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GENETIC STUDIES IN A DEMOGRAPHIC FRAMEWORK: ANNUAL VARIATION, INBREEDING HISTORY, AND EFFECTS OF ISOLATION IN A RARE GROUP OF AMPHIBIANS

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GENETIC STUDIES IN A DEMOGRAPHIC FRAMEWORK: ANNUAL VARIATION, INBREEDING HISTORY, AND EFFECTS OF ISOLATION IN A RARE GROUP OF AMPHIBIANS

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Abstract

Anthropogenic habitat fragmentation and reduction are major causes of population declines and extinction. As these processes intensify, our ability to rescue imperiled taxa is critically dependent on an understanding of historical, demographic, and genetic parameters of shrinking populations. Opportunities to study all three and their interactions in natural populations are rare. Such opportunity existed in an isolated population of the US endangered Dusky Gopher Frog, *Rana sevosa*. With rapid contraction of its historic range, one might predict a substantial reduction in genetic variation. However, the actual effects of rapid decline on population size, genetic diversity, and population processes were unknown. Microsatellite DNA markers were used to address four research objectives: (1) to compare genetic variation in the isolated population of *R. sevosa* to non-isolated populations of its two sister taxa; (2) to address inbreeding history of the isolated population; (3) to determine temporal change versus stasis (over a ten-year period) in population polymorphism of *R. sevosa*; and (4) to address preservation of genetic variability via fitness-related consequences of inbreeding.

Rana sevosa had significantly lower genetic variation than non-isolated populations of its sister taxa. In fact, the isolated population had an observed heterozygosity that was 72% and allelic richness that was 61% of the average non-isolated population. Seven alleles were lost from the population during 1995–2004. No differences were found for heterozygosities over this period, but significant population structuring among years evidenced by F_{ST} and R_{ST}, corroborated previous demographic data of high annual

population turnover and small population size. Additionally, it appears that a historic bottleneck (pre 1997) and a more recent bottleneck (between 1997 and 2001) occurred based on genetic signatures. Finally, mechanisms of enhanced fitness were explored (1) differential survival of more genetically diverse offspring, (2) mass mortality of egg clutches based on high relatedness of parents, and (3) pairing of more genetically different mates. A positive relationship was found between genetic variability and fitness for individuals surviving to metamorphosis and for survival of embryos within egg clutches, which suggests that more outbred individuals had higher fitness. No evidence was found for non-random mating with respect to genotype.

CHAPTER 1

GENETIC CONSEQUENCES OF POPULATION REDUCTION AND ISOLATION IN THE CRITICALLY ENDANGERED FROG, *RANA SEVOSA*, WITH COMPARISON TO NON-ISOLATED POPULATIONS OF TWO SISTER TAXA, *RANA AREOLATA* AND *RANA CAPITO*

Introduction

A fundamental issue in conservation biology is applying our knowledge of metapopulation biology to systems in which inter-population dynamics have been reduced because of habitat fragmentation. As human populations continue to expand and encroach on the natural landscape, the severity of habitat fragmentation intensifies and habitat-patch sizes diminish. Concurrently, populations of most non-human organisms that were once large and connected by gene flow become subdivided, reduced in size, and typically confined to discrete, small habitat patches (Ovaskainen & Hanski 2003). Thus, subdivided subpopulations tend to become inbred and genetically differentiated (e.g., Aldrich et al. 1998; Lucchini et al. 2004; Van Rossum et al. 2004). This anthropogenic habitat fragmentation and reduction are major causes of population declines and local

extinctions (Saccheri et al. 1998). Remedying situations in which populations have become completely isolated is necessary for conservation because gene flow acts to ameliorate the negative effects of inbreeding (e.g., Spielman & Frankham 1992; Saccheri et al. 1998) and migration between subpopulations allows those with local extinctions to be recolonized (Etienne & Heesterbeek 2001).

Few studies have addressed the ecology and genetic dynamics of metapopulations in amphibians (Sjögren 1991), but understanding worldwide amphibian declines may rely on these types of studies (Alford & Richards 1999). Many amphibians, especially those that breed in ponds, tend to have clumped distributions across the landscape. Maintenance of interconnectedness and population dynamics within this metapopulation setting (in the sense of "ponds as patches"; Marsh & Trenham 2001) is critical if populations are to persist (Joly et al. 2001), particularly for rare species (Ficetola & De Bernardi 2004; Moore et al. 2004). Although patch size is positively related to genetic variability and persistence (e.g., Knaepkens et al. 2004), the dynamics and connections among patches more severely affect extinction probabilities of populations and species (Drost & Fellers 1996; Joly et al. 2001; Semlitsch 2002).

At least two life history characteristics of amphibians predispose them to failure in fragmented habitats. First, populations, especially those of pond-breeding amphibians, are characterized by episodic annual reproductive success consisting of fairly infrequent "boom" years of high reproductive output interspersed among "bust" years (e.g., Semlitsch et al. 1996; Richter et al. 2003). In fact, amphibian populations tend to decrease in size more often than they increase because their inherent annual reproductive output is highly variable (Alford & Richards 1999). Also, amphibians typically do not

move great distances across the landscape, especially compared to other vertebrates (Dodd & Smith 2003). However, many studies have found that a few individuals move much greater distances than the population mean would suggest (see Appendix 1 of Semlitsch & Bodie 2003).

Low levels of migration are sufficient to maintain genetic homogeneity among populations in the absence of selection (Spielman & Frankham 1992; Hartl & Clark 1997). As one might predict, numerous studies have found that amphibian populations separated by great distances and unimpeded by barriers to migration have little genetic differentiation among them (e.g., Hitchings & Beebee 1997; Squire & Newman 2002). The opposite is true for studies of amphibian populations impeded by barriers (Hitchings & Beebee 1997; Vos et al. 2001). For example, Hitchings and Beebee (1997) found a high degree of genetic differentiation between two urban, fragmented populations of the European common frog (*Rana temporaria*) separated geographically by only a short distance (2.3 km). What is more striking is that populations separated by an average of 41 km had low genetic differentiation between them, which indicates a much greater rate of gene flow between populations separated by large geographic distances (range = 11–93 km) than those separated by < 3 km (see Table 4 of Hitchings & Beebee 1997).

Genetic variation of populations is naturally influenced by evolutionary, demographic, and stochastic processes (e.g., selection, mutation, effective population size, and bottlenecks). Contemporary genetic variability is typically eroded most severely by unnatural processes including loss and subdivision of local populations. At one extreme of the range in severity of subdivision, populations in isolation and with small size have reduced variation as a result of individual and synergistic effects of inbreeding

(Charlesworth & Charlesworth 1987; Ellegren 1999; Hedrick & Kalinowski 2000), population bottlenecks (Landergott et al. 2001; Wahl & Gerrish 2001), lack of gene flow (Hitchings & Beebee 1997), and genetic drift (Palo et al. 2001). For small populations, drift will reduce allelic diversity at a much higher rate than mutations arise, which then disrupts the mutation-drift equilibrium (see below). Additionally, inbreeding will have severe negative effects on genetic variability, will increase the expression of deleterious recessive mutations, and reduce fitness in small populations (Lynch et al. 1995; Harper et al. 2003; Knaepkens et al. 2004).

Not all studies of small or isolated populations discovered low genetic variability. For example, high variation has been found in populations founded by a small number of individuals (Signer et al. 1994) and in small, insular populations (Nichols et al. 2001). Conversely, low genetic variation has been found in populations with large size (Travis et al. 1997).

For non-isolated populations, genetic variation is maintained by at least two factors, which are not mutually exclusive— (1) a population of multiple subpopulations, which allows for larger total population size; and (2) semi-isolation of individual (sub)populations with periodic gene flow between them, which results in population substructure. Variation can be enhanced further if periodic gene flow occurs between habitat patches that vary in quality and localized genetic processes (e.g., Ovaskainen & Hanski 2003). Variability can be maintained in isolation by factors including annual dynamics of selection pressures (e.g., annual variation in predator presence and abundance; Richter 2000), differential survival of heterozygotes (Coltman et al. 1998; Rossiter et al. 2001), and population substructure (Nichols et al. 2001).

At least two major groups of measures of within-population genetic variation exist and reveal different historical information. One quantifies variation in terms of genotypes (e.g., heterozygosity) and the other in terms of genotypic components (e.g., allelic richness). Heterozygosity can be measured as the observed heterozygosity (H_O), which is the proportion of individuals observed in the population with different alleles at a locus or as expected heterozygosity (H_E), which is the expected proportion of heterozygous individuals based on observed allele frequencies under Hardy-Weinberg equilibrium (HWE). Expected heterozygosity [Nei's (1973) gene diversity] is the probability that two randomly chosen alleles from the population are different and is the proportion of heterozygotes expected if the population is at HWE (Nei 1973). Reduction in population size negatively impacts allelic richness (mean number of alleles per locus) more severely than it does expected heterozygosity (Leberg 1992; Garner et al. 2003; Hinten et al. 2003). Additionally, allelic richness more effectively reveals long-term evolutionary potential of a population (e.g., Petit et al. 1998). Because a loss in the number of alleles can limit future response to selection, allelic richness should be given higher priority for conservation measures than other measures of genetic variability (e.g., observed heterozygosity).

Anthropogenic habitat fragmentation and reduction are major causes of population declines and extinction. As these processes intensify, our ability to rescue imperiled taxa is critically dependent on an understanding of historical, demographic, and genetic parameters of shrinking populations. Opportunities to study all three and their interactions in natural populations are rare. Such opportunity existed in an isolated population of Dusky Gopher Frogs, *Rana sevosa*. *Rana sevosa* is listed as Endangered

under the US Endangered Species Act and Critically Endangered on the Red List of the International Union for Conservation of Nature and Natural Resources (USFWS 2001; IUCN 2004). They are rare amphibians whose historic geographic range once extended throughout the coastal plain of Louisiana, Mississippi, and western Alabama in upland, longleaf pine (*Pinus palustris*) forests (Goin & Netting 1940). *Rana sevosa* breed in temporary, upland ponds and spend the non-breeding season in adjacent longleaf pine forests in small mammal burrows, holes associated with dead trees, and other below-ground refugia (Richter et al. 2001). Annual breeding-migration events between the forest and pond facilitate sampling adults of the population.

Critical habitat has been reduced across the geographic distribution of gopher frogs due to logging and conversion of longleaf pine ecosystems to slash pine (*P. elliottii*) plantations. In addition to habitat loss, gopher frogs are threatened by habitat fragmentation, fire suppression, introduction of fish to breeding ponds, and road mortality (Richter & Jensen *in press*). The number of breeding populations of gopher frogs has been greatly reduced. *Rana sevosa* is considered extirpated in Louisiana and Alabama. Although once abundant in coastal Mississippi (Allen 1932), only two breeding populations are known to exist. Extensive ecological and demographic data exist for one of the populations (Glen's Pond; Harrison County, Mississippi) (Richter & Seigel 2002; Richter et al. 2003), whereas the other population, which is located ca. 32 km east of Glen's Pond, was only recently discovered (March 2004; Mike's Pond; Jackson County, Mississippi). The status of the Mike's Pond population is unknown.

Interpretation of genetic studies of isolated populations using neutral markers is facilitated by having at least two factors removed from consideration that affect genetic

diversity: gene flow and selection (e.g., Hinten et al. 2003). However, complications arise when no population-level genetic information exists prior to isolation and no non-isolated populations of the focal species are known (e.g., Taylor et al. 1994; Palo et al. 2003). Because this is true for *R. sevosa*, non-isolated populations of its sister species (other gopher frogs, *R. capito*) and of the sister species to *R. capito* and *R. sevosa* (crawfish frogs, *R. areolata*) serve as the next best means of comparison.

All three species are rare. Whether this rarity is due to the secretive nature of the frogs or to loss of populations is unknown for many portions of the range. *R. sevosa* is imperiled and is only known to occur in two counties (Jackson and Harrison Counties, Mississippi) from its historic three-state distribution (Figure 1). Although *R. areolata* and *R. capito* are relatively secure in some portions of their geographic distributions (Figure 1), populations are in severe decline in other regions. Causes for decline in *R. capito* are the same as for *R. sevosa* (Jensen & Richter *in press*; Parris & Redmer *in press*; Richter & Jensen *in press*).

Small population size and isolation should cause reduced genetic variation primarily as a result of the synergism of genetic drift and inbreeding. With rapid contraction of the historic range of *Rana sevosa*, one might predict a substantial reduction in genetic variation. However, the actual effects of rapid decline on population size, genetic diversity, and population processes were unknown. Microsatellite DNA markers were used to address four research objectives: (1) to compare genetic variation in the isolated population of *R. sevosa* to non-isolated populations of its two sister taxa; (2) to address inbreeding history of the isolated population; (3) to determine temporal change versus stasis (over a ten-year period) in population polymorphism of *R. sevosa*; and (4) to

address preservation of genetic variability via fitness-related consequences of inbreeding.

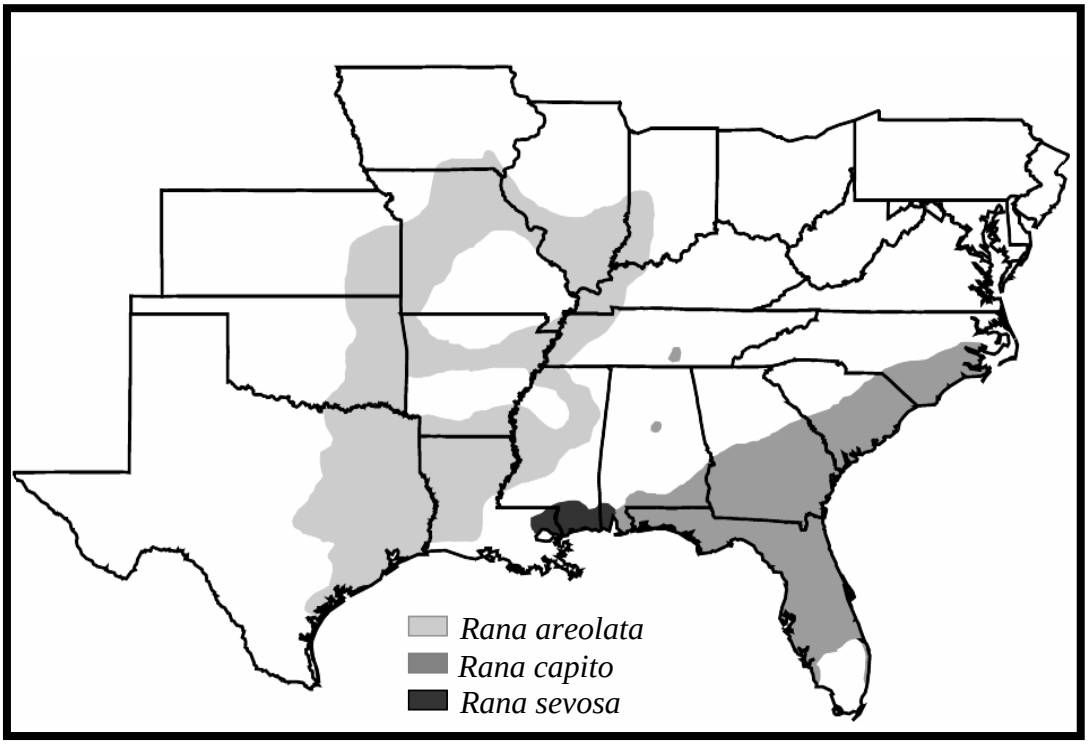


Figure 1. Geographic distribution of gopher frogs (*Rana capito* and *R. sevosa*) and crawfish frogs (*R. areolata*).

Methods

Sample collection

Three populations, an isolated population of *R. sevosa* and non-isolated populations of *R. areolata* and *R. capito*, were sampled. The *R. sevosa* (*Rs*) site is located at the northern edge of United States Forest Service (USFS) property in De Soto National Forest (Harrison County, Mississippi). In fact, the breeding pond is only 250 m south of the USFS boundary line (see Richter et al. 2001). Based on visual and audio surveys

performed since the late 1980s, this population is completely isolated and geographically separated by ca. 32 km from the only other known site. The *R. capito* (*Rc*) site is located in contiguous, protected habitat with many breeding ponds at Archbold Biological Station (Highlands County, Florida). The *R. areolata* (*Ra*) site is on private lands within contiguous pastureland with many breeding ponds (Atoka County, Oklahoma).

Toe-clip samples were collected from 46 *R. sevosa*, 37 *R. capito*, and 32 *R. areolata*. Individuals were captured by hand (*R. areolata*), via a drift fence with pitfalls that completely encircles the breeding pond (*R. sevosa*), or by hand and with pitfall-trap arrays (*R. capito*). Samples of *R. sevosa* were collected prior to the species being listed as endangered, so no special permits were required. All samples were stored in 95% ethanol until DNA was extracted.

Microsatellite library development

Microsatellite DNA loci, which are short tandem-repeat motifs of nucleotides (usually 2-4 bases long), are an ideal tool for use in individual- and population-level genetic studies primarily due to their abundance in the genome, high level of allelic variation (due to their inherent instability), and codominance (i.e., heterozygotes are distinguishable from homozygotes) (Goldstein & Schlötterer 1999). Microsatellite genetic variation can be quantified for individuals not only based on heterozygosity as is true for other molecular markers (e.g., allozymes and quantitative trait loci) but also by exploiting their size-information content. Specifically, microsatellite alleles are distinguished by size, which results from differences in the number of the repeat motifs.

To discover microsatellite loci for *R. sevosa*, *R. capito*, and *R. areolata*, a

microsatellite library was created for *R. sevosa* and screened for loci that would amplify in all three species. DNA was extracted from a single *R. sevosa* individual using a Qiagen® DNEasy tissue kit. The library was developed following the protocol of T. Glenn and M. Schable (unpubl. data; see Hauswaldt & Glenn 2003), which was modified from Hamilton et al. (1999).

Genomic DNA (ca. 3.3 µg) was digested using the restriction enzyme *RsaI*. Resulting DNA fragments were then ligated to linkers (SuperSNX24 forward: GTTTAAGGCCTAGCTAGCAGAATC; SuperSNX24+4p reverse: GATTCTGCTAGCTAGGCCTTAAACAAAA). The ligated linkers provided a primer binding site for use in the polymerase chain reaction (PCR), which is an artificial means of replicating large numbers of copies of specific DNA fragments (for more details, see McPherson & Møller 2000). Ligated genomic fragments were then allowed to hybridize to a mixture of the following biotinylated probes: $(TG)_{12}$, $(AG)_{12}$, $(AAG)_8$, $(ATC)_8$, $(AAC)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACCT)_6$, $(ACAG)_6$, $(ACTC)_6$, $(ACTG)_6$, $(AAAT)_8$, $(AACT)_8$, $(ACAT)_8$, $(AGAT)_8$, $(AACC)_5$, $(AACG)_5$, $(AAGC)_5$, $(AAGG)_5$, $(ATCC)_5$. Following hybridization, the biotin on each probe was attached to streptavidin-coated magnetic beads (Dynabeads; Dynal Biotech, Inc.). Magnetism was used to selectively retain microsatellite-containing fragments, and unbound fragments were discarded. PCR employing the linker priming site was used to increase the number of copies of each microsatellite fragment. These were ligated into pGEM®-T Easy vectors (Promega Corporation), and transformed into *Escherichia coli* competent cells. Clones were transferred to agar plates and incubated overnight at 37° C. Next, insert-containing colonies were "picked" and incubated overnight at 37° C in LB

broth. Following incubation, plasmid DNAs were isolated and sequenced using an ABI Prism® 310 Genetic Analyzer (Advanced Biosystems).

Of the 192 fragments screened, 27 microsatellite loci were identified with flanking regions sufficiently large to allow primer development. Of these, 17 appeared appropriate for population genetic work (i.e., the microsatellite repeat was the only repetitive sequence in the fragment). Primers were designed for these 17 and tested using 3-temperature touchdown PCRs with the highest temperatures ranging from 60 to 46° C (Don et al. 1991). For example, the 60° touchdown PCR had annealing temperatures of 60 for 5 cycles, 58 for 5 cycles, and 55 for 25 cycles. Fourteen loci amplified for *R. sevosa* only, 12 loci amplified for *R. sevosa* and *R. capito*, and 3 amplified for all 3 species. These loci were screened for polymorphism by first amplifying them with PCR and then visualizing the PCR products on a 3% agarose gel (n = 15 for each species). For loci found to be polymorphic, another PCR was performed using a fluorescently labeled forward primer for each locus for *R. sevosa* (n=46), *R. capito* (n=37), and *R. areolata* (n=32). An aliquot of each PCR product was loaded into an ABI Prism® 310 Genetic Analyzer (Applied Biosystems), and allele lengths were scored using GeneScan® Analysis Software version 3.1.2 (Applied Biosystems). Following data collection, number of alleles, observed heterozygosity (H_O), and heterozygosity expected under Hardy Weinberg equilibrium (H_E) were determined. H_E was calculated using FSTAT Version 2.9.3.2 (Goudet 1995, 2002).

Of the loci that amplified, 8 of 14 were polymorphic in *R. sevosa* (Table 1). Of these 8, 7 amplified and were polymorphic in *R. capito*, and 3 amplified and were polymorphic

Table 1. Description of 8 microsatellite loci developed for *Rana sevosa*. Size range of alleles is shown as total size range (bp) and as range in number of repeat motifs. Allelic data and genetic variation were derived from 46 individuals. Annealing temperatures (T_m) are presented as the highest temperature in a three-temperature touchdown PCR with 2 degree decrements.

