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RESEARCH ARTICLE

Population structure and conservation genetic assessment of the endangered Pugnose Shiner, *Notropis anogenus*

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Abstract The Pugnose Shiner is a small minnow with a fragmented distribution across the Great Lakes and Upper Mississippi River in North America. The species is listed federally as endangered in Canada, and in the United States its status varies by state, from Special Concern to Endangered (as well as Extirpated). We conducted a thorough genetic assessment of the Pugnose Shiner using both microsatellite loci and mitochondrial DNA collected for samples across the species range. Our results indicate high levels of population differentiation suggesting restricted dispersal, in some cases at very small geographical scales. We also found strong evidence of small effective population sizes and one case of a genetic bottleneck. Although significant range-wide genetic variation was observed in both microsatellite loci and mitochondrial DNA, the species is best characterized as a single evolutionarily significant unit for conservation purposes.

Keywords Microsatellites · mtDNA · Population structure · Freshwater fish · Bottleneck

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Introduction

Many freshwater species are vulnerable to extinction (Abell 2002) with 39 % of described freshwater fish species in North America considered imperiled (Jelks et al. 2008). Within the family Cyprinidae (carps and minnows), as many as 46 % of species are imperiled, largely due to human activities, including habitat degradation, fragmentation, and loss, and introduced species (Jelks et al. 2008). The Pugnose Shiner, *Notropis anogenus* Forbes 1885 occupies a fragmented range across the Great Lakes and Upper Mississippi River (Fig. 1) and is considered to be one of the rarest cyprinids in the northern United States and southern Canada (Bailey 1959; Becker 1983; Holm and Mandrak 2002). The Pugnose Shiner is found in areas with exceptional water quality and abundant submerged vegetation (Becker 1983; Holm and Mandrak 2002), including some of the most pristine habitat across its distribution. Unfortunately, the species appears to be declining in virtually all states and provinces where it is found, most likely due to increased turbidity, reduction in aquatic vegetation, and the presence of invasive species (DFO 2010). Most research on fish species in the Great Lakes region has focused on larger-bodied sport fishes, and relatively little research has addressed conservation questions related to non-game species. Moreover, as many of the locations where the Pugnose Shiner occurs are occupied by other species at risk, efforts to understand and protect this species will likely aid in the conservation of other threatened and endangered aquatic taxa. The specific habitat requirements of the Pugnose Shiner also make it a potentially valuable environmental indicator of habitat quality.

North American fish experts consider the Pugnose Shiner to be 'Threatened' (Jelks et al. 2008), the IUCN list the species as 'Near Threatened' (IUCN 2013), and

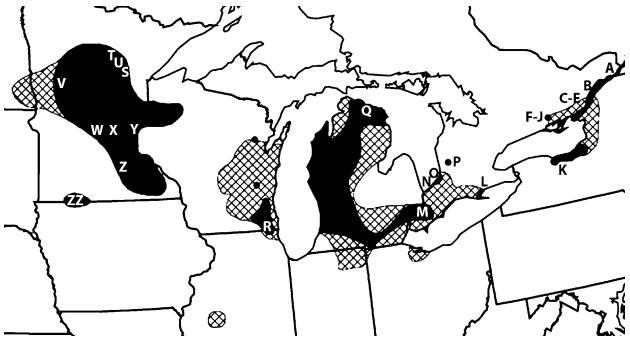

Fig. 1 The distribution of the Pugnose Shiner and sampling localities. Solid and cross-hatched distributions together represent the distribution according to Page and Burr (2011), while the solid distribution only is considered the current distribution according to COSEWIC (2013). Sampled sites are indicated by letters (see Table 1 for names)

NatureServe (2013) considers the species to be 'Nationally Vulnerable' (category N3) in the United States and 'Nationally Imperiled' in Canada (category N2). In the United States, the species is not listed federally, but state status varies from 'Endangered' to 'Special Concern', and it is considered extirpated in Ohio. In Canada, the species has a particularly fragmented distribution and is currently listed as 'Endangered' under the Canadian Species at Risk Act (SARA). The official conservation status of species in Canada is determined by the Committee on the Status of Endangered Wildlife (COSEWIC) and is based on criteria including recent population declines, area of occupancy, population size, and the number of distinct populations. Unfortunately, conservation efforts to protect the Pugnose Shiner, including determinations of appropriate conservation status for the species, have been hindered by limited genetic information. To date, no range-wide surveys of genetic variation have been completed for this rare and vulnerable species, greatly limiting our understanding of population structure, levels of dispersal and barriers to gene flow, and appropriate management units.

Here, we present a range-wide survey and analysis of genetic variation in the Pugnose Shiner, made possible by several years of targeted sampling for this elusive fish. Our primary objectives were three-fold. First, we determined the genetic population structure for the Pugnose Shiner across its North American range. Genetic differentiation among sample sites can indicate whether populations experience high levels of gene flow with neighboring populations or whether they are relatively isolated. Second, we estimated effective population size (N_e) and tested for bottlenecks in identified populations. These results provide insight into population vulnerability to inbreeding depression, mutation accumulation, and reduced adaptive potential (Frankham 2005; Spielman et al. 2004; Willi et al. 2006). Finally, we evaluated deeper patterns of intra-specific divergence using mitochondrial DNA (mtDNA). A core priority of conservation genetics is the characterization and protection of significant genetic divergence below the species level. Many definitions of evolutionarily significant units (ESUs) exist in the literature, typically based on deep genetic divergence that has arisen over a long period of time and representing a significant component of the evolutionary legacy of the species (Waples 1991; Moritz 1994; Green 2005; de Guia and Saitoh, 2007). Overall, our results provide valuable insight regarding the biology and conservation of the Pugnose Shiner.

