

Low variation but strong population structure in mitochondrial control region of the plains topminnow, *Fundulus sciadicus*

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The plains topminnow *Fundulus sciadicus* is a freshwater killifish endemic to the Great Plains of North America. Rising concerns for future viability of this species have prompted recent changes in its conservation status. In this study, the results of a range-wide population genetic survey based on the sequence of entire mitochondrial control region (CR) are presented. A total of 181 fish were collected from 11 sites in Nebraska and 10 sites in Missouri spanning the distribution range of the species. Seven polymorphic sites were found in the 966 base pairs of the CR, and only nine unique haplotypes were detected among all fish. Phylogenetic analysis and statistical parsimony networks identified two distinct clades. The first included fish in the Osage, Gasconade and Spring River drainages in Missouri, while the second included individuals from Nebraska and the Lamine River in Missouri, although the Lamine River is much closer to the other Missouri sites than to the Nebraska sites. Pair-wise F_{ST} and average population distances indicated that populations from most drainages are genetically distinct, as 93% of the total molecular variance was attributed to among-drainage effects. Four sites within the main distributions of this species and a highly disjunct site from the south-western portion of the range are suggested as potential targets for conservation.

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INTRODUCTION

The plains topminnow *Fundulus sciadicus* Cope, Fundulidae, Cyprinodontiformes inhabits clear, cold, slow-moving water adjacent to or within beds of vegetation along the shoreline (Pflieger, 1997). It avoids interspecific competition and predation pressure by colonizing shallow pools and streams, where other species of *Fundulus* usually are not found (Brinkman, 1994). *Fundulus sciadicus* occurs in two major disjunct areas (Fig. 1). The first area spans

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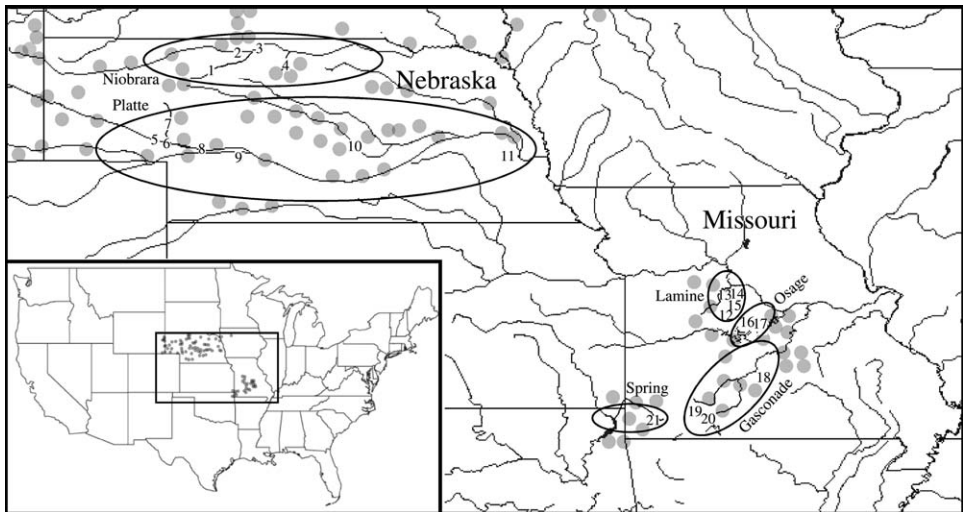


FIG. 1. Map of native range and sampling sites for *Fundulus sciadicus* in this study. Sampling locations correspond to those in Table I. Oval outlines inscribe the six drainages surveyed in this study. Shading represents the historical distribution of *F. sciadicus* based on museum records following Shute (1979). The inset illustrates the sampling locations in the context of a map of the U.S.A.

Central Missouri, south-east Kansas and north-west Oklahoma (Shute, 1979; Pflieger, 1997), including a disjunct offshoot in the Spring River drainage (Arkansas River basin) of south-west Missouri. The second area is in Nebraska and includes sparse areas in northeastern Colorado, eastern Wyoming, southern South Dakota and north-western Iowa (Shute, 1979; Pflieger, 1997). In Nebraska, it is widely distributed but sporadic and is more abundant in Sandhills streams than elsewhere (Stribley & Stasiak, 1982; Kaufmann & Lynch, 1991; Lynch & Roh, 1996).