Locus	Primer sequence	Repeat motif	T_m (^oC)	size range (bp)	size range (motifs)	# alleles	H_E	H_O
*Rsev*B12	TATAAGTGTGCCAACGCAGAC	ATAG	55	208–236	10–17	7	0.779	0.826
	CAATCATTTCCAAAAACAC							
*Rsev*C02	TGCATGACTGAGTAATTGTC	AC	55	165–169	5–7	2	0.502	0.435
	GATGTATAGTAAGCCATCCG							
*Rsev*A05	CCATGTCACTAATGCACCTG	CT	48	263–269	15–15	3	0.423	0.435
	AGCTTTGCTGTATATTGAGC							
*Rsev*C05	TACATTAGTGTGATGGTCAG	TAT	55	172–178	2–4	2	0.196	0.174
	GATTGTAAGCTCTTCTGAGC							

(*Table 1* cont.)

Locus	Primer sequence	Repeat motif	T_m (^oC)	size range (bp)	size range (motifs)	# alleles	H_E	H_O
*Rsev*E03	ATCTCGGCTTCACTGATTGC	GA	55	276–304	9–23	6	0.727	0.391
	GCCTACTATGTAACTACTAT							
*Rsev*F01	GTGGCGTAACATGCCAGTC	ATAG	55	163–195	9–17	6	0.729	0.609
	CTGTGGATTGAAAGTGTACGC							
*Rsev*G11	GTCTTCCATTACAAGGCTGC	TCTA	55	226–276	8–21	8	0.856	0.869
	ACTTCTGACAGTCTAGTTAA							
*Rsev*Ms3	ATGTAAGCAATGCTTGTCC	CA	55	274–306	14–30	6	0.787	0.761
	AAGGACATTGCCACTCAGGC							

Table 2. Microsatellite loci data for *Rana capito* and *R. areolata*. Allelic data and genetic variation were derived from 37 individuals for *R. capito* and 32 individuals for *R. areolata*. Annealing temperatures are the same as for *R. sevosa* (Table 1).

Species	Locus	Repeat motif	Size range (bp)	Size range (motifs)	# alleles	H_E	H_O
Rana capito	*Rsev*B12	ATAG	212–240	11–18	7	0.791	0.838
	*Rsev*C02	AC	163–171	4–8	5	0.777	0.703
	*Rsev*A05	CT	255–283	8–22	9	0.681	0.487
	*Rsev*C05	TAT	172–184	2–6	4	0.531	0.649
	*Rsev*E03	GA	278–308	10–25	10	0.622	0.595
	*Rsev*F01	ATAG	163–195	9–17	9	0.847	0.946
	*Rsev*Ms3	CA	266–292	10–23	9	0.801	0.703
Rana areolata	*Rsev*A05	CT	255–265	8–13	6	0.650	0.688
	*Rsev*E03	GA	286–308	14–25	7	0.732	0.625
	*Rsev*F01	ATAG	163–195	9–17	9	0.841	0.875

in *R. areolata* (Table 2). Thus, differences in the number of suitable loci among species were caused by lack of amplification and not a lack of polymorphism. All loci had moderate to high genetic variation in terms of allelic richness (4–10 alleles per locus), H_O (0.595–0.946), and H_E (0.531–0.856) (Tables 1 & 2).

Genetic data collection

DNA was extracted from each individual using Qiagen® DNEasy tissue kits. For each individual, DNA was PCR amplified for seven microsatellite loci for *Rs* and *Rc* and for three loci in *Ra*. Genetic data were collected using an ABI Prism® 310 Genetic Analyzer (Applied Biosystems) by pooling samples of PCRs for three loci per individual using different fluorescently labeled primers for each multiplexed locus. Allele lengths were scored using GeneScan® Analysis Software version 3.1.2 (Applied Biosystems). A total of 137 individuals from four populations was analyzed for seven loci (*Rs* and *Rc*) or three loci (*Ra*).

Genetic analyses

The following genetic calculations and analyses were performed using FSTAT Version 2.9.3.2 (Goudet 1995, 2002). Observed heterozygosity (H_O), allelic richness (*A*), expected heterozygosity (H_E), and Wright's inbreeding coefficient (F_{IS}) were calculated. For all except H_O, unbiased estimators were calculated by standardizing each to the size of the smallest sample (Nei 1987; El Mousadik & Petit 1996). For loci that amplified only in *Rs* and *Rc*, the smallest sample size was for *Rc* (n = 37). For the loci that amplified in all three species, *Ra* had the smallest sample size (n = 32). To evaluate

deviations from HWE for each locus and population, global tests (1000 permutations) with Bonferroni corrections were used (Weir 1996).

The following measures of genetic variability were compared among populations as follows. For each measure, differences were qualitatively compared among all three species due to low sample size (3 loci) in *Ra* and statistically evaluated between *Rs* and *Rc*. Differences in unbiased H_E were evaluated using a paired *t*-test on arcsine-transformed data (Nei 1987). Differences in F_{IS} over all loci were compared based on 28,000 randomizations and Bonferroni corrections. For allelic richness (*A*), one must be careful in making comparisons between populations because these data can be biased by variation in sample sizes, especially for more polymorphic loci (Leberg 2002). Therefore, a rarefaction technique was first used to account for unequal sample sizes in estimating *A* as recommended by Leberg (2002). Differences were then evaluated using Wilcoxon's signed-rank test. Rarefaction standardizes *A* to the smallest sample size in a comparison using the frequency distribution of alleles to estimate the number of alleles that would occur for smaller samples (El Mousadik & Petit 1996; Leberg 2002).

A measure similar to allelic richness is allelic dropout, which focuses on loss of alleles rather than simply the number per locus. Garza and Williamson (2001) developed a population-level statistic (*M*) that quantifies allelic dropout by dividing the number of alleles by the range in size of alleles for each locus averaged over all loci (i.e., *M* takes advantage of the number of alleles being reduced at a higher rate than the size range). They calculated *M* for populations from the literature with different demographic histories and found that values of *M* consistently predicted the reported history of

populations (Garza & Williamson 2001). *M* was evaluated in the context of the literature review of Garza & Williamson (2001).

Two analyses implemented in BOTTLENECK Version 1.2.02 (Cornuet & Luikart 1996; Piry et al. 1999) were used to test for recent population bottlenecks. The first test was designed to take advantage of allelic diversity being reduced at a faster rate than heterozygosity subsequent to severe reductions in population size. For each locus, heterozygosity excess was evaluated by calculating H_E, which is based on allele frequencies, and then estimating heterozygosity expected at mutation-drift equilibrium (H_{EQ}), which is based on observed number of alleles and sample size. To emphasize the difference, tests of excess H_E (i.e., $H_O > H_E$) examine data for excess heterozygotes, and tests of mutation-drift equilibrium, where the hypothesis is $H_E > H_{EQ}$, examine data for excess heterozygosity (Piry et al. 1999). Populations that have not undergone a recent change in population size will have $H_E = H_{EQ}$.

H_{EQ} was estimated under three models of mutation: infinite alleles model (IAM), stepwise mutation model (SMM), and two-phase mutation model (TPM) based on 5000 iterations. The TPM was used with 95% single-step mutations (5% multi-step) and a variance among multiple steps of 12% as recommended by Piry et al. (1999). The IAM, SMM, and TPM are all possibly appropriate models of mutation for microsatellite DNA. However, the TPM is apparently most appropriate (Di Rienzo et al. 1994; Piry et al. 1999). Following estimation of H_{EQ}, a one-tailed Wilcoxon's signed-ranks test was used to test the prediction that $H_E > H_{EQ}$ in *Rs* and not *Rc* or *Ra*. This test was recommended by Piry et al. (1999) because it is most powerful and is robust for tests using < 20 polymorphic loci.

The second analysis was the qualitative evaluation of allele frequency distributions, which was originally described by Luikart et al. (1998). This mode-shift indicator test was used to determine the shape of the frequency distribution of alleles and inspect it for a signature distortion. Allelic frequency distributions in healthy populations should be L-shaped because of the presence of many low-frequency alleles. The distortion to this L-shape in populations that have undergone a bottleneck results from a greater probability of loss for low-frequency alleles (Luikart et al. 1998).

Results

Severe reductions in polymorphism were found in three loci for *R. sevosa* (two loci with only two alleles and one with three) compared to much higher levels in the non-isolated populations of *R. areolata* and *R. capito* (Table 3). For individual loci, genetic variation was generally less in the isolated population (*Rs*) compared to the non-isolated populations (*Ra* and *Rc*). Observed heterozygosity (H_O) and unbiased estimates of expected heterozygosity (H_E) were lower for six of seven loci in *Rs*. H_O ranged from 0.174 to 0.826 for *Rs*, 0.595 to 0.946 for *Rc*, and 0.625 to 0.875 for *Ra* (Table 3). H_E ranged from 0.196 to 0.783 for *Rs*, 0.531 to 0.847 for *Rc*, and 0.650 to 0.841 for *Ra* (Table 3). No loci deviated significantly from HWE at the 5% significance level after Bonferroni correction. Mean H_E and H_O were much lower in *Rs* than *Ra* or *Rc* (Table 4). Differences were found between *Rs* and *Rc* for H_O (marginally significant; paired *t*-statistic 2.22, df = 6, $P = 0.068$) and H_E (significant; paired *t*-statistic 2.46, df = 6, $P = 0.049$).

The number of alleles per locus (allelic richness; *R*) was fewer in *Rs* than *Ra* or *Rc* for

Table 3. Genetic variability at each microsatellite locus for *Rana sevosa* at Glen's Pond, Harrison County, Mississippi (*Rs*; n = 46), *R. capito* at Archbold Biological Station, Highlands County, Florida (*Rc*; n = 37), and *R. areolata* at Atoka County, Oklahoma (*Ra*; n = 32). Data are as follows: *A* = observed number of alleles per locus; *R* = corrected (via rarefaction) number of alleles per locus; range = size range of alleles.

Populations		Locus						
		*Rs*B12	*Rs*C02	*Rs*C05	*Rs*Ms3	*Rs*F01	*Rs*E03	*Rs*A05
Rs	*A (R)*	7 (6.8)	2 (2.0)	2 (2.0)	5 (4.8)	4 (3.6)	5 (4.8)	3 (3.0)
	range	8	3	3	17	4	5	3
	H_E	0.779	0.502	0.196	0.783	0.715	0.678	0.423
	H_O	0.826	0.435	0.174	0.761	0.609	0.674	0.435
Rc	*A (R)*	7	5	4	9	9 (8.8)	10 (9.7)	9 (8.8)
	range	8	5	5	14	9	16	15
	H_E	0.791	0.777	0.531	0.801	0.847	0.622	0.681
	H_O	0.838	0.703	0.649	0.703	0.946	0.595	0.487
Ra	*A*	–	–	–	–	9	7	6
	range	–	–	–	–	9	12	6
	H_E	–	–	–	–	0.841	0.732	0.650
	H_O	–	–	–	–	0.875	0.625	0.688

Table 4. Mean data and inbreeding coefficient (F_{IS}) for seven microsatellite loci for *Rana sevosa* at Glen's Pond, Harrison County, Mississippi (*Rs*), *R. capito* at Archbold Biological Station, Highlands County, Florida (*Rc*), and *R. areolata* at Atoka County, Oklahoma site (*Ra*). *N* = # of individuals sampled; A_{TOT} = total # alleles observed for all loci; *A* = mean # of observed alleles per locus; *PA* = mean # of private alleles per locus; *R* = corrected (via rarefaction) mean # of alleles per locus; *S* = mean size range of alleles; *M* = ratio of corrected mean # alleles to size range of alleles (see Garza & Williamson 2001); Standard errors are indicated in parentheses. *Because only three loci amplified for *R. areolata*, $A_{TOT} = A$ x 7 for this population.

Population	*N*	A_{TOT}	*A*	*R*	*PA*	*S*	*M*	H_O	H_E	F_{IS}
Rs	46	28	4.00 (0.69)	3.85 (0.66)	1.3 (0.7)	8.3 (2.2)	0.661 (0.088)	0.518 (0.086)	0.598 (0.084)	0.122
Rc	37	53	7.57 (0.87)	7.48 (0.84)	3.7 (1.0)	10.3 (1.8)	0.784 (0.066)	0.703 (0.040)	0.729 (0.057)	0.026
Ra	32	51*	7.33 (0.88)	7.33 (0.88)	1.7 (0.9)	9.0 (1.7)	0.861 (0.138)	0.729 (0.055)	0.746 (0.075)	0.016

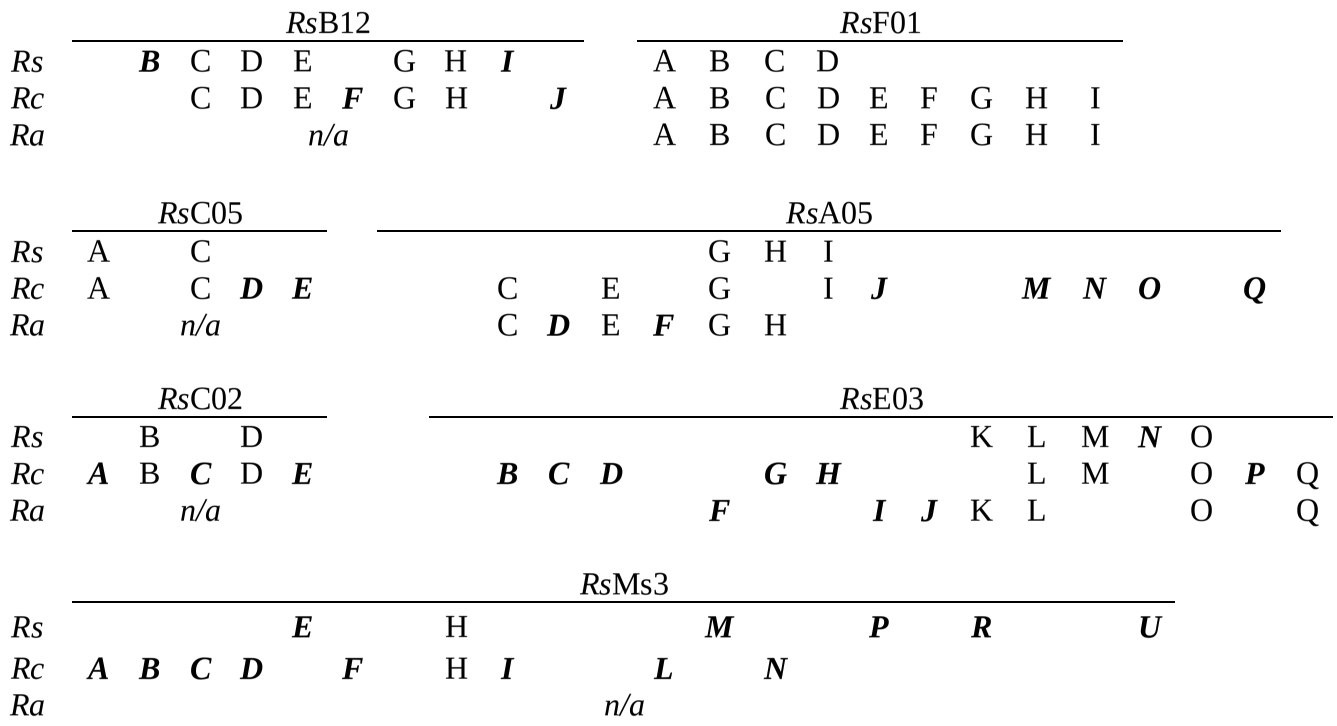
all loci, both for observed numbers and estimates of *R* corrected via rarefaction (Table 3).

Corrected *R* was significantly lower in *Rs* than in *Rc* (Wilcoxon's signed-rank test; Z = 2.37; *P* = 0.018). Allelic size ranges varied widely among loci and populations, and no clear pattern was detected among populations (Table 3). A total of 67 distinct alleles were revealed for the 7 loci. Of the 67 total alleles, 38 (57%) were unique to individual populations— 7 (10%) to *R. sevosa*, 26 (39%) to *R. capito*, and 5 (7%) to *R. areolata*.

Although populations differed considerably in allelic composition, only one locus (*Rs*Ms3) had private alleles that were outside the allelic range of other species by >1 repeat motif (Figure 2). Private alleles were represented at fairly low frequencies within each population (mean $\pm$ SE = 0.111 $\pm$ 0.03 for *Rs*, 0.114 $\pm$ 0.03 for *Rc*, and 0.110 $\pm$ 0.03 for *Ra*). The high number of private alleles can be explained in part by the apparent high frequency of allelic dropout in *Rs* (Figure 2). Allelic dropout was more severe in *Rs* in terms of allelic richness and *M* (Table 4).

Analyses of heterozygote excess indicated that a recent population bottleneck occurred in *Rs*. Under the IAM and TPM, *Rs* showed a significant heterozygote excess. All tests of mutation-drift equilibrium in *Ra* and *Rc* were not significant. *Rs* (but not *Rc* or *Ra*) exhibited an allele frequency shift as a result of loss of low-frequency alleles, which also indicated that a recent bottleneck occurred in *Rs* (Table 5). This is evidenced by a comparison of a frequency distribution of allelic proportions in the populations of *Ra*, *Rc*, and *Rs* (Figure 3).

Figure 2. Alleles for each microsatellite locus in *Rana sevosa* at Glen's Pond (*Rs*), *R. capito* at Ross Prairie (RP) at Archbold Biological Station (*Rc*), and *R. areolata* at Atoka County (*Ra*). Loci not amplifying in *R. areolata* are indicated by *n/a.* Unique alleles are indicated in bold italics.



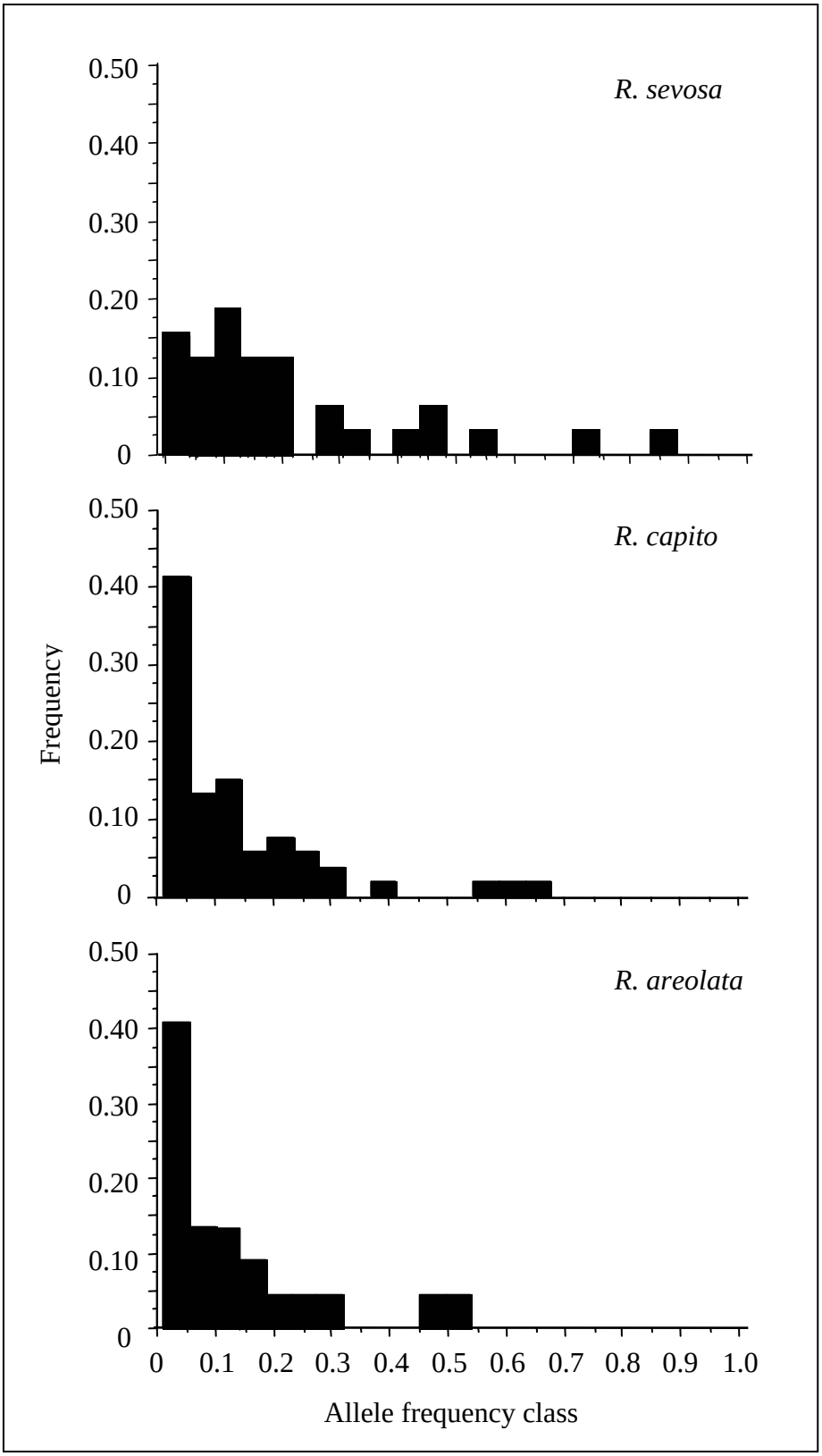


Figure 3. Frequency distribution of alleles for *Rs*, *Ra*, and *Rc* demonstrating a loss of low frequency alleles and mode-shift for *Rs*, which is indicative of a recent bottleneck.

