

Genetic evidence of contemporary hybridization in one of North America's rarest anurans, the Florida bog frog

J. D. Austin¹, T. A. Gorman², D. Bishop^{2,*} & P. Moler³

¹ Department of Wildlife Ecology and Conservation, University of Florida, Gainesville, FL, USA

² Department of Fisheries and Wildlife Sciences, Virginia Tech, Blacksburg, VA, USA

³ Florida Fish and Wildlife Conservation Commission, Gainesville, FL, USA

Keywords

introgression; amphibian conservation; steephead; microsatellite.

Correspondence

James D. Austin, Department of Wildlife Ecology and Conservation, University of Florida, Gainesville, FL 32611, USA.

Email: austinj@ufl.edu

Editor: Trent Garner

Associate Editor: Robert Jehle

*Current address: US Fish and Wildlife Service, Back Bay National Wildlife Refuge, Virginia Beach, VA, USA.

Received 4 January 2011; accepted 20 March 2011

doi:10.1111/j.1469-1795.2011.00461.x

Introduction

Hybridization is among the demographic and genetic factors thought to pose risks to rare species (Allendorf *et al.*, 2001). General conservation scenarios include previously isolated species that have come into contact and hybridized due to range expansions in response to habitat modification or climate change (Keller *et al.*, 2008; Garroway *et al.*, 2010), or due to the breakdown of reproductive isolation correlated with environmental disturbance (Heath, Bettles & Roff, 2010). In such examples, when one species is very rare and the other is common, recurrent gene flow poses the risk of extinction for the former (Allendorf *et al.*, 2001). This can occur via genetic swamping, where the occurrence of hybrids eventually replaces the numerically less abundant species. Alternatively, if hybrid fitness is low relative to conspecific breeding, frequent hybridization can decrease the population growth rate of the numerically inferior species (Levin, Francisco-Ortega & Jansen, 1996). Theory also predicts that hybridization can lead to an influx of novel and beneficial genetic variation that may restore variation in rare, relatively inbred species (Arnold, 1997; Dowling &

Abstract

When a widespread species is sympatric with a rare, geographically restricted conspecific, recurrent gene flow can pose the risk of extinction for the latter. This can occur via genetic swamping, where the occurrence of hybrids eventually replaces the numerically less abundant species. We took a molecular genetic approach to quantify the occurrence and degree of contemporary hybridization between two species of frog co-occurring in a small geographic area in northwest Florida, USA. The Florida bog frog *Lithobates okaloosae* is a small ranid limited in distribution to a few acidic seepage and steephead streams that feed the Yellow, Shoal and East Bay river drainages in Walton, Okaloosa and Santa Rosa counties, Florida. The bronze frog *Lithobates clamitans* is a widespread (Eastern North America) congener. Data from nine microsatellite loci and 350 frogs were analyzed using Bayesian clustering (STRUCTURE) and a Bayesian hybrid classification method implemented in NEWHYBRIDS. Power to detect hybrids with the dataset was assessed through simulations. Both methods detected hybrids in similar proportions (5–10%), including congruent confirmation and rejection of samples previously classified as putative hybrids based on phenotypic characteristics. However, the nine loci lacked sufficient power to differentiate among F₁ and backcrossed individuals, leaving open the question of the extent of genetic introgression. Longitudinal genetic monitoring is recommended to evaluate whether the observed level of hybridization represents a long-term threat to the distinction of one of North America's most geographically restricted frogs.

Secor, 1997). Hybridization can also lead to greater divergence through reinforcement of reproductive barriers (e.g. hybrid inviability, Arnold, 1993).

Understanding the nature of ongoing hybridization is an important aspect of any long-term management plan. Here we take a molecular genetic approach to quantify the occurrence and degree of contemporary hybridization between two species of frog co-occurring in a small geographic area in northwest Florida, USA. The Florida bog frog *Lithobates okaloosae* is a small ranid limited in distribution to a few acidic seepage and steephead streams that feed the Yellow, Shoal and East Bay river drainages in Walton, Okaloosa and Santa Rosa counties, Florida (Fig. 1). The Florida bog frog is currently considered a Species of Special Concern in Florida and is classified as Vulnerable by the IUCN. This listing is due to the limited range (<20 km²) and the fact that it is uncommon throughout its range. Approximately 90% of the entire distribution is found within the boundary of Eglin Air Force Base (Eglin AFB) (Gorman, 2009). With rapid development of lands surrounding the AFB, Eglin plays an important role in maintaining habitat for this and numerous other species of