Earlier ichthyological surveys (Lynch & Roh, 1996; Rahel & Thel, 2004) have documented a reduced incidence of the species at Nebraska sites, where it was once locally abundant. Other reports indicate that it may also be declining in Missouri (Pflieger, 1971) and may be extirpated in Oklahoma (Collier, 1998). *Fundulus sciadicus* populations may have been challenged by drought, water diversion and the introduction of exotic competitors, such as western mosquitofish, *Gambusia affinis* (Baird & Girard) (Lynch & Roh, 1996). *Fundulus sciadicus* has recently been listed as a Tier 1 species at risk in Nebraska because of its declining status and peculiar disjunct distribution (Schneider *et al.*, 2005). Although conservation concern for *F. sciadicus* is increasing, little is known about its population structure (O'Hare, 1985). *Fundulus sciadicus* are typically found in patchy headwater or backwater habitat (Lynch & Roh, 1996; Pflieger, 1997) and spawn eggs with thin filaments that entangle with submerged vegetation (Kaufmann & Lynch, 1991). The patchy distribution and adhesive eggs are conducive to population fragmentation of *F. sciadicus* within and among river drainages. The only population study published on this species to date is a survey among populations in Nebraska and Missouri using morphology and allozyme data (O'Hare, 1985). Contrary to expectations, O'Hare found extreme genetic

homogeneity within and between populations, suggesting high gene flow within drainages. This conclusion, however, may not be representative for the species as a whole as it was based on samples from a limited number of localities and on a small number of allozyme loci.

In this study, the first range-wide genetic survey of *F. sciadicus* is reported using the mtDNA control region (CR) as a genetic marker. The CR is one of the most frequently used markers for population studies because it mutates rapidly and usually sorts more quickly than nuclear loci because of its smaller effective population size. Thus, it may provide better resolution and reflect clearer population structure than allozymes. In this study, the phylogenetic relationship among disjunct populations is inferred, and the level of fragmentation (amount of gene flow) within and among drainages of the species, effective population size and population growth or decline rates are estimated. Another focus of this study is to identify populations with the highest genetic diversity as candidates for efforts of conservation.

MATERIALS AND METHODS

SAMPLE COLLECTION AND DNA EXTRACTION

Thirty-two sites in Nebraska and 20 sites in Missouri were surveyed where *F. sciadicus* collections had been previously documented. Dip-nets and a small beach seine were used, where workable, to catch the fish in typical minnow habitat (Lynch & Roh, 1996; Pflieger, 1997). The species was found at only 11 sites in Nebraska and 10 sites in Missouri (Table I and Fig. 1). A total of 86 fish from Nebraska and 95 fish from Missouri were collected (Table I). In addition, a small number of *Fundulus zebrinus* Jordan & Gilbert and *Fundulus catenatus* (Storer) were collected and used as outgroups. Specimens were stored in 95% ethanol. DNA was extracted from muscle tissue using the DNeasy Tissue Kit (Qiagen; www.qiagen.com).

POLYMERASE CHAIN REACTION AND SEQUENCING

The mtDNA CR was amplified following the method of Sivasundar *et al.* (2001). Polymerase chain reaction (PCR) products were sequenced using BigDye Terminator cycle sequencing (Applied Biosystems Inc.; www.appliedbiosystems.com) on a BaseStation 5100 fragment analyzer (MJ Research; www.mj-research.com). In addition to this standard experimental approach, the possibility that fragments amplified by this procedure could have originated from nuclear copies of mtDNA (Numts) was tested. It is well known that Numts can mislead genetic studies based on mtDNA (Zhang & Hewitt, 1996; Bensasson *et al.*, 2001). Although rare, Numts have also been recorded in fish (Telletchea *et al.*, 2006). For this control experiment, a long PCR approach was used to amplify the complete mtDNA genome in a few individuals to verify that the sequences originated from mtDNA (Thalmann *et al.*, 2004). Primers used for long PCR were 16S135F: 5'-GCAATAGAVAWAGTACCGCAAGG and 12S54R: 5' YCCAAGYG-CACCTTCCGGTA (Li & Orti, 2007). The mtDNA genome was amplified in a 10 µl reaction containing 1 µl Ex Taq buffer (Mg²⁺ plus), 0.8 µl deoxyribonucleotide triphosphate (dNTP) mixture buffer, 0.32 µl of each primer (20 µM), 0.05 µl TaKaRa Ex Taq™ Hot Start version, 0.4 µl template DNA (100 ng µl⁻¹) and 7.11 µl distilled water. The long PCR included 30 cycles of denaturing at 98° C for 10 s, annealing at 57° C for 15 s and extension at 72° C for 15 min, followed by a final extension for 15 min. Products from the long PCR were diluted 1:50 and used as a template for subsequent amplification of the mtDNA CR as described above (Sivasundar *et al.*, 2001).

