Genetic diversity in the fountain darter *Etheostoma fonticola*

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ABSTRACT

The endangered fountain darter *Etheostoma fonticola* is an obligate spring endemic distributed in only the Comal and San Marcos Rivers in the Guadalupe River drainage in central Texas. Comal River fountain darters were extirpated following a severe drought in the 1950s and reintroduced in the early 1970s using 457 darters from the San Marcos River. We conducted genetic analyses for this species using nine microsatellite loci in order to describe genetic population structure, assess if putative barriers (low-head dams) impede gene flow within each river and determine if a 457 fish reintroduction was sufficiently large to maintain genetic diversity. Bayesian analysis of individual genotypes supported two distinct genetic groups concordant with the two rivers. Estimates of genetic divergence (F-statistics) also revealed significant differences between the two rivers with a higher proportion of divergence due to differences among aggregations from different rivers than differences among aggregations from the same river. Results from a variety of statistical tests indicated that some of the dams may be reducing gene flow, but most of the results were inconclusive and additional analyses are warranted. Our results indicate that there has been a reduction in genetic diversity in the Comal River. Samples collected in the Comal River are lower in allelic richness and have fewer private alleles than those collected in the San Marcos River. Assuming the genetic diversity in the fountain darters reintroduced into the Comal River was representative of the San Marcos River, 457 individuals appears to have been insufficient to maintain the full array of genetic diversity that we observed in the San Marcos River.

INTRODUCTION

The fountain darter *Etheostoma fonticola* is a small, spring-endemic percid distributed only in the upper San Marcos and Comal Rivers (Guadalupe River drainage, Gulf of Mexico) in central Texas and is listed as endangered pursuant to the U.S. Endangered Species Act as amended (ESA 1973). Essential habitat for the species is strongly influenced by the amount of spring water emerging from the Edwards Aquifer at the headwaters of each river, and the discharge is determined by the amount of precipitation over aquifer recharge areas and the amount of water extracted from the aquifer for human use.

Droughts in central Texas occur at least once a decade, and during a severe drought in the 1950s, Comal Springs ceased flowing for five months (Brune 2002). Fountain darter was likely extirpated from this location following this drought, but a reintroduction occurred in the early 1970s, with a small number of darters (457) artificially transferred from the San Marcos River to the Comal River (USFWS 1984), which now contains a fairly large population (n > 150,000; Linam *et al*. 1993; Ed Oborny 2011 pers. comm.). Both rivers contain impounded headwaters and numerous low-head dams and water diversion structures for recreational uses. Dams can have dramatic effects on lotic habitat by altering water chemistry and flow (Baxter 1977), river geomorphology (Ligon *et al*. 1995), fish and macroinvertebrate communities (Lessard and Hayes 2003; Santucci *et al*. 2005; Tiemann *et al*. 2004) and can cause a reduction of gene flow within species, leading to reduced genetic diversity and increased genetic differentiation (Wofford *et al*. 2005; McCraney *et al*. 2010). Understanding genetic structure in imperiled species is particularly important because limited genetic diversity may inhibit an organism's ability to survive by reducing its ability to adapt (Roman and Darling 2007).

Here we use nine microsatellite loci to conduct population genetic analysis for fountain darters. The objectives are to describe population structure of fountain darters in the Comal and San Marcos Rivers, determine if low-head dams impede gene flow within each river and determine if 457 fish reintroduction was sufficiently large to maintain the genetic diversity present in the San Marcos River. The results are assessed with regard to on-going and future conservation efforts.

METHODS

Sample collection

Fountain darters were collected from seven locations on the Comal River (n = 147) and nine locations on the San Marcos River (n = 180, Table 1, Figure 1). Each river consists of four segments separated by dams blocking upstream movement. For most analyses, the collections within each segment were pooled and termed aggregations (Table 1). Fish were anesthetized with tricane methane-sulfonate (MS-222; Finquel, Argent Chemical Laboratories, Inc., Redmond, Washington) and preserved individually in vials of 95% ethanol. Fin tissue samples were collected for genetic analysis, preserved in 95% ethanol, and sent to the Conservation Genetics Laboratory, Alaska Region, U.S. Fish and Wildlife Service for analyses.

Laboratory analyses

Nine microsatellite loci were used to estimate genetic variation in the 327 fountain darter samples (Table 2). Total genomic DNA was extracted from fin tissue (~25mg) using proteinase K with the DNeasy™ DNA isolation kit (Qiagen Inc. Valencia, CA), quantified with fluorometry and diluted to a standard concentration. An MJResearch DNA Engine® thermal cycler was used to perform polymerase chain reactions (PCR) in 10 µl volumes; general conditions were: 2.5 mM $MgCl_2$, 1X PCR buffer (20 mM Tris-HCl pH 8.0, 50 mM KCl), 200 µM of each dNTP, 0.40µM fluorescently labeled forward primer, 0.40 µM unlabeled reverse primer, 0.008 units *Taq* polymerase, and 1 µl of DNA (30ng/µl). Standard thermal cycling conditions were: initial denaturation cycle of 92°C for 2 min, followed by 92°C for 15 sec, 52-60°C for 15 sec (locus-specific sequences and annealing temperature are given in Table 3), 72°C for 30 sec, (30 cycles) with a final single cycle of 72°C for 10 min. One-half µl of PCR product was electrophoresed and visualized with the Applied Biosystems 3730 Genetic Analyzer utilizing a polymer denaturing capillary system. Microsatellite allele sizes were estimated and scored by the computer program GeneMapper® version 4.0. Applied Biosystems GeneScan™-600 LIZ® size standards, 20-600 bases, were loaded in all lanes as an internal lane standard. Two independent researchers verified all scores manually, with discrepancies being resolved by replicating the analysis for the samples in question and repeating the double scoring process until scores

matched (unresolved scores were excluded from further analysis). Data for samples from at least one row in each 96-well sample plate was automatically replicated to confirm that proper plate orientation was maintained throughout genotyping efforts for the project.

Statistical analyses

The samples collected at multiple sites within river segments were pooled as aggregations for most analyses (see aggregations, Table 1). However, to evaluate if this approach was reasonable, and to assess if significant genetic population structure exists within aggregations, an initial analysis was performed among samples without regard to aggregations. First, we used the program STRUCTURE version 2.3.1 (Pritchard *et al*. 2000) which is a Bayesian, model-based algorithm that groups individuals in order to estimate the most likely aggregation scenario that satisfies Hardy-Weinberg equilibrium. We tested K = 2 to 16 clusters (aggregations) assuming admixture and correlated allele frequencies (between the K clusters) and using a burn-in of 20,000 replications followed by 50,000 Markov chain Monte Carlo replications. We also used the LOCPRIOR model developed by Hubisz *et al*. (2009) that accounts for weak population structure by allowing locations to be used as priors in the clustering algorithm. Ten replications were performed to confirm the consistency of the log-likelihood probabilities and to estimate the variance. The most likely aggregation scenario was the one that produced the largest penalized likelihood value (the mean of the likelihood values from each replicate minus half the variance). Second, we used the computer program FSTAT version 2.9.3 (Goudet 2001) to conduct a *G*-test of allele frequency homogeneity to test for genetic differentiation between all pairs of sample locations within each river.

