RESEARCH ARTICLE

Inbreeding and strong population subdivision in an endangered salamander

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Abstract Studies of genetic population structure and genetic diversity are often critical components of endangered species conservation and management plans. Genetic studies are thus particularly important for amphibians, which are in global decline. We studied genetic variation and population structure among 276 individuals from approximately half of the known localities of the endangered Sonora tiger salamander, *Ambystoma mavortium stebbinsi*, using ten microsatellite loci. Allelic diversity was generally low (2.7 alleles per locus per population) and overall observed heterozygosity (0.191) was significantly lower than expected (0.332). Most populations showed significant departures from Hardy–Weinberg equilibrium, which are likely due to inbreeding. In addition, evidence of recent bottlenecks was suggested by shifted allele frequency distributions in 5 of 16 populations, and ratios of allele number to allele size range (M) values lower than critical values in all populations. A high degree of genetic subdivision ($\theta = 0.133$) was found over all populations, and nearly all pairwise population combinations were genetically subdivided. Thus, gene flow is limited even over small distances, perhaps because high desert grassland throughout the study area limits the efficacy of inter-pond movement of salamanders. Further, population sizes and gene flow of Sonora tiger salamanders are likely compromised by several contemporary ecological threats, including: frequent die-offs due to an infectious virus, introductions of non-native species, and continuing cattle grazing. Overall, these genetic data support the endangered status of the Sonora tiger salamander and suggest the subspecies exists in small, inbred populations.

Keywords Inbreeding · Genetic structure · Microsatellites · Salamander · *Ambystoma tigrinum*

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Introduction

Amphibians are declining globally, and studies of genetic diversity and population genetic structure are crucial for conservation and management planning (Blaustein and Kiesecker 2002; Collins and Storfer 2003; Frankham 2003; Stuart et al. 2004). Amphibian populations are increasingly threatened by land use change, climate change, introduction of non-native, invasive species and emerging infectious diseases for which transport is enhanced by human activities (Collins and Storfer 2003; Schloegel et al. 2009, 2010). Amphibian populations fluctuate widely in numbers from year to year, and consequently, many years of census data are necessary for accurately detecting population trends (Pechmann et al. 1991; Alford and Richards 1999). However, studies of genetic variation facilitate relatively rapid inferences of population declines or even recent bottlenecks (i.e., drastic reductions in population size; see Cornuet and Luikart 1996; Luikart et al. 1998a, b; Garza and Williamson 2001). Thus, genetic studies facilitate

management decisions over much shorter time scales than census studies, which may be critical for saving rapidly declining or endangered species.

Studies of genetic variation are a critical component of management programs because genetic variation underlies the potential for adaptation to future environmental change (Frankel and Soulé 1981; Frankham 2003). Genetic analyses can be used to prioritize conservation of small populations that are prone to the potential negative impacts of genetic drift or inbreeding (Lande 1995; Reed et al. 2003). Small, genetically depauperate populations may consequently become targets of management programs with the goal of augmenting genetic variation (Hedrick 1995; Storfer 1999; Frankham 2003).

Genetic studies also determine the extent of gene flow and consequent population genetic structure. Gene flow is often seen as a creative force that maintains genetic variation among populations (Slatkin 1987). However, anthropogenic forces such as development can restrict gene flow among populations and, in turn, reduce genetic diversity (Slatkin 1987; Frankham 2003). Data on gene flow among amphibian populations can also help researchers determine if metapopulation structure exists (Marsh and Trenham 2001; Storfer 2003; Smith and Green 2005). Identification of source and sink populations can, in turn, lead to appropriate management strategies, such as artificial colonization of locally extirpated populations or augmentation of sink populations via translocations (Vitt et al. 2009). Alternatively, if amphibian populations are not acting as metapopulations because gene flow is low, translocations may not be warranted due to the possibility of disrupting local adaptation (Storfer 1999).

The endangered Sonora tiger salamander, *Ambystoma mavortium stebbinsi* (Lowe) is subject to many of the factors that threaten amphibians globally, including introduction of exotic species, habitat alteration, and frequent epizootics due to an iridoviral disease (Jancovich et al. 1997, 2005; Storfer et al. 2007). Previous molecular studies support the distinctiveness of *A. m. stebbinsi* from other tiger salamander subspecies (Jones et al. 1988; Storfer et al. 2004). The subspecies is only found within the San Rafael Valley (SRV) of south-central Arizona (Fig. 1), where the predominant land use is cattle grazing. Sonora tiger salamanders breed exclusively in human-made, earthen stock ponds ("cattle tanks") and have not been collected from non human-modified habitats. However, it is thought that prior to European settlement, *A. m. stebbinsi* occupied natural cienegas (marshes) that historically covered parts of the SRV (Jones et al. 1988, 1995). The southern boundary of *A. m. stebbinsi* ends at the US-Mexico border because no *A. m. stebbinsi* have been confirmed in Mexico despite repeated surveys over the last 20 years (Jones et al. 1995, unpubl.).
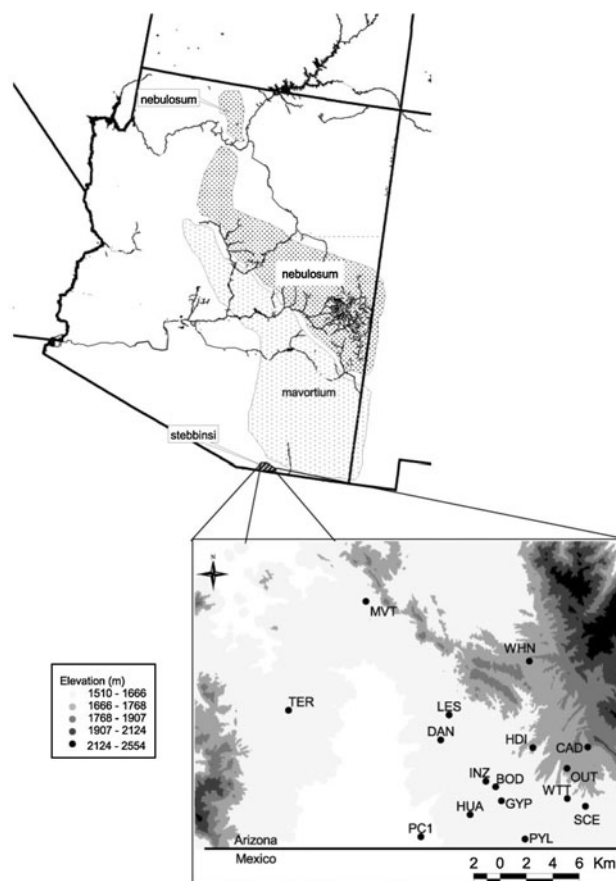

Fig. 1 Tiger salamander subspecies found in Arizona. *Dark stippling* indicates the geographic range of *A. m. nebulosum*, *light stippling* indicates the range of *A. m. mavortium*, and cross-hatching shows the range of *A. m. stebbinsi*. *Inset* shows the localities sampled for genetics within the range of *A. m. stebbinsi*

Our goal in this study were to estimate levels of genetic variability and genetic population structure among populations of *A. m. stebbinsi* using microsatellite markers. Due to the contemporary threats of exotic species introductions, viral epizootics, and habitat alteration due to cattle grazing, rapidly evolving microsatellite markers are a particularly appropriate tool for assessing genetic structure in *A. m. stebbinsi*. Therefore, we conducted analyses of genetic diversity and population structure at 10 microsatellite loci from 276 samples collected throughout the range of the subspecies.

