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Experimental evaluation of the usefulness of microsatellite DNA for detecting demographic bottlenecks

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Abstract

Evolutionary and conservation biologists often use molecular markers to evaluate whether populations have experienced demographic bottlenecks that resulted in a loss of genetic variation. We evaluated the utility of microsatellites for detection of recent, severe bottlenecks and compared the amounts of genetic diversity lost in bottlenecks of different sizes. In experimental mesocosms, we established replicate populations by releasing 1, 2, 4 or 8 pairs of the western mosquitofish, *Gambusia affinis* (Poeciliidae). Using eight polymorphic microsatellite loci, we quantified seven indices of genetic diversity or change that have been used to assess the effects of demographic bottlenecks on populations. We compared indices for the experimentally bottlenecked populations to those for the source population and examined differences between populations established with different numbers of founders. Direct count heterozygosity and the proportion of polymorphic loci were not very sensitive to genetic changes that resulted from the experimental bottlenecks. Heterozygosity excess and expected heterozygosity were useful to varying degrees in the detection of bottlenecks. Allelic diversity and temporal variance in allele frequencies were most sensitive to genetic changes that resulted from the bottlenecks, and the temporal variance method was slightly more correlated with bottleneck size than was allelic diversity. Based on comparisons to a previous study with allozymes, heterozygosity, temporal variance in allele frequencies and allelic diversity, but not proportion of polymorphic loci, appear to be more sensitive to demographic bottlenecks when quantified using microsatellites. We found that analysis of eight highly polymorphic loci was sufficient to detect a recent demographic bottleneck and to obtain an estimate of the magnitude of bottleneck severity.

Keywords: bottleneck, *Gambusia*, genetic diversity, heterozygosity, mesocosm, microsatellites, mosquitofish

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Introduction

Demographic bottlenecks occur when populations experience severe, temporary reductions in size. Because bottlenecks may influence the distribution of genetic variation within and among populations, the genetic effects of reductions in population size have been studied extensively by evolutionary biologists (Wright 1931; Nei *et al*. 1975). More recently, conservation biologists have become concerned with the effects of demographic bottlenecks on the viability of small populations. Loss of genetic diversity may reduce the potential of small populations to respond to selective pressures (Allendorf & Leary 1986), and increased inbreeding may reduce population viability (Leberg 1991; Newman & Pilson 1997; Westemeier *et al*. 1998).

A common theme in conservation genetics has been the use of genetic variation to identify populations that have experienced demographic bottlenecks. Populations known to have experienced a reduction in demographic size often show reduced genetic diversity (e.g. Bonnell & Selander 1974; Bouzat *et al*. 1998). Numerous threatened or endangered species and populations have been found to have low levels of genetic variation (Gottelli *et al*. 1994; O'Brien 1994; Taylor *et al*. 1994; Vrijenhoek 1994; Mundy *et al*.

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1997; Gibbs *et al*. 1998). Using the converse of the theory that bottlenecks result in the loss of genetic diversity, low genetic diversity has often been taken as evidence that a population has experienced a bottleneck. However, not all populations that have been reduced to small sizes show measurably lower levels of nuclear genetic diversity (e.g. Bowling & Ryder 1987; Waldman *et al*. 1998). Clearly, an evaluation of approaches to assess bottlenecks with molecular markers would aid conservation and evolutionary geneticists in studying reductions in population size.

The most common measures of genetic diversity are heterozygosity (proportion of heterozygous individuals in the population), allelic diversity (number of alleles at a locus in the population), and proportion of polymorphic loci (Nei *et al*. 1975; Leberg 1992). Changes in heterozygosity are often measured to detect bottlenecks; however, in an experimental assessment of allozyme polymorphisms in fish populations, it was found that estimates of heterozygosity sometimes increased after severe bottlenecks (Leberg 1992). Simulations have shown that, after a founder event, the estimated heterozygosity at one or a small number of loci may increase due to chance shifts towards more equal allele frequencies (Leberg 1992; Neigel 1996). Allelic diversity is expected to be more sensitive than heterozygosity to the effects of demographic bottlenecks (Nei *et al*. 1975), a result verified in an experimental study of allozyme variation in fish (Leberg 1992).

Another index of the effects of bottlenecks on genetic variation is the temporal change in allele frequencies (Nei & Tajima 1981; Waples 1989; Luikart *et al*. 1999). Simulations and empirical observations of allozyme variation show that temporal variance is likely to increase as a result of reductions in population size, but is likely to be underestimated in populations experiencing the most severe bottlenecks (Richards & Leberg 1996). The weak relationship between estimates of temporal variance and bottleneck severity is due to the loss of alleles during the bottleneck, which places an absolute limit on drift of allele frequencies (Richards & Leberg 1996).

Cornuet & Luikart (1996) recently introduced heterozygosity excess as an alternative method for detection of demographic bottlenecks. This method is based on the premise that populations experiencing recent reductions in size develop an excess of heterozygosity at selectively neutral loci relative to the heterozygosity expected at mutation-drift equilibrium (Cornuet & Luikart 1996). Heterozygosity excess offers an advantage over measurements of average heterozygosity, allelic diversity and temporal change in allele frequencies: no genetic data from the population prior to the bottleneck are needed (Cornuet & Luikart 1996). This method has successfully detected demographic bottlenecks in a series of natural populations (Luikart & Cornuet 1998), but has the disadvantage of requiring large numbers of polymorphic loci.

