Sexual selection drives speciation in an Amazonian frog

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ELECTRONIC SUPPLEMENTARY MATERIAL (ESM)

(b) ***Phonotaxis experiments (continued)***

In phonotaxis experiments, female preferences are tested by broadcasting to females different male calls and allowing them to choose between calls; choice is indicated by close approach to a speaker broadcasting a call. Phonotaxis experiments are an accurate bioassay for anuran mate choices under natural settings. This is because females only show phonotaxis to choose a mate. This is virtually true for all anuran species that have been studied to date (Gerhardt & Huber 2002). In addition, the reproductive behavior of *P. petersi* is strikingly similar, as one might expect, to that of its sister species, *P. pustulosus*. That mating system has been documented more exhaustively than that of almost any other vertebrate (Ryan 1985). *Physalaemus* frogs show the canonical anuran female choice behavior. Females approach a calling male with no interference from "scrambling" males that sometimes interfere with female movements in other taxa. Females exhibit phonotaxis to the calling male and initiate amplexus by making physical contact with him. The pair remains in amplexus for several hours before depositing eggs.

We prepared stimuli for the phonotaxis experiments using Sound Edit 16 (Version 2) and Canary 1.2.4. We randomly chose calls from four individual frogs in Yasuní and from three individuals in La Selva. A total of six calls were selected per individual. We tested females from Yasuní with simple calls from each population, and with simple versus complex calls from their own population. La Selva females were tested with the same set of simple calls, and with their simple calls versus an artificial complex call that was produced by digitally appending a squawk from a Yasuní call to a simple La Selva call.

For female choice tests, amplexed pairs were collected in the field, taken back to a field laboratory, and placed in a large cooler for 10–20 minutes prior to initiation of experiments. We conducted phonotaxis experiments in a rectangular chamber 92 cm wide, 188 cm long, and 92 cm high. The walls of the chamber consisted of a frame of PVC piping 2 cm in diameter fitted with mattress foam to minimize sound reflection. We mounted two infrared cameras on the PVC piping in the upper corner of the chamber. One camera was used to shine infrared light in the chamber and the other had a wide-angle lens attached in order to view the entire chamber. A real-time image of the chamber was then displayed on a Macintosh iBook computer. A black plastic tarp covered the top and sides of the outside of the chamber to ensure uniform darkness.

A Sony Sports CD Walkman (Model No. D-SJ15) and two Saul Mineroff Field Speakers (SME-AFS; frequency response=100–12,000 Hz) were placed in the middle of either end of the chamber and broadcast stimuli antiphonally. Test females were restrained under a funnel of 15 cm in diameter prior to the initiation of the experiment. The amplitude of the call at the release site was 80 dB SPL (re 20 µP).

Water was sprayed on the floor prior to the initiation of the experiments to provide a moist environment. The temperature of the chamber was always 23–26º C, within the temperature range experienced by breeding aggregations of these frogs. Preceding each trial, the female was placed under the funnel and two stimuli were broadcast antiphonally

for three minutes, after which time the funnel was lifted. A choice was made when the female came within 11 cm of either speaker. The trial was ended and a no choice was recorded if the female remained at her initial position for 5 minutes, if the female moved and then remained in one place for 2 minutes, or if the female climbed the chamber wall for 2 minutes. A no choice was also recorded if the female reached the speaker by following the chamber wall since this is typical of escape behavior. For each preference experiment, we calculated the exact binomial probability of the null hypothesis of no preference. Fisher's exact tests were used to test whether female preferences for squawks were significantly different between populations.

(c) ***Phylogenetic analysis (continued)***

Parsimony analyses were conducted in PAUP* 4.0b10 (Swofford 2000) using a heuristic search with 1,000 random addition-sequence replicates and TBR branch swapping. Nodal support was assessed through nonparametric bootstrap analysis using 1,000 bootstrap replicates with 100 random addition-sequence replicates per bootstrap replicate. The score of the 6 most parsimonious (MP) trees was 874.

The most appropriate model of sequence evolution for the likelihood analysis (GTR + Γ + I) was selected using Akaike's information criterion (AIC; Akaike 1974) for the complete sequence (12S, tRNA-Val, and 16S) using Modeltest 3.7 (Posada & Crandall 1998). Likelihood analysis was then conducted in PAUP* 4.0b10 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. Only two iterations were required to reach convergence of tree topologies and branch lengths. The negative log-likelihood score for our tree was 7442.2.