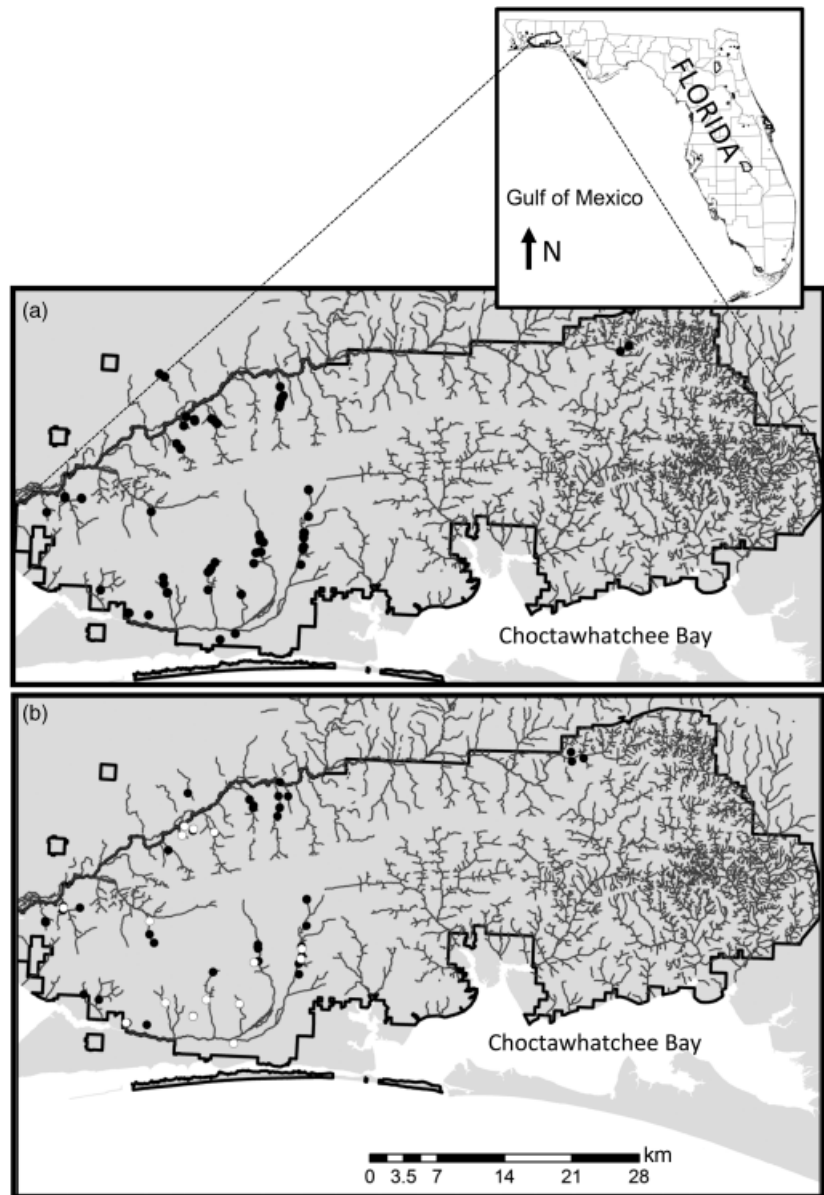


Figure 1 Distribution of samples used to examine the frequency of hybridization. Outlines represent military lands in Florida. (a) Distribution of Florida bog frog *Lithobates okaloosae* samples. Distribution reflects the known range of the Florida bog frog, including the isolated northeast portion of the range. (b) Bronze frog *Lithobates clamitans* in black and putative hybrids based on molecular results in white.

conservation concern (Sutter *et al.*, 2001). The bronze frog *Lithobates clamitans* is a widespread (eastern North America), closely related species (Austin *et al.*, 2003) and it is syntopic with the Florida bog frog. These frogs can be differentiated phenotypically by size [bronze frog 54–75 mm SVL (Conant & Collins, 1998); Florida bog frog 34–49 mm SVL (Moler, 1993)], the extent of toe webbing (highly reduced in Florida bog frogs), presence of paired vocal sacs in the Florida bog frog, and by their distinct calls (Moler, 1993).

Increasing attention has focused on the possibility of hybridization between the Florida bog frog and the bronze frog based on the observation of putative phenotypic intermediates (Moler, 1993; Bishop, 2005). Mitochondrial DNA (mtDNA) phylogeography has demonstrated that

these frogs share mtDNA polymorphisms (Austin & Zamudio, 2008). However, the interpretation of hybridization based on mtDNA alone is potentially misleading as the pattern of shared polymorphism could also be the result of incomplete lineage sorting or ancient introgression. However, widespread mtDNA replacement under persistent, low-frequency hybridization (e.g. Babik, Szymura & Rafinski, 2003; Babik *et al.*, 2005) is plausible in this system given the presumed small population size of Florida bog frogs.

Of conservation interest is whether ongoing hybridization is occurring and, if so, whether it is introgressive. Despite the lack of unique mtDNA in Florida bog frogs, both bog frogs and bronze frogs continue to exhibit distinct morphological differences. Using rapidly evolving, bi-parentally inherited

microsatellites, we assess the level of introgression and hybridization between these two species. We also ask whether the reported intermediate phenotypes can be genetically distinguished as hybrids, and whether the rate of hybridization (if detected) is high enough to pose a long-term threat to Florida bog frogs.

Methods

Sampling and marker selection

We obtained 206 Florida bog frogs, 131 bronze frog, and 13 additional samples that were characterized as 'hybrids' or 'unknown', reflecting the difficulty in making out diagnostic characteristics or based on uncharacteristic calls. Samples were collected opportunistically from much of the Florida bog frog's range during the summer months of 2003–2007 (Fig. 1). Frogs were hand-caught and had one partial digit or larval tail clip removed using disinfected microscissors. Tissue samples were preserved in 95% ethanol or tissue buffer, and frogs were released at the point of capture. Although samples were collected opportunistically, efforts were made to search creeks that had few or no samples but where Florida bog frogs were expected to occur. Samples represent much of the geographic distribution of the Florida bog frog and reflect the patchy distribution of breeding aggregations and individuals.

