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Gene Flow and Genetic Structure of Two of Arkansas's Rarest Darter Species (Teleostei: Percidae), the Arkansas Darter, *Etheostoma cragini*, and the Least Darter, *E. microperca*

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Running Title: Genetic Structure of Arkansas's Rarest Darter Species

Abstract

Distinguishing the effects of naturally caused historical fragmentation from those of contemporary landscape modification is critically important to understanding the consequences of human influences on patterns of gene flow and population dynamics. Nonetheless, relatively few recent studies focusing on this issue have dealt with species that showed evidence of historical fragmentation. In the current study, we disentangled the effects of fragmentation operating over separate timescales on two darter species, *Etheostoma cragini* and *E. microperca*, from the Ozark Highlands. Formerly more wide-spread within this region in Arkansas, these species now occur only in highly isolated habitats (*i.e.*, spring-runs). We separated fragmentation effects at distinct spatial and temporal scales by using several molecular loci (i.e., mtDNA/nuclear DNA/nuclear microsatellite DNA), as well as a variety of analytical approaches. Sequence divergence among Ozark and northern populations of *E. microperca* indicate long-standing isolation resulting from vicariant events. Both species were further isolated in unique 'island' habitats, sometimes at fine spatial scales, as shown by sequence divergence among Ozark Highland populations of *E. cragini*. Microsatellite data also revealed additional subdivision among Arkansas populations with *E. cragini* divided into three distinct populations and *E. microperca* into two. Overall, migration rates were similar among contemporary and historical time periods although patterns of asymmetric migration were inverted for *E. cragini*. Estimates of contemporary effective population size (N_e) were substantially lower for both species than past population sizes. Overall, historical processes involving natural fragmentation have had long-lasting effects on these species, potentially making them more susceptible to current anthropogenic impacts.

Introduction

Habitat fragmentation operating both over historical time scales and over more recent timescales results in species with highly fragmented distributions, significantly compromising the maintenance of genetic diversity and population viability (Keyghobadi *et al.* 2005; Zellmer and Knowles 2009). Distinguishing between these time scales is important to conservation efforts because knowledge of historical population structure is essential to assessing the impact of current anthropogenic effects. Several recent studies comparing past and current patterns of gene flow among populations have revealed that recent human activities have substantially altered connectivity among populations, resulting in increased bottlenecks and high levels of inbreeding (Reed *et al*. 2011; Apodaca *et al.* 2012; Blakney *et al.* 2014); others suggest the high levels of structure observed among populations reflect long-standing limited dispersal of the species rather than recent habitat fragmentation (Chiucchi and Gibbs, 2010). These two causes of fragmentation may also act synergistically, such that the historically fragmented populations of a species become reduced in number or each experience declines in membership due to anthropogenic effects. Populations that are both highly fragmented and exhibit reduced population sizes have high rates of local extinction and therefore higher probability of global extinction (Templeton *et al.* 1990). Recent fragmentation may also substantially influence metapopulation dynamics, which may play a critical role in contributing to adaptive differences observed among populations even at small spatial scales (Zellmer and Knowles 2009).

An ideal setting for studying the consequences of natural fragmentation occurs in the Ozark Highlands region extending from southern Missouri to northern Arkansas, USA. This region is well known for having

historical climatic fluctuations and complex geology and topography, including distinctive karst topography (Templeton *et al.* 1990). It is hypothesized that many aquatic species entered this region during a cooler and wetter Pleistocene and later became isolated in fragmented habitats as the region dried during arid conditions of the Late Wisconsin (Cross *et al.* 1986). The detrimental effects of recent habitat loss and fragmentation may be amplified for these species because populations often display disjunct distributions and are associated with habitats displaying unique features. For example, species closely associated with groundwater-fed springs in this region exhibit patchy distributions and high endemism due to the sporadic location of habitat islands (Robison and Buchanan 1988; Pflieger 1997). The stable environment provided by spring ecosystems (Hubbs 1995) may have facilitated the persistence of these fragmented populations over time, despite potentially facing reduced genetic diversity and small population sizes (Fluker *et al.* 2010). These habitats are, however, easy targets for anthropogenic impacts which have resulted in further population fragmentation and increased imperilment of spring species (*see*, *e.g.*, Fluker *et al.* 2009).

Although the genetic consequences of habitat fragmentation are well known (Keyghobadi 2007), relatively few studies have disentangled the effects of both past and recent events when assessing the impact on species having naturally fragmented distributions (although *see* Apodaca *et al.* 2012). In this study, we apply a variety of analytical approaches including genetic assignment and coalescent methods to several molecular loci (mtDNA/nuclear DNA/nuclear microsatellite DNA) having different mutation rates and levels of variability to detect genetic structure at distinct spatial and temporal scales. Using this comprehensive approach, we assess the genetic diversity and population structure of two of Arkansas's rarest darter species, the Arkansas Darter, *Etheostoma cragini* Gilbert, and the Least Darter, *Etheostoma microperca* Jordan and Gilbert. Both of these species have highly fragmented distributions in the Ozark Highland region of northwestern Arkansas, with their presence critically dependent on availability of spring-runs filled with abundant aquatic vegetation, and their existence under significant and immediate threats from rapid urban and suburban development (Wagner *et al.* 2011,2012). We evaluate genetic diversity and historical isolation of disjunct populations in Arkansas compared to other populations with sequences from the mitochondrial cytochrome *b* gene and nuclear S7 intron. Analyses of more rapidly-evolving nuclear microsatellite data allow us to independently examine current genetic diversity, and to identify fine-scale subdivision among Ozark populations. Using microsatellites, we estimate contemporary and historical migration rates among populations and effective population sizes to separate the influence of fragmentation processes operating over different time scales. Similarly, we evaluate the significance of any potential recent or historical reduction in population size using several methods to detect population bottlenecks. Our results allow us to make comparisons with spring-endemic taxa elsewhere and to make inferences about metapopulation dynamics of species confronted with reduced habitat connectivity as in the Ozark Highlands.

