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SHORT COMMUNICATION

Assessing fine-scale genetic structure and relatedness in the micro-endemic Florida bog frog

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Abstract The Florida bog frog (*Rana okaloosae*) is restricted to approximately 25 seepage drainages on the Florida Panhandle, southeastern USA. We evaluated fine-scale (<1–10 km) genetic structure among 80 samples from a long-term study area in one portion of its range. We also included co-distributed samples ($N = 48$) from bronze frogs (*R. clamitans*). Individual *R. okaloosae* were significantly more related to one another than expected under panmixia, though significant patterns of isolation-by-distance were detected reflecting limited dispersal. Bayesian clustering failed to identify discrete genetic clusters within species. Subtle, though important differences in genetic structuring between *R. okaloosae* and *R. clamitans* suggests that future efforts to predict the impact of landscape changes on *Rana okaloosae*, should focus on the species itself rather than *R. clamitans* as a surrogate species.

Keywords Microsatellites · Correlogram · Mantel's tests · Relatedness · Isolation-by-distance

Introduction

The Florida Panhandle contains unique seepage and steephead stream habitats that are host to a high proportion of temperate North America's endemic species (Means 2000; Stein et al. 2000). Species with limited geographic ranges may be more susceptible to habitat loss and environmental change (Lande 1993). Thus, examining population processes within species having limited ranges is important for understanding the ecological factors required for population persistence and subsequently for making scientifically informed conservation decisions (Beebee 2005).

The Florida bog frog [*Rana (Lithobates) okaloosae*] is one of the most geographically restricted and, until recently (Bishop 2005; Gorman 2009), one of the least studied frog species in North America (Moler 1993). *Rana okaloosae* uses acidic streams that originate from seepage and steephead habitats. *Rana okaloosae* is patchily distributed within its small range (Gorman 2009), with only a few large breeding congregations (e.g. >50 calling males) identified over the past 20 years. Distributional surveys have detected *R. okaloosae* at approximately 25 streams in Walton, Okaloosa and Santa Rosa counties, Florida. Most, (>90%) of the known distribution is on Eglin Air Force Base (Fig. 1). Nothing is known about the scale of dispersal or genetic structuring in *R. okaloosae*, even though its habitat associations suggest that fine-scale dispersal might be limited to low-lying, seepage streams.

Here, we apply individual-based analytical approaches to quantify the fine-spatial scale of genetic structure in

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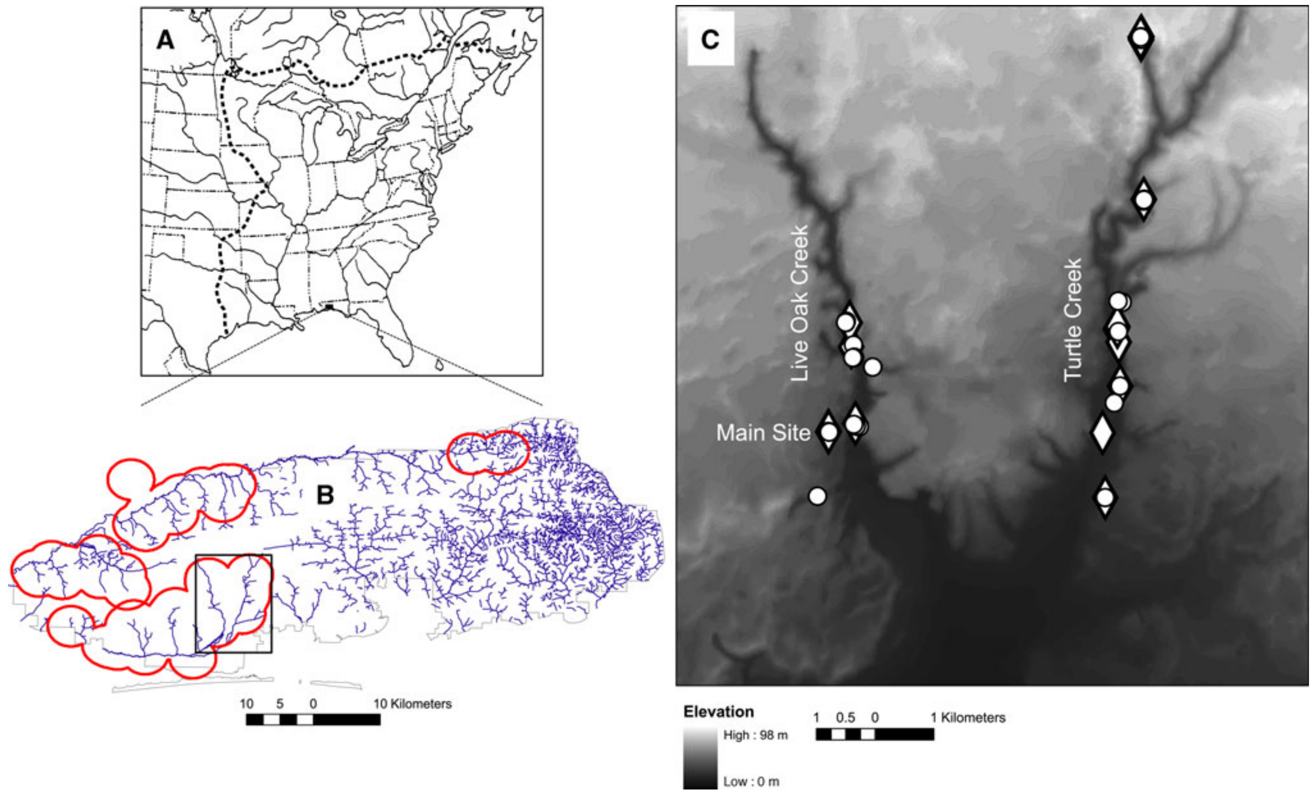

