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Phylogeography and Population Structure of the Imperiled Redtail Splitfin (Goodeidae: *Xenotoca eiseni*): Implications for Conservation

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The river drainages of Central Mexico have a high degree of freshwater diversity, and are subsequently a focal point for many freshwater fish conservation studies. The livebearing subfamily Goodeinae (Teleostomi: Goodeidae) is a diverse endemic group, under threat from many anthropogenic factors. *Xenotoca eiseni*, the Redtail Splitfin, a member of this subfamily, has a fragmented distribution in the western basins of the Pacific Coast including the Ríos Grande de Santiago, Compostela, Ayuquila, Coahuayana, and the endorheic Lago de Magdalena and Etzatlán-San Marcos basins. Previous studies have noted high levels of genetic differentiation between the endorheic Lago de Magdalena and Etzatlán-San Marcos basins and surrounding areas which may be indicative of more taxonomic diversity within *X. eiseni* than currently recognized. The objectives of this study were to use mitochondrial (cytochrome *b*) and nuclear (ITS-1) DNA sequences and microsatellite data to assess phylogeography, genetic differentiation, and population structure between and within populations of this species. Analysis of the sequence data resulted in two deeply divergent clades, with a mean nucleotide difference of 2.51% within cytochrome *b* and 0.88% within ITS-1 between populations in the endorheic Lago de Magdalena and Etzatlán-San Marcos basins and all other locations. Microsatellite data also found significant structuring within these two clades of *X. eiseni* and identified multiple operational conservation units (OCUs). Each of these units contains a proportion of the total variation within the species and requires conservation attention and protection.

EFFECTIVE management and conservation of species requires a thorough understanding of genetic structure and diversity (Frankham et al., 2010). This is especially true with the ongoing anthropogenic manipulation of habitats and environmental degradation that have negatively impacted the distribution and survival of species worldwide. Genetic approaches to the study of conservation enable resolution of taxonomy and the measurement of diversity within and between populations of threatened or endangered species. One of the first steps in conserving and protecting distinct populations or species is to identify and define management units (Moritz, 1994). This information can be used to manage populations and design captive breeding and re-introduction programs (Vrijenhoek, 1998).

An operational conservation unit (OCU) is ''a continuous area limited by geographical boundaries and inhabited by one or more populations sharing the same genetic pattern'' (Doadrio et al., 1996). Each OCU requires conservation protection as it contains a proportion of the total variation within a species. Re-introduction of individuals within these OCUs can be a vital strategy in the recovery and maintenance of threatened and endangered freshwater fish species (Domínguez-Domínguez et al., 2007). Identification of OCUs requires an understanding of the genetic structure of a species, as well as distributional, historical, ecological, and social information (Crandall et al., 2000).

Throughout the western Hemisphere, many biologically diverse areas contain numerous imperiled species. The country of Mexico possesses extremely high freshwater fish diversity, with approximately 520 freshwater fish species, of which 163 are endemic (Miller et al., 2005). Neotropical and Nearctic faunas interact in the center of the country at the Mesa Central, referred to as Central Mexico for biogeographic purposes (Domínguez-Domínguez and Pérez-Ponce de Léon, 2009), which is spanned by the Trans-Mexican Volcanic belt. The topology of this region was dramatically changed during the Pliocene and Pleistocene and has experienced sporadic activity since (Barbour, 1973) leading to substantial changes in watersheds and fragmentation of basins.

Xenotoca eiseni (Rutter, 1896), the Redtail Splitfin or mexcalpique cola roja, is a member of the subfamily Goodeinae (Teleostomi: Goodeidae) and endemic to this region. There is current debate over its taxonomic status, as the genus *Xenotoca* (Fitzsimons, 1972) has been found to be paraphyletic (Webb et al., 2004). However, for the purposes of this study, *Xenotoca* is being used in the broad sense to avoid further taxonomic confusion, though a taxonomic revision of this genus is required (Grudzien et al., 1992; Doadrio and Domínguez, 2004; Webb et al., 2004; Domínguez-Domínguez et al., 2010). *Xenotoca eiseni* occupies a variety of habitats but generally is found in areas of water with little vegetation. Historically *X. eiseni* inhabits the Río Grande de Santiago and its tributaries near Tepic in the state of Nayarit. South of Tepic, it occurs in Pacific tributaries north of Río Balsas (Miller et al., 2005). It is also found internally in the endorheic Lago de Magdalena and Etzatlán-San Marcos endorheic area, in the state of Jalisco. The interior basin and Pacific basin tributaries are hypothesized to have been isolated during the period of heavy tectonic activity during the Pliocene and Pleistocene (Barbour, 1973), and the fragmentation of these drainages would result in disruption of gene flow between populations and subsequent genetic differentiation within this species. This hypothesis has been supported by phylogenetic analyses (Doadrio and Domínguez, 2004), based on a limited number of individuals, which found high genetic divergence in cytochrome *b* (mtDNA) between populations from the Pacific tributaries and Etzatlán-San Marcos and Magdalena endorheic basins. Doadrio and Domínguez (2004) stated that a comprehensive study incorporating morphological and nuclear DNA is needed to resolve the taxonomic status of the populations in their study.

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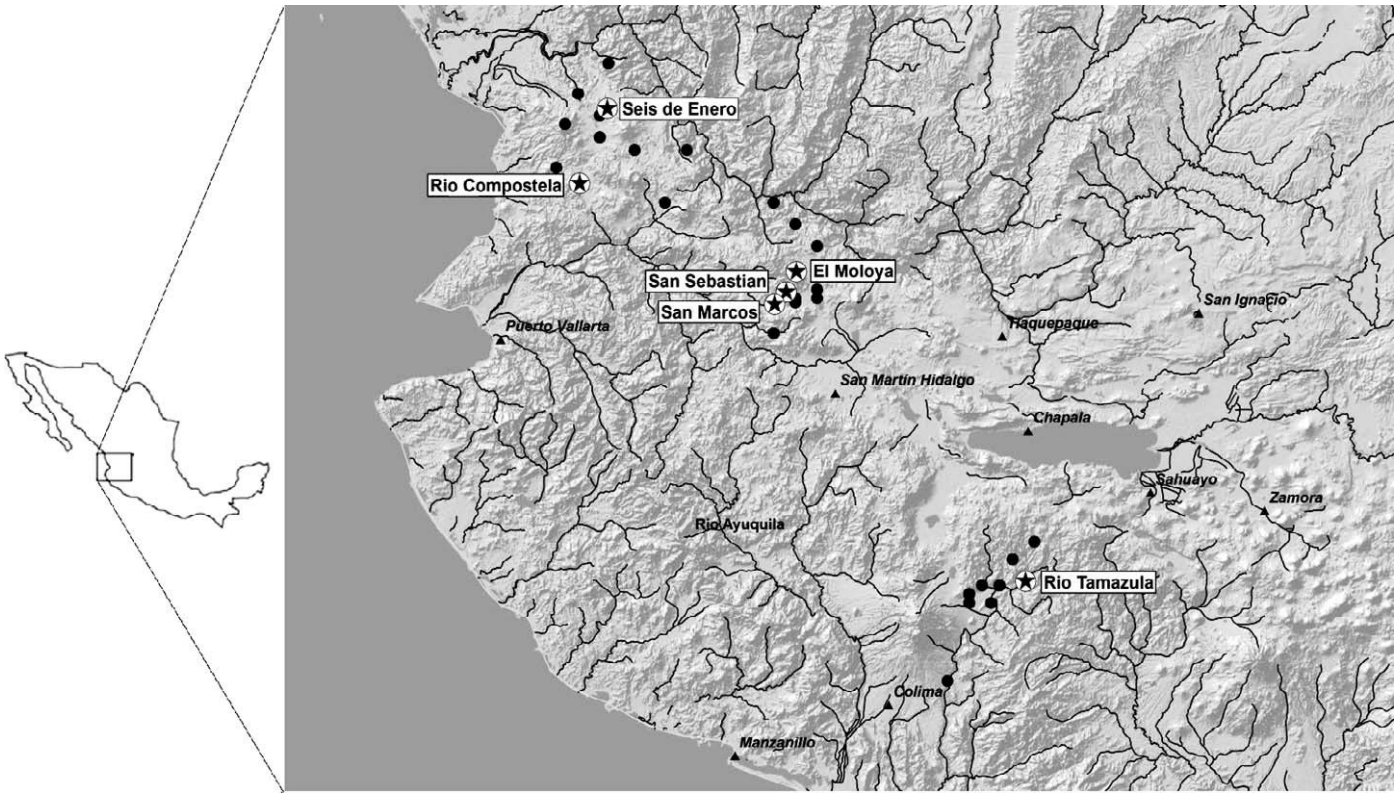