Allozymes have often been used to assay genetic diversity in populations that have experienced bottlenecks (Leberg 1996). In most populations, the relatively low numbers of highly polymorphic loci and alleles per locus limit the utility of allozymes for this purpose. Marker loci with higher levels of allelic diversity might be more sensitive than allozymes for detecting bottlenecks in natural populations (Richards & Leberg 1996; Luikart *et al*. 1998, 1999). One example of such a highly polymorphic marker is microsatellite DNA. Microsatellites are short, tandemly repeating, 1–5 bp units of DNA that occur throughout the genome of many organisms (e.g. Scribner *et al*. 1996; Bensch *et al*. 1997; Wyttenbach *et al*. 1997). These loci often have larger numbers of alleles and higher heterozygosity than allozymes. For example, microsatellites developed for the western mosquitofish, *Gambusia affinis*, have an average of 18.8 alleles per locus and exhibited much higher direct count heterozygosities ($\bar{H}_{obs} = 0.79$ for seven loci) than allozyme loci ($\bar{H}_{obs} = 0.11$ for 17 loci) from the same population (Spencer *et al*. 1999).

Our objective was to evaluate the utility of microsatellites for detecting demographic bottlenecks. Our experiments addressed short-term bottlenecks created from a single founder event after which the population rapidly recovered. We compared the relative sensitivity of seven measures of genetic diversity or change that might be valuable for detection of demographic bottlenecks. Furthermore, by experimentally manipulating bottleneck sizes, we examined how severe a reduction in population size was needed to generate a measurable change in the genetic characteristics of a population. Our results provide useful guidance to investigators attempting to use molecular markers to assess the severity of demographic bottlenecks.

Materials and methods

The western mosquitofish, *Gambusia affinis* (Poeciliidae), is a small (20–50 mm) live-bearer able to tolerate a wide range of habitats, temperatures and water qualities (Meffe & Snelson 1989). This species is a close relative of *G. holbrooki*, which has been used in experimental assessment of the effects of bottlenecks on allozyme variation (Leberg 1992; Richards & Leberg 1996). In experimental mesocosms, successful mosquitofish populations produce two to three generations over the course of a field season (April to September), with population sizes ranging from approximately 100–250 individuals (Leberg 1993; Rogowski 1997).

Experimental populations

Mesocosms were located in an open field at the University of Louisiana's Center for Ecology and Environmental Technology, located in Carencro, Louisiana. Each large

plastic pool was approximately 2.2 m in diameter and 86 cm deep. Pools were filled with approximately 2800 L of well water. To provide a quasi-natural environment, 2 kg of pond sediments were deposited in each mesocosm in April 1997. Two artificial cover objects (plastic pom-pons, approximate diameter 66 cm) were added to provide cover for offspring. Mesocosms were established 14 days before the introduction of *Gambusia* to allow for colonization by insects and zooplankton. No supplemental feeding was conducted, and water levels were maintained either by natural rainfall or by addition of well water. Each mesocosm represented a replicate aquatic community with complex interactions involving primary producers and vertebrate and invertebrate predators and prey (Leberg 1993; Rogowski 1997).

The source of *G. affinis* for our experiments was the Bayou Ile des Cannes, Lafayette Parish, Louisiana (Spencer *et al*. 1999). In April 1997, we established three replicates of each of four bottleneck sizes in mesocosms, following the design of Leberg (1992). These treatments consisted of unrelated founders of 1, 2, 4 and 8 pairs. All founders were of approximately the same length to avoid variation in population growth resulting from length-specific fecundity (Leberg 1993). Female founders were obtained from a general laboratory stock and may have been inseminated prior to release. Random selection of females from a source stock results in a better approximation of the natural bottlenecks that occur in many wild populations of *Gambusia* (Robbins *et al*. 1987) than does use of virgin females as founders (e.g. Leberg 1992). However, the number of fish released to establish populations should be viewed as the minimum rather than absolute number of founding individuals.

Populations were allowed to expand from their initial small sizes over the summer field season and were assessed in October 1997, approximately 2–3 generations after the founder event. Population censuses were conducted with a 1/8-inch mesh seine; each mesocosm was seined seven times to ensure that all fish large enough to be seined were counted. Population size differences among treatments were evaluated with an analysis of variance (ANOVA). We used Spearman's rank correlation (R) to analyse the relationship between population size and bottleneck size.

DNA isolation and amplification, and microsatellite analysis

DNA from tail muscle, or from entire fish in the case of smaller fish, was isolated from 20 males and 20 females from each mesocosm and from the source population according to the Gentra Puregene™ DNA isolation protocol. Seven loci (Gafμ1–Gafμ7) described by Spencer *et al*. (1999) and one (Mf-13) described by Zane *et al*. (1999) were amplified from each DNA isolate. Conditions identical to those described by Zane *et al*. (1999) were used to successfully amplify locus Mf-13 in *G. affinis*. Amplifications for the loci described by Spencer *et al*. (1999) were performed in a 15 μL final reaction volume containing at least 100 ng of genomic DNA template, 5.4 pmol forward primer and 0.6 pmol end-labelled forward primer (ABI Fluorescent Label, Perkin-Elmer), 6.0 pmol reverse primer, 100 μM each dNTP, 0.6 U Amplitaq Gold DNA polymerase (Perkin-Elmer™), and PCR buffer (Perkin Elmer: 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% w/v gelatin final concentration) overlaid with mineral oil. Loci were amplified by PCR using a Perkin-Elmer 480 thermal cycler. The cycling conditions for all loci began with an initial 12 min denaturation at 94 °C followed by 25 cycles of 40 s denaturation at 94 °C, 40 s at an optimum annealing temperature for each primer pair, a 1 min ramp to 72 °C, and 1 min extension at 72 °C, and concluded with a 30 min final extension at 72 °C. Annealing temperatures are listed in Zane *et al*. (1999) and Spencer *et al*. (1999).

Fluorescent-labelled PCR products were size-separated and analysed by an ABI Prism 310 Genetic Analyser using the GENESCAN software package. We identified allele peak profiles at each locus and assigned each individual a genotype, with alleles designated by their size in base pairs. For each population we tested the observed frequencies of heterozygotes for deviation from Hardy–Weinberg expectations using Fisher's exact test, computed in Genetic Data Analysis (GDA, Lewis & Zaykin 1997). Data are available on request from the corresponding author and may be found in Spencer (1998).