TABLE I. Sampling localities for *Fundulus sciadicus*

Site*	Location	Drainage	State	n†	Haplotypes‡
1	Arkansas Flats	Niobrara River	NE	17	H1, H2
2	Niobrara River	Niobrara River	NE	4	H1
3	Minnechadua Creek	Niobrara River	NE	15	H1
4	Bone Creek	Niobrara River	NE	20	H1
5	North Platte, Lisco	Platte River	NE	2	H1
6	North Platte, Oshkosh	Platte River	NE	4	H1, H3
7	Blue Creek	Platte River	NE	5	H1, H3
8	Cedar Creek	Platte River	NE	2	H1
9	Fremont Slough East	Platte River	NE	5	H1
10	Middle Loup River	Platte River	NE	5	H1
11	Clear Creek	Platte River	NE	7	H1
12	Haw Creek	Lamine River	MO	10	H4, H5
13	Gabriel Creek	Lamine River	MO	10	H4
14	Middle Richland Creek	Lamine River	MO	10	H4
15	Richland Creek	Lamine River	MO	10	H4
16	Gravois Creek	Osage River	MO	9	H6, H8
17	Saline Creek	Osage River	MO	10	H6–H8
18	Mill Creek	Gasconade River	MO	9	H6
19	Osage Fork	Gasconade River	MO	9	H6
20	Prairie Brook	Gasconade River	MO	8	H6
21	Shoal Creek	Spring River	MO	10	H9
			Total	181	9

*Localities indicated by number in Fig. 1.

†Number of individuals used in this study.

‡Haplotypes network is shown in Fig. 2.

SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSIS

DNA sequences were aligned using Clustal X (Thompson *et al.*, 1997). Phylogenetic relationships among distinct haplotypes were assessed with the neighbour-joining (NJ) algorithm and Kimura 2-parameter (K2P) distances and with maximum parsimony (MP) as implemented in PAUP* 4.0 (Swofford, 2003). The K2P model was selected with Akaike information criteria (AIC) with ModelTest (Posada & Crandall, 1998). Bootstrap analyses with 1000 replications were performed for both MP and NJ methods. Statistical parsimony (SP) networks were constructed using TCS 1.21 (Clement *et al.*, 2000). To estimate divergence time between the major clades, a relaxed molecular clock model (Drummond *et al.*, 2006) implemented in BEAST 1.4.7 (Drummond & Rambaut, 2007) was used. This method accounts for uncertainty in rate variation among branches. To calibrate the absolute time of divergence, a molecular rate based on published estimates of CR divergences for percoid fishes of $1.8 \pm 0.23\%$ per million years was used (Donaldson & Wilson, 1999). The rate reference was used to set the lower and upper boundary for the prior of the mean rate in the log-normal relaxed molecular model.

INTRAPOPULATION AND INTERPOPULATION SEQUENCE ANALYSES

Samples were grouped into 'populations' according to river drainage (Fig. 1 and Table I) because no genetic structure was found among sampling localities within drainages. Pair-wise F_{ST} and average pair-wise population distances (Nei & Li, 1979) were

calculated based on the K2P distance using Arlequin (Schneider *et al.*, 2000). The distribution of genetic variance among and within populations was assessed with analysis of molecular variance (AMOVA) as implemented in Arlequin (Excoffier *et al.*, 1992). Within populations, mismatch distributions were checked against the null hypothesis of expanding population size (Schneider & Excoffier, 1999), and Tajima's *D* (Tajima, 1989) was calculated to test for neutrality of the CR sequences.