Table 5. Tests of recent population bottlenecks for the isolated population of *R. sevosa* (*Rs*) and non-isolated populations of *R. areolata* (*Ra*) and *R. capito* (*Rc*). Data were evaluated using Bottleneck Version 1.2.02. Presented in the table are *P*-values for Wilcoxon's signed-ranks tests under three mutation models (IAM, SMM, and TPM). Shape of distribution represents a qualitative evaluation of allele frequency distributions. L-shaped = distribution expected under mutation-drift equilibrium; Shifted = characteristic mode-shift distortion of bottlenecked populations (Luikart et al. 1998).

Population	IAM	TPM	SMM	Shape of Distribution
Rs	0.004	0.027	0.406	Shifted
Rc	0.188	0.656	0.852	L-shaped
Ra	0.063	0.875	0.938	L-shaped

Discussion

The results indicate a negative genetic consequence of recent population reduction and isolation in the endangered Dusky Gopher Frog, *Rana sevosa*. All genetic analyses that were performed support this, including observed and expected heterozygosity, allelic richness, allelic dropout, *M* statistic, test of heterozygote excess, and mode-shift of allele frequency distribution. Finding a relationship between reduction in size of an isolated population and diminished genetic variation may seem trivial, but conservation implications are more general in terms of amphibians with similar life histories and for many other organisms that use the same breeding sites.

Species will respond differently to various levels of habitat fragmentation. Habitat specialists, which are typically rare, will be impacted more severely than habitat generalists, which are typically common. Whereas generalists may lose connections among a few populations as the habitat is subdivided, specialists have a much greater probability of having populations that are completely isolated. This study addressed a worst-case extreme in the range of habitat fragmentation— complete isolation. Populations that become completely isolated have high probabilities of extinction (e.g., Richter et al. 2003). In fact, other highly fragmented populations of *R. sevosa* in the vicinity of Glen's Pond have gone extinct in the past ca. 20–50 years (G. N. Johnson, pers. comm.).

Although habitat specialists are predisposed to extinction by human encroachment, non-isolated populations are buffered more against demographic, genetic, or cataclysmic extinctions than isolated species with similar life histories. In a multi-year, multi-pond study of *R. capito* in the Ocala National Forest (Ocala, Florida), Greenberg (2001) found

that *R. capito* had some level of recruitment within a complex of ponds in all five years of the study. Annual variation in recruitment was high among the eight ponds, although a few ponds had reproductive success in all five years and others had success in only two of five years. Many unmarked, recently metamorphosed frogs entered most ponds in years with high landscape-level reproductive success. Even though reproductive success was variable within individual ponds, the presence of multiple ponds allowed reproductive recruitment into the population for all five years. *R. capito* at the Savannah River Site (Aiken, South Carolina) appears subject to rapid local extinction due to small population sizes and infrequent recruitment of juveniles (Semlitsch et al. 1995). Nevertheless, these populations are not isolated and continue to persist (J. W. Gibbons, unpubl. data).

Amphibian breeding populations that are part of a larger metapopulation complex should have higher genetic variation and would be less likely to go extinct than isolated populations where no recruitment from outside sources is possible. Causes of extinction could be a result of genetics (e.g., mutational meltdown or reduction of genetic variation and consequently fitness), demographics (e.g., severe reduction in reproductive recruitment and population size), or catastrophes (e.g., single event that extirpates entire population). In a metapopulation, populations have reduced inbreeding, increased recruitment from nearby populations, and can be recolonized from a nearby source following a local extinction (Skelly et al. 1999; Marsh & Trenham 2001). These features contrast strongly to the population of *R. sevosa* at Glen's Pond because isolation of this population eliminates the possibility of a rescue effect occurring naturally and enhances inbreeding and demographic difficulties.

Allelic richness and dropout are more sensitive indicators of population bottlenecks and genetic erosion in small populations than is heterozygosity (i.e., small population size reduces heterozygosity less than it does allelic richness) (Allendorf 1986; Cornuet & Luikart 1996; Garner et al. 2003). That is, heterozygosity is relatively insensitive to the number of different genotypes at a locus (Allendorf 1986). In fact, populations could have similar levels of heterozygosity and greatly different allelic diversity and patterns of allelic dropout (Barker 2001). The number and identity of alleles at a locus contain more information than variation in terms of proportion of heterozygous individuals. While sufficient heterozygosity is necessary for short-term population survival, long-term persistence requires allelic diversity (i.e., frequencies and numbers) because this is the variation upon which future selection can act (Kimura & Crow 1964; Allendorf 1986).

Rana sevosa exhibited a high allelic dropout and consequently a significant heterozygote excess (see Cornuet & Luikart 1996), mode-shift in allele frequencies (see Luikart et al. 1998), and low *M* (mean ratio of number of alleles to range in size of alleles; Garza & Williamson 2001). These three analyses indicated a recent population bottleneck. Following population bottlenecks, populations will return to a balanced state in which allelic diversity and frequency distributions are in mutation-drift equilibrium (i.e., they result from an equilibrium between mutations and genetic drift). Alleles that are typically lost during bottlenecks (or at least have a higher likelihood of being lost) are those in low frequency. The heterozygote excess test examined the data for an imbalance in heterozygosities, whereas the mode-shift indicator examined the frequency distribution of alleles, primarily for the signature loss of low frequency alleles as a signal of a recent bottleneck. Neither of the non-isolated populations (*Rc* and *Ra*) exhibited significant

heterozygote excess under the IAM, SMM, or TPM mutation model. Extent of heterozygote excess in *Rs* was much greater than *Ra* and *Rc* and was significantly different from that expected under mutation-drift equilibrium. In other words, a higher heterozygosity existed in the *Rs* population than one would expect if the population were stable and the level of heterozygosities were as expected under mutation-drift equilibrium.

Garza & Williamson (2001) surveyed the literature and calculated *M* for microsatellite studies of species with known demographic histories to evaluate the utility of their statistic as an indicator of population status. *M* for stable populations ranged from 0.823–0.926 (mean $\pm$ SE = 0.873 $\pm$ 0.011), whereas reduced populations ranged from 0.599–0.693 (mean $\pm$ SE = 0.641 $\pm$ 0.010). In this study, *M* for *Rs* (0.645) was just above the average for reduced populations and well within the range. Conversely, *M* for *Rc* (0.784) and *Ra* (0.861) were much higher, well above the range for reduced populations, and just below (*Rc*) or within (*Ra*) the range for stable populations.

Interpretation of patterns in private alleles (i.e., those that are unique to single populations) can be problematic. Most microsatellite studies within single species find unique alleles among populations (e.g., Goodman et al. 2001; Burridge & Gold 2003; Lucchini et al. 2004). Because nearly all genetic studies only sample populations and are not able to determine every allele, private alleles can often be attributed to sampling error, especially because they are typically in low frequency. When one must use sister species to evaluate population genetic variation for rare species, it is difficult if not impossible to determine if private alleles are a result of species-specific differences. Most private alleles in this study were within the allelic range of other populations (all

except locus *Rs*Ms3), which preliminarily suggests that these alleles are not due to interspecific differences but to sampling artifacts or loss due to genetic drift and/or bottlenecks.

A previous population genetic allozyme study of *R. sevosa* found an average heterozygosity of 0.077 (Young & Crother 2001). This is much lower than for microsatellites, but heterozygosity for allozymes is relatively low for vertebrates (typically < 0.10; (Nei 1987). Young & Crother (2001) found an average heterozygosity for 17 *R. capito* and 3 *R. areolata* populations of 0.072 (0.010 SE; range = 0–0.15; N = 1–10). Most populations sampled were isolated to semi-isolated across the geographic distribution, but many (including *R. sevosa*) fell within the range of "typical" for vertebrates (Young & Crother 2001). *R. sevosa* also was at average for allelic richness (33; overall mean $\pm$ SE = 32.5 $\pm$ 0.63) and for number of polymorphic loci (6 of 26; overall mean $\pm$ SE = 6.1 $\pm$ 0.50). Genetic comparisons among populations can be biased by unequal sample sizes (Leberg 2002). Therefore, genetic variability in *R. sevosa* (N = 10) is likely to be inflated relative to other populations, which had much lower sample sizes (mean $\pm$ SE sample size = 4.1 $\pm$ 0.6; range = 1–8). Overall, genetic variation for *R. sevosa* in 1995 was at or just below the average of other gopher and crawfish frogs and within the range "typical" for vertebrates, but this is probably an optimistic interpretation potentially biased by unequal sampling.

Implications for Conservation and Management

Following the listing of *R. sevosa* as US Endangered, a recovery team was assembled to develop conservation priorities and recovery plans. The team is composed of research

scientists and other individuals from diverse fields (vertebrate and plant ecology, forest management, evolutionary biology, population genetics, parasitology, and epidemiology) and multiple organizations, including the US Fish and Wildlife Service, the US Forest Service, the Memphis Zoo, the Mississippi Department of Wildlife, Fisheries, and Parks, and multiple universities (University of Memphis, University of New Orleans, University of Oklahoma, University of Southern Mississippi, and Towson University). Funding for research and management is derived primarily through the US Fish and Wildlife Service and the US Forest Service.

Current conservation efforts being implemented through the recovery team include monitoring the demographic status of populations, continuing surveys for unknown populations, translocating eggs to a newly constructed pond (if experiments demonstrate the pond is suitable), restoring of historic sites with potential for translocations, implementing periodic controlled burning of ponds and upland habitats, maintaining buffer zones around current and potential breeding ponds, using ground water to augment pond levels when they reach a critical minimum, and "farming" tadpoles in cattle tanks to be released at breeding sites to supplement natural reproductive recruitment. A future goal is to use stock from the two known extant populations to reintroduce the species to historic localities in which it has gone locally extinct.

Based on the neutral genetic variation of the Glen's Pond population, the following management strategies should be incorporated into the long-term recovery plan for *R. sevosa*. An outbreak of a *Perkinsus*-like disease during the 2003 breeding season caused a high (> 90%) rate of larval mortality. Eggs selected for rearing tadpoles in cattle tanks to avoid complete reproductive failure due to disease outbreaks should be hosen so as to

maximize genetic diversity by taking a portion from each of every egg mass deposited in the pond. Genetic diversity of the eggs should be determined using microsatellite loci from this study. The other population of *R. sevosa* (Mike's Pond), which was discovered recently (May 2004), is located in an upland that is bisected by a road and other human development. It has many genetic affinities with the Glen's Pond population but also has a few unique alleles (SCR, unpubl. data). Because of this, more genetic comparisons between the populations need to be performed followed by careful consideration of transplanting eggs from Mike's Pond to Glen's Pond to enhance genetic variability at the primary breeding site. Captive populations are being maintained in zoos. The decision of whether to use these populations to supplement natural populations will depend on future circumstances. Regardless, captive individuals should be genotyped as they are brought into captivity in the event that reintroduction or supplementation occurs.

Conclusions

Population genetic diversity in *Rana sevosa* has been eroded by isolation (lack of gene flow), a recent and apparently ongoing population bottleneck, loss of alleles via genetic drift, and inbreeding due to reduction in population size. A long-standing debate questions the importance of and interaction between genetic and demographic factors for species' survival (Lande 1988; Frankham 1995). For example, bottlenecks severely reduce population sizes and have demographic and genetic consequences. Genetic consequences include increased inbreeding, expression of deleterious mutations, and loss of genetic variability. Demographic stochasticity increases and extremes in population fluctuations have a higher likelihood of approaching zero, especially in amphibians

(Alford and Richards 1999). These genetic data will greatly enhance conservation planning of the species, and management decisions can be made that alleviate both demographic and genetic concerns. For example, establishing subpopulations nearby should decrease the likelihood of extinction due to catastrophe, allow for differential juvenile recruitment into the population among ponds, and maintain higher genetic variation over time.

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CHAPTER 2

TEMPORAL GENETIC VARIATION IN A BOTTLENECKED POPULATION OF ENDANGERED FROG, *RANA SEVOSA*

Introduction

Fluctuations in genetic variation over time can have consequences for populations, especially those with small size. Even when demographic history is well documented, genetic consequences are difficult to predict and interpretations of contemporary genetic variation rely on comparison with other populations of known demographic history or status. For example, assessment of loss in genetic variability following population size reduction is difficult when preexisting data are absent. Insight can be gained from temporal studies that focus on stability of genetic structure through loss and addition of alleles, changes in allele frequencies, dynamics of rate of immigration and emigration (i.e., the proportion of current population composed of descendents from the historic population), and relative importance of demographic factors such as population contraction or expansion on genetic variation (Jordan et al. 1992; Tessier & Bernatchez 1999; Pertoldi et al. 2001; Guinand et al. 2003; Arnaud & Laval 2004).

The advent of the polymerase chain reaction (PCR) in the 1990s enabled DNA studies using historic samples. A limited number of temporal genetic studies exist and most are of fish populations, primarily salmonids (e.g., Jordan et al. 1992; Miller & Kapuscinski 1997; Nielsen et al. 1997; Tessier & Bernatchez 1999; Ruzzante et al. 2001; Hansen 2002; Heath et al. 2002; Meldgaard et al. 2003; Østergaard et al. 2003; Bartron & Scribner 2004). Temporal studies within natural populations exist for only a few other taxa— isopods (Lessios et al. 1994), *Daphnia* (Limburg & Weider 2002), mosquitoes (Huber et al. 2002), snails (Arnaud & Laval 2004), mammals (Taylor et al. 1994; Pertoldi et al. 2001), and birds (Mundy et al. 1997). Additionally, many studies only assay two years during the study period (beginning and end) and extrapolate to other years, which assumes a linear relationship over the span of time between the two years (e.g., Lessios et al. 1994; Tessier & Bernatchez 1999; Meldgaard et al. 2003; Arnaud & Laval 2004; Bartron & Scribner 2004).

DNA studies of temporal genetic variation are possible for many organisms because DNA is not degraded by many methods of specimen or tissue preservation and can be PCR amplified. For example, target DNA has been successfully amplified for population genetic studies from dried fish scales (85 years old; Hansen et al. 2002), mammalian skin (ca. 106 years old; Taylor et al. 1994) and skulls (101 years old; Pertoldi et al. 2001), and resting eggs of *Daphnia* (ca. 200 years old; Limburg & Weider 2002). A potential disadvantage of historic sampling is that interpretation is often limited by small sample sizes ($n < 10$).

Amphibians are particularly problematic for these types of studies because specimens are typically liquid preserved and formalin fixed prior to long-term storage in

ethanol (Simmons 2002). Some amphibian tissues (e.g., eggs and larvae) are permanently stored in formalin. Unlike dried tissues, which generally have recoverable DNA, tissues stored in liquid potentially have many associated sources of DNA degradation or impediments to extraction, including fluctuations in pH, exposure to formalin, and diluted solutions that lose buffering capacity. Only one genetic study exists that used historical amphibian tissues (Hoffman & Blouin 2004). Population genetic variation was compared between extant and historic populations in the periphery of the range of *Rana pipiens*, which was possible because ethanol-preserved liver and dried skin tissues were available through two museum collections (Hoffman & Blouin 2004).

Studies of genetic variation differ in their temporal resolving power, especially relative to spatial resolution. The relationship between temporal and spatial structure is complex and causes for differences are frequently system specific. In a genetic study of population structure in Atlantic cod (*Gadus morhua*), Ruzzante et al. (2001) found that populations were structured more spatially than temporally. This demonstrates the effectiveness of temporal genetic analyses in studying site fidelity and structure of localized demes. Although Ruzzante et al. (2001) found no temporal effect for structure, a finer-scale study within demes might have revealed previously unapparent changes in allele frequencies and allelic compositions.

The temporal component of genetic variability may not be as obvious or important in large, wide-ranging populations as for restricted and small populations (Richards & Leberg 1996; Miller & Kapuscinski 1997; Nielsen et al. 1997, 1999; but see Heath et al. 2002). Studies of spatial structure in large populations often assume that genetic

structure is stable over time (Nielsen et al. 1999; Heath et al. 2002), and this should hold as long as no major deviation from mutation-drift equilibrium occurs. This assumption might be valid for short-term studies of large populations but has been shown to be erroneous over long time periods (e.g., Nielsen et al. 1997; Limburg & Weider 2002). Over sufficient time, significant genetic differentiation can occur among years (Limburg & Weider 2002). Under these circumstances, populations tend to be more temporally than spatially structured (Nielsen et al. 1997; Hansen et al. 2002). In small populations, temporal changes in heterozygosity and allelic richness are often more severe (e.g., Nielsen et al. 1997; Miller & Kapuscinski 1997; Giunand et al. 2003).

The context of hypotheses will drive what temporal scale is appropriate for particular studies. If one is interested in conservation genetics of rare taxa, differences in genetic variation should be obvious at a much finer temporal scale, especially compared to studies of common species with large population sizes. In populations with low population size, temporal genetic structure could vary drastically among years due to enhanced effects of genetic drift and inbreeding. Therefore even short-term studies of small populations should reveal inbreeding history, population status, and temporal trend of genetic variation. For example, a genetic study of Atlantic salmon (*Salmo salar*) found that a contemporary sample and a sample collected 60 years earlier of an endangered population clustered together when compared to the spatially closest existing population (Nielsen et al. 1997). Nielsen et al. (1999) found that *S. salar* populations with moderate gene flow sampled from 1913 to 1989 had greater spatial relative to temporal structure.

Dusky Gopher Frogs, *Rana sevosa*, are listed as Endangered under the US

Endangered Species Act and Critically Endangered on the Red List of the International Union for Conservation of Nature and Natural Resources (USFWS 2001; IUCN 2004). Although their geographic range once extended throughout the coastal plain of Louisiana, Mississippi, and western Alabama, the frogs have been reduced to only two known populations. Extensive ecological and demographic data exist for one of the populations (Glen's Pond; Harrison County, Mississippi) (Richter & Seigel 2002; Richter et al. 2003), whereas the status of the other population (Mike's Pond; Jackson County, Mississippi) is unknown because it was only recently (March 2004) discovered.

Annual breeding-migration events between the forest (non-breeding season habitat) and pond facilitate sampling adults of the population. Adults are capable of returning to breed over five seasons but the average rate of return among years is low (mean = 1.2 years; Richter & Seigel 2002). Based on the number of egg masses deposited annually over the past 10 years (mean = 38 $\pm$ 11.5; range = 0–130), the numbers of adults annually migrating to the pond to breed over a three-year period (mean = 91; range = 67–110), and the high annual population turnover, mean annual adult population size is approx. 60–100 individuals (Richter & Seigel 2002; Richter et al. 2003).

In genetic studies and conservation, the number of adults that breed and contribute to future generations (effective population size, N_e) is more important than the total adult population size because N_e is what drives genetic dynamics in populations. Many factors influence N_e, including total population size, migratory pattern (i.e., between populations), and mating system. Because individual females deposit a single, obvious clutch per year, the female component of N_e is measurable, and the number of breeding males is the unknown. Males are known to interact aggressively (Doody et al., 1995),

which suggests territoriality or at least potential for associated behaviors that permit multiple mating by males, as is known for other ranids (e.g., Wells, 1978). Based on the number of egg masses deposited from 1995–2004 (Table 1) and the number of males that contributed, range of annual N_e would be 22–131 if one male fertilized every clutch, which is highly unlikely (range = number of clutches [thus females] plus one male), or 42–260 if every clutch was fertilized by a different male, which is also unlikely (range = number of clutches multiplied by two) (Table 1). Regardless, effective population size is small, fluctuates over time because of high annual population turnover and annual variation in reproductive success, and is declining overall. In fact, a fairly large decline occurred between 1998 and 2004, the effects of which were most obvious after 2001. The decline appears to be caused by a three year drought (gopher frogs rely heavily on annual recruitment; Richter et al. 2003) and on the establishment of two diseases whose effects are yet to be realized. How this affects the dynamics of annual genetic variation will determine long-term population viability. This study addressed temporal variation of genetic structure across this period of known demographic disturbance (1995–2004) in an isolated population of dusky gopher frogs, *Rana sevosa*.

Genetic variation of microsatellite DNA loci was examined four times over ten years using historical tissue samples from within the same population. Previous genetic analyses revealed that a population bottleneck occurred prior to 2001 and was possibly ongoing (Chapter 1). The present study had two primary objectives. One was to examine levels and stability of temporal genetic polymorphism. The second was to address the timing of the bottleneck, and whether the genetic signature predicts an

ongoing and intensifying bottleneck. Temporal shifts in allele frequency, allele richness, and heterozygosity due to demographic disturbance were assessed for 1995, 1997, 2001, and 2004. Additionally, population structure and tests of bottlenecks will be compared among years. The effects of population decline on genetic variation will be discussed in context of demographic data of this population and species.

Table 1. Range of annual effective population sizes (N_e) in *Rana sevosa* in Harrison County, Mississippi based on egg mass counts from 1995 to 2004 (Thurgate et al. 2002; Richter et al. 2003; T. Mann unpubl. data). Range of N_e = # clutches + 1 male (lower limit) to # clutches X 2 (upper limit).

Year	# Clutches	Range of N_e
1995	130	131–260
1996	37	38–74
1997	58	59–116
1998	37	38–74
1999	5	–
2000	0	–
2001	36	37–72
2002	28	29–56
2003	21	22–42
2004	28	29–56

Methods

Collection of samples

Samples of *R. sevosa* (N = 232) were collected from an isolated population as follows. Adults were collected by hand in 1994–95 (n = 10) and via a drift fence completely encircling the breeding pond with pitfall traps in 1997 (n = 106) and 2001 (n = 46). In 2004, eggs were collected by hand from each of 15 egg masses (n = 72) and reared to free swimming larvae in the laboratory. Samples of adults were collected by clipping one toe except in 1995 (collection of whole frogs; see Young & Crother 2001). All samples except 2004 were collected prior to the species being listed as Endangered. Samples in 2004 were collected under Department of Interior, US Fish and Wildlife Service, Federal Fish and Wildlife Permit number TE 072081-2.