Estimates of allele frequency, allelic richness (Ar, the number of alleles adjusted for differences in sample size) and observed (Ho, number of heterozygotes at a locus/number of individuals collected) and expected heterozygosity (He, number of heterozygous for randomly chosen locus/number of individuals) were computed for each locus and aggregation using FSTAT. Randomization tests were used to test for conformity to Hardy-Weinberg expectations (HWE) for each locus and aggregation combination and to test for composite genotypic disequilibrium among locus pairs over all aggregations. These tests were performed using

FSTAT and the threshold for statistical significance ($\alpha = 0.05$) was corrected (α/k) using the sequential Bonferroni method (Rice 1989) for k simultaneous tests and the value of k decreased sequentially by removing significant tests until no tests were judged significant. Two initial values of k were used for the HWE test to evaluate each aggregation over all loci ($k = 9$) and each locus over all aggregations ($k = 8$).

We tested if genetic diversity in fountain darters from the Comal River was lower than in fountain darters from the San Marcos River; randomization tests in FSTAT were used to compare mean values of Ar and He. We also computed estimates of private allele richness (pAr) for each locus in each river using the computer program HP-RARE version 1.0 (Kalinowski 2005). A Wilcoxon paired-sample test implemented in the computer software R 2.12.0 (http://www.r-project.org/) was used to assess if estimates of pAr differed between the two rivers over all loci.

The computer program FSTAT was used to conduct a *G*-test of allele frequency homogeneity to test for genetic differentiation between all pairs of aggregations within and among each river and to determine if the dams impede gene flow. The level of population divergence was estimated with F_{ST} (Wright 1943), which was computed over all aggregations and for each pair of aggregations, over all loci, according to Weir and Cockerham (1984). Fst-values can range from 0 to 1, where a high F_{ST} implies a considerable degree of differentiation among populations. Analysis of Molecular Variation (AMOVA) was used to quantify the relative level of genetic variation between rivers (Fct) and within rivers (Fsc) and to test for statistical significance of each value. AMOVA was performed using ARLEQUIN version 3.5 (Excoffier *et al*. 2005).

RESULTS

The analysis of individual genotypes using STRUCTURE indicated that the most likely solution was K = 2 clusters. These two clusters clearly aggregated individuals by river (Figure 2). Further analysis was conducted using individuals from each river separately (the hierarchical method, e.g. Vähä *et al*. 2007) to assess if genetic structure was evident that may conflict with

the a-priori aggregations in Table 1. The results revealed no evidence of structuring within the rivers (data not shown). The *G*-tests of allele frequency homogeneity did not reveal evidence of population structure among the collection locations within each aggregation. Collectively, the STRUCTURE and *G*-test results did not support treating sample locations separately for the analysis; all further analyses were performed using the aggregations described in Table 1.

With the exception of *Eos*C6, *Esc*132b and *Esc*26b, all loci had fewer than five alleles (Table 2). The estimates of genetic diversity within aggregations as measured by average heterozygosity (He) and allelic richness (Ar) were lowest in aggregation CR4 at 0.446 and 5.21 and highest in aggregation SMR4 at 0.530 and 6.33, respectively (Table 2). Tests of Hardy-Weinberg equilibrium initially revealed a deficit of heterozygote genotypes ($P < 0.05$) at locus *Eche*002 in aggregations CR1 and CR4 and at locus *Esc*26bC3 in aggregation SMR1 (Table 2). However, these tests were not statistically significant after correction for multiple tests.

The randomization tests in FSTAT indicated that the estimates of mean heterozygosity (Hs) did not differ significantly among aggregations from the two rivers however estimates of mean Ar were significantly larger in aggregations from the San Marcos River compared to the Comal River ($P = 0.009$, Table 4). The Wilcoxon paired-sample test showed that the estimates of private allele richness (pAr) were larger across loci in the San Marcos River compared to the Comal River. The differences were especially apparent at the most variable loci, *Esc*132b and *Esc*26b.

Genetic diversity within collections from both rivers is relatively high at two loci (He > 0.8, Ar > 10). The remaining seven loci exhibited relatively low polymorphism. The estimates of genetic variation among aggregations as measured by pairwise Fst ranged from 0.0012 (SMR3 x SMR4) to 0.0342 (CR1 x SMR3) and were generally largest when the aggregations were from different rivers (Table 5). Eighteen of 28 pairs of aggregations showed statistically significant differences in allele frequencies when the *G*-test *P*-values were adjusted for multiple tests. Most of the significant results occurred when the aggregations were from different rivers (Table 5). However, fountain darters collected from the impounded headwaters of the San Marcos River were significantly different from those collected at one of the lower sites, separated by two dams (one creating the impoundment and one low-head dam). Furthermore, fountain darters collected

from the impounded headwaters of the Comal River were significantly different from those collected at the lowermost site, separated by one dam and two water-control structures.

The estimate of Fst over all aggregations was 0.0195 (Table 6). The estimates of genetic variation among aggregations within rivers (Fsc) and among rivers (Fct) were 0.0084 and 0.0112, respectively (Table 6). The estimates of Fst, Fsc, and Fct were all significantly larger than zero. Finally, the estimate of Fst was larger among the Comal River aggregations than among the San Marcos River aggregations but the difference was not statistically significant (Table 4).

The number of private alleles found among individuals collected from both the San Marcos and Comal River differed (Table 7). The San Marcos River (n = 17) had a greater number of private alleles than the Comal River (n = 4) at more loci (n = 4) than the Comal River specimens (n = 4 alleles; n = 2 loci). The San Marcos River fish had private alleles at loci *Eos*C6 (n = 3), *Esc*132b (n= 7), *Eos*C112 (n = 1), and *Esc*26b (n = 6). The Comal River fish had private alleles, at loci *Esc*132b (n = 3), and *Esc*26b (n = 1).

DISCUSSION

Analyses of nine microsatellite markers reveal that some population structure does exist in fountain darters. Some divergence of neutral genetic markers has occurred since the reintroduction into the Comal River during the 1970's. Genetic differentiation is primarily due to differences among aggregations from the two rivers (Fct). However, there are significant differences in allele frequencies between the most distant aggregations within each river. We cannot determine if these differences are due to reduction in gene flow from artificial barriers, absolute distance, or a combination of these and other factors. Even though the *P*-values between the uppermost Comal River site (CR1) and two adjacent sites (CR2 and CR3), and the uppermost San Marcos site and the adjacent site (SMR2) do not indicate significant differences at the 0.05 level after a Bonferroni adjustment, the unadjusted *P*-values are less than 0.05, indicating that there is likely some reduction in gene flow between adjacent aggregations. Some adjacent aggregations separated by barriers (e.g. CR1xCR2, SMR1xSMR2) exhibited low, but not statistically significant, *P*-values for the test of pairwise genetic divergence. It is possible

that adding more loci to the analysis would reveal significant divergence between these adjacent aggregations.

