

Molecular evidence for historical and recent population size reductions of tiger salamanders (*Ambystoma tigrinum*) in Yellowstone National Park

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Abstract

Population declines caused by natural and anthropogenic factors can quickly erode genetic diversity in natural populations. In this study, we examined genetic variation within 10 tiger salamander populations across northern Yellowstone National Park in Wyoming and Montana, USA using eight microsatellite loci. We tested for the genetic signature of population decline using heterozygosity excess, shifts in allele frequencies, and low ratios of allelic number to allelic size range (*M*-ratios). We found different results among the three tests. All 10 populations had low *M*-ratios, five had shifts in allele frequencies and only two had significant heterozygosity excesses. These results support theoretical expectations of different temporal signatures among bottleneck tests and suggest that both historical fish stocking, recent, sustained drought, and possibly an emerging amphibian disease have contributed to declines in effective population size.

Introduction

The maintenance of genetic variation in a population is recognized as important for long-term persistence, especially in the face of novel selective pressures (Amos and Balmford 2001; Hedrick 2004). Population bottlenecks, or large reductions in effective population size, can reduce genetic variation, thus compromising the ability of a population to respond to environmental change (Amos and Balmford 2001). As anthropogenic influences are creating novel environmental conditions, it is important to detect population declines before genetic variation is greatly reduced.

The use of genetic markers has great potential for detecting signatures of population declines. Using neutral markers such as microsatellites, statistical tests can detect recent severe population size reductions (Cornuet and Luikart 1996; Garza and Williamson 2001). One method tests across all

loci for a significant observed heterozygosity excess relative to heterozygosity expected from the number of observed alleles (Cornuet and Luikart 1996). This is a transient signature of declines in effective population size and has been shown to detect bottlenecks accurately when past demographic history is known (Beebee and Rowe 2001). A second test examines the frequency of different alleles. In a large population, most alleles will be in low frequency (<0.1), but as a population bottleneck occurs and rare alleles are lost, alleles with intermediate frequency will be most abundant (Luikart et al. 1998). Therefore, populations that have the majority of alleles with a frequency greater than 0.1 likely have recently experienced declines. Third, the *M*-ratio (calculated as $M = k/r$ where k = number of alleles and r = overall range in fragment sizes) also tests for population size reductions (Garza and Williamson 2001). A declining population will have a smaller *M*-ratio than a stable one

because k is expected to decrease faster than r in small populations due to genetic drift causing loss of rare alleles (Garza and Williamson 2001). The three tests described here should provide evidence of population decline and recovery over different time scales, which would allow placement of the genetic impact of known population disturbances into a temporal framework. Specifically, the M -ratio is expected to have a long recovery time while heterozygosity excess and allele frequency distributions will recover relatively quickly (Garza and Williamson 2001). This is because recovery is characterized by the addition of new rare alleles. Any addition of rare alleles will show recovery in heterozygosity excess and allele frequency tests. However, new alleles may not increase the M -ratio because they might increase the overall size range. As such, populations that have primarily been influenced by historic decline(s) will show low M -ratios but non-significant heterozygosity excess and allele shifts, while populations recently or currently in decline will not have had time to recover from the genetic signatures associated with any of the bottleneck detection methods.

Molecular studies of genetic variability may be preferable to demographic studies for detecting declines in species such as amphibians, which are generally difficult to locate and tend to fluctuate widely in population size from year to year (Pechmann et al. 1991; Semlitsch et al. 1996). As a result, most demographic monitoring methods of amphibians tend to have low statistical power to detect declines (Alford and Richards 1999). It is increasingly important to monitor amphibian populations due to increasing evidence that amphibian populations are declining globally (Collins and Storfer 2003; Semlitsch 2003; Stuart et al. 2004), and genetic methods are likely to detect declines more quickly than demographic studies.