Total genomic DNA was extracted using DNeasy Tissue Extraction Kits (Qiagen, Valencia, CA, USA). We used nine nuclear microsatellite loci, six loci developed for Florida bog frog and bronze frog (*Roka194*, *Roka195*, *Roka205*, *Roka249*, *Roka253*, *Roka299*; Austin, Gorman & Bishop, 2011) and three (*J8*, *J21* and *J54*) previously published bullfrog *Rana catesbeiana* markers (Austin *et al.*, 2003). We generated fluorescently labeled PCR products using FAM, HEX, or TAMRA dyes attached to the 5' end of an M13 (5'-CACGACGTTGTAACGAC-3') sequence tag. Amplifications were performed in 25 μ L reactions containing 20 ng genomic DNA, 1.0 μ L 5 \times buffer (GoTaq[®] Flexi, Promega, Madison, WI, USA), 2.5 mM MgCl₂, 0.25 mM of dNTP, 0.02 μ M of each forward M13 primer, 0.02 μ M of each reverse primer, 0.4 μ M of fluorescently labeled, 1.0 U of Taq DNA polymerase (GoTaq[®] Promega). Thermal cycling parameters for all amplifications were: 95 °C for 5 min, then nine cycles each of 95 °C for 30 s, 90 s at locus appropriate annealing temperature, and 72 °C for 30 s, then followed by 29 cycles each of 95 °C for 30 s, 48 °C for 1 min 30 s and 72 °C for 30 s, followed by one final elongation step for 72 °C for 30 min. Amplified products were run on an ABI 3730 with a ROX 500 size standard (ABI, Carlsbad, CA, USA). Allele scoring was performed using GENEMARKER[®] software (SoftGenetics, State College, PA, USA) and all alleles were manually confirmed. MICRO-CHECKER version 2.2 (van Oosterhout *et al.*, 2004) was used to address possible issues associated with null alleles and scoring error.

We used GENEPOP version 4 (Rousset, 2008) to test for linkage disequilibrium using Fisher's global test among each pair of loci. Markov chain parameters for all comparisons

used 10 000 dememorization steps, 500 batches, and 5000 iterations per batch. Deviations from Hardy–Weinberg equilibrium (HWE) were examined previously at the one large population sampled (Main Site, Austin *et al.*, 2011). We further tested the range-wide samples for deviations from HWE even though we expected deviations due to the admixture of multiple genetic demes in the dataset. HWE was tested with the exact tests available in GENEPOP, using a Markov chain algorithm with 10 000 dememorization steps, 200 batches, and 10 000 iterations. FSTAT version 2.9 (Goudet, 1995) was used to quantify allelic diversity.

Quantifying hybridization

Evidence for recent hybridization between Florida bog frog and sympatric bronze frog was examined using a Bayesian clustering algorithm implemented in STRUCTURE version 2.3 (Pritchard, Stephens & Donnelly, 2000). The admixture model implemented in STRUCTURE allows for portions of individual genomes (q) to be probabilistically assigned to specific populations and reflects recent or current gene flow at rates that are sufficient to reflect ancestry from more than one population. We applied the correlated model that assumes populations diverged from a common ancestor and that some of the differences in allele frequencies are due to genetic drift as well as possible gene flow. We examined the fit of $K=1$ and 2 (where K refers to the number of assumed species) to confirm that STRUCTURE was differentiating between the two species. Additional runs ($K=3-5$) were examined to determine additional substructure with the dataset. We determined the appropriate K by examining the pattern of likelihood and associated variance among replicates (high variance indicates weaker convergence) and using ΔK which identifies the best K by finding a breakpoint in the slope of the distribution of likelihood scores. (Evanno, Regnaut & Goudet, 2005). Analyses were run without prior specification of species. We used 100 000 burn-in generations followed by one million generations to estimate posterior distributions. For each value of K we ran 10 independent replicates (using different starting seeds) and consensus analyses were performed using the full search option in CLUMPP version 1.1 (Jakobsson & Rosenberg, 2007) to avoid issues associated with stochastic differences among replicate runs (Jasra, Holmes & Stephens, 2005). CLUMPP output was visualized using DISTRUCT version 1.1 (Rosenberg, 2004). Following the suggestions of Vähä & Primmer (2006) and the results from simulations, individual genetic assignment to clusters was based on a minimum posterior probability threshold (T_q) of 0.90. Individuals displaying $0.1 \leq q_i \leq 0.90$ were considered of admixed ancestry. Subsequent estimates of differentiation (F_{ST}) and allelic diversity between pure Florida bog frog and pure bronze frog populations ($T_q > 0.9$) were calculated in GENEPOP version 4 (Rousset, 2008).

Our second approach used Anderson & Thompson's (2002) method of hybrid identification that is more specifically aimed at detecting hybrids between species. In

this model, each individual's genotype frequency class or hybrid category is inferred, thus providing a posterior probability to reflect the level of certainty that an individual belongs to a certain hybrid category (e.g. F_1 , backcross, purebred). Rather than treating the parameter of interest (q) as a random continuous variable as is done in STRUCTURE, q is a discrete variable with up to six genotype frequency classes (i.e. purebreds, F_1 , F_2 , backcrosses). The Bayesian method implemented by NEWHYBRIDS version 1.1 (Anderson & Thompson, 2002) was used to assign individuals into pure *Rana okaloosae*, pure bronze frog, and hybrids (F_1 , F_2 and backcrosses with Florida bog frog or bronze frog). The approach of Jeffrey's priors was used (following preliminary runs that found congruent results with uniform priors), and results were based on the average of five independent runs each with 10^6 iterations following 10^5 burn-in steps.

The performance of NEWHYBRIDS to detect purebred and hybrid individuals with the nine-loci microsatellite dataset was assessed using simulations. From the initial NEWHYBRIDS analysis, pure Florida bog frog and bronze frog genotypes were selected based on a $q_i > 0.90$. From these genotypes we generated 1000 new genotypes of each parental population by randomly drawing alleles from the allele frequency distribution of each 'pure' sample. These new parental genotypes were then used to simulate F_1 , F_2 and backcrossed populations. All simulations were generated using the program HYBRIDLAB version 1.0 (Nielson, Bach & Kotlicki, 2006). These simulated genotypes were subsequently analyzed in NEWHYBRIDS. Power (number of correctly identified individuals for a category over the actual number of individuals of that category) and accuracy (number of correctly identified individuals for a category over the total number of individuals assigned to any category) were calculated for five T_q values (0.5, 0.6, 0.7, 0.8 and 0.9). Analysis was based on the mean of five replicates of simulated datasets.