Fig. 1. Map showing the historical distribution of *Xenotoca eiseni* within the Mesa Central of Mexico based on fishnet records (Accessed through the Fishnet2 Portal, www.fishnet2.org, April 2014) and collection localities for specimens used in this study. Historical localities are indicated by black circles, whereas collection sites are indicated by stars.

Increased water use, poor land-use practices, and introduction of exotic species continuously threaten the ichthyofauna of Central Mexico (Contreras-Balderas and Lozano-Villano, 1994; Lyons et al., 1998; Miller et al., 2005), and much of the area occupied by *Xenotoca eiseni* has become degraded in the last two decades. Currently, *X. eiseni* is not listed under any formal endangered species classifications; however, recent sampling has shown that *X. eiseni* is decreasing in number at localities where it was previously abundant (Domínguez-Domínguez et al., 2006; Kenway-Lynch et al., 2010; Piller, unpubl.), and some populations are in danger of extirpation (Domínguez-Domínguez et al., 2005a, 2005b; Domínguez-Domínguez and Pérez-Ponce de Léon, 2009). Restoration, reintroduction, and stocking programs have been initiated for other species in Mexico (Martínez Palacios et al., 2006), and obtaining information on genetic variation is critical to protecting and conserving the long term viability of *X. eiseni* and guiding restoration efforts that may need to be implemented in the future. The general objectives of this study were to assess the genetic structure of *X. eiseni* using both mitochondrial (cytochrome *b*) and nuclear (ITS-1 and microsatellite loci) DNA. Specifically the goals were to 1) identify genetically distinct populations and/or lineages of *X. eiseni* and 2) examine inter- and intrapopulation genetic diversity from multiple populations across the range of *X. eiseni*. The results from this study have important conservation implications, as the baseline genetic information derived from this study may be useful if conservation actions or genetic restoration programs need to be implemented in the future.

MATERIALS AND METHODS

Study areas and specimen collection.—Specimens of *Xenotoca eiseni* were obtained from all basins from which it is known except for the Río Ayuquila (Río Armería drainage), in which the species has historically been rare (Fig. 1). Samples (n = 219) were obtained from six different localities (Table 1) including Río Compostela, Seis de Enero, and Río Tamazula (Río Compostela, Río Grande de Santiago, and Río Coahuayana basins, respectively), and El Moloya, San Sebastian, and San Marcos (endorheic Lago de Magdalena and Etzatlán-San Marcos areas). Specimens were obtained with a standard seine, dip net, and/or electrofisher. Fin clips were taken from each specimen and preserved in 95–100% ethanol, and genomic DNA was extracted using the DNeasy Kit (Qiagen, Inc.) following the manufacturer's instructions. The majority of the specimens were released near the point of capture. However, some voucher specimens were preserved in the Southeastern Louisiana University Vertebrate Museum or the Colección de Peces de la Universidad Michoacana.

Sequence data.—Cytochrome *b* (GenBank KP00058966–KP059034) and the nuclear ITS-1 (GenBank KM973039–KM973056) region were amplified from 28 and 19 specimens, respectively, with representatives from from all six localities in both datasets (Table 1). PCR primers identified in Doadrio and Domínguez (2004) for cyt*b* (GluF and ThrR) and Hillis and Dixon (1991) for ITS-1 (VIIIF and IIIR) were used. The cyt*b* amplification conditions were as follows: initial denaturation at 94°C for 2 min, 30 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 1 min 30 s, and a final

Table 1. Locality information for populations of *Xenotoca eiseni*. SLU-TC# refers to catalog numbers for fin-clipped specimens stored in the Southeastern Louisiana University Vertebrate Museum.

			Individuals					GenBank ID	
Population	Site	Basin	Microsatellites	Cyt *b*	ITS	Total	SLU-TC #	Cyt *b*	ITS
Compostela	Río Compostela	Río Compostela	42	5	3	50	0651, 0705, 0710, 0715, 0720, 0725, 0730, 0735, 0740, 2493–2542	KP058996, KP058997, KP059004, KP059005, KP059007	KM973043, KM973044, KM973045
Seis de Enero	Outlet channel in Seis de Enero	Río Grande de Santiago	50	6	4	60	0653–0655, 0701–0703, 0706–0708, 0711–0713, 0716–0718, 0721–0723, 0726–0729, 0731–0734, 0736–0739, 0741–0743, 0747–0749, 2563–2592	KP058998, KP058999, KP059008, KP059022, KP059023, KP059024	KM973046, KM973049, KM973050
Tamazula	Río Tamazula	Río Coahuayana	30	7	2	39	2435–2470, 2543–2562	KP059006, KP059020, KP059021, KP059025, KP059028, KP059029, KP059030	KM973040, KM973039
El Moloya	Manantial El Moloya	Lago de Magdalena	7	4	4	15	0652, 0704, 0709, 0714, 0719, 0724, 0745, 0750	KP059002, KP059003, KP059018, KP059019	KM973041, KM973054, KM973055, KM973056
San Sebastian	El Tanque at Hacienda San Sebastian Rancho Dos Hermanos	Lago de Magdalena	32	4	4	40	0526–0555, 0656, 0657, 0746	KP059000, KP059015, KP159016, KP059017	KM973042, KM973051, KM973053
San Marcos	Road San Marcos-Etzatlan in Granja Sahuaripa	Lago de Magdalena	11	2	2	15	0324, 0409, 2481–2492	KP059026, KP059027	KM973048, KM973052
Total	—	—	172	28	19	219	—	—	—

extension at 72°C for 5 min. The ITS-1 amplification procedure was modified from Hillis and Dixon (1991) as follows: an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 59°C for 15 s and extension at 72°C for 30 s, and a final extension at 72°C for 1 min. Amplified products were purified using ExoSAP-IT (Affymetrix, Inc.) and sequenced by the Pennington Biomedical Research Center, Louisiana State University. Sequences were aligned using Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI) and submitted to GenBank (Table 1).