Materials and methods

Sampling and DNA isolation

Repeated surveys have identified a total of ~40 SRV ponds between the eastern side of the Patagonia Mountains and the western and southern sides of the Huachuca

Mountains in southern Arizona that contain *A. m. stebbinsi*. We sampled 16 of these ponds from 1997 to 1999, and collected tissue samples from *A. m. stebbinsi* by walking the perimeter of each stock tank with a 6 m seine net or the center of the tank using a 30′ deep-water seine (see Fig. 1; Table 9 in Appendix for locations and sample sizes). When we caught a salamander, we cut a piece of tissue (∼1 cm²) from the posterior end of the tail and clipped one toe (for marking purposes). We suspended tissue samples in 95 % ethanol in sterile 1.5 ml centrifuge tubes on ice, and stored them at −20 °C upon return to the laboratory.

We extracted DNA from samples collected prior to 2002 using Puregene® DNA isolation kits (Gentra, Inc., Minneapolis, MN) with a modified version of the standard animal tissue isolation protocol. Our modifications followed three basic steps, all using 20 mg/ml Proteinase-K solution: (1) tissues were homogenized in Puregene® cell lysis buffer and digested at 55 °C for 1 h with 10 μl of Proteinase-K; (2) tissues were re-homogenized and digested at 55 °C overnight; (3) after incubation, samples were homogenized a third time before proceeding with the Puregene® protocol. All extracted DNA was suspended in Puregene® DNA hydration solution and stored at −20 °C. For samples collected during 2002, we extracted DNA with a standard phenol–chloroform protocol following Sambrook et al. (1989).

Microsatellite characterization

We performed microsatellite genotyping via PCR reactions of genomic DNA (PCR conditions in Mech et al. 2003) on 10 polymorphic microsatellite loci developed for *A. m. stebbinsi* (ATS 4-11; ATS 4-20; ATS 4-25; ATS 5-6; ATS 5-7; ATS 5-8; ATS 10-7; ATS 12-3; ATS 13-1 and ATS 14-3); we used fluorescently labeled heavy strand PCR primers and ran products on an ABI 377 automated sequencer with GeneScan Version 3.1 software (Life Technologies, Foster City, CA). To prevent sample cross-contamination, we used filter tips and negative controls in all PCR reactions. We scored microsatellite alleles using Genotyper Version 2.5 (Life Technologies). Alleles were scored blind by S.G.M., and a known sample standard was run on every gel to ensure scoring consistency. Alleles that were much larger or much smaller than the next largest or smallest allele were confirmed by re-amplification, sequencing, and verification of the appropriate repeat structure. All unique alleles (alleles found in only 1 individual) were confirmed by re-amplification and re-scoring.

We showed the possibility of limited hybridization between *A. m. stebbinsi* and *A.m. mavortium* (Storfer et al. 2004), and hybridization between *A. m. mavortium* and the California tiger salamander, *A. californiense* has been well-documented (Riley et al. 2003; Fitzpatrick and Shaffer

2007). As a result, we ensured that our analyses for this study only included pure *A. m. stebbinsi,* by using two analytical methods to exclude potential hybrids. First, we examined all individuals for nine microsatellite alleles (all those included herein except 13-1) unique to 47 *A. m. mavortium* from seven sites analyzed previously (Storfer et al. 2004), and any individual with such an allele was removed from the analysis. Second, for all individuals collected from four ponds known to have possible hybrid populations (SCE, WHN, GYP, and HDI), we used a maximum likelihood based assignment test in Whichrun (Banks and Eichert 2000) to assign individuals to subspecies using their multilocus microsatellite genotype. "Pure" reference populations of each of the two subspecies were used as potential parental populations, whereby Whichrun then computed likelihood ratios of belonging in one parental population versus another. Only those individuals assigned to *A. m. stebbinsi* with likelihood ratios exceeding 10^5 (i.e., that an individual was at least 100,000 times more likely to be *A. m. stebbinsi* than *A. m. mavortium*), were used in the following analyses. In total, 39 individuals were excluded due to unique alleles and another 9 were excluded due to the assignment test.

Statistical analysis

Genetic diversity

We estimated allelic diversity, overall observed and expected heterozygosity, and tested for linkage disequilibrium between all possible locus pairs using Fisher's exact tests in Genepop 3.4 (Raymond and Rousset 1995). In Genepop, we also tested for deviations from Hardy–Weinberg frequencies, reflected as whether the inbreeding coefficient (f, Weir and Cockerham 1984) is significantly different from zero; exact p-values are calculated with the Markov chain method (dememorization 10000; batches 100; iterations per batch 5000; Guo and Thomson 1992). We performed a sequential Bonferroni correction to minimize type-I errors by adjusting p-values to correct for multiple table-wide tests (Rice 1989).

For loci that deviated from Hardy–Weinberg expectations, we estimated the frequency of null alleles using Cervus 2.0 (Marshall et al. 1998). Cervus uses the method proposed by Summers and Amos (1997), which allows inclusion of double null homozygotes, to compute the frequency of null alleles (see Garner et al. 2004). To determine whether null alleles were problematic for loci that showed significant departures from Hardy–Weinberg expectations, we calculated the frequency of expected double null allele genotypes by squaring the expected null frequency as calculated by Cervus. We then compared our observed frequency of double null allele genotypes

(individual samples that did not amplify) at each locus with the expected frequency using a binomial test. Note that this test should be conservative because some samples may not amplify due to problems in the DNA template, thus inflating the apparent number with null alleles.

Next, we tested for evidence of recent bottlenecks in each population with two different methods. The first method used the program BOTTLENECK (Piry et al. 1999), which tests whether there are a significant number of loci that exhibit a heterozygote excess. This excess is expected to be transient in a population because recent, severe reductions in effective population size will result in reductions of allelic diversity faster than heterozygosity (Cornuet and Luikart 1996). We used the Wilcoxon sign-rank test in BOTTLENECK to test for heterozygote excesses. This test is more powerful than the sign test when average sample size per site is 30 or less, and the other available test—the standardized differences test—requires a minimum of 20 loci (Luikart et al. 1998b). We used a two-phase mutation model (TPM), which is modified from the strict stepwise mutation model (SMM), because it allows for (a user-defined percentage of) multi-step mutations. Consequently, the TPM was shown to be best fit for most microsatellite datasets (Di Rienzo et al. 1994; Piry et al. 1999). In BOTTLENECK, we chose 5 % multistep mutations (Piry et al. 1999), which approximated the frequency of multistep mutations in our dataset (Mech et al. 2003).