Methods

Samples were collected primarily by seining during 2009-2011, although some samples dated as far back as 2005 (Old Ausable Channel) and 1996 (Lake St. Clair) (Table 1; see Supp. Table i. for geographic coordinates). Fin clips were taken for genetic analysis with the exception of the 2005 scale samples from the Old Ausable Channel. In total, we collected 959 individuals from 27 sites, with temporal replicates from West Lake, Long Point Bay, and Lake St. Clair (Fig. 1; Table1).

We tested 27 microsatellite loci originally isolated in *Notropis* or *Dionda species*, however, most loci either failed to amplify or were monomorphic in the Pugnose Shiner. We genotyped eight dinucleotide polymorphic microsatellite loci in the Pugnose Shiner that were originally isolated in other cyprinids. These included six loci originally isolated in the Cape Fear Shiner, *Notropis mekistocholas* (Burridge and Gold 2003; Gold et al. 2004), and two loci originally isolated in the genus *Dionda* (Renshaw et al. 2009) (see Supp. Table iia for locus details). We extracted DNA with DNeasy Blood & Tissue kits (QIAGEN). We conducted PCR amplification of 10-μL volumes containing 20–100 ng DNA, 2.0 mM $MgCl_2$, 50 μM each dNTP, 0.5 U *Taq* DNA polymerase, 0.3–0.5 μM each primer, and 1× PCR buffer (10 mM Tris–HCl, pH 8.3; 50 mM KCl). Thermal cycling conditions were as follows: 95 °C for 3 min, followed by 35 cycles of denaturing at 95° C for 30 s, primer-specific annealing (see Supp. Table iia for primer-specific T_A) for 30 s, and extension at 72° C for 30 s, with a final extension at 72° C for 3 min. PCR products were sent to The Centre for Applied Genomics (TCAG) at the Hospital for Sick Children (Toronto, Ontario) for genotyping on an ABI 3,100 system. To assess scoring accuracy, we ran duplicates of approximately 10 % of samples across all loci and determined scoring error following Bonin et al. (2004).

The mitochondrial cytochrome b gene was amplified with PCR using primers developed from a published sequence of the Pugnose Shiner (Schonhuth and Doadrio

Table 1 Sample sites, map id, and year of collection are listed for all sites

Map ID	Location	Year collected	Lake or river system	n-micros	Ave H_e	Ave A_R	N-mtDNA	H	π
A	Long Sault	2011	St. Lawrence	16	0.39	2.81			
B	St. Lawrence East	2011	St. Lawrence	50	0.44	3.02			
C	Mallorytown	2009	St. Lawrence	42	0.41	2.90			
D	Thompson's Bay	2009	St. Lawrence	55	0.41	3.04	10	0.38	0.025
E	Goose Bay	2009	St. Lawrence	43	0.42	2.91			
F	Smith Bay	2010	Lake Ontario	50	0.36	2.37			
G	Trent River	2011	Lake Ontario	47	0.40	2.82			
H	East Lake	2010	Lake Ontario	48	0.38	2.64			
Ia	West Lake	2009	Lake Ontario	32	0.47	3.12			
Ib	West Lake	2010	Lake Ontario	53	0.46	3.09			
J	Weller Bay	2010	Lake Ontario	50	0.39	2.57			
K	Sodus Bay	2009	Lake Ontario	42	0.41	2.84	8	0.54	0.022
La	Long Point Bay	2009	Lake Erie	24	0.48	3.31	6	0.33	0.028
Lb	Long Point Bay	2011	Lake Erie	24	0.53	3.34			
Ma	L. St. Clair	1996, 2007	Lake St. Clair	9	0.58	3.54	7	0.86	0.083
Mb	St. Clair R, L. Bear Ck	2010	Lake St. Clair	33	0.58	3.77			
N	Mouth Lake	2010	Lake Huron	31	0.36	1.97			
O	Old Ausable Channel	2005–9	Lake Huron	65	0.49	3.07			
P	Teeswater River	2010	Lake Huron	25	0.23	2.01	8	0.43	0.018
Q	Black River	2010	Lake Superior	57	0.40	2.75	7	0.71	0.067
R	Cross Lake	2009	Mississippi R.	32	0.51	3.07	11	0.18	0.008
S	Floodwood Lake	2009	Lake Superior	6	0.39	2.47			
T	Cameron Lake	2009	Red River	8	0.32	2.51			
U	Nashwauk Lake	2009	Mississippi R.	29	0.44	2.84	8	0.00	0.000
V	Little Floyd River	2009	Red River	11	0.41	3.26	8	0.46	0.021
W	Long Lake	2009	Mississippi R.	6	0.58	4.34			
X	Limestone Lake	2009	Mississippi R.	8	0.42	3.41			
Y	Forest Lake	2009	Mississippi R.	22	0.39	3.27	8	0.71	0.060
Z	Fish Lake	2009	Mississippi R.	32	0.57	4.17			
ZZ	West Okoboji Lake	2009, 11	Mississippi R.	9	0.50	3.28	4	0.83	0.042