Phylogeographic analysis.—Maximum parsimony (MP) analysis was performed using a heuristic search in PAUP* v4.0b10 (Swofford, 2002) and a bootstrap analysis with 1,000 replicates. Uncorrrected pairwise sequence divergence was calculated using MEGA 4.0 (Tamura et al., 2007). MODELTEST v3.7 (Posada and Crandall, 1998) was used to determine the most appropriate model of DNA substitution for both datasets using the Akaike information criterion (AIC) for each codon position. Bayesian Inference (BI) analysis was carried out with MrBayes v3.1.1 (Huelsenbeck and Ronquist, 2001). 10,000,000 Markov Chain Monte Carlo (MCMC) repetitions were run with every 100 trees sampled. The ln likelihood scores of the sampled trees were plotted to determine the burn-in period, and the first 5,000 trees were discarded. A consensus tree was created using 50% majority rule. Other goodeids were included in the cyt*b* analysis to assess the monophyly of the species, including *Xenotoca melanosoma*, *Skiffia bilineata*, *Ameca splendens*, *Allodontichthys tamazulae*, *Goodea atripinnis*, and *Characodon audax*. Three outgroup species, *Fundulus heteroclitus*, *F. notatus*, and *Gambusia affinis* were used to root the tree.

Microsatellite characteristics and genetic diversity.—Eight microsatellite loci were selected via a cross-priming strategy (Estoup and Angers, 1998) and genotyped using primers designed for *Zoogoneticus tequila* (*Zt1.2*, *Zt1.43*, *Zt1.9*; Boto and Doadrio, 2003), *Ameca splendens* (*As2*, *As5*), *Ilyodon whitei* (*Iw196*), and *Xenoophorus captivus* (*Xc18*, *Xc25*; Hamill et al., 2007). Primers were fluorescently labeled using the DS-30 dye set (6-FAM, HEX, NED; Applied Biosystems). The loci were analyzed in two multiplexes using Qiagen PCR Multiplex Kit (Qiagen, Inc.). Multiplex I (*Zt1.43*$^{6\text{-FAM}}$, *As5*$^{\text{HEX}}$, *Iw196*$^{6\text{-FAM}}$, *As2*$^{\text{NED}}$, and *Zt1.9*$^{6\text{-FAM}}$) was amplified as follows: an initial denaturation at 95°C for 15 min, 30 cycles at 94°C for 30 s, 59°C for 1 min 30 s, and 72°C for 1 min, and a final extension at 60°C for 30 min. Multiplex II (*Xc25*$^{6\text{-FAM}}$, *Xc18*$^{\text{HEX}}$, and *Zt1.2*$^{\text{NED}}$) was amplified with the same conditions as multiplex I, but with an increased annealing temperature of 60°C. The products were analyzed on a 3730xl 96-Capillary Genetic Analyzer with a GeneScan 500 ROX size standard (Applied Biosystems) by the DNA Analysis Facility on Science Hill (Yale University, New Haven, CT). Alleles were determined using Peak Scanner v1.0 (Applied Biosystems).

The original microsatellite dataset consisted of 172 individuals (Table 1, Appendix 1) genotyped for eight microsatellite loci. The program MICRO-CHECKER v2.2.3 (van Oosterhout et al., 2004) was used to detect for typographic and genotyping errors caused by null alleles and/or large allele dropout. The program GENEPOP v.4.2.2 (Rousset, 2008) was used to test for deviations from Hardy-Weinberg equilibrium (HWE) with exact tests using a MCMC algorithm with 10,000 dememorisations, 100 batches, and 10,000 iterations per batch. Tests for linkage disequilibrium (LD) across all loci were also implemented in GENEPOP using 10,000 dememorisations, 500 batches, and 5,000 iterations per batch. A sequential Bonferroni correction (Rice, 1989) was applied to both HWE and LD tests to correct for multiple testing. Inbreeding coefficients (F_{IS}) were determined using FSTAT v.2.9.3.2 (Goudet, 2002). GENALEX v.6.5.0.1 (Peakall and Smouse, 2006, 2012) was used to calculate number of alleles (A_T), mean number of alleles (A_M), unbiased expected heterozygosity (H_E), and observed heterozygosity (H_O) per locus and per population. Allelic richness was assessed using HP-Rare (Kalinowski, 2005) to estimate the number of alleles, assuming equal sample sizes, per population across loci and per locus across populations.

Population structure.—Pairwise population differentiation (pairwise F_{ST}) and associated significance was assessed with FSTAT v.2.9.3.2 (Goudet, 2002). Pairwise F_{ST} estimates were adjusted for null alleles (ENA method) using the program FREENA (Chapuis and Estoup, 2007) to account for any overestimation bias caused by minor null allele frequencies. Confidence intervals (95%) for the unadjusted and adjusted pairwise F_{ST} estimates were also calculated with FREENA.

Population genetic structure assuming *a priori* two separate clades (Pacific and endorheic), and populations was assessed separately by hierarchical analysis of molecular variance (AMOVA; Excoffier et al., 1992) with 10,000 reps implemented in Arlequin v.3.5.1.3 (Excoffier and Lischer, 2010). The program STRUCTURE v.2.3.4 (Pritchard et al., 2000) was used to assess population genetic structure without *a priori* groupings. The analysis was conducted with 15 replications for each *K* ranging from 1 to 8 (200,000 burnin; 500,000 iterations after burnin) under an admixture ancestry model and with correlated allele frequencies among populations (Falush et al., 2003). Appropriate values of *K* were estimated using the conservative *ad hoc* summary statistic ΔK (Evanno et al., 2005), calculated using the web-based program STRUCTURE HARVESTER v0.6.93 (Earl and von Holdt, 2012). Cluster outputs of each run of the appropriate *K* were permuted with the program CLUMPP v.1.1.2 (Jakobsson and Rosenberg, 2007) using the full search method which produces a single cluster output that aligns replicates as close as possible. The resulting single output was graphically displayed using the program DISTRUCT v1.1 (Rosenberg, 2004).

RESULTS

Phylogenetic analysis.—Cytochrome *b* sequences used for the analysis were slightly truncated, at 1,119 bp in length. There were 52 polymorphic sites, 39 of which were parsimony informative. Both MP and BI analyses recovered congruent tree topologies among the ingroup populations; however, it should be noted that sample sizes for some populations are small. The following discussion will be based on the results from the BI analysis (Fig. 2). *Xenotoca eiseni* was recovered as monophyletic (98% Bayesian support) and sister to *X. melanosoma*. Two geographically defined clades were recovered within *X. eiseni*, with a mean interclade sequence divergence of 2.51%. Clade I comprised two groups (88% Bayesian support) including individuals located in the more northerly Pacific drainages: Río Compostela and Rio Santiago (Seis de Enero population) and the other from Río Tamazula. Clade II is in the endorheic Magdalena and Etzatlán-San