Because allele size range is reduced more slowly than allelic diversity in cases of reduced effective population size, the second method we used to test for bottlenecks was calculating M (where $M = k/r$, where k = # of alleles per locus and r = allelic size range for that locus across all samples; Garza and Williamson 2001). To test significance of M-values generated for each population, we used Critical_M.exe software (Garza 2001), which generates a critical M value (that which 5 % of simulations were below) based on number of individuals sampled and number of loci using 10,000 replicates. Thus, observed M values below the critical value suggest significant bottlenecks. In Critical_M.exe, we parameterized the general SMM model (Garza and Williamson 2001) with the proportion of one-step mutations and average size of multi-step mutations (Δ) that were obtained empirically for each sampling site. Also needed to parameterize Critical_M.exe are estimations of long term effective population sizes, which we estimated based on a coalescent approach using MIGRATE 1.7.6 (Beerli and Felsenstein 2001; Beerli 2002). Specifically, a parameter (Θ—not to be confused with the F-statistic analog by Weir and Cockerham 1984) equal to $4N_e\mu$ (where N_e = effective population size μ = mutation rate), is generated by MIGRATE using a Monte Carlo Markov Chain (MCMC) approach. We used a mutation rate estimated for microsatellites at 10^{-5} per locus per generation (Goldstein and Schlötterer 1999). Each population was analyzed separately due to the known low rates of gene flow among tiger salamander populations (e.g., Spear et al. 2005, 2006). We analyzed each locus separately using a Brownian approximation of the SMM (note that, although TPM is preferred for microsatellites, it is not an available option in this program), with MCMC searches for 10 short chains (500 gene trees each) and two long chains (5000 gene trees each). In each of the five replicate runs, the first 10,000 steps were discarded to allow "burn in" and stationarity of parameters was ensured by comparing standard deviations of likelihood values among replicate runs.

Genetic population structure

We analyzed population genetic structure using analysis of molecular variance (AMOVA; Excoffier et al. 1992) in ARLEQUIN 2.0 (Excoffier and Lischer 2010). We also used the Mantel test (Mantel 1967) in ARLEQUIN to test for a significant isolation-by-distance relationship. We then used GDA (Lewis and Zaykin 2001) to estimate Weir and Cockerham's (1984) analogs of F-statistics between all possible population pairs. We did not use R_{ST} (Slatkin 1995), which was developed for microsatellites and follows the SMM, because seven of our 10 loci violate assumptions of the SMM (four of ten loci were imperfect, and three of the remaining six loci had large jumps in allelic size distributions see Table 9 in Appendix). F-statistics were bootstrapped with 10,000 replicates to determine confidence intervals and significance from zero. Because some of our sample sizes were small (because we were sampling an endangered species), we also jackknifed the θ estimates over populations using GDA to determine whether sampling error in small populations skewed results. We also repeated calculations of overall F-statistics for populations with at least 15 individuals sampled and again for populations with at least 20 individuals sampled. Loci that showed significant departures from Hardy–Weinberg equilibrium in multiple populations (due to the possibility of null alleles) were excluded from the analyses and overall results were calculated on the remaining loci, as well as the full 10-locus dataset.

Results

Genetic diversity

A total of 276 individuals were genotyped for an average of 17.2 ± 2.2 (mean $\pm$ SE) individuals per population. An average of 274.1 ± 0.8 individuals were genotyped at each

Table 1 Basic genetic information. Measures of genetic variation among 10 microsatellite loci in *A. m. stebbinsi* among 16 sites in SRV, AZ

Population	Locus										Overall		
	ATS 4-11	ATS 4-20	ATS 4-25	ATS 5-6	ATS 5-7	ATS 5-8	ATS 10-7	ATS 12-3	ATS 13-1	ATS 14-3	A	Ap	N
BOD	2	3	4	2	3	4	3	3	5	4	3.3	3.3	33.2
CAD	2	3	2	2	3	1	3	3	4	3	2.6	2.8	10.0
DAN	1	2	3	2	1	1	3	2	2	2	1.9	2.3	17.9
GYP	2	5	2	3	4	2	2	3	5	4	3.2	3.2	17.0
HDI	4	2	3	2	2	1	3	3	5	2	2.7	2.9	11.0
HUA	3	3	4	3	1	3	2	5	6	4	3.4	3.7	21.9
INZ	3	2	3	3	3	2	5	4	3	5	3.3	3.3	10.0
LES	3	4	2	1	2	2	5	2	2	2	2.5	2.7	10.0
MVT	3	6	2	2	1	3	2	1	4	2	2.6	3.0	14.0
OUT	3	3	3	2	2	1	2	4	5	5	3.0	3.2	17.0
PC1	2	2	1	4	4	2	1	2	3	3	2.4	2.8	17.0
PYL	3	2	2	2	1	2	2	2	3	4	2.3	2.4	12.6
SCE	3	4	3	5	4	3	4	3	4	4	3.7	3.7	39.6
TER	2	2	1	2	1	1	2	2	3	3	1.9	2.3	11.0
WHN	3	4	2	3	6	3	1	2	3	4	3.1	3.3	20.9
WTT	2	3	2	1	2	1	1	3	2	3	2.0	2.4	11.0
Mean	2.6	3.1	2.4	2.4	2.5	2.0	2.6	2.8	3.7	3.4	2.7	3.0	17.1
Overall	8	11	6	8	9	8	8	7	11	5			
N	268	275	276	276	276	275	272	275	273	275			

Allelic diversity within each sampling locality (A), number of alleles per polymorphic locus (Ap), average sample size for each locality (N), and per locus means across all localities

locus yielding an average of 8.1 ± 0.6 alleles per locus overall (Table 1). However, within population allelic diversity was low, with a mean of 2.7 alleles per locus. Allele frequencies and sample sizes are given in Table 9 in Appendix. Observed heterozygosity was 0.191, significantly lower than the expected heterozygosity of 0.332 ($f = 0.433$; $p < 0.0001$; 1000 iterations; Table 2). Most populations showed significant heterozygote deficiencies as well (Table 2), suggesting the possibility of non-random mating. Note that we removed a total of 48 individuals from the analyses because they were potential hybrids between *A. m. stebbinsi* and *A. m. mavortium* based on previous work (Storfer et al. 2004). When these individuals are included in the analysis, results do not differ appreciably, and in many cases f values increased. Thus, although our inclusion criteria for "pure" *A. m. stebbinsi* were stringent, they did not bias results appreciably.

We did not find any significant linkage disequilibrium among loci. However, five loci (ATS 4-20, ATS 5-7, ATS 10-7, ATS 13-1 and ATS 14-3) showed significant departures from H–W equilibrium overall (Table 2); four of these five loci showed significant heterozygote deficiency in at least four sampling localities. Null allele frequencies were estimated for each of these loci, and observed double null frequencies were significantly lower than expected proportions for all five loci (Table 3). This suggests that

departures from Hardy–Weinberg equilibrium (reflected as significant heterozygote deficiencies) were not a result of null alleles. Nonetheless, in our overall analyses of population structure below, we conducted two sets of analyses: with these five loci excluded, as well as with all ten initial loci.