We sampled populations of the Blotched Tiger Salamander (*A. t. melanostictum*) across the northern range of Yellowstone National Park and used eight microsatellite markers to test for evidence of genetic bottleneck signatures. Tiger salamanders are pond-breeding amphibians with an average generation time of 4–5 years, although this is variable dependent on environmental conditions. They have low dispersal, as populations

across northern Yellowstone are highly subdivided ($F_{ST}=0.24$) (Spear et al. 2005). As a result, low gene flow among sites may make salamanders vulnerable to stochastic disturbances that could lead to population bottlenecks, and possibly local extinctions.

We expect that three primary factors have likely contributed to reductions in populations of tiger salamanders in northern Yellowstone. First, the region is experiencing a prolonged drought, with below average rainfall since 1998 and in 15 of the last 20 years (Western Regional Climate Center data). Drought of greater than 4 years has been suggested as sufficient to cause declines in tiger salamanders (Buhlmann and Mitchell 2000). Second, epizootics due to an iridoviral disease have been detected in Yellowstone (Patla and Peterson 2004; Spear and Storfer unpubl. data). *Ambystoma tigrinum* virus can cause mass mortalities of tiger salamanders within a given year, leading to apparent local extirpation (Jancovich et al. 1997, 2005; Brunner et al. 2004). However, some animals have sublethal infections and return to ponds to breed in the following year (Brunner et al. 2004) thus maintaining epizootics across years and potentially leading to sustained low population size. Third, trout were stocked in many Yellowstone lakes between 1900 and 1950 (Varley 1981). Trout almost certainly cause reductions to salamander populations in sites where they have been introduced, and ambystomatid salamanders generally survive poorly when fish are present (Tyler et al. 1998; Dunham et al. 2004).

In this study, we examine whether Yellowstone populations exhibit both historical and recent bottleneck signatures. We expect that historic perturbations associated with trout stocking in permanent sites would still be reflected in low M -ratios but that measures of heterozygosity excess and allele frequency shifts would have normalized during the number of generations that have passed. On the other hand, populations at ephemeral sites would be expected to be particularly vulnerable to recent drought and should still show significant bottleneck signatures in both slowly and rapidly recovering measures. Finally, disease can impact both permanent and ephemeral sites, and therefore would be expected to show significant reductions in all tests at sites where infections have occurred.

Materials and Methods

We collected tissue samples from 199 individuals at 10 sites across the northern range of Yellowstone National Park (Figure 1). Within our study area, two sites, Buck Lake and Gardiner 3, each had hundreds of thousands of trout stocked 55–70 years ago (Figure 1; Varley 1981). In addition, Slough Creek 38 and Everts 4 are adjacent to water bodies that were stocked, and therefore may have had trout present through flooding events. All sites are currently fishless. There are also several sites that are ephemeral across the area. Information regarding permanence is contained in Figure 1.

We used eight microsatellite loci (ATS 10–7, ATS 12–3, ATS 14–3, ATS 4–11, ATS 4–20, ATS 5–6, ATS 5–7, ATS 5–8; see Mech et al. 2003). Primers and PCR conditions are found in Mech et al. (2003). In short, microsatellites were

amplified using PCR on a Bio-Rad thermocycler with fluorescently labeled heavy strand primers. PCR products were then run on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA) and then scored using Genotyper 2.5 (Applied Biosystems). To test for heterozygosity excess, we used BOTTLENECK version 1.2.02 (Cornuet and Luikart 1996) using a two-phase mutation model (TPM) with 10% multistep mutations, which is believed to be a likely mutation scenario for microsatellites (Di Rienzo et al. 1994). To determine which sampling locations had a significant heterozygote excess across loci, a Wilcoxon signed rank test was used. This test is most appropriate for our analysis because it achieves high statistical power even if the average sample size per site is 30 or fewer (Cornuet and Luikart 1996). BOTTLENECK was also used to determine if there was a normal allele frequency distribution at each site.