Sample sizes used in the analysis of seven microsatellite loci are indicated (n-micros), as well as expected heterozygosity (He) and allelic richness (based on five individuals, AR). Samples sizes for mtDNA (n-mtDNA) are also provided, as well as haplotype (H) and nucleotide diversity (π) (see Fig. 1 for location of samples)

2003; Supp. Table iib). We conducted PCR amplification of 25-μL volumes containing 50–200 ng DNA, 2.0 mM $MgCl_2$, 50 μM each dNTP, 1.25 U *Taq* DNA polymerase, 2.0 μM each cyt b primer, and 1× PCR buffer (10 mM Tris–HCl, pH 8.3; 50 mM KCl). Thermal cycling conditions were as follows: 95 °C for 3 min, followed by 35 cycles of denaturing at 95 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 3 min. PCR products were purified with a QIAquick PCR Purification Kit (Qiagen) and sequenced in both forward and reverse directions. Sequencing was performed at the TCAG DNA Sequencing Facility at SickKids (Toronto, Ontario). Sequences were compiled and edited with Sequencher 4.6 (Gene Codes Corp., Ann Arbor, MI, USA). In total, we sequenced 815 bp from 85 individuals across 11 sites (ranging from 4 to 11 individuals per site).

Genetic diversity, heterozygosity

Microsatellite loci were assessed for null alleles, stutter, and large allele drop-out with Microchecker v2.2.1 (Van Oosterhout et al. 2004). The frequency of null alleles was also estimated with FreeNA (Chapuis and Estoup 2007). Hardy–Weinberg equilibrium and genotypic equilibrium were assessed with Genepop (Raymond and Rousset 1995). Standard diversity indices such as expected heterozygosity (H_e), number of alleles (N_A), and allelic richness (A_R) were evaluated in FSTAT v2.9.3.2 (Goudet 1995). For mtDNA, we evaluated haplotype (H) and nucleotide (π) diversity

per population in Arlequin v3.5 (Excoffier and Lischer 2010).

Population structure

Population structure was evaluated using microsatellite loci with pair-wise F_{ST} in FSTAT v2.9.3.2 (Goudet 1995), and significance was evaluated with contingency tests of allele frequencies in TFPGA (Miller 1997). As the presence of null alleles can affect F_{ST} estimates, we also calculated F_{ST} using a correction for null alleles with FreeNA (Chapuis and Estoup 2007). We performed a clustering analysis with the program POPULATIONS (Langella 1999) based on Chord distance, Dc (Cavalli-Sforza and Edwards 1967), which has been found to perform well with microsatellite data for closely related populations and species (Takezaki and Nei 1996). Significance was evaluated by bootstrapping across loci using 10,000 replicates.

We performed a hierarchical analysis of population structure with STRUCTURE 2.3.3 (Pritchard et al. 2000), which uses Hardy–Weinberg and linkage equilibrium to determine the number of distinct genetic clusters. We identified the number of clusters with the strongest statistical support using the ΔK method of Evanno et al. (2005). As fine-scale patterns can be difficult to discern, we subdivided the dataset wherever possible to facilitate greater resolution (Rosenberg et al. 2002). Where populations or groups of populations were easily differentiated, we reran STRUCTURE on progressively smaller subsets of the data in order to detect differentiation on finer scales. If subdivision was not straightforward with the first peak ΔK, we used the next highest peak ΔK value. We used sample locations as priors (Hubisz et al. 2009), implementing a burn-in of 10^5 followed by 10^6 iterations to determine the number of clusters (from $k = 1$ to the maximum number of populations per run) using the admixture model. We ran STRUCTURE multiple times for all iterations to assess convergence and consistency. Results were visualized with the program DISTRUCT (Rosenberg 2004).

N_e estimates and bottlenecks

We used several methods to estimate effective population size (N_e) and bottlenecks, and we restricted all analyses to sample sites with at least 20 individuals. Our first approach was to estimate current N_e with the linkage disequilibrium method in LDNe (Waples and Do 2008). The linkage disequilibrium method assumes that populations are isolated and that the species has discrete generations. The Pugnose Shiner has overlapping generations; however, Waples and Do (2010) speculated that the LD method can provide a rough approximation of N_e per generation if the sample represents a random collection of the cohorts that constitute a generation. We removed low frequency alleles using the 0.02 cut-off point, as recommended by Waples and Do (2010) in order to reduce upward bias of N_e estimates.