We also inferred the level of introgression via estimates of long-term gene flow (N_m) using a maximum likelihood (ML) approach implemented in MIGRATE 3.0 (Beerli & Felsenstein, 1999, 2001). MIGRATE simultaneously estimates the historical average effective population size parameter (θ) and rates of gene migration (M) using a coalescent approach. M quantifies the number of new variants introduced into the population by immigration relative to mutation. MIGRATE was run a minimum of four times; the first run used F_{ST} -based estimates of θ and M as the start point. Subsequent runs used the results of the previous run as start values. The program was run until θ and M estimates were consistent between runs, either reaching an asymptote or having broadly overlapping 95% confidence intervals (CI). The final migration rate (M) was converted into the effective number of migrants per generation (N_m) by multiplying M by θ and then dividing by 4. Ten short chains were run with 10 000 genealogies and three long chains with 400 000 genealogies; the burn-in was set to 10 000 and we used a five chain-heating scheme with the following temperatures 1.0, 7.6, 20.8, 47.2 and 100.0.

Results

Microsatellite variation

After sequential Bonferroni correction for multiple tests, linkage disequilibrium was detected for two of 93 independent tests, involving loci *Roka249* and *Roka194* in *R. okaloosae*, and *Roka195* and *J54* in bronze frog (both $P < 0.0005$). Because these significant comparisons involved different sets of loci, and because the same loci comparisons were not significant in the other species, we treat these loci as functionally unlinked. Tests for HWE found significant heterozygote deficiency at seven of nine loci for Florida bog frog and eight of nine for bronze frog (Table 1). These results are not unexpected, due to the sampling from multiple creeks, and may reflect the large amount of substructure (Nei, 1977) in the populations of these frogs. MICRO-CHECKER detected possible null alleles in bronze frogs at loci *Roka249*, *Roka205*, *Roka253*, *Roka194* and *Roka195*. Previous reamplification of these loci indicate that genotyping error is low ($< 1\%$) (Austin *et al.*, 2011). Null alleles were not detected in Florida bog frogs.

Variation at the nine loci varied considerably between bog and bronze frog. Average allelic richness was higher in bronze frog ($A_R = 22$) than in bog frog ($A_R = 12$). Differentiation between bog frog and bronze frog was high and significantly greater than zero ($F_{ST} = 0.223$, 95% CI = 0.127–0.347).

Initial STRUCTURE results comparing $K = 1$ and $K = 2$ models support a separation between Florida bog and bronze frog species. The likelihood of the $K = 1$ model ($\text{Ln} = -12\,272 \pm 0.295 \text{ SD}$) relative to $K = 2$ ($\text{Ln} = -10\,293 \pm 0.391$), and the clustering of genotypes into the appropriate species (Fig. 2) supports the $K = 2$ model. A number of genotypes appeared to contain mixed ancestry, including a number of the putative hybrid individuals identified in the field. Additional STRUCTURE runs at $K = 3$ through $K = 5$ did not provide strong support for more than two genetic clusters. The likelihood after $K = 2$ levels out, with a concomitant increase in the standard deviation among replicates at each K (see supporting information). The ΔK analysis strongly supported $K = 2$ as being a better fit than $K = 3$ or 4. Because ΔK detects the highest level of population structure when hierarchical structure exists (Evanno *et al.*, 2005) we subsequently examined the Florida bog frog using the same model parameters to determine whether additional substructure exists. The result was good support for at least two, possibly three additional clusters range-wide (data not shown).

The Bayesian clustering analyses performed by NEWHYBRIDS on simulated genotypes are shown in Table 2. Maximum accuracy was achieved for most T_q (except $T_q = 0.5$) for *R. okaloosae*, but there were variations in power, meaning nearly all assigned genotypes were correctly assigned to *R. okaloosae*. However, not all simulated Florida bog frog genotypes were assigned. Accuracy remained at or near 0.9 across all classes examined for more conservative T_q values (i.e. ≥ 0.8), though power tended to be low for F_2 and

Table 1 Summary of variation at nine microsatellite loci in Florida bog frog *Lithobates okaloosae* and bronze frog *Lithobates clamitans* samples

Locus	<i>N</i>	<i>N_a</i>	<i>A_R</i>	<i>H_o</i>	<i>H_e</i>
<i>Lithobates okaloosae</i>					
Roka299	208	7	5.4	0.038*	0.093
Roka249	206	18	15.8	0.548*	0.784
Roka205	208	21	19.5	0.740*	0.874
Roka253	206	10	7.2	0.437*	0.509
J8	207	8	6.9	0.534	0.451
J54	208	9	8.7	0.942	0.595
J21	208	4	3.8	0.986	0.513
Roka195	204	32	28.6	0.721*	0.872
Roka194	206	16	14.4	0.519*	0.832
mean	206.8	13.9	12.3	0.607	0.614
<i>Lithobates clamitans</i>					
Roka299	131	14	13.9	0.366*	0.717
Roka249	131	38	37.9	0.740*	0.905
Roka205	129	39	39.0	0.396*	0.952
Roka253	130	21	21.0	0.740*	0.913
J8	131	13	13.0	0.595*	0.804
J54	131	14	14.0	0.664*	0.769
J21	131	5	5.0	0.679	0.532
Roka195	129	32	32.0	0.702*	0.894
Roka194	129	24	24.0	0.541*	0.906
mean	130.2	22.2	22.2	0.603	0.826

Sample size (*N*), number of alleles (*N_a*) and allelic richness (*A_R*) standardized to a common sample size of 129, observed (*H_o*, *Significant at $\alpha=0.05$) and expected (*H_e*) heterozygosities.