Four replicate Bayesian analyses were conducted with MrBayes 3.04b (Huelsenbeck & Ronquist 2001) on an 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. Plots of model parameters and likelihood versus generation number indicated that stationarity had been reached by generation 100,000. Bipartition posterior estimates obtained from all samples after removal of a conservative burn-in period of 5,000 (equivalent to 500,000 generations) also converged in pairwise comparisons between runs (using the comparetree command in MrBayes). Therefore, the last 4.5 million generations (equivalent to 45,000 sampled trees) of each of the four runs were combined to yield 180,000 trees for the final Bayesian posterior probabilities.

An assumption of our phylogenetic analysis is that our phylogeny represents evolutionary relationships among populations, and that the distribution of complex and simple calls on the phylogeny demonstrates multiple gains and/or losses of squawks (figure 1*c*). An alternative interpretation of our phylogeny is that there are two species with different call types that have hybridized, such that the gene tree reflects introgression of mitochondria rather than relations among populations. However, this second possibility would require two events which we argue are unlikely: (i) introgression between a species with complex calls and another species with simple calls; and (ii) subsequent fixation of foreign haplotypes in each population in which

introgression has occurred. There are three reasons why introgression between a species with complex calls and another species with simple calls is improbable. First, previous allozyme work (Gascon *et al.* 1998) and our microsatellite data demonstrate that gene flow among populations of *Physalaemus petersi* is highly restricted. Second, there is little evidence for major range shifts in the Amazon basin (e.g., Lessa *et al.* 2003). These first two points, then, suggest that there is low potential for different populations or species of *P. petersi* to come into contact. Third, even if individuals of a species with complex calls came into contact with individuals of a species with simple calls, it is unlikely that they would mate with each other given the strong behavioral isolation between frogs with these different call types demonstrated in this study.

The second event that would have to occur in order for our phylogeny to represent introgression, rather than evolutionary relations among populations, is fixation of foreign haplotypes in each population in which introgression has occurred. The probability of a new haplotype becoming fixed in a population is $1/N$, where N is the effective number of females (Hartl & Clark 1989). Thus, the probability of fixation of new haplotypes from a foreign species would be very small. If introgression between two species with different call types had occurred, one would expect to see very divergent haplotypes (e.g., haplotypes from the two major north and south clades in figure 1*c*) within single populations. However, this is not observed in our phylogeny. Thus, the evidence suggests that it is unlikely that our phylogeny reflects introgression between two species with different call types.

(d) ***Coalescent analysis (continued)***

As with any simulation analysis, our coalescent analysis makes assumptions which we argue are reasonable for the current analysis. First, this method assumes that the mtDNA sequences used, 12S and 16S, evolve neutrally. We could not explicitly test this assumption using the McDonald-Kreitman (McDonald & Kreitman 1991) test because 12S and 16S are non-coding genes. Although it is possible that secondary structure constrains the evolution of some regions of 12S and 16S, this form of selection would not be expected to increase or decrease the rate of lineage sorting of haplotypes, and therefore should not affect our results. A second assumption is that the ancestral population was polymorphic for call types prior to population divergence. This assumption is also reasonable, because variation in call types is distributed fairly evenly across the phylogeny of *P. petersi* (Ron *et al.* 2006), instead of being restricted to a single node which would suggest a single gain or loss of complex calls. Moreover, even if the ancestral population was monomorphic for call type, the observed pattern of among-population variation would be even less likely under neutrality because call variation would have to evolve de novo. The third assumption is that discordance between gene trees and populations is primarily due to incomplete lineage sorting, rather than gene flow among populations. This assumption is likely valid too, as our microsatellite analysis and a previous allozyme analysis indicated very low levels of gene flow among populations of *P. petersi*, even over short geographic distances (e.g., tens of kilometers; Gascon *et al*. 1998). The last assumption is that the fixation of alleles at one or more nuclear genes underlies the fixation of call types. As nothing is known about the genetic basis of call variation in *P. petersi* or any other anuran, it is possible that this assumption does not hold (e.g., if call type is a polygenic trait with thresholds). However, the combination of

complete sorting of call types despite relatively recent population divergence (as indicated by substantial incomplete lineage sorting of mtDNA haplotypes) and strong female preferences for squawks at Yasuní makes a compelling argument that sexual selection is responsible for the observed pattern of call variation.