Genetic data collection

DNA was extracted from each individual using Qiagen® DNEasy tissue kits. For each individual, DNA was PCR amplified for eight microsatellite loci. Genetic data were collected using an ABI Prism® 310 Genetic Analyzer (Applied Biosystems) by pooling samples of PCRs for three loci per individual using different fluorescently labeled primers for each multiplexed locus. Allele lengths were scored using GeneScan® Analysis Software version 3.1.2 (Applied Biosystems).

Genetic analyses

Statistical and descriptive genetic analyses were performed for 1997, 2001, and 2004. Because of the low sample size for 1995 (n = 3–8 per locus), these data were

descriptively (and not statistically) evaluated and primarily were included to potentially identify alleles lost. Therefore, 1995 data were restricted to figures and tables only to examine provisional allele frequencies and heterozygosity. Genetic calculations and analyses were performed using FSTAT v. 2.9.3.2 (Goudet 1995, 2002). Observed heterozygosity (H_O), allelic richness (*R*), expected heterozygosity (H_E), and Wright's inbreeding coefficient (F_{IS}) were calculated. For all except H_O, unbiased estimators were calculated by standardizing each to the size of the smallest sample (2001; n = 46) as described in Chapter 1. Deviations from Hardy-Weinberg equilibrium (HWE) for each locus and population were examined using exact tests (10000 permutations) with Bonferroni corrections (Guo & Thompson 1992; Raymond & Rousset 1995).

The following measures of genetic variability were compared among years. Loci were evaluated for linkage disequilibrium (i.e., non-independent assortment during meiosis) because loci must be independent or statistical analyses may not be valid. Differences in unbiased H_E were evaluated using an ANOVA on arcsine-transformed data (Nei 1987). Statistical analyses will have low power due to low sample sizes (n= 8), and could potentially violate the assumption of independence of samples because sampling was of the same population over eight years. However, *R. sevosa* has a low rate of return of adults among years, an even lower rate across eight years, and a heavy reliance on recruitment of juveniles for sustained population size and persistence (Richter & Seigel 2002; Richter et al. 2003). Differences in F_{IS} over all loci were compared based on 28,000 randomizations and Bonferroni corrections. Correlation coefficients were calculated for frequencies of each allele observed in the population between each year.

For microsatellite loci, measures of genetic differentiation can be based on allelic states (e.g., F_{ST}; Wright 1951; Weir & Cockerham 1984) or based on differences in allele sizes (e.g., R_{ST}; Slatkin 1995). F_{ST} assumes the infinite alleles model of mutation, and R_{ST} assumes the stepwise mutation model (see Chapter 3 for details about models). Although R_{ST} was developed because F_{ST} was thought to be a biased estimator of population structure, the appropriateness of each measure depends on the mutation model under which each locus evolved (e.g., Broughton & Gold 1997). Thus, both measures were used.

The following calculations and statistics were performed using ARLEQUIN v. 2.000 (Schneider et al. 2000). To assess the component of gene diversity due to allelic variation between temporal samples, an analysis of molecular variance (AMOVA; Michalakis & Excoffier 1996) was performed using each of F_{ST} and R_{ST} in distance calculations. Also, pairwise F_{ST} (Weir & Cockerham 1984) and R_{ST} (Slatkin 1995) calculations were compared to test for genetic differentiation among years.

Two analyses implemented in BOTTLENECK v. 1.2.02 (Cornuet & Luikart 1996; Piry et al. 1999) were used to test for recent population bottlenecks as described in Chapter 1. Briefly, the first test takes advantage of allelic diversity being reduced at a faster rate than heterozygosity subsequent to severe reductions in population size. For each locus, heterozygosity excess was evaluated by comparing H_E to heterozygosity expected at mutation-drift equilibrium (H_{EQ}). Populations that have not undergone a recent change in population size will have $H_E = H_{EQ}$. As for Chapter 1, H_{EQ} was estimated under three models of mutation: infinite alleles model (IAM), stepwise mutation model (SMM), and two-phase mutation model (TPM) based on 5000

iterations. The TPM was used with 95% single-step mutations (5% multi-step) and a variance among multiple steps of 12% as recommended by Piry et al. (1999). Following estimation of H_{EQ}, a two-tailed Wilcoxon's signed-ranks test was used to test the hypothesis that $H_E = H_{EQ}$ in 1997, 2001, and 2004. For the second analysis, the mode-shift indicator test was used to determine the shape of the frequency distribution of alleles. Populations that have undergone bottlenecks have distorted distributions caused by alleles at low frequencies being lost at higher rates than others (Luikart et al. 1998).

Results

Allele frequencies of the eight microsatellite loci qualitatively varied among years (Figure 1). Some of the alleles exhibited linear temporal trends in frequency (i.e., steadily increased or decreased over the entire study period), the most obvious being alleles of the two biallelic loci (Figure 1). No obvious shifts in the rarest or most common alleles occurred across the study period. Based on an examination of correlation coefficients of allele frequencies between years, alleles exhibited temporal stability in frequency and one to one relationships of allele frequency between each year, except for locus *Rsev*B12 (Figure 2). When correlations of allele frequency between years were examined for loci separately, this correlation held for all loci except *Rsev*B12— mean $\pm$ SE correlation coefficient between years for *Rsev*B12 = 0.625 $\pm$ 0.130 (range = 0.366–0.766) and for all other loci = 0.939 $\pm$ 0.014 (range = 0.836–1.000).

Locus *Rsev*F01 was not in HWE in 1997 (Table 2), and this single locus deviation

Table 2. Genetic variability at each microsatellite locus for 1995 (n = see table), 1997 (n = 106), 2001 (n = 46), and 2004 (n = 72). *A* = observed number of alleles per locus; *R* = corrected (via rarefaction for n = 46) number of alleles per locus; H_E = unbiased expected heterozygosity; H_O = observed heterozygosity; n = sample size. *Significant deviation from Hardy-Weinberg equilibrium (after Bonferroni correction).

		Locus							
		*Rs*B12	*Rs*C02	*Rs*C05	*Rs*Ms3	*Rs*F01	*Rs*E03	*Rs*A05	*Rs*G11
1995	*A*	4	2	2	3	2	3	2	3
	H_E	0.800	0.458	0.571	0.733	0.429	0.439	0.733	0.800
	H_O	0.800	0.625	0.000	0.800	0.500	0.500	0.667	0.333
	n	5	8	7	5	4	6	3	3
1997	*A (R)*	8 (7.4)	2 (2.0)	2 (2.0)	8 (6.9)	4 (4.0)	4 (4.0)	3 (3.0)	9 (8.4)
	H_E	0.824	0.486	0.289	0.735	0.687*	0.652	0.342	0.828
	H_O	0.792	0.543	0.302	0.667	0.529*	0.582	0.333	0.770
2001	*A*	7	2	2	5	4	5	3	8
	H_E	0.779	0.502	0.196	0.783	0.715	0.678	0.423	0.856
	H_O	0.826	0.435	0.174	0.761	0.609	0.674	0.435	0.870
2004	*A (R)*	6 (6.0)	2 (2.0)	2 (2.0)	5 (5.0)	4 (4.0)	4 (4.0)	3 (3.0)	8 (7.7)
	H_E	0.782	0.502	0.220	0.732	0.643	0.579	0.312	0.818
	H_O	0.786	0.375	0.250	0.676	0.542	0.611	0.319	0.750

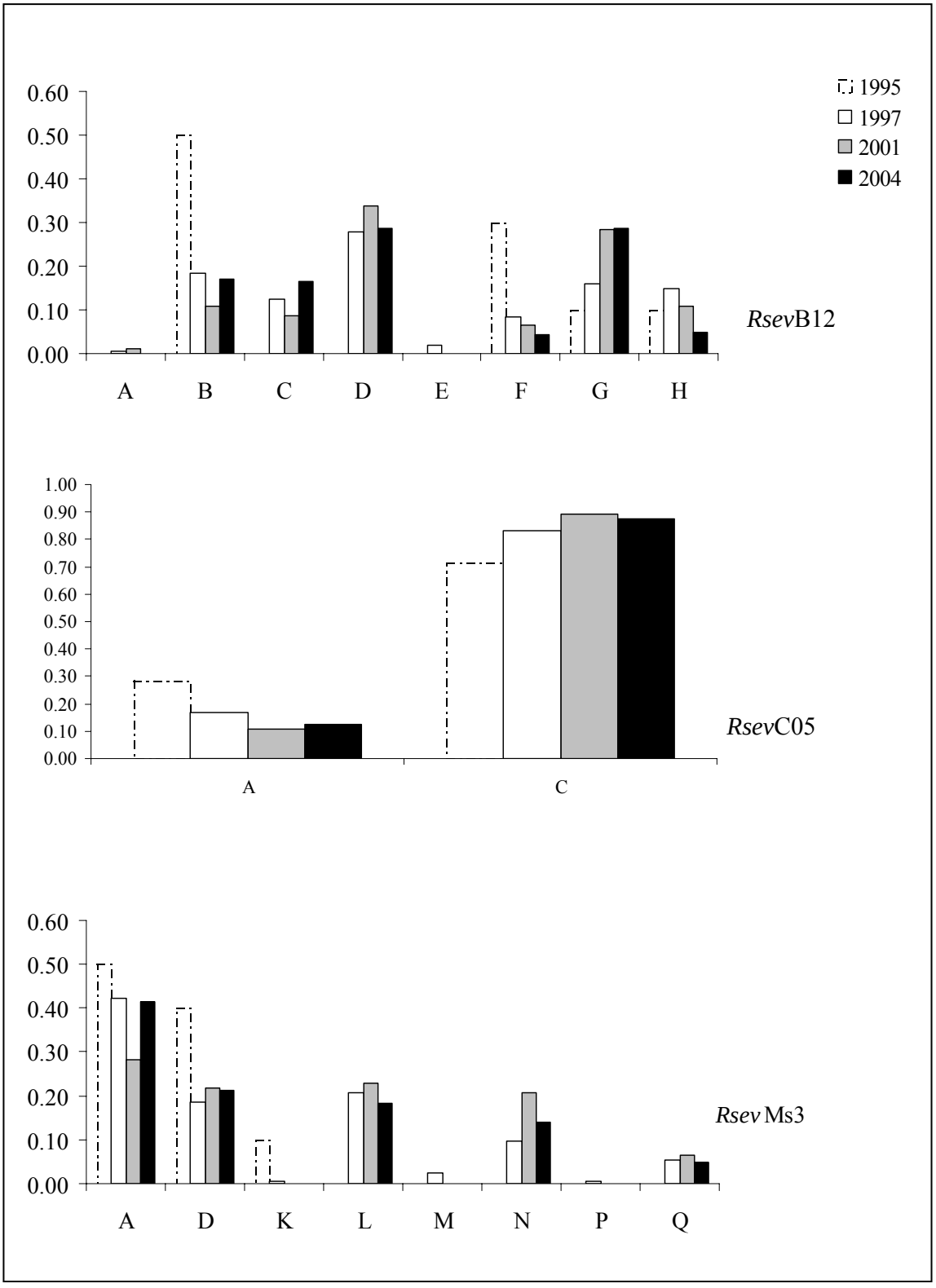

Figure 1. Allele frequencies of eight microsatellite loci for 1995, 1997, 2001, and 2004.

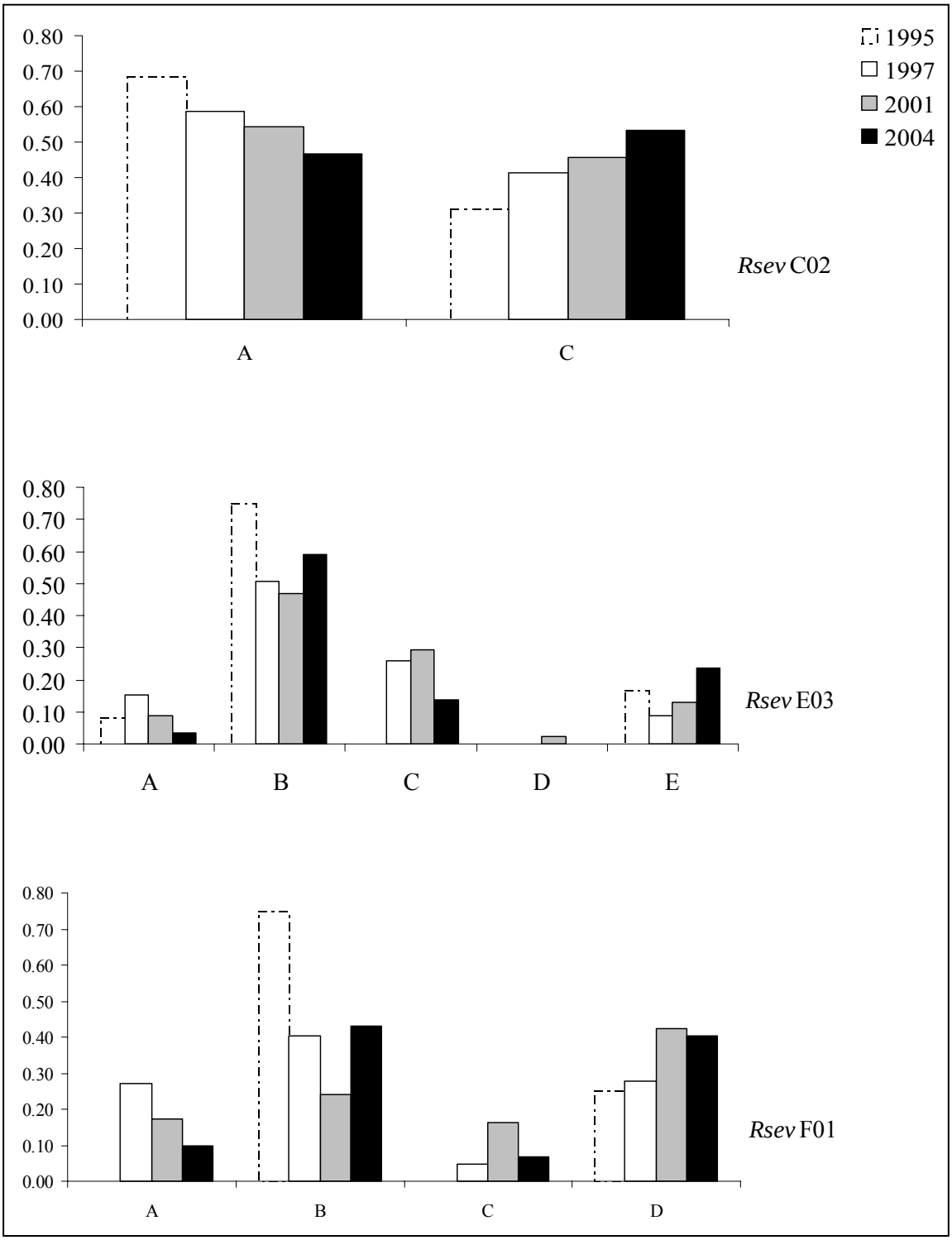

Figure 1. (continued)

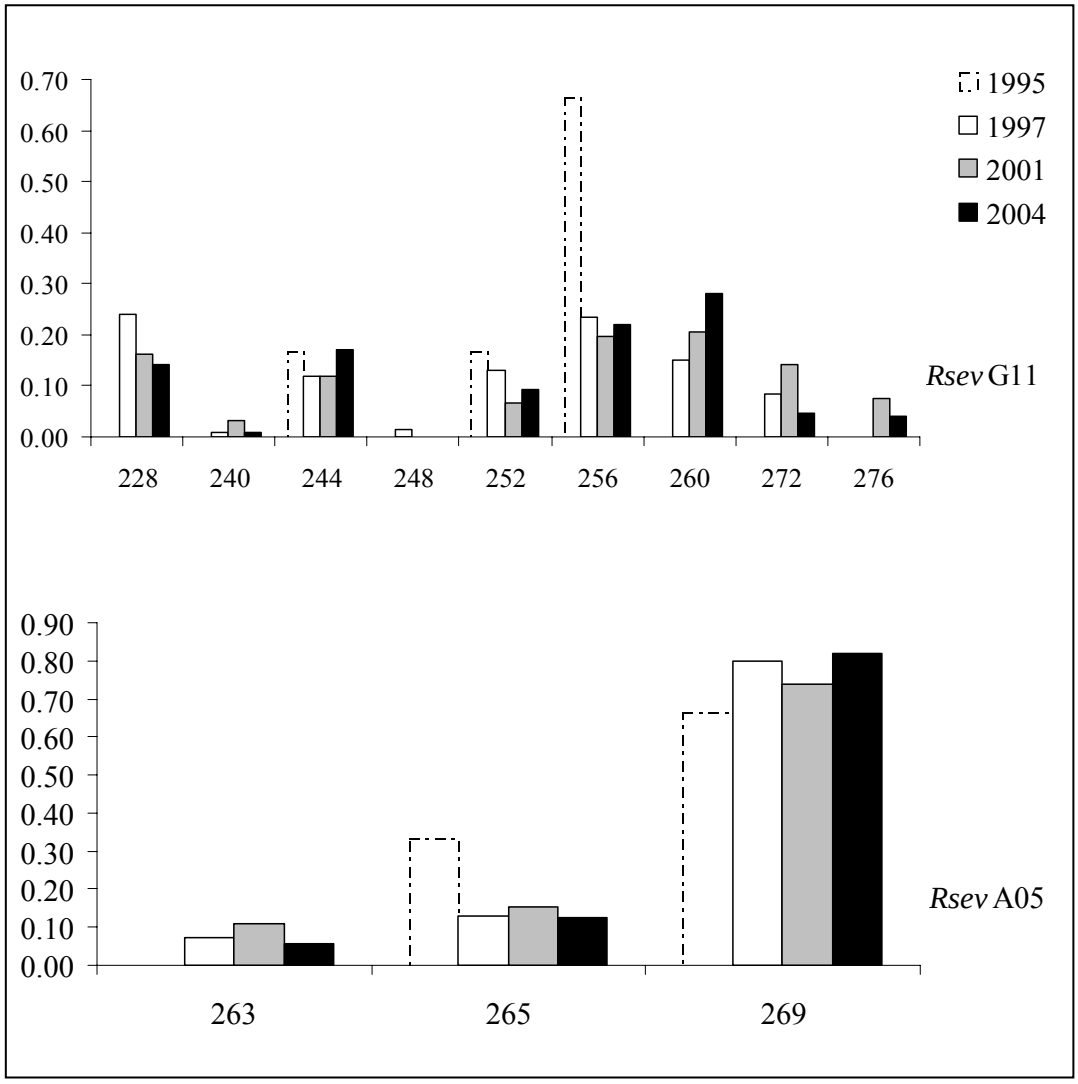

Figure 1. (continued)

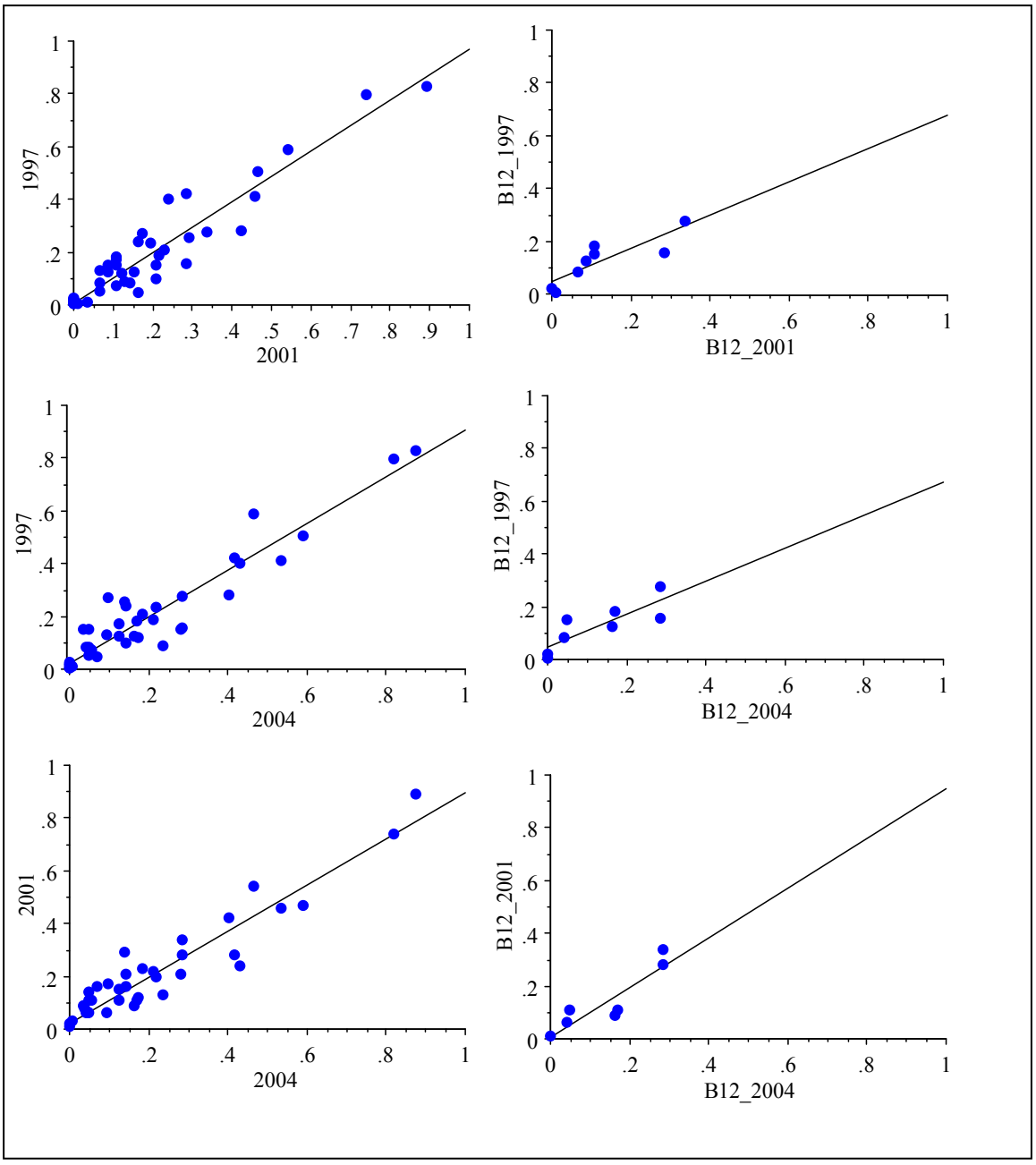

Figure 2. Bivariate regression graphs for correlations of allele frequencies between years. Alleles were pooled across loci.

drove the significant F_{IS} found for 1997 (Table 3). One pair of loci were in linkage disequilibrium for every year— *Rsev*F01 and *Rsev*B12. Temporal trends in observed and expected heterozygosity varied among loci (Table 2), but overall both H_E and H_O increased from 1997 to 2001 and decreased from 2001 to 2004 (Figure 3). This trend was found for all loci except *Rsev*C02 and *Rsev*C05 for H_O and *Rsev*C05 for H_E (Figure 4), which are both biallelic. However, no significant difference was found for either H_O ($F_{2,21} = 0.22$; $P = 0.803$) or H_E ($F_{2,21} = 0.10$; $P = 0.907$).