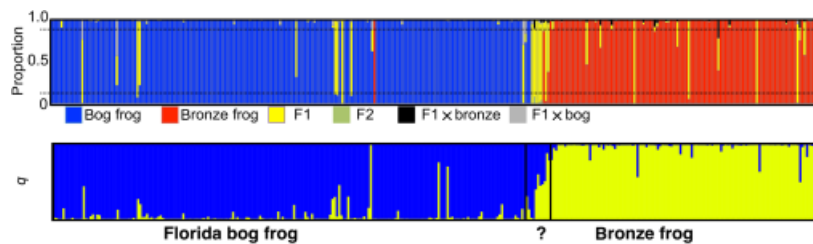


Figure 2 (a) Bayesian assignment of Florida bog frog *Lithobates okaloosae* and bronze frog *Lithobates clamitans* into pure and hybrid classes using NEWHYBRIDS. Each individual (column) is partitioned into probability of assignment to each of six possible classes: pure bog frog, pure bronze frog, F₁ hybrid, F₂ hybrid and F₁ backcrossed with both parental species. Dashed lines highlight probability thresholds used to assign individuals to classes. (b) STRUCTURE results depicting the admixture coefficient (*q*) averaged across 10 independent runs (*K*=2). Each vertical column represents one individual genotype. Question mark indicates frogs that were thought to be hybrids based on phenotype.

backcrosses. When all hybrid classes were considered together the simulations showed high accuracy and power in distinguishing hybrids from pure classes. This is not unexpected given the difficulty in detecting F₂ and backcrossed genotypes with a small number of loci. As a result we summed the posterior probabilities of hybrid classes to use as an estimate for the detection of hybrids but without definition of their admixture ancestry. We used a threshold of 0.9 to assign individuals as pure or hybrid. At this threshold, 19 individuals (~5%) were categorized as hybrids, including seven of the 11 putative hybrids, five putative 'bronze', and six putative 'bog' frogs (Fig. 2). If non-assigned genotypes (at $T_q > 0.9$) are included as hybrid ancestry, the per cent of hybrids rises to 10%. These

results were very similar to that from our STRUCTURE analysis (Table 3).

ML estimates of long-term gene migration estimated using MIGRATE revealed a pattern of asymmetrical migration between Florida bog and bronze frogs. Florida bog frog allelic migration (*M*) into bronze frog genomes was estimated at 0.10 (95% CI = 0.09–0.12), whereas bronze frog migration into bog frogs was greater at 0.16 (95% CI = 0.13–0.19). MIGRATE detected highly divergent mutation-scaled population sizes (bog frog $\theta = 8.91$, 95% CI = 8.62–9.21; bronze frog $\theta = 28.05$, 95% CI = 26.88–29.29), reflecting known species distributions and abundance. Our MIGRATE estimates translate into average long term gene flow estimates that reflect limited gene flow from Florida bog frogs into bronze

Table 2 Power and accuracy of NEWHYBRIDS to detect purebred and hybrid simulated individuals across five threshold (T_q) values

Class	$T_q=0.9$		$T_q=0.8$		$T_q=0.7$		$T_q=0.6$		$T_q=0.5$	
	Power	Accuracy	Power	Accuracy	Power	Accuracy	Power	Accuracy	Power	Accuracy
Bog	0.77	1.00 (0.99)	0.85	1.00 (0.99)	0.90	1.00 (0.99)	0.91	1.00 (0.98)	0.95	0.96 (0.95)
Bronze	0.80	0.96 (0.94)	0.83	0.93 (0.93)	0.85	0.92 (0.91)	0.88	0.91 (0.90)	0.89	0.90 (0.89)
F ₁	0.71	1.00	0.80	0.98	0.83	0.96	0.86	0.95	0.90	0.95
F ₂	0.43	0.95	0.48	0.86	0.55	0.80	0.57	0.74	0.64	0.71
Bog × F1	0.35	0.98	0.66	0.94	0.77	0.92	0.82	0.92	0.87	0.89
Bronze × F1	0.43	0.92	0.69	0.91	0.81	0.90	0.85	0.88	0.87	0.88
Hybrid	0.96	>0.99	0.97	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99

Accuracy is calculated for individual classes against the combined hybrid class (in parentheses). For example, all $T_q \geq 0.9$ for bog frog consisted of correct assignments, until hybrid classes were summed, which led to one incorrect assignment (0.99).

Florida bog frog *Lithobates okaloosae*, and bronze frog *Lithobates clamitans* 'pure' classes and F₁ and F₂ and backcross classes consisted of 100 genotypes. The 'hybrid' category represents the sum of the assignment probabilities over the four hybrid categories. Power was estimated as the number of correctly identified individuals for a class and threshold over the actual number of individuals of that class in the sample. Accuracy: number of correctly identified individuals for a class over the total number of individuals assigned to that class at a specified T_q . Values in parentheses reflect accuracy measured against hybrid class.

Table 3 Frequencies and proportions of pure and admixed Florida bog frog *Lithobates okaloosae* and bronze frog *Lithobates clamitans* individuals inferred by Bayesian clustering (STRUCTURE) and assignment (NEWHYBRIDS) methods

	Bog frog	Admixed	Bronze frog
NEWHYBRIDS	196 (57.8%)	35 (10.3%) ^a	108 (31.9%)
STRUCTURE	195 (57.5%)	33 (9.7%)	111 (32.7%)

We considered strict assignments to parental species only ($T_q \geq 0.9$).

^a11 of 35 individuals at the $T_q=0.5$ threshold and 14 of 35 at the $T_q=0.7$ threshold were not assigned to a specific class and were, therefore, considered of undetermined hybrid origin.

frogs ($N_m = 0.356$, 95% CI = 0.28–0.43), and from bronze to Florida bog frogs ($N_m = 0.701$, 95% CI = 0.60–0.88).