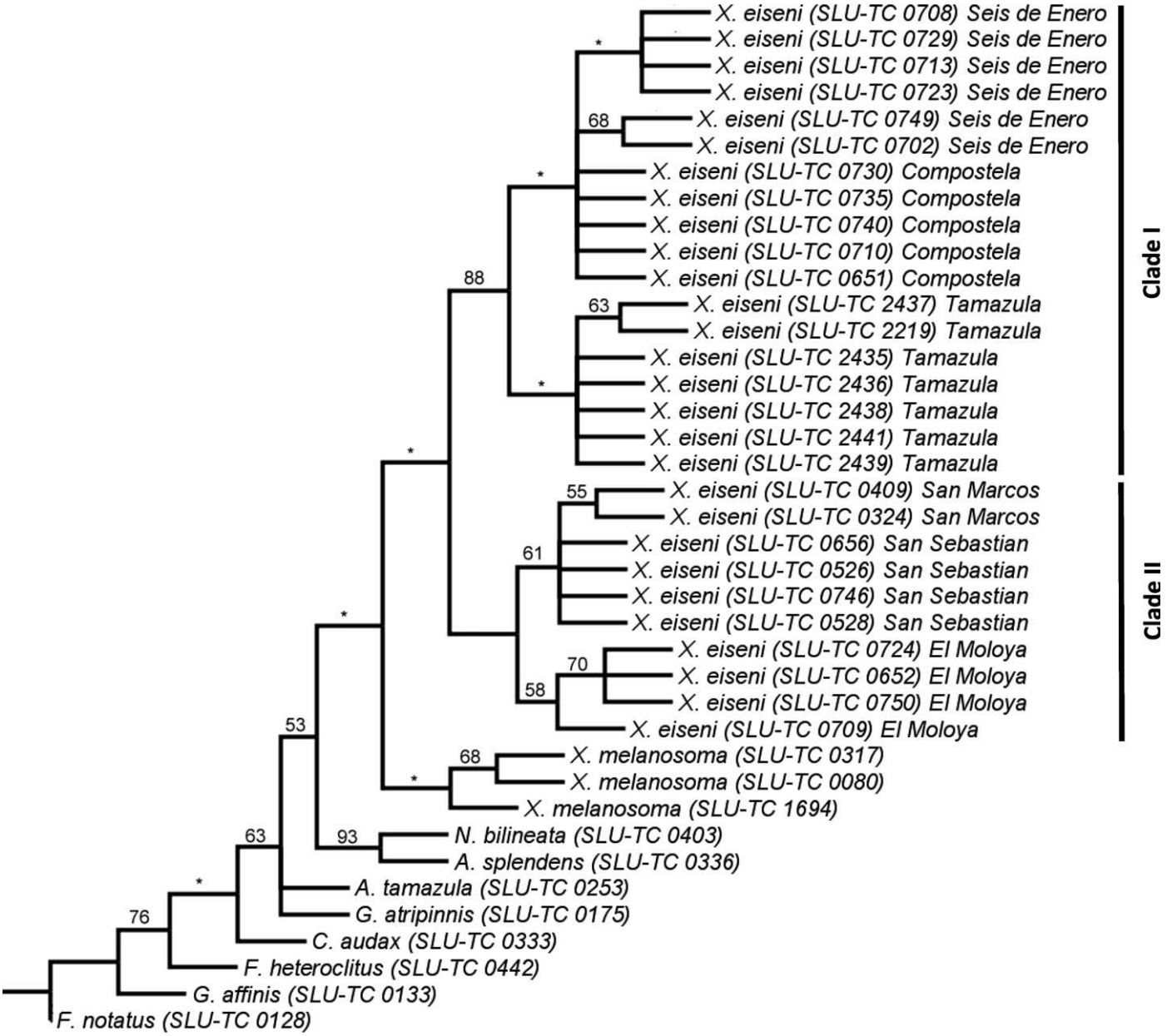

Fig. 2. Phylogram of relationships of *Xenotoca eiseni* based upon cytochrome *b* sequence data inferred from Bayesian Inference analysis. The numbers above the branches represent posterior probabilities. Asterisks represent values ≥95. Bars represent the two deeply divergent (2.51%) clades of this species.

Marcos basins (San Sebastian, El Moloya, and San Marcos sites). The optimal BI tree also shows two clades in clade II, albeit weakly supported (Bayesian support = 0.58–0.61), including one group from San Marcos and San Sebastian (Etzatlán-San Marcos area) and the other containing individuals from El Moloya.

Sequences for the nuclear ITS-1 were truncated at 636 bp in length, with 19 polymorphic sites, 16 of which were parsimony informative. The unrooted parsimony network (not shown) was similar to the Bayesian network (Fig. 3) and both were generally congruent with the cyt*b* tree. Cytochrome *b* clades I and II were represented by separate clusters in the ITS-1 network, with a mean interclade sequence divergence of 0.88%. Unlike cyt*b*, individuals from Río Tamazula grouped with those from Seis de Enero rather than with those from Río Tamazula, but this is not a robust relationship (52% Bayesian support). In clade II, San Sebastian and San Marcos individuals grouped together, as in cyt*b* analysis.

Microsatellite variation.—Eight different loci were initially included in the study, and all were polymorphic and easily scored. Locus *Zt1.43* was polymorphic for only two alleles, and all individuals were heterozygotic for these two alleles. Therefore, locus *Zt1.43* was removed from subsequent analyses. MICRO-CHECKER detected the possibility of null alleles for some populations at loci *As5*, *As2,* and *Zt1.2* in consequence of significant homozygote excess. Of the 42 tests for HWE, 11 showed significant heterozygote deficiencies at loci *As5*, *As2*, *Zt1.9,* and *Zt1.2,* following sequential Bonferroni correction. However, the majority (eight out of 11 significant tests) involved only loci *As5* and *Zt1.2* with

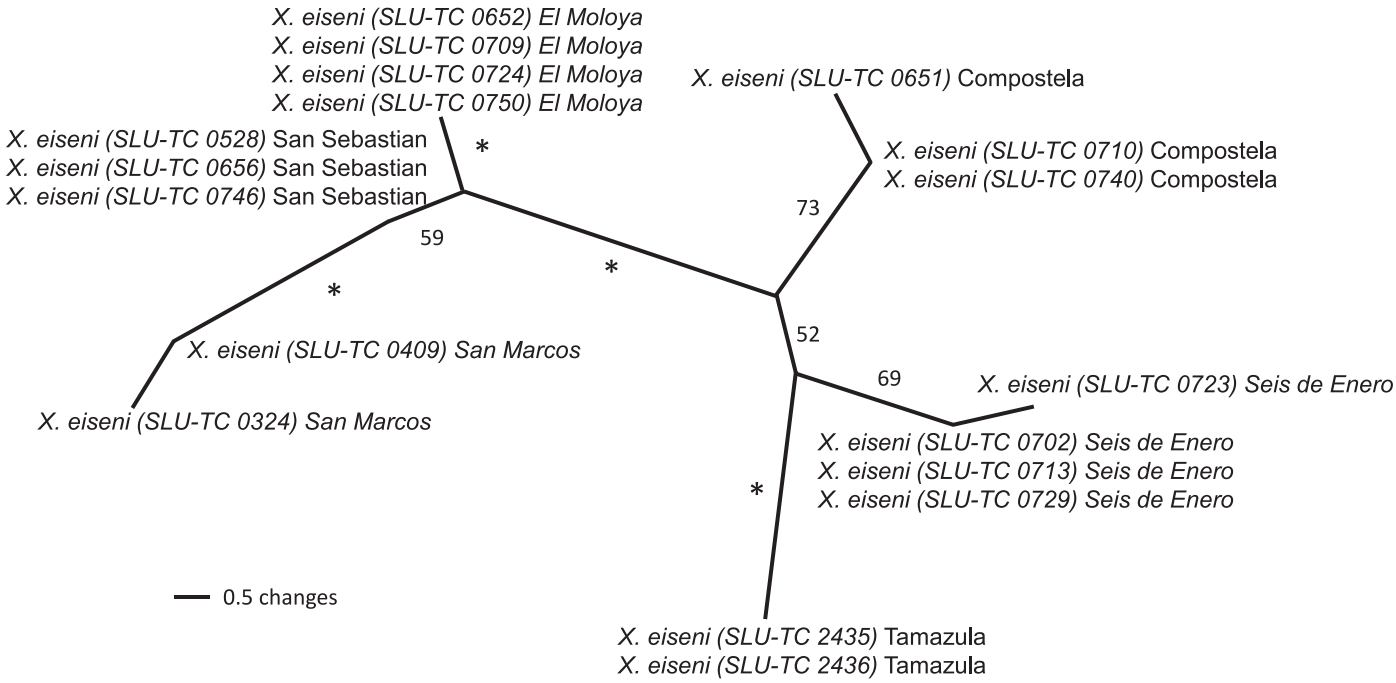

Fig. 3. Network phylogram of relationships within *Xenotoca eiseni* based upon ITS-1 sequence data inferred from Bayesian Inference analysis. The numbers above the branches represent posterior probabilities. Asterisks represent values ≥95.

deviations occurring at almost every sampled site. Tests for LD between loci across all samples revealed evidence for significant linkage between loci *As5* and *Iw196* and between *Zt1.2* and *Iw196*. Based on all the aforementioned evidence, loci *As5* and *Zt1.2* were also removed and the remaining five loci (*Iw196*, *As2*, *Zt1.9*, *Xc25*, and *Xc18*) were utilized in further analyses.

