on opposite sides of a river may have historically been on the same side (Räsänan *et al.*, 1987). Fewer studies have tested the effects of elevational gradients on genetic divergence in Amazonian and Andean taxa. Elevational gradients may be important in speciation of poison frogs (Graham *et al.*, 2004), but elevation does not seem to play a role in speciation in the rodents and birds studied (Patton & Smith, 1992; da Silva & Patton, 1993; Dingle *et al.*, 2006).

We also did not find a significant relationship between genetic divergence and geographical distance in *P. petersi*. In other words, there was no evidence for isolation-by-distance. There are at least three possible explanations for a lack of isolation-by-distance: (1) gene flow is not limited by distance so that the amount of gene flow among populations does not depend on distance; (2) populations have recently expanded and there has not been sufficient time for distance-limited dispersal to generate a correlation between genetic and geographical distance; and (3) there is little or no gene flow among some populations regardless of the distance between them. The first two explanations seem unlikely in the case of *P. petersi*. First, the maximum distance between sites in this study is 609 km between ACTS and Cando, almost 2 orders of magnitude greater than the maximum documented dispersal distances for frogs (Marsh & Trenham, 2001; Funk *et al.*, 2005b). Thus dispersal rates are not equivalent between all sites in this study. Second, the high F_{ST} estimates among some *P. petersi* populations found here (mean pairwise $F_{ST} = 0.280$; Table 4) suggest that populations have not recently expanded. In the case of recent expansion, F_{ST} values should be substantially lower. Moreover, Funk *et al.* (2007) found little evidence for recent population expansion in *P. petersi* in the Napo region using several different analyses. The last hypothesis, little or no gene flow among some populations, seems most plausible for *P. petersi*. F_{ST} values are low for some geographically close populations with similar call dominant frequencies (e.g. Yasuní and Tiputini; see Tables 1 and 4, Fig. 2), suggesting high gene flow among these populations. But populations with divergent call dominant frequencies separated by small distances and populations with similar call dominant frequencies separated by even moderate geographical distances likely have little or no gene flow which should result in a breakdown in isolation-by-distance. Thus strong isolation among populations seems like the most likely explanation for the lack of isolation by distance at microsatellite loci in *P. petersi*.

In *P. pustulosus*, the sister species of the clade containing *P. petersi* and *P. freibergi*, there is no significant relationship between genetic divergence and call differences among populations despite significant variation in calls (Ryan *et al.*, 1996, 2007). This observation is consistent with differences between *P. petersi* and *P. pustulosus* in the strength of female preferences for local calls. In *P. petersi*, females strongly prefer the calls of local

males and discriminate against the calls of foreign males when they differ significantly in dominant frequency (Boul *et al.*, 2007; Guerra & Ron, 2008). For example, the proportion of La Selva and Yasuní females preferring their local call when presented with calls from the other population was 0.89 and 1.00 respectively (Boul *et al.*, 2007). In *P. pustulosus* females from Gamboa, Panama, the mean proportion preferring their local call to calls of 27 other populations from throughout the species' range was 0.66 and ranged from 0.30 to 0.85 (Ryan *et al.*, 2007). Pröhl *et al.* (2006) showed a similar result in a more fine-scale study in an area of parapatry between two genetic groups within *P. pustulosus*. Strong discrimination against calls with different dominant frequencies in *P. petersi* should result in the accumulation of genetic differences in proportion to the difference in dominant frequency. But in *P. pustulosus*, lack of such strong discrimination should not result in such a relationship, as observed. The reason for this difference in the strength of preferences for local calls between these closely related species is currently unknown.

Fine-scale genetic structure within sites

The two methods we used to test for fine-scale genetic structure within sites, Hardy–Weinberg tests and the Bayesian clustering algorithm in program Structure, gave inconsistent results. Hardy–Weinberg tests revealed homozygote excess at multiple loci in all sites except the one in which only a single breeding aggregation was sampled, La Selva. Only an approximately 50 m stretch of shoreline was sampled at La Selva, whereas 1–2 km areas were sampled at other sites to find large enough sample sizes. This suggests genetic substructure over small (1–2 km) spatial scales within sites. Homozygote excess at multiple loci is predicted when multiple populations are combined in a single sample, the well-known Wahlund effect (Hartl & Clark, 1989). Null alleles, on the other hand, typically result in homozygote excess at one or two loci in multiple populations. Thus the large number of loci with potential null alleles identified by Microchecker is likely primarily due to a Wahlund effect rather than to null alleles. In contrast, program Structure did not detect multiple populations in any site, suggesting either lack of fine-scale genetic structure or low power to detect structure with our sample sizes of 30 to 38 individuals. In other studies with similar or even larger sample sizes, Structure tended to be conservative at detecting differences among populations (Funk *et al.*, 2005a; Elmer *et al.*, 2007; see below), suggesting that the failure of Structure to find multiple populations in *P. petersi* and *P. freibergi* sites here may be due to low power.