DEMOGRAPHIC ANALYSIS

To estimate demographic parameters of populations, such as effective population size and population growth rate, the coalescence approach implemented in LAMARC was used (Kuhner & Smith, 2007). Coalescent estimations were performed for each drainage separately because of the fragmented pattern among drainages. The Gasconade Fork and Shoal Creek samples were excluded from this analysis because they had a single haplotype. Simulations were run under the coalescent model with exponential growth to estimate the population parameters g (the exponential growth rate in unit of μ^{-1}) and θ ; $\theta = 2N_f\mu$, where N_f is female effective population size and μ is the per-site mutation rate. Final estimates were based on a run of 10 short chains, each sampling 500 trees and two long chains, each sampling 10 000 trees; the sampling interval was 20. Approximate CIs for these parameters were obtained using the percentile method.

RESULTS

SEQUENCE VARIATION AND THE PARSIMONY HAPLOTYPE NETWORK

A total of 181 individuals from 21 localities spanning the distributional range of the species were sequenced for this study. All *F. sciadicus* CR sequences were easily aligned as no alignment gaps were required; total length of the alignment was 966 base pairs (bp). Only seven sites were polymorphic, defining a total of nine unique haplotypes, H1–H9. Haplotype sequences were deposited in GenBank (EU182721–EU182729). All variable characters were parsimony informative.

The nine haplotypes were split into two major subclades in the parsimony network (Fig. 2). The first subclade included haplotypes from only the southern portion of the distribution, the Osage River (H6, H7 and H8), the Gasconade River (H6) and the Spring River (H9) in Missouri. The second subclade included individuals from the Niobrara River (H1 and H2) and the Platte River (H1 and H3) in Nebraska and the Lamine River (H4 and H5) in Missouri. Haplotypes from the Lamine River in Missouri were more closely related to haplotypes from Nebraska than to other haplotypes from Missouri (Fig. 2). Phylogenetic analysis of the nine *F. sciadicus* haplotypes plus outgroup taxa revealed the same genetic structure as the parsimony network (data not shown). The estimated divergence time between the two major clades was recent, at *c.* 0.622 (0.109–1.399) million years ago (MYA) by the relaxed molecular-clock model.

INTRAPOPULATION AND INTERPOPULATION SEQUENCE DIVERGENCE

Most samples from Nebraska harboured a single mtDNA haplotype (H1). Only the samples from two localities, Arkansas Flats (Niobrara River