Materials and Methods

Distribution and sampling

All historic localities for *E. cragini* and *E. microperca* in Arkansas and additional nearby sites were sampled during 2009-2011 (*see* Wagner *et al.* 2011, 2012 for more details). For *E. cragini*, caudal fin clips were taken from a total of 117 individuals from 13 locations, representing the entire range of the species in Arkansas (*see* Fig. 1a). Fin clips from two additional locations were obtained from Missouri, in Shoal Creek and the headwaters of Spring River. For *E. microperca*, caudal fin clips or in some cases whole specimens were taken of 235 individuals from 29 locations. Samples were obtained from three major areas in Arkansas: Little Osage Creek/Osage Creek; Flint Creek; and Clear Creek (Fig. 2a). We obtained comparative material from 22 individuals from northern populations in Illinois (1), Indiana (3), Ohio (5), Michigan (11), Ontario (1), and Wisconsin (1). For both species, total genomic DNA was isolated from each individual using DNeasy Tissue Extraction Kits (Qiagen, Valencia, CA, USA) following manufacturer's instructions.

DNA sequencing and microsatellite genotyping

The complete mitochondrial cytochrome *b* (cyt *b*) gene (1140 base pairs) was amplified using primers located in flanking $tRNA^{GLU}$ and $tRNA^{THR}$ genes (Schmidt and Gold, 1993) for a subset of individuals from populations selected for each species (*E. cragini*, n = 32; *E. microperca*, n = 65). PCR was performed in 25 µl reactions containing 10-50 ng DNA, 0.8 mM dNTP, 0.4 µM each primer, 1.5 mM $MgCl_2$, and 1 Unit Taq polymerase (Promega, Madison, WI). Cycling conditions were 4 min at 94° C followed by 40 cycles at 94° C (1 min), 48° C (1 min), 72° C (2 min), with a final extension at 72° C for 7 min. The first intron of the

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nuclear S7 ribosomal protein intron 1 was amplified with universal primers (Chow and Hazama, 1998) for the same individuals amplified for cyt *b*; the thermal profile for cyt *b* was modified to include an annealing temperature of 59° C for S7 amplifications. Sequencing reactions were performed by htSEQ High-Throughput Genomics Unit (University of Washington, USA). The first 57 and last 78 nucleotides of the cyt *b* gene were excluded prior to analyses due to poor quality, resulting in the final cyt *b* fragment length of 1005 nucleotides and a S7 fragment length of 540 nucleotides (GenBank accession numbers KC445320-KC445462).

Nine microsatellite loci used previously in studies of darters were identified as having potential variability in *E. cragini*: EosC117, EosC6, EosC112, EosC208, EosD108, EosD11, EosD107, EosC2 (Switzer *et al.* 2008) and Esc26b (Gabel *et al.* 2008). Seven microsatellite loci were chosen for *E. microperca* based on ease of amplification and allele calling: EosC3, EosC6, EosD108, EosC208, EosC2, EosC124 (Switzer *et al.*, 2008) and Esc26b (Gabel *et al.* 2008). PCR for microsatellite loci was performed in 10 µl reactions containing ~10 ng DNA, 0.8 mM dNTP, 0.2 µM each primer, 1.5 mM $MgCl_2$, and 1 Unit Taq polymerase (Promega, Madison, WI) under the thermal cycling conditions of Switzer *et al.* (2008). Labeled PCR products were loaded into ≈ 20 µl reactions with 20 µl SLS and 0.25 µl 400 size STD (Beckman Coulter, Saint Louis, MO, USA) and genotyped using a Beckman CEQ8000 Genetic Analysis System (Department of Biology, Saint Louis University). Alleles within the designated range for each locus were called by eye.

Genetic data analysis

All analyses were conducted independently for each species. Genetic diversity estimates from cyt *b* were computed using ARLEQUINv3.11 (Excoffier *et al.* 2005), including the mean number of pairwise differences (π, nucleotide diversity, Nei, 1987) and the probability that two randomly chosen haplotypes are different (*h*, equivalent to gene diversity, Nei 1987). We performed an Analysis of Molecular Variance (AMOVA) in ARLEQUINv3.11 to test for hierarchical partitioning of genetic structure among populations (*see* supplementary data at the Journal website). Significance was assessed using 1000 permutations for all calculations. Haplotype networks were constructed using the median-joining method in NETWORKv4.610 (Bandelt *et al.*, 1999). Haplotype reconstruction for sequences of the S7 intron with ambiguous sites was conducted using PHASEv2.1 (Stephens *et al.* 2001; Stephens and Donnelly 2003). Missing data and invariable sites were excluded when constructing median-joining networks.