We also estimated N_e using a full-likelihood Bayesian method implemented by MSVAR v1.3 (Beaumont 1999; Storz and Beamont 2002). MSVAR uses a Markov chain Monte Carlo approach to estimate the current effective population size (N_0), ancestral effective population size (N_1), mutation rate (μ), and time when the population started to expand or decline (t_a). We set priors for, N_0, N_1, and t_a to 10^3, and μ to 10^{-4}, using an exponential model. We chose a variance of 3 for N_0, N_1, and t_a, and a variance of 2 for μ to explore a wide range of parameter space (see Supp. Table iii for more detail). MSVAR assumes a stepwise mutation model (SMM) and, therefore, we removed one locus from our dataset that exhibited large gaps in allele sizes particularly in Minnesota. We ran the program multiple times for each population, with each run producing a minimum of 10^9 iterations. We discarded the first 10 % of each run as burn-in and results were combined across runs. Convergence was assessed with Tracer v1.5 (Rambaut and Drummond 2009) by examining posterior distributions of all parameters (normal curve), auto-correlation in the trace file, consistency across runs, and ESS values (>100). Although MSVAR has been widely used to examine changes in population size and may be more sensitive to changes in population size than BOTTLENECK (Girod et al. 2011), recent simulation studies have shown it to be biased towards detecting declines with violations of assumptions about the mutation model (Faurby and Pertoldi 2012) and population structure (Chikhi et al. 2010). Faurby and Pertoldi (2012) found N_0 to be the most robust parameter to departure from the SMM model in their simulation studies, and when interpreted in a relative sense to compare N_0 across populations, it was even more robust. Therefore, we focused on estimates of N_0 and relative N_0 across populations.

To evaluate the possibility of population bottlenecks, we used two moment-based methods. The number of alleles is expected to decrease more rapidly than heterozygosity when populations contract, and we tested for statistical support for heterozygosity excess with the one-tailed Wilcoxon test in BOTTLENECK (Cornuet and Luikart 1996). We also used the program M_P_Val to implement the M-Ratio test for bottlenecks (Garza and Williamson 2001). The M-Ratio test identifies reductions in population size based on the expectation that population contraction will decrease the number of alleles more rapidly than the range of allele sizes (Garza and Williamson 2001). We used M_P_Val to calculate the M-ratio and the probability of detecting the observed value if the population was at equilibrium. For both the heterozygosity excess and M-Ratio analyses, we used the two-phase model (TPM) of

mutation with conditions recommended by Garza and Williamson (2001) (proportion of non-stepwise mutations, $p_g = 0.1$, and average non-stepwise mutational size change, $\delta_g = 3.5$), as well as those recommended by Peery et al. (2012) ($p_g = 0.22$, $\delta_g = 3.1$).

Conservation units

We assessed patterns of divergence in the Pugnose Shiner by creating an mtDNA haplotype network using statistical parsimony in Network 4.611 (Fluxus Technology Ltd.). We then visually examined the distribution of haplotypes across the range of the species to determine if the species has a single or multiple evolutionarily distinct units.

Results

Genetic diversity, heterozygosity

Departures from HWE were found in nine instances ($p < 0.05$) with only one remaining significant after correcting for number of loci ($p < 0.007$). Null alleles were identified in two instances by Microchecker, although the frequency of null alleles in both cases was estimated to be <0.1 with FreeNA. The frequency of null alleles per locus (averaged across populations) was low (<0.02), and no consistent trends were found across loci or populations. Genotypic disequilibrium was not significant among loci, so loci were assumed to be segregating independently. Heterozygosity, H_e, and number of alleles varied widely among loci, and one locus with particularly low H_e (<0.1) was removed from further analysis (Supp. Table ii). Our results are, therefore, based on the remaining seven loci, with average H_e across loci ranging from 0.23 to 0.58 among sample sites (Table 1). We found an average scoring error rate of 1 % across the seven microsatellite loci. Mitochondrial DNA (mtDNA) sequencing analysis identified 20 transitions and 5 transversions among a total of 24 distinct haplotypes (Genbank Accession Numbers KF744250–KF744334). Haplotype diversity ranged from 0 to 0.86, and nucleotide diversity ranged from 0 to 0.07 (Table 1).

Population structure

Population allele frequencies were significantly different in almost all pair-wise comparisons, with the exception of several sites within the St. Lawrence River, all temporal replicates, and several sites from Minnesota with very small sample sizes. F_{ST} values among sites ranged from 0.002 to 0.510 with the highest values found between Minnesota sites and the rest of the range (see Supp. Table iv for all pairwise F_{ST} and p-values). The overall average F_{ST} value estimated both with and without a correction for null alleles was 0.23, and we found a correlation between the two estimates (with and without correcting for null alleles) of 0.999 across all pairwise comparisons. Therefore, we do not expect that null alleles significantly affected our results. Population clustering based on Dc identified three main groups, identified as 'western' (Minnesota/Iowa), 'central (samples from Wisconsin to Lake Erie), and 'eastern' (Lake Ontario and the St. Lawrence River), although bootstrap values were generally low (Fig. 2). A hierarchical STRUCTURE analysis provided additional evidence of population differentiation on both regional and local scales (Fig. 3; see Supp. Fig. i for full results from the hierarchical approach). The STRUCTURE analysis confirmed the three regional groups identified in the clustering analysis ('western', 'central', and 'eastern'; Fig. 2). The hierarchical analysis provided strong support for genetic differentiation of nearly all sample sites, with the exceptions of sites within the St. Lawrence River, all temporal replicates, and several sites from Minnesota with very small sample sizes.