Fountain darters from the San Marcos and Comal Rivers exhibited alleles not found in any individuals from the other river (private alleles). While it is possible the private alleles found in the Comal River collection originated from fountain darters that survived the drought in the early 1970s, it may not be probable. Alternatively we hypothesize that the private alleles in the Comal River collection are likely the outcome of neutral mutations (mutations that do not influence survival) that have occurred since the re-introduction, as would be expected given a conservative estimate of the number of generations since reintroduction, the effective population size, and standard rates for microsatellite mutation (data not shown). Nevertheless, additional genetic research is needed to refute or corroborate this hypothesis.

The Comal River fish exhibited lower diversity as measured by allelic richness and private allele richness than the fish from the San Marcos River. Assuming the genetic diversity in the fountain darters reintroduced into the Comal River was representative of the San Marcos River, 457 individuals appears to have been insufficient to maintain the full array of genetic diversity present in the San Marcos River. However, if the sample of 457 darters reintroduced into the Comal was not representative (e.g., consisted of closely related individuals), the reduction in genetic diversity may be a result of insufficient sampling and not a result of a reduction in genetic diversity occurring after the reintroduction. Currently, San Marcos NFHTC houses 300 fountain darters each from the San Marcos and Comal Rivers. Our results indicate that if it became necessary to reintroduce fountain darters into habitat where they have been extirpated, 300 would not be sufficient to avoid some reduction in genetic diversity. We do not have sufficient information to determine exactly how many fish would be required to maintain the full suite of genetic diversity present in the wild populations. There are many factors (e.g. ecological, morphological, behavioral etc.) that must be considered in addition to genetics to make this determination. In addition to further genetic analyses of more samples (e.g., additional loci, progeny from free spawning vs. pairwise spawning), collection procedures (spatial and temporal), husbandry techniques, spawning practices, reintroduction methods, and other factors

must be taken into account in order to determine the best course of action in preparation for potential droughts and future reintroductions.

RECOMMENDATIONS

The recommendations are predicated on the assumption that the Comal River fountain darter population was extirpated in the 1950s prior to reintroduction efforts.

- Continue to maintain stocks as outlined in the San Marcos/Comal/Edwards Aquifer Rare, Threatened, and Endangered Species Contingency Plan (1996) until the following recommendations have been completed and vetted.
- Establish a review team to evaluate the fountain darter conservation program and recommend next steps; as indicated above many factors must be considered in addition to genetics. An adaptive management approach should be used to clearly define objectives and the various actions that would be necessary based on the results of future monitoring.
- The program should consider increasing the refuge population size, more conservatively on the order of 1,000 individuals. Although other factors should also be considered, our results along with a simple theoretical genetic evaluation (see Appendix 1) suggests 300 is not enough to prevent the loss of significant genetic diversity.
- Develop additional genetic markers to evaluate and monitor genetic diversity in the wild and captive populations to better inform future conservation efforts.
- Conduct additional genetic analyses of the two populations to support/refute the finding of this single genetic study.
- Taking into account the assumption that the selective pressures in the two rivers are nearly identical (due to similarities in habitat, environmental variables, etc.), the reality of limited resources to house an infinite number of fish in captivity, and the results of this study, maintaining a large refugium population from the San Marcos River as opposed to two smaller populations from both rivers may be merited if additional genetic work supports this recommendation.

- Based solely on this single genetic study and its limited results it appears that the best source of fish for potential introduction into the Comal River may be the San Marcos River if additional genetic work supports this recommendation.
- If there are data that indicate otherwise, the recommendations above, especially to maintain a single source from the San Marcos River and to stock the Comal River with individuals from the San Marcos River, must be reevaluated.

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INCLUDED DATA:

Genotype data for all sixteen collections and for the sixteen collections pooled into eight aggregations is shown in FSTAT format in Appendices 2 and 3.

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Table 1. Sample locations and aggregation designation used to examine genetic diversity in fountain darter collections from the San Marcos and Comal Rivers 2009-2010; n = sample size.

River	Location	n	Date	Aggregation
Comal	Houston St., Landa Lake	15	02/25/10	CR1
Comal	Liberty Ave., Landa Lake	15	02/25/10	CR1
Comal	Landa Lake, Landa Park	9	03/09/10	CR1
		21	04/14/10	
Comal	Elizabeth Ave., Old Channel	1	02/25/10	CR2
		13	02/26/10	
		5	04/16/10	
		6	04/21/10	
Comal	Hinman Island Park, New Channel	9	03/31/10	CR3
		12	04/09/10	
		9	04/14/10	
Comal	Above Hinman Weir, Old Channel	2	04/14/10	CR3
Comal	Garden St.	10	03/31/10	CR4
		9	04/14/10	
		11	04/16/10	
San Marcos	Spring Lake, near hotel	15	11/23/09	SMR1
		15	02/05/10	
San Marcos	Spring Lake, near outflow dam	15	11/23/09	SMR1
		15	02/05/10	
San Marcos	Sewell Park	15	03/19/10	SMR2
		14	11/23/09	
San Marcos	City Park	15	03/17/10	SMR2
		15	04/09/10	
San Marcos	Rio Vista Park	14	03/17/10	SMR2
San Marcos	Cheatum St.	15	03/17/10	SMR3
San Marcos	I-35	15	03/19/10	SMR3
San Marcos	Cypress Tree, Lower	4	10/28/09	SMR4
San Marcos	Todd Island	3	10/28/09	SMR4
		4	04/09/10	
		6	04/23/10	

Table 2. Genetic diversity estimates for nine microsatellite loci in eight fountain darter aggregations from the Comal (CR) and San Marcos (SMR) Rivers. Estimates include expect heterozygosity (He), observed heterozygosity (Ho, and allelic richness (Ar). Estimates of Hc lower than expected based on Hardy-Weinberg expectations are underlined and in bold ($P < 0.05$).