Among loci across populations, mean number of alleles (A_M) varied in range (4.3–12.1) with *Xc18* and *Iw196* showing the lowest and highest A_M, respectively (Table 2). Similarly, total number of alleles (A_T) varied between loci, ranging from nine (*Xc25*) to 33 (*As2*), with an average of 21.6 globally. Among populations across loci, the range of A_M was not quite as variable (6.0–11.2) with both the lowest and highest levels occurring in the endorheic group (El Moloya and San Sebastian, respectively; Table 3). El Moloya and San Marcos had the lowest A_M among populations (6.0 and 6.4), but this may be an artifact of small sample size (n = 7 and 11, respectively). However, allelic richness (AR) did not highly vary (4.5–6.0). Allelic richness is a better estimate of allelic variation in this case because it accounts for differences in sample size. Measurements of observed heterozygosity (H_O) did not highly vary between populations (0.495–0.782).

Genetic structure.—Pairwise fixation indices unadjusted (F_{ST}) and adjusted (F_{ST}) for null alleles were relatively consistent, indicating that null allele frequencies are minor and do not severely bias estimates of population subdivision (Table 4). All pairwise F_{ST} estimates were significant except between El Moloya and San Sebastian (F_{ST} = 0.02). Regardless of statistical significance, which is inherently dependent on sample size, F_{ST} estimates were much larger between Pacific and endorheic populations (0.23–0.41) than between populations of the same group (Pacific: 0.06–0.12; endorheic: 0.02–0.10).

To assess population structure with *a priori* groupings, two hierarchical AMOVAs were produced assuming two separate groups (Pacific and endorheic clades) and separate populations independent of groups (Table 5). The analysis assuming separate populations independent of groups revealed significant, relatively high genetic differentiation among

Table 2. Observed genetic diversity at five microsatellite loci averaged over six populations of *Xenotoca eiseni*. A_{SR}, allele size range; A_T, total number of alleles per locus; AR, mean allelic richness per locus across all populations; A_M, mean number of alleles per locus; H_E, unbiased expected heterozygosity; H_O, observed heterozygosity.

							Standard error		
Locus	A_{SR}	A_T	AR	A_M	H_E	H_O	A_M	H_E	H_O
Iw196	193–267	32	7.48	12.17	0.864	0.878	2.23	0.038	0.017
As2	213–355	33	7.40	11.67	0.845	0.664	1.23	0.020	0.043
Zt1.9	343–507	23	6.95	10.33	0.873	0.868	1.54	0.014	0.094
Xc25	164–186	9	2.83	4.67	0.346	0.404	0.56	0.082	0.121
Xc18	258–278	11	2.45	4.33	0.281	0.330	0.88	0.065	0.079
Group mean	—	21.6	5.42	8.63	0.642	0.629	0.87	0.054	0.054

Table 3. Observed genetic diversity of six populations averaged over five microsatellite loci for *Xenotoca eiseni*. *N*, number of individuals; A_T, total number of alleles per population; AR, allelic richness per population across loci; A_M, mean number of alleles per population; H_E, unbiased expected heterozygosity; H_O, observed heterozygosity; F_{IS}, inbreeding coefficient.

							Standard error			
Population	*N*	A_T	AR	A_M	H_E	H_O	A_M	H_E	H_O	F_{IS}
Compostela	42	46	4.592	9.200	0.545	0.495	2.92	0.181	0.178	0.092
Seis de Enero	50	48	5.261	9.600	0.660	0.672	1.60	0.122	0.112	−0.012
Tamazula	30	47	5.600	9.400	0.688	0.627	1.60	0.101	0.102	0.091
El Moloya	7	30	6.000	6.000	0.659	0.629	1.58	0.141	0.132	0.050
San Sebastian	32	56	5.639	11.200	0.579	0.569	3.18	0.183	0.178	0.019
San Marcos	11	32	5.450	6.400	0.719	0.782	1.08	0.105	0.102	−0.065
Group mean	28.67	43.17	5.424	8.633	0.642	0.629	0.87	0.054	0.054	0.029

populations (F_{ST} = 0.24; P = <0.0001) and accounted for about 25% of the total observed variation. The analysis assuming two separate groups revealed relatively high levels of genetic differentiation between the Pacific and endorheic groups that accounted for about 28% of the total variation, but estimates were not significant (F_{CT} = 0.27; P = 0.0977). Similar to the pairwise F_{ST} estimates, levels of genetic differentiation among populations within groups showed low, but significant, estimates that accounted for only about 7% of the total variation (F_{SC} = 0.09; P = <0.0001). However, estimates within populations (i.e., among populations among groups) displayed the largest level of differentiation which was significant and accounted for about 65% of the total variation (F_{ST} = 0.34; P = <0.0001).

A Bayesian clustering method (STRUCTURE) was used to independently assess population structure without *a priori* groupings. The STRUCTURE analysis showed significant population structuring, with the best estimate of K at K = 3 as indicated by the ΔK values (Table 6). Group assignments for K = 3 (Fig. 4) showed that Seis de Enero and Tamazula had high proportions of membership (0.982 and 0.931) to cluster 1 (yellow). El Moloya, San Sebastian, and San Marcos all had high proportions of membership (0.966, 0.981, and 0.937) to cluster 2 (green), while Compostela had a high proportion of membership (0.951) to cluster 3 (red).

DISCUSSION

Phylogeography and evolutionary history.—Results of this study indicate that *X. eiseni* consists of two clades. Clade I includes individuals from the river basins along the Pacific coast of Central Mexico, and clade II consists of individuals in the endorheic Magdalena and Etzatlán-San Marcos basin supporting the findings of previous studies (Doadrio and Domínguez, 2004). Both clades appeared in the Bayesian inference tree for cyt*b*, although there was little statistical support (posterior probabilities <0.95) for each clade. Additionally, the ITS-1 network recovered the same two groups, with the longest branch separating the clade I and II populations (cyt*b*). Furthermore, pairwise F_{ST} values from the microsatellite analysis indicated a greater divergence between these clade I and II populations than among populations within the clades.

The cyt*b* divergence between clades I and II is higher than that observed in other goodeid species pairs, which have interspecific genetic divergences within the same genera of

Table 4. Pairwise fixation indices unadjusted for null alleles (F_{ST}) and adjusted for null alleles (F_{ST}). Upper and lower bounds of 95% confidence interval (CI) are listed for each estimate. Significance (P < 0.05) is denoted with an asterisk (*).