Microsatellite loci were examined for evidence of null alleles and scoring errors using MICROCHECKERv2.2 (van Oosterhout *et al.* 2004). To test for conformity to Hardy-Weinberg equilibrium (HWE), exact tests were performed for all loci using GENEPOPv4.010 (Guo and Thompson 1992) with significance assessed using Markov chains with 1000 dememorizations and 100 batches with 1,000 iterations per batch. Standard genetic diversity was measured in GENALEXv6.2 (Peakall and Smouse 2006) as the total number of alleles (N_A), mean number of alleles per locus (A_M), Shannon's Information Index (I), observed heterozygosity (H_O), unbiased heterozygosity (H_E), and number of private alleles unique to a population (PA). Allelic richness (AR) and private allelic richness (PAR) independent of sample size were also calculated using HP-RAREv1.0 (Kalinowski, 2005). Using the microsatellite DNA data, the Bayesian clustering method in STRUCTUREv2.3.3 (Pritchard *et al.* 2000) was implemented to determine the appropriate number of genetic clusters without any *a priori* group assignment. A model allowing admixture of genotypes and correlated allele frequencies between populations was used to assess the best value of K, the number of discrete populations (Falush *et al.* 2003). Twenty replicate runs consisted of a burn-in of 100,000 generations followed by an additional 500,000 iterations for each K ranging 1 to 10. The best value of K was chosen by determining ΔK, whereby the rate of change in the log likelihood values between successive K values was assessed (Evanno *et al.* 2005). The final results for chosen K values were visualized with DISTRUCT (Rosenberg 2004).

Three methods were used to detect genetic signatures of changes in population size due to bottlenecks using the microsatellite DNA data. First, the M-ratio test (Garza and Williamson 2001) was used to detect bottlenecks that occurred over relatively long periods of time (>100 generations). The program M P Val was used to estimate M-ratios of the number of alleles to the range in allele size and compare them to population specific critical M values (M_c) estimated using the program Critical M (Garza and Williamson 2001). The M-ratio was estimated using the two-phase model (TPM) with 90% single-step mutations, mean size of non-stepwise mutations=3.5, and pre-bottleneck θ value of 4, as suggested by Garza and Williamson (2001). A total of 10,000 simulation replicates were run for the calculation of M_c (Garza and Williamson 2001). Second, the Wilcoxon's sign rank test was used to detect

bottlenecks occurring over approximately the last $<4N_e$ generations. Third, the mode-shift test was used to detect population declines that may have occurred within the last few dozen generations (Cornuet and Luikart 1996; Luikart *et al.* 1998). Both of these tests were performed using BOTTLENECKv1.2.02 (Piry *et al.* 1999). A total of 10,000 replicates were run under a two-phase model (TPM) with 95% single step mutations and 5% multi-step mutations, variance for mutation size was 12, as suggested by Piry *et al.* (1999). For all analyses, populations were defined based on the results obtained with STRUCTURE.

The Bayesian coalescent approach of the program Migrate-*n* v3.2.11 (Beerli 2009) was used to estimate the historical effective population size ($\theta = 4N_e\mu$, where μ = mutation rate) and past (roughly $4N_e$ generations) migration rates between populations ($M = m/\mu$, where m = migration rate) from the microsatellite datasets (Beerli 2009). The Bayesian inference method was used with uniform priors (range = 0 - 100, delta = 10) and slice sampling with one long chain and a sample increment of 1000 for 50,000 recorded steps, with 500,000 discarded as initial burn-in. Five replicates were run using a static heating scheme (1, 1.5, 3.0, 10000) with a swapping interval of 1, for a total of 250,000,000 visited parameter values. Final priors and starting values for θ and M were chosen based on results of multiple trial runs. Final runs were performed in parallel on the bioserv cluster at the University of Nebraska-Lincoln.

We used the program BayesAss v3.03 (Wilson and Rannala 2003), which implements a Bayesian MCMC approach and genetic assignment method, to estimate contemporary rates of migration within the last five generations. For each species three independent runs were conducted with 10 million iterations and a 1 million generation burn-in, sampling every 1,000 generations. To compare estimates of historical migration rates from Migrate ($M = m/\mu$) with contemporary gene flow estimates from BayesAss (m) we multiplied all M values by the mutation rate (μ), where $\mu = 5.0 \times 10^{-4}$ (Yue *et al.* 2007). To estimate contemporary effective population sizes (N_e), we performed the sibship assignment method implemented in COLONY V2.0 (Wang 2009a). The sibship assignment method is more accurate than e.g., the heterozygote excess method, the linkage disequilibrium method, and the temporal method at estimating N_e (Wang 2009a).

Results

Etheostoma cragini

Only two unique haplotypes of cyt *b* were recovered from 16 individuals from Arkansas (Fig. 1b). Consequently, mean uncorrected cyt *b* divergence among Arkansas samples was low (0.102%); however, divergence among Arkansas and Missouri populations was relatively high (2.086%). For the nuclear S7 intron, thirteen unique haplotypes were detected from 34 phased sequences. Five S7 haplotypes representing Arkansas populations were separated by 6 mutation steps from the nearest Missouri haplotypes. Genetic diversity (π and h) estimates from cyt *b* for Arkansas populations were low compared to Missouri populations (unpaired t-test, P = 0.0001 and P = 0.5309, respectively; *see* supplementary data at the Journal website).

For the microsatellite DNA data there was no evidence of scoring errors or allelic dropout detected by MICRO-CHECKER. Four localities deviated from HWE: Clabber Creek AR, Healing Spring AR, Osage Creek AR, and Shoal Creek MO. Following Bonferroni correction, only one locus (EosD11) for Shoal Creek MO was significant. The number of alleles per locus was generally low, ranging from 1-16 with an average of 2.496 (±0.2) alleles per locus. Microsatellites also indicate low genetic diversity of Arkansas populations (Table 1). Estimates of allelic richness were the highest for Missouri populations (avg. 3.68). Missouri populations also had a high number of private alleles (15 and 17, respectively), indicating isolation and reduced gene flow among populations.