Discussion

Hybridization among Florida bog and bronze frogs has been assumed based on phenotypic 'intermediates' (Moler, 1992, 1993; Bishop, 2005), and has been inferred (Gorman, Bishop & Haas, 2009) based on the pattern of shared mtDNA haplotypes (Austin *et al.*, 2003). There are various explanations for shared variation at mtDNA including ancestral introgression from bronze frogs, or incomplete lineage sorting (Austin *et al.*, 2003). As with mtDNA a pattern of microsatellite allele sharing could be the product of retained ancestral polymorphism. However two arguments suggest that recent hybridization accounts for most introgressed alleles in these frogs. First is that our analyses of our microsatellite data using two different Bayesian approaches (cluster vs. hybrid classification analysis) has provided robust support for the hybridization claim, as a proportion (5–10%) were inferred with high power to represent mixed Florida bog–bronze frog genotypes, with decreasing power to detect backcrossed or F₂ hybrids. Thus, it seems unlikely that F₁ hybrids would be inferred

from alleles representing ancestral polymorphism. Second, most of the detected hybrids include animals that were identified *a priori* as putative hybrids. If ancestral polymorphism were causing the pattern of shared alleles then one would expect a random assignment of F₁ hybrids, and not primarily phenotypic outliers. Our analyses did detect at least one misidentified individual (a putative Florida bog frog i.e. really a bronze frog). This specimen was a metamorph, a stage that is likely to be the most difficult to distinguish among Florida bog and bronze frogs based on gross morphology. Otherwise, these frogs are phenotypically distinct in many ways (e.g. relative size as adults, extent of webbing, call structure).

As a basic rule, N_m of <1 per generation is on average insufficient to prevent differentiation among populations at neutral loci (Franklin, 1980; Frankel & Soulé, 1981). Gene flow has been greater from bronze frogs into Florida bog frogs as might be predicted based on the relative population sizes of the two species. However, N_m has been less than one-migrant-per-generation, contradicting the seemingly common pattern of contemporary hybridization detected here. Though the amount of hybridization detected here is not high relative to some other amphibian species (Cousineau & Rogers, 1991), it is at a level that could produce a higher level of historic gene flow. That inference assumes that the current level of hybridization has been consistent through time and that F₁ hybrids are able to produce viable offspring. Alternatively, the level of hybridization may be variable through time and current levels not typical of the recent past. Another explanation for the contrast between historical N_m estimates and F₁ hybridization is Haldane's rule (Haldane, 1922). Almost all frogs samples here were males (due to conspicuous calls). Males are the heterogametic sex (Elinson, 1981) and therefore may have highly reduced fitness relative to hybrid females, similar to that seen in other frogs (Lemmon & Lemmon, 2010). This would also help to explain the pattern of shared mitochondrial haplotype variation between these two species, where hybrid

female offspring carrying bronze frog mtDNA could sweep through the small population.

Crossing experiments among closely related ranids have demonstrated that pre- and post-zygotic barriers tend to be complete (Elinson, 1974, 1977, 1981). However, in some studies (e.g. Berger, 1967; Moore, 1950) artificial interspecific hybridization demonstrated that conspecific frogs from similar habitats can hybridize. The level of hybridization observed here may be a typical pattern that, all things equal, does not pose any obvious threat to the genetic integrity of Florida bog frogs. Studies on the genomics of hybridization demonstrate that species can retain their distinction despite frequent introgression of neutral markers and adaptive loci (Lexer *et al.*, 2010). So, although our microsatellite data reveal hybridization, the demographic threat to Florida bog frogs remains ambiguous without further effort to study the ecological mechanisms that might be driving hybridization, and the extent to which hybrids are backcrossing with parental species.

We recommend genetic monitoring of selected syntopic populations of Florida bog and bronze frogs to determine the frequency and environmental correlates of hybridization. Introgressive hybridization between a rare species and an abundant congener could drive population extinction via genetic assimilation, assuming F_1 individuals are fertile, which can occur within a few generations (Ellstrand, Prentice & Hancock, 1999). Future research could determine the viability of F_1 crosses between these two species. Even with significant levels of hybridization, divergent selection for ecologically important and distinguishing traits can prevail over the homogenizing effects of gene flow (Nosil & Yukilevich, 2008), as has been argued to have occurred for a number of species (e.g. Danley *et al.*, 2000; Senar, Domelech & Camerino, 2005). Therefore, continued homogenization of neutral genetic variation (e.g. microsatellite loci) may have little short-term impact on Florida bog frog distinctiveness. However little is known about the adaptive differences between these two frogs. Hence, it would be useful to study the rate and pattern of hybridization and introgression in stream populations that are adjacent to highly disturbed and relatively pristine locations to test whether there is evidence of anthropogenic factors in driving inter-species dynamics. Experimental crosses would also be a cost effective (though long-term) means of testing hybrid viability and the potential for introgression.

Declines in abundance alone can cause increased rates of hybridization among species due to decreased densities of individuals of one species (Allee, 1949). Habitat modification on parts of Eglin AFB over the past 70 years could directly or indirectly cause increased rates of hybridization among these species (e.g. Gottelli *et al.*, 1994; Gutierrez *et al.*, 2007). Our dataset did not have sufficient power to accurately distinguish among F_1 , F_2 , or backcrossed individuals, an important step in elucidating the importance of contemporary hybridization in causing genetic introgression and genetic swamping. In general, the ability to distinguish F_1 from other hybrid classes requires large numbers (~20) of non-diagnostic markers (Anderson & Thompson, 2002).

Distinguishing between F_1 and other hybrid classes should provide greater insight into the importance of hybridization on Florida bog frog persistence (Levin, 2004). A better picture of whether hybridization is correlated with population densities would be possible by systematically sampling replicate creeks with relatively high and low densities of Florida bog frogs. With the increasing human demographic pressures related to the recent and ongoing population growth associated with the mission expansion of Eglin AFB, it is recommended that detailed and systematic demographic and genetic monitoring be established in selected creeks across the Air Force Base.