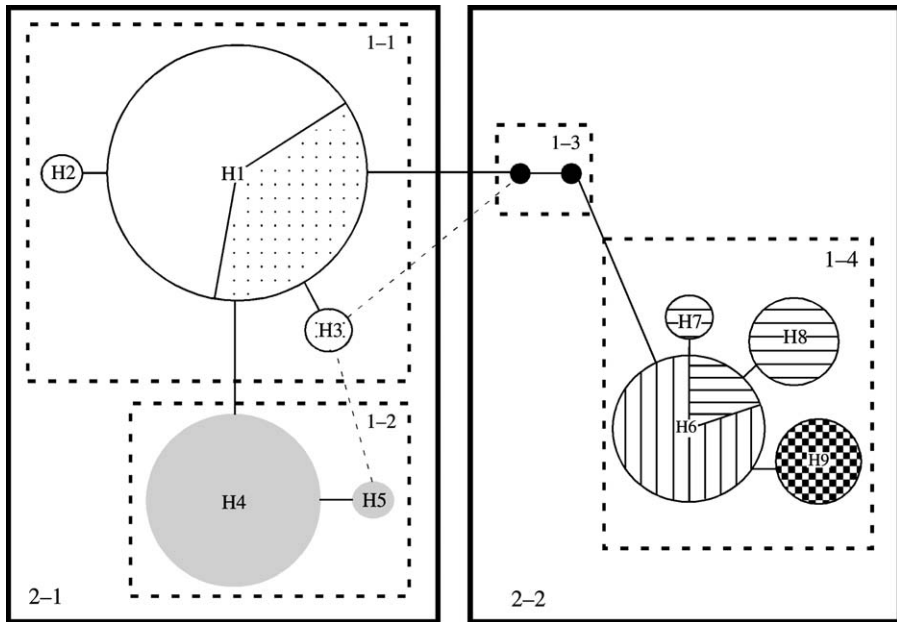


FIG. 2. Statistic parsimony network of nine haplotypes (H1–H9) in *Fundulus sciadicus*. Solid lines represent a single mutation. Dotted lines indicate ambiguity in resolving the haplotype network. Circle sizes are proportional to the number of individuals found for each haplotype (e.g. H1 was identified in 81 individuals and H2 in two individuals). (○) Niobrara, NE; (⊙) Platte, NE; (●) Lamine, MO; (⊖) Osage, MO; (⊕) Gasconade, MO; (⊗) Spring, MO; and (●) Missing haplotypes.

drainage) and Blue Creek (Platte River drainage), contained additional haplotypes (H2 and H3, respectively; Table I). In contrast, most of the variation (six haplotypes, H4–H9) was found among fish collected in Missouri. Saline Creek (Osage River drainage) showed the most diversity with three haplotypes (H6–H8), while samples from the other sites had one or two haplotypes (Table I). Because of the low level of variation in each site and the similarity among sites within the same drainage, sequences were grouped according to drainage for population analysis (Tables I and II).

The number of individuals sampled for each river basin varied between 10 and 56, with only 1–3 haplotypes in each drainage (Table II). The Osage sample had the highest within-population sequence divergence at 0.028% (Table II), while the Gasconade and Spring river samples each had a single haplotype. The overall average pair-wise sequence difference for all samples was 0.19%. Tests of the mismatch distribution did not reject the null hypothesis of a sudden population expansion in the Platte, Niobrara, Lamine and Osage drainages (Table II). All estimates of Tajima's D were negative, but none was significant ($P > 0.05$; Table II).

Pair-wise F_{ST} values indicated substantial differentiation among drainages, with values ranging from 0.84 to 1.00 ($P < 0.001$). Two exceptions were the Platte *v.* Niobrara ($F_{ST} = 0.07$) and the Osage *v.* Gasconade ($F_{ST} = 0.14$; Table III). The structure inferred from pair-wise F_{ST} also was supported by the

TABLE II. Within drainage descriptive values and neutrality statistics for *Fundulus sciadicus*

Populations	Platte	Niobrara	Lamine	Osage	Gasconade	Spring
Number of individuals	30	56	40	19	26	10
Number of haplotypes	2	2	2	3	1	1
Average pair-wise differences	0.019	0.007	0.010	0.028	0.000	0.000
Mismatch Ssd	0.00030	0.00001	0.00006	0.00223	—	—
P (Sim. Ssd \geq Obs. Ssd)	0.402	0.208	0.264	0.606	—	—
Tajima's D	-0.40885	-0.88483	-0.83603	-0.03486	—	—
P (D Sim. $\leq D$ Obs.)	0.36213	0.20005	0.21584	0.50036	—	—

Ssd, sum of squares deviation; Sim., stimulated; Obs., observed.

average pair-wise sequence divergences between drainages (Table III). To further assess the population structure, an AMOVA indicated that most of the total variance was explained by differences among drainages (93.14%), while only 0.98 and 5.88% of the variance was attributed to among sites within drainages and among individuals within sites, respectively (Table IV).

HISTORICAL DEMOGRAPHY

Because the mismatch distribution did not reject a population expansion model for all drainages, the coalescent model with exponential population growth in LAMARC was used to estimate demographic parameters for populations in the Platte, Niobrara, Lamine and Osage drainages separately. Estimates of theta (θ) and exponential population growth (g) appear in Table V. High growth rates were estimated for all populations, although the 95% CI was broad and included zero for each drainage.