STRUCTURE analysis identified two distinct genetic clusters: (1) Arkansas populations and (2) Missouri populations. Secondary runs containing only Arkansas populations identified an additional four genetic clusters: (1) Clabber Creek/Wilson Spring, (2) Turentine Spring, (3) Chamlin-Wise Spring/Osage Creek, and (4) Lick Creek/Unnamed Spring; with remaining individuals having mixed assignment to either the third or fourth cluster preventing these clusters from being clearly separated (Fig. 1c).

Overall, migration rates averaged across all comparisons were similar during both time periods (0.0225 vs. 0.0297, two-tailed P = 0.6167; *see* supplementary data at the Journal website). However, contrasting patterns of asymmetric migration were observed (Fig. 3). Historically, migration rates from Osage Creek to Clabber Creek/Wilson Spring and Turentine Spring were substantially higher (0.0296 and 0.0375, respectively) than rates from these populations to Osage Creek (0.0016 and 0.0026, respectively).

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Table 1. Genetic diversity estimates from nuclear microsatellite loci of Arkansas Darter, *Etheostoma cragini*, and Least Darter, *E. microperca*, genetic clusters identified by STRUCTURE

Species/Population	N	N_A	A_M	I	H_O	H_E	PA	AR (PAR)
Etheostoma cragini								
Spring River, MO	24	50	5.556(1.608)	1.042(0.280)	0.489(0.118)	0.493(0.107)	17	3.81(1.40)
Shoal Creek, MO	26	45	5.000(1.658)	1.013(0.296)	0.432(0.134)	0.495(0.126)	15	3.56(1.28)
Osage Creek, AR	52	38	4.222(0.830)	0.854(0.214)	0.311(0.090)	0.446(0.107)	11	2.96(0.69)
Clabber Creek/								
Wilson Spring, AR	8	19	2.111(0.423)	0.383(0.131)	0.088(0.037)	0.227(0.074)	3	2.07(0.44)
Turentine Spring,AR	7	14	1.556(0.242)	0.294(0.123)	0.206(0.104)	0.210(0.088)	0	1.56(0.10)
Etheostoma microperca								
OsageCk/FlintCk, AR	54	71	10.143(4.194)	1.221(0.522)	0.395(0.179)	0.430(0.174)	30	6.11(2.61)
Trudell Spring, AR	32	30	4.286(1.507)	0.783(0.336)	0.366(0.144)	0.364(0.146)	4	3.55(0.97)
Northern populations								
(IL, IN, OH, MI, WI)	63	72	10.286(3.037)	1.299(0.459)	0.397(0.168)	0.475(0.159)	27	6.23(2.04)
Deer Creek, OH	16	31	4.429(1.716)	0.799(0.362)	0.384(0.168)	0.362(0.158)	2	4.03(0.68)
Mallet River, ON/								
Doke Lake, OH	16	20	2.857(0.937)	0.594(0.287)	0.265(0.134)	0.314(0.149)	2	2.72(0.35)
Tenmile Creek, OH	12	17	2.429(0.841)	0.458(0.252)	0.190(0.097)	0.246(0.133)	1	2.43(0.22)

N – Number of genotypes examined, N_A – Total number of alleles, A_M – mean number of different alleles, I – Shannon's Information Index, H_O – observed heterozygosity, H_E – Nei's (1987) unbiased heterozygosity, PA – Number of alleles unique to a single population, AR – Allelic Richness with rarefaction, PAR – Private Allelic Richness with rarefaction.

Estimates of contemporary migration, however, exhibit a reversed pattern, with substantially higher rates to Osage Creek (0.0386 and 0.0586) versus from Osage Creek (0.0071 and 0.0104).

Contemporary effective population sizes (N_e = 12 – 31) were consistently an order of magnitude lower than historically (N_e = 208 – 544) (Table 2). Evidence of historical population bottlenecks were observed with *M*-ratios below critical M_c values (M_c = 0.62 – 0.72). More recent population declines were detected by the Wilcoxon's sign rank test for Clabber Creek/Wilson Spring ($P < 0.05$) and by the mode-shift test for Clabber Creek/Wilson Spring and Turentine Spring.

Etheostoma microperca

Two cyt *b* haplotypes were recovered from nine individuals from Arkansas. These haplotypes were separated by more than 62 mutation steps from other populations, representing substantial cyt *b* divergence (7.57%) and indicating long-term isolation from northern populations (Fig. 2b). For the nuclear S7 intron, ten unique haplotypes were detected from 44 phased sequences. Three S7 haplotypes representing Arkansas populations were separated by 9 mutation steps (1.78% uncorrected divergence) from haplotypes of northern populations. Results from cyt *b* indicate significantly lower genetic diversity (π and *h*) of populations from Arkansas relative to northern populations (unpaired t-test, P = 0.0001 and P = 0.0025, respectively; *see* supplementary data at the Journal website).

For the microsatellite DNA data there was no evidence of scoring errors or allelic dropout detected by MICRO-CHECKER. Three localities deviated from HWE: Nichols Lake MI, Spring Creek IL, and Healing Spring AR. Following Bonferroni correction, one locus (EosC6) for Nichols Lake and one locus (EosC2) for Healing Spring were significant. The number of alleles ranged from 1-23 with an average of 3.444 (±0.335) alleles per locus. Analyses of microsatellite data indicate lower genetic diversity (A_M, I, H_E, AR) of Arkansas populations identified by STRUCTURE (Table 1). Comparisons among individual Arkansas localities revealed several having relatively moderate genetic diversity (*see* supplementary data at the Journal website). In particular, Healing Spring had higher genetic diversity with A_M = 8.429, Shannon's I = 1.138, and 11 private alleles; however, after accounting for sample size, this locality had only the fifth highest estimate for allelic richness (2.13). STRUCTURE analysis identified two distinct genetic clusters: (1) Arkansas populations and (2) northern populations. Additional runs containing only Arkansas populations identified an additional two genetic clusters: (1) Trudell