Table 3. Mean data and inbreeding coefficient (F_{IS}) for eight microsatellite loci for *Rana sevosa* at Glen's Pond during 1995, 1997, 2001, and 2004. N = # of individuals sampled; A_{TOT} = total # alleles observed for all loci; A = mean # of observed alleles per locus; R = corrected (via rarefaction) mean # of alleles per locus. Standard errors are indicated in parentheses. *Significant deficit of heterozygotes detected based on 28,000 randomizations and Bonferroni correction.

	N	A_{TOT}	A	R	H_O	H_E	F_{IS}
1995	8	21	2.63 (0.03)	–	0.528 (0.094)	0.621 (0.058)	0.149
1997	106	40	5.00 (1.02)	4.71 (0.89)	0.565 (0.064)	0.605 (0.074)	0.079*
2001	46	36	4.50 (0.78)	4.50 (0.78)	0.598 (0.084)	0.617 (0.079)	0.030
2004	72	34	4.25 (0.73)	4.21 (0.70)	0.539 (0.072)	0.574 (0.077)	0.061

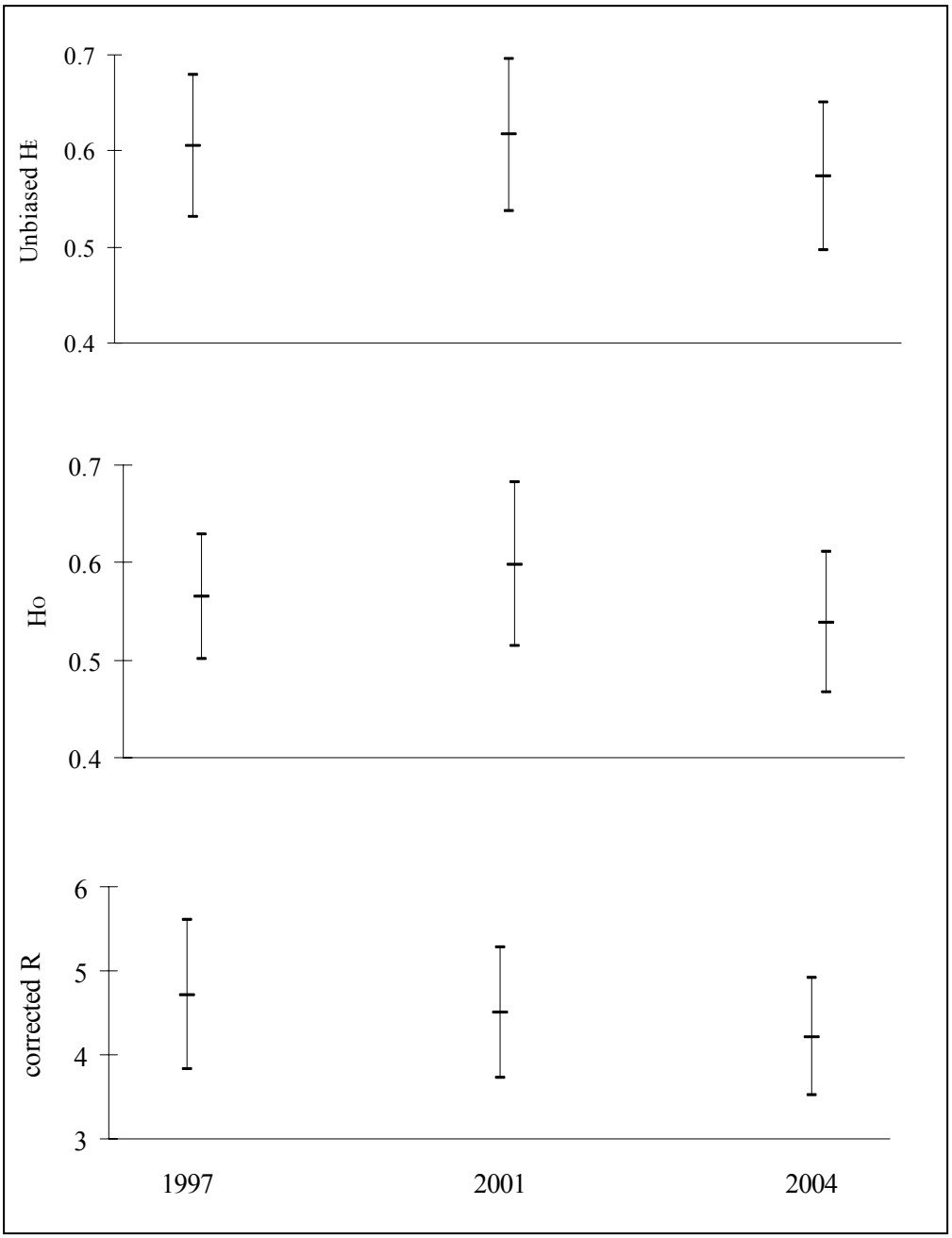

Figure 3. Mean ± SE of unbiased H_E, observed heterozygosity (H_O), and corrected mean number of alleles (*R*) for 1997, 2001, and 2004.

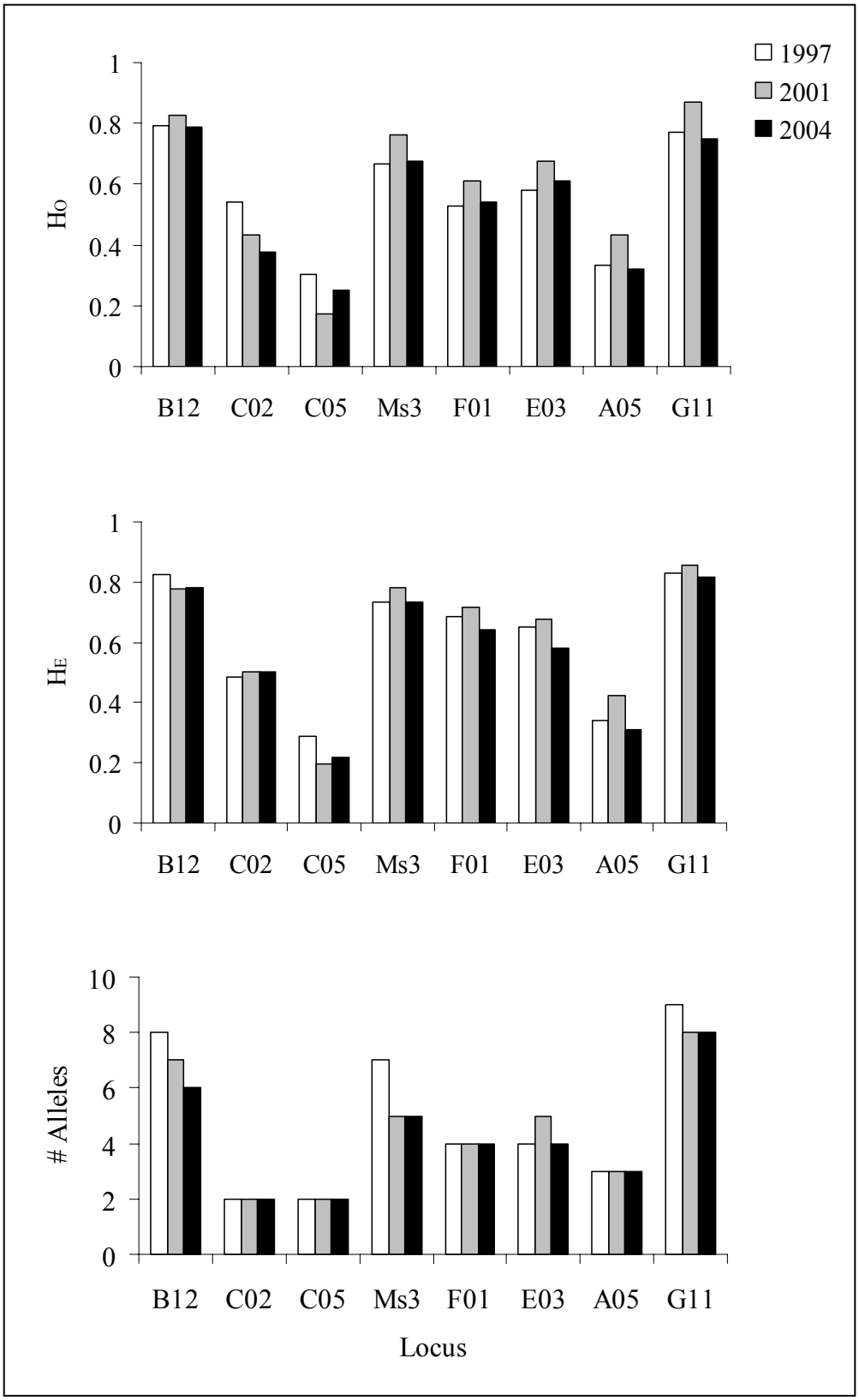

Figure 4. Temporal trends for H_E, H_O, and number of alleles for 1997, 2001, and 2004.

The mean corrected number of alleles per locus decreased over the study period (Figure 3; Table 3), but no significant difference was detected ($F_{2,21} = 0.069$; $P = 0.933$). A total of six alleles from three loci were only detected in 1997, and one other was only detected in 1997 and 2001 (Figure 5). Thus, seven alleles from three loci were lost over this eight-year period, and one was gained.

Figure 5. Alleles for eight microsatellite loci in *Rana sevosa* at Glen's Pond for 1997, 2001, and 2004. A "●" indicates a missing allele.

	*Rsev*B12							
1997	A	B	C	D	E	F	G	H
2001	A	B	C	D	●	F	G	H
2004	●	B	C	D	●	F	G	H

	*Rsev*F01			
1997	A	B	C	D
2001	A	B	C	D
2004	A	B	C	D

	*Rsev*C05		
1997	A		C
2001	A		C
2004	A		C

	*Rsev*E03				
1997	A	B	C		E
2001	A	B	C		E
2004	A	B	C		E

	*Rsev*C02		
1997	A		C
2001	A		C
2004	A		C

	*Rsev*A05			
1997	A	B	C	
2001	A	B	C	
2004	A	B	C	

	*Rsev*G11									
1997	A		C	D	E	F	G	H	I		L	●
2001	A		●	D	E	●	G	H	I		L	M
2004	A		●	D	E	●	G	H	I		L	M

	*Rsev*Ms3									
1997	A			D			K	L	M	N		P	Q
2001	A			D			●	L	●	N		●	Q
2004	A			D			●	L	●	N		●	Q

A significant overall structure was found due to variation between temporal samples using the AMOVA based on F_{ST} (0.0137) and R_{ST} (0.0136) estimates (both $P < 0.001$). These differences appear to be primarily due to two loci, *Rsev*E03 and *Rsev*F01, which were the only two loci that contributed to significance after Bonferroni correction (Table 4). For pairwise F_{ST} and R_{ST} comparisons, all comparisons were significant for each pair of years except for R_{ST} between 1997 and 2001 (Table 5).

Table 4. F_{ST} and R_{ST} values for individual and all loci. Bonferroni-adjusted alpha = 0.0063. Values shown in bold were statistically significant.

Locus	F_{ST}	*P*-value	R_{ST}	*P*-value
*Rsev*B12	0.009	0.016	0.009	0.014
*Rsev*C02	0.011	0.096	0.011	0.100
*Rsev*Ms3	0.004	0.159	0.004	0.168
*Rsev*C05	0.002	0.278	0.002	0.277
***Rsev*E03**	**0.025**	**< 0.001**	**0.025**	**< 0.001**
***Rsev*F01**	**0.032**	**< 0.001**	**0.032**	**< 0.001**
*Rsev*G11	0.010	0.017	0.010	0.019
*Rsev*A05	0.000	0.478	0.000	0.480
Overall	**0.014**	**< 0.001**	**0.014**	**< 0.001**

Table 5. Pairwise F_{ST} (above diagonal) and R_{ST} (below diagonal) values between years 1997, 2001, and 2004 in the isolated population of *R. sevosa*. Values shown in bold were statistically significant.

	1997	2001	2004
1997	-	**0.0106**	**0.0163**
2001	**0.0235**	-	**0.0126**
2004	0.0039	**0.0204**	-

For each year, analysis of heterozygote excess revealed a recent population bottleneck occurred under IAM and TPM mutation models but not based on the SMM (Table 6). However, only 2001 and 2004 exhibited the allele-frequency shift characteristic of recently bottlenecked populations (Table 6). This shift results from a loss of low-frequency alleles (Figure 6). Data for a non-isolated population of *R. capito*, the sister species of *R. sevosa*, was presented to demonstrate the expectation for a non-bottlenecked population (Table 6; Figure 6).

Table 6. Tests of recent population bottlenecks for 1997, 2001, and 2004 in the isolated population of *Rana sevosa* and for a non-isolated population of *R. capito* (see Chapter 1). Presented in the table are *P*-values for Wilcoxon's signed-ranks tests under three mutation models (IAM, SMM, and TPM). Shape of distribution represents a qualitative evaluation of allele frequency distributions. L-shaped = expected under mutation-drift equilibrium; Shifted = characteristic mode-shift distortion of bottlenecked populations (Luikart et al. 1998).

Year	IAM	TPM	SMM	Shape of Distribution
1997	0.004	0.040	0.250	L-shaped
2001	0.004	0.020	0.074	Shifted
2004	0.004	0.039	0.074	Shifted
R. capito	0.188	0.656	0.852	L-shaped

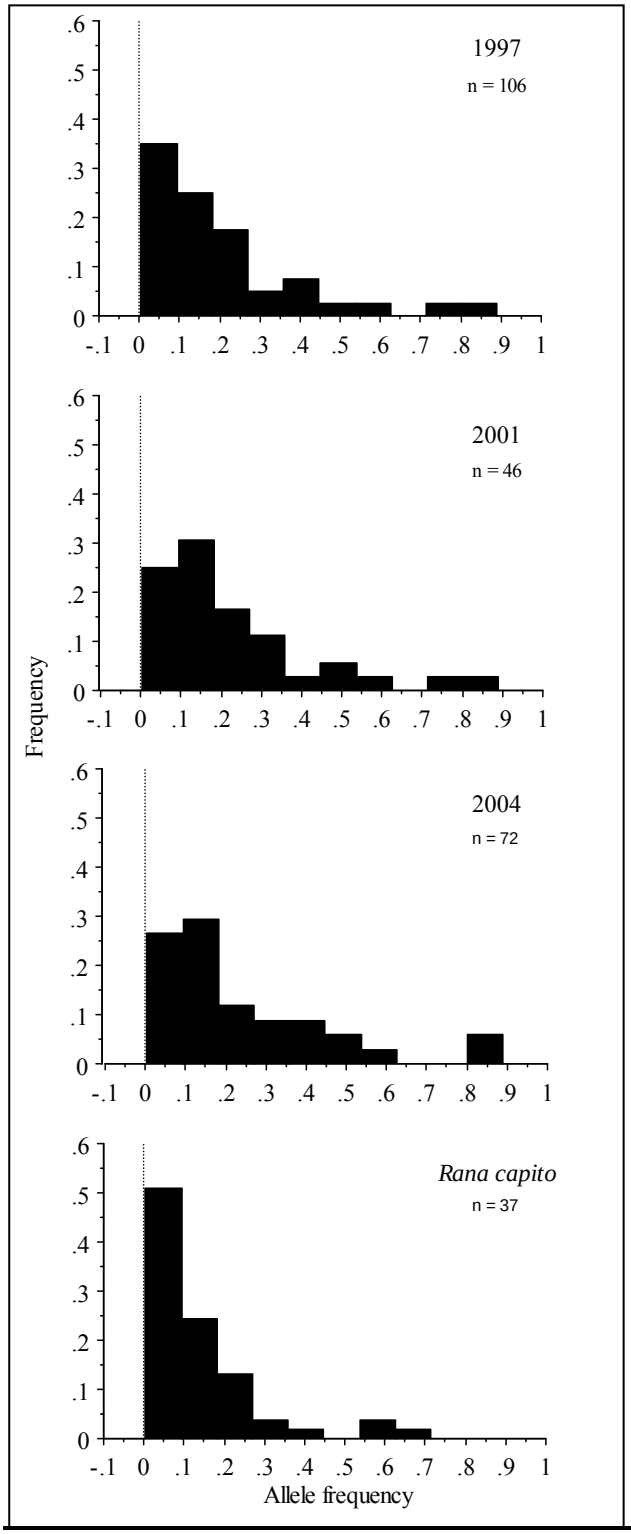

Figure 6. Frequency distribution of alleles for *Rana sevosa* in 1997, 2001, and 2004 and for a non-isolated population of *R. capito*. The loss of low frequency alleles and mode-shift for 2001 and 2004 is indicative of a recent population bottleneck.

Discussion

No significant differences were found for measures of genetic variability (H_E, H_O, or allelic richness) across years. However, non-significance of at least one of these (allelic richness) was due to low power of the analysis. The biological significance should take precedence over statistical interpretation. Seven alleles were lost over an eight-year period, and the signal of genetic erosion intensified. Loss of genetic variation can lead to lower adaptive potential, lowered resistance to disease, and increased risk of extinction (Kimura & Crow 1964; Allendorf 1986; Frankham 1995; Lacy 1997). Decreased heterozygosity is an obvious signal of genetic health of a population, but allelic richness more effectively reveals long-term evolutionary potential of populations as loss in the number of alleles can limit future response to selection (Allendorf 1986). As population size declines, the frequency of inbreeding increases, heterozygosity decreases, and alleles are lost more readily due to the increased effects of drift.

One would not predict that heterozygosity would be greatly reduced over an eight-year period because longer time periods are generally necessary unless reduction in population size is severe (e.g., Hutchinson et al. 2003). However, loss of alleles might be expected over 2–3 generations for small populations due to adult mortality and genetic drift. The temporal stability of the *R. sevosa* population was difficult to predict *a priori*. But, shifts in allele frequencies are expected because the effects of genetic drift are enhanced in small populations. Palm et al. (2003) provided estimates for the probability of detecting significant allele frequency differences across a range of years when in fact a difference existed (i.e., power). Estimates were calculated using allozyme data of two small populations ($N_E < 50$) of brown trout (*Salmo trutta*). They

concluded that the probability is "fairly small" for studies of only a few years even in populations with small effective population sizes (Palm et al 2003). However, the relationship was not linear and can vary depending on marker variability, sample sizes of individuals and loci, sampling interval, effective population size, and inbreeding history.

Heterozygosity in *R. sevosa* was low but appeared to be fluctuating rather than continuing to decline. In fact, observed heterozygosity increased by 5.5 % from 1997 to 2001, and decreased by 9.9 % from 2001 to 2004. This stresses the complications of studies that only sample the beginning and end of a temporal period and the need for at least one intermediate sampling year. The variation decreased from the first to last year (by 4.6%) but increased between them indicating that variation may be fluctuating rather than linearly decreasing as would be assumed if only the first and last year were sampled. In contrast, the number of alleles declined linearly over the study period. Overall, seven alleles were lost from three loci from 1997 to 2004. This temporal pattern of genetic variation might be expected because reductions in effective population size more severely impact allelic richness than heterozygosity (Leberg 1992; Luikart et al. 1998).