			95% CI			95% CI	
Pairwise comparisons		F_{ST}	Upper	Lower	F_{ST}	Upper	Lower
Compostela	Seis de Enero	0.1157*	0.1557	0.0739	0.1059*	0.1371	0.0696
	Tamazula	0.1269*	0.1703	0.0751	0.1235*	0.1674	0.0776
	El Moloya	0.3986*	0.7130	0.0911	0.3942*	0.7033	0.0913
	San Sebastian	0.4154*	0.7439	0.0729	0.4090*	0.7369	0.0697
	San Marcos	0.3489*	0.6713	0.0802	0.3419*	0.6626	0.0725
Seis de Enero	Tamazula	0.0633*	0.1169	0.0213	0.0637*	0.1177	0.0221
	El Moloya	0.3111*	0.5524	0.0639	0.3176*	0.5546	0.0690
	San Sebastian	0.3530*	0.6132	0.0803	0.3567*	0.6194	0.0801
	San Marcos	0.2808*	0.4961	0.0919	0.2823*	0.4972	0.0850
Tamazula	El Moloya	0.2675*	0.4884	0.0299	0.2861*	0.4888	0.0511
	San Sebastian	0.3219*	0.5786	0.0596	0.3312*	0.5794	0.0648
	San Marcos	0.2335*	0.4286	0.0604	0.2426*	0.4314	0.0636
El Moloya	San Sebastian	0.0205	0.0552	−0.0060	0.0207	0.0556	−0.0060
	San Marcos	0.0756*	0.1030	0.0435	0.0741*	0.1024	0.0435
San Sebastian	San Marcos	0.1047*	0.1963	0.0623	0.1027*	0.1970	0.0626

Table 5. Analysis of molecular variance (AMOVA) testing comparisons among all putative populations together and comparisons of Pacific (Clade I) and endorheic (Clade II) groupings for *Xenotoca eiseni*. Estimates are based on microsatellite DNA loci. Asterisks represent AMOVAs with significant results ($P < 0.05$).

Testing assumptions	Source of variation	df	SS	% of variance	Fixation index	*P*-value
Assuming separate populations	Among populations	5	146.12	24.68	$F_{ST} = 0.247$	<0.0001*
	Within populations	338	528.34	75.32	—	—
	Total	343	674.46	100.00	—	—
Assuming two groups	Among groups	1	104.90	27.64	$F_{CT} = 0.276$	0.0977
	Among populations within groups	4	41.23	6.92	$F_{SC} = 0.096$	<0.0001*
	Within populations	338	528.33	65.44	$F_{ST} = 0.346$	<0.0001*
	Total	343	674.46	100.00	—	—

1.7 to 11% (Doadrio and Domínguez, 2004). Divergence of the two clades of *X. eiseni* began approximately 2.8 Mya based upon the cyt*b* molecular clock of 0.9% divergence per million years for the Goodeinae (Webb, 1998). This appears to be correlated with the predicted isolation time for the endorheic Magdalena region, which was isolated in the late Pliocene to early Pleistocene (Barbour, 1973; Doadrio and Domínguez, 2004). The fact that clade II is not nested within clade I indicates the possibility of gene flow along the coastal regions (clade I) subsequent to the isolation of the Magdalena region. Similar patterns of apparent gene flow among coastal drainages subsequent to isolation of endorheic drainages can be seen in other members of the subfamily Goodeinae, such as *Xenotoca variata* and *Zoogoneticus quitzeoensis* that are divided into multiple clades and show relatively high levels of intraspecific variation (Doadrio and Domínguez, 2004; Domínguez-Domínguez et al., 2008a, 2008b).

Intracladal and intrapopulational genetic diversity.—Within clade I, the two subclades identified by the phylogenetic analysis of cyt*b* correspond to two different geographical regions. One subclade is in the northern Pacific Coast drainages including the Río Compostela and Río Santiago (Seis de Enero site), and the other subclade is in the Río Tamazula, a more southern Pacific Coast drainage. The event separating the northern clade from the southern clade cannot be inferred, as specimens from the Río Ayuquila were not available for this study. ITS-1 provided no resolution of relationships within clade I, but microsatellites, in contrast to the cyt*b* tree, indicated that the Río Compostela population is the divergent member of the clade. It is possible that the geographic pattern of similarity in microsatellite variation is confounded by genetic drift resulting from degradation of the surrounding waterways and decreases in population sizes. Most goodeid populations of Central Mexico have undergone anthropogenic declines in abundance, but the Río Compostela population of *X. eiseni* in particular appears to have been severely impacted.

Many other species have similar distributions along the Pacific coast of Central Mexico, including the cyprinids of the genus *Algansea* (Miller et al., 2005; Pérez-Rodríguez et al., 2009), which have a similar distribution to *X. eiseni*. However, *Algansea* has undergone speciation events between regions, whereas *X. eiseni* only shows marginal intraclade subdivision along the coast. There are additional examples where occurrences such as erosion events appear to have allowed contact between isolated basins on the Pacific coast. An example from goodeids is the presence of *Girardinichthys multiradiatus* in the Rio Lerma and northern tributaries of the Rio Balsas. This distribution probably represents recent capture of southern streams of the Rio Lerma by the Rio Balsas (Doadrio and Domínguez, 2004). Subsequently, relatively low levels of divergence among the coastal populations of *X. eiseni* could be attributed to similar drainage change events. However, because *X. eiseni* is

Table 6. Maximum likelihood scores for Bayesian clustering analysis of each number of clusters (*K*) over 15 reps produced in STRUCTURE. The likeliest "true" cluster based on ΔK is shaded ($K = 3$). *LnP(K)*, mean estimated likelihood of the data; SD *LnP(K)*, standard deviation of the likelihood scores; ΔK, *ad hoc* estimator of the true *K* (Evanno et al., 2005).

K	*LnP(K)*	SD *LnP(K)*	ΔK
1	−3820.88	0.4004	—
2	−3363.31	0.2711	533.476
3	−3050.39	0.2642	789.913
4	−2946.17	0.3634	178.530
5	−2906.85	13.7900	0.364
6	−2872.54	9.6055	4.703
7	−2883.41	83.0239	0.176
8	−2879.65	46.2600	—

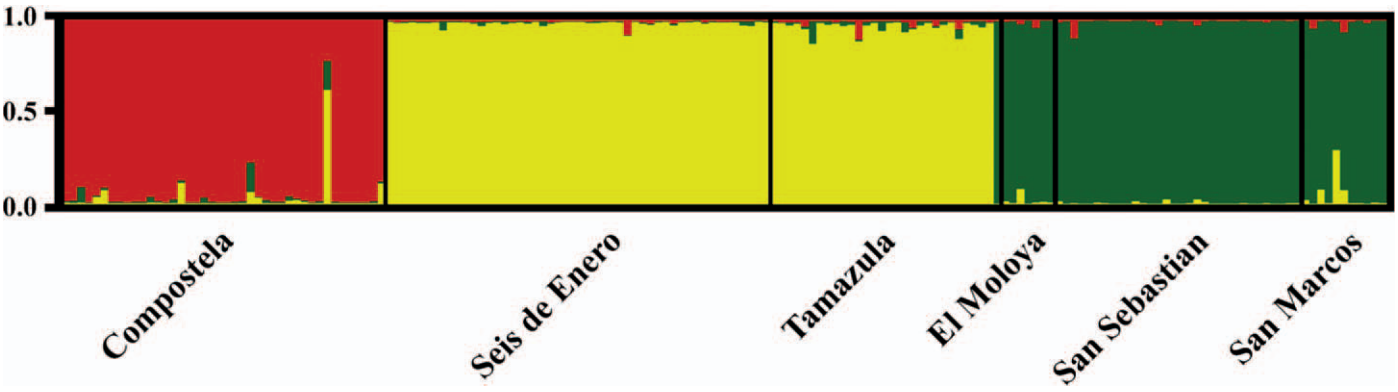

Fig. 4. STRUCTURE plots depicting results from the microsatellite analysis of *Xenotoca eiseni*. Colors represent the probability of ancestry to each cluster inferred from $K = 3$, following the methods of Evanno et al. (2005).

decreasing in numbers and becoming more fragmented, if this trend continues, events of gene flow within basins on the Pacific coast will decrease, leading to further fixation of alleles.