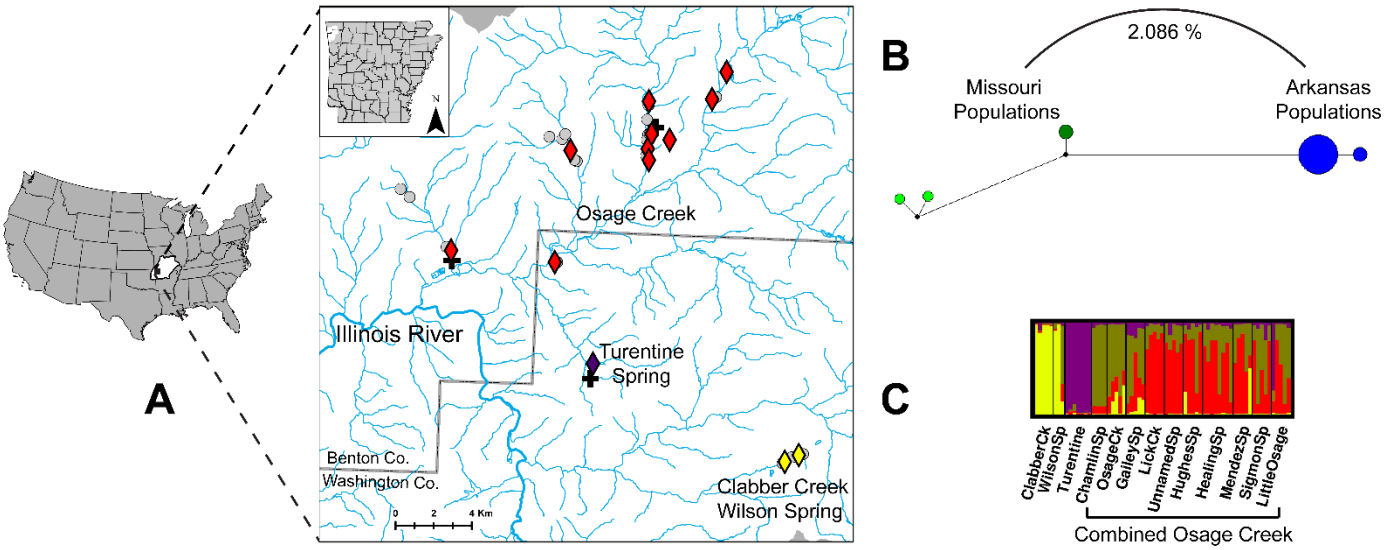

Figure. 1 (A) Map showing collection sites and major river drainages for the Arkansas Darter, *Etheostoma cragini*, in Arkansas, USA with inset map highlighting the Ozark Highlands outlined in white. Symbols designating genetic sites are colored according to populations identified by STRUCTURE analyses (See online version for full color). Extirpated sites are indicated by a cross (*see* Wagner *et al.* 2011 for additional information). (B) Median-joining haplotype network for *E. cragini* based on data from the mitochondrial cytochrome *b* gene. Mean uncorrected sequence divergence is shown among populations. Circle sizes reflect relative frequency of haplotypes, with smallest circles representing extinct or unsampled haplotypes. (C) Results from STRUCTURE analysis of Arkansas localities of *E. cragini*. Shown are individual assignment proportions to four clusters with each vertical bar corresponding to a single individual in the dataset.