Another population genetic study of a frog (*Pristimantis ockendeni*) in Amazonian Ecuador also found fine-scale genetic structure within a single site over spatial scales similar to those analysed here (Elmer *et al.*, 2007). As in *P. petersi* and *P. freibergi* here, Elmer *et al.* (2007) found homozygote excess at all loci and evidence for null alleles with Microchecker, but lack of genetic structure according to the Structure results, in *P. ockendeni*. The authors attributed the lack of genetic structure to a significant pattern of isolation by distance among individual frogs. Although there were not distinct populations, frogs farther apart were less closely related to each other. We did not have geographical coordinates for individual frogs, thus we could not test the relationship between geographical distance and genetic relatedness between frogs. But the suggestion of a Wahlund effect in *P. petersi* and *P. freibergi* in our study and in *P. ockendeni* suggests that limited dispersal and fine-scale genetic structure may be common in some Amazonian frogs.

One factor that may contribute to limited dispersal in *P. petersi*, *P. freibergi* and *P. ockendeni* is their reproductive modes. *Physalaemus petersi* and *P. freibergi* have foam nests which they deposit at the margins of rainforest pools and oxbow lakes. Because these breeding habitats are often scarce and distant from each other, this may result in isolated breeding populations with limited among-population dispersal and gene flow. *Pristimantis ockendeni* and other *Pristimantis* species have direct development (no aquatic larval stage) in which they lay their eggs in the leaf litter or on leaves in the forest such that migration to breeding sites is unnecessary. Low migration and dispersal in direct developing frogs should also lead to restricted gene flow. In contrast, populations of rainforest frog species which breed in more common and widespread aquatic habitats (e.g. the many hylid species which breed in large lakes or ponds, often in disturbed habitats) may be more connected due to a higher density of ponds, lower inter-pond distance, and greater migration and dispersal abilities. Comparative analyses of gene flow in amphibians with different reproductive modes and life histories would shed light on variation among these groups in patterns and rates of gene flow.

Effective population sizes and bottlenecks

We found substantial variation in effective population sizes (N_e) among populations of *P. petersi* and *P. freibergi* (Table 3). Small effective population sizes may partly explain low genetic variation observed within La Selva and Cando, although Puerto Bolívar also had a small N_e but average levels of diversity (Table 1). Moreover, small effective population sizes in some sites suggest that genetic drift may potentially play a role in divergence in calls and female preferences. For example, La Selva has the smallest N_e and also has call and whine dominant frequencies substantially higher than other sites. It is possible that genetic drift has facilitated this divergence. Analysis of mtDNA data using a coalescent approach showed that call evolution in *P. petersi* has proceeded faster than expected by genetic drift, implying divergent selection on calls (Boul *et al.*, 2007). This coalescent

analysis, however, considers the effects of drift in all populations simultaneously, essentially averaging over populations. Although average effective population sizes may be too large to invoke genetic drift as a general mechanism causing call divergence, small effective population sizes may influence call and preference divergence in some individual populations.

Although effective population sizes were small in some sites, there was no evidence from bottleneck tests for reductions in N_e. Thus bottlenecks, which can increase divergence estimates (Hedrick, 1999), are not a confounding factor in our analysis. In some sites, however, there were significant tests for population expansion. In particular, we found evidence for expansion at Tiputini and ACTS in *P. petersi* and at TRC in *P. freibergi*. Evidence for expansion in ACTS and TRC is consistent with mtDNA data, although mtDNA analysis did not find evidence for expansion at Tiputini (Funk *et al.*, 2007). Thus most of the populations analysed appeared to be fairly stable over time, but small effective population sizes suggest that genetic drift could play a role in call and preference evolution in some populations.

Conclusions

Our results add to previous evidence (Boul *et al.*, 2007; Guerra & Ron, 2008) that behavioural isolation stemming from divergence in male calls and female preferences is causing genetic divergence and speciation among populations of *P. petersi* and *P. freibergi*. Boul *et al.* (2007) also found genetic divergence at microsatellite loci between *P. petersi* populations with divergent calls and preferences, but genetic divergence was potentially confounded by the intervening Río Napo. Here we found a strong positive relationship between genetic divergence and differences in whine dominant frequency in *P. petersi* and no significant relationship between genetic divergence and the Río Napo in *P. petersi* or the Río Tambopata in *P. freibergi*, supporting the hypothesis that behavioural isolation, not landscape features, is causing speciation.

All evidence to date indicates that behavioural isolation plays an important role in speciation in *P. petersi* and *P. freibergi*, but several questions remain about the ultimate and proximate mechanisms causing behavioural isolation. For example, what combination of selective forces has generated such striking among-population variation in male calls and female preferences? Is variation in male calls driven by runaway sexual selection, divergent ecological selection acting directly on calls or on correlated traits, or a combination? And what are the genetic mechanisms that underlie among-population variation in male calls and female preferences? *Physalaemus petersi* and *P. freibergi* should be excellent species for addressing these fundamental evolutionary questions for several reasons including divergence in calls and preferences over small spatial and evolutionary time scales, at least two independent centres of call divergence (one in *P. petersi* and the other in *P. freibergi*), robust assays for testing preferences, and a wealth of background information on communication and sexual selection in *Physalaemus* frogs (e.g. Ryan, 1985; Ryan & Rand, 1999).

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