The endorheic populations, clade II (El Moloya, San Sebastian, and San Marcos), were not significantly differentiated based on cyt*b* and ITS-1, and only weakly divergent for microsatellite DNA (Fig. 4). This may be due to recent secondary contact due to man-made connections between these two areas. Similarly, the goodeid *Zoogoneticus quitzeoensis*, which also occurs in both El Moloya and San Sebastian, shows minimal amounts of mtDNA divergence between these areas (Domínguez-Domínguez et al., 2007). The recent modifications to these drainages due to construction of drainage channels may therefore have enabled a gene flow between the areas of El Moloya and San Sebastian while reducing San Marcos to a low-water ditch and therefore isolating it from the other waterways. Although El Moloya is a relatively healthy spring system with high water flow, San Sebastian has been, and still is, heavily impacted by human activities. The anthropogenic modifications and drainage of the Lago de Magdalena, leaving only irrigation channels (Camacho, 1998), drastically impounded this system, and land use changes and water diversions for irrigation, pollution, and the introduction of exotic species are present threats to the species in the system (Domínguez-Domínguez et al., 2007). El Moloya, which remained isolated after Magdalena was drained, is also at risk of introduction of exotic species, and both systems are suffering from modifications for recreational usage. The difference in genetic structuring based upon these sequences (cyt*b* and ITS-1) and microsatellites implies that phylogeographic history and recent events have both played different roles in shaping the structure of this clade.

Overall, although there are limitations in the application of the data from this study due to small sample sizes for some locations, the results of this study indicate that the current structure of *X. eiseni* has been highly impacted by historical changes in drainages during the Pliocene and Early Pleistocene, and further modified by more recent changes in drainage structure caused largely by anthropogenic factors.

Conservation implications.—The genetic structure of *X. eiseni* appears to have been significantly affected by the drastic changes which have occurred within the basins of Central Mexico. One of the major risks for *X. eiseni*, other than the continued degradation of its habitat (Domínguez-Domínguez et al., 2007), is that the small population sizes may lead to losses of local populations via demographic stochasticity and increase the risk of extinction due to inbreeding, decreases in genetic variation, and fixation of deleterious alleles (Lande, 1998; Saccheri et al., 1998). Therefore, to prevent decreases within the genetic fitness of these populations, habitat restoration with subsequent retention of genetic diversity and increases in population size may be one of the most important strategies for this species (Palmer et al., 1997).

At a minimum, conservation plans for this species should aim to protect the two primary clades of *X. eiseni*, which are monophyletic and potentially represent different species. A less conservative approach, based on all of the data, suggests that there are, at a minimum, three identifiable OCUs within *X. eiseni*, which correspond to the Río Compostela, Río Tamazula/Seis de Enero, and the endorheic Lago de Magdalena localities (Fig. 4). Each OCU contains a proportion of the total variation within this species, which suggests they are each worthy of conservation. The low levels of genetic divergence among these groups of populations indicate that extinction to any one of them would result in the loss of a significant portion of the remaining genetic diversity in the species.

A low level of immigration into small endangered populations, termed 'genetic rescue,' has been shown to have fitness benefits above and beyond those that can be predicted theoretically, but reduced fitness can also result if the immigrants are genetically divergent from the already established population (Tallmon et al., 2004). In the absence of translocation, other strategies must be implemented to help protect these OCUs. One of the most important conservation tools may be protection of their current habitat by stopping or reducing practices which are causing degradation, such as drainage of streams, agricultural activities, modification of streams for recreational usage, pollution of waterways, and introduction of non-native species (Domínguez-Domínguez et al., 2007). However, given the high levels of degradation in the surrounding areas, other approaches may need to be implemented. Captive breeding programs to increase population numbers with subsequent re-introduction into the wild may also be beneficial. These captive management programs can prevent different lineages from being mixed and subsequently avoid or limit negative fitness consequences to the wild populations.

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Appendix 1. Estimates of allele frequencies and measures of genetic variability for populations of *Xenotoca eiseni* by locus. *N* = Number of individuals per population; N_A = Number of alleles sampled per population; AR = Allelic richness; PAR = Private allelic richness; GD = Gene diversity; F_{IS} = Inbreeding coefficient; H_O = Observed heterozygosity.

Locus and statistic	Allele	Populations						All weighted	All unweighted
		Compostela	Seis de Enero	Tamazula	El Moloya	San Sebastian	San Marcos		
Iw196	125	0	0	0	0	0	0.045	0.003	0.008
	193	0	0.090	0	0	0	0	0.026	0.015
	197	0	0.010	0	0	0	0	0.003	0.002
	199	0	0.110	0	0	0	0	0.032	0.018
	201	0.024	0	0.033	0.143	0.063	0	0.029	0.044
	203	0	0	0.417	0.071	0.078	0.273	0.108	0.140
	205	0	0.110	0.050	0	0.016	0	0.044	0.029
	207	0.024	0.210	0.350	0	0.016	0	0.131	0.100
	211	0.012	0	0.117	0	0.016	0.227	0.041	0.062
	213	0.107	0.010	0	0	0	0	0.029	0.020
	215	0.012	0.220	0	0	0.047	0	0.076	0.046
	217	0	0.040	0	0	0	0	0.012	0.007
	219	0.012	0.040	0	0	0	0	0.015	0.009
	221	0.167	0	0	0.071	0	0	0.044	0.040
	223	0.179	0	0.017	0	0.063	0	0.058	0.043
	227	0	0.160	0	0	0.063	0.227	0.073	0.075
	229	0	0	0	0	0.094	0	0.017	0.016
	231	0.024	0	0	0.071	0.063	0	0.020	0.026
	233	0.024	0	0	0	0.109	0	0.026	0.022
	235	0.024	0	0	0.071	0.141	0	0.035	0.039
	237	0.012	0	0	0.143	0.016	0	0.012	0.028
	239	0.048	0	0	0.071	0.047	0	0.023	0.028
	241	0.155	0	0	0	0.125	0	0.061	0.047
	243	0.071	0	0	0	0	0	0.017	0.012
	245	0.012	0	0	0	0.016	0	0.006	0.005
	249	0.036	0	0	0	0	0.045	0.012	0.014
	251	0.024	0	0	0	0	0	0.006	0.004
	255	0.024	0	0	0.071	0	0	0.009	0.016
	257	0	0	0.017	0.143	0	0.045	0.012	0.034
	259	0.012	0	0	0	0.016	0.045	0.009	0.012
	261	0	0	0	0.143	0	0.091	0.012	0.039
	267	0	0	0	0	0.016	0	0.003	0.003
N		42	50	30	7	32	11		
N_A		20	10	7	10	18	8		
AR		8.416	6.487	4.299	10.000	9.278	6.420		
PAR		2.543	3.262	0.096	2.489	1.954	1.532		
GD		0.903	0.854	0.694	0.964	0.931	0.841		
F_{IS}		0.104	−0.077	−0.296	0.111	0.061	−0.081		
As2	213	0	0.010	0	0	0	0	0.003	0.002

Appendix 1. Continued.