Acknowledgments

We thank C. Haas for logistical support and S. Eddy, K. Jones, B. Rincon and S. Ritchie for field assistance. Funding came from a Nongame Research Grant [Florida Fish and Wildlife Conservation Commission (FFWCC) NG07-007 to J.D.A.], and the Natural Resources Branch (Jackson Guard) of Eglin Air Force Base, and the Department of Fisheries and Wildlife Sciences, Virginia Tech (for T.A.G.). This research was conducted under FFWCC Permits WV01232, WV04037 and WX08005A and Virginia Tech Institutional Animal Care and Use Committee Protocols 02-084-F&W, 04-012-F&W and 07-033-FIW.

References

- Allee, W.C. (1949). *Principles of animal ecology*. Philadelphia: WB Saunders Co.
- Allendorf, F.W., Leary, R.F., Spruell, P. & Wenburg, J.K. (2001). The problems with hybrids: setting conservation guidelines. *Trends Ecol. Evol.* **16**, 613–622.
- Anderson, E.C. & Thompson, E.A. (2002). A model-based method for identifying species hybrids using multilocus genetic data. *Genetics* **160**, 1217–1229.
- Arnold, J. (1993). Cytonuclear disequilibria in hybrid zones. *Ann. Ecol. Syst.* **24**, 521–553.
- Arnold, M.L. (1997). *Natural hybridization and evolution*. New York: Oxford University Press.
- Austin, J.D., Gorman, T.A. & Bishop, D. (2011). Assessing fine-scale genetic structure and relatedness in the micro-endemic Florida bog frog. *Conserv. Gen.* Online DOI: 10.1007/s10592-010-0176-7.
- Austin, J.D., Loughheed, S.C., Moler, P. & Boag, P.T. (2003). Phylogenetics, zoogeography, and the role of vicariance and dispersal in the evolution of the *Rana catesbeiana* (Anura: Ranidae) species group. *Biol. J. Linn. Soc.* **80**, 601–624.
- Austin, J.D. & Zamudio, K.R. (2008). Incongruence in the pattern and timing of intra-specific diversification in bronze frogs and bullfrogs (Ranidae). *Mol. Phylog. Evol.* **48**, 1041–1053.
- Babik, W., Branicki, W., Crnobrnja-Isailovic, J., Cogălniceanu, D., Sas, I., Olgun, K., Poyarkov, N.A., Garcia-París,