Evidence of recent bottlenecks was at least partially supported in separate analyses. Although BOTTLENECK showed that no populations had a significant proportion of loci that were in heterozygote excess, five populations (CAD, DAN, INZ, LES and PC1) showed allelic distribution shifts, which suggests recent bottleneck events (Luikart et al. 1998a). That is, bottlenecks cause transient (fewer than a few dozen generations) distortions in allele frequency distributions, whereby low frequency alleles (<0.1) decline in occurrence relative to "intermediate" frequency alleles (0.1–0.2). In addition, seven populations (BOD, GYP, HDI, HUA, INZ, OUT, and SCE) had a significant proportion of loci in heterozygote deficiency under the TPM. Migrate results generally showed Θ values that were consistent across loci within populations, and mean values are presented in Tables 4, 5. Calculations of M statistic were also generally low, ranging from 0.272 to 0.741 (Table 5). All populations showed evidence of recent bottlenecks because M values were lower than the corresponding critical M value (M_c) for each population.

Table 2 Measures of genetic variation among 10 microsatellite loci in *A. m. stebbinsi* among 16 sites in SRV

Locus	Statistic	BOD	CAD	DAN	GYP	HDI	HUA	INZ	LES	MVT	OUT	PC1	PYL	SCE	TER	WHN	WTT	Mean
ATS4-11	He	0.032	0.505	0	0.299	0.398	0.172	0.358	0.416	0.5	0.169	0.337	0.495	0.545	0.368	0.376	0.091	
	f	0	−0.2	–	−0.185	0.091	−0.057	0.455	0.04	0	−0.043	−0.231	0.231	**0.289**	0.268	0.07	0	0.073
ATS4-20	He	0.394	0.353	0.514	0.408	0.247	0.138	0.189	0.721	0.762	0.444	0.428	0.517	0.166	0.485	0.549	0.606	
	f	0.331	0.446	**1**	0.286	−0.111	**0.661**	**1**	0.176	0.065	**0.871**	**1**	**1**	**0.851**	**1**	0.573	**1**	**0.617**
ATS4-25	He	0.437	0.337	0.298	0.299	0.537	0.174	0.532	0.395	0.495	0.426	0	0.212	0.344	0	0.502	0.247	
	f	**0.667**	−0.2	**1**	0.22	0.672	0.485	0.449	−0.286	0.286	0.034	–	−0.091	−0.091	–	0.245	0.111	0.266
ATS5-6	He	0.058	0.337	0.056	0.221	0.091	0.376	0.195	0	0.452	0.114	0.357	0.148	0.19	0.173	0.138	0	
	f	1	−0.2	0	1	0	−0.214	−0.029	0	−0.444	1	0.01	−0.043	0.211	−0.053	−0.034	–	0.029
ATS5-7	He	0.165	0.484	0	0.223	0.173	0	0.358	0.526	0	0.114	0.59	0	0.383	0	0.535	0.416	
	f	**0.824**	0.6	–	0.742	1	–	1	1	–	1	0.609	–	**0.936**	0	0.561	1	**0.806**
ATS5-8	He	0.414	0	0	0.059	0	0.21	0.1	0.1	0.442	0	0.428	0.077	0.097	0	0.181	0	
	f	0.198	–	–	0	–	−0.082	0	0	−0.138	–	0.731	0	−0.03	–	0.481	–	0.195
ATS10-7	He	0.425	0.416	0.381	0.214	0.255	0.304	0.574	0.668	0.254	0.214	0	0.212	0.165	0.173	0	0	
	f	0.218	0.769	1	0.458	0.655	**1**	**0.833**	**0.857**	0.447	0.214	–	−0.091	−0.06	−0.053	–	–	**0.637**
ATS12-3	He	0.36	0.484	0.108	0.219	0.394	0.362	0.505	0.505	0	0.608	0.114	0.159	0.648	0.091	0.438	0.329	
	f	0.021	0.6	1	−0.076	0.318	0.753	1	1	–	0.033	1	−0.048	0.114	0	0.022	−0.111	0.372
ATS13-1	He	0.468	0.7	0.475	0.647	0.658	0.671	0.532	0.505	0.701	0.617	0.506	0.468	0.419	0.481	0.585	0.416	
	f	**0.752**	**0.864**	−0.055	0.279	0.32	**0.667**	0.449	0.617	0.398	**0.531**	0.773	0.795	0.704	0.444	**0.841**	0.574	**0.559**
ATS14-3	He	0.42	0.679	0.214	0.508	0.485	0.656	0.732	0.526	0.389	0.69	0.586	0.705	0.474	0.394	0.735	0.325	
	f	**0.792**	0.274	**1**	0.076	−0.132	**0.728**	0.045	0.25	0.458	**0.834**	0.506	**0.681**	**0.686**	0.551	0.161	−0.127	**0.418**
Overall	He	0.317	0.429	0.205	0.31	0.324	0.307	0.407	0.436	0.399	0.34	0.335	0.299	0.343	0.216	0.404	0.243	0.332
	Ho	0.17	0.27	0.056	0.229	0.227	0.15	0.19	0.23	0.35	0.165	0.153	0.157	0.209	0.118	0.259	0.127	0.191
	f	**0.469**	**0.384**	**0.734**	**0.265**	0.308	**0.516**	**0.547**	0.486	0.128	**0.523**	**0.551**	0.484	0.394	0.466	**0.365**	**0.488**	**0.433**

Included is expected heterozygosity (He), inbreeding coefficient, *f* (Weir and Cockerham 1984) for each locus within each populations and overall. Bolded numbers depict significant departures from Hardy–Weinberg proportions after Bonferroni correction for multiple tests. A – means a population is fixed at a locus and thus, *f*, is undefined

Table 3 Five loci with significant heterozygote deficiencies

Locus	Null frequency	Expected null homozygotes	Observed null homozygotes	p value
ATS 4-20	0.5105	0.2607	0	<0.0001
ATS 5-7	0.7124	0.5075	0	<0.0001
ATS 10-7	0.4029	0.1623	0.0145	<0.0001
ATS 13-1	0.4604	0.212	0.0108	<0.0001
ATS 14-3	0.4232	0.1791	0	<0.0001

For each locus, the frequency of null alleles was estimated by Cervus 2.0 (Marshall et al. 1998). Expected null homozygotes were calculated by squaring the estimated null frequency. Observed null homozygotes were calculated by dividing the number of individuals with double null genotypes (did not amplify) by total number of samples (276). p-values were computed by comparing observed versus expected proportions using a binomial test

Table 4 Results from Bottleneck

Population	Deficient/excess	p-value	Distribution shape
BOD	10/0	0.0005	Normal
CAD	4/5	0.5449	Shifted
DAN	5/2	0.3437	Shifted
GYP	8/2	0.0020	Normal
HDI	7/2	0.0185	Normal
HUA	7/2	0.0049	Normal
INZ	7/3	0.0068	Shifted
LES	3/6	0.1797	Shifted
MVT	3/5	0.1250	Normal
OUT	9/0	0.0009	Normal
PC1	3/5	0.4720	Shifted
PYL	7/2	0.8496	Normal
SCE	8/2	0.0068	Normal
TER	5/2	0.1875	Normal
WHN	5/4	0.3262	Normal
WTT	4/3	0.2344	Normal