While no statistically significant differences were found for genetic variability among years, values for temporal genetic heterogeneity were significant, although small (F_{ST} and $R_{ST} < 0.020$). AMOVA revealed significant temporal population structure between all years based on F_{ST} and R_{ST}. However, power for the analysis of variation among years was limited by sample size because the sampling unit was H_E per locus per year ($n = 8$; $N = 24$). Power for analysis of genetic structure should be high because the

sampling unit was alleles per year (n = 92–212; N = 448). Additionally, a sample size of at least 50 individuals (n = 100) should yield sufficient power to detect population differentiation for microsatellite loci with a large number of allelic variants (Ruzzante 1998). Power is further enhanced for *R. sevosa* because allelic richness per locus is low (range = 2–9), especially compared to the loci used in Ruzzante's (1998) calculations (range = 10–30).

Pairwise comparisons of F_{ST} and R_{ST} between years were significant for all but R_{ST} between 1997 and 2004. Because the study was over only 2–3 generations, mutation should be negligible, and differences more likely will be due to genetic drift. Under these circumstances, F_{ST} and R_{ST} should minimally converge. In fact, F_{ST} might be more appropriate and effective for a short time scale because it partitions variance of allele frequencies among groups whereas R_{ST} incorporates an additional component into its estimation of differentiation (variance of allele sizes; SMM), which might act to lower power (Slatkin 1995; Ruzzante 1998).

Maltagliati & Camilla (2000) found the same pattern as for this population of *R. sevosa* (i.e., no difference in heterozygosity but significant F_{ST}) in an allozyme study of cyprinodontid fish. They attributed the temporal structure to genetic drift but could not exclude the potential effects of selection on two loci. For *R. sevosa*, genetic drift is presumably the primary source of temporal structuring. This is supported by the following observations: (1) inbreeding does not change allele frequencies (i.e., natural selection must occur), (2) microsatellite alleles are considered selectively neutral whereas the neutrality of other markers cannot always be assumed (van Oosterhout et al. 2004), (3) effective population size is small (<< 150), and (4) seven alleles were lost

over an eight-year period.

Recent demographic changes for *R. sevosa* have contributed to temporal instability and changes in genetic structure between 1997 and 2004 and prior to this period. Based on significant deviation from mutation-drift equilibrium for each year of the study, and a mode-shift of allele frequencies for 2001 and 2004 only, this population of *R. sevosa* underwent a historic bottleneck (pre-1997) and is currently undergoing another (post-1997). This conclusion was based on the genetic data and on historical and demographic data.

The population at Glen's Pond was once a subpopulation of a much larger metapopulation, and its isolation was fairly recent. A male was heard calling in a pond approximately 3.5 km from Glen's Pond in 1988 (Glen Johnson, pers. comm.), which implies that this pond may have supported gopher frogs in the last 20 years. Also, other ponds in the vicinity of Glen's Pond apparently supported gopher frogs in the last ca. 50 years based on habitat characteristics and a few instances of single calling males (SCR, pers. obs.; G. N. Johnson, pers. comm.). The heterozygote excess test ($H_E = H_{EQ}$) can detect bottlenecks that occurred many generations in the past (Cornuet & Luikart 1998). The mode-shift indicator should be less informative about historic bottlenecks and lose information as time elapses following the bottleneck because low frequency alleles will become reestablished in the population as mutations occur or more common alleles become rare via drift. After sufficient time passes following the bottleneck, rare alleles will "accumulate" and the L-shape of the frequency distribution of alleles will form. If genetic structure was evaluated at this time, H_E would not equal H_{EQ} but the frequency distribution would be as expected (L-shaped). Assuming no other demographic

disturbances, the population eventually would return to mutation-drift equilibrium and lose all signatures of the bottleneck.

A previous population genetic allozyme study of gopher frogs, which included individuals from this population of *R. sevosa* from 1995, can further elucidate changes in temporal variation (Young 1997; Young & Crother 2001). Average heterozygosity for allozymes is relatively low for vertebrates (typically < 0.10), especially compared to other organisms (Nei 1987). This potentially results from allozyme loci being fixed (i.e., having only one allele and a heterozygosity of zero) and being under different levels of selection. Because it is difficult to interpret genetic variation of allozyme loci in the context of microsatellite variation, comparative data will be used from populations of *R. capito* and *R. areolata*, which were calculated based on Table 3 of Young (1997).

Young & Crother (2001) found an average heterozygosity for 17 *R. capito* and 3 *R. areolata* populations of 0.072 (0.010 SE; range = 0–0.15; N = 1–10). If only populations with N >1 are included, average heterozygosity is 0.078 (0.010 SE; range = 0.013–0.14; N = 2–10). Average heterozygosity of the *R. sevosa* population in 1995 was 0.077, which is just at the average for all populations sampled. The large variation around the mean (SE = 13% of mean) is partly due to sample size variation, but can also be attributed to variation in demographic history of populations. Most populations are isolated to semi-isolated across the geographic distribution but many (including *R. sevosa*) fell within the range of "typical" for vertebrates (Young & Crother 2001). *R. sevosa* also was at average for allelic richness (33; overall mean $\pm$ SE = 32.5 $\pm$ 0.63) and for number of polymorphic loci (6 of 26; overall mean $\pm$ SE = 6.1 $\pm$ 0.50). Genetic

comparisons among populations can be biased by unequal sample sizes (Leberg 2002). Therefore, genetic variability in *R. sevosa* (N = 10) is likely to be inflated relative to other populations from Young (1997) with much lower sample sizes (mean $\pm$ SE sample size for populations with N > 1 = 5.1 $\pm$ 0.6; range = 2–8). Overall, genetic variation for *R. sevosa* in 1995 was at or just below the average of other gopher and crawfish frogs and within the range "typical" for vertebrates, but this is probably an optimistic interpretation potentially biased by unequal sampling.

Conclusions

Although no statistically significant differences in heterozygosity or allelic richness were found over the study period, seven alleles were lost from 1997 to 2004. Populations were significantly structured among years, which supports previous demographic data of high annual population turnover and small population size (Richter & Seigel 2002). Further resolution could be gained via an allozyme study of contemporary variation to compliment the data from previous studies (Young 1997; Crother & Young 2001). The longer this population remains isolated, the greater the effects of inbreeding, drift, and future bottlenecks will be intensified. Even though individuals could be transplanted from the other known population or from Zoo populations to supplement genetic variation (i.e., artificial gene flow), the only long-term sustainable option is to establish populations nearby. Even if N_e remains low in the Glen's Pond population following the establishment of nearby populations, genetic variation should stabilize and be maintained by gene flow (Østergaard et al. 2003). Creating a system of interconnected breeding ponds would not only remedy genetic

erosion but would also increase the probability of surviving disease outbreaks and environmental catastrophes.

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CHAPTER 3

RELATIONSHIP BETWEEN FITNESS AND INDIVIDUAL GENETIC VARIABILITY IN AN ISOLATED POPULATION OF DUSKY GOPHER FROGS, *RANA SEVOSA*

Introduction

Genetic studies of isolated populations typically discover reduced genetic variation and make recommendations for alleviating genetic deficiencies (e.g., Chapter 1; Frankham et al. 2002; Machon et al. 2002; Lecis & Norris 2004). However, very few studies attempt to address the maintenance of genetic diversity in isolation (Nichols et al. 2001). For small isolated populations, genetic variation is reduced by interactions between inbreeding, lack of gene flow, and genetic drift (Hitchings & Beebee 1997; Ellegren 1999; Landergott et al. 2001; Palo et al. 2001; Harper et al. 2003; Al-Rabab'ah & Williams 2004). In isolation, the only mechanism that generates variation (i.e., new alleles) is mutation because immigration of new alleles is not possible.

Alleles are lost from isolated populations via genetic drift and inbreeding. The intensity of genetic drift is primarily a consequence of effective population size, whereby

alleles are more likely to be lost or become fixed at low population sizes. Mating sometimes occurs between relatives in natural populations because population size is finite. Population size and probability of breeding with a relative are inversely related. The extent to which inbreeding negatively affects a population is dependent upon many factors, which are not mutually exclusive. Some of these include population size, frequency of inbreeding, degree of relatedness of mates, and time interval over which inbreeding has occurred. As populations approach a critical minimum size, the effects of inbreeding are expressed and can intensify to the extent of threatening population persistence (e.g., Sjögren 1991; Saccheri et al. 1998; Nilsson 2004).

Genetic variation can be maintained in isolated populations if genomic diversity acts to enhance fitness. Thus, severity and rate of genetic loss can be counteracted by many fitness-genetic associations, including negative assortative mating (Reusch et al. 2001), non-random survival of adults with greater genetic variability (Bancroft et al. 1995; Saccheri et al. 1998), avoidance of mating among closely related individuals (Amos et al. 2001; Garner & Schmidt 2003), extra-pair matings and sperm storage (Blomqvist et al. 2002; Foerster et al. 2003; Arnaud & Laval 2004), and increased fitness of individuals with higher genetic variability via heterosis or balancing selection (Nei 1987; Coltman et al. 1998; Rossiter et al. 2001; Shikano & Taniguchi 2002).

Demonstrating negative effects of inbreeding historically required a known pedigree. Therefore most early studies that addressed the consequences of inbreeding were on laboratory, captive, or domestic populations (Charlesworth & Charlesworth 1987; Frankham 1995; but see Keller 1998). Since that time, many genetic measures of relatedness have been developed that allow one to assess inbreeding-fitness relationships

for populations without known pedigrees (Coulson et al. 1998; Coltman et al. 1998; Amos et al. 2001). Similarly, these measures have been used to address the relationship between genetic variation and fitness not linked to inbreeding via analysis of siblings (Hansson et al. 2001).

Neutral molecular measures of genetic diversity are not always in accordance with quantitative-trait genetic diversity (e.g., Pfrenderet al. 2000). However, most of these studies lack statistical power to detect an effect. Many studies have found significant, positive relationships between genetic diversity of neutral loci and fitness-related traits (Coltman et al. 1998; Coulson et al 1998; Coltman & Slate 2003; Hannson et al. 2004). Three hypotheses to explain genetic-fitness correlations are (1) functional overdominance in which the markers are directly under positive selection for more diverse individuals, (2) linkage of the marker with genes under selection, and (3) the marker reflects diversity in fitness-related loci throughout the genome (Hannson & Westerberg 2002; Slate & Pemberton 2002; Coltman & Slate 2003).

The positive relationship between genome-level heterozygosity and fitness, although perhaps difficult to quantify, is not a controversial issue (Britten 1996). However, identifying genes that affect the fitness trait under study is seldom possible. Additionally, difficulty arises when one attempts to apply genetic markers whose functions in selection are unknown or neutral. One alternative is to survey as many genes as possible and identify a subset that correlates with fitness. A more realistic approach is to use a neutral genetic marker that has been shown previously to correlate with genome-wide diversity (e.g., microsatellite DNA).

Microsatellite DNA loci, which are short tandem-repeat motifs of nucleotides

(usually 2–4 bases long), are an ideal tool for use in individual- and population-level genetic studies primarily due to abundance in the genome, high level of allelic variation (due to an inherent instability), and codominance (i.e., heterozygotes are distinguishable from homozygotes). Because most microsatellites are selectively neutral, the first hypothesis to explain genetic-fitness correlations (functional overdominance) can be disregarded.

The inherent instability of microsatellite DNA is a consequence primarily of slipped-strand mispairing during DNA replication but has also been shown to occur via unequal crossover during recombination (reviewed in Eisen 1999). Both mechanisms result in the insertion or deletion of repeat units. This instability causes increased variability. In fact, mutation rate of microsatellites (10^{-3} to 10^{-6}) is much higher than rates for nucleotide substitutions (10^{-9} to 10^{-10}) (reviewed in Hancock 1999). Because microsatellites are composed of multiple copies of repeats, replication slippage more often results in misaligned strands and the likelihood of homologue misalignment and unequal crossover is increased (Eisen 1999). Most mutations of microsatellites result in a change of one to three repeat units (stepwise mutation model). However, a more realistic model is a two-phase mutation model, which allows stepwise mutations in addition to infrequent large increases in repeat number (Jeffreys et al. 1988; Di Rienzo et al. 1994; Fu & Chakraborty 1998).

Microsatellite genetic variation can be quantified for individuals not only based on heterozygosity as is true for other molecular markers (e.g., allozymes and quantitative trait loci) but also by exploiting the size-information content. Specifically, microsatellite alleles are distinguished by size, which results from differences in the number of repeat

motifs. Three primary measures of the genetic background of individuals have been developed, each of which exploit different information contained in microsatellite DNA loci.

Multilocus heterozygosity (MH; Coltman et al. 1999) is simply the heterozygosity of an individual derived by averaging its heterozygous state across loci. Mean d^2 (MD^2; Coulson et al. 1998) estimates the degree of outbreeding or inbreeding for an individual by assessing the relatedness of an individual's parents based on the difference in size of microsatellite alleles across loci (= width) where high MD^2 = relatively outbred and low MD^2 = relatively inbred. Therefore, MD^2 is an individual internal distance measure that uses the information content of microsatellite allele sizes to estimate the genetic distance of parental gametes (Coltman et al. 1998; Coulson et al. 1998; Pemberton et al 1999). It is calculated as the difference in size of two alleles at each microsatellite locus and then averaged over loci. As mean d^2 decreases, the time since parents shared a common ancestor decreases and level of inbreeding increases. Detecting this relationship does not rely on a stepwise mutation process *sensu stricto* because most if not all mutations at microsatellite loci give rise to alleles with lengths similar to the ancestral allele (Goldstein et al. 1995). However, microsatellite loci that have evolved via stepwise mutation should have a tighter correlation between time since divergence and width of alleles. Internal relatedness (IR; Amos et al. 2001) incorporates potential rare-allele effects by combining data of individual heterozygosity and frequency of an individual's alleles in the population.

Formulas for each are as follows.

(1) $$MH = het_N / N$$

where het_N = number of loci for which an individual was heterozygous and N = the number of loci genotyped

(2) $$MD^2 = \sum (a_1 - a_2)^2 / N$$

where a_1 = the larger of the two alleles at each locus, a_2 = the smaller allele at each locus, and N = number of loci genotyped

(3) $$IR = (2H - \sum f_i) / (2N - \sum f_i)$$

where H = number of homozygous loci, N = number of loci genotyped, and f_i = the frequency of the i^{th} allele in the genotype (i.e., $\sum f_i$ = the sum of the population allele frequencies for each allele within an individual)

Internal relatedness incorporates a correction for missing data, but MD2 and MH need standardization when all individuals have not been genotyped for each locus (Coltman et al. 1999; Amos et al. 2001). To standardize MD2, individual d2 scores for each locus are divided by the maximum value for that locus. This prevents individuals with missing data from being biased by loci with allelic size ranges greatly different from others. MH is standardized by dividing the heterozygosity of individuals by the average heterozygosity scores of genotyped loci. This removes the bias of loci differing greatly in levels of polymorphism. Standardized mean d2 (SMD2; Amos et al. 2001) and

standardized multilocus heterozygosity (SMH; Coltman et al. 1999) are calculated as follows.

(4) $$SMD^2 = 1/N \sum (d^2 / d^2_{max})$$

where $d^2 = (a_1 - a_2)^2$ from formula *(2)*, d^2_{max} = the maximum d^2 for each locus, and N = number of loci genotyped

(5) $$SMH = het_{individ} / het_{loci}$$

where $het_{individ}$ = the proportion of loci for which an individual was heterozygous and het_{loci} = the mean heterozygosity of loci genotyped for the individual

Recent and historic genetic effects can drive the genetic-fitness relationship. Parental relatedness is a consequence of historical events (i.e., ancestry), whereas genotypes of offspring are due to more recent events. Thus, it is possible to have different levels of resolution when using measures based on individual heterozygosity (MH, SMH, and IR) and based on width of alleles (MD^2 and SMD^2). This resolution is lost if population size has been small for many generations. If the incidence of inbreeding has been increasing, one might expect that both types of measures would covary. Additionally, if part or all of the microsatellite alleles used in a study do not follow the stepwise mutation model, then the linear relationship between mean d^2 and time attenuates and is possibly lost. At some level of deviation, an individual's mean d^2 will not correlate with time since divergence of two alleles, and size-information content will be reduced to simply an identity state (i.e., heterozygous state).

Previous studies have yielded results with varying levels of support or lack of support

for the prediction that individuals with higher heterozygosity and wider alleles will have a fitness advantage over less genetically diverse individuals (reviewed in Neff 2004b). This could be due to a lack of effect, however, it is difficult to separate confounding variables including the limited resolution and unknown role allozyme loci have in selection (for studies that used allozymes), low power, variation in effect size depending on level of inbreeding or outbreeding in the population, and simultaneous deleterious effects of extreme outbreeding and inbreeding within the same population (e.g., Waser & Price 1989; Neff 2004a). The *R. sevosa* system provides an opportunity to study the genetic-fitness relationship in that it has a genetic signature of high inbreeding (Chapter 1), so the effect size should be large and consequences of inbreeding should have severe consequences on fitness.

General characteristics of this group of amphibians further increase their suitability as a study system for genetic-fitness relationships. Anuran amphibians are known for having exceptional diversity in life histories, especially in reproductive traits and modes. The generalized (and presumed ancestral) mode of reproduction and life cycle is for adults to return to water to reproduce and deposit eggs (Duellman & Trueb 1986). Following development of eggs and aquatic larvae, metamorphosis to a terrestrial morph occurs. Many amphibians with this life history have typical "*r*-selected" characters, including large clutch size, lack of parental care, and high mortality of eggs, larvae, and metamorphic frogs (metamorphs) relative to adults. The aquatic stage thus provides an arena for selection to occur, especially for many species of *Rana*, which have clutch sizes of a thousand to many thousand individuals (e.g., the average clutch size of *R. sevosa* is ca. 1200 eggs; Richter et al. 2003). Recruitment can be strongly tied to fitness because

most offspring (> 90%) die before sexual maturity (Turner 1960; Herreid and Kinney 1966; Calef 1973; Richter et al. 2003; Ulmar Grafe et al. 2004).

Rowe et al. (1999) assessed survival and growth rates of natterjack toad (*Bufo calamita*) larvae that varied in population size, degree of isolation, and distance from the edge of the geographic distribution. They found that expected heterozygosity (H_E) was strongly correlated with larval growth rate. However, this relationship was confounded because the smallest and most isolated populations exhibited low larval growth and low H_E. Although important conclusions were made at the level of geographic distribution, this example highlights a problem of population-level approaches to fitness-related consequences of genetic variability. Separating population-level effects from individual effects is complex.

The goal of the present study was to address mechanisms for preservation of genetic variability in an isolated population of the US endangered dusky gopher frog, *Rana sevosa*. The primary question was "Do more genetically diverse individuals have higher fitness?" Three specific mechanisms of enhanced fitness were explored: (1) differential survival of more genetically diverse offspring through development, (2) mass mortality of egg clutches based on parental genetic background and relatedness, and (3) pairing of more genetically distinct mates. To address these mechanisms, genotypes for eight microsatellite loci were evaluated for three life-history stages from a single year (1997)—breeding adults, eggs, and emerging metamorphs.

Methods

Sample collection and demographic data

Tissue samples were collected from three life-history stages during 1997— from adults that migrated to the pond to breed, egg clutches deposited, and emerging metamorphs. Adults and metamorphs were captured using a drift fence with pitfall traps that completely encircled the breeding site (see Richter & Seigel 2002). Single toes from each adult and metamorph were removed and placed in 95% ethanol for genetic analyses. Following breeding events, the pond was surveyed for egg clutches to monitor reproductive output (see Richter et al. 2003). During visual surveys, a total of 250 eggs were collected by removing 4–8 eggs (mean $\pm$ SE = 4.4 $\pm$ 0.1) from each of 57 egg clutches and placing each in 95% ethanol.

During egg surveys, individual clutches were flagged and monitored throughout development. Egg mortality was determined for each clutch by counting the number of undeveloped eggs after all surviving larvae hatched. Percent mortality was calculated per clutch by dividing the number of undeveloped eggs by the estimated total clutch size. Clutch size was estimated using a water displacement method by first counting the number of eggs required to displace 1 ml and then determining how much volume the entire clutch displaced.

Genetic data collection

DNA was extracted from each individual using Qiagen® DNEasy tissue kits. For each individual, DNA was PCR amplified for eight microsatellite loci as previously described (see Chapter 1). Genetic data were collected using an ABI Prism® 310 Genetic Analyzer

(Applied Biosystems) by pooling samples of PCRs for three loci per individual using different fluorescently labeled primers for each locus. Allele lengths were scored using GeneScan® Analysis Software version 3.1.2 (Applied Biosystems). A total of 470 individuals were genotyped for eight microsatellite loci (106 adults, 209 metamorphs, and 155 eggs).

Genetic analyses

Following genotyping of eight microsatellite loci, individuals were given two scores per locus: heterozygous state and width. Heterozygous state was scored as a '0' for homozygotes and a '1' for heterozygotes. Width refers to the difference in size of alleles and was scored by ($|a_1 - a_2|$) / length of repeat motif. For example, for *Rsev*B12, the repeat motif is ATAG. So, for alleles 220 and 232, width would equal ($|220 - 232|$) / 4 = 3. Scores of '0' width were given to homozygotes. These scores were used to derive individual measures of genetic variation within life history stages (width, MH, SMH, MD^2, SMD^2, and IR).

Offspring fitness can be predicted using genetic diversity of the parents (Primmer et al. 2003). For *Rana sevosa*, survival at the egg stage is highly variable (0–100% of eggs per clutch) with a fairly low average survival per clutch (as low as 63%; Richter et al. 2003). To test for a genetic effect on egg mortality, clutch-level analyses of mortality were performed using the genotypes from clutch subsamples to determine average width, MH, SMH, MD^2, SMD^2, IR of clutches by calculating individual scores for each egg and averaging them within clutches. Mortality data for individual clutches were taken from

Richter (1998). Additionally, the number of alleles per clutch, which could range from 1 to 4, was averaged over loci genotyped for each clutch (MR).

