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Tests of biogeographic hypotheses for diversification in the Amazonian forest frog, *Physalaemus petersi*

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Abstract

Several hypotheses have been proposed to explain the biogeographic processes that generate the high species richness of the Amazon basin. We tested two of them in a terra firme (upland) forest frog species, *Physalaemus petersi*: (1) the riverine barrier hypothesis; and (2) the elevational gradient hypothesis. Mitochondrial DNA sequence data (2.4 kb) from the 12S, 16S, and intervening valine tRNA genes were obtained from 65 *P. petersi* individuals and 4 outgroup taxa and analyzed with a combination of phylogenetic and population genetic approaches. Moderate support for the riverine barrier hypothesis was found for one of the three rivers examined, but little evidence was found for the elevational gradient hypothesis. Phylogenetic analyses revealed that high levels of sequence divergence (an average of 4.57–4.79%) separate three well-supported clades from the northwestern, southwestern, and eastern Amazon. Strong evidence for recent population expansion in *P. petersi* in the southwestern region of the Amazon basin was also uncovered.

Keywords: *Physalaemus*; Frog; Amazon basin; Mitochondrial DNA; Phylogeography; Speciation; Population expansion

1. Introduction

The processes that generate the high species richness of the Amazon basin have fascinated evolutionary biologists and naturalists since the 19th century (Bates, 1863; von Humboldt, 1808; Wallace, 1853). Historically, investigations of these processes relied primarily on distributional data of extant taxa (e.g., Haffer, 1969; Wallace, 1852). However, molecular phylogeographic studies of populations and recently diverged species (e.g., Aleixo, 2004; Cheviron et al., 2005; da Silva and Patton, 1993; Glor et al., 2001; Hoffman and Baker, 2003; Kronauer et al., 2005; Lessa et al., 2003; Lougheed et al., 1999; Lovette, 2004; Marks et al., 2002; Patton et al., 2000; Zamudio and Greene, 1997), coupled with hypothesis testing, promise to illuminate our understanding of speciation processes in the Amazon basin.

Several biogeographic mechanisms have been proposed to explain diversification in the Amazon, including allopatric speciation via riverine barriers (Wallace, 1852), forest refugia (Haffer, 1969, 1997), marine incursions (Nores, 1999; Webb, 1995), historic mountain ridges (Räsänan et al., 1990), or climatic disturbance (Bush, 1994; Colinvaux et al., 1996; Colinvaux, 1998), as well as parapatric speciation caused by divergent selection across ecological gradients (Endler, 1977). Some sites in the Amazon Basin have the highest known species richness of amphibians (Duellman, 1978, 1988, 1999), yet studies of amphibian speciation in an explicit phylogeographic framework have just started (Chek et al., 2001; Lougheed et al., 1999; Symula et al., 2003).

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According to the riverine barrier hypothesis, large Amazonian rivers isolate populations of terra firme (unflooded upland) forest, thereby restricting gene flow and allowing populations to diverge via divergent selection and/or drift (Capparella, 1988; Sick, 1967; Wallace, 1852). Four specific predictions of this hypothesis can be tested using phylogenetic and population genetic approaches: (1) populations reciprocally monophyletic for mitochondrial DNA haplotypes will occur on opposite sides of major Amazonian rivers after sufficient time has elapsed for lineage sorting; (2) genetic divergence between populations will be positively correlated with the presence of intervening rivers after removing the effects of geographic distance; (3) genetic divergence between populations separated by a river will be greater in the river's lower section than in the headwaters; and (4) rivers will act as areas of primary differentiation rather than secondary contact of lineages. Evidence for the riverine barrier hypothesis is mixed. Some studies of birds and butterflies showed strong support that some Amazonian rivers are important barriers (Aleixo, 2004; Cheviron et al., 2005; Hall and Harvey, 2002; Hayes and Sewlal, 2004; Höglund and Shorey, 2004), but other studies of birds, mammals, and amphibians demonstrated little or no barrier effect (Aleixo, 2004; da Silva and Patton, 1993; Gascon, 1996; Gascon et al., 1996, 1998, 2000; Lougheed et al., 1999; Symula et al., 2003).

Unlike the riverine barrier hypothesis, the ecological gradients hypothesis does not require isolation of populations by vicariance. Instead, this hypothesis posits that divergent selection across environmental gradients, such as changes in precipitation and elevation, causes populations to diverge despite ongoing gene flow (Endler, 1977; Moritz et al., 2000). This mechanism predicts that genetic divergence between populations will be significantly correlated with ecological differences between populations after removing the effects of geographic distance. Elevational gradients may be important in speciation of poison frogs (Graham et al., 2004), in contrast to rodents and birds (da Silva and Patton, 1993; Dingle et al., 2006; Patton and Smith, 1992).

This study tests these two biogeographic hypotheses in the Amazonian frog *Physalaemus petersi* (Jiménez de la Espada, 1872), an excellent focal species for two reasons. First, *P. petersi* has a wide distribution in the Amazon basin, extending from the Andean foothills of Ecuador, Peru, and Bolivia, up to approximately 1200 m, eastward through much of the Colombian, Brazilian, and French Guianan Amazon basin to the Atlantic (Cannatella and Duellman, 1984; Fig. 1). The range includes several large rivers and the elevational gradient of the Andean foothills, which allows testing of the riverine barrier and ecological gradient (or more specifically, the elevational gradient) hypotheses. Second, *P. petersi* is a terra firme forest species (Aichinger, 1992; Duellman, 1978; Toft and Duellman, 1979), so riverine barriers are predicted to restrict gene flow (Gascon et al., 1998). We did not test other hypotheses (e.g.,