The TPM was used with 5 % mutations greater than one-step. Included are the number of loci that are heterozygote deficient versus those that show heterozygote excesses, the p-value based on the Wilcoxon test, and whether the allelic frequency distribution shape was normal or shifted

Table 5 Estimates of M, as an estimate of putative bottlenecks, for each population (Garza and Williamson 2001)

Population	$4N_e\mu$ (Θ)	M	M_C
BOD	0.145	0.327	0.843
CAD	0.417	0.399	0.717
DAN	0.092	0.697	0.802
GYP	0.074	0.402	0.798
HDI	0.148	0.307	0.725
HUA	0.177	0.272	0.657
INZ	0.897	0.392	0.586
LES	0.193	0.488	0.61
MVT	0.519	0.456	0.682
OUT	0.119	0.577	0.704
PC1	0.076	0.595	0.713
PYL	0.068	0.522	0.771
SCE	0.157	0.443	0.816
TER	0.099	0.741	0.762
WHN	0.172	0.46	0.647
WTT	0.295	0.489	0.62

$4N_e\mu$ was calculated for each population using a coalescent approach in Migrate (Beerli 2002). M was calculated in each population as the ratio of number of alleles per locus (k) divided by allelic size range for that locus (r). M_c was estimated using software from J. C. Garza (M_crit.exe) and generates a critical value below which M values indicate significant bottlenecks in their respective populations

Genetic population structure

The AMOVA showed significant subdivision at all levels: $\Phi_{SC} = 0.433$, $\Phi_{CT} = 0.133$, and $\Phi_{ST} = 0.509$ ($p < 0.0001$ for all three statistics, Table 6), with the highest proportion of variation (49.43 %) explained among individuals. Results were nearly identical and did not differ significantly for all three F-statistics (f, F and θ) whether all populations were sampled, populations with greater than 15 individuals were sampled, or populations with a minimum of 20 individuals were sampled (Table 7). Further, no single population was responsible for the large overall θ value (jackknife θ values averaged 0.118 ± 0.001). When

the five loci that showed significant departures from Hardy–Weinberg proportions were excluded, both f and F decreased significantly, but remained significantly higher than zero (Table 7). Theta (θ), however, did not change significantly with exclusion of these five loci.

Pairwise θ estimates were, with two exceptions, significantly greater than zero, indicating significant population structure among all but two population pairs (Table 7). The exceptions were INZ–OUT (6.45 km apart; $\theta = 0.021$; $p = 0.09$) and TER–WTT (22.92 km apart; $\theta = 0.04$; $p = 0.052$), although p-values for these two pairs were

Table 6 AMOVA of 10 microsatellite loci among 16 localities in SRV, Arizona

Source	d.f.	Sum of squares	Variance component	% Variation	p-value	Φ-Statistic
Among populations	15	164.007	0.253	13.31	<0.00001	$\Phi_{CT} = 0.133$
Among individuals within populations	260	611.823	0.707	37.27	<0.00001	$\Phi_{SC} = 0.433$
Within individuals	276	276	0.938	49.43	<0.00001	$\Phi_{ST} = 0.509$
Total	551	1034.83	1.898			

Results are from Arlequin Version 2.0 for all locations

Table 7 Resampling of F-statistics using all localities, localities with a minimum of 15 individuals sampled and localities with a minimum of 20 individuals sampled

Sampling localities/loci included	F-statistic	Overall	Upper 95 % CI	Lower 95 % CI	p-value
All localities; all 10 loci	f	0.433	0.547	0.287	<0.0001
	F	0.509	0.609	0.367	<0.0001
	θ	0.133	0.166	0.1	<0.0001
All localities; 5 loci	f	0.203	0.272	0.103	<0.0001
	F	0.293	0.364	0.188	<0.0001
	Θ	0.112	0.132	0.088	<0.0001
Localities w/≥15 samples	f	0.454	0.577	0.307	<0.0001
	F	0.523	0.625	0.391	<0.0001
	θ	0.125	0.174	0.077	<0.0001
Localities w/≥20 samples	f	0.429	0.57	0.269	<0.0001
	F	0.506	0.628	0.367	<0.0001
	θ	0.135	0.19	0.078	<0.0001

Also included were F-statistics for 5 loci, excluding loci ATS 4-20, ATS 5-7, ATS 10-7, ATS 13-1 and ATS 14-3, which showed departures from Hardy–Weinberg equilibrium over all populations

both below 0.1. There was no significant isolation-by-distance pattern revealed by the Mantel test (Matrix correlation = 0.230, Z = 168.1, P = 0.117, 15000 permutations). Nei's (1978) genetic distances ranged from 0.0097 (INZ–OUT) to 0.2906 (LES–SCE). These same pairs had the lowest and highest θ values, respectively (Table 8).

Discussion

Genetic diversity

Overall, *A. m. stebbinsi* has low genetic diversity and shows evidence of population bottlenecks among some populations. The level of genetic diversity based on allelic richness and heterozygosity is lower than that typical of other amphibians (Scribner et al. 2001; Garner et al. 2003; Hoffman and Blouin 2004; Spear and Storfer 2008; Murphy et al. 2010). However, similar levels of heterozygosity have been documented in a few cases, including *A. m.*

melanostictum in Yellowstone (Spear et al. 2006), the Pacific giant salamander, *Dicamptodon tenebrosus*, in a fragmented landscape in British Columbia (Curtis and Taylor 2004) and the hellbender, *Cryptobranchus alleganiensus* (Sabatino and Routman 2009; Crowhurst et al. 2011).

While no bottlenecks were reflected in heterozygote excesses, *A. m. stebbinsi* showed evidence of population bottlenecks in two ways. First, several populations (CAD, DAN, INZ, LES and PC1) showed shifted allele frequency distributions. Second, estimates of *M* ranged from 0.272 to 0.741, and all were lower than critical values estimated for each particular population. In addition, nearly all of these values were lower than 0.68, or the critical value proposed by Garza and Williamson (2001) for concluding decline in population numbers with at least seven loci.