Genetic diversity indices

We calculated seven genetic diversity measures that might reflect each population's history of bottlenecks. Four of the measures of genetic diversity were the same as those examined by Leberg (1992): average proportion of heterozygotes per locus determined by direct counts (H_{DC}), the average proportion of heterozygotes per locus based on Hardy–Weinberg expectations (H_{HW}), the proportion of polymorphic loci (P) and the average number of alleles (A) per locus. A Wilcoxon signed ranks test was used to compare H_{DC}, H_{HW} and A from each treatment population to the source population. In these comparisons, the values of H_{HW}, H_{DC} and A for each locus were the units of replication (Leberg 1992). Fisher's exact test was used to compare P for each mesocosm population to that for the source population.

We also calculated a measure of temporal change in allele frequencies (F_c, Tajima & Nei 1984; Waples 1989) for each population. To determine whether experimental populations had drifted significantly from the source population, we calculated the expectation of F_c, $E(F_c)$,

assuming that no genetic drift not due to the experimental bottleneck had occurred. This assumption is not true as some drift occurs even in large populations; however, based on past simulations (Leberg 1992; Richards & Leberg 1996), we can conclude that drift in post-bottleneck populations of *Gambusia* is small relative to drift resulting from the bottleneck events. Under this assumption, $E(F_c)$ becomes the difference in allele frequencies expected to result from errors in allele frequency estimates associated with sampling 40 individuals from each of the source and experimental populations (Richards & Leberg 1996). We calculated 95% confidence intervals for estimates of F_c (Eqn. 16 in Waples 1989) for each population and determined whether $E(F_c)$ fell within the confidence intervals. If $E(F_c)$ was less than the lower 95% confidence limit of F_c, we rejected the hypothesis that there was no evidence of genetic drift resulting from the experimental bottleneck. If $E(F_c)$ was greater than or equal to the lower 95% confidence limit, temporal differences in allele frequencies can be explained by sampling error without invoking genetic drift (Waples 1989).

Finally, we calculated the heterozygosity excess (*HE*) for each locus and population (Cornuet & Luikart 1996). Calculations were performed by the program BOTTLENECK (Piry *et al*. 1999). The program computes the standardized difference for each locus $[(H_{HW} - H_{exp})/SD = HE]$, where H_{HW} is the heterozygosity under Hardy–Weinberg expectations, H_{exp} is an expectation of heterozygosity based on the number of alleles per locus in the population, and SD is the standard deviation of H_{exp}. The program then simulates a distribution of expected heterozygosities using the coalescent process of number of loci (8) under two possible mutation models, the infinite allele model (IAM) and the stepwise mutation model (SMM).

Statistical analyses

BOTTLENECK automatically reports statistics for both HE_{IAM} and HE_{SMM} (listed as *DH*/SD in the output). The program then performs a Wilcoxon signed ranks test of the hypothesis that the average standardized difference across loci for each population is not significantly different from zero. Because the standardized differences test is parametric and assumes a normal distribution of heterozygosity across loci, we also used Wilcoxon signed ranks to test the hypothesis that values of *HE* from the source and each of the experimental populations were not different.

In addition to evaluating whether indices of microsatellite variation indicated that the experimental populations had experienced a bottleneck, we examined differences in the values of indices among experimental populations founded with different numbers of individuals. This analysis was conducted to determine which indices were most sensitive to small differences in bottleneck size. We used a nonparametric Friedman's test, with locus as the unit of replication, to determine whether A, H_{HW}, $H_{DC,}$ F_c, HE_{IAM} and HE_{SMM} differ between experimental populations. If the results of this test indicated that an index of microsatellite variation differed among experimental populations, we used a multiple range test, controlling for type II error, to evaluate which populations were different. Because there is no expectation for *HE* in cases when a locus is fixed, the Friedman's analysis was modified slightly in comparisons involving the three populations that had lost variation at one locus (populations A9, B6 and A10). In comparing each of these populations to the other populations, the multiple range test was conducted with only the seven loci that were polymorphic in each population, rather than all eight loci. We assessed the relationship of each genetic measure within bottleneck size using Spearman's R correlation analysis. All statistical tests, with the exception of the Wilcoxon signed ranks test conducted in BOTTLENECK and the Friedman's test, were conducted using SAS procedures (SAS Institute Inc. 1990), and the significance level was set at $\alpha = 0.05$.

Results

Population bottleneck and recovery

Of the 12 experimental populations, one of the three mesocosm replicates with two founders became extinct due to death of the female founder and was thus not available for analysis. Populations grew rapidly in the remaining mesocosms (Fig. 1). No significant variation in population size was detected among the treatments after 2–3 generations of growth following the bottleneck ($F_{3,7} = 2.02$, $p = 0.200$). The population sizes were similar to those obtained by Rogowski (1997), and are believed to be at or near the carrying capacity for the mesocosms during the summer and autumn in Louisiana. Rapid growth of the populations with the smallest numbers of founders means that most of the genetic differences between treatments will be due to founder number and not the population sizes in subsequent generations (Leberg 1992).