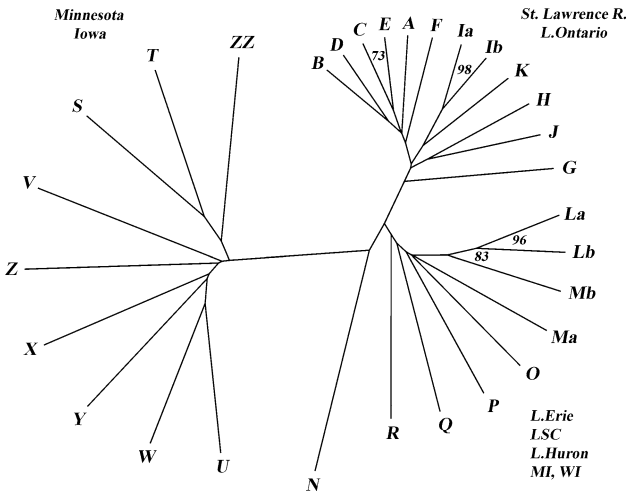

Fig. 2 Cluster relationships for Pugnose Shiner populations, determined with the program POPULATIONS (Langella 1999), using UPGMA of chord distances (Dc) for 7 microsatellite loci. Bootstrap values >70 are indicated (10 000 replicates, bootstrapping across loci). See Table 1 for locality names

N_e estimates and bottlenecks

LDNe produced negative N_e estimates for many populations, indicating that the level of linkage disequilibrium observed could be explained by sampling error alone (Waples and Do 2010). We found non-negative N_e estimates in nine sites ranging from 39 to 2,040, with three sites (Old Ausable Channel, Teeswater River, Cross Lake) having N_e estimates below 100 (Supp. Table v). However,

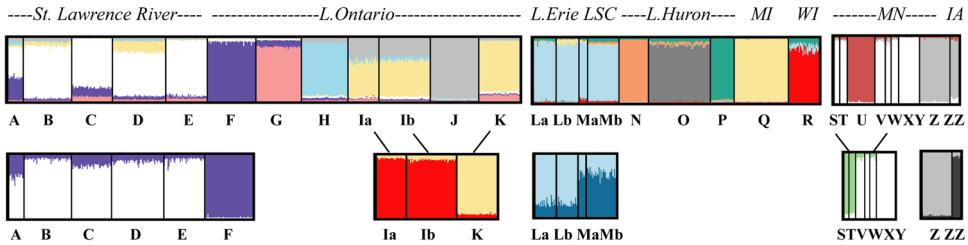

Fig. 3 Structure results for seven microsatellite loci genotyped across 27 sites, including three temporal replicates. Population differentiation was exhibited for almost all sites via progressive subdivision of the data. Further subdivision of sites within the St. Lawrence River (*A–E*), between temporal replicates (*Ia, Ib; La, Lb; Ma, Mb*), and among sites in Minnesota with small sample sizes (*S*, *T*, *V*, *W*, *X*, *Y*) was not possible. For complete results of hierarchical analysis, see Supp. Fig. ii). See Table 1 for locality names

even when N_e estimates are large, lower confidence limits can still provide valuable information (Waples and Do 2010), and both Teeswater River and Mouth Lake had particularly low Lower CIs (<10).

MSVAR produced a normal distribution and demonstrated convergence in all parameters for eleven populations (Table 2). MSVAR failed to reach convergence for four populations, and six additional populations exhibited bimodal distributions for the temporal parameter—results from these ten populations are not reported here. Estimates of current effective population size (N_0) ranged from 2 to 647 across sample sites (Table 2), and N_0 estimates at four sites were below 30 (Weller Bay, Mouth Lake, Black River, and Nashwauk Lake). Several other sites had N_0 estimates of $\sim$100 (Old Ausable Channel, Teeswater River, Fish Lake). In contrast, the highest N_0 estimates were found along the St. Lawrence, particularly within the Thousand Islands Park region (Mallorytown, $N_0 > 600$).

The heterozygosity excess test for bottlenecks provided a significant result for one site: Mouth Lake. Results were significant for all mutation models, including both TPM models, IAM and SMM ($p < 0.05$) (Supp. Table vi). The program M_P_Val produced M-ratios estimates ranging from 0.70 to 0.91, with significant evidence of bottlenecks at several sites at $p < 0.05$ using the mutation model more closely approximating SMM (East Lake, Old Ausable Channel, and Nashwauk Lake; Supp. Table vii). However, significant results were not found when the mutation model recommended by Peery et al. (2012) was used.

Conservation units

The mtDNA haplotype network resembled a "star-phylogeny", with most haplotypes differing from the central haplotype (haplotype 1) by one or two mutations (Fig. 4). Haplotype 1 was found in 35 % of individuals and was distributed across the range, from the St. Lawrence River to Iowa. Most haplotypes were not shared among localities, indicating limited gene flow. Regional patterns were observed across the range of the Pugnose Shiner, similar to that found with microsatellites. In the 'central group', Long Point Bay and Lake St. Clair shared haplotype 7 as well as

Table 2 Results from Msvar analysis, including mean and 95 % highest posterior distributions (HPD)