Locus and statistic	Allele	Populations						All weighted	All unweighted
		Compostela	Seis de Enero	Tamazula	El Moloya	San Sebastian	San Marcos		
	215	0	0.080	0	0	0	0	0.023	0.013
	219	0	0.040	0	0	0	0	0.012	0.007
	229	0	0	0.017	0	0	0	0.003	0.003
	231	0	0	0.050	0	0	0	0.009	0.008
	233	0	0.030	0	0	0	0	0.009	0.005
	235	0	0.120	0.033	0.071	0	0	0.044	0.037
	239	0	0	0	0	0.031	0	0.006	0.005
	241	0	0	0.133	0	0.016	0	0.026	0.025
	243	0	0.030	0.133	0	0	0.045	0.035	0.035
	245	0	0.110	0.083	0.071	0.141	0	0.076	0.068
	247	0	0.120	0.167	0.429	0.422	0.091	0.166	0.205
	249	0	0.320	0.117	0.071	0.031	0	0.122	0.090
	251	0	0.090	0.033	0	0	0	0.032	0.021
	253	0.024	0.020	0.083	0.071	0	0	0.029	0.033
	255	0.036	0.020	0.033	0.071	0	0	0.023	0.027
	257	0.333	0	0.017	0	0.016	0	0.087	0.061
	259	0.250	0	0	0.071	0.094	0	0.081	0.069
	261	0.060	0	0	0	0	0	0.015	0.010
	263	0.024	0	0.017	0	0	0	0.009	0.007
	265	0	0	0.017	0	0.031	0	0.009	0.008
	267	0.024	0	0.067	0	0.016	0.182	0.032	0.048
	269	0.155	0	0	0	0.094	0.227	0.070	0.079
	271	0.095	0	0	0	0	0	0.023	0.016
	273	0	0	0	0	0.016	0.182	0.015	0.033
	277	0	0	0	0	0	0.091	0.006	0.015
	279	0	0	0	0	0.016	0	0.003	0.003
	289	0	0.010	0	0.071	0	0.091	0.012	0.029
	293	0	0	0	0	0.047	0	0.009	0.008
	295	0	0	0	0.071	0	0.045	0.006	0.019
	313	0	0	0	0	0.016	0	0.003	0.003
	343	0	0	0	0	0	0.045	0.003	0.008
	355	0	0	0	0	0.016	0	0.003	0.003
N		42	50	30	7	32	11		
N_A		9	13	15	9	15	9		
AR		5.649	7.002	8.502	9.000	6.723	7.524		
PAR		2.226	2.137	2.106	0.936	1.904	2.525		
GD		0.799	0.849	0.914	0.857	0.791	0.905		
F_{IS}		0.285	0.269	0.089	0.333	0.052	0.296		
Zt1.9	343	0	0.020	0	0	0	0	0.006	0.003
	403	0	0.010	0	0	0	0	0.003	0.002

Appendix 1. Continued.

Locus and statistic	Allele	Populations						All weighted	All unweighted
		Compostela	Seis de Enero	Tamazula	El Moloya	San Sebastian	San Marcos		
	407	0	0.100	0	0	0	0	0.029	0.017
	411	0	0.010	0	0	0	0	0.003	0.002
	415	0	0.090	0	0	0	0	0.026	0.015
	419	0	0.050	0.067	0	0	0	0.026	0.019
	423	0.012	0.240	0.067	0.143	0	0.136	0.099	0.100
	427	0.036	0.020	0.067	0	0.016	0.182	0.041	0.053
	431	0.250	0	0	0	0.047	0.273	0.087	0.095
	435	0.179	0.050	0.083	0	0.047	0.091	0.087	0.075
	439	0.214	0.150	0.083	0	0.203	0.136	0.157	0.131
	443	0.214	0.170	0.183	0.286	0.063	0.136	0.166	0.175
	447	0.071	0.060	0.117	0.071	0.141	0	0.084	0.077
	451	0.012	0.030	0.083	0.143	0.125	0.045	0.058	0.073
	455	0	0	0.133	0.214	0.094	0	0.049	0.074
	459	0	0	0.083	0.143	0.094	0	0.038	0.053
	463	0.012	0	0	0	0.016	0	0.006	0.005
	467	0	0	0.033	0	0.016	0	0.009	0.008
	471	0	0	0	0	0.031	0	0.006	0.005
	475	0	0	0	0	0.016	0	0.003	0.003
	479	0	0	0	0	0.047	0	0.009	0.008
	483	0	0	0	0	0.031	0	0.006	0.005
	507	0	0	0	0	0.016	0	0.003	0.003
N		42	50	30	7	32	11		
N_A		9	13	11	6	16	7		
AR		5.494	7.243	8.128	6.000	8.442	6.396		
PAR		0.138	2.276	0.635	0.082	2.063	0.118		
GD		0.815	0.870	0.917	0.857	0.906	0.859		
F_{IS}		−0.139	−0.080	0.564	−0.167	−0.035	−0.164		
Xc25	164	0	0	0.017	0	0	0	0.003	0.003
	166	0	0	0.017	0	0	0	0.003	0.003
	168	0.036	0.150	0.217	0	0	0.045	0.093	0.075
	170	0.929	0.810	0.683	0	0	0.091	0.587	0.419
	174	0	0.010	0.017	0.071	0	0.318	0.029	0.069
	176	0.024	0.020	0.033	0.857	0.906	0.500	0.253	0.390
	178	0	0.010	0.017	0.071	0.047	0.045	0.020	0.032
	180	0.012	0	0	0	0.031	0	0.009	0.007
	186	0	0	0	0	0.016	0	0.003	0.003
N		42	50	30	7	32	11		
N_A		4	5	7	3	4	5		
AR		1.899	2.456	3.329	3.000	2.141	4.152		
PAR		0.102	0.004	0.484	0.113	0.546	0.001		
GD		0.138	0.323	0.491	0.274	0.178	0.655		

Appendix 1. Continued.

Locus and statistic	Allele	Populations						All weighted	All unweighted
		Compostela	Seis de Enero	Tamazula	El Moloya	San Sebastian	San Marcos		
F_{IS}		0.309	−0.175	−0.155	−0.043	−0.054	−0.389		
Xc18	258	0	0	0.017	0	0	0	0.003	0.003
	260	0	0	0.033	0	0	0	0.006	0.006
	262	0	0.010	0.083	0.786	0.953	0.818	0.279	0.442
	264	0	0	0.050	0.214	0.031	0.091	0.029	0.064
	266	0.012	0.140	0.050	0	0	0.091	0.058	0.049
	268	0.964	0.760	0.750	0	0	0	0.587	0.412
	270	0	0.020	0	0	0	0	0.006	0.003
	272	0	0.010	0.017	0	0	0	0.006	0.004
	274	0	0	0	0	0.016	0	0.003	0.003
	276	0.012	0.050	0	0	0	0	0.017	0.010
	278	0.012	0.010	0	0	0	0	0.006	0.004
N		42	50	30	7	32	11		
N_A		4	7	7	2	3	3		
AR		1.500	3.116	3.744	2.000	1.611	2.758		
PAR		0.221	0.974	0.855	0.033	0.219	0.033		
GD		0.071	0.403	0.431	0.357	0.092	0.327		
F_{IS}		−0.012	−0.191	−0.005	−0.200	−0.022	−0.111		
All loci									
N		42	50	30	7	32	11		
N_A		46	48	47	30	56	32		
AR		4.592	5.261	5.600	6.000	5.639	5.450		
PAR		1.046	1.731	0.835	0.731	1.337	0.842		
H_O		0.495	0.672	0.627	0.629	0.569	0.782		
F_{IS}		0.092	−0.012	0.091	0.050	0.019	−0.065		