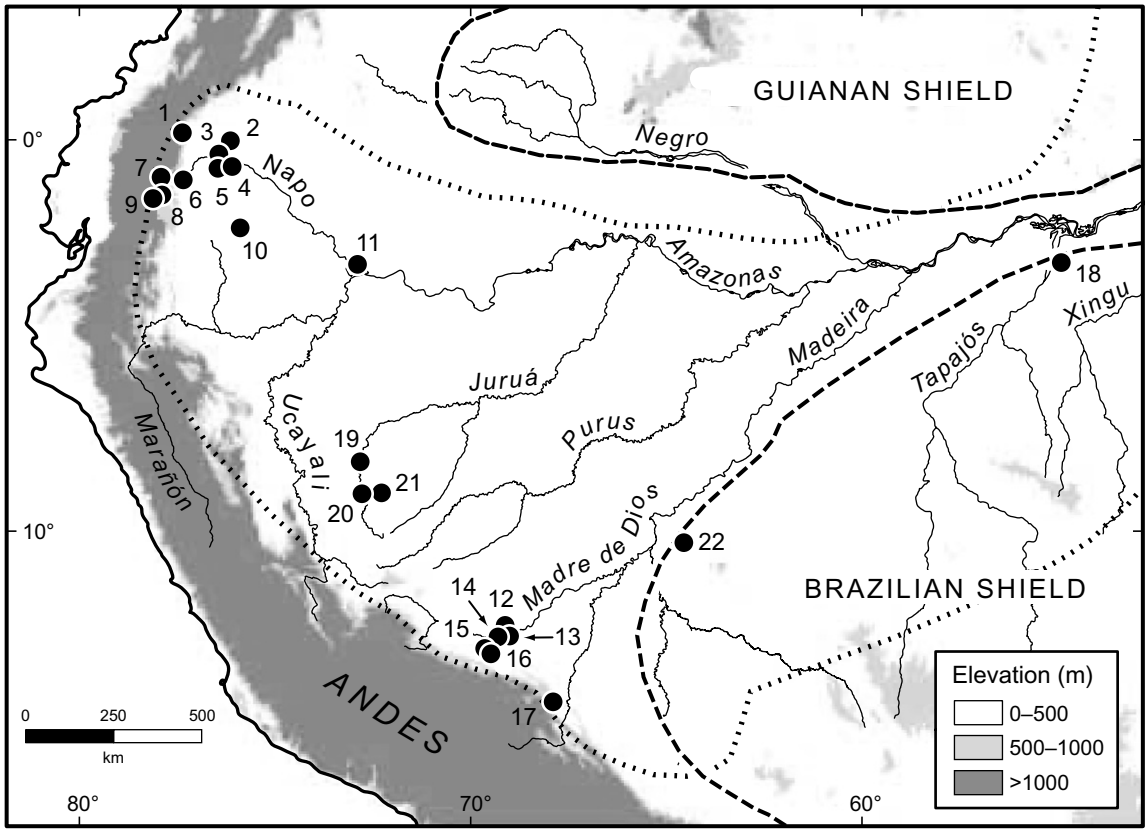

Fig. 1. Distribution of sampling sites for *Physalaemus petersi*. Site numbers correspond to those used in Figs. 2 and 3, Tables 1 and 3, and Appendix A. The dotted outline shows the approximate range of *P. petersi*. Dashed outlines show the Guianan Shield and Brazilian Shield.

forest refugia, marine incursion, etc.) because of inadequate samples in crucial regions.

2. Materials and methods

2.1. Taxon sampling

Samples from 4 outgroup species and from 65 *Physalaemus petersi* individuals from 22 sites across the species' distribution were analyzed (Fig. 1; Appendix A). Tissue samples (liver, muscle, and/or toes) were either frozen or stored in 95% ethanol or DMSO buffer. Voucher specimens for most samples are available in several museums (Appendix A) except in cases in which permits did not allow collection of vouchers. Cannatella et al. (1998) treated *P. freibergi* as distinct from *P. petersi*, but they did not define species boundaries and distributions. Therefore, we refer to both species as *P. petersi*. *Physalaemus pustulosus* is the sister-species of *P. petersi*, and the two form a well-supported clade (clade name Edentulus) that is the sister-group to a clade (clade name Duovox) containing all other species in the *P. pustulosus* species group (Ron et al., 2005, 2006). Therefore, for outgroup taxa, we used two *P. pustulosus* specimens and one each of two species in the Duovox clade, *P. pustulatus* and *P. coloradorum*. Although Nascimento et al. (2005) resurrected the genus *Engystomops* for the *Physalaemus pustulosus* group, that action is not consistent with their own analysis of relationships. Ron et al. (2006) uncritically followed the use of *Engystomops*. However, one of the authors of the latter paper (DCC) agrees that the 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.

2.2. DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from tissue samples using DNeasy Tissue Kits (Qiagen, Inc., Valencia, CA). Overlapping sets of primers were used to amplify approximately 2.4 kb of the mitochondrial 12S and 16S ribosomal RNA genes and the intervening valine tRNA gene using the polymerase chain reaction (PCR). Primers, PCR conditions, and sequencing protocol were described in Pauly et al. (2004). Editing and assembly of contigs was completed using Sequencher 4.5 (Gene Codes Corp., Ann Arbor, MI).

2.3. Alignment and phylogenetic analyses

Initial alignment of DNA sequences was completed in ClustalX (Thompson et al., 1997). Manual adjustments were then made in MacClade 4.06 (Maddison and Maddison, 2000) so as to minimize the number of changes required across taxa. Only unique haplotypes were included in matrices used for phylogenetic analyses. Parsimony analyses were conducted in PAUP* 4.0b10 (Swofford, 2000) using a heuristic search with 1000 random addition-sequence replicates and TBR branch swapping. Nodal support was assessed through nonparametric bootstrap analysis using 1000 bootstrap replicates with 100 random addition-sequence replicates per bootstrap replicate.

The most appropriate model of sequence evolution for the likelihood analysis was selected using Akaike's information criterion (AIC; Akaike, 1974) for the complete sequence (12S, tRNA-Val, and 16S) using Modeltest 3.7 (Posada and Crandall, 1998). Likelihood analysis was then conducted in PAUP* using successive iterations with starting parameters based on estimates from the previous tree, a method shown to perform well (Sullivan et al., 2005). Parameters for the first iteration were estimated from the most-parsimonious tree with the best likelihood score. Iterations were continued until successive searches yielded identical trees.

Four replicate Bayesian analyses were conducted with MrBayes 3.04b (Huelsenbeck and Ronquist, 2001) on a NPACI Rocks cluster (http://www.rockscluster.org). Four Markov chains were used in each replicate, and the chain was sampled every 100 generations. Each replicate was run for 5 million generations.

Parametric bootstrap tests (Hillis et al., 1996; Huelsenbeck et al., 1996a,b; termed the SOWH test by Goldman et al., 2000) were used to test the riverine barrier hypothesis. Specifically, we tested the null hypothesis of reciprocal monophyly of haplotypes on opposite sides of rivers. This hypothesis was tested separately for the Napo, Juruá, and Madre de Dios Rivers, using unique haplotypes from all sites located within the interfluves (regions bounded by major rivers) adjacent to each river (Table 1). The riverine barrier hypothesis predicts that lower portions of rivers will be more effective barriers than the headwaters; therefore, we also performed separate tests for the upper (U.) and lower (L.) Napo River, dividing the Napo River between sites 5 and 6 because the river narrows substantially at this point.

To implement the parametric bootstrap tests, we: (1) used AIC in Modeltest 3.7 (Posada and Crandall, 1998) to determine the most appropriate model of sequence evolution for each reduced-taxon dataset; (2) found the most-parsimonious tree without a topological constraint; (3) found the most-parsimonious tree with the topological constraint for the given hypothesis; (4) estimated likelihood parameters under the constraint tree using the selected model of sequence evolution; (5) simulated 1000 replicate datasets with Seq-Gen v.1.3.2 (Rambaut and Grassly, 1997) using the model parameters from step 4 and the best constraint tree; (6) for each replicate dataset, calculated the difference in parsimony scores between the best tree under no constraints and the best constrained tree; (7) compared the observed difference in tree scores with the null distribution of tree score differences obtained in step 6. The null hypothesis was rejected when the observed tree score difference was greater than 95% of the values in the null distribution.