Previous genetic work on *A. m. stebbinsi* suggests evidence of a historical bottleneck due to low allozyme variation (Jones et al. 1988), as well as low mitochondrial DNA variation (Jones et al. 1995; Storfer et al. 2004). We suggest there also have been recent bottlenecks because microsatellites are generally reflective of more recent population-level processes than allozymes or mtDNA data (Goldstein and Schlötterer 1999; Jehle and Arntzen 2002). There are several ecological factors that could explain recent bottlenecks as opposed to natural fluctuations among ponds containing *A. m. stebbinsi*. First, non-native species such as fish and bullfrogs have been introduced sporadically throughout ponds in SRV, causing local extirpation in some sites and population size reductions in others (Gerst 1997; Collins et al. 1988). During the study period, we observed at least three predatory fish introductions (including mosquito fish, *Gambusia affinis* and green sunfish, *Lepomis cyanellus*) that resulted in local extirpations of tiger salamanders (Maret et al. 2006). Several other studies have shown correlations between fish introductions and amphibian declines (see Kats and Ferrer 2003). For example, declines of *A. macrodactylum* were correlated with trout stocked in lakes in the North Cascades of Washington (Tyler et al. 1998). Bullfrogs, which are excellent competitors and predators of other amphibian species (Kats and Ferrer 2003), have also been introduced into the SRV. Recent survey data show presence of

Table 8 Genetic distance

	BOD	CAD	DAN	GYP	HDI	HUA	INZ	LES	MVT	OUT	PC1	PYL	SCE	TER	WHN	WTT
BOD	*	0.1357	0.0651	0.0480	0.0623	0.0806	0.0521	0.2496	0.1969	0.0572	0.1332	0.1585	0.1906	0.1185	0.1489	0.1326
CAD	0.0848	*	0.1844	0.0403	0.0819	0.0728	0.0425	0.1394	0.1031	0.0676	0.0799	0.0739	0.1623	0.1181	0.0965	0.1242
DAN	0.0239	0.0837	*	0.0722	0.1336	0.1087	0.1487	0.3071	0.2619	0.0845	0.1543	0.1884	0.2755	0.0733	0.2221	0.0919
GYP	0.0229	0.0173	0.0258	*	0.0309	0.0251	0.0403	0.1941	0.1288	0.0241	0.0568	0.0612	0.1685	0.0318	0.1104	0.0527
HDI	0.0315	0.0510	0.0503	0.0146	*	0.0724	0.0409	0.1945	0.1415	0.0617	0.1357	0.1232	0.1625	0.1404	0.1174	0.1577
HUA	0.0413	0.0368	0.0462	0.0125	0.0361	*	0.0536	0.2381	0.1785	0.0495	0.0991	0.0878	0.1509	0.0689	0.1420	0.1273
INZ	0.0271	0.0323	0.0609	0.0183	0.0223	0.0249	*	0.1793	0.1533	**0.0207**	0.1183	0.1221	0.1265	0.1474	0.0595	0.1606
LES	0.1947	0.1319	0.1893	0.1395	0.1543	0.1851	0.1733	*	0.1473	0.2113	0.2103	0.1928	0.3085	0.2573	0.1983	0.2239
MVT	0.1359	0.0836	0.1525	0.0807	0.0983	0.1218	0.1302	0.1303	*	0.1722	0.1516	0.1178	0.2588	0.2008	0.1301	0.2035
OUT	0.0278	0.0406	0.0329	0.0117	0.0335	0.0258	0.0097	0.1741	0.1274	*	0.0988	0.1008	0.1413	0.0813	0.0802	0.0726
PC1	0.0741	0.0486	0.0669	0.0289	0.0806	0.0539	0.0772	0.1702	0.1067	0.0574	*	0.1136	0.2370	0.0789	0.1688	0.0596
PYL	0.0990	0.0480	0.0809	0.0343	0.0760	0.0527	0.0814	0.1480	0.0773	0.0631	0.0615	*	0.2019	0.1058	0.1506	0.1184
SCE	0.1212	0.1115	0.1675	0.1242	0.1013	0.0880	0.0800	0.2906	0.2132	0.0864	0.1694	0.1433	*	0.2537	0.1097	0.2651
TER	0.0536	0.0516	0.0215	0.0113	0.0597	0.0293	0.0699	0.1660	0.1159	0.0342	0.0328	0.0401	0.1573	*	0.2063	**0.0401**
WHN	0.0936	0.0778	0.1310	0.0694	0.0810	0.0930	0.0435	0.1919	0.1060	0.0514	0.1259	0.1125	0.0710	0.1306	*	0.1819
WTT	0.0610	0.0627	0.0284	0.0217	0.0753	0.0619	0.0873	0.1464	0.1276	0.0327	0.0260	0.0498	0.1733	0.0124	0.1159	*

Pairwise F_{st} values (above diagonal, from Arlequin 2.0) and Nei's (1978) genetic distance (below diagonal, from GDA). Bolded F_{st} values are not significantly different from zero at the $\alpha = 0.05$ level

bullfrogs in several SRV ponds (including BOD, INZ, OUT and PC1; Storfer unpubl.). Note that two of these ponds (INZ, PC1) showed shifted allelic distributions.

The second ecological reason that may explain recent bottlenecks is disease. Salamanders in SRV ponds regularly experience epizootics due to an iridovirus (*A. tigrinum* virus or ATV), and evidence suggests this virus has been recently introduced to SRV (Jancovich et al. 1997, 2005; Storfer et al. 2007). Although a chytrid fungus is apparently the pathogen most closely linked to amphibian declines (Stuart et al. 2004; Murray et al. 2009; McCallum 2012), iridoviruses are responsible for the majority of amphibian epizootics in North America (Green et al. 2002; Miller et al. 2011). ATV is a virus that is highly infectious, and frequently lethal, even at low doses (Jancovich et al. 1997, 2005; Brunner et al. 2004, 2007; Storfer et al. 2007). Die-offs have occurred in at least seven populations studied herein (BOD, HDI, HUA, INZ, OUT, PC1 and SCE).

During an epizootic event, larval mortality rates can approach 100 % in a given year, thus potentially causing population-level bottlenecks (Brunner et al. 2007; Schock et al. 2009; Greer et al. 2009). Other populations show evidence of repeated fluctuations in population size, which is also a source of bottlenecks. That is, repeated temporal surveys have shown that animals can often return, and mark-recapture data suggest that these animals are survivors, rather than re-colonists from neighboring ponds (Brunner et al. 2004). Coupling the low rates of gene flow observed in this study, die-offs resulting in few survivors, and unlikely recolonization, it is expected that

ATV-induced epizootics contribute to the observed declines in genetic diversity.

The third ecological reason for potential bottlenecks is that the SRV is a human-dominated landscape that has been drastically modified for cattle grazing over the past 150 years. Extensive grazing throughout the valley has lowered water tables, removing most cienegas that were the likely historical habitat for *A.m. stebbinsi* (Collins et al. 1988). The fact that *A. m. stebbinsi* now relies exclusively on human-made habitats for breeding and that human disturbance has generally made SRV drier apparently limits gene flow among ponds, thus limiting genetic variability. Other work shows lower genetic variability in disturbed versus undisturbed sites, suggesting gene flow is limited by disturbance. For example, in Britain the common frog, *Rana temporaria*, had higher inbreeding levels disturbed, urban sites as opposed to in rural areas (Hitchings and Beebee 1997). In addition, giant salamanders showed a significant positive correlation of genetic variation with forest stand age after clear-cutting (Curtis and Taylor 2004).

Taken together, the above arguments suggest that within-population departures from Hardy–Weinberg equilibrium are a result of inbreeding resulting from population bottlenecks and not due to null alleles. Our data showed a significantly lower proportion of null homozygotes than expected, several populations showed evidence of shifted allele frequency distributions, and all populations had *M* values below the critical value. Evidence of inbreeding depression is reflected in lab-rearing experiments that show

lower embryonic survival, lower larval survival, and lower disease resistance in *A. m. stebbinsi* relative to *A. m. nebulosum* (Storfer unpubl.), which has higher genetic variation and lower levels of inbreeding (Jones et al. 1988, 1995).