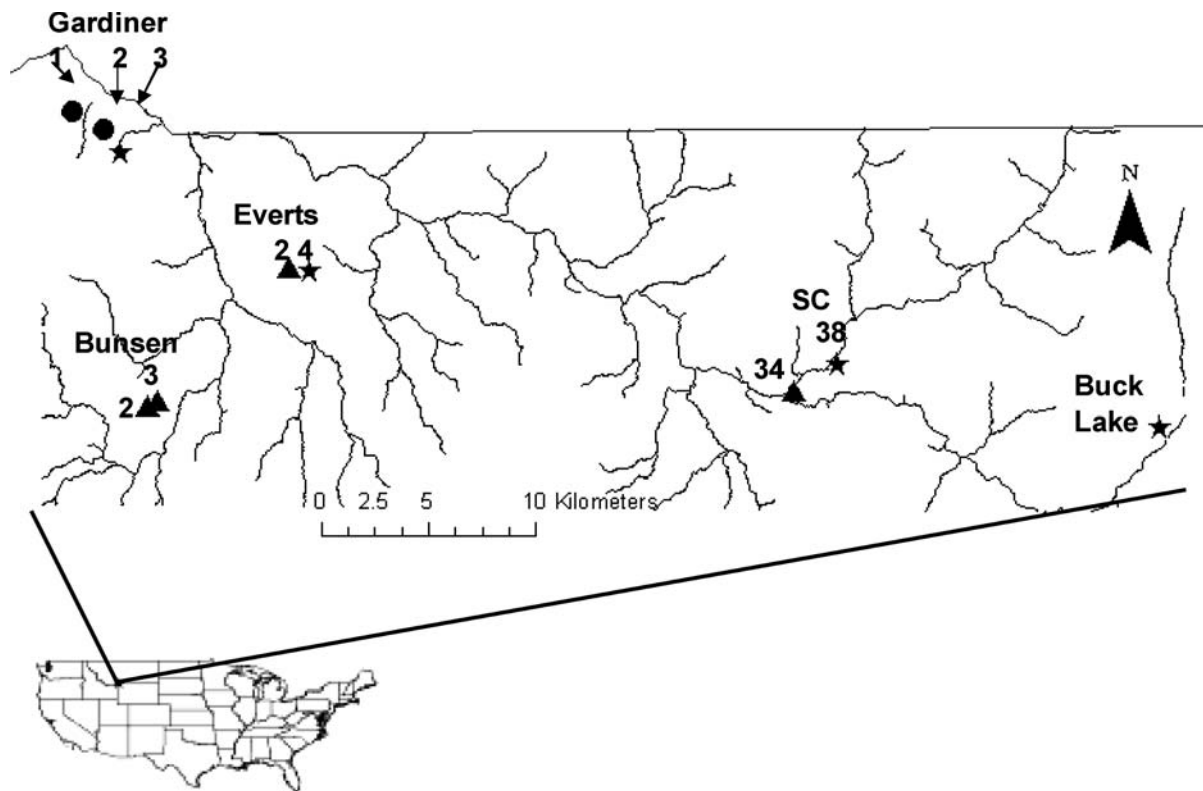


Figure 1. Map of sampling sites in Northern Yellowstone. Sites represented by a triangle are ephemeral sites, those represented by stars are sites that were either directly stocked with trout or directly adjacent to stocked sites, and circles represent sites with permanent water with no recorded fish history. Gray dashed lines are rivers and streams across the area and the top line is the national park boundary. The black lines at bottom extend to location of the study area within the United States.

To estimate M -ratios, we used AGARST version 2.9 (Harley 2002). One locus was omitted from this analysis because it did not conform to the stepwise mutation model. To determine the critical M -ratio value (below which population declines are inferred) for these populations, we used the M -crit program developed by Garza and Williamson (2001). Three parameters are needed for this program: effective population size, percentage of mutations greater than one step, and average size of a non one-step mutation. Effective population size was estimated for each site using MIGRATE 1.7.3 (Beerli 2003), which is based on a stepwise mutation model. We then used 0.12 for the proportion of non one-step mutations and 2.8 for the average size of non-one step mutations as mean values found in a literature survey (Garza and Williamson 2001). Each of these tests assumes Hardy–Weinberg equilibrium, no linkage, and low immigration. These assumptions are satisfied, as all populations were in H–W equilibrium, there was no linkage disequilibrium, and they exhibited significant genetic subdivision (Spear et al. 2005) and can thus be considered closed populations.