		Comal River				San Marcos River			
Locus	Stat	CR1 (60)	CR2 (25)	CR3 (32)	CR4 (30)	SMR1 (60)	SMR2 (73)	SMR3 (30)	SMR4 (17)
*Eche*001[a]	He	0.546	0.381	0.607	0.346	0.424	0.313	0.505	0.524
	Ho	0.533	0.400	0.531	0.333	0.433	0.288	0.500	0.471
	Ar	3.26	2.68	3.78	3.63	3.61	3.49	3.97	4.00
*Eche*002[a]	He	0.307	0.256	0.177	0.129	0.187	0.143	0.128	0.404
	Ho	**0.233**	0.240	0.188	**0.067**	0.167	0.123	0.133	0.294
	Ar	3.18	2.96	3.02	2.49	2.73	2.64	2.49	4.00
*Eos*C112[b]	He	0.000	0.000	0.031	0.000	0.017	0.027	0.033	0.000
	Ho	0.000	0.000	0.031	0.000	0.017	0.027	0.033	0.000
	Ar	1.00	1.00	1.53	1.00	1.28	1.47	1.57	1.00
*Eos*C2 [b]	He	0.342	0.528	0.478	0.582	0.405	0.523	0.452	0.557
	Ho	0.350	0.600	0.469	0.533	0.400	0.479	0.467	0.588
	Ar	2.64	3.00	2.99	3.00	2.96	2.95	2.00	3.00
*Eos*C3 [b]	He	0.325	0.431	0.441	0.357	0.382	0.407	0.506	0.467
	Ho	0.317	0.440	0.438	0.367	0.400	0.425	0.600	0.588
	Ar	2.82	2.90	2.78	2.82	2.28	2.66	2.57	2.00
*Eos*C6 [b]	He	0.835	0.762	0.829	0.783	0.887	0.881	0.851	0.875
	Ho	0.817	0.720	0.875	0.667	0.917	0.890	0.833	0.882
	Ar	7.05	7.26	6.96	7.02	10.22	9.90	9.33	9.00
*Esc*132b[c]	He	0.937	0.928	0.957	0.940	0.956	0.957	0.959	0.949
	Ho	0.950	1.000	0.969	0.933	0.967	0.932	0.967	0.941
	Ar	16.02	16.70	18.79	16.59	18.35	19.15	19.81	18.00
*Esc*26b[c]	He	0.872	0.885	0.903	0.881	0.913	0.925	0.925	0.932
	Ho	0.883	0.840	0.938	0.933	**0.817**	0.890	0.833	0.882
	Ar	10.03	11.81	11.67	9.33	13.13	13.27	13.82	14.00
*Esc*57[c]	He	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.059
	Ho	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.059
	Ar	1.28	1.00	1.00	1.00	1.00	1.00	1.00	2.00
Avg	He	0.465	0.463	0.491	0.446	0.463	0.464	0.484	0.530
	Ho	0.456	0.471	0.493	0.426	0.457	0.451	0.485	0.523
	Ar	5.25	5.48	5.84	5.21	6.17	6.28	6.28	6.33

[a] Khudamrongsawat *et al*. (2007), [b] Switzer *et al*. (2008), [c] Gabel *et al*. (2008).

Table 3. Locus-specific primer sequences, repeat motif, GenBank number, annealing temperature, size and citation source used for genetic analyses for the fountain darter. M13 sequence for *Eche*001 and 002 is spelled out at the start of the forward sequence as opposed to being spelled as in the original citation.

Locus		Primer Sequence (5'-3')	Repeat Motif	GenBank No.	Annealing Temperature °C	Size	Citation
*Eche*001	F:	GTA AAA CGA CGG CCA GTT CGG TGA CAG ATC AGA TTA G	(TAC)21	EF117312	58	151-163	Khudamrongsawat *et al*. (2007)
	R:	TCA AAC AAA GCA GCA GC					
*Eche*002	F:	GTA AAA CGA CGG CCA GCC CTT CCT GAG ATG GTA TAA T	(CAT)14(CTT)5	EF117313	52	143-167	Khudamrongsawat *et al*. (2007)
	R:	CCA AAG CTG CAG ATA CTG ATG					
*Eos*C112	F:	CAT GCA GGT ATG CAC ACG TA	(CATC)11	EF570437	60	167-175	Switzer *et al*. (2008)
	R:	GGC AGT GGT GAG ACA GAA AC					
*Eos*C2	F:	GCT CTC ACA AAC ACA CAC AAA C	(CATC)11	EF570433	58	93-105	Switzer *et al*. (2008)
	R:	ATC GAC TCA ACC CCA GAT TAG					
*Eos*C3	F:	CAG CAT TTT CAG GTC ATA CCA T	(CATA)2(CATC)9(CATA)1(CATC)2	EF570434	58	183-212	Switzer *et al*. (2008)
	R:	GCTTTGGTTTCTCAGCTACTCC					
*Eos*C6	F:	AAA GCC TGA GGG ACA ATT ACA C	(CATC)13	EF570435	58	223-275	Switzer *et al*. (2008)
	R:	CCT TTG CTG GTA AAT CTC ACA C					
*Esc*132b	F:	GAA GCA CCT CAC CAA ACA GCG	(CTAT)33	EF421255	58	148-272	Gabel *et al*. (2008)
	R:	CCA CAC TGA CAC TGT GGC TGA C					
*Esc*57	F:	CCT GTG GAG GCT GAA GTG AG	(GATA)12	EF421251	58	101-105	Gabel *et al*. (2008)
	R:	GGT ACC TCG CTG AAG ACA CC					

Table 3-Continued. Locus-specific primer sequences, repeat motif, GenBank number, annealing temperature, size and citation source used for genetic analyses for the fountain darter.

Not Used in Analysis							
*Eche*005	F:	AGC AGA ATC ACG TTT TCC CAG	(CA)2(GACA)5	EF117314	60	131	Khudamrongsawat *et al*. (2007)
	R:	ACC GTC GGG ATG GATG					
*Eos*D11	F:	ACCAGATGCAGTGGATGAATAT	(TAGA)18	EF570443	58	142-212	Switzer *et al*. (2008)
	R:	GCGGTATCTAATGCTATTTCCC					
*Eos*D131	F:	AAA AAG GGG GAC AGT GTG TC	(TAGA)17	EF570447	58	82-95	Switzer *et al*. (2008)
	R:	GCA TCA GCA AAT AGG CAG AG					
*Esc*18	F:	CTG GCA GGC TTA TTG TGC TG	(GATA)11	EF421249	58	66-88	Gabel *et al*. (2008)
	R:	CAT TGT ACT CTC CCA TTG TTT GGG					
*Esc*26b	F:	CAA TGC GCC ACA TTG AGA AGG	(TAGA)27	EF421250	60	160-294	Gabel *et al*. (2008)
	R:	GCA CAA CAT ATG TCG TTA AGC TCC					

Table 4. Comparison of estimates of mean allelic richness (Ar), mean heterozygosity (Hs), and the proportion of the total genetic variance contained in a subpopulation (the s subscript) relative to the total genetic variance (the *T* subscript; Fst) for fountain darter aggregations from the Comal and San Marcos Rivers (each consisting of four aggregations). Fst-values can range from 0 to 1, where a high F_{ST} implies a considerable degree of differentiation among populations.

River	Ar	Hs	Fst
San Marcos	6.27	0.473	0.006
Comal	5.44	0.467	0.011
P-value	0.009	0.592	0.412

Table 5. Pairwise estimates of Fst (below diagonal) and the *P*-values from the *G*-test frequency homogeneity (above diagonal) for sample aggregations from the Comal an Marcos Rivers. A bold and underlined *P*-value indicates a statistically significant dif allele frequencies using an alpha-level of 0.002 (adjusted for multiple tests).