(6) $$MR = \sum r_{in} / N$$

where r_{in} = the number of alleles at the ith locus, which can range from 1 to 4, and N = the number of loci genotyped

To address the potential for non-random association of breeding pairs in terms of genetic background, average clutch scores of the six individual genetic measures above were compared to those of the adult population. If nonrandom mating resulted in more diverse offspring, then individual egg clutches should have higher average width, MH, SMH, MD^2, and SMD^2 and lower IR scores.

Statistical analyses

All measures of individual genetic background were calculated using EXCEL® 2002 (Microsoft Corp.) with programs written by the author. Allele frequencies used in calculating IR were generated using ARLEQUIN v. 2.000 (Schneider et al. 2000). Statistical analyses were performed using STATVIEW® version 5.0 (SAS Institute, Inc.).

Differences among life-history stages were evaluated using ANOVAs followed by Games-Howell multiple comparisons (GH) on log (n+1) transformed width, MH, SMH, MD^2, SMD^2, and IR. The GH procedure is appropriate when sample sizes are unequal (Toothaker, 1991).

To test for genetic effects on mortality of eggs, which was measured as the proportion of eggs that did not hatch for each clutch, regression analyses were performed on arcsine transformed percent mortality estimates and each of log (n+1) transformed MR, width, MH, SMH, MD^2, SMD^2, and IR.

To test for nonrandom association of breeding pairs, the difference in genetic measures between the adult population and individual egg clutches was evaluated using ANOVAs of log (n+1) transformed width, MH, SMH, MD^2, SMD^2, and IR.

Results

Survival to metamorphosis

All measures (both corrected and uncorrected) of individual genetic background except MD^2 were highly correlated with each other ($r > |0.650|$ excluding MD^2) for life-history stages such that individuals with higher heterozygosity had wider alleles and more negative IR scores (Table 1; Figure 1). Eggs were not significantly different from adults in terms of individual genetic background based on any measure (Tables 2 &3; Figure 2). Metamorphs were significantly more diverse than either adults or eggs (Tables 2 & 3; Figure 2). A potential sample size effect occurred for these analyses. Both sample size and magnitude of measures of genetic variability were such that metamorphs were greater than eggs, which were greater than adults (Table 2). Because of this potential bias due to sample size, 106 metamorphs were randomly selected for analysis, and the difference in effect size for all measures actually increased rather than decrease (as would be expected in the effect was driven by sample size (Table 2).

Table 1. Correlation coefficients between the six estimates of average individual genetic background for life history classes. Numbers above the diagonal are the correlation coefficient (*r*) and below the diagonal are p-values for the coefficient.

	Width	MD^2	SMD^2	MH	SMH	IR
Width	-	0.900	0.782	0.687	0.653	-0.712
MD2	< 0.001	-	0.581	0.427	0.399	-0.461
SMD2	< 0.001	< 0.001	-	0.723	0.697	-0.737
MH	< 0.001	< 0.001	< 0.001	-	0.990	-0.942
SMH	< 0.001	< 0.001	< 0.001	< 0.001	-	-0.913
IR	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	-

Table 2. Genetic measures for three life history stages: width, mean d^2 (MD^2), standardized mean d^2 (SMD^2), mean heterozygosity, standardized multilocus heterozygosity (SMH), and internal relatedness (IR). Data for each class are presented as mean $\pm$ SE in the first row and the range of individual measures in the second row. Sample size (N) is given below the class name.

	Width	MD^2	SMD^2	MH	SMH	IR
Adults	1.83 $\pm$ 0.09	11.322 $\pm$ 1.014	0.244 $\pm$ 0.132	0.558 $\pm$ 0.019	0.996 $\pm$ 0.033	0.071 $\pm$ 0.029
N = 106	(0.17–4.25)	(0.167–38.875)	(0.002–0.678)	(0.125–1.000)	(0.222–1.778)	(-0.505–0.783)
Eggs	2.05 $\pm$ 0.08	12.650 $\pm$ 0.736	0.278 $\pm$ 0.011	0.577 $\pm$ 0.016	0.997 $\pm$ 0.028	0.055 $\pm$ 0.025
N = 155	(0.00–4.13)	(0.000–32.000)	(0.000–0.583)	(0.000–1.000)	(0.000–1.754)	(-0.486–1.000)
Metamorphs	2.44 $\pm$ 0.04	16.222 $\pm$ 0.373	0.328 $\pm$ 0.008	0.609 $\pm$ 0.009	1.000 $\pm$ 0.016	-0.134 $\pm$ 0.016
N = 209	(1.00–3.88)	(2.250–27.125)	0.065–0.690)	(0.250–0.875)	(0.410–1.436)	(-0.658–0.546)
Metamorph subsample	2.52 $\pm$ 0.05	17.291 $\pm$ 0.484	0.332 $\pm$ 0.011	0.624 $\pm$ 0.012	1.026 $\pm$ 0.020	-0.155 $\pm$ 0.020
N = 106	(1.13–3.88)	(2.875–27.125)	0.065–0.690)	(0.375–0.875)	(0.616–1.436)	(-0.618–0.357)

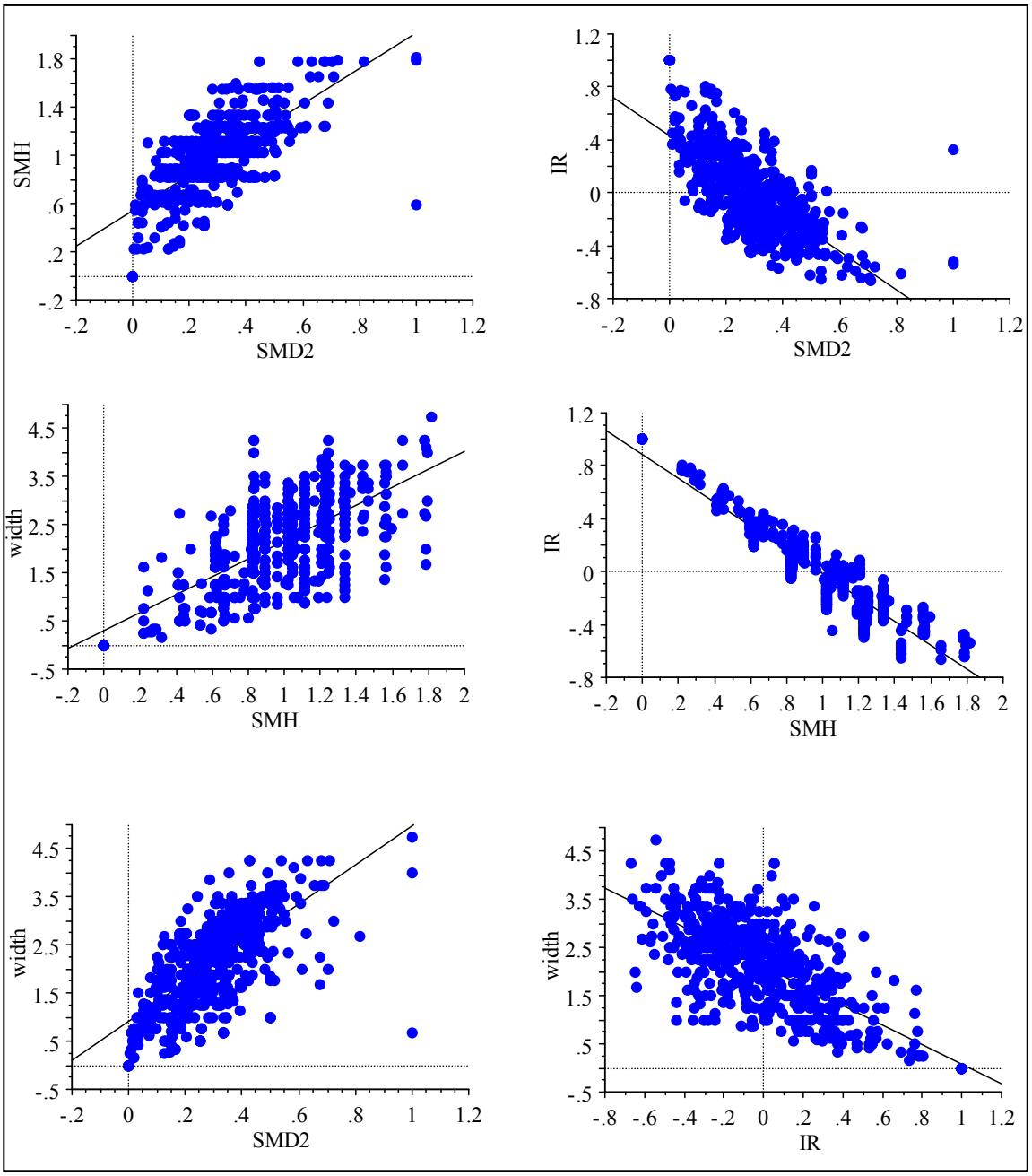

Figure 1. Bivariate regression graphs for correlations between genetic measures for life history stage analyses. See Table 1 for correlation coefficients and p-values.

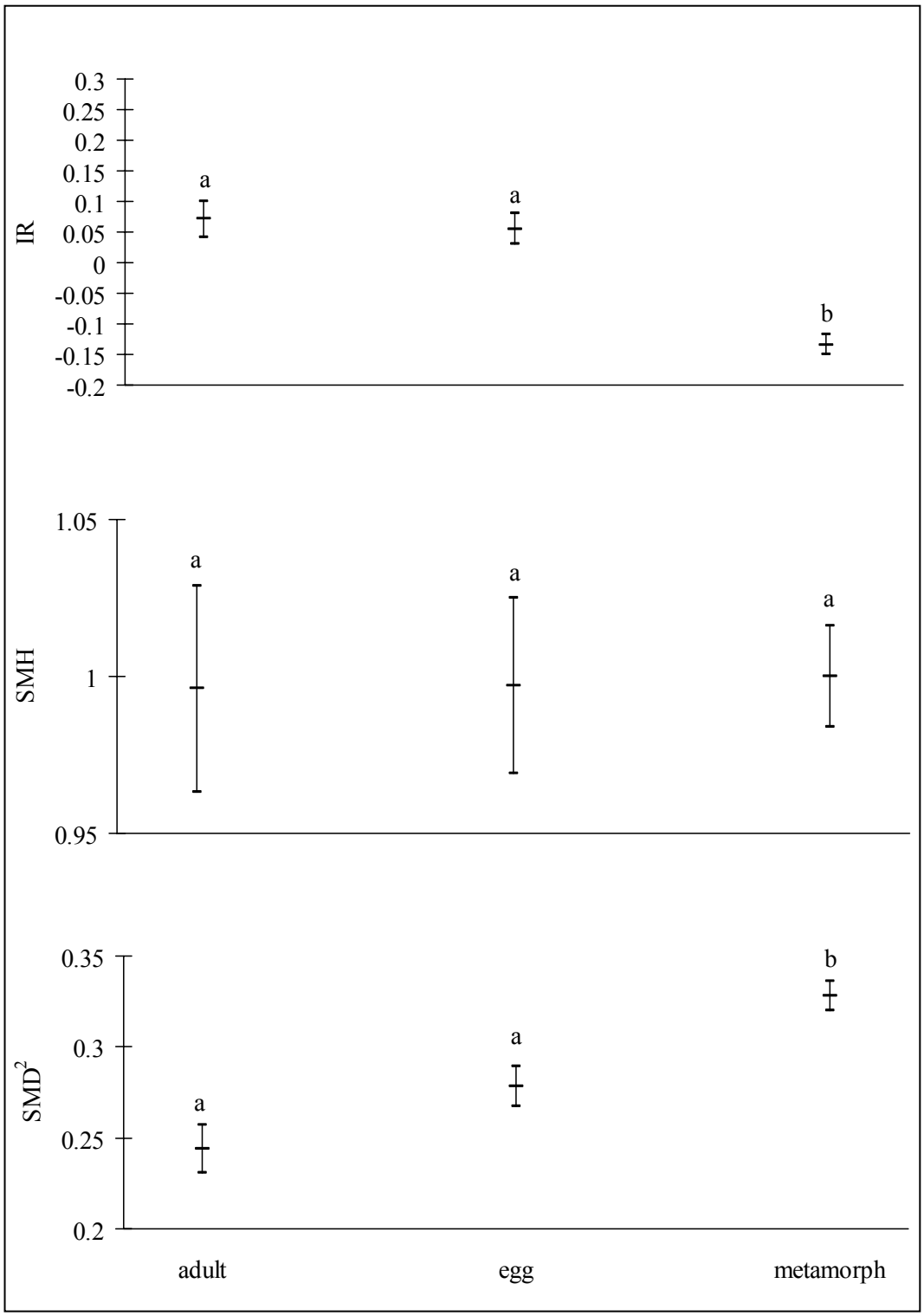

Figure 2. Mean ± SE of internal relatedness (IR), standardized mean d^2 (SMD^2), and standardized mean heterozygosity (SMH) for each life history class. Letters represent among-class comparisons where a different letter indicates a significant difference determined with GH multiple comparisons following ANOVAs (see Table 3).

Table 3. Results of one-way ANOVA evaluating differences in genetic background for individuals of each life-history class. Following the ANOVA analyses, multiple comparison statistics were calculated using the Games-Howell procedure. These results are presented in the bottom three rows (see also Figure 2).

	Width	MD^2	SMD^2	MH	SMH	IR
$F_{2, 467}$	28.33	36.93	17.23	4.31	0.30	28.03
P-value	< 0.0001	< 0.0001	< 0.0001	0.014	0.743	< 0.0001
Adult - egg	NS	NS	NS	NS	NS	NS
Adult - metamorph	S	S	S	S	NS	S
Egg - metamorph	S	S	S	NS	NS	S

Mortality in egg clutches

All genetic measures for egg clutches except MR were correlated with each other ($r >$ |0.610| for all excluding MR and MD^2; Table 4; Figure 3). Although there was a negative trend between mortality and SMR and SMD^2, the relationship was not significant (Table 5; Figure 4). A significant relationship was found between mortality and SMH (positive) and IR (negative), but the variation explained by each was low ($R^2 < 0.160$ for both; Table 5; Figure 4).

Table 4. Correlation coefficients between the six estimates of average individual genetic background for individual egg clutches. Numbers above the diagonal are the correlation coefficient (*r*) and below the diagonal are p-values for the coefficient.

	Width	MR	MD^2	SMD^2	MH	SMH	IR
Width	-	0.250	0.891	0.706	0.638	0.611	-0.624
MR	0.142	-	0.180	0.388	0.246	0.250	-0.229
MD2	< 0.001	0.297	-	0.517	0.384	0.361	-0.381
SMD2	< 0.001	0.019	0.001	-	0.814	0.812	-0.736
MH	< 0.001	0.150	0.020	< 0.001	-	0.992	-0.965
SMH	< 0.001	0.143	0.030	< 0.001	< 0.001	-	-0.972
IR	< 0.001	0.180	0.021	< 0.001	< 0.001	< 0.001	-

Table 5. Results of regression analyses evaluating the relationship between measures of genetic background for individuals and egg clutch mortality. Variable names are presented, but each was log (n+1) transformed prior to statistical analyses.

	MR	Width	MD^2	SMD^2	MH	SMH	IR
$F_{1,32}$	0.578	5.623	3.541	1.845	5.394	5.72	4.874
P-value	0.453	0.024	0.069	0.184	0.027	0.023	0.035
R^2	0.018	0.149	0.100	0.054	0.144	0.152	0.132

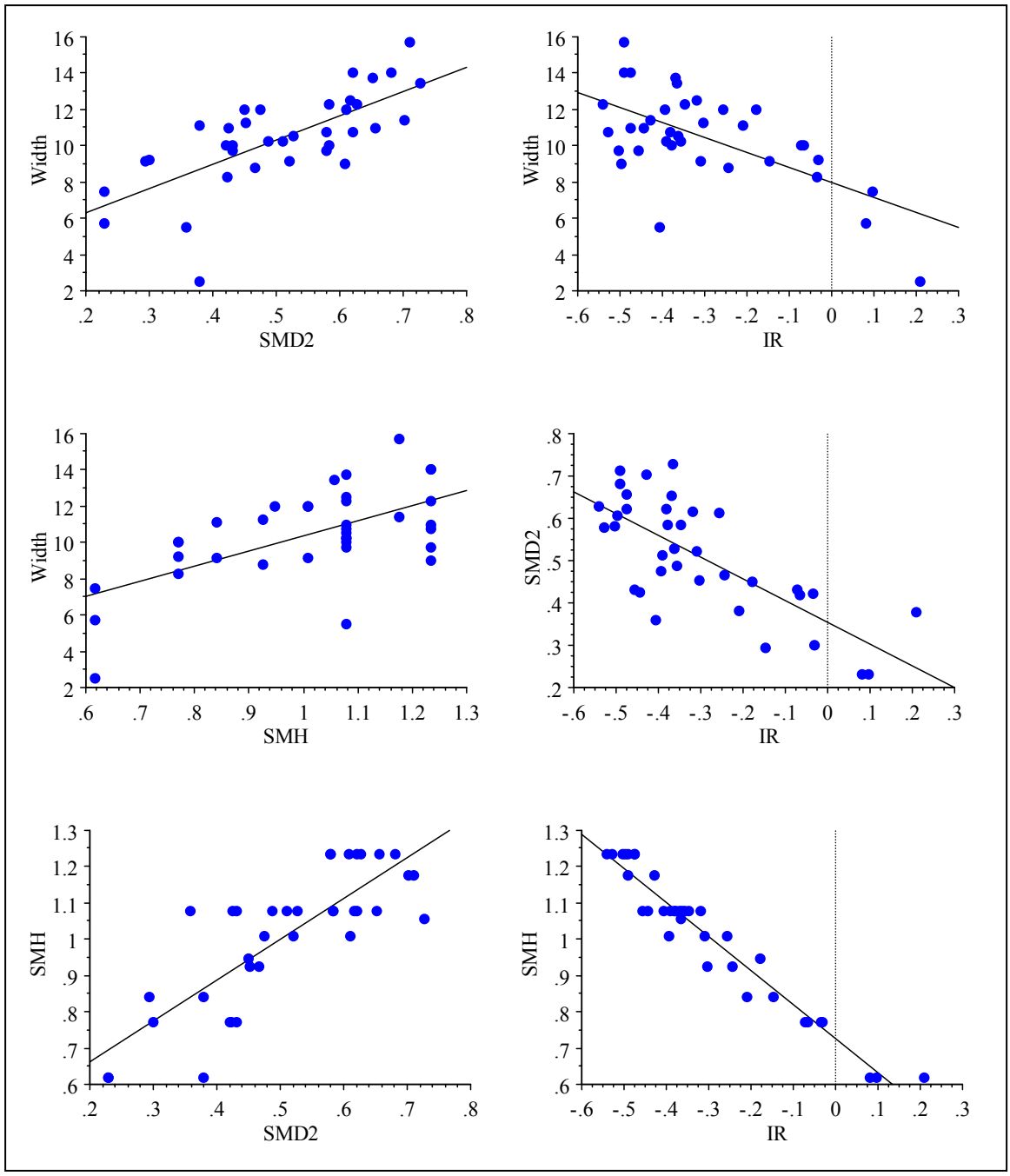

Figure 3. Bivariate regression graphs for correlations between genetic measures for egg clutch level analyses. See Table 4 for correlation coefficients and p-values.