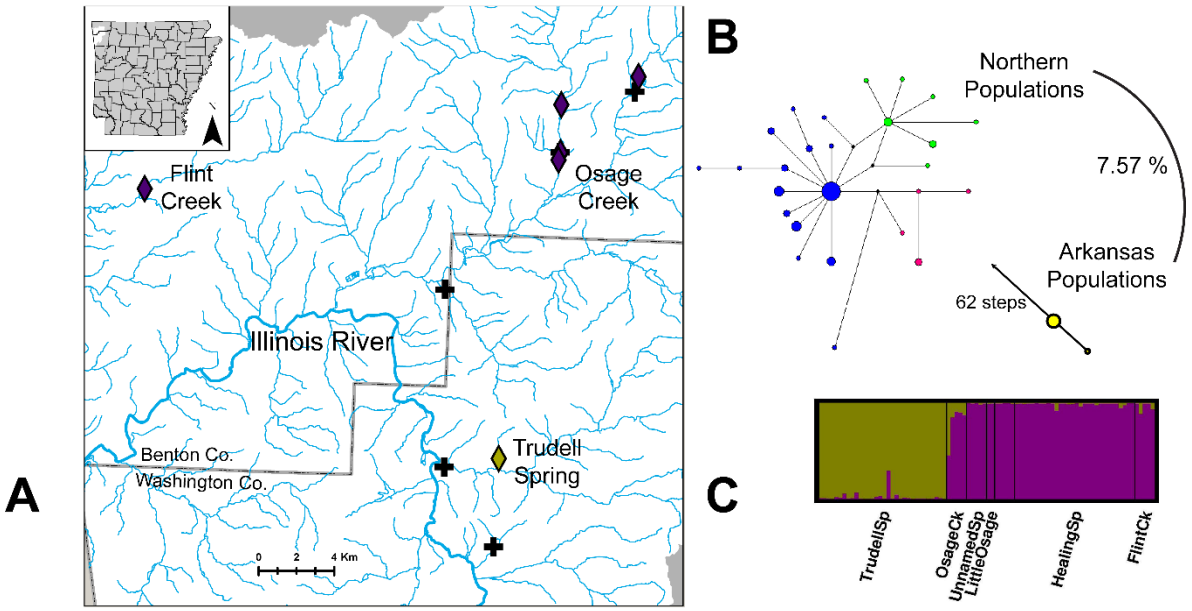

Figure 2. (A) Map showing collection sites and major river drainages for the Least Darter, *Etheostoma microperca*, in Arkansas, USA. Symbols designating genetic sites are colored according to populations identified by STRUCTURE analyses (See online version for full color). Extirpated sites are indicated by a cross (*see* Wagner *et al.* 2012 for additional information). (B) Median-joining haplotype network for *E. microperca* based on data from the mitochondrial cytochrome *b* gene. Mean uncorrected sequence divergence is shown among populations. Circle sizes reflect relative frequency of haplotypes, with smallest circles representing extinct or unsampled haplotypes. (C) Results from STRUCTURE analysis of Arkansas localities of *E. microperca*. Shown are individual assignment proportions to two genetic clusters with each vertical bar corresponding to a single individual in the dataset.

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Spring and (2) Osage Creek/Flint Creek (Fig. 2c). Overall, migration rates averaged across all comparisons were similar during both time periods (0.0194 vs. 0.0137, two-tailed $P = 0.7906$), although contrasting migration patterns were observed (Fig. 3; *see* supplementary data at the Journal website). Historical migration rates were asymmetric with a much higher rate ($m = 0.0383$) from Osage Creek/Flint Creek to Trudell Spring versus the opposite direction ($m = 0.0006$). However, contemporary migration rates between populations were more similar (0.0117 and 0.0157).

Contemporary effective population sizes ($N_e = 19 – 35$) were consistently an order of magnitude lower than values historically ($N_e = 660 – 2715$) (Table 2). Evidence of historical population bottlenecks were observed with *M*-ratios below critical M_c values < 0.7 ($M_c = 0.65 – 0.68$), with more recent bottlenecks detected by the Wilcoxon's sign rank test for Osage Creek.

Table 2. Estimates of contemporary and historical effective population sizes (N_e) of the Arkansas Darter, *Etheostoma cragini*, and Least Darter, *E. microperca*, with 95% confidence intervals shown in parentheses

Species/Population	N_e (MIGRATE)	N_e (COLONY)
Etheostoma cragini		
Osage Creek	544 (0-1360)	31 (19-53)
ClabberCk/WilsonSp	349 (0-1160)	19 (7-∞)
Turentine Spring	208 (0-960)	12 (5-130)
Etheostoma microperca		
OsageCk/FlintCk	2715 (810-4040)	35 (22-59)
Trudell Spring	660 (0-1460)	19 (11-38)

Discussion

In this study we assessed gene flow and population structure of two of Arkansas's rarest darter species. Complete sampling of all known extant localities of *E. cragini* and *E. microperca* in Arkansas, as well as use of several molecular loci (mtDNA/nuclear DNA/nuclear microsatellite DNA) having different mutation rates and levels of variability have allowed a comprehensive analysis of these populations at multiple temporal scales. There are three major results of this study. (1) Historical fragmentation has led to the isolation and genetic distinctness of Arkansas populations, particularly for *E. microperca*. (2) Arkansas populations were isolated in spring habitats at fine spatial scales and exhibited low contemporary and historical migration rates. (3) Both species have experienced significant population declines due to recent fragmentation that may impact overall metapopulation stability. We elaborate on each of these conclusions below and their implications on future conservation strategies.

Historical fragmentation

In other fishes not associated with spring habitats, ancient vicariant events have been suggested to explain congruent phylogeographic patterns revealing a deep split between the Ozark and Eastern Highlands (Near *et al.* 2001; Berendzen *et al.* 2003,2008). More recent geological events (~130,000 YBP) associated with the development of the present Arkansas River contributed to further vicariance, resulting in the isolation of the Ozark and Ouachita Highland regions (Mayden 1985). In addition to being affected by these historical vicariant events, populations of *E. microperca* and *E. cragini* were further fragmented by the availability of favorable spring habitats. This additional level of fragmentation may have resulted in reproductively isolated populations exhibiting substantial differentiation on a small geographic scale.

Prior to the Pleistocene, populations of *E. microperca* from the Ozark Highlands were separated from northern populations, as evident from divergence observed at cyt *b* and S7 loci. Deep divergence between Ozark and northern populations suggests historical vicariance, as opposed to scenarios involving recent dispersal, which predict shallow population divergence with high rates of migration. Comprehensive morphological analyses also confirm the divergence of Ozark populations relative to northern populations, despite relatively high disparity among characters of Ozark populations (Burr 1978). A recent systematic analysis of northern and Ozark populations of *E. microperca*, as well as the disjunct population from the Blue River, Oklahoma revealed additional unrecognized diversity, with evidence of long-term isolation of Illinois River populations representing an ancient lineage (Echelle *et al.* 2015). Levels of sequence divergence among *E. cragini* populations also indicate historical isolation at a fine geographic scale among the Illinois and Neosho River basins of the Arkansas River drainage. In contrast, other fish species exhibit only shallow divergence among Arkansas River samples, which form a separate clade relative to other Ozark drainages (Berendzen et al. 2008). Overall, these results suggest a variety of historical factors, involving vicariance, as well as further fragmentation and isolation in unique spring environments, may have

allowed gradual, adaptive, allopatric speciation to occur at small spatial scales. This may be the norm rather than the exception for aquatic organisms associated with spring habitats that are often geographically and genetically isolated (Lucas *et al.* 2009; Timpe *et al*, 2009; Wang 2009b; Fluker *et al.* 2010).