Fig. 1 **a** Geographic range of *Rana clamitans* in eastern North America and the range of *R. okalossae* is indicated on the Florida Panhandle. **b** Boundary of Eglin Air Force Base with stream networks. Bubble outlines the distribution of *R. okaloosae*, and rectangle indicates portion of the range studied herein. **c** Sample locations of *R. okaloosae* (*circles*) and *R. clamitans* (*diamonds*). The Main Site on Live Oak Creek is denoted and was the only aggregation of frogs sampled (Main site: *R. okaloosae* $N = 46$; *R. clamitans* $N = 27$). Other locations were represented by one to four samples

R. okaloosae and the closely related, sympatric *R. clamitans*. Our working hypothesis is that less spatial structure will be evident in *R. clamitans* given its greater niche-breath, greater localized movement (Gorman et al. 2009), and dispersal potential. The latter assumption is based upon the greater body size in *R. clamitans* (Moler 1993) that may lead to greater dispersal ability, and high gene flow detected in closely related, similarly wide-spread species (*Rana catesbeiana*, Austin et al. 2004).

Materials and Methods

Toe clips were collected haphazardly during distributional surveys during summer of 2003–2008 (80 *R. okaloosae* and 48 *R. clamitans*). We concentrated our genetic analysis on the southeast portion of the range of *R. okaloosae* due to the relatively high densities of *R. okaloosae*. Genomic DNA was extracted using a standard phenol–chloroform method (Sambrook and Russell 2001). A microsatellite library was constructed using an enrichment procedure described in Chan (2007). Sequencing of clones and PCR conditions are described elsewhere (Online Supplementary File 1). We designed primers for eight new loci and we screened previously published *Rana catesbeiana* primers (Austin et al. 2003) of which three (J8, J21, and J54) were variable and easy to score. PCR products were run on an ABI 3130 Genetic Analyzer (Applied Biosystems) and scored using GENEMARKER® software (SoftGenetics, State College PA).

Evidence of null alleles and scoring error was examined using MICROCHECKER version 2.2 (van Oosterhout et al. 2004). We used GENEPOP version 4 (Rousset 2008) to test for linkage disequilibrium among pairs of loci. Heterozygote deficiency and excess were tested with the exact tests available in GENEPOP. Posthoc sequential Bonferroni tests (Rice 1989) were applied. We calculated Weir and Cockerham's (1984) and Robertson and Hill's (1984) bias-corrected F_{IS}, the latter being ideal for low genetic differentiation (Raufaste and Bonhomme 2000). Observed (H_O) and expected heterozygosities (H_E) were calculated in MSA version 4.05 (Dieringer and Schlötterer 2003). Departures from Hardy–Weinberg equilibrium (HWE) were examined among samples from the largest sample site. The effective number of alleles (A_E) was calculated following Weir (1990).

We ran STRUCTURE version 2.3 (Pritchard et al. 2000) on intra- and inter-specific data sets, the latter to confirm species-level structure exists. In all cases we applied the admixture model with correlated allele frequencies. We tested number of clusters (K) from one to five. We ran 1.0×10^6 iterations following 3.0×10^5 burn-in iterations, repeating 10 times per K.