Genetic population structure

A. t. stebbinsi shows a high degree of population genetic structure, with nearly all population pairs significantly subdivided from one another, despite some inter-pond distances that are less than one kilometer. In addition, there was no significant isolation-by-distance pattern, probably because nearly all populations were significantly subdivided from each other. Levels of gene flow documented herein are generally lower than microsatellite studies of pond breeding frogs (Newman and Squire 2001; Garner et al. 2003; Emel and Storfer 2012) but similar to that reported for the hellbender (Crowhurst et al. 2011). Sonora tiger salamanders also show lower gene flow than their sister taxon, *A. californiense*, which has a high degree of genetic connectivity and dispersal on a local scale (Trenham et al. 2001).

Several factors could limit gene flow among ponds in SRV. First, SRV ponds are generally separated by open, dry grassland (Jones et al. 1988), and movement across grassy fields in SRV could increase risk of both desiccation and predation. Drying of these habitats has been exacerbated by cattle grazing, which has lowered the water table in SRV (Gerst 1997). Several studies have shown ambystomatids tend to avoid open habitats (deMaynadier and Hunter 1999; Trenham 2001; Rothermel and Semlitsch 2002; Spear et al. 2006). A radiotelemetry study of the eastern tiger salamander, *A. tigrinum*, showed avoidance of grassy fields (Madison and Farrand 1998), and genetic subdivision was found among populations of *A. tigrinum* separated by fields in Nebraska (Routman 1993). Another study showed that juvenile amphibians (including *A. maculatum*) moved less and were recaptured less often in fields as compared to forested areas (Rothermel and Semlitsch 2002).

Second, because habitats in SRV are managed and relatively permanent, presence of perennibranchiate (sexually mature, gilled) adults is common in *A. m. stebbinsi* (Jones et al. 1988). These individuals are unable to disperse among ponds, thus limiting gene flow and potentially genetic

variability among ponds. Support for this prediction comes from a study of among populations of *A. m. melanostictum* in northern Yellowstone, where presence of aquatic adults appears to limit gene flow (Spear et al. 2005, 2006). Another study showed that perennibranchiate tiger salamander populations had lower genetic variability than metamorphic populations (Shaffer and Breden 1989).

Small population sizes could also contribute to differentiation among SRV populations via genetic drift. Low effective population sizes that are likely caused by a combination of disease, habitat disturbance and introduction of exotic species may mean that the magnitude of genetic drift is sufficiently large to mask gene flow. Thus, among populations separated by short distances that are apparently subdivided genetically, gene flow may be swamped by genetic drift.

In summary, the Sonora tiger salamander shows evidence of a high degree of inbreeding and strong genetic population structure. The high level of inbreeding and low genetic variability are of particular concern if there is no longer sufficient genetic variability for the subspecies to survive, for example, introduction of new viral strains (Jancovich et al. 2005; Picco et al. 2010). The subspecies appears to be particularly prone to drastic reductions in population size and, consequently, local extinction. In addition, the high degree of genetic structuring among populations suggests low probability of re-colonization following local extinction events. Therefore, unlike the endangered California tiger salamander (Trenham et al. 2001; Smith and Green 2005), Sonora tiger salamanders apparently do not operate as a metapopulation, and continued management may include artificial re-colonizations. Based on the genetic data presented herein, the endangered status of the species is supported.

Appendix

See Table 9.

Table 9 Sample sizes (*N*) and allele frequencies for each population

Locus	Allele	BOD	CAD	DAN	GYP	HDI	HUA	INE	LES	MVT	OUT	PC1	PYL	SCE	TER	WHN	WTT
ATS4-11	(N)	31	10	18	17	11	22	10	10	14	17	17	13	36	11	20	11
	175	–	–	–	–	0.045	–	–	–	–	–	–	–	–	–	–	–
	183	–	–	–	–	–	–	–	–	–	–	–	0.038	–	–	–	–
	187	–	–	–	–	–	–	–	–	–	–	–	–	–	0.227	–	–
	189	0.984	0.600	1.000	0.824	0.773	0.909	0.800	0.750	0.643	0.912	0.794	0.654	0.556	0.773	0.775	0.955
	191	–	–	–	–	–	–	–	0.050	0.036	0.029	–	–	0.056	–	0.050	–
	193	0.016	0.400	–	0.176	0.136	0.068	0.100	0.200	0.321	0.059	0.206	0.308	0.389	–	0.175	0.045
	195	–	–	–	–	0.045	–	0.100	–	–	–	–	–	–	–	–	–
	223	–	–	–	–	–	0.023	–	–	–	–	–	–	–	–	–	–
ATS4-20	(N)	34	10	18	17	11	21	10	10	14	17	17	13	40	11	21	11
	329	–	–	–	–	–	–	–	–	0.071	–	–	0.538	0.025	–	–	0.091
	331	0.750	0.800	0.500	0.765	0.864	0.929	0.900	0.250	0.429	0.706	0.706	0.462	0.912	0.636	0.643	0.455
	333	0.221	0.150	0.500	0.118	–	0.048	–	–	0.143	0.265	0.294	–	0.050	0.364	0.190	0.455
	335	0.029	–	–	–	–	–	–	–	–	0.029	–	–	–	–	–	–
	337	–	0.050	–	0.059	0.136	0.024	–	–	0.071	–	–	–	0.013	–	0.071	–
	339	–	–	–	–	–	–	–	–	0.214	–	–	–	–	–	–	–
	343	–	–	–	–	–	–	0.100	0.400	–	–	–	–	–	–	–	–
	345	–	–	–	–	–	–	–	0.300	–	–	–	–	–	–	0.095	–
	361	–	–	–	0.029	–	–	–	0.050	–	–	–	–	–	–	–	–
	363	–	–	–	0.029	–	–	–	–	–	–	–	–	–	–	–	–
	374	–	–	–	–	–	–	–	–	0.071	–	–	–	–	–	–	–
ATS4-25	(N)	34	10	18	17	11	22	10	10	14	17	17	13	40	11	21	11
	223	0.029	–	–	–	–	0.045	0.100	–	–	0.059	–	–	–	–	–	–
	225	–	–	–	–	–	0.023	–	–	–	–	–	–	–	–	–	–
	229	–	–	0.111	–	0.273	–	–	–	–	–	–	–	–	–	–	–
	231	0.721	0.800	0.833	0.824	0.636	0.909	0.650	0.750	0.607	0.735	1.000	0.885	0.787	1.000	0.429	0.864
	233	0.029	–	0.056	–	0.091	0.023	0.050	–	–	–	–	–	0.013	–	–	–
	235	0.221	0.200	–	0.176	–	0.023	0.250	0.250	0.393	0.206	–	0.115	0.200	–	0.571	0.136
ATS5-6	(N)	34	10	18	17	11	22	10	10	14	17	17	13	40	11	21	11
	193	0.029	0.200	0.028	–	0.045	0.045	0.050	–	0.321	–	–	–	0.013	–	0.048	–
	195	–	–	–	–	–	–	–	–	–	–	–	–	0.025	–	–	–
	197	–	–	–	0.059	–	0.182	–	–	–	–	–	0.077	0.037	0.091	0.024	–
	199	0.971	0.800	0.972	0.882	0.955	0.773	0.900	1.000	0.679	0.941	0.794	0.923	0.900	0.909	0.929	1.000
	201	–	–	–	–	–	–	–	–	–	0.059	0.147	–	0.025	–	–	–
	203	–	–	–	0.059	–	–	–	–	–	–	–	–	–	–	–	–
	207	–	–	–	–	–	–	–	–	–	–	0.029	–	–	–	–	–
	209	–	–	–	–	–	–	–	–	–	–	0.029	–	–	–	–	–