	Comal River				San Marcos River			
Aggregation	CR1	CR2	CR3	CR4	SMR1	SMR2	SMR3	SMR4
CR1	-	0.050	0.038	**<u>>0.002</u>**	**<u>>0.002</u>**	**<u>>0.002</u>**	**<u>>0.002</u>**	**<u>>0.002</u>**
CR2	0.0104	-	0.391	0.043	**<u>>0.002</u>**	**<u>>0.002</u>**	**<u>>0.002</u>**	**<u>>0.002</u>**
CR3	0.0032	0.0087	-	0.232	**<u>>0.002</u>**	**<u>>0.002</u>**	**<u>>0.002</u>**	**<u>>0.002</u>**
CR4	0.0230	0.0063	0.0129	-	**<u>>0.002</u>**	**<u>>0.002</u>**	**<u>>0.002</u>**	**<u>>0.002</u>**
SMR1	0.0144	0.0161	0.0101	0.0221	-	0.021	**<u>>0.002</u>**	0.021
SMR2	0.0265	0.0177	0.0169	0.0090	0.0031	-	0.216	0.050
SMR3	0.0342	0.0285	0.0138	0.0284	0.0092	0.0054	-	0.146
SMR4	0.0243	0.0216	0.0092	0.0261	0.0107	0.0081	0.0012	-

Table 6. Estimates of the level of population structure among all aggregations (Fst), among aggregations within the San Marcos and Comal Rivers (Fsc), and among the two rivers (Fct). An asterisks indicates the value is significantly larger than zero ($P < 0.05$).

Population	Statistic	Value
Among all aggregations	Fst	0.0195*
Among aggregations within rivers	Fsc	0.0084*
Among rivers	Fct	0.0112*

Table 7. Allele frequency by locus for pooled collections from the San Marcos and Comal Rivers. Private alleles are in bold and denoted by an astrerisk.

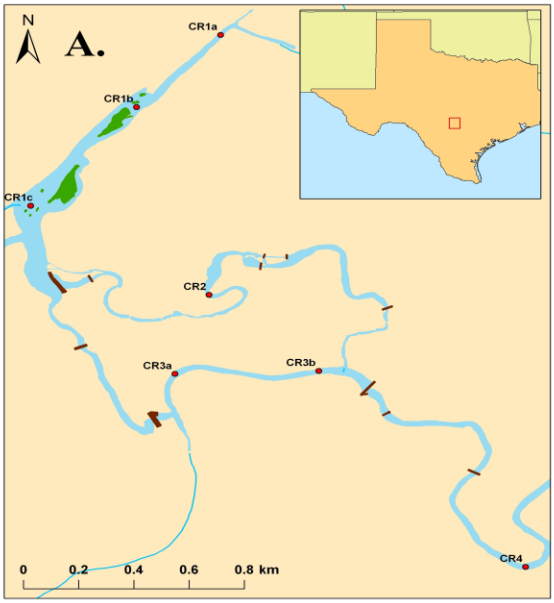
		River	
Locus	Allele	Comal	San Marcos
*Eche*001	151	0.017	0.036
	157	0.252	0.139
	160	0.656	0.756
	163	0.075	0.069
EosC2	93	0.224	0.269
	99	0.078	0.064
	105	0.697	0.667
*Eos*C3	183	0.037	0.017
	187	0.765	0.706
	189	0.197	0.278
*Eos*C6	223	0.010	0.064
	227	0.020	0.047
	231	0.054	0.039
	235	0.000	**0.006***
	239	0.150	0.169
	243	0.286	0.117
	245	0.000	**0.008***
	247	0.221	0.192
	251	0.109	0.169
	255	0.133	0.072
	259	0.014	0.072
	263	0.000	**0.022***
	275	0.003	0.022
*Esc*132b	158	0.000	**0.006***
	170	0.007	0.008
	174	0.051	0.028
	178	0.003	0.011
	182	0.054	0.033
	186	0.109	0.033
	190	0.010	0.019
	191	**0.007***	0.000
	194	0.065	0.028
	198	0.146	0.056

Table 7-continued. Allele frequency by locus for pooled collections from the San Marcos and Comal Rivers. Private alleles are in bold and denoted by an astrerisk.

		River	
Locus	Allele	Comal	San Marcos
	199	0.000	**0.006***
	202	0.031	0.069
	203	0.000	**0.014***
	206	0.058	0.025
	207	0.000	**0.014***
	210	0.017	0.039
	211	0.000	**0.008***
	214	0.010	0.064
	215	0.000	**0.003***
	218	0.024	0.058
	219	**0.017***	0.000
	222	0.020	0.039
	223	0.007	0.019
	226	0.014	0.106
	227	0.003	0.008
	230	0.024	0.033
	234	0.037	0.039
	235	0.003	0.003
	238	0.048	0.067
	239	**0.024***	0.000
	242	0.054	0.039
	246	0.017	0.028
	250	0.027	0.042
	254	0.024	0.014
	258	0.071	0.011
	264	0.014	0.017
	268	0.003	0.011
	272	0.000	**0.003***
*Esc*57	101	0.997	0.997
	104	0.003	0.003
*Eche*002	143	0.007	0.006
	158	0.003	0.003
	161	0.068	0.036
	164	0.871	0.903
	167	0.051	0.053

Table 7-continued. Allele frequency by locus for pooled collections from the San Marcos and Comal Rivers. Private alleles are in bold and denoted by an astrerisk.

		River	
Locus	Allele	Comal	San Marcos
*Eos*C112	167	0.000	**0.003***
	171	0.997	0.989
	175	0.003	0.008
*Esc*26b	206	**0.003***	0.000
	210	0.003	0.006
	214	0.000	**0.008***
	218	0.000	**0.003***
	222	0.000	**0.022***
	226	0.024	0.075
	230	0.017	0.019
	234	0.051	0.031
	238	0.119	0.069
	242	0.133	0.125
	246	0.116	0.100
	250	0.133	0.064
	254	0.068	0.081
	258	0.010	0.058
	262	0.173	0.111
	266	0.037	0.083
	270	0.088	0.058
	274	0.017	0.028
	278	0.003	0.019
	282	0.003	0.025
	286	0.000	**0.008***
	290	0.000	**0.003***
	294	0.000	**0.003***



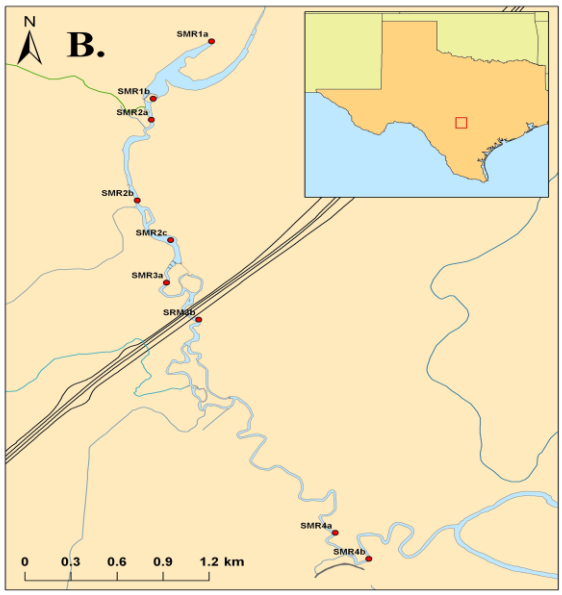


Figure 1. Genetic collection sites on the (A) Comal (CR) and (B) San Marcos (SMR) Rivers, Guadalupe River Basin, in Texas. Collection sites (red circles) are named and grouped by their respective relationship to in-stream barriers where a, b, and c indicate sites located above or between structures presumed to effect fish passage. These structures are represented by straight black lines perpendicular to river flow.