For each species, we ran 1000 permutations in IDENTIX version 1.1 (Belkhir et al. 2002) using the $\mathrm{rxy}_{\mathrm{Identity}}$ (Belkhir et al. 2002) and $\mathrm{rxy}_{\mathrm{LR}}$ (Lynch and Ritland 1999) estimators of relatedness. The permutation procedure allows us to test whether deviations from HWE are the result of sampling bias (Belkhir et al. 2002).

Mantel's tests were conducted in GenAlEx 6.3 (Peakall and Smouse 2006) to examine the correlation between the pairwise individual-by-individual Ln genetic (Codom-genotypic option) and Ln geographic distances. We used straight-line Euclidian distances and we measured distances between samples along streams using the measure tool in ArcGIS 9.3 (Environmental Systems Research Institute, Redlands, CA) at a scale of 1:15,000. Significance was tested using 10,000 permutations. We also conducted spatial autocorrelation analysis in GenAlEx to provide a measure of genetic correlation as a function of distance. We generated frequency distributions of pair-wise distances in GenAlEx and inspected these to select distance class boundaries that reflected sensible biological classes (Peakall et al. 2003). We subsequently used class sizes of 0–100 m, >100–1000 m, >1,000–2,000 m, >2,000–5,000 m, >5,000–7,000 m, and >7,000–9,000 m for Euclidian metrics. Aquatic distance classes were the same with the following exceptions to reflect the distances along aquatic corridors: >5,000–9,000 m, >9,000–30,000 m, and >30,000–36,000 m. Note that there is no requirement for distance classes to be even in spatial autocorrelation analysis (Smouse and Peakall 1999). The 10,000 permutations provided an estimate of r and 95% confidence intervals about the null hypothesis of no spatial genetic structure ($r = 0$). The 95% confidence interval around empirical r was estimated via 10,000 bootstraps.

We calculated the kinship coefficients of Loiselle et al. (1995) and Rousset's A inter-individual distance (Rousset 2000) and compared with distances in the software SPAGeDi (Hardy and Vekemans 2002). These individual kinship/relationship estimators do not assume HWE. Genes and locations were permuted 10,000 times to create a null distribution. The coefficients were regressed against the logarithm of Euclidian and stream distance to determine whether the slopes were significantly different from zero.

Results

Two of the eight new loci were invariable for 30–50 individuals in both species (Table 1) and were abandoned. Three of 36 linkage contrasts from *R. okaloosae* (253 and 194, 8 and J8, and 194 and 195) and three from *R. clamitans* (J54 and 195, J8 and J21, and 253 and 21) were significant (all P $0.05 < 0.001$). Following sequential Bonferroni, only one (J54 and 195 in *R. clamitans*) remained significant. We therefore treated all loci as independent. In *R. okaloosae* sampled at the Main Site, three loci had excess homozygosity, and excess heterozygosity was detected in two (Table 2). In *R. clamitans* all but one locus deviated from HWE. Microchecker identified patterns suggestive of null alleles at five loci in *R. clamitans* (all but 299, J54, J8 and J21); no null alleles were detected in *R. okaloosae*. Re-amplification of 32 homozygous *R. clamitans* at these five loci suggested a genotyping error rate of 3% at J54 (1 new allele detected in one genotype), and an overall error rate of <1%.

Bayesian clustering analysis on the combined *R. okaloosae* + *R. clamitans* data showed that a $\mathrm{K} = 2$ was supported, reflecting species-level differentiation, Structure failed to detect multiple clusters in *R. clamitans*. In *R. okaloosae* Ln probability values increased between $\mathrm{K} = 1$ and $\mathrm{K} = 2$ though inter-run variance also increased, suggesting that these K values failed to converge on the true K (Supplemental Data File 2).

Allelic diversity was greater in *R. clamitans* (paired $t = 3.65$, $\mathrm{df} = 8$, $P = 0.007$), as was inbreeding (Table 2). For *R. okaloosae* the mean and variance of each relatedness estimator was significantly greater than the null distribution: $\mathrm{rxy}_{\mathrm{Indentity}}$ mean 0.245, $P = 0.001$, variance 0.044, $P = 0.001$; $\mathrm{rxy}_{\mathrm{LR}}$ mean -0.008, $P = 0.025$, variance 0.029, $P = 0.001$. For *R. clamitans* the means were not significant (mean $\mathrm{rxy}_{\mathrm{Indentity}}$ 0.189, $P = 0.880$; $\mathrm{rxy}_{\mathrm{LR}}$ 0.021, $P = 0.15$) though the variances were (variance $\mathrm{rxy}_{\mathrm{Indentity}}$ 0.130, $P = 0.028$; $\mathrm{rxy}_{\mathrm{LR}}$ 0.008, $P = 0.005$).