DISCUSSION

LOW VARIATION IN MITOCHONDRIAL CR

In most species, the CR is the most variable portion of the mtDNA genome, presumably because of the lack of coding constraints (Simon, 1991);

TABLE III. Pair-wise F_{ST} and average pair-wise distances between river drainages. Above diagonal, pair-wise F_{ST} between river drainages; below diagonal elements, corrected average pair-wise distances [$P_{ixy} - (P_{ix} + P_{iy})/2$]; P values shown in parentheses

Population	Platte	Niobrara	Lamine	Osage	Gasconade	Spring
Platte		0.07 (0.04)	0.88 (0.00)	0.93 (0.00)	0.97 (0.00)	0.96 (0.00)
Niobrara	0.00 (0.05)		0.92 (0.00)	0.96 (0.00)	0.98 (0.00)	0.99 (0.00)
Lamine	0.10 (0.00)	0.10 (0.00)		0.96 (0.00)	0.98 (0.00)	0.98 (0.00)
Osage	0.29 (0.00)	0.30 (0.00)	0.40 (0.00)		0.14 (0.00)	0.84 (0.00)
Gasconade	0.29 (0.00)	0.30 (0.00)	0.40 (0.00)	0.00 (0.00)		1.00 (0.00)
Spring	0.39 (0.00)	0.40 (0.00)	0.50 (0.00)	0.10 (0.00)	0.10 (0.00)	

TABLE IV. Results of a hierarchical analysis of molecular variance in *Fundulus sciadicus*

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	<i>P</i> value
Among drainages	5	160.39	1.1106	93.14	0.0000
Among sites within drainages	15	2.47	0.0117	0.98	0.0195
Within sites	160	11.22	0.0701	5.88	0.0000
Total	180	174.07	1.1924		

consequently, it has been widely used in population studies, including three *Fundulus* species (Bernardi & Talley, 2000; April & Turgeon, 2006; Bernardi *et al.*, 2007). Average CR sequence divergence between haplotypes in *F. sciadicus* (0.19%) is strikingly low compared with other species such as *Fundulus parvipinnis* Girard (Bernardi & Talley, 2000), and *Fundulus lima* Vaillant (Bernardi *et al.*, 2007), with values of 5.8 and 1.94%, respectively. April & Turgeon (2006) also reported 20 variable positions in only 389 bp in the CR of *Fundulus diaphanus* (Lesueur). In contrast, in this study, only seven variable sites appeared in 966 bp of the CR of 181 individuals of *F. sciadicus* covering the whole species distribution. Compared with other *Fundulus* species, *F. sciadicus* has 7–30 times less variation at this locus. Exceptionally, low sequence variation in the mtDNA CR has been found in a few other freshwater species, such as pike, *Esox lucius* L. in Central and Southern Europe (Nicod *et al.*, 2004) and yellow perch, *Perca flavescens* (Mitchill) in Lake Erie (Ford & Stepien, 2004). Authors of both studies attributed the low genetic variation to bottleneck effects (Ford & Stepien, 2004; Nicod *et al.*, 2004) or, alternatively, to the stocking effects in the case of *E. lucius* (Nicod *et al.*, 2004).

Possible explanations for the low variation in *F. sciadicus* include selective sweeps or bottlenecks in population size. Negative Tajima's *D* values (Table II) suggest that selection or population bottleneck effects are plausible (Table II); however, none of these values is significant, probably because of the extremely low variation. Mismatch distributions are consistent with population expansions, as the sudden population expansion model cannot be rejected in any of the four drainages tested (Table II). However, if the demographic events (expansions after bottlenecks) shaped the current genetic structure of *F. sciadicus*, the putative population fluctuations have to be reconciled with the limited

TABLE V. Theta and population growth rate estimates using coalescent method in LAMARC; 95% CI indicated in parentheses

Population	Theta (θ)	Growth rate (<i>g</i>)
Niobrara	0.000341 (0.000196–0.001057)	8301 (–5000–8301)
Platte	0.000789 (0.000136–0.00201*)	15 000 (–5000–25 000)
Lamine	0.000638 (0.000242–0.00186*)	15 000* (–5000–15 000)
Osage	0.000369 (0.000078–0.001679)	3955 (–5000–25 000)

*This profile value had a warning from the maximizer, probably a failure to converge.

dispersal capabilities of *F. sciadicus* and its wide distribution within drainages. These results should be interpreted with caution, and additional data from nuclear loci (e.g. microsatellites) should be collected to improve the power of the analysis and to provide more robust tests to distinguish between selection and demographic history as the dominant force shaping the population structure in *F. sciadicus*.