Map ID	Location	95 % HPD lower	N_0	95 % HPD upper	95 % HPD lower	N_1	95 % HPD upper	95 % HPD lower	T (gen)	95 % HPD upper	μ	log (N_0/N_1)
B	St. Lawrence East	0	229	3.5×10^5	9	11 830	1.6×10^7	0	214	3.4×10^6	2.4×10^{-4}	−1.7
C	Mallorytown	0	647	5.6×10^6	0	4 721	5.6×10^7	0	805	1.1×10^9	4.7×10^{-4}	−0.9
H	East Lake	0	184	3.6×10^5	8	11 912	1.9×10^7	0	242	5.7×10^6	2.2×10^{-4}	−1.8
J	Weller Bay	0	27	4.0×10^4	17	13 804	1.1×10^7	0	60	1.6×10^5	5.8×10^{-5}	−2.7
N	Mouth Lake	0	2	3.0×10^3	177	127 057	1.3×10^8	0	47	5.8×10^4	4.8×10^{-5}	−4.8
O	Old Ausable Ch	0	94	1.1×10^5	25	17 989	9.1×10^6	0	155	2.5×10^5	1.8×10^{-4}	−2.3
P	Teeswater River	0	106	1.3×10^5	5	8 147	7.4×10^6	0	610	4.9×10^6	2.3×10^{-4}	−1.9
Q	Black River	0	12	1.9×10^4	69	50 466	3.6×10^7	0	37	6.0×10^4	6.1×10^{-5}	−3.6
R	Cross Lake	0	195	1.7×10^5	15	10 447	5.4×10^6	0	375	5.4×10^5	2.9×10^{-4}	−1.7
U	Nashwauk Lake	0	25	4.1×10^4	19	16 293	1.6×10^7	0	101	2.2×10^5	7.7×10^{-5}	−2.8
Z	Fish Lake	0	86	1.1×10^5	24	18 197	1.3×10^7	0	68	1.6×10^5	1.2×10^{-4}	−2.3

For current population size, N_0, ancestral population size, N_1, and timing of declines (t), mutation rate, μ, and log (N_0/N_1)

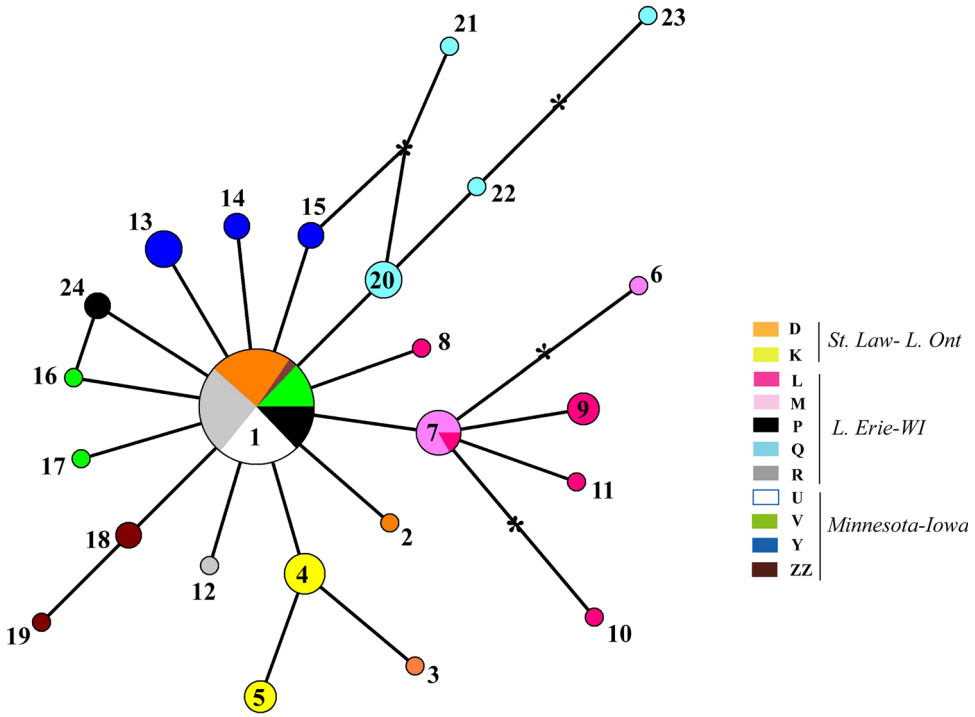

Fig. 4 MtDNA haplotype network based on cytochrome b sequences from across the range of the Pugnose Shiner. Twenty-four haplotypes were found in total, with the size of each circle representing haplotype frequency. Each line segment represents a single mutation, and each sample site is represented by a unique colour. 'Missing' haplotypes are indicated by asterisks. See Table 1 for locality names

related haplotypes 6, 7, 9–11 (Fig. 4). In the 'eastern' group, related haplotypes 3, 4, and 5 were found in the St. Lawrence River and Lake Ontario.

Discussion

Population connectivity

The small body size of the Pugnose Shiner, its presumed weak swimming ability, the rarity of the species, and its disjunct distribution all suggest that populations should be highly differentiated from one another, although this has never been shown. Genetic data provide strong support for very limited connectivity among populations. Population structure was pronounced across the range of the Pugnose Shiner with microsatellites (overall average F_{ST} of 0.23, maximum F_{ST} of 0.51). Although inferring precise levels of gene flow from the degree of genetic differentiation can be problematic (Whitlock and McCauley 1999), genetic data can provide approximate indications of demographic independence (Waples and Gaggiotti 2006). In this study, all localities with at least 20 individuals were significantly different from one another (or marginally significant, $p < 0.004$, Supp. Table iv), with the exception of three sites on the St. Lawrence River less than 10 km apart, suggesting demographic independence among most sites.