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Table 1. List of specimens sequenced for phylogenetic analysis. (All specimens are *Physalaemus petersi* unless otherwise noted. No. in tree refers to number in the phylogenetic tree in figure 1*c*. TRC=Tambopa Research Center (north TRC is on the north side of the Tambopata River; south TRC is on the south side of the Tambopata River); DCC=collection of D. C. Cannatella; KM=collected at Kent's Marsh, Gamboa, Panama; LW=collection of L. Weigt; MJR=collection of M. J. Ryan; MUSM=Universidad Nacional Mayor de San Marcos, Museo de Historia Natural; QCAZ=Museo de Zoología de la Pontificia Universidad Católica del Ecuador; USNM=Smithsonian National Museum of Natural History; ZUEC=State University of Campinas, Museum of Zoology, Brazil.)

Site	No. in tree	Voucher no.	GenBank accession no.
Cando, EC	1	DCC 3699	EF011516
Cando, EC	2	DCC 3701	EF011517
Cando, EC	3	DCC 3710	EF011518
Cando, EC	4	DCC 3711	EF011519
Cando, EC	5	DCC 3712	EF011520
Cando, EC	6	QCAZ 11965	DQ337231
Jatun Sacha, EC	1	QCAZ 24045	EF011521
Jatun Sacha, EC	2	MJR 004	EF011522
Jatun Sacha, EC	3	MJR 005	EF011523
Jatun Sacha, EC	4	MJR 006	EF011524
Jatun Sacha, EC	5	MJR 008	EF011525
Jatun Sacha, EC	6	MJR 001	EF011526
La Selva, EC	1	QCAZ 28578	EF011527
La Selva, EC	2	QCAZ 28577	EF011528
La Selva, EC	3	QCAZ 28576	EF011529
La Selva, EC	4	QCAZ 23975	EF011530

La Selva, EC	5	QCAZ 24029	EF011531
La Selva, EC	6	DCC 3705	EF011532
Tiputini, EC	1	QCAZ 28620	EF011533
Tiputini, EC	2	QCAZ 28611	EF011534
Tiputini, EC	3	QCAZ 28610	EF011535
Tiputini, EC	4	QCAZ 28608	EF011536
Tiputini, EC	5	QCAZ 28607	EF011537
Tiputini, EC	6	QCAZ 28612	EF011538
Yasuní, EC	1	QCAZ 11863	EF011539
Yasuní, EC	2	DCC 3685	EF011540
Yasuní, EC	3	DCC 3682	EF011541
Yasuní, EC	4	QCAZ 12128	DQ337233
Yasuní, EC	5	QCAZ 15138	EF011542
Yasuní, EC	6	QCAZ 15136	EF011543
Boca, BR	1	ZUEC 9511	DQ337229
Restauraçao, BR	1	ZUEC 9523	EF011544
Explorer’s Inn, PE	1	USNM 343264	EF011545
Explorer’s Inn, PE	2	USNM 343260	EF011546
North TRC, PE	1	MUSM 19368	EF011547
North TRC, PE	2	MUSM 19403	EF011548
North TRC, PE	3	MUSM 19404	EF011549
North TRC, PE	4	MUSM 19363	EF011550
South TRC, PE	1	MUSM 19348	EF011551

South TRC, PE	2	MUSM 19381	EF011552
South TRC, PE	3	MUSM 19380	EF011553
South TRC, PE	4	MUSM 19382	EF011554
P. pustulosus	(not shown in tree)	KM 91	DQ337239
P. pustulosus	(not shown in tree)	LW 1033	DQ337247
P. pustulatus	(not shown in tree)	QCAZ 23420	DQ337215
P. coloradorum	(not shown in tree)	QCAZ 19418	DQ337222

Table 2. Primer sequences of eight variable microsatellite loci developed in *Physalaemus petersi*. (Repeat motif and allele size (number of bp) refer to alleles of the sequenced clones. The number of observed alleles was determined from all 90 *P. petersi* individuals included in the current analysis.)