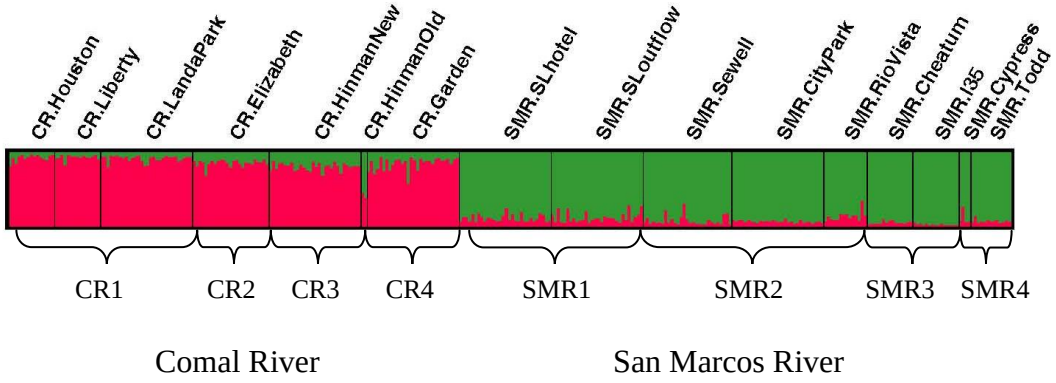


Figure 2. Results of the cluster analysis from STRUCTURE showing the most likely cluster scenario (K = 2). Each individual is represented as a vertical line and the color indicates the proportion of the individual genotype from the putative Comal cluster (red) and San Marcos cluster (green). The black vertical lines separate the sample locations (top labels) and the horizontal brackets denote the aggregations in Table 1.

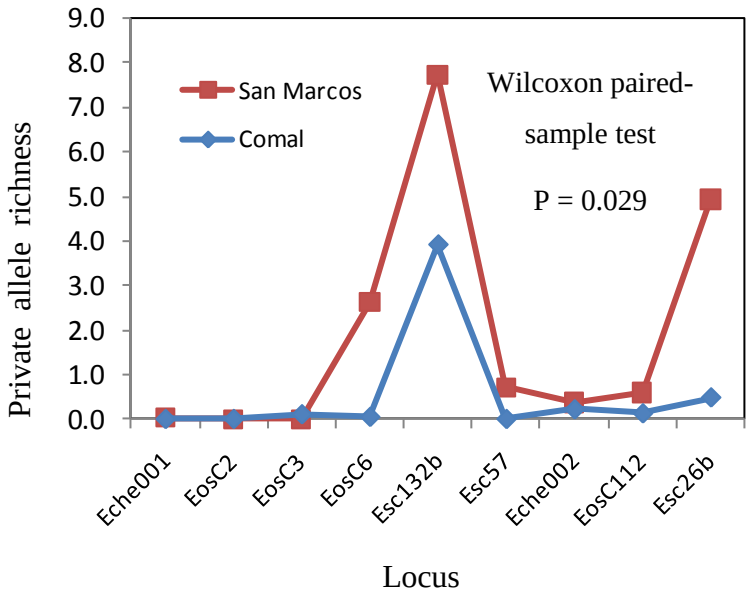


Figure 3. Estimates of private allele richness for nine microsatellite loci in fountain darters from the Comal and San Marcos Rivers.

Appendix 1:
A simple theoretical evaluation of genetic diversity in the fountain darter refugium program at the San Marcos National Fish Hatchery and Technology Center

INTRODUCTION

Presently, the San Marcos National Fish Hatchery and Technology Center (SMTC) rears approximately 750 fountain darters in captivity. In this refugium program the fish are collected (and replaced) annually from the naturally spawning population in the San Marcos and Comal Rivers and are intended to be used for restoration/recovery in the event of a localized extinction. For example, if all fountain darter habitat is temporarily lost in the Comal River due to drought, then the fish from the SMTC could be used to re-populate the river when habitat becomes available.

In the simple theoretical evaluation below we examine the potential for loss of genetic diversity in the current refugium program at the SMTC. The intent of this evaluation is to help guide managers in assessing the current refugium program and population but not to recommend a single number for the refugium population. Many factors can influence the changes in genetic diversity that result from a restoration program and the size of the refugium (or hatchery) population is just one of those factors. Here, we evaluate the expected loss of genetic diversity after re-introduction under different refugium population sizes and assuming different population growth rates in the recovering population.

METHODS

For this simple theoretical evaluation we used the following formula to determine the expected loss of heterozygosity (1-Ht/Ho) in the re-introduced population relative to a source population (e.g., San Marcos) after 50 generations:

$$Ht/Ho = \Pi^{t}_{i=} 1[1-1/(2Nei)]$$

where,

Ht/Ho = the fraction of heterozygosity at time t (in generations) relative to the initial heterozygosity (in this case Ht is the heterozygosity in the re-populated population at time t and Ho is the heterozygosity in the San Marcos population prior to collection of the refugium fish).

t = time in generations (maximum of 50 in this analysis)

Nei = the effective population size at time i.

We examined four scenarios reflecting four refugium population sizes (N = 300, 500, 750, 1000). Nested within each scenario, we examined three different population growth rates (25% each generation, 50% each generation, 100% each generation).

We made the following four assumptions in addition to the assumptions associated with the formula above:

1 The census size of the population (San Marcos) is 200,000.
2 The carrying capacity of the target habitat is 200,000. In other words, following re-population, the target habitat will allow the population to increase (at one the rates above) until reaching a census size of 200,000.
3 The ratio of effective population size to census size (Ne/N) is 0.1 derived from Frankham (1995).
4 The refugium population is not spawned in captivity. In other words, the refugium population is maintained by sampling each generation from the population (e.g., San Marcos).