Table 1
Parametric bootstrap results of tests of the riverine barrier hypothesis for different Amazonian rivers

River	Constraint tree	*n*	Tree length		*P*
			No. constr.	Constr.	
Napo	(((1–3, 7, 11), (4–6, 8–10)), outgroups)	21	858	885	<0.001
U. Napo	(((1, 7),(6, 8–9)), outgroups)	9	741	749	0.105
L. Napo	(((2–3, 11), (4–5, 10)), outgroups)	12	817	835	0.001
Juruá	(((19–20), 21), outgroups)	4	729	732	0.315
Madre de Dios	((12, (13–17)), outgroups)	13	753	753	1.000

Numbers in constraint trees refer to sites (see Fig. 1). Sites not shown in a constraint tree were not included for the given test. *n* is the number of unique *Physalaemus petersi* haplotypes available for a given test (not including outgroups). *P* is the probability of observing a difference in tree lengths between the unconstrained and constrained trees as large or larger than that observed under the null hypothesis (as defined by the given constraint tree).

2.4. Population genetic analyses

Outgroups were not used for population genetic analyses. All 65 *Physalaemus petersi* sequences were used for estimation of diversity statistics and tests of population expansion (including haplotypes shared by more than one individual). In contrast, only unique haplotypes were used for Mantel tests so that sequence divergence was greater than zero for all pairwise comparisons (this conservative approach reduces the number of pairwise comparisons and, therefore, statistical power). Diversity statistics, including the number of haplotypes, the number of polymorphic sites (s), and nucleotide diversity (π_n), were calculated using Arlequin 2.000 (Schneider et al., 2000).

Mantel tests (Mantel, 1967) and partial Mantel tests (Smouse et al., 1986) were used to examine the effects of straight-line geographic distance, intervening rivers, and elevational differences among sites on corrected sequence divergence using Fstat ver. 1.2 (Goudet, 1995). Partial Mantel tests, which measure the correlation between a variable and genetic distance after controlling for another variable, were used to test the effects of intervening rivers and elevational differences after removing the effects of geographic distance. Separate analyses were conducted for four different regions: the Napo River, the U. Napo River, the L. Napo River, and the Madre de Dios River. The effects of geographic distances and intervening rivers were examined in all regions, but the effect of elevational differences was examined only in the Napo River region, in which sites varied substantially in elevation (102–1069 m). Natural-log-transformed geographic distances were used for three regions (Napo River, L. Napo River, and Madre de Dios River) to improve the fit between sequence divergence and geographic distance. A pair of haplotypes was considered to be separated by a river if a straight line between the two sites crossed the river of interest.

We used three different assessments of population expansion in order to test the fourth prediction of the riverine barrier hypothesis, that rivers are areas of primary differentiation rather than areas of secondary contact. Population expansion near a given river leaves open the possibility that clades diverged via processes other than riverine barriers and only recently met at the river. The first method was Harpending's (1994) raggedness index of mismatch distributions, implemented in Arlequin. Rapid population expansion results in smooth, unimodal mismatch distributions. A smaller raggedness index indicates a smoother mismatch distribution. One thousand bootstrap replicates were used to test the probability of a raggedness index as large as observed under a null hypothesis of a sudden population expansion. The second method was Fu's (1997) F_s, also in Arlequin. Large negative values of F_s are predicted under population expansion. The significance of F_s was also tested using 1000 bootstrap simulations. The third method was a coalescent approach for estimating population growth (g), implemented in Fluctuate Version 1.4 (Kuhner et al., 1998). In each run, we used 10 short chains (sampling increments of 10; 1000 steps per chain), 10 long chains (sampling increments of 10; 20,000 steps), a random starting tree, and a starting value of $g = 1$, following Lessa et al. (2003). Large values of g, and small coefficients of variation of g, provide evidence for population expansion. The evidence for population expansion for a given clade was considered strong if all three methods indicated expansion.

3. Results

3.1. Sequence alignment and variation

The DNA sequences are deposited in GenBank (Appendix A). In the final alignment, 19 nucleotide positions had an apparent deletion in the majority of individuals. Exclusion of these 19 sites did not affect the topology of the parsimony tree. Therefore, we excluded these characters from all analyses. Two apparent cases of heteroplasmy, or multiple mitochondrial genomes in an individual, were also detected. The first case was for individual 1 (see Appendix A), which had both C and T nucleotides at position 2000. All other individuals, including outgroups, had a C at this position. Individual 28 had both C and T at position 2112, but all other individuals had C. These bases were coded as pyrimidines (Y) for these individuals in all analyses. In the final alignment, 619 out of 2380 characters were variable, and 445 were parsimony-informative. A total of 44 unique haplotypes was found for the 65 *P. petersi* individuals.

3.2. Phylogenetic analyses

The maximum likelihood analysis used a GTR + Γ + I model of sequence evolution and only required two iterations to reach convergence of tree topologies and branch lengths. The likelihood score of our final tree was 8674.2069 (estimated base frequencies: A 0.3640, C 0.1826, G 0.1651, T 0.2883; rate matrix: A–C 17.3959, A–G 73.4668, A–T 38.7195, C–G 1.1600×10^{-12}, C–T 224.4711, G–T 1.0000; shape parameter for gamma distribution: 0.6574; proportion of invariant sites: 0.4784; Fig. 2). Parsimony analysis generated six most-parsimonious trees of 1076 steps (CI = 0.725, RI = 0.879). The parsimony tree is not shown because it was consistent with and almost identical to the likelihood tree (see following description of differences).

For the Bayesian analysis, plots of model parameters and likelihood versus generation number indicated that stationarity had been reached by generation 100,000. Moreover, bipartition posterior estimates obtained from all samples after removal of a conservative burn-in period of 500,000 generations appeared to converge in pairwise comparisons between runs (using the comparetree command in MrBayes). Therefore, the last 4.5 million generations of each of the four runs were combined to yield 180,000 trees for the final Bayesian posterior probabilities (bpp). The 50% majority-rule consensus tree of all 180,000 Bayesian samples was identical to the maximum likelihood topology. Bayesian posterior probabilities for all nodes with bpp greater than 50% are shown on the maximum likelihood tree (Fig. 2).

Parsimony, likelihood, and Bayesian analyses consistently identified many of the same well-supported clades (Figs. 2 and 3). All analyses identified three main clades within *Physalaemus petersi*, which we call the northwestern