The level of population structure observed in the Pugnose Shiner was similar to other population genetic surveys of threatened and endangered fish species (Sousa et al. 2010; DeHaan et al. 2012), although the geographic scale over which genetic differentiation occurred was not as extreme as has been observed in some studies (Austin et al. 2011; Fluker et al. 2010, Robinson et al. 2013, Sterling et al. 2012). In comparison to similarly sized species within the Great Lakes, F_{ST} values were slightly higher than that found in the stream-dwelling Greenside Darter (Beneteau et al. 2009). Most larger-bodied species within the Great Lakes exhibit considerably less population structure than the Pugnose Shiner, including Walleye (Stepien et al. 2009), Bloater (Favé and Turgeon 2008) and Lake Whitefish (Bernard et al. 2009). Interestingly, comparable F_{ST} values (to the Pugnose Shiner) have been observed in the Smallmouth Bass and Yellow Perch within the Great Lakes, possibly related to strong spawning site fidelity (Stepien et al. 2007; Sepulveda-Villet and Stepien 2011).

Population differentiation in the Pugnose Shiner was more pronounced in some areas than others, likely illustrating physical barriers to dispersal which were often related to human alterations of the landscape or hydrology. For example, Mouth Lake and Old Ausable Channel were highly distinct ($F_{ST} = 0.16$) despite being separated by <15 km. The Old Ausable Channel and Mouth Lake used to be connected within the Ausable River drainage system via intermittent flooding. However, dam construction and alterations to the hydrology starting in the 1,800ws effectively isolated both populations. Mouth Lake is now completely isolated from other water bodies as it has no outlet channel. Similarly, the Trent River population, first discovered in 2011, was highly genetically differentiated

from nearby populations (F_{ST} ~0.2), likely due to dam construction and the presence of several locks on the Trent River. Artificial barriers that isolate populations and increase genetic differentiation are not uncommon, though they can be particularly damaging in species at risk that already suffer from fragmentation (McCraney et al. 2010). East Lake and West Lake, were also significantly different (F_{ST} = 0.03–0.05) despite being approximately ~15 km from one another via outlet channels. However, in this case dispersal between sites is likely limited by natural conditions, including intervening regions of exposed beach, cold water, and little submerged vegetation which the species depends on for food and protection (DFO 2010).

Population structure was also observed on a broader scale, possibly reflecting historical patterns of connectivity. The regional structure among 'eastern', 'central', and 'western' sample sites (Supp. Table iv; Figs. 2, 3) illustrates the obvious separation between the Upper Mississippi River and the Great Lakes basin, and also suggests that the Niagara Falls has been a significant barrier to gene flow between Lake Erie and Lake Ontario. Interestingly, the relatively low level of differentiation between samples from Lake Erie and Lake St. Clair (F_{ST} of 0.02–0.07, Supp. Table iv), which are currently more than 400 km apart, may also reflect historical patterns of connectivity. These populations may have been connected by gene flow in the past, as the species used to be found at several additional sites along northern Lake Erie, which are now considered extirpated (DFO 2010). mtDNA provided additional support for restricted gene flow among populations, as well as regional structure.

Another surprising finding was the very limited genetic differentiation among sites from three distinct drainage systems in Minnesota that are not currently connected (Great Lakes drainage basin, Red River of the North, and Mississippi River. These populations tended to cluster with geographically proximate populations rather than by watershed, suggesting relatively recent colonization. In particular, the two Red River samples clustered with different groups (Fig. 3), suggesting multiple colonization routes into the Red River drainage (one into Rainy River/Lake of the Woods and one into the Red River proper). Headwaters of the major drainage systems in Minnesota can occur very close to one another in low-lying marshy areas, which may allow for inter-basin dispersal during intermittent flooding events (Underhill 1957). However, with a small-bodied species like the Pugnose Shiner, another possibility is introduction by anglers via bait bucket transfer.

Evidence of small effective population size and population bottlenecks

Genetic factors, such as inbreeding depression, mutation accumulation, and reduced adaptive potential, may contribute to the vulnerability of the Pugnose Shiner. Identifying populations most at risk of deleterious genetic effects is critical for appropriate management decisions. Small N_e and severe reductions in N_e (genetic bottlenecks) can lead to a reduction in genetic variation, increases in inbreeding, and a reduction in long-term evolutionary potential (Frankham et al. 2002). If N_e remains small for a long period of time, mutation accumulation can further erode population fitness (Willi et al. 2013). The 50/500 rule in conservation biology states that an effective population size of 50 individuals is required for short-term viability to reduce the effect of inbreeding depression and an N_e of 500 individuals is necessary to ensure the adaptive potential over the long-term (Franklin 1980). More recent estimates suggest that an N_e of 1,000 may be required for long-term evolutionary potential (Franklin and Frankham 1998).