Metapopulation structure and gene flow patterns

Additional subdivision and genetic structure was observed among Arkansas populations of both species with variable, albeit low, levels of historical asymmetric migration among populations (Figs. 1-3). Low levels of gene flow would have permitted the spread of highly advantageous alleles, enabling fragmented Arkansas populations to evolve as a single cohesive unit while simultaneously differentiating at neutral loci (Morjan and Rieseberg 2004). Migration would have also been important in colonizing new habitat islands and will continue to be in the future, especially considering the number of local extinctions observed in past and recent surveys of Arkansas populations (Figs. 1, 2; Harris and Smith 1985; Hargrave and Johnson 2003; Wagner and Kottmyer 2006; Wagner *et al.* 2011,2012). Periodic local extinction and recolonization events may have been common features throughout the evolutionary history of these species, as shown by surveys of *E. cragini* elsewhere in Colorado (Labbe and Fausch 2000), Kansas (Eberle and Stark 2000), and Oklahoma (Blair 1959). Rates of local extinction can be explained, in part, by natural drying of intermittent streams during summer months, although recent anthropogenic impacts including gravel mining, urbanization, and cattle farming have intensified these effects (USFWS 2016). Consequently, recent fragmentation events may be substantially altering metapopulation dynamics by decreasing the number of available habitat islands and altering migration patterns and rates.

Overall comparisons of historical versus contemporary gene flow rates were similar between the focal species; although discordant migration patterns were observed between periods for *E. cragini*, potentially reflecting effects from anthropogenic impacts (Fig. 3). These results could be an artifact of small sample sizes of *E. cragini* from Clabber Creek/Wilson Spring and Turentine Spring rather than from historic and/or contemporary differences. Alternatively, temporal variation in migration patterns could be caused by natural phenomena or may be a successful survival strategy. Distinguishing among these explanations requires a detailed understanding of metapopulation dynamics and landscape changes over time. In the present study, fine-scale genetic structure and asymmetric rates of gene flow among Arkansas populations of both species suggest a hybrid metapopulation model which combines characteristics of the patchy population and source-sink models (*see* Schlosser and Angermeier 1995). In particular, *E. cragini* localities from the Osage Creek drainage have a mosaic of genotypes that display patchy population dynamics caused by rates of dispersal among populations

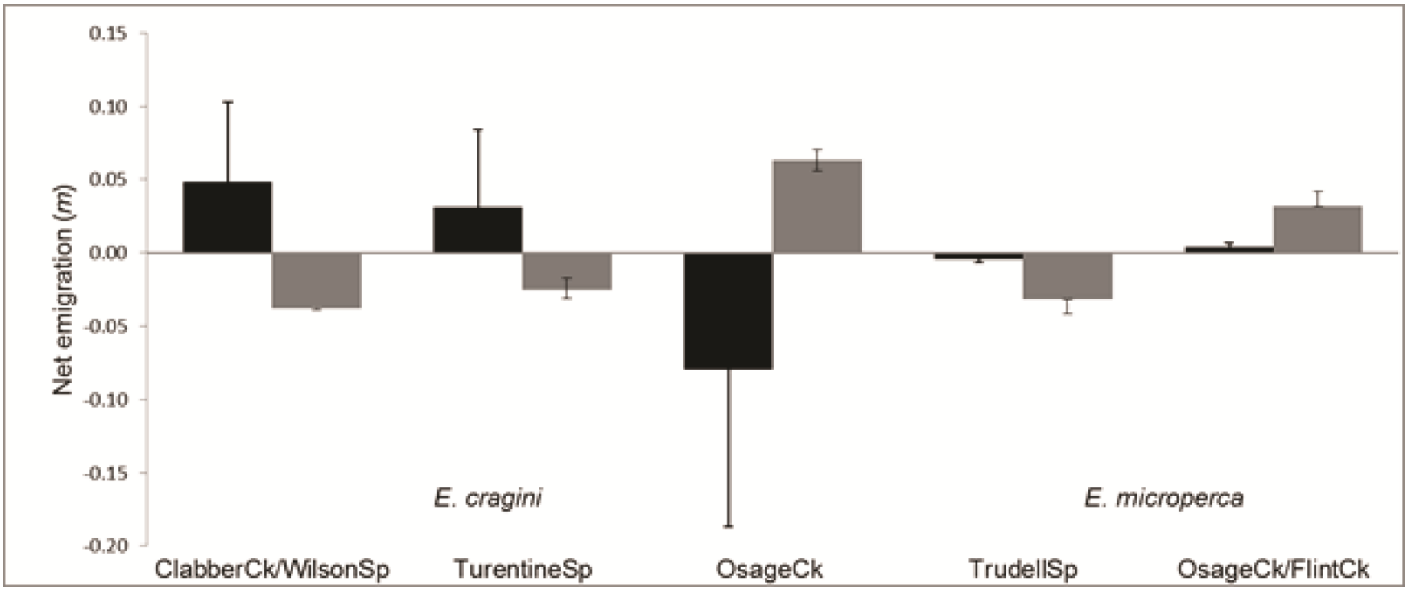

Figure 3. Estimates of net emigration rates (*m*) for Arkansas populations of the Arkansas Darter, *Etheostoma cragini*, and Least Darter, *E. microperca*, calculated by subtracting total immigration rates from total emigration rates for each population. Estimates of contemporary rates are shown in black while historical rates are shown in gray with error bars depicting 95% confidence interval.