Indices of genetic diversity

Estimates of H_{HW} for six of the 11 experimental populations were significantly less than for the source population (Table 1). All of the populations established with two fish, and two-thirds of those established with four fish had significant loss of H_{HW} compared to the source, as did one of each of the populations established with 8 and 16 individuals (Table 1). A Friedman's test indicated that populations established with two individuals usually had

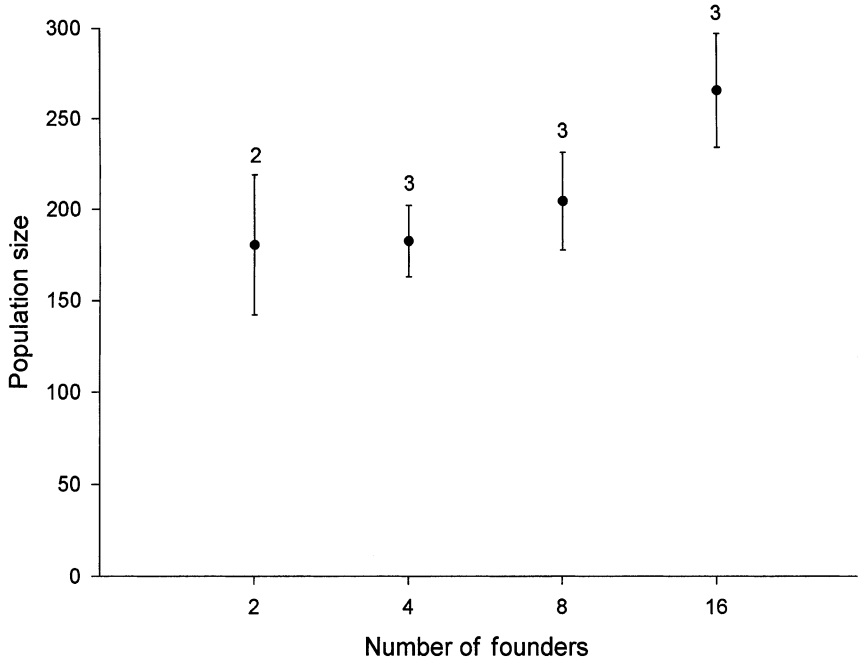

Fig. 1 Population sizes of *Gambusia affinis* in 11 experimental mesocosms measured after four months of recovery from four different initial population sizes. Bars represent standard errors. Numbers above the bars indicate the number of replicates.

Table 1 Wilcoxon signed ranks tests (WSR) for expected heterozygosity (H_{HW}), direct count heterozygosity (H_{DC}) and allelic diversity (A) for 11 experimentally bottlenecked populations of *Gambusia affinis* at eight microsatellite loci

		H_{HW}			H_{DC}			A			P
Number of founders	Population	$\bar{H}_{HW}$	WSR	p	$\bar{H}_{DC}$	WSR	p	$\bar{A}$	WSR	p	
2	A5	0.513	18	0.008	0.559	6.0	0.375	3.5	14.0	0.016	1.000
2	B6	0.524	18	0.008	0.644	4.0	0.641	3.6	18.0	0.008	0.875
4	A9	0.546	18	0.008	0.657	3.0	0.742	5.5	18.0	0.008	0.875
4	B2	0.595	13	0.078	0.769	–12.0	0.109	3.9	14.0	0.016	1.000
4	F8	0.610	17	0.016	0.657	6.0	0.461	5.6	14.0	0.016	1.000
8	A10	0.632	9	0.250	0.695	–5.0	0.547	6.5	17.0	0.016	0.875
8	D6	0.641	16	0.023	0.670	2.5	0.773	6.8	14.0	0.016	1.000
8	F6	0.641	13	0.078	0.651	5.0	0.547	9.6	14.0	0.016	1.000
16	B4	0.640	16	0.023	0.697	–1.0	0.945	9.9	14.0	0.016	1.000
16	F1	0.732	10	0.195	0.752	–8.0	0.313	11.3	14.0	0.016	1.000
16	F5	0.681	10	0.195	0.699	–6.0	0.461	9.8	10.5	0.031	1.000
	Source	0.732			0.686			15.1			

Values of p represent the probability that the measures of genetic diversity are not different from those of the source population. The proportion of polymorphic loci (P) was not different between any of the populations and the source based on Fisher's exact test ($p > 0.30$).

lower estimates of H_{HW} than populations established with 8 or 16 fish, and several of the populations established with four individuals had lower estimates of H_{HW} than populations established with 16 individuals (Table 2). Estimates of H_{HW} were positively correlated with the number of founders ($R = 0.914$, $p = 0.0001$, Fig. 2).

None of the estimates of H_{DC} for the experimental populations were significantly different from that for the source population (Table 1). There was a significant positive correlation between H_{DC} and the number of founders ($R = 0.661$, $p = 0.027$, Fig. 2). Friedman's test detected no differences in H_{DC} among any of the experimental populations.

The differences in the results of comparisons of estimates of H_{DC} and H_{HW} between the source and experimental populations and between the various experimental

Table 2 Number of significant differences* between experimental populations over the total number of pair-wise comparisons for each index of genetic change†

Genetic index	Number of founders			
	2	4	8	16
Heterozygosity (H_{HW})				
2	0/1	0/6	5/6	6/6
4		0/3	0/9	5/9
8			0/3	0/9
16				0/3
Allelic diversity (A)				
2	0/1	2/6	6/6	6/6
4		0/3	6/9	9/9
8			0/3	2/9
16				0/3
Temporal variance method (F_c)				
2	0/1	4/6	6/6	6/6
4		2/3	7/9	9/9
8			0/3	5/9
16				1/3
Heterozygote excess (HE_{SMM})				
2	0/1	1/6	4/6	2/6
4		0/3	2/9	1/9
8			0/3	1/9
16				0/3

There were no significant differences in proportion of polymorphic loci (P), direct count heterozygosity (H_{DC}), and heterozygote excess based on the infinite allele model (HE_{IAM}), so these indices are not presented.

*Based on multiple comparison procedure for Friedman's non-parametric ANOVA. This procedure was used to maintain a type I error of 0.05 when making non-independent pairwise comparisons.

†For example, comparing the three populations with 8 founders to the three with 16 founders implies that nine pairs of populations might differ for an index of genetic change. Significantly lower post-bottleneck values of A occurred in populations established with eight individuals in two of the nine comparisons (2/9). In five of the nine (5/9) comparisons, populations founded with eight individuals experienced more drift (higher values of F_c) than populations with 16.