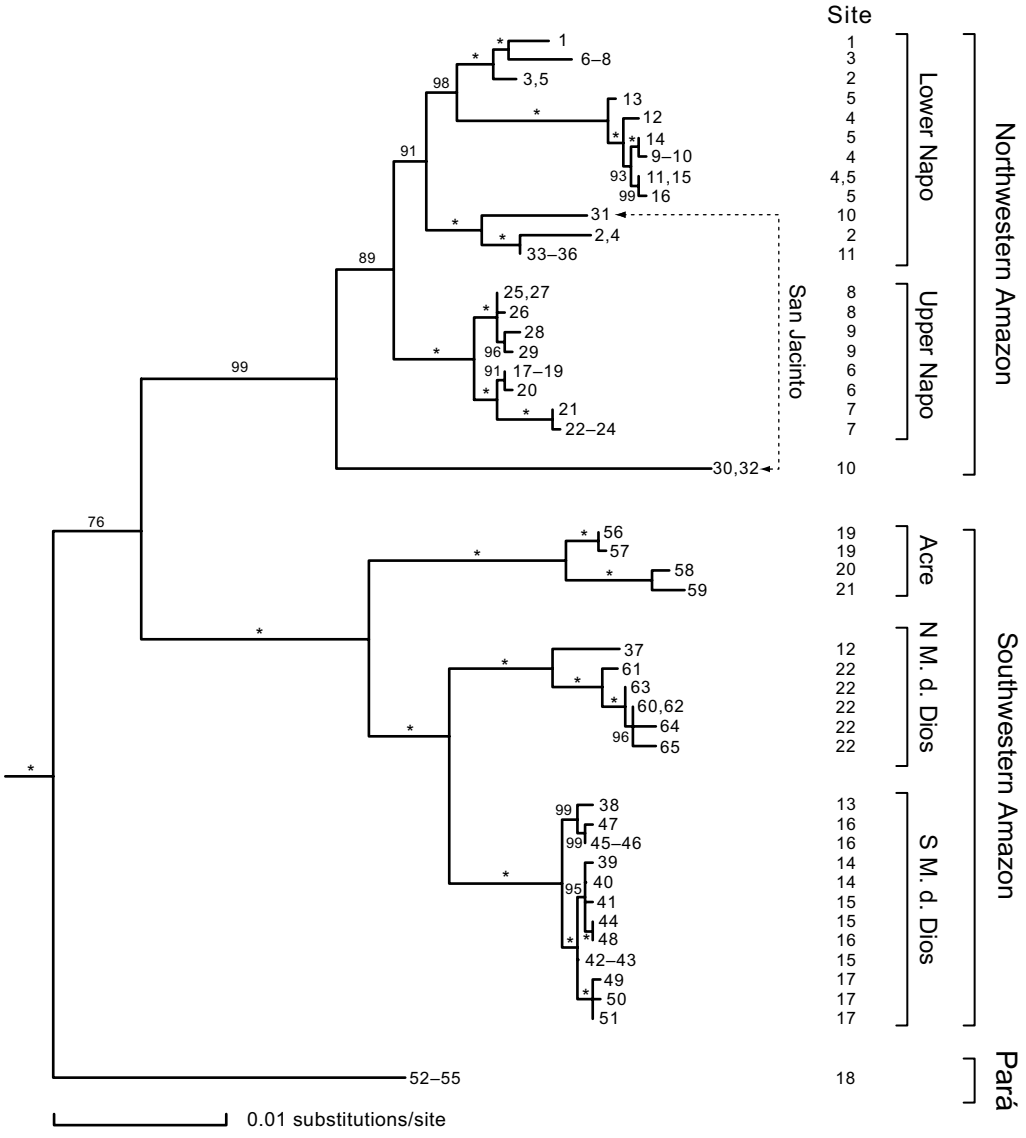

Fig. 2. Maximum likelihood topology. Numbers on branches are Bayesian posterior probabilities from 180,000 sampled trees; asterisks indicate posterior probabilities of 100%. Site numbers correspond to those in Figs. 1 and 3, Tables 1 and 3, and Appendix A. Individual specimen numbers are shown by terminal nodes and correspond to those in Appendix A. Outgroup taxa are not shown.

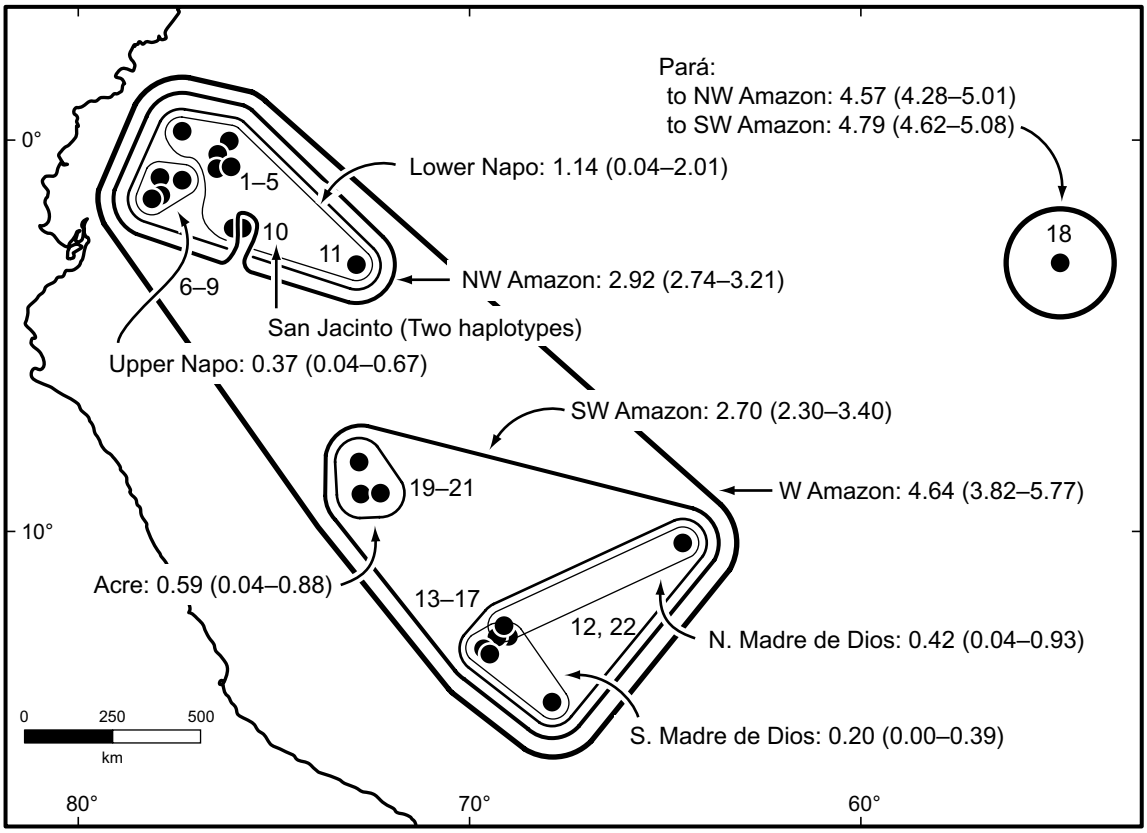

Fig. 3. Geographic distribution of major clades identified in phylogenetic analyses, with average percent corrected sequence divergence (and ranges) shown within and among clades (using a GTR + Γ + I model). Clade names correspond to those in Fig. 2 and Tables 2, 4, and site numbers correspond to site numbers in Figs. 1 and 2, Tables 1 and 3, and Appendix A. Site 10 is included in two different clades (the San Jacinto and the lower Napo clades) because it contained two divergent haplotypes (see text for details).