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that exceed rates of local extinction (Harrison 1991; Schlosser and Angermeier 1995). Historically, this "single" Osage Creek population may have acted as a source for colonizing other favorable habitat islands during high flood events, including Clabber Creek/Wilson Spring and Turentine Spring (Fig. 3). However, comparisons with contemporary migration rates reveal reversed patterns, with *E. cragini* populations shifted from genetic sources to sinks and vice versa (Fig. 3). Moreover, substantial declines in current effective population sizes relative to historical conditions have significantly reduced the effective number of migrants (Table 2).

Metapopulation stability

The combined effects of small population sizes and reduced number of migrants per generation have been associated with the accumulation of deleterious mutations, potentially leading to significant increases in the probability of population extinctions (Couvet 2002). However, other studies have suggested the pattern of migration can be more important than the number of migrants, with conditions under asymmetric migration resulting in reduced fitness (Bouchy *et al.* 2005) and having direct consequences on adaptive evolution (Kawecki and Holt 2002). Contemporary patterns and rates of gene flow among *E. cragini* populations suggest unstable metapopulation dynamics, whereas the current similarity in migration observed among *E. microperca* populations may in theory allow the maintenance of metapopulation viability. Most empirical studies of asymmetric migration indicate dispersal is density dependent, with patterns of higher gene flow from large into small populations (Fraser *et al*, 2004; Manier and Arnold 2005; Jehle *et al.* 2005). Patterns of asymmetric gene flow that reverse over evolutionary time may involve a variety of factors including population dynamics, local adaptation, behavioral and life-history strategies, and environmental stochasticity (Palstra *et al.* 2007). These factors may be involved in the discordant patterns observed among *E. cragini* populations, where directionality of gene flow was not always contingent on population size. Future studies involving resampling of Arkansas populations could determine whether current migration rates and patterns vary over time and their influence on changing metapopulation dynamics.

All Arkansas populations of *E. cragini* and *E. microperca* have experienced relatively severe past bottlenecks as indicated by M-ratio tests. Moreover, recent genetic bottlenecks and population declines were detected for Clabber Creek/Wilson Spring and Turentine Spring for *E. cragini* and Osage Creek for *E. microperca*. Estimates of current effective population sizes for both species were extremely small (N_e = 12 – 35), in addition to being an order of magnitude lower than during historical times (Table 2). Current effective population estimates for *E. cragini* at 12 localities in Colorado were also relatively small, ranging from 20 to 47 (average ± STD = 35 ± 9), though no recent bottlenecks were detected at these localities (Fitzpatrick *et al.* 2014). Corresponding estimates of current and long-term effective population sizes for the Watercress Darter, *Etheostoma nuchale*, a spring-endemic of the south-eastern United States were considerably larger than those for *E. cragini* and *E. microperca*, with the exception of the Osage Creek/Flint Creek historical population of *E. microperca* (Fluker *et al.* 2010). Similarly, estimates of contemporary effective population size were substantially larger for the federally endangered Fountain Darter, *Etheostoma fonticola*, another spring-endemic and close relative of *E. microperca* from the Comal River (N_e = 899) and San Marcos River (N_e = 9,234) in Texas, USA (Olsen *et al.* 2016). Whereas discrepancies may be influenced by different methods employed, particularly for contemporary estimators (i.e., ONeSAMP, LD-N_e and N_EESTIMATOR versus COLONY, *see* Wang 2009a; Luikart *et al.* 2010), they indicate both *E. cragini* and *E. microperca* have experienced significant population declines compared with similar darters dependent on spring habitats. Recent signatures of population bottlenecks, as well as contrasting histories, with *E. nuchale* colonizing a series of springs and diverging from a widespread stream-dwelling relative (Fluker *et al.* 2010) versus *E. cragini* and *E. microperca* that were previously more widespread but affected by vicariance and further fragmentation, may explain the extremely small effective population sizes observed. These results suggest the impacts of natural fragmentation can have long-lasting effects on species, potentially making them more susceptible to contemporary influences from human-imposed changes.

Conservation implications

Conservation and management efforts usually focus on protecting 'source' populations that are deemed important for providing migrants for a particular region. However, the results of this study suggest 'source-sink' dynamics (Pulliam 1988; Dias 1996) can become reversed over evolutionary timescales. Whether this 'source-sink inversion' occurred via demographic changes and adaptive evolution as Dias (1996) originally proposed or through complex interactions involving a variety of mechanisms (*see*, *e.g.*, Palstra *et*

al. 2007) warrants further attention beyond the scope of this study. However, the lack of permanent 'source' populations identified as having higher net emigration rates suggests conservation efforts should focus on conserving as many habitat islands as possible. Similarly, concentrating efforts on specific localities or populations believed to maintain overall higher genetic diversity may be unreliable, since positive correlations between neutral and adaptive variation might not be particularly high (Hedrick 2001). Moreover, for *E. craigini* and *E. microperca*, uniformly small contemporary effective population sizes defy efforts to prioritize conservation of any single population. The best management strategies for these species would ideally involve protection of all Arkansas populations, as well as protection and enhancement of additional unoccupied habitats that may be important for maintaining connectivity among currently isolated islands. Successful management strategies must address a variety of stressors (e.g. urbanization and development, water depletion, water quality degradation) impacting populations across these species' ranges. Water depletion has already resulted in lowering of aquifers from groundwater withdrawals (Juracek *et al.* 2017) and led to decreases in perennial stream refugia and local extirpations of *E. cragini* localities (USFWS, 2016). Thus, habitat restoration at a landscape-level may be necessary for species inhabiting naturally fragmented landscapes within the Ozark Highland region, independent of whether they rely on disturbance-generated habitats or constantly stable environments (Neuwald and Templeton 2013).

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