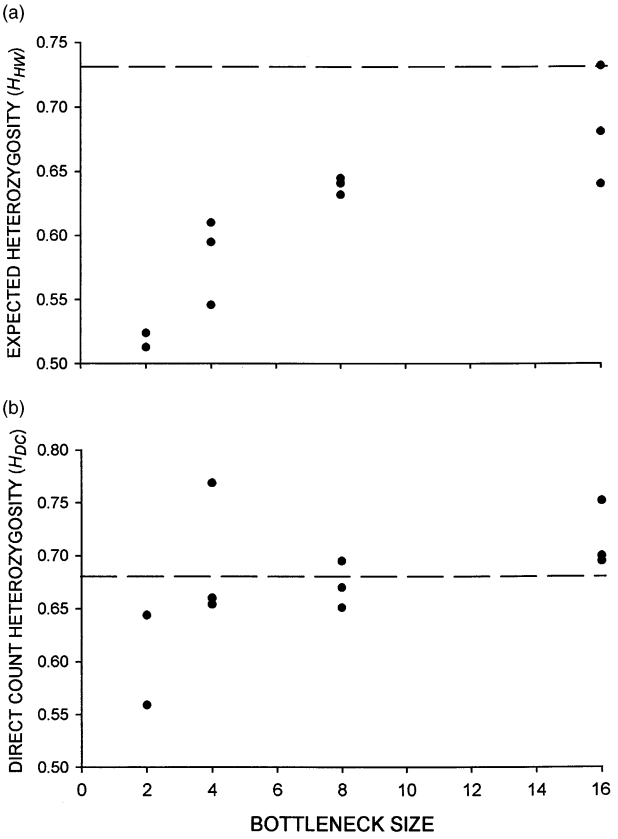

Fig. 2 Estimates of expected Hardy–Weinberg heterozygosity (a) and direct count heterozygosity (b) for 11 experimental populations of *Gambusia affinis* calculated from eight polymorphic microsatellite loci plotted against the number of founders for each population. Estimates for the source population are represented by dashed lines.

populations suggested that the genotype frequencies might not be in Hardy–Weinberg equilibrium. Observed frequencies of heterozygotes deviated from Hardy–Weinberg expectations in the populations founded with 2, 4 and 8 individuals at 3–5 loci per population ($p < 0.05$). The source population and the populations founded with 16 individuals were in Hardy–Weinberg equilibrium for all loci. Differences in H_{DC} and H_{HW} can also be explained by binomial sampling error of maternal and paternal gametic allele frequencies, which seems likely because of the small numbers of founders in our experimental populations (Pudovkin *et al*. 1996).

The estimate of number of alleles per locus (A) for each of the experimental populations was significantly less than for the source (Table 1). All populations established with 8 or 16 individuals had higher allelic diversity than did populations established with two individuals. Likewise, A was different in one-third of the comparisons between populations established with 2 or 4 individuals. Significant differences in estimates of A were observed between populations established with 4 and 16 individuals; differences were also observed between many of the populations founded with 4 and 8 fish (Table 2). Over half of the comparisons of the populations established with eight founders had lower estimates of A than populations established with 16 individuals. One comparison between two populations each founded by eight individuals also detected a difference in A. There was a strong positive

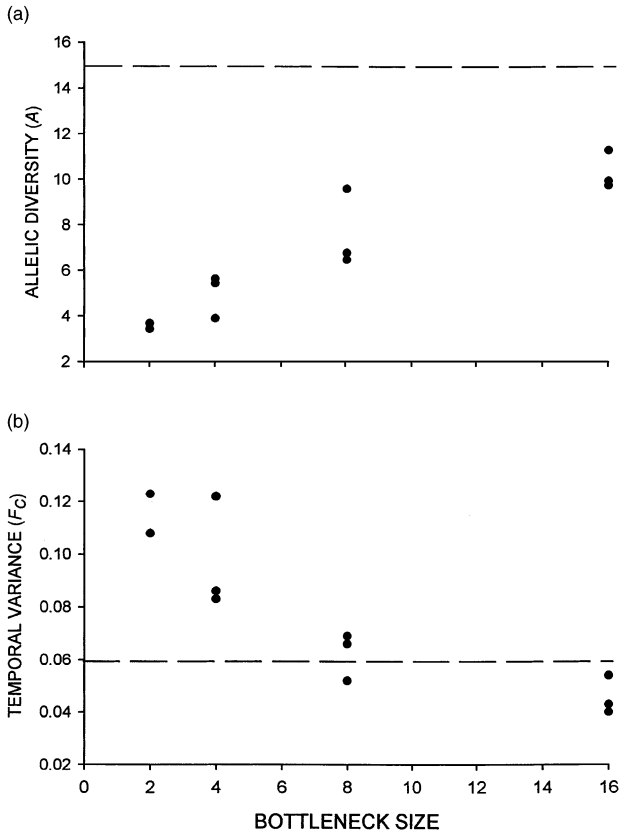

Fig. 3 Estimates of allelic diversity (a) and temporal variance in allele frequencies (b) for 11 experimental populations of *Gambusia affinis*, calculated from eight polymorphic microsatellite loci, and plotted against the number of founders for each population. For allelic diversity, the dashed line represents the estimate for the source population; for temporal variance, it is the variance in allele frequencies expected to result from sampling error.

correlation between A and bottleneck size ($R = 0.905$, $p = 0.0001$; Fig. 3).

The proportion of polymorphic loci was largely unaffected by bottlenecks. The average P for each bottleneck size ranged from 0.93 to 1.00 (Table 1). In only three populations did allele frequencies for any locus reach fixation; in each of these populations only one locus became monomorphic. There were no differences between the proportions of polymorphic loci in the source and any of the experimental populations (Table 1). There was no correlation between bottleneck size and P ($R = 0.266$, $p > 0.2$), and there was no difference in P among the experimental populations.