Amazon clade, the southwestern Amazon clade, and the Pará clade. Within the northwestern Amazon clade, all analyses also identified an U. Napo clade, consisting of four sites west of the confluence of the Napo and Coca Rivers in eastern Ecuador, and the San Jacinto haplotype, representing two individuals from a single site in northern Peru. Within the southwestern Amazon clade, all analyses identified three clades, hereafter referred to as the Acre clade, consisting of three sites in the state of Acre, Brazil; the northern (N.) Madre de Dios clade, consisting of one site on the north bank of the Madre de Dios River in southeastern Peru and one site in the state of Rondônia, Brazil; and the southern (S.) Madre de Dios clade, consisting of four sites south of the Madre de Dios River in southeastern Peru and one site in northwestern Bolivia.

There were two main differences between the topology of the strict consensus parsimony tree and the likelihood tree. The strict consensus parsimony tree had a polytomy for the node that contains the northwestern Amazon, southwestern Amazon, and Pará clades, because five of the six most-parsimonious trees placed the Pará clade as the sister-group to a clade containing all other *P. petersi*, and the other most-parsimonious tree placed the Pará clade as the sister-group to the southwestern Amazon clade. In contrast, in the likelihood tree the Pará clade was the sister-group to a clade containing all other *P. petersi* (same topology as five of the six most-parsimonious trees). However, the support for this topology is not strong, as 15.2% of Bayesian samples placed the Pará clade as the sister-group to the southwestern Amazon clade (as seen in one of the six parsimony trees). Another difference is that sites 1–5 and 10–11 form a fairly well-supported lower Napo clade in the likelihood tree (Bayesian posterior probability = 91%), but this clade occurs only in three of the six MP trees.

Corrected sequence divergence between *P. petersi* and outgroup taxa under a GTR + Γ + I model of sequence evolution averaged 29.13% (22.28–34.72%). Within *P. petersi*, average sequence divergence varied substantially among clades, ranging from 1.63% (1.15–2.07%) between the L. and U. Napo clades and 1.88% (1.56–2.13%) between the S. and N. Madre de Dios clades to 4.79% (4.62–5.08%) between the southwestern Amazon and Pará clades (Fig. 3). Average sequence divergence among haplotypes within clades with more than one haplotype (L. Napo, U. Napo, Acre, N. Madre de Dios, and S. Madre de Dios clades) ranged from 0.20% to 1.14% (Fig. 3). In two cases, we observed very divergent haplotypes within a single site. At site 10, sequence divergence was 3.21% between individuals 30 and 32 (the San Jacinto haplotype) and individual 31 (grouping with the L. Napo clade). At site 2, sequence divergence was 1.20% between the haplotype of individuals 2 and 4 and the haplotype of individuals 3 and 5 (all in the L. Napo clade).

Results of the parametric bootstrap tests are shown in Table 1. We could not reject the hypothesis of reciprocal monophyly for the Madre de Dios River ($P = 1.000$) or the Juruá River ($P = 0.315$), but the hypothesis was strongly rejected for the Napo River ($P < 0.001$). Moreover, the hypothesis was strongly rejected ($P = 0.001$) for the L. Napo River, but not for the U. Napo River ($P = 0.105$). It is likely that failure to reject the riverine barrier hypothesis for the Juruá River and the U. Napo River was due to the limited number of haplotypes available for these tests ($n = 4$ and 9, respectively); however, a P of 1.000 for the Madre de Dios River strongly supports a barrier effect.

3.3. Population genetic analyses

Diversity statistics varied substantially among clades (Table 2). There was a general pattern of an increase in the number of haplotypes, but a decrease in the number of polymorphic sites (s) and nucleotide diversity (π_n), from north to south, even after considering variation in the number of sites and individuals sampled. For example, 7 sites and 21 individuals were sampled in both the L. Napo and Madre de Dios clades, but the L. Napo has fewer haplotypes (12 in the L. Napo versus 18 in Madre de Dios), more polymorphic sites ($s = 75$ in the L. Napo versus 65 in Madre de Dios), and higher nucleotide diversity ($\pi_n = 0.0106$ in the L. Napo versus 0.0091 in Madre de Dios). Similarly, the number of polymorphic sites and nucleotide diversity are higher in the Acre clade than in the S. Madre de Dios clade ($s = 22$ and $\pi_n = 0.0057$ in Acre versus $s = 16$ and $\pi_n = 0.0019$ in S. Madre de Dios), despite a smaller number of sites and individuals and a smaller geographic area sampled in Acre (number of sites = 3 and number of individuals = 4 in Acre versus number of sites = 5 and number of individuals = 14 in S. Madre de Dios). This suggests that although more haplotypes occur in southern clades compared to northern clades, average sequence divergence among haplotypes (as measured by s and π_n) is lower in southern clades than average sequence divergence among haplotypes in northern clades.

Table 2
Diversity statistics by clade

Clade	No. sites	No. individs.	No. hap.	s	π_n
Napo	11	34	20	98	0.0117 ± 0.006
U. Napo	4	13	8	20	0.0034 ± 0.002
L. Napo	7	21	12	75	0.0106 ± 0.005
San Jacinto	1	2	1	0	0.0000 ± 0.000
Acre	3	4	4	22	0.0057 ± 0.004
Madre de Dios	7	21	18	65	0.0091 ± 0.005
N. Madre de Dios	2	7	6	26	0.0035 ± 0.002
S. Madre de Dios	5	14	12	16	0.0019 ± 0.001
Pará	1	4	1	0	0.0000 ± 0.000

Clades correspond to those defined in Figs. 2 and 3. No. hap. is the number of haplotypes; s is the number of polymorphic sites; and π_n is nucleotide diversity. The San Jacinto clade includes individuals 30 and 32 from site 10; individual 31, also from site 10, grouped with the L. Napo clade.

Mantel tests and partial Mantel tests demonstrated that the correlation between patristic distance (corrected sequence divergence) and geographic distance, presence/absence of intervening rivers, and elevation also varied substantially among regions (Table 3). Sequence divergence was strongly correlated with geographic distance in the Napo, U. Napo, and L. Napo regions ($P = 0.0001$–0.0004), but not in the Madre de Dios region ($P = 0.697$). Conversely, sequence divergence was strongly correlated with the presence of the Madre de Dios River before ($P = 0.0001$) and after ($P = 0.0001$) removing the effects of geographic distance, but sequence divergence was not correlated with intervening rivers in the other three regions before ($P = 0.058$–0.746) or after ($P = 0.243$–0.959) removing the effects of geographic distance. In the Napo region, the only region with both low and high elevation sites, the correlation between sequence divergence and elevational differences before removing the effects of geographic distance was not significant ($P = 0.135$), but there was a small, but significant negative correlation between sequence divergence and elevational differences after correcting for geographic distance ($P = 0.031$).