There were positive correlations between genetic distances and Euclidean (*R. okaloosae,* $\mathrm{Rxy} = 0.472$, $P = 0.001$; *R. clamitans,* $\mathrm{Rxy} = 0.757$, $P = 0.002$) and stream distances (*R. okaloosae,* $\mathrm{Rxy} = 0.428$, $P = 0.002$; *R. clamitans,* $\mathrm{Rxy} = 0.666$, $P = 0.005$). However, in both species significant inter-individual autocorrelation was found only among individuals within the first distance class (0–100 m) (Euclidean—*R. okaloosae* $\Omega = 54.9$, $P = 0.001$; *R. clamitans* $\Omega = 33.6$, $P = 0.003$; stream distance—*R. okaloosae* $\Omega = 60.8$, $P = 0.001$; *R. clamitans* $\Omega = 32.5$, $P = 0.015$).

Loiselle's kinship coefficient (Loiselle et al. 1995) had a weak but significant slope (b) of -0.002 ($P < 0.0001$) in *R. okaloosae* and -0.001 in *R. clamitans* ($P = 0.007$), indicating that average kinship decreased over geographic distance. Rousset's distance estimator had a weakly positive regression slope of 0.008 in *R. okaloosae* ($P = 0.0002$), and in *R. clamitans* $b = 0.004$ ($P = 0.028$).

Table 1 Characterization of microsatellite loci and PCR conditions

Locus	Repeat motif	Primers (5′–3′)	(C°)	$[MgCl_2]$	Allele size range (bp)	Genbank #
299	$(AGT)_{10}$	F-TGTAGCAGCCAAATATCCTGAA R-CTGTGTGTATGTGGCCAACC	60	2	*okaloosae* 130–157 *clamitans* 133–160	HQ439092
249	$(CA)_{11}$	F-CCATGGAATTTGCAGAATGA R-GGCAAAAACCTGGCCTAAA	60	2	*okaloosae* 153–187 *clamitans* 127–215	HQ439093
205	$(TA)_6(AC)_8AGT(AG)_{36}$	F-GGAACCCTCATTGAATTTCG R-GCGTCCCGATACTTTTGACA	53	1.2	*okaloosae* 105–187 *clamitans* 105–183	HQ439094
253	$(AG)_{43}$	F-CGCACTATGGAAACACAACG R-TGGACCATTGTCTGAATTTCATT	50	1.2	*okaloosae* 180–234 *clamitans* 180–262	HQ439095
195	$(CA)_{33}$	F-GGAGCCGAGTGTAACCTGAG R-TGGACTCTATGCCAACACCA	55	2	*okaloosae* 165–221 *clamitans* 163–215	HQ439097
194	$(TTTG)_5$	F-CTGCTGTGGTATCTATGCTTTTT R-CAACTGACATTTGCCCAACA	55	2	*okaloosae* 163–258 *clamitans* 163–258	HQ439096
219	$(TC)_{28}$	F-ACTGGTGCCCAAAACTGAAC R-GTTTCCAAAGCGAGCAAAGT	55	1.3	*okaloosae* 133 *clamitans* 133	HQ439098
229	$(ACA)_5$	F-CCATCAACACCACCTACCAA R-GCGCCACTAAGTCTGCTGTT	53	1.5	*okaloosae* 193 *clamitans* 193	HQ439099
J8	$(GT)_{18}$	F-CCATAGGAATCAAAACAACCCTC R-GGGATATGTGATGGACCCAAG	60	2	*okaloosae* 85–107 *clamitans* 85–109	AY323931
J21	$(GT)_{25}$	F-CCCATCTTATCCTGTGTACT R-CAAGCCTCCATCTCACCTTACC	60	2	*okaloosae* 160–166 *clamitans* 160–168	AY323929
J54	$(CA)_{18}$	F-TCATTACCACTGCCTTCTGC R-TGCTGCTGTCCTATTGCTAG	60	2	*okaloosae* 130–160 *clamitans* 130–166	AY323930

Discussion

Jackson (2006) speculated that *R. okaloosae* movement likely occurs along stream corridors and floodplains although bog frogs have been observed upslope (D. Bishop, personal observation). We found no suggestion that stream-distance better explains the pattern of isolation-by-distance than did Euclidian distance between locations. We observed similar small-scale spatial autocorrelation patterns between *R. okaloosae* and *R. clamitans* though subtle differences in genetic structure (e.g. greater consanguinuity in *R. clamitans*, see below).