The $E(F_c)$ associated with source and experimental sample sizes of 40 individuals was 0.025. This value falls below the lower confidence limit for all populations with two and four founders and two of three populations with eight founders (Table 3). The expectation falls within the confidence interval for the remaining population with eight founders and all three populations with 16 founders. Estimates of F_c were significantly different among the populations that experienced experimental bottlenecks. Populations established with two individuals had significantly higher estimates of F_c than most of the populations established with four individuals and all of the populations established with eight and 16 fish (Table 2). Populations established with four fish usually had significantly higher estimates of F_c than populations established with eight or 16 fish. Over half of the comparisons of populations founded with eight fish had higher estimates of F_c than populations established with 16 individuals (Table 2). In several cases, estimates of F_c differed among populations founded with identical numbers of fish (Table 2). Estimates of $\bar{F}_c$ were negatively correlated with the number of founders ($R = -0.981$, $p = 0.0001$; Fig. 3).

Under the IAM, estimates of heterozygosity excess were significantly greater than zero for all but two populations (Table 3). Four of the populations had different values of HE_{IAM} than the source, although these differences were not related to bottleneck size. There was no difference in estimates of HE_{IAM} among the populations that experienced experimental bottlenecks. Number of founders and HE_{IAM} were not significantly correlated ($R = -0.501$, $p = 0.116$, Fig. 4). The source populations exhibited no heterozygosity excess relative to IAM expectations.

Unlike the case for the IAM, the source population exhibited significantly less heterozygosity than expected under the SMM (Table 3; $p > 0.975$). This suggests that the SMM does not apply to our data as well as the IAM. In practice, most investigators will not have access to a source population when examining heterozygosity excess in natural populations. Therefore, we present the analysis of SMM estimates for the experimental populations in spite of the apparent lack of fit of this model to our data.

Estimates of heterozygosity excess under the SMM were only significantly greater than zero for populations founded with two fish (Table 3). Six of the populations had estimates of HE_{SMM} that were significantly greater than that of the source; differences were scattered across treatments (Table 3). Values of HE_{SMM} were significantly different among the populations that experienced experimental bottlenecks. Populations founded with two individuals often had significantly higher estimates of HE_{SMM} than did populations established with eight. In fewer cases, populations established with two individuals had significantly higher estimates than did populations established with 4 or 16 fish (Table 2). In less than a third of the pairwise comparisons, populations started with four individuals had estimates of HE_{SMM} that were significantly higher than populations established with 8 or 16 individuals. The number of founders and HE_{SMM} were negatively correlated ($R = -0.661$, $p = 0.027$; Fig. 4).

Table 3 Results of temporal variance ($\bar{F}_c$) and heterozygosity excess (*HE*) for both the IAM and SMM mutation models for 11 experimentally bottlenecked populations of *Gambusia affinis* at eight microsatellite loci

Number of founders	Population	Temporal variance method			Heterozygote excess			Heterozygote excess		
		$\bar{F}_c$	Lower	Upper	HE_{IAM}	p^e	p^s	HE_{SMM}	p^e	p^s
2	A5	0.108*	0.049	0.398	0.915	0.010	0.123	0.597	0.027	0.025
2	B6	0.123*	0.056	0.452	1.135	0.004	0.019	0.685	0.039	0.063
4	A9	0.083*	0.038	0.304	0.489	0.148	0.310	−0.608	0.711	0.068
4	B2	0.122*	0.056	0.447	1.090	0.004	0.025	0.546	0.320	0.012
4	F8	0.086*	0.039	0.315	0.709	0.006	0.093	0.045	0.422	0.025
8	A10	0.066*	0.030	0.243	0.986	0.008	0.018	0.298	0.188	0.028
8	D6	0.069*	0.031	0.252	0.547	0.037	0.093	−0.451	0.844	0.025
8	F6	0.052	0.024	0.190	0.272	0.191	1.000	−1.009	0.990	0.263
16	B4	0.054	0.025	0.199	0.351	0.027	0.674	−0.899	1.000	0.327
16	F1	0.040	0.018	0.147	0.800	0.004	0.036	−0.216	0.809	0.025
16	F5	0.043	0.020	0.157	0.628	0.020	0.208	−0.515	0.770	0.123
	Source				0.094	0.422		−2.241	1.000	

Average temporal variance ($\bar{F}_c$) and the upper and lower limits of a 95% confidence interval are given for each population. An asterisk indicates that the expected amount of variance in allele frequencies [$E(F_c) \approx 0.025$] due to sampling error alone lies below the 95% confidence interval for the $\bar{F}_c$ estimated for the experimental population (and thus drift was not detected). Values of p^e represent the probability that a population's value of *HE* is not different from the expected value for a population near mutation-drift equilibrium. Values of p^s represent the probability that a population's value of *HE* is not different from the source value.