In addition to selective sweeps and bottleneck effects, these results could also arise from mistakenly sampling Numts because Numts can exhibit extremely low variation (Zhang & Hewitt, 1996; Zhang *et al.*, 2006). This possibility is ruled out by the experimental control using long PCR to amplify the complete mtDNA genome and to use this as a template for a second round of PCR with CR sequence primers. All sequences amplified from whole mtDNA genome templates were the same as those directly amplified from genomic DNA with a single PCR reaction, thus no Numts were identified in this study.

POPULATION STRUCTURE AND CONSERVATION

Although sequence variation in the CR is low in *F. sciadicus*, the level of divergence among drainages is noteworthy. Except for the Platte *v.* Niobrara and the Osage *v.* Gasconade comparisons, all pair-wise F_{ST} and average sequence differences between samples show significant divergences (Table III). AMOVA corroborates these results as most of the variance is attributed to among drainage effects (Table IV). Population structure is also revealed by the phylogeny and the parsimony network, which indicates four distinct populations: Niobrara–Platte, Lamine, Osage–Gasconade and Spring River (Fig. 2). One important result supported by both SP (Fig. 2) and phylogenetic analysis (data not shown) is that the Lamine population is more closely related to Nebraska samples than to the rest of the Missouri samples, even though the Lamine River is 500 km closer to other Missouri sites than it is to the closest Nebraska site (Fig. 1). O'Hare (1985) also found that the level of divergence among *F. sciadicus* populations within Missouri is similar to that between Nebraska and Missouri using morphology and allozymes. O'Hare attributed the divergence between Lamine and Gasconade populations to habitat differentiation and selection, but the results indicate that the oldest vicariant event within the current distribution of *F. sciadicus* lies between Lamine + Nebraska and Osage + Gasconade. This suggests that differences between the Lamine and the Gasconade drainages may be caused by other historical mechanisms.

Events associated with Pleistocene glaciations were previously invoked to explain the peculiar distribution of *F. sciadicus* (Metcalf, 1966; Cross, 1970; Pflieger, 1971). Stream captures following glaciation may have provided dispersal routes to disjunct portions of the range currently in Missouri (O'Hare, 1985), in particular the Spring River drainage. Increases in water temperature and siltation following late Pleistocene warming may have caused the extirpation of *F. sciadicus* from large portions of a more continuous historical range. Presently, spring-fed streams in Sandhill and Ozark highlands provide cool, clear water for extant populations. The estimated divergence time between the two primary clades of 0.622 MYA is consistent with a late Pleistocene fragmentation. Similar disjunct distributions have been seen in other small species

in the same region. For example, the southern redbelly dace, *Phoxinus erythrogaster* (Rafinesque) (Stasiak, 2007) and the Ozark minnows, *Notropis nubilus* (Forbes) (Mayhew, 1987) have a major distribution in the Ozark Plateau and in the isolated populations in the north. The historical forces that shaped the current distributions of the plains topminnow could also affect the two species in the same way.

Based on the current fragmented distribution, conservation status and the structure apparent in this study, candidate populations that should be targeted for conservation are found in the Arkansas Flats of the Niobrara drainage, Blue Creek in the Platte drainage, Haw Creek in the Lamine drainage and Saline Creek in the Osage drainage. These sites are important because they hold a larger number of haplotypes than other intra-drainage areas. Within the highly disjunct Spring River drainage of south-west Missouri, samples were found only from a single site. Populations in this area have probably suffered from frequent introductions of exotic salmonids (Turner, 1979). Indeed, the single population found occurred in a remote pool apparently inaccessible by downstream salmonids. All 10 individuals from this site possessed an identical endemic haplotype; thus, it should be considered as an additional target area for *F. sciadicus* conservation.

Although limited variation exists in the CR of *F. sciadicus*, the results reveal a strong signal of population structure among drainages and indicate a possible population expansion in the past. Most importantly, the results clearly suggest target regions for potential conservation, which has the urgency to act given the declining status and the rising conservation concern for this species.

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