Table 9 continued

Locus	Allele	BOD	CAD	DAN	GYP	HDI	HUA	INE	LES	MVT	OUT	PC1	PYL	SCE	TER	WHN	WTT
ATS5-7	(N)	34	10	18	17	11	22	10	10	14	17	17	13	40	11	21	11
	242	–	–	–	0.059	–	–	–	–	–	0.059	0.265	–	0.125	–	–	0.273
	244	0.912	0.700	1.000	0.882	0.909	1.000	0.800	0.500	1.000	0.941	0.588	1.000	0.775	1.000	0.667	0.727
	246	–	–	–	–	–	–	–	–	–	–	0.088	–	–	–	–	–
	248	–	–	–	–	–	–	–	–	–	–	0.059	–	–	–	–	–
	250	0.074	–	–	–	–	–	–	0.500	–	–	–	–	–	–	0.095	–
	254	–	0.100	–	0.029	–	–	0.100	–	–	–	–	–	0.037	–	0.024	–
	256	–	0.200	–	0.029	–	–	0.100	–	–	–	–	–	0.063	–	0.048	–
	258	0.015	–	–	–	0.091	–	–	–	–	–	–	–	–	–	0.143	–
	260	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.024	–
ATS5-8	(N)	33	10	18	17	11	22	10	10	14	17	17	13	40	11	21	11
	356	–	–	–	–	–	–	–	–	–	–	–	–	0.013	–	–	–
	360	–	–	–	–	–	0.023	–	–	–	–	–	0.038	0.037	–	0.048	–
	362	–	–	–	–	–	0.091	–	–	–	–	–	–	–	–	–	–
	364	0.030	–	–	0.029	–	–	–	–	–	–	–	–	–	–	0.048	–
	366	0.742	1.000	1.000	0.971	1.000	0.886	0.950	0.950	0.714	1.000	0.706	0.962	0.950	1.000	0.905	1.000
	368	0.197	–	–	–	–	–	0.050	0.050	0.250	–	0.294	–	–	–	–	–
	370	–	–	–	–	–	–	–	–	0.036	–	–	–	–	–	–	–
	372	0.030	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ATS10-7	(N)	30	10	18	17	11	22	10	10	14	17	17	13	40	11	21	11
	292	–	–	–	–	–	–	–	0.100	–	–	–	–	–	–	–	–
	294	–	–	–	–	–	–	–	0.200	–	–	–	–	0.013	–	–	–
	296	–	0.200	0.111	–	–	–	–	0.100	–	–	–	0.115	0.013	–	–	–
	298	0.200	0.050	0.111	0.118	0.045	–	0.050	0.050	0.143	–	–	–	0.063	0.091	–	–
	300	0.733	0.750	0.778	0.882	0.864	0.818	0.650	0.550	0.857	0.882	1.000	0.885	0.912	0.909	1.000	1.000
	302	–	–	–	–	–	0.182	0.100	–	–	0.118	–	–	–	–	–	–
	304	0.067	–	–	–	0.091	–	0.100	–	–	–	–	–	–	–	–	–
	314	–	–	–	–	–	–	0.100	–	–	–	–	–	–	–	–	–
ATS12-3	(N)	34	10	18	17	11	22	10	10	14	17	17	12	40	11	21	11
	191	0.191	–	–	–	–	0.023	–	–	–	–	–	–	–	–	–	–
	193	0.029	–	–	–	–	0.023	–	–	–	–	–	–	–	–	–	–
	213	–	–	–	0.029	0.136	–	–	–	–	0.118	–	0.083	0.200	–	–	–
	221	–	0.100	–	0.088	0.091	0.068	0.100	–	–	0.088	–	–	0.412	0.045	0.310	0.091
	223	0.779	0.700	0.944	0.882	0.773	0.795	0.700	0.600	1.000	0.206	0.941	0.917	0.388	0.955	0.690	0.091
	225	–	0.200	0.056	–	–	0.091	0.100	0.400	–	0.588	0.059	–	–	–	–	0.818
	227	–	–	–	–	–	–	0.100	–	–	–	–	–	–	–	–	–

Table 9 continued

Locus	Allele	BOD	CAD	DAN	GYP	HDI	HUA	INE	LES	MVT	OUT	PC1	PYL	SCE	TER	WHN	WTT
ATS13-1	(N)	34	10	18	17	11	22	10	10	14	17	17	10	40	11	21	11
	327	–	–	–	–	–	–	–	–	–	–	–	–	0.013	–	–	–
	329	0.706	0.200	0.639	0.412	0.545	0.409	0.650	–	0.179	0.500	0.294	0.250	0.738	0.273	0.571	0.273
	331	0.029	–	–	–	0.045	0.068	–	–	–	–	–	–	–	–	–	–
	333	–	–	–	–	–	–	–	–	0.071	0.029	–	–	–	–	–	–
	335	–	–	–	–	–	–	–	–	–	–	–	–	–	0.045	–	–
	337	–	–	–	0.029	–	–	–	–	–	–	–	–	–	–	–	–
	339	0.044	–	–	0.059	0.227	0.023	0.100	0.600	0.321	0.029	0.059	0.050	0.050	–	0.143	–
	341	–	–	–	–	0.045	0.045	–	–	–	–	–	–	–	–	–	–
	367	–	0.050	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	369	0.191	0.450	0.361	0.441	0.136	0.409	0.250	0.400	0.429	0.382	0.647	0.700	0.200	0.682	0.286	0.727
	371	0.029	0.300	–	0.059	–	0.045	–	–	–	0.059	–	–	–	–	–	–
ATS14-3	(N)	34	10	17	17	11	22	10	10	14	17	17	13	40	11	21	11
	103	0.088	0.300	–	0.206	0.364	0.114	0.100	0.500	0.750	0.059	0.206	0.269	0.163	0.091	0.310	0.136
	105	0.044	0.250	–	0.059	–	0.091	0.350	–	–	0.206	0.206	0.115	0.013	–	0.310	–
	121	–	–	–	–	–	–	0.100	–	–	0.176	–	–	–	–	–	–
	127	0.118	–	0.118	0.059	–	0.295	0.050	–	–	0.059	–	0.154	0.700	0.136	0.286	0.045
	129	0.750	0.450	0.882	0.676	0.636	0.500	0.400	0.500	0.250	0.500	0.588	0.462	0.125	0.773	0.095	0.818

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