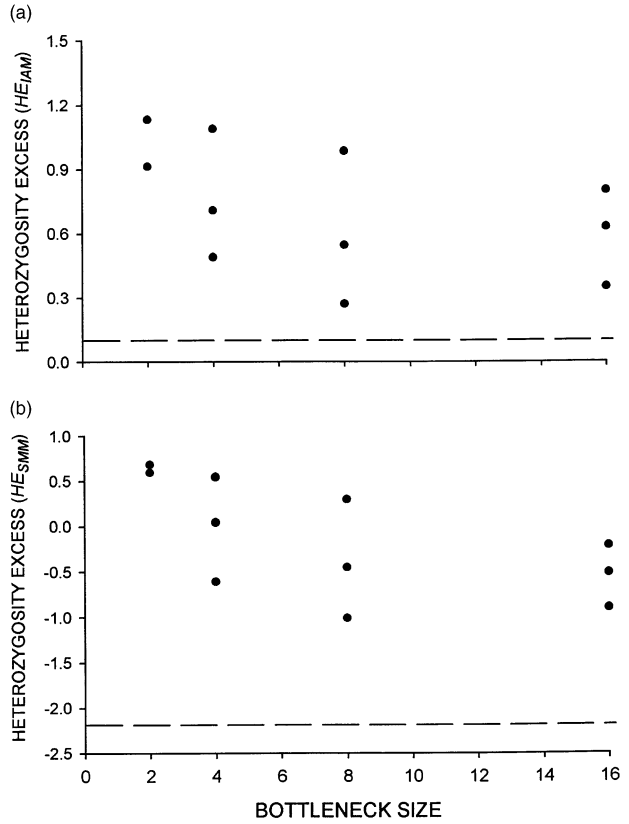

Fig. 4 Estimates of heterozygosity excess under the IAM (a) and the SMM (b) for 11 experimental populations of *Gambusia affinis* calculated from eight polymorphic microsatellite loci and plotted against bottleneck size for each population. Estimates for the source population are represented by dashed lines.

Discussion

For the assessment of demographic bottlenecks, a genetic index should (1) change as the result of the bottleneck, (2) differ between bottlenecks of differing severity, and (3) be correlated with bottleneck magnitude. We assessed these three criteria in the comparison of source and experimental populations, the comparison of experimental populations, and the correlation of index values with founder number. For comparison of the seven indices, it is useful to assign them relative sensitivities to the effects of demographic bottlenecks based on the results of our study (Table 4). To examine the hypothesis that microsatellite variation will be more sensitive to the effects of bottlenecks than allozymes, we also assigned relative sensitivities to H_{DC}, H_{HW}, *P*, *A* and F_c, based on an allozyme study of experimental bottlenecks that was very similar to our study system (Leberg 1992; Richards & Leberg 1996).

Heterozygosity is probably the most widely used measure of genetic diversity in natural populations. Using allozymes, Leberg (1992) found that both direct count and expected heterozygosity only weakly reflected a population's history of bottlenecks (Table 4). With microsatellites, we found that direct count heterozygosity for the experimental populations was not distinguishable from that of the source, and that it did not differ significantly among populations with different numbers of founders. The expected heterozygosity of the bottlenecked populations was less than that of the source population in some cases, but was only a sensitive indicator of bottlenecks when

Table 4 Three criteria of relative sensitivity of genetic indices used to assess bottleneck severity: differences between source and bottlenecked populations, differences between populations experiencing different levels of bottlenecks, and relationship of index with bottleneck size

	Difference from source population*		Differences in bottleneck severity†	Correlation with bottleneck size‡	
Genetic index	Microsatellites	Allozymes§	Microsatellites	Microsatellites	Allozymes§
Direct count heterozygosity	Low	Moderate	Low	Moderate ($R = 0.661$)	Low ($R = 0.397$)
Expected heterozygosity	Moderate	Moderate	Moderate	High ($R = 0.914$)	Low ($R = 0.397$)
Allelic diversity	High	High	High	High ($R = 0.905$)	Moderate ($R = 0.769$)
Proportion of polymorphic loci	Low	High	Low	Low ($R = 0.266$)	Low ($R = 0.586$)
Temporal variance method (F_c)	Moderate	Moderate	High	High ($R = -0.981$)	Low ($R = 0.162$)
Heterozygote excess (IAM)	Moderate	Low	Low	Low ($R = -0.501$)	Low ($R = -0.131$)
Heterozygote excess (SMM)	Moderate	Low	Low	Moderate ($R = -0.661$)	Low ($R = -0.154$)

*Relative sensitivity is based on proportion of populations experiencing a bottleneck that had index values different from an expectation based on sampling error at the $\alpha = 0.05$ level: high, > 0.70; moderate, > 0.30 but < 0.70; low < 0.30 of the populations.
†Relative sensitivity is based on proportion of comparisons between populations experiencing different sizes of bottlenecks that significantly differ at the $\alpha = 0.05$ level: high, > 0.70; moderate, > 0.30 but < 0.70; low < 0.30 of the populations. Comparisons of indices among populations were not made for allozymes in Leberg (1992) and Richards & Leberg (1996) and so are not included here.
‡Relative sensitivity is based on the strength of the correlation of the index estimate of genetics variation with the number of founders: high, > 0.80; moderate, > 0.60 but < 0.80; low, < 0.60.
§Information from allozymes is taken from Leberg (1992) and Richards & Leberg (1996); data from these studies were re-analysed to obtain estimates of heterozygote excess; data are available from the corresponding author.

populations were established with four or fewer fish. The correlation with founder number was much stronger for expected than direct count heterozygosity; this difference could occur because of gametic sampling error with a small number of founders (Pudovkin *et al*. 1996), inbreeding depression (Saccheri *et al*. 1999) or selection at linked loci (Saccheri *et al*. 1999; Coltman *et al*. 1999), among other explanations. In concordance with observed estimates from both allozyme loci (Leberg 1992) and simulations (Neigel 1996), microsatellite-based estimates of direct count heterozygosity in several of the experimental populations were actually higher than that of the source population.

For every bottlenecked population, there was a significant reduction in allelic diversity relative to the source population. Many of the populations that had the severest bottlenecks had less genetic diversity than populations founded with larger numbers of fish, and there was a strong positive correlation between the number of founders and allelic diversity. Using allozymes, Leberg (1992) concluded that allelic diversity was the most effective of the four measures that he examined for detecting severe reductions in population size. Our observations of microsatellite variation support the contention that measuring changes in allelic diversity is one of the most sensitive methods for detecting demographic bottlenecks (Table 4).