Here, we estimated N_e with the LDNe method knowing that sample size and number of loci used in this analysis may not provide sufficient power to determine N_e in all cases. For several populations, this proved to be the case. However, the analysis did provide evidence of relatively small N_e for Old Ausable Channel and Teeswater River, as well as extremely small lower CIs for Mouth Lake. MSVAR provided additional evidence of small effective population sizes in a number of populations, (N_e <30 in Weller Bay, Mouth Lake, Black River, Nashwauk Lake; N_e ~ 100 in Fish Lake, Old Ausable Channel, Teeswater River). Some uncertainty is associated with our N_e estimates, and Faurby and Pertoldi (2012) even documented a slight downward bias in current N_e estimates from MSVAR with pronounced departure from SMM. Nevertheless, our results suggest that multiple Pugnose Shiner populations may be at, or close to, the point at which inbreeding depression may significantly affect extinction risk.

Mouth Lake was the only population to show a significant result with the BOTTLENECK test. The program BOTTLENECK has only rarely shown conclusive evidence of genetic bottlenecks, failing to identify bottlenecks even in well-known population collapses such as the Scandinavian lynx, California sea otter, and Amur tiger (reviewed in Peery et al. 2012). Therefore, the finding of a bottleneck in Mouth Lake is noteworthy and suggests a strong reduction in genetic diversity in this population. Broquet et al. (2010) have shown that significant genetic bottlenecks using the program BOTTLENECK can result from either demographic decline or reduction in gene flow, therefore, the significant result in Mouth Lake may be related to recent isolation rather than population collapse. However, both demographic decline and a reduction in gene flow can have negative consequences for populations, reducing genetic variation, increasing the role of drift, and increasing inbreeding coefficients, particularly in a population as small as Mouth Lake (~5 ha lake with small N_e estimates, Table 2).

The M-Ratio test also showed evidence of population bottlenecks in three populations (Nashwauk Lake, Old Ausable Channel, East Lake), although only with the mutation model more closely resembling SMM. The M-Ratio test is often considered to be more powerful than BOTTLENECK; however, power is strongly reduced if genetic variation in the original population is low ($\theta \sim 1$), and if the mutation model departs from SMM (Peery et al. 2012). Peery et al. (2012) advise that when significant results are not found with all mutation models (including $p = 0.22$, also tested in our study), results should be interpreted as 'potential' cases of decline only. Therefore, we view our results with the M-Ratio test as tentative. As mentioned above, both Mouth Lake and Old Ausable Channel used to be part of the larger Ausable River system drainage but hydrological alteration has effectively isolated both populations. Our results illustrate the negative impacts of significant hydrological alteration on Pugnose Shiner populations. Significantly, a population contraction has been documented for another species in the Old Ausable Channel, the Greenside Darter (*Etheostoma blennioides*) (Beneteau et al. 2012), indicating that the genetic impacts of hydrological changes may be a general phenomenon in this region.

Identifying conservation units

Both Canada and the United States recognize genetic divergence below the species level in conservation legislation. Canada recognizes Designatable Units, DUs (Green 2005) and the United States recognizes Evolutionarily Significant Units, ESUs (Waples 1998). Both concepts seek to identify deep divergence within the species representing distinct evolutionary history and potential, and they both rely on genetic and ecological criteria. The genetic criterion used by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) to define DUs is 'fixed' genetic differences (i.e. a mutation that can be used to differentiate one region from another) as determined by 'slowly-evolving' genetic regions, such as mtDNA. Frequency differences at 'rapidly-evolving' loci such as microsatellites, for example, are not considered sufficient. In the United States, the criteria are similar. Waples (1991) defines ESUs as "substantially reproductively isolated from other conspecific population units" that represents "an important component in the evolutionary legacy of the species". In practice, ESUs are often identified by distinct genetic variation in mtDNA, and one of the more commonly applied criteria (de Guia and Saitoh 2007) is reciprocal monophyly of mtDNA lineages (Moritz 1994).

In this study, we did not find evidence that Pugnose Shiner populations exhibit either 'fixed genetic differences' or reciprocal monophyly of mtDNA across geographic regions. Biogeographic divisions (e.g. Mississippi River populations versus Great Lakes drainage populations) were not characterized by reciprocally monophyletic haplotype clades. We suggest that the Pugnose Shiner should be characterized as a single DU or ESU across its range. However, we note that ecological considerations were not taken into account here, making this a 'molecular-based' or 'partial' ESU (see de Guia and Saitoh 2007).

Conclusion

In summary, genetic data demonstrate that the Pugnose Shiner is characterized by small and relatively isolated populations. Barriers to gene flow (including geographic distance) appear to be common and most sites were found to be genetically differentiated from one another. Hydrological and other habitat alterations have likely had a strong effect on the species, most notably in Mouth Lake as well as the Old Ausable Channel. In contrast, the St. Lawrence River probably represents some of the best habitat for the Pugnose Shiner, supporting a greater density of occupied sites, greater opportunities for gene flow, and larger effective population sizes than elsewhere.

Population fragmentation leading to high levels of population differentiation is common in species at risk. The genetic differentiation and N_e and estimates we documented are typical of threatened and endangered species, although the most extreme case (e.g. Mouth Lake) appears to be unusual. The fragmented distribution and low dispersal potential of this species make it an unlikely candidate for 'genetic rescue' of small populations (e.g. Ingvarsson 2001; Vila et al. 2003). As such, management should emphasize protection and restoration of local habitat, with a focus on minimizing sedimentation and changes to hydrology to minimize turbidity and promote native submerged aquatic macrophyte growth.

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