RESULTS/DISCUSSION

The results of the evaluation are shown in figure A1 for each scenario. A few trends are worth noting. First, most of the heterozygosity is lost early (in the first 5-15 generations) depending upon the refugium population size and the population growth rate. This result reflects the fact

that we assume the populations continue to grow at a steady rate. If growth is slower or declines, the loss of heterozygosity will be greater. Second, the loss of heterozygosity is lowest when the growth rate is largest. Less than 4% of heterozygosity is lost in the smallest refugium population (N = 300) if the population doubles each generation until reaching carrying capacity (N = 200,000). This result suggests that small refugium populations may be adequate provided the re-introduced population grow rapidly. On the other hand, over 8% of the heterozygosity is lost in the smallest population if the population growth rate is 25% per generation. Third, the variation in loss of heterozygosity is lowest (1.0%-2.5% for growth rates of 1.25-2.0) for the largest refugium population (N = 1,000). This result indicates that the larger refugium populations in this analysis are influenced less by growth rate of the re-introduced population than are the smaller refugium populations.

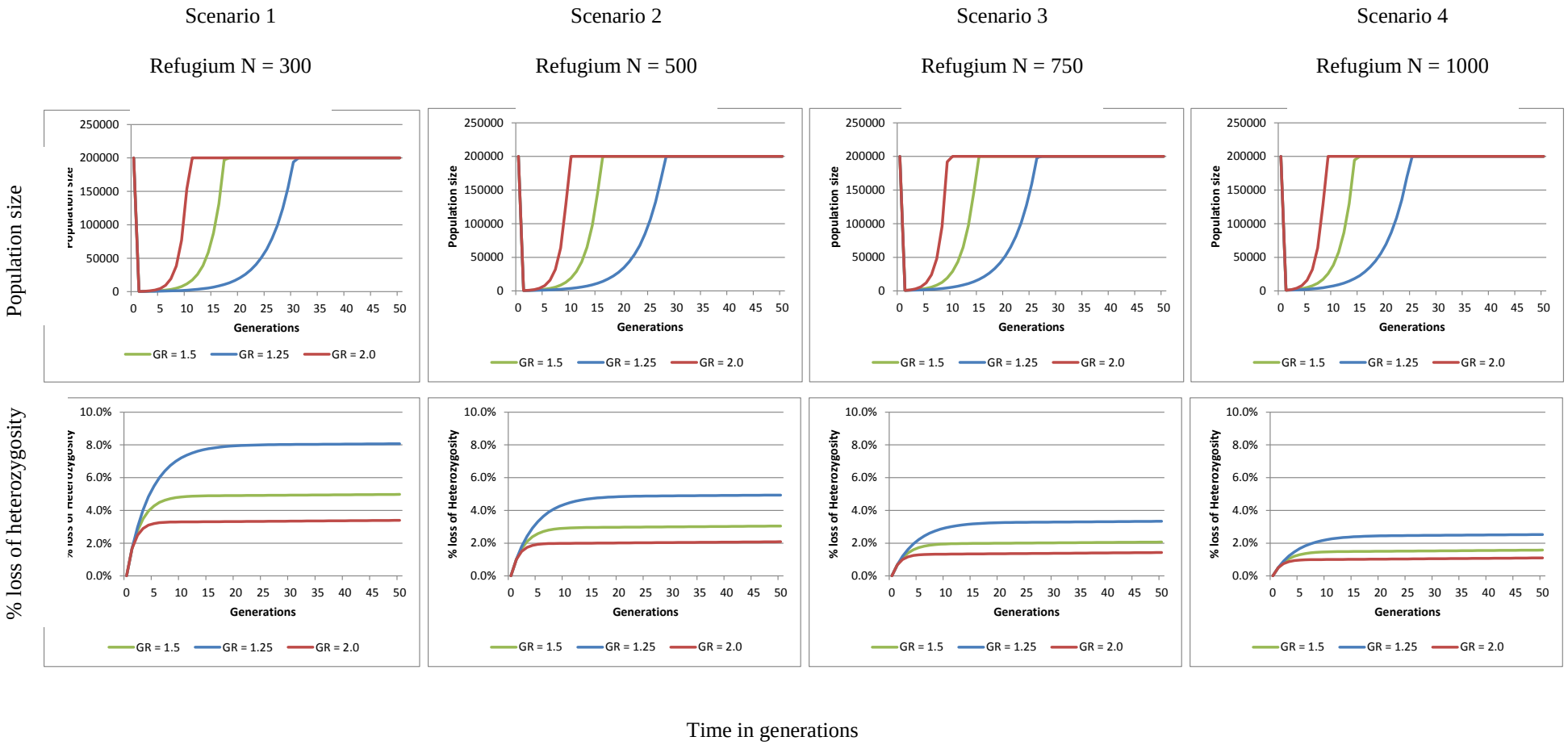


Figure A1. Estimates of the percent loss of heterozygosity for four refugium populations at three population growth rates after re-introduction.

Appendix 2:
Genotype data for the sixteen sample locations in FSTAT format

Populations 1-16
CRHouston
CRLiberty
CRLanda
CRElizabeth
CRHinman
CRAboveHinman
CRGarden
SMRSpringLHotel
SMRSpringLdam
SMRSewell
SMRCityPark
SMRRioVista
SMRCheatum
SMRI35
SMRCypress
SMRTodd

FSTAT Input File
16 9 294 3
*Eche*001-m13
*Eos*C2
*Eos*C3
*Eos*C6
*Esc*132b
*Esc*57
*Eche*002-m13
*Eos*C112
*Esc*26b
1 160160 105105 187187 239239 238238 101101 164164 171171 262266
1 157160 105105 187189 247251 239250 101101 164164 171171 242242
1 160160 105105 187187 231239 186191 101101 164164 171171 226274
1 160160 093105 187187 243243 191258 101101 164164 171171 246250
1 160160 105105 187187 247255 186206 101101 164164 171171 262270
1 157160 105105 187187 247259 182194 101101 164164 171171 242262
1 160160 105105 187187 239255 198202 101101 164164 171171 242262
1 157160 105105 187187 243255 186258 101101 164164 171171 246262
1 160160 105105 187187 247247 202258 101101 164164 171171 250250
1 157163 099105 187187 247255 194206 101101 164164 171171 238250
1 160160 093105 183187 243255 234264 101101 164164 171171 242246
1 157160 093105 187189 255255 198214 101101 164164 171171 262270
1 157160 105105 187189 251255 230238 101101 164164 171171 242262
1 157160 093105 187187 239255 186258 101101 164164 171171 238266
1 160160 093105 183187 239243 234239 101101 161161 171171 234238
2 157160 093105 187189 247255 194198 101101 158161 171171 242250
2 160160 105105 183187 227243 198238 101101 167167 171171 234242
2 157157 105105 187187 243247 198230 101101 161161 171171 238238
2 160160 093093 187189 227255 190242 101101 164164 171171 238262
2 157160 105105 187187 243247 186198 101101 164164 171171 238270
2 160160 105105 187187 231247 242254 101101 164164 171171 238262
2 157163 105105 187189 227243 234258 101101 164164 171171 242250
2 160160 093105 187187 243255 174186 101101 164164 171171 230262