Results and Discussion

As expected, evidence for reductions differed dependent on the bottleneck test. First, under the

two-phase mutation model that had 10% multi-step mutations in BOTTLENECK, only two of the ten sites showed significant heterozygote excess. The two populations were Bunsen 2 ($P=0.02$), and Slough Creek 34 ($P=0.008$) (Table 1). Neither of these sites had a previous fish stocking history. Instead, both were ephemeral sites, and Slough Creek 34 dried out both years it was sampled, with no apparent breeding success. This supports recent, prolonged drought (since 1998) as the cause of these bottlenecks. The analysis for allele frequency shifts indicated that five populations had undergone recent size reductions. These populations were Bunsen 2, Bunsen 3, Everts 2, Slough Creek 34 and Gardiner 2 (Table 1). Note that the two sites that were significant for heterozygosity excess also had frequency shifts. Again, none of these sites were previously stocked with trout. Beebe and Rowe (2001) found that all known reduced populations of natterjack toads were significant for heterozygosity excess and frequency shifts, but that additional populations which had recent translocations had only allele frequency shifts. If this is a general pattern, then the three sites with only allele frequency shifts but not heterozygosity excess may be due to a founder effect and these sites may have started to recover.

Finally, the M -ratios suggested that bottlenecks had occurred in all ten sites. Across all sites and loci, the M -ratio ranged from 0.474 to 0.671,

Table 1. Results based on heterozygosity excess test, allele frequency analysis and M -ratios

Site	Avg Het excess	P value no excess	Allele freq	M -ratio	M -ratio variance	M_c	θ	θ Lower CL	θ Upper CL
Bunsen 2	0.135	0.02	Shifted	0.602	0.143	0.81	0.44	0.4	0.48
Bunsen 3	0.064	0.188	Shifted	0.602	0.143	0.81	0.5	0.447	0.552
Buck	-0.085	0.891	Normal	0.507	0.114	0.82	0.33	0.295	0.363
Everts 2	-0.062	0.902	Shifted	0.596	0.158	0.82	0.34	0.307	0.37
Everts 4	-0.068	0.852	Normal	0.474	0.108	0.81	0.51	0.464	0.57
Gardiner 1	0.035	0.289	Normal	0.606	0.139	0.82	0.39	0.356	0.429
Gardiner 2	0.005	0.5	Shifted	0.538	0.116	0.81	0.58	0.541	0.629
Gardiner 3	0.015	0.406	Normal	0.505	0.165	0.8	0.47	0.422	0.524
SC 34	0.109	0.008	Shifted	0.671	0.163	0.81	0.42	0.382	0.456
SC 38	0.017	0.344	Normal	0.567	0.096	0.8	0.75	0.679	0.824

For the heterozygosity excess test, we used a two-phase mutation model with 10% multistep mutations. Normal allele frequency means that the majority of alleles had frequencies less than 0.1, whereas shifted indicates that the most frequent alleles were intermediate to common. M -ratio average and variance were calculated across loci. Locus 5–7 was omitted because it violates the assumption of the model. M_c is the critical M -value calculated through the M -crit program developed by Garza and Williamson (2001). θ is the effective population sizes estimated by MIGRATE along with the upper and lower 95% confidence limits.

which, in each case, was lower than the critical value for individual sites calculated by M -crit (Table 1). Further, all values were lower than the critical value of 0.68 proposed by Garza and Williamson (2001). This method did seem to reflect the influence of fish stocking, as sites that were stocked or near stocked areas had a mean M -ratio of 0.513, whereas sites without any fish had a mean M -ratio of 0.602. These values are also lower than the only other published amphibian study that used M -ratios (European tree frogs; Anderson et al. 2004), which had a mean value of 0.678 for populations that had known demographic bottlenecks. We also have M -ratio data using the same loci in monitored populations of the federally endangered Sonora tiger salamander subspecies *A. t. stebbinsi* (Storfer et al, unpublished). The Sonora tiger salamander exists only in approximately 50 ponds in south-central Arizona, primarily in earthen stock ponds used for cattle drinking (Collins et al. 1988). Sonora tiger salamanders face the same threats (i.e., fish stocking and disease) as Yellowstone populations (Collins et al. 1988), with the exception of drought, because pond levels are artificially maintained. In a demographic study that began in the late 1980s and has continued through the present, two of the sampled *A. t. stebbinsi* populations had no history of any demographic reductions or evidence of fish introductions or disease and can thus serve as reference populations. These two populations did not have heterozygosity excess and had M -ratios of 0.697 and 0.741, which are larger than any of the values in this study (Storfer, unpublished).