Locus	Repeat motif	Primer sequences (5´–3´)	Allele size	No. alleles	T_a (°C)	GenBank accession no.
Ppet1	$(TAGA)_9$	F-GAGGCACTTCATCTACACAGTC	254	5	57	DQ995212
		R-CCGCCACATACACTTGTC				
Ppet4	$(CA)_{12}$	F-ATCCAACCGTAAATCACAA	157	7	55	DQ995213
		R-GCAAGTCTCCTCACACTATTG				
Ppet7	$(TAGA)_{18}TACAA(TAGA)_{15}$	F-CCTTGGAGTCTTTGTCATTG	235	23	57	DQ995214
		R-CACCACTTTCGTTTTTGAAC				
Ppet11	$(TG)_{21}$	F-ACCATTAAAGAACATCCACCAC	128	12	56	DQ995215
		R-AAGAGCAGATCCTGCAAGAG				
Ppet114	$(TG)_{13}$	F-TTGGTCCTGTGATGTCAGTG	280	9	58	DQ995216
		R-GACTCCGATTGGTTTGTCTC				
Ppet118	$(TAGA)_6TTAGATAA(TAGA)_{10}$	F-GAACTGGGATGGATGATAGAC	196	27	57	DQ995217

		RGAGGCTGCATATAATGGAATT				
Ppet125	$(TCTA)_{27}TCAA(TCTA)_{28}$	F-CCTTGAAGTATTGATTGAGGAT	389	18	58	DQ995218
		R-TAGGCAATGAGCATAAGACAG				
Ppet131	$(TAGA)_{24}$	F-GGAACAACAAGTACACATCAAA	287	29	57	DQ995219
		R-TGGGTTACAATGAGCAGTG				

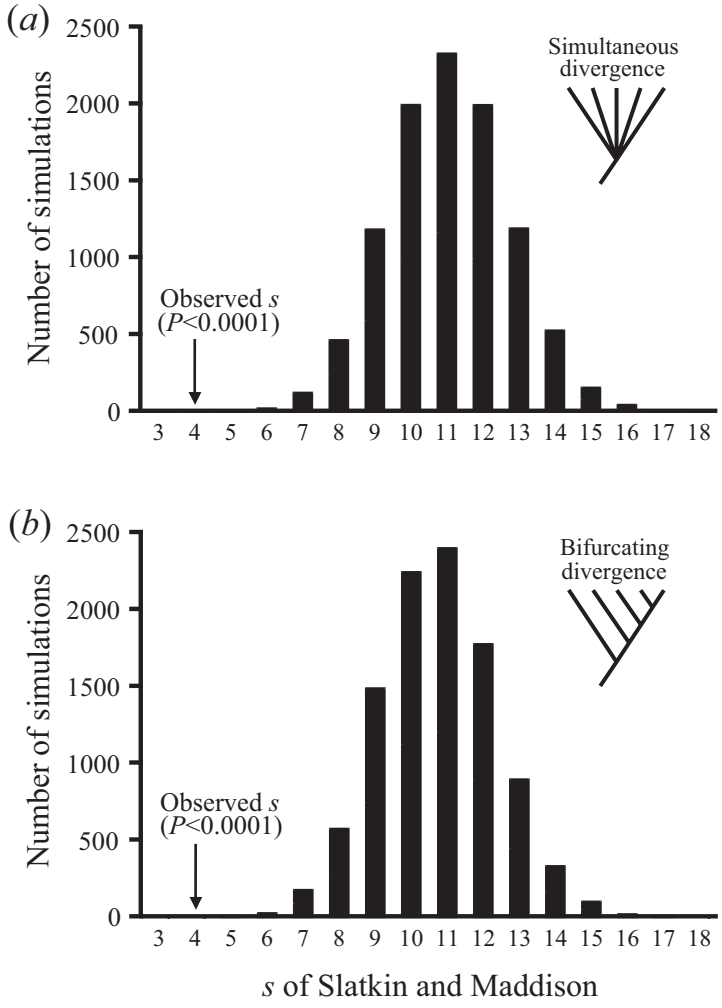

Figure 3. Results of coalescent simulations to test the probability of complete sorting (fixation) of call types (complex versus simple) among five Ecuadorian populations of *Physalaemus petersi* under the assumption of neutrality. *s* of Slatkin and Maddison is a measure of incomplete lineage sorting among populations, with larger values of *s* indicating greater discordance between the mtDNA gene tree and populations (indicating more recent population divergence) and smaller values of s indicating less discordance between the gene tree and populations (indicating older population divergence). Under neutrality, the probability of complete sorting of call types (equivalent to s=4), as observed, is $P<0.0001$, regardless of whether populations are assumed to have diverged simultaneously (*a*) or through a bifurcating branching process (*b*). This suggests that divergent selection, not genetic drift, has caused the observed pattern of fixation of call types.