- M. & Arntzen, J.W. (2005). Phylogeography of two European newt species – discordance between mtNDA and morphology. *Mol. Ecol.* **14**, 2475–2491.
- Babik, W., Szymura, J.M. & Rafinski, J. (2003). Nuclear markers, mitochondrial DNA and male secondary sexual traits variation in a newt hybrid zone (*Triturus vulgaris* × *T. montandoni*). *Mol. Ecol.* **12**, 1913–1930.
- Beerli, P. & Felsenstein, J. (1999). Maximum-likelihood estimation of migration rates and effective population numbers in two populations using a coalescent approach. *Genetics* **152**, 763–773.
- Beerli, P. & Felsenstein, J. (2001). Maximum likelihood estimation of a migration matrix and effective population sizes in subpopulations by using a coalescent approach. *Proc. Natl. Acad. Sci. USA* **98**, 4563–4568.
- Berger, L. (1967). Embryonal and larval development of F₁ generation of green frogs different combinations. *Acta Zool. Cracov.* **12**, 123–160.
- Bishop, D.C. (2005). *Ecology and distribution of the Florida bog frog and flatwoods salamander on Eglin Air Force Base*. PhD Dissertation, Virginia Polytechnic Institute and State University, Blacksburg.
- Conant, R. & Collins, J.T. (1998). *Reptiles and amphibians: Eastern/Central North America*. Boston: Houghton Mifflin Co.
- Cousineau, M. & Rogers, K. (1991). Observations on sympatric *Rana pipiens*, *R. blairi*, and their hybrids in Eastern Colorado. *J. Herpetol.* **25**, 114–116.
- Danley, P.D., Markert, J.A., Arnegard, M.E. & Kocher, T.D. (2000). Divergence with gene flow in the rock-dwelling cichlids of Lake Malawi. *Evolution* **54**, 1725–1737.
- Dowling, T.E. & Secor, C.L. (1997). The role of hybridization and introgression in the diversification of animals. *Ann. Rev. Ecol. Syst.* **28**, 593–619.
- Elinson, R.P. (1974). A block to cross-fertilization located in the egg jelly of the frog *Rana clamitans*. *J. Embryol. Exp. Morph.* **32**, 325–335.
- Elinson, R.P. (1977). Amphibian hybrids: a genetic approach to the analysis of their developmental arrest. *Differentiation* **9**, 3–9.
- Elinson, R.P. (1981). Genetic analysis of developmental arrest in an amphibian hybrid *Rana catesbeiana*, *Rana clamitans*. *Dev. Biol.* **81**, 187–196.
- Ellstrand, N.C., Prentice, H.C. & Hancock, F.C. (1999). Gene flow and introgression from domesticated plants into their wild relatives. *Ann. Rev. Ecol. Syst.* **30**, 539–563.
- Evanno, G., Regnaut, S. & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol. Ecol.* **14**, 2611–2620.
- Frankel, E.O. & Soulé, M.E. (1981). *Conservation and evolution*. Cambridge: Cambridge University Press.
- Franklin, I.R. (1980). Evolutionary change in small populations. In *Conservation biology: an evolutionary ecological perspective*: 135–150. Soulé, M.E. & Wilcox, B.A. (Eds). Sunderland: Sinauer Associates.
- Garraway, C.J., Bowman, J., Cascaden, T.J., Holloway, G.L., Mahan, C.G., Malcolm, J.R., Steele, M.A., Turner, G. & Wilson, J.J. (2010). Climate change induced hybridization in flying squirrels. *Glob. Change Biol.* **16**, 113–121.
- Gorman, T.A. (2009). *Ecology of two rare amphibians of the Gulf Coastal Plain*. PhD Dissertation, Virginia Polytechnic Institute and State University, Blacksburg.
- Gorman, T.A., Bishop, D.C. & Haas, C.A. (2009). Spatial interactions between two species of frogs: *Rana okaloosae* and *R. clamitans clamitans*. *Copeia* **2009**, 138–141.
- Gottelli, D., Sillero-Zubiri, C., Applebaum, G.D., Roy, M.S., Girman, D.J., Garcia-Moreno, J., Ostrander, E.A. & Wayne, R.K. (1994). Molecular genetics of the most endangered canid: the Ethiopian wolf *Canis simensis*. *Mol. Ecol.* **3**, 301–312.
- Goudet, J. (1995). FSTAT (version 1.2): a computer program to calculate F-statistics. *J. Hered.* **86**, 485–486.
- Gutierrez, R.J., Cody, M., Courtney, S. & Franklin, A.B. (2007). The invasion of Barred Owls and its potential effects on the spotted owl: a conservation conundrum. *Biol. Invasions* **2007**, 181–196.
- Haldane, J.B.S. (1922). Sex ratio and unisexual sterility in hybrid animals. *J. Genet.* **12**, 101–109.
- Heath, D., Bettles, C.M. & Roff, D. (2010). Environmental factors associated with reproductive barrier breakdown in sympatric trout populations on Vancouver Island. *Evol. Appl.* **3**, 77–90.
- Jakobsson, M. & Rosenberg, N.A. (2007). CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* **23**, 1801–1806.
- Jasra, A., Holmes, C.C. & Stephens, D.A. (2005). Markov chain Monte Carlo methods and the label switching problem in Bayesian mixture modeling. *Stat. Sci.* **20**, 50–67.
- Keller, B., Wolinska, J., Manca, M. & Spaak, P. (2008). Spatial, environmental and anthropogenic effects on the taxon composition of hybridizing *Daphnia*. *Phil. Trans. Roy. Soc. Lond. B: Biol. Sci.* **363**, 2943–2952.
- Lemmon, E.M. & Lemmon, A.R. (2010). Reinforcement in chorus frogs: lifetime fitness estimates including intrinsic natural selection and sexual selection against hybrids. *Evolution* **64**, 1748–1761.
- Levin, D.A. (2004). The congener as an agent of extermination and rescue of rare species. In *Evolutionary conservation biology*: 344–355. Ferrière, R., Dieckmann, U. & Couvet, D. (Eds). Cambridge: Cambridge University Press, Cambridge studies in adaptive dynamics.
- Levin, D.A., Francisco-Ortega, J. & Jansen, R.K. (1996). Hybridization and the extinction of rare plant species. *Conserv. Biol.* **10**, 10–16.
- Lexer, C., Joseph, J.A., Van Loo, M., Barbara, T., Heinze, B., Bartha, D., Castiglione, S., Fay, M.F. & Buerkle, C.A. (2010). Genomic admixture analysis in *Populus* spp. reveals

- unexpected patterns of reproductive isolation and mating. *Genetics* **186**, 699–712.
- Moler, P.E. (1992). Florida bog frog, *Rana okaloosae* Moler. In *Rare and endangered biota of Florida, vol. 3: amphibians and reptiles*: 30–33. Moler, P.E. (Ed). Gainesville: University Press of Florida.
- Moler, P.E. (1993). *Rana okaloosae*. Moler, Florida bog frog. *Cat. Am. Amphib. Reptil.* **561**, 1–3.
- Moore, A.-B.C. (1950). The development of reciprocal androgenetic frog hybrids. *Biol. Bull.* **99**, 88–111.
- Nei, M. (1977). F-statistics and analysis of gene diversity in subdivided populations. *Ann. Hum. Genet.* **41**, 225–233.
- Nielson, E.E.G., Bach, L.A. & Kotlicki, P. (2006). Hybridlab (version 1.0): a program for generating simulated hybrids from population samples. *Mol. Ecol. Notes* **6**, 971–973.
- Nosil, P. & Yukilevich, R. (2008). Mechanisms of reinforcement in natural and simulated polymorphic populations. *Biol. J. Linn. Soc.* **95**, 305–319.
- van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M. & Shipley, P. (2004). MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* **4**, 535–538.
- Pritchard, J.K., Stephens, M. & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–959.
- Rosenberg, N.A. (2004). Distruct: a program for the graphical display of population structure. *Mol. Ecol. Notes* **4**, 137–138.
- Rousset, P. (2008). Genepop'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Mol. Ecol. Res.* **8**, 103–106.
- Senar, J.C., Domenech, J. & Camerino, M. (2005). Female siskins choose mates by the size of the yellow wing stripe. *Behav. Ecol. Soc.* **57**, 465–469.
- Sutter, R.D., Bachant, J.J., Gordon, D.R. & Litt, A.R. (2001). An assessment of the desired future conditions for focal conservation targets on Eglin Air Force Base. Available at http://www.tncfire.org/documents/USfln/USFL-N2_EglinDFC.pdf (accessed October 26, 2010).
- Vähä, J.-P. & Primmer, C.R. (2006). Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different number of loci. *Mol. Ecol.* **15**, 63–72.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Plots of mean (st. dev.) of likelihood scores for K values from 1 to 5 and corresponding delta K values for values ranging from 2 to 4.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.