The M -ratio test is expected to show a significant bottleneck pattern longer than the other two tests (Cornuet and Luikart 1996; Garza and Williamson 2001), which is consistent with the result that five permanent sites had low M -ratios but neither heterozygosity excesses nor allele frequency shifts. Two of these sites (Gardiner 3 and Buck Lake) almost certainly underwent declines between 1900 and 1950 due to trout stocking (Varley 1981), and Everts 4 and Slough Creek 38 may have as well with flooding events. We expect fish stocking would have caused severe population declines due to the poor ability of salamanders to coexist with trout (Tyler et al. 1998; Dunham et al. 2004). The fact that declines due to trout are not

detected as heterozygosity excess or frequency shifts strongly suggests that these two measures have a recovery period of less than 50 years. On the other hand, four of the five sites that had either heterozygosity excess or allele frequency shifts along with low M -ratios were ephemeral sites that are susceptible to drought. The one permanent site, Gardiner 2, had low water levels during sampling, and therefore populations may have had drought pressures.

Another possible explanation of recent bottlenecks is disease. We confirmed iridovirus infection at Gardiner 3 using PCR amplification of a portion of the major capsid protein of the *Ambystoma tigrinum* virus (Jancovich et al. 1997) using primers designed by Mao et al. (1996). Surprisingly, Gardiner 3 only showed a significant reduction using the M -ratio. However, a genetic bottleneck based on heterozygosity excess is less likely to be detected if the effective population size is very small (Cornuet and Luikart 1996). Therefore, it is possible that this population has not recovered from the previous reduction due to fish stocking.

This study provides evidence that genetic bottlenecks have occurred across northern Yellowstone and suggest there may have been both recent and historic population reductions. Drought is likely responsible for contemporary reductions, while it is unclear what effect disease may have in this system. However, there is some evidence that low genetic diversity increases the susceptibility of amphibians to disease (Pearman and Garner 2005). We reviewed several salamander microsatellite studies and found that the average expected heterozygosity was 0.519 (range 0.14–0.937; Curtis and Taylor 2003; Myers and Zamudio 2004; Adams et al. 2005; Jehle et al. 2005; Storfer, unpublished). The expected heterozygosity for Yellowstone populations was 0.317 (Table 2), the second lowest of any of these studies. This low diversity may mean that iridoviral disease may be a strong threat in the future. This research also supports theoretical expectations that the M -ratio has a longer recovery time, while allele frequency shifts and heterozygosity excess tests are only sensitive to recent events. Therefore, these tests could be used in conjunction when suspected bottlenecks may have occurred on different temporal scales.

Table 2. Genetic diversity estimates of sampled populations based on eight loci

Site	<i>N</i>	Avg alleles	H_e	H_o	F_{IS}
Bunsen 2	20	2	0.373	0.406	-0.089
Bunsen 3	20	2	0.316	0.317	-0.004
Buck	16	2.125	0.212	0.219	-0.031
Everts 2	18	2.5	0.313	0.261	0.165
Everts 4	17	2.25	0.263	0.217	0.176
Gardiner 1	20	2.375	0.358	0.280	0.218
Gardiner 2	31	2.125	0.263	0.230	0.124
Gardiner 3	16	2.375	0.347	0.301	0.131
SC 34	18	2	0.356	0.347	0.024
SC 38	23	2.625	0.371	0.368	0.010
Mean	19.9	2.238	0.317	0.295	0.072

N represents sample size, avg alleles is the average number of alleles per locus, H_e is expected heterozygosity, H_o is observed heterozygosity, and F_{IS} is the inbreeding coefficient.

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