Tests of population expansion revealed a consistent signal of expansion only in the S. Madre de Dios clade (Table 4). In this clade, results of all three tests were consistent with the predictions of an expanding population. Specifically, the mismatch distribution was smooth as indicated by

Table 3
Results of simple and partial Mantel tests to investigate the relationship between patristic distance, geographic distance, rivers, and elevation

Region	n	Mantel test	r	P
Napo (1–11)	21	Patr dist × ln geo dist	0.510	0.0001
		Patr dist × river	0.023	0.746
		Patr dist × elev	0.107	0.135
		(Patr dist × river).ln geo dist	0.004	0.959
		(Patr dist × elev).ln geo dist	−0.154	0.031
U. Napo (1, 6–9)	9	Patr dist × geo dist	0.835	0.0001
		Patr dist × river	0.347	0.058
		(Patr dist × river).geo dist	0.214	0.243
L. Napo (2–5, 10–11)	12	Patr dist × ln geo dist	0.443	0.0004
		Patr dist × river	0.157	0.247
		(Patr dist × river).ln geo dist	0.138	0.313
Madre de Dios (12–17)	13	Patr dist × ln geo dist	0.049	0.697
		Patr dist × river	0.983	0.0001
		(Patr dist × river).ln geo dist	0.982	0.0001

Patr dist × geo dist, patr dist × river, and patr dist × elev are simple Mantel tests between patristic distance (corrected sequence divergence) and geographic distance, presence/absence of intervening rivers, and elevational differences between sites, respectively. (patr dist × river).geo dist and (patr dist × elev).geo dist are partial Mantel tests between patristic distance and rivers and elevation, respectively, after controlling for the effects of geographic distance. Natural-log-transformed geographic distances were used for three regions to improve the linear fit between patristic distance and geographic distance. Regions were defined a priori and do not correspond to the clades identified in the phylogenetic analyses. Numbers in parentheses indicate which sites (see Fig. 1) were included in each region; n is the number of unique haplotypes available for the given test; r is the standardized Mantel test statistic which is equivalent to a Pearson product-moment correlation coefficient; P was estimated from 10,000 randomizations.

Table 4
Results of tests of historical population expansion

Clade	n	Raggedness	P (raggedness)	Fu's F_s	P (Fu's F_s)	g	SD (g)	CV (g)
U. Napo	13	0.0459	0.510	−6.713	<0.001	234.4	243.5	1.04
L. Napo	21	0.0413	0.013	−6.801	0.004	37.8	62.5	1.65
Acre	4	0.1667	0.937	0.678	0.388	305.9	186.9	0.61
N. Madre de Dios	7	0.1383	0.374	−1.912	0.085	434.7	181.8	0.42
S. Madre de Dios	14	0.0463	0.647	−11.392	<0.001	1757.2	691.4	0.39

Clades correspond to the clades defined in Figs. 2 and 3. Raggedness values are measures of the smoothness of mismatch distributions, with lower raggedness values indicating smoother distributions. Smooth Poisson mismatch distributions are characteristic of rapid population expansion. P (raggedness) is the probability of observing a distribution with higher raggedness under a null hypothesis of population expansion based on 1000 bootstrap replicates. Negative F_s values also indicate population expansion. P (Fu's F_s) is based on 1000 simulations. g is a coalescent-based estimate of population expansion; $CV(g) = SD(g)/g$. n is the number of individuals in each clade.

a low raggedness value (0.0463) and a large probability of observing a raggedness value this large or larger under the null hypothesis of population expansion ($P = 0.647$); Fu's F_s was large and negative ($F_s = -11.392$) and was significantly smaller than 0 ($P < 0.001$); and g estimated by Fluctuate was very large ($g = 1757.2$) and the coefficient of variation was relatively small (0.39) given the limited number of samples available for the test ($n = 14$). In two other clades, the U. Napo and N. Madre de Dios, two out of three tests supported population expansion (mismatch distributions for both clades, and F_s for the U. Napo and g for the N. Madre de Dios), providing some support for expansion. In the remaining two clades, the L. Napo and Acre, only one test supported population expansion (F_s for the L. Napo and the mismatch distribution for Acre).

4. Discussion

4.1. Tests of biogeographic hypotheses for diversification

Our results provide little support for an effect of the elevational gradient of the Andes in structuring mtDNA haplotype diversity in *Physalaemus petersi*, but provide some support for the riverine barrier hypothesis for one of the three rivers examined, the Madre de Dios. For the Napo River, we found no support for the first three predictions of the riverine barrier hypothesis, namely that: (1) reciprocally monophyletic populations will occur on opposite sides of the river (rejected using parametric bootstrapping; Table 1); (2) genetic divergence between populations will be positively correlated with the presence of intervening rivers after removing the effects of geographic distance (rejected using a partial Mantel test; Table 3); and (3) genetic divergence between trans-riverine populations will be greater in the river's lower part than in the headwaters (rejected using both parametric bootstrapping and partial Mantel tests in separate analyses for the L. and U. Napo River; Tables 1 and 3). Therefore it is clear that the Napo River has not acted as a biogeographic barrier for *P. petersi*. Interestingly, genetic divergence was actually higher between populations separated by the U. Napo River than between populations separated by the L. Napo River (opposite the prediction of the riverine barrier hypothesis), perhaps because of greater topographic relief surrounding the upper Napo River, which passes through the Andean foothills. Despite the lack of a barrier effect of the Napo River, populations 3 and 5, which lie on opposite sides, are strongly isolated based on variation in male calls and female preferences (Boul et al., 2007).

For the Madre de Dios River, we could not reject the first two of the above hypotheses (Tables 1 and 3), suggesting that the Madre de Dios separates two divergent clades, in support of the riverine barrier hypothesis. However, strong evidence for population expansion in the southern Madre de Dios clade (Table 4) leaves open the possibility that the Madre de Dios is an area of secondary contact of expanding lineages rather than an area of primary differentiation. Nevertheless, the observation of a well-supported divergent clade south of the Madre de Dios suggests that, at the very least, this river currently acts as a barrier to gene flow. For the Juruá River, we were not able to reject reciprocal monophyly of populations on opposite sides of the river using parametric bootstrapping, probably because of the small number of unique haplotypes ($n = 4$; Table 1).

Previous research on *P. petersi* and other taxa supports our finding that the barrier effect of Amazonian rivers varies. Gascon et al. (1998) found that the Juruá River is not an important barrier for *P. petersi*. Several other studies also show little support for the riverine barrier hypothesis for Amazonian rivers in amphibians, birds, and mammals (Aleixo, 2004; da Silva and Patton, 1993; Gascon, 1996; Gascon et al., 1996, 1998, 2000; Lougheed et al., 1999; Symula et al., 2003). In contrast, some studies of Amazonian butterflies and birds have found support for the riverine barrier hypothesis (Aleixo, 2004; Cheviron et al., 2005; Hall and Harvey, 2002; Hayes and Sewlal, 2004; Höglund and Shorey, 2004). The Chagres River in Panama also acts as a barrier to gene flow in *P. pustulosus* (Lampert et al., 2003), the sister species of *P. petersi* (Ron et al., 2006).

Contrary to the elevational gradient hypothesis, genetic divergence among sites was not positively correlated with elevational differences after removing the effects of geographic distance (Table 3). This result in *P. petersi* contrasts with recent work in poison frogs (Dendrobatidae) suggesting that elevational speciation may be important at higher elevations (Graham et al., 2004), but is in agreement with Patton and Smith (1992), who rejected the elevational gradient hypothesis for mice on the Peruvian Andean slopes,

and Dingle et al. (2006), who did not find support for the hypothesis in birds in northwestern Amazonia. *Physalaemus petersi* extends from sea level up to approximately 1200 m, a range which may not be sufficient to provide divergent selection pressures strong enough to cause parapatric speciation. Surprisingly, we found that genetic divergence among sites was significantly negatively correlated with elevational differences after removing the effects of geographic distance (Table 3).