There was low polymorphism in *R. okaloosae* relative to *R. clamitans*, consistent with expectations for rare species. We could expect greater levels of genetic variation despite greater inbreeding in *R. clamitans* due to the long-term effect of gene flow from populations outside of the study area. Few data sets exist that compare homologous markers among related, sympatric species. *Rana okaloosae* at the SE portion of its range represents a non-panmictic, though continuous genetic population, represented by weak isolation-by-distance. The observed fine-scale structure reflects the possible importance of habitat connectivity in maintaining populations, an issue of particular importance in these rare and important stream habitats. However, our results suggest that stream connectivity itself does not explain the existing structure better than geographic distance.

The lack of genetic clustering within species may reflect the scale of sampling (Pritchard et al. 2000), the number of loci used, or both. Structure results provide some support for two clusters (higher Ln probability) though it is more conservative to interpret the best support for $K = 1$ given the relatively low variance among replicates. At $K = 2$ clusters did not consist of samples representing distinct drainages, but were mixed (not shown).

Null alleles may also be influencing departures from HWE in *R. clamitans*, though similar evidence of null alleles were lacking in *R. okaloosae*. Relatedness estimators that are not influenced by departures from HWE, had higher than expected average relatedness in *R. okaloosae*, suggesting small N_e at the scale examined. In *R. clamitans*, the lack of deviation of the mean relatedness estimators from their null expectation was in contrast to the significantly high variances, a pattern that may reflect that *R. clamitans* samples consist of several independent consanguineous groups (Belkhir et al. 2002). Though the inclusion of *R. clamitans* was a minor part of our objective,

Table 2 Number of alleles (*A*), effective number of alleles (A_E) for the total dataset (*R. okaloosae* $N = 81$; *R. clamitans* $N = 48$)

	A	A_E	Main site			
			H_O	H_E	W&C	R&H
R. okaloosae						
299	4	1.07	0.022	0.022	–	–
249	12	2.29	0.522^{a}	0.579	0.050	0.392
205	16	7.28	0.870^{a}	0.881	0.013	0.155
253	6	2.09	0.543	0.486	−0.119	−0.121
195	16	8.17	0.848	0.860	0.014	0.038
194	10	5.06	0.609^{a}	0.676	0.101	0.340
J8	5	1.76	0.413	0.353	−0.174	−0.055
J54	8	2.44	0.956^{b}	0.594	−0.620	−0.142
J21	3	2.09	1^{b}	0.536	−0.883	−0.494
Mean	8.4	3.58		0.554	−0.202	0.014
R. clamitans						
299	6	3.25	0.481^{a}	0.739	0.353	0.324
249	25	17.47	0.704^{a}	0.927	0.244	0.137
205	22	10.68	0.296^{a}	0.903	0.661	0.498
253	12	9.44	0.667^{a}	0.877	0.243	0.225
195	20	8.82	0.593^{a}	0.869	0.288	0.141
194	14	8.77	0.519^{a}	0.841	0.388	0.316
J8	11	5.45	0.667^{a}	0.792	0.161	0.165
J54	8	2.66	0.481	0.516	0.069	0.054
J21	4	2.42	0.852^{b}	0.603	−0.424	−0.233
Mean	13.3	7.66		0.785	0.220	0.181

Observed (H_O) and expected (H_E) heterozygosity, and Weir and Cockerham's (W&C) and Robertson and Hill's (R&H) F_{IS} for each locus from Main Site (*R. okaloosae* $N = 46$; *R. clamitans* $N = 27$)

[a] Excess homozygosity $P \leq 0.05$; [b] excess heterozygosity

we were interested in exploring the ability of using the closely related species as a surrogate to the rare *R. okaloosae*. Without greater sample sizes reflecting similar patterns of genetic structure (e.g. reduced consanginuity in *R. clamitans*) we believe that future effort to understand the landscape level patterns of genetic structure across Eglin AFB and adjacent lands should focus on *R. okaloosae* rather than a surrogate species.

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