Leberg (1992) found that the proportion of polymorphic allozyme loci often reflected a population's history of bottlenecks. Relative to allozyme loci, the large number of alleles at microsatellite loci may explain why the proportion of polymorphic microsatellite loci was not sensitive to the effects of our experimental bottlenecks. The most polymorphic allozyme loci examined by Leberg (1992) had only three alleles, and it was a relatively common event for all alternative alleles of a locus to be lost in a bottleneck. In our source population, the average number of alleles for eight microsatellite loci was 15; this large number of alleles at a locus made it unlikely that all allelic diversity would be lost. The increased polymorphism provided by microsatellites actually diminished the usefulness of this measure of genetic diversity for detection of bottlenecks.

Temporal variance in allele frequencies values were typically greater than expected based on sampling error for populations established with two or four individuals. This index often differed among populations experiencing different sizes of bottlenecks and was highly correlated with bottleneck magnitude. Relative to other indices,

temporal variance in allele frequencies had the strongest ability to discriminate between populations experiencing bottlenecks of different magnitudes. For very severe bottlenecks (< 6 founders), Richards & Leberg (1996) examined genetic drift in mosquitofish and found no correlation between the expected and observed values of temporal variance of allozyme frequencies, the same estimator of drift as we used with microsatellites. They attributed the lack of relationship between drift and bottleneck severity to use of allozyme loci with only a few alleles per locus. In severe bottlenecks, the loss of alleles places an absolute limit on the amount that allele frequencies can change from one generation to the next (Richards & Leberg 1996). This bias in estimates of drift due to allele extinction may be less for loci with large numbers of alleles such as microsatellites than it would be for allozymes, as multiple alleles are more likely to survive the bottleneck.

Our results support a simulation study by Luikart *et al*. (1999) which led to the conclusion that the higher polymorphism of microsatellite loci, relative to allozymes, should improve the sensitivity of F_c for detecting bottlenecks. In fact, temporal variance in allele frequencies had a strong ability to discriminate between different bottleneck sizes and to detect the less severe bottlenecks (i.e. those of 8 and 16 founders). This ability for temporal variance in allele frequencies to discern all levels of bottlenecks indicates that it is a highly sensitive index for detecting a population's history of bottlenecks. For our microsatellite data, this index outperforms even the extremely sensitive allelic diversity index.

The two measures of heterozygosity excess were moderately sensitive to the experimental bottlenecks we examined. In many cases, estimates of *HE* in the populations that experienced bottlenecks were higher than estimates for the source population or the expectation for a large population. However, the relationship of estimates of *HE* with founder number was not strong enough to make the indices good predictors of bottleneck severity. This lack of sensitivity of HE_{SMM} and HE_{IAM} may be due to a lack of fit of the models to microsatellite evolution (G. Luikart, personal communication). While the SMM may predict microsatellite evolution better than the IAM, a hybrid model that incorporates a portion of IAM processes into the basic SMM framework would be more appropriate (Di Rienzo *et al*. 1994). Such a model of microsatellite mutation is currently being explored for incorporation with procedures for estimating heterozygosity excess (G. Luikart, personal communication). Identification of the correct mutation model may increase the sensitivity of heterozygote excess to bottleneck severity, which would be desirable, as *HE* has an advantage over all approaches discussed here. Because this approach is based on an expectation generated from data obtained solely from the putatively bottlenecked population and does not require knowledge of the population's pre-bottleneck genetic diversity (Cornuet & Luikart 1996), it expands the number of situations where genetic markers might be useful in detecting bottlenecks.

When considering the relative sensitivity of microsatellites and allozymes for detecting bottlenecks, it is important to recognize differences between the design of our study and those of Leberg (1992) and Richards & Leberg (1996). These earlier experimental evaluations of allozyme variation examined bottlenecks that were generally more severe than those in our study. Furthermore, while the populations studied by Leberg (1992) and Richards & Leberg (1996) were founded by virgin females, it is likely that some of the females used in the current study were inseminated by males not included in the fish released into the pool. Multiple insemination increases the effective number of founders in populations of *Gambusia* (Robbins *et al*. 1987); this process probably reduced the severity of the bottlenecks we examined. Moreover, multiple insemination has the potential for increasing variance in founder number among populations receiving the same number of released fish. These factors should have made it more difficult to detect differences between the source and the experimental populations and to find correlations between the number of known founders and the genetic indices in our study relative to Leberg (1992) and Richards & Leberg (1996). Because bottlenecks examined with microsatellites were on average less severe than those examined with allozymes, it is likely that the increase in sensitivity of microsatellites over allozymes that we observed for most genetic indices is actually less than the true difference in the sensitivity of these two molecular marker systems.

Our design focused on single, severe bottlenecks and examined genetic diversity after only a few generations. We recognize that this is an idealized situation when compared to examination of natural populations. Most field researchers do not know whether they have examined a natural population within the 2–3 generations immediately following a bottleneck; rather, natural populations are surveyed at an unknown time with respect to putative bottlenecks. It is quite possible, if not probable, that the relative sensitivity of different indices and molecular markers will change as the number of generations between the bottleneck event and the point of sampling is increased. In addition, Nei *et al*. (1975) concluded that not only bottleneck size but also rate of recovery, or bottleneck duration, is an important factor in the ability of a population to recover heterozygosity at neutral loci. If our experiments had examined prolonged bottlenecks or serial bottlenecks, then the relative sensitivity of the various indices of genetic change to bottleneck severity could well have been different from the relative sensitivity we observed in this study of single, severe bottlenecks.

Issues related to the detection of bottlenecks and their severity are relevant to conservation and evolutionary biology. Not every index or class of molecular marker that has been used to assess a population's history provides consistent or readily interpretable information about reductions in population size. We found that microsatellites generally provided more power for distinguishing between different sizes of bottlenecks than did allozymes. When the objective is to obtain an estimate of the relative magnitude of a recent bottleneck, it is important that the population be examined with at least one class of markers that displays considerable allelic diversity, and that loss of this diversity as measured via temporal variances in allele frequencies be considered as one of the primary measures of bottleneck history.

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