High levels of divergence between the northwestern Amazon, southwestern Amazon, and Pará clades remain to be explained (Figs. 2 and 3). Other studies have found similar north-south and east-west genetic breaks in Amazonia, although exact break points vary (Banguera-Hinestroza et al., 2002; Cheviron et al., 2005; Glor et al., 2001; Hoffman and Baker, 2003; Lovette, 2004; Marks et al., 2002; Symula et al., 2003). Potential vicariant barriers between the northwestern and southwestern Amazon clades include the Amazon and Marañon Rivers and historical mountain ridges running east-west in northern Peru (Hoorn et al., 1995; Lundberg et al., 1998; Räsänan et al., 1987; Räsänan et al., 1990). The western clades and the Pará clade are also separated by several major Amazonian rivers and historical ridges running north-south, which may also have acted as potential barriers. Additional sampling in these three clades will be necessary to test these hypotheses.

4.2. Patterns of population expansion

We found a strong genetic signal of population expansion in the S. Madre de Dios clade in southeastern Peru and northwestern Bolivia. Results from all three methods matched the expectations of an expanding population in the S. Madre de Dios clade, including a smooth mismatch distribution, a large negative F_s value, and a large positive g value (Table 4). There was also some support for population expansion in the U. Napo and N. Madre de Dios clades, with two out of three of the above tests supporting population expansion. In contrast, there was limited support for population expansion in the L. Napo and Acre clades, with only one of three tests supporting expansion.

The diversity indices (Table 2), Mantel tests (Table 3), and tree topologies (Fig. 2) also strongly supported the hypothesis of population expansion in the S. Madre de Dios clade. Specifically, this clade had a large number of haplotypes, low number of polymorphic sites (s), and low nucleotide diversity (π_n) compared to clades to the north, even after accounting for differences in the number of individuals and sites sampled (Table 2). This pattern is expected in an expanding population, because less time will have elapsed for lineage sorting (resulting in a relatively large number of haplotypes) and for sequence differences to evolve (resulting in low s and π_n). Additionally, only the Madre de Dios region lacked a significant correlation between genetic divergence and geographic distance in Mantel tests (Table 3). This pattern is also expected in a recently expanding population, because not enough time will have elapsed for dispersal-limited gene flow to result in isolation-by-distance of haplotypes. Finally, visual inspection of likelihood trees revealed a star-like branching pattern for the S. Madre de Dios clade, characteristic of population expansion. Also, the topology of the N. and S. Madre de Dios clades relative to the more basal Acre clade suggests a pattern of north-to-south population expansion. Phylogeographic studies of birds have also found evidence for population expansion in southwestern Amazonia (Aleixo, 2004; Cheviron et al., 2005), but not in small mammal populations (Lessa et al., 2003). These results are consistent with the idea that lowland rainforest suitable for these species has expanded into southwestern Amazonia after the Pleistocene when precipitation, and rainforest, was reduced along the periphery of the Amazon basin (Bush, 1994).

4.3. High sequence divergence among haplotypes at single localities

Surprisingly, highly divergent haplotypes were found at two sites in the L. Napo clade. At site 10 (see Fig. 1), sequence divergence between the two haplotypes was 3.21%, and at site 2, divergence between the two haplotypes was 1.20%. These numbers are similar to those seen in the same genes among *Physalaemus* species that are well-differentiated by call characteristics (Ron et al., 2005). Two potential explanations are: (1) incomplete lineage sorting, and (2) gene flow from a distant lineage. The observation that one of the divergent haplotypes in sites 2 and 10 is closely related to the haplotype observed at site 11 (Fig. 2) is consistent with a pattern of secondary gene flow from site 11 to sites 2 and 10 (see pp. 68–70, Avise, 2000). Thus, although there is not a strong signal of population expansion for the entire lower Napo clade, there is evidence for secondary population expansion or dispersal on a smaller geographic scale within this clade.

4.4. Systematic implications

The high level of sequence divergence observed among the northwestern Amazon, southwestern Amazon, and Pará clades (Fig. 3) suggests the presence of more than one species of Amazonian *Physalaemus*. Four species names have been applied to Amazonian *Physalaemus* in the *P. pustulosus* species group: *P. petersi* (Jiménez de la Espada, 1872), *P. paraensis* (Müller, 1923), *P. schereri* (Myers, 1942), and *P. freibergi* (Donoso-Barros, 1969), but only *P. petersi* and *P. freibergi* are currently recognized (Cannatella and Duellman, 1984; Cannatella et al., 1998). The type locality of *P. petersi* is "Napo-Pastaza, Ecuador", which generally corresponds to sites 6–9 (Fig. 1). Since these sites are part of a well-supported northwestern Amazon clade including sites 1–11, possibly the name *P. petersi* corresponds to this clade. The type locality of *P. freibergi* is Rurrenabaque, Departmento El Beni, Bolivia, which is only 38 km from site 17 (Fig. 1). The well-supported southwestern Amazon clade includes

sites 12–17 and 19–22, and *P. freibergi* may correspond to this clade. It is possible that the Pará lineage, represented by site 18, corresponds to *P. paraensis*, but the type locality for *P. paraensis*, Peixe-boi, Estado Pará, Brazil, is approximately 870 km east of site 18, so additional sampling near the type locality is needed. Similarly, the type locality for *P. schereri*, Pevas (Pebas), Departamento Loreto, Peru, is 120 km from site 11, so additional sampling near the type locality is needed to assess the validity of this taxon. We are currently working on an analysis of morphological and call variation in Amazonian *Physalaemus* in the *P. pustulosus* species group to assess species differences and geographic ranges. Additional assessment of behavioral isolation, such as that provided by Boul et al. (2007), would also be important in assessing species distinctness.

5. Conclusions

Few other studies have explicitly tested a priori biogeographic hypotheses for Amazonian diversification using a phylogeographic framework (for other examples, see Aleixo, 2004; Cheviron et al., 2005; Patton and Smith, 1992; Patton et al., 1994, 2000). This is also one of the first phylogeographic studies of an Amazonian amphibian (also see Chek et al., 2001; Lougheed et al., 1999; Symula et al., 2003). Our results provide little evidence for the elevational gradient hypothesis for *P. petersi*, but provide some support for the riverine barrier hypothesis for the Madre de Dios River. Moreover, we observed distinct, well-supported clades in the northwestern Amazon, southwestern Amazon, and eastern Amazon (Pará, Brazil), separated from each other by high levels of sequence divergence. Similar north-south and east-west genetic breaks have been found in other Amazonian taxa, but sampling is not sufficient to determine whether there exist breakpoints that are concordant among taxa or with any present or historical geographic features. Additionally, our results provide strong support for population expansion in the southwestern corner of the Amazon basin, in agreement with two other phylogeographic studies of birds that have also found evidence for population expansion in southwestern Amazonia, but contrast with work in mammals in western Amazonia showing little evidence for population expansion. Although this phylogeographic study represents one of the most geographically well-sampled for any Amazonian species, large regions remain, primarily in the Brazilian state of Amazonas that should be sampled to provide a more general picture of diversification across the Amazon basin.