2 157157 093105 189189 247251 194254 101101 164164 171171 254262
2 157160 105105 187187 239255 174182 101101 164164 171171 246262
2 157160 105105 187189 231247 202239 101101 164164 171171 246246
2 157160 105105 187187 247247 218234 101101 161164 171171 238242
2 160163 105105 187187 251251 198206 101101 164164 171171 238262
2 157163 093105 187187 239243 198254 101101 161164 171171 246262
2 157163 105105 187187 231239 198218 101101 164164 171171 242274
3 160160 093105 187189 239243 198198 101101 164164 171171 262262
3 157160 105105 187187 239247 174182 101101 164167 171171 238250
3 157160 105105 187187 243247 202238 101101 164164 171171 210270
3 157157 105105 187189 227243 198258 101101 164164 171171 238242
3 157160 105105 187189 243247 242264 101101 161164 171171 238246
3 160160 093105 187187 243243 174258 101101 164164 171171 242250
3 160160 105105 187187 231255 186222 101101 164164 171171 234270
3 160160 105105 187187 239243 186198 101101 164164 171171 226246
3 160160 105105 187187 239239 182258 101101 161164 171171 242262
3 160163 093105 187187 239247 186226 101101 164164 171171 238250
3 151157 105105 187187 243255 174246 101101 164164 171171 238270
3 157160 105105 187189 243247 222242 101101 164164 171171 238262
3 157160 105105 187187 243247 258258 101101 164167 171171 246270
3 157160 105105 187189 239239 182234 101101 164164 171171 242266
3 160160 093105 187187 251255 182230 101101 161164 171171 206238
3 160160 105105 183187 247247 222226 101101 164167 171171 242262
3 157160 105105 183187 243251 194222 101101 164164 171171 246262
3 160160 093093 187187 247255 174186 101101 164167 171171 238238
3 157163 105105 187187 247251 186226 101101 164164 171171 238242
3 157160 093105 187187 227251 238258 101101 161164 171171 238274
3 160163 093105 189189 243251 198242 101101 164164 171171 246250
3 160160 105105 187187 243243 198234 101101 161164 171171 262262
3 160160 099105 187187 231243 194202 101101 164164 171171 246262
3 157157 105105 187187 231239 198234 101101 164164 171171 262266
3 157163 099105 187187 231243 194234 101101 164167 171171 242270
3 160160 105105 187189 231239 194206 101104 161164 171171 238242
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3 160163 105105 187187 247251 186239 101101 164164 171171 234262
3 160160 093105 187187 231255 198242 101101 164164 171171 254270
4 160160 105105 187187 243247 186198 101101 164164 171171 262274
4 160160 093105 187187 239243 218230 101101 164164 171171 242246
4 160160 105105 187187 243255 230239 101101 164164 171171 242262
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4 160160 093105 187187 239275 218223 101101 164164 171171 262262
4 157160 099105 189189 239243 198206 101101 164164 171171 258262
4 157160 105105 187189 243255 198254 101101 164167 171171 242246
4 160160 105105 187187 243243 210222 101101 164164 171171 246262
4 157160 093105 183189 231243 186194 101101 161164 171171 230242
4 157160 093105 187189 243243 182186 101101 164164 171171 234238
4 160160 099105 187187 243251 206258 101101 164164 171171 242250
4 157160 093105 187187 251255 198250 101101 164164 171171 230238
4 160160 105105 187187 243255 235254 101101 164164 171171 242254
4 157157 093105 187189 243243 182194 101101 164164 171171 262266
4 160160 105105 187187 243243 186198 101101 164164 171171 254254
4 157160 093099 187189 247251 198206 101101 161167 171171 226242
4 160160 093105 187189 243251 210250 101101 164164 171171 242250
4 160160 093093 187189 243243 186234 101101 164164 171171 246254
4 160160 093105 187187 243247 186242 101101 164167 171171 226262
4 157160 093105 187187 223239 194242 101101 164164 171171 262262

4 157160 099105 187189 231255 186198 101101 164164 171171 238246
4 160160 105105 187187 247251 182198 101101 164164 171171 234254
4 160163 105105 187187 247255 186258 101101 164164 171171 258270
4 160160 105105 183187 243243 186194 101101 164167 171171 262262
4 160160 099105 187189 239251 194238 101101 161164 171171 250274
5 157157 105105 187187 223247 186238 101101 164164 171171 254266
5 157163 099105 187187 231243 186198 101101 161164 171171 262270
5 160160 105105 187189 239243 182198 101101 143164 171171 246266
5 160160 093105 187189 247255 194198 101101 164164 171171 234246
5 160163 093105 187187 239243 198206 101101 164167 171171 246262
5 160160 099105 187189 247255 234238 101101 164164 171171 238262
5 157160 105105 187189 239247 186202 101101 164164 171171 234234
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5 160160 105105 183187 239247 206250 101101 164164 171171 262282
5 160160 093105 189189 239243 210234 101101 164164 171171 234262
5 163163 093105 187187 243247 219250 101101 164164 171171 250270
5 151157 093105 187189 247247 198218 101101 164164 171171 238250
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5 157160 105105 189189 247247 174178 101101 164164 171171 242246
5 157163 105105 187187 231243 182258 101101 164164 171171 238254
5 160160 099105 187189 243251 182219 101101 161164 171171 254266
5 157160 093105 187187 251255 194194 101101 164164 171171 242250
5 160160 093105 187189 247255 186218 101101 164164 171171 254278
5 160163 093093 187187 243251 190258 101101 164164 171171 250262
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5 157160 105105 187189 255255 190230 101101 164164 171171 250254
5 157160 105105 187187 243247 194218 101101 161164 171171 226270
5 157160 105105 187187 239251 198246 101101 164164 171171 254270
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6 160163 099105 187189 243247 170182 101101 164164 171171 262270
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16 157157 093105 187189 243247 214238 101101 164164 171171 242266
16 160160 093105 189189 243255 218242 101101 164164 171171 266274
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16 157160 099099 187187 239259 230230 101101 164167 171171 270270
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16 157160 105105 187189 223251 198210 101101 164164 171171 226266
16 151163 099105 187187 247255 186226 101101 164164 171171 242282
16 157160 105105 187187 239259 202206 101101 164164 171171 226274

Appendix 3:
Genotype data for the eight population aggregations sample in FSTAT format

Populations 1-8
CR1
CR2
CR3
CR4
SMR1
SMR2
SMR3
SMR4

FSTAT Input File
8 9 294 3
*Eche*001-m13
*Eos*C2
*Eos*C3
*Eos*C6
*Esc*132b
*Esc*57
*Eche*002-m13
*Eos*C112
*Esc*26b
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1 157160 105105 187189 247251 239250 101101 164164 171171 242242
1 160160 105105 187187 231239 186191 101101 164164 171171 226274
1 160160 093105 187187 243243 191258 101101 164164 171171 246250
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1 157160 105105 187187 247259 182194 101101 164164 171171 242262
1 160160 105105 187187 239255 198202 101101 164164 171171 242262
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1 157160 093105 187189 255255 198214 101101 164164 171171 262270
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