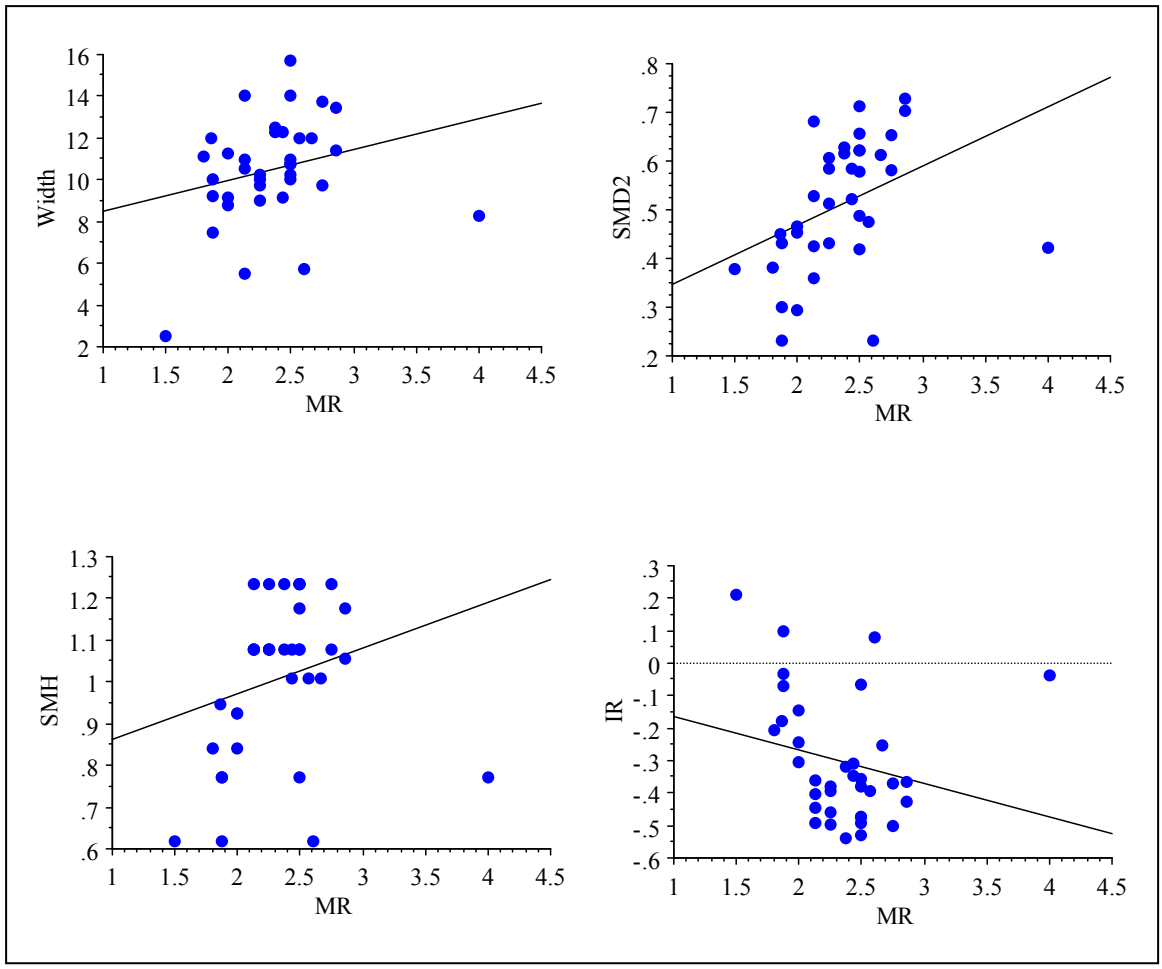

Figure 3. (continued)

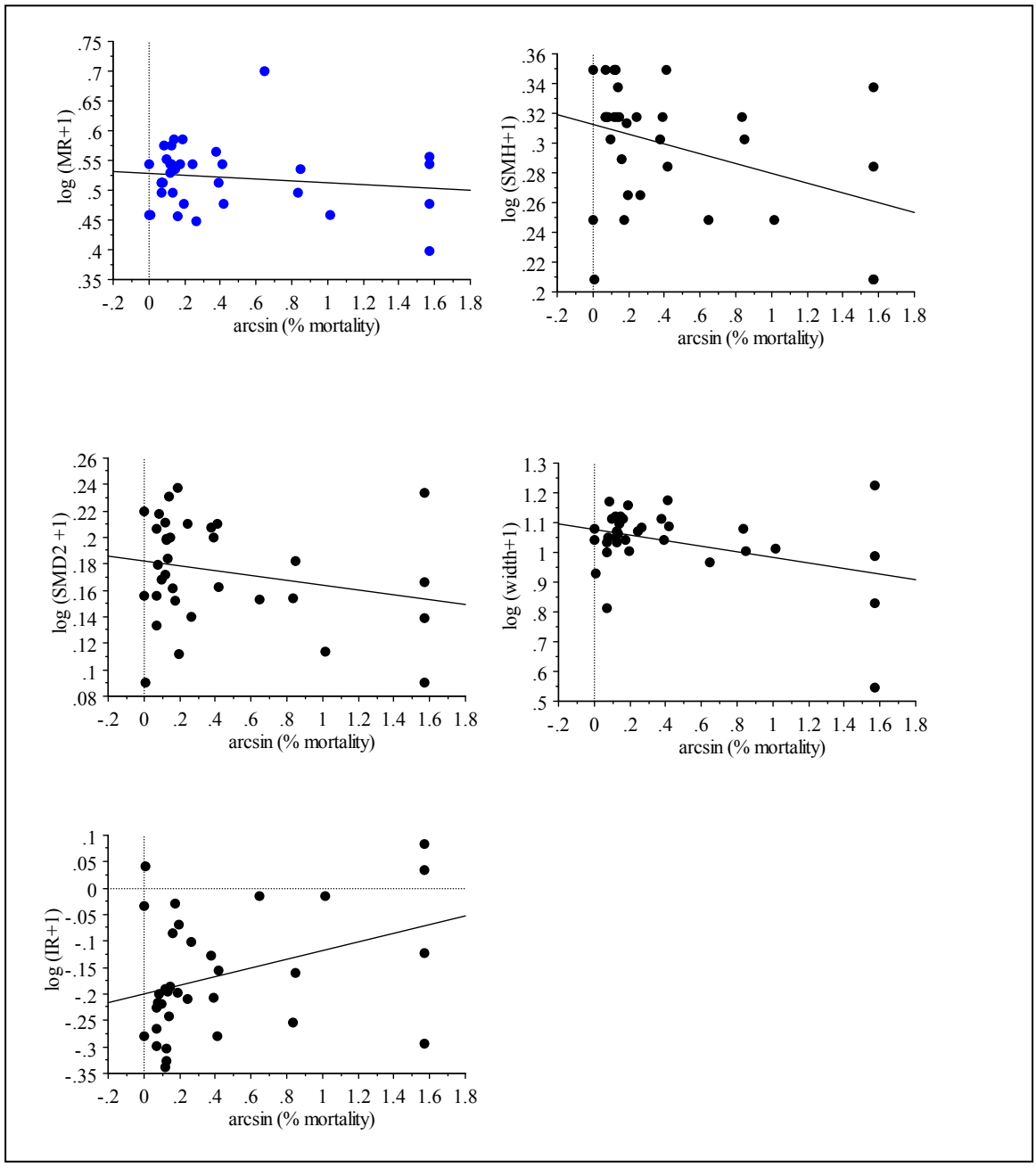

Figure 4. Regression plots depicting the relationship between egg clutch mortality and mean number of alleles (MR), standardized mean heterozygosity (SMH), standardized mean d^2 (SMD^2), and internal relatedness (IR).

Relatedness of breeding pairs

No significant differences were found between egg clutches and adults for any genetic measure, although egg clutches exhibited a trend toward higher d^2 compared to adults (Table 6; Figure 5).

Table 6. Results of one-way ANOVA evaluating differences in genetic background between adults (N = 106) and egg clutches (N = 36).

	Width	MD^2	SMD^2	MH	SMH	IR
$F_{1,140}$	1.94	3.60	1.97	0.31	0.01	0.10
P-value	0.166	0.060	0.163	0.579	0.928	0.748

Discussion

Six genetic measures were presented in the results for individuals (width, multilocus heterozygosity [MH], standardized multilocus heterozygosity [SMH], mean d^2 [MD^2], standardized mean d^2 [SMD^2], and internal relatedness [IR]) and seven for clutches (six measures as for individuals and mean allelic richness [MR]). However, only the four that were standardized or needed no standardization (SMH, SMD^2, IR, and MR) will be discussed because the others do not correct for missing data at different loci. Those not standardized were presented simply as a demonstration that all measures converged on the same results. This might be expected because many of the measures employ the same

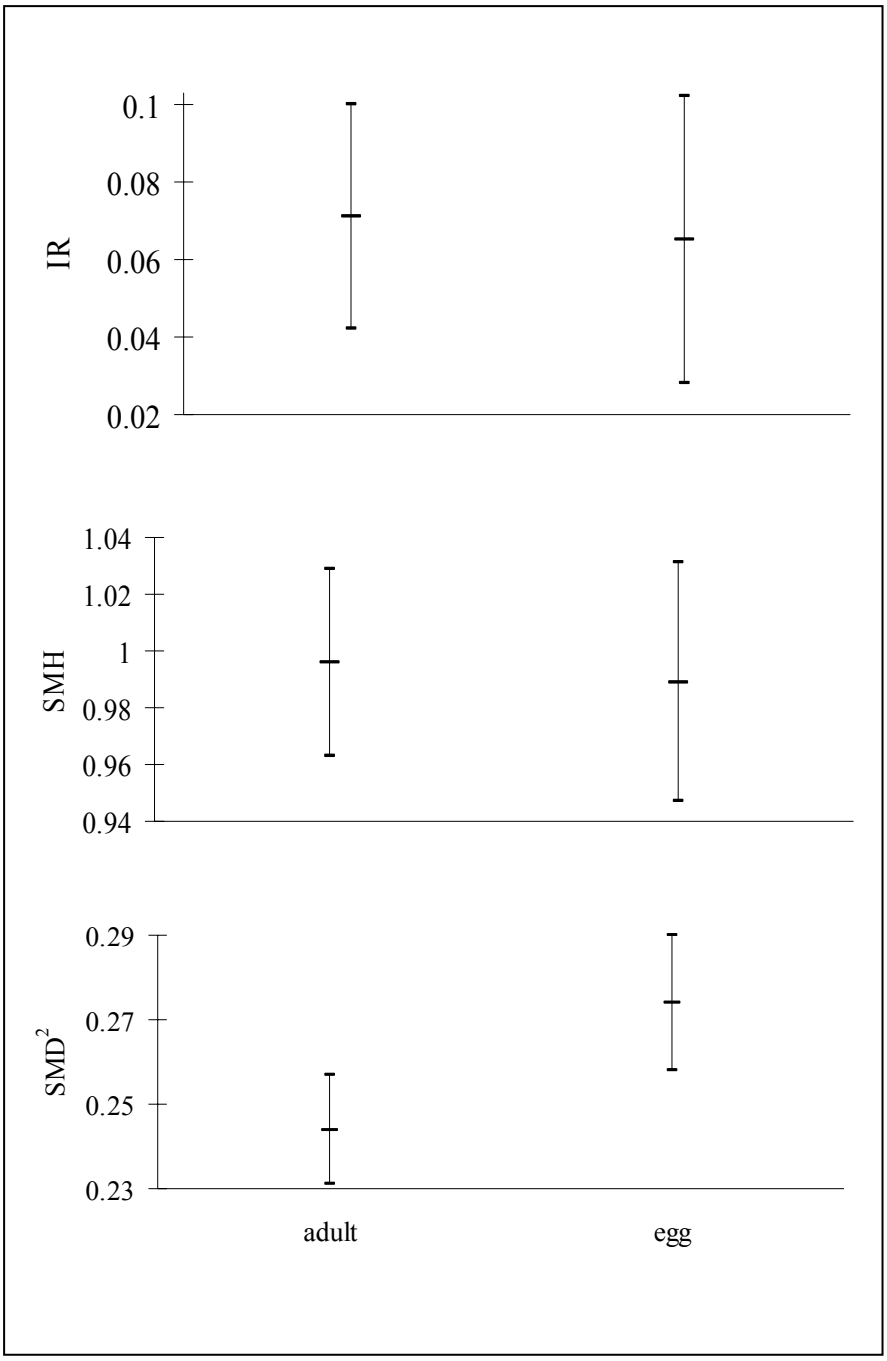

Figure 5. Mean ± SE of internal relatedness (IR), standardized mean d^2 (SMD^2), and standardized mean heterozygosity (SMH) for adults and for individual egg clutches. None of the differences were significant (see Table 6).

data in their calculations. However, deviations are expected depending on the amount of missing data and on the true relationship between genetic variability, width of alleles, and fitness.

Survival to metamorphosis

This study demonstrates a positive genetic-fitness relationship for individuals through metamorphosis. Metamorphs had significantly greater SMD^2 values and significantly lower IR scores than did eggs. Large differences in heterozygosity can drive width relationships (i.e., homozygotes receive a width of zero and greatly lower measures of d^2). However, SMH was similar among groups. This suggests that more outbred individuals had higher fitness than inbred individuals. Significantly higher standardized d^2 values for metamorphs than eggs indicate that individuals surviving to metamorphosis (i.e., those with higher SMD^2) had parents that were more genetically divergent than their non-surviving counterparts. Significantly lower IR, which is calculated based on homozygosity and allele frequencies, indicates that metamorphs had more low frequency alleles than eggs that did not develop into froglets, especially since no significant difference was found between the groups for heterozygosity (SMH). For highly variable genetic markers such as microsatellite DNA, measures of heterozygosity are often not different among treatments and do not correlate with fitness because the high levels of polymorphism decrease population variance in individual heterozygosity (Slate & Pemberton 2002).

Increased survival to metamorphosis of individuals with higher SMD^2 and lower IR appears to be driven by the width and not the rareness of alleles. This conclusion is based

on the following rationale. Alleles at the extreme of the range for a locus tend to be in low frequency, which results in individuals with a greater allele width having rarer alleles on average. Significant IR was most likely a corollary of width effects. Thus, individuals with high SMD^2 and low IR have higher fitness because their parents are more genetically different and not because they possess rarer alleles.

The development from a single-celled egg to metamorphosis of most amphibians with a larval stage is a likely time for selection to occur because the proportion of eggs that result in metamorphs is low << 0.10 (Turner 1960; Herreid and Kinney 1966; Anderson et al. 1971; Calef 1973; Shoop 1974; Semlitsch 1987; Richter et al. 2003). Amphibian populations should benefit greatly if selection favors more outbred individuals during this time rather than if mortality were random with respect to genotype.

Metamorphs also had significantly greater SMD^2 and lower IR scores than adults. The relationship between genetic measures in adults and metamorphs is difficult to predict but should vary among years depending on factors including annual variation in intensity of selection at the egg and larval stage, stochastic mortality events of any life history stage, genetic effect on survival from metamorphosis to maturity, and genetic-fitness relationships within adults. It is improbable that metamorphs would be more outbred (i.e., have wider [SMD^2] and rarer [IR] alleles) than adults every year. This pattern would only be possible if survival from metamorph to adult was inversely related to width and rarity of alleles, which is highly unlikely. The cause appears to be stochastic variation in survival and selection during larval development.

Increased selection at the larval stage during 1997 may have caused greater differences found in genetic background between adults and metamorphs. During this

breeding year, metamorph recruitment was low relative to reproductive investment— 58 egg clutches:221 metamorphs compared to 37:2488 during 1998 (Richter et al. 2003). In fact, the ratio of reproductive investment to output in 1997 (0.004) was much lower than for 1998 (0.054) and for other species of *Rana* (range = 0.037–0.050) (reviewed in Richter et al. 2003).

Individual genetic background at the egg stage was not significantly different from the adult stage. Genetic variation of adults on average should be reflected in the eggs if most or all adults breed. However, sampling methods could bias results. Because it is not possible to genotype every egg without sacrificing the entire year class, subsamples were removed from each egg clutch with the assumption that 4–6 eggs would capture most of the genetic variation within the clutch. If there is assortative pairing of mates, genetic measures at the egg stage may deviate from adult measures, but this would be more evident if eggs were examined as grouped by egg clutch instead of as a single sample (see below).

An apparent correlation between sample size and magnitude of measures existed, so metamorphs, which had the largest sample size and was the only group significantly greater than others, were randomly sub-sampled. This valuable demonstration showed that the effect was not driven by sample size but was not completely necessary because of the nature of the data. For adults, the annual population was 110 individuals, and 106 of them were genotyped. The metamorph sample was 209 of the 214 that emerged from the pond. For both, nearly the entire population (in a statistical sense) was sampled, and so statistical estimation should closely approximate the population value.

Mortality in egg clutches

Data for individual egg clutches suggested a negative association between survival at the egg stage and genetic variability of clutches. However, caution should be taken in interpretation. The difference was significant for both SMH and IR and was only a non-significant trend for SMD^2 and MR. Additionally, the association of variables in the regressions was low ($R^2 < 0.16$ for all). For all measures, wide variation in genetic background of clutches with high mortality increased statistical (unexplained) noise and potentially weakened the power of the analyses. These analyses preliminarily suggest a genetic-fitness effect to explain *R. sevosa* egg mortality, but more detailed ecological genetic studies are required to determine the robustness of these data.

Relatedness of breeding pairs

Although genetic measures for all eggs in the population should approximate measures for adults, one might expect deviations when examining measures for individual egg clutches. Genetic measures of egg clutches based on unbiased sampling of all eggs should approximate the effective population of adults. If the effective population size is greatly different from the census population size or if non-random mating occurs with respect to genotype, then measures for individual egg clutches should differ from the adult population. Non-random mating via choice of mates with higher genetic quality (i.e., "good genes;" Welch et al. 1998) or reproduction between less-related mates (e.g., Garner et al. 2003) has been demonstrated for anuran and caudate amphibians. Although the mating system of *R. sevosa* is not well-studied, males are known to interact aggressively (Doody et al., 1995), which suggests territoriality or at least potential for

associated behaviors that permit multiple mating by males as is known for other ranids (e.g., Wells 1978).

No significant differences in genetic measures were found between the adult population and egg clutches. This suggests that mating was random and that most adults actually bred. Egg clutches had higher SMD^2 and lower IR, which suggested non-random mating. However, results were inconclusive, and analyses potentially suffered from low power because only a small fraction of eggs for each clutch (< 0.1 %) was sampled. Genetic variation of the entire egg clutch potentially would not be sampled, especially for such highly polymorphic genetic markers as microsatellites.

Genetic-fitness relationships

Offspring of genetically divergent parents often have higher fitness (Rossiter et al. 2001; Blomqvist et al. 2002; Foerster et al. 2003; Primmer et al. 2003; Hansson et al. 2004). Individuals with a more varied genetic background should have a higher survival probability than those homozygous for many loci (Mather 1953; reviewed in Møller & Swaddle 1997). The relationship between genetic variability and fitness could result from numerous sources. Inbreeding increases the number of homozygous loci throughout the genome in offspring. As the frequency and severity of inbreeding reaches a critical level, negative consequences on fitness ensue (inbreeding depression) via at least two mechanisms: increased occurrence of offspring inheriting deleterious recessive alleles from each parent and loss of heterozygote advantage (Charlesworth & Charlesworth 1999).

The population-level effects of mortality in early life history stages (eggs, larvae, and

metamorphs) of amphibians with similar life histories to *R. sevosa* are much less than at later stages (juveniles and adults) (Vonesh & De la Cruz 2002). That is, high levels of mortality naturally occur at early stages in these amphibians without affecting long-term population persistence. Therefore, under certain circumstances elevated levels of egg mortality may benefit a population, especially if the probability of survival is positively correlated with genetic variation (i.e., genetic background positively affects developmental stability; e.g., Hoelzel et al. 2002). In the case of *R. sevosa*, enhanced fitness of more outbred individuals should act to counter the effects of genetic drift, which are most severe for small populations. More specifically, these individuals have wider and rarer alleles (as evidenced by comparisons of SMD^2 and IR scores). Drift will have more severe effects on alleles in low frequency because these are the most likely to go extinct due to stochasticity (Nei 1987). Therefore, increased survival of individuals possessing rarer alleles should minimally delay the negative consequences of drift and help to maintain allelic richness.

Assuming microsatellites evolve under a step-wise or two phase mutation model, d^2 should be related to the time to coalescence of alleles (i.e., time since two alleles evolved from a common ancestor) (Coulson et al. 1998; Slate et al. 2000). When averaged over many loci, d^2 should reflect the genetic distance between parental gametes (Slate et al. 2000). Therefore, measures of heterozygosity should reveal more recent breeding history whereas width-based measures focus on events deeper in the pedigree (Slate et al. 2000; Neff 2004b). In fact, many studies have found an association between fitness and d^2 with no corresponding association between fitness and heterozygosity (Rossiter et al. 2001; Höglund et al. 2002; Neff 2004b).

Another potential mechanism for differential genetic-fitness relationships of measures is that power of statistical analyses, which is equally affected by low sample size and number of loci, is more severely impacted for width-based measures than for heterozygosity-based measures (Höglund et al. 2002; Slate & Pemberton 2002; Coltman & Slate 2003). Therefore meta-analyses that address fitness-genetic relationships for heterozygosity and width based measures could be biased by studies that commit type II statistical errors because they used less than 10 loci and lacked the power to detect small (and actual) effect sizes (Britten 1996; Slate & Pemberton 2002; Coltman & Slate 2003).

Previous studies vary in which measures were more robust and powerful in detecting a fitness-genetic relationship (reviewed in Slate & Pemberton 2002). Width-based measures have often been implicated as a more robust indicator of outbreeding depression rather than inbreeding depression, primarily because of the small effect size relative to the sample size necessary to detect genetic effects of inbreeding depression (Slate & Pemberton 2002; Neff 2004b). However, the consequences of inbreeding are more obvious for isolated and small populations, and power of these measures should increase greatly for studies conducted in these populations (Höglund et al. 2002). Population genetic analyses of *R. sevosa* indicated that the population has undergone a bottleneck and has a genetic signature indicative of inbreeding (Chapters 1 and 2). Negative fitness consequences of inbreeding could explain the large effect size for the genetic-fitness relationship in this population. Therefore, only a few loci were necessary to detect a pattern for width-based measures. This hypothesis is difficult to test because it requires a non-inbred population of *R. sevosa*, and none is known to exist. An examination of survival through development of a non-inbred population of *R. capito*, the

sister species of *R. sevosa*, would be the best surrogate study. Perhaps more appropriate would be a genetic and laboratory experiment of survival and fitness-related traits (e.g., performance) for inbred individuals of *R. sevosa* and non-inbred *R. capito*.

Conclusions

Genetic variation in small, isolated populations will continue to decline unless populations become established nearby by natural or artificial means. However, selection for more outbred individuals should help to alleviate genetic stress and should prolong (at least temporarily) the persistence of isolated populations. The strongest evidence for increased fitness of more outbred individuals of *Rana sevosa* was for survival to metamorphosis. The egg and larval stage of the amphibian life cycle is one of high mortality. Thus, non-random survival of offspring with respect to width (and thus rareness) of alleles should oppose the effects of drift and ultimately maintain allelic richness, which may extend the time to extinction due to genetic factors.

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