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Appendix A

Site information, specimens sampled, and GenBank accession numbers

Site information				Specimen information		
No.	Location	Coordinates	Elev (m)	No.	Museum No.	Accession No.
1	Ecuador: Sucumbíos: Lumbaquí	N 0°6′41″; W 77°22′28″	610	1	QCAZ 25790	EF470253
2	Ecuador: Sucumbíos: Puerto Bolivar	S 0°5′19″; W 76°8′31″	240	2	QCAZ 27813	EF470254
				3	QCAZ 28169	EF470255
				4	QCAZ 28172	EF470256
				5	QCAZ 28178	EF470257

Appendix A (*continued*)

Site information				Specimen information		
No.	Location	Coordinates	Elev (m)	No.	Museum No.	Accession No.
3	Ecuador: Sucumbíos: La Selva Lodge	S 0°29′20″; W 76°22′29″	226	6	QCAZ 23975	EF011530
				7	QCAZ 24029	EF011531
				8	(DCC 3705)	EF011532
4	Ecuador: Orellana: Tiputini Biodiversity Station	S 0°38′14″; W 76°9′54″	208	9	QCAZ 28610	EF011535
				10	QCAZ 28611	EF011534
				11	QCAZ 28612	EF011538
				12	QCAZ 28620	EF011533
5	Ecuador: Orellana: Estación Científica Yasuní	S 0°40′41″; W 76°23′48″	250	13	QCAZ 11863	EF011539
				14	QCAZ 12128	DQ337233
				15	QCAZ 15136	EF011543
				16	QCAZ 15138	EF011542
6	Ecuador: Napo: Jatun Sacha Biological Station	S 1°2′24″; W 77°21′36″	450	17	QCAZ 24045	EF011521
				18	(MJR 005)	EF011523
				19	(MJR 006)	EF011524
				20	(MJR 008)	EF011525
7	Ecuador: Napo: Cando	S 1°4′1″; W 77°55′59″	702	21	QCAZ 11965	DQ337231
				22	(DCC 3699)	EF011516
				23	(DCC 3701)	EF011517
				24	(DCC 3710)	EF011518
8	Ecuador: Pastaza: Puyo	S 1°26′35″; W 77°59′48″	954	25	QCAZ 26210	DQ337230
				26	QCAZ 26211	EF470258
				27	QCAZ 28857	EF470259
9	Ecuador: Pastaza: Shell	S 1°30′; W 78°3′	1069	28	QCAZ 25038	EF470260
				29	QCAZ 25039	EF470261
10	Peru: Loreto: San Jacinto	S 2°18′45″; W 75°51′46″	180	30	KUNHM 222069	EF470262
				31	KUNHM 222070	EF470263
				32	KUNHM 222071	EF470264
11	Peru: Loreto: Amazon Conservancy for Tropical Studies	S 3°15′34″; W 72°54′10″	102	33	MUSM 21546	EF470265
				34	MUSM 21556	EF470266
				35	MUSM 21562	EF470267
				36	MUSM 21564	EF470268
12	Peru: Madre de Dios: Cusco Amazónico	S 12°35′; W 69°5′	200	37	KUNHM 215534	EF470269
13	Peru: Madre de Dios: Trail between Madre de Dios River and Lago Sandoval	S 12°36′; W 69°5′	200	38	KUNHM 215133	EF470270
14	Peru: Madre de Dios: Explorer's Inn	S 12°50′18″; W 69°17′45″	207	39	USNM 343260	EF011546
				40	USNM 343264	EF011545
15	Peru: Madre de Dios: Tambopata Research Center	S 13°8′6″; W 69°36′23″	167	41	MUSM 19363	EF011550
				42	MUSM 19368	EF011547
				43	MUSM 19403	EF011548
				44	MUSM 19404	EF011549
16	Peru: Madre de Dios: south side of Tambopata River across from Tambopata Research Center	S 13°8′36″; W 69°35′51″	201	45	MUSM 19348	EF011551
				46	MUSM 19380	EF011553
				47	MUSM 19381	EF011552
				48	MUSM 19382	EF011554
17	Bolivia: La Paz: Chalalán Ecolodge	S 14°25′29″; W 67°55′14″	400	49	MNCN/ADN 2823	EF470271
				50	MNCN/ADN 2845	EF470272
				51	MNCN/ADN 2846	EF470273
18	Brazil: Pará: Agropecuária Treviso	S 3°9′; W 54°50′	122	52	LSUMZ 18728	EF470274
				53	LSUMZ 18729	EF470275
				54	LSUMZ 18730	EF470276
				55	LSUMZ 18731	EF470277
19	Brazil: Acre: Porto Walter	S 8°15′31″; W 72°46′37″	219	56	LSUMZ 13649	EF470278
				57	LSUMZ 13687	EF470279
20	Brazil: Acre: Mouth of Tejo River	S 9°3′; W 72°44′	260	58	ZUEC 9511	DQ337229
21	Brazil: Acre: Restauraçao	S 9°3′; W 72°17′	272	59	ZUEC 9523	EF011544
22	Brazil: Rondônia: Parque Estadual Guajará-Mirim	S 10°19′17″; W 64°33′48″	151	60	LSUMZ 17422	EF470280
				61	LSUMZ 17427	EF470281
				62	LSUMZ 17459	EF470282
				63	LSUMZ 17467	EF470283
				64	LSUMZ 17489	EF470284
				65	LSUMZ 17523	EF470285
P. pustulosus	Panama: Panama: Gamboa	—	—	66	(KM91)	DQ337239
	Mexico: Chiapas: Puerto Madera	—	—	67	(LW1033)	DQ337247

(*continued on next page*)

Appendix A (*continued*)

Site information				Specimen information		
No.	Location	Coordinates	Elev (m)	No.	Museum No.	Accession No.
P. pustulatus	Ecuador: Guayas: Cerro Blanco	—	—	68	QCAZ 23420	DQ337215
P. coloradorum	Ecuador: Pichincha: La Florida	—	—	69	QCAZ 19418	DQ337222

All specimens are *Physalaemus petersi* unless otherwise noted. Site numbers correspond to Figs. 1–3 and specimen numbers correspond to the numbers shown by terminal nodes in Fig. 2. Voucher numbers in parentheses are field identification numbers for specimens in which museum numbers are not available. Museum abbreviations are as follows: KUNHM, University of Kansas Natural History Museum and Biodiversity Research Center; LSUMZ, Louisiana State University Museum of Natural Science; MNCN/ADN, Museo Nacional de Ciencias Naturales, Spain; MUSM, Universidad Nacional Mayor de San Marcos Museo de Historia Natural, Peru; QCAZ, Museo de Zoología de la Pontificia Universidad Católica del Ecuador; USNM, Smithsonian National Museum of Natural History, Washington, DC; ZUEC, State University of Campinas Museum of Zoology, Brazil.

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