STATE WILDLIFE GRANT PROJECT - FINAL REPORT

GENE FLOW AND GENETIC STRUCTURING OF YELLOWCHEEK DARTERS (*ETHEOSTOMA MOOREI*) IN THE LITTLE RED RIVER WATERSHED

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Date

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INTRODUCTION

Yellowcheek darters, *Etheostoma moorei* (Raney and Suttkus), are endemic to the four headwater streams of the Little Red River in north central Arkansas (Robison and Harp 1981). Construction of Greers Ferry Dam by the United States Army Corps of Engineers and the inundation of the Little Red River watershed above the dam beginning in 1962 resulted in much of the known range of *E. moorei* being converted from the riverine habitat required by this species to reservoir habitat. Prior to the filling of Greers Ferry Reservoir the four headwater streams were connected via continuous riverine habitat, which may have allowed individuals from each fork to interact as a single metapopulation. Since the filling of Greers Ferry Reservoir, Beech Fork and Middle Fork flow directly and singly into the reservoir while Archey Fork converges with the South Fork prior to the South Fork entering the reservoir. These tributaries flow through the Boston Mountains of the Ozark Plateau in north central Arkansas, which is of Pennsylvanian age and surfaced in sandstone and shale (ADP 1974). The landscape is characterized by steep relief and relatively impermeable soils (ADP 1974).

Genetic Basis of Population Analysis

The first genetic study of the yellowcheek darter was performed by Wood (1996), who by way of allozyme analysis studied a single population of *E. moorei* from the Middle Fork in attempting to better understand the *Nothonotus* species complex. This was followed shortly by Mitchell et al. (2002), who also used allozyme analysis to study seven sites containing *E. moorei* within the Little Red River drainage system. Their analysis of 85 individuals from the Middle, South and Turkey Forks using seventeen loci and six meristic characters showed conflicting results (Mitchell et al. 2002). Genetic distances based on allozyme analysis ranged from 0.000 to 0.213, with each stream population partitioning into distinct subpopulations. Turkey Fork

individuals had high genetic distance values from Middle and South Fork individuals (0.213 and 0.205, respectively), but with a small sample size of six. These genetic differences for these differing stream populations are far greater than found for darters of other stream systems. For example, Buth et al. (1980) found little conspecific genetic difference (0.00 - 0.06) for E. *microperca* populations separated by geographic distances of up to 800 km. Additionally, Karlin and Rickett (1990) found average intraspecific genetic distances of 0.004 to 0.006 for two darter species (*E. collettei* and *E. whipplei*) within streams of the Saline River drainage of Arkansas. Ecological and life history differences also have been observed for the *E. moorei* of Turkey Fork versus Middle and South forks (McDaniel 1984). In contrast to genetic data, none of the six meristic features studied by Mitchell et al. (2002) demonstrated significant differences between stream sites. Our genetic findings and these previous ecological and life history studies suggest that the Turkey Fork and Middle/South Fork populations be treated as unique management and breeding units. The declining numbers of individuals as reported by Mitchell (1999) and later by Wine et al. (2001) led to efforts by the USF&WS in association with Conservation Fisheries Incorporated to develop a captive-breeding program for *E. moorei*. An effective breeding program cannot be established without a good understanding of the genetic structuring of these populations.

Bottlenecking, a sudden and dramatic decline in numbers, has been associated with genetic drift and rapid species change (Nei 1987). Wood (1996) studied a single population of *E. moorei* from the Middle Fork in addition to other *Etheostoma* species. A comparison of the Mitchell et al. (2002) study with that of Wood showed no decline in genetic diversity in the 7 y between studies (collections in 1998 and 1991, respectively). Despite the lack of reduction in

genetic diversity (*e.g.*, Wood 1996; Mitchell et al. 2002), there were high levels of Hardy-Weinberg disequilibrium (Johnson et al. 2006), and the long-term impact of this bottleneck will be the random loss of rare alleles through genetic drift (Nei 1987) and greater genetic partitioning of stream populations. Last, Johnson (2005) performed a preliminary study using high resolution amplified fragment length polymorphisms (AFLP) analysis to demonstrate genetic structuring among sites and streams. Non-lethal techniques such as AFLPs can provide higher resolution as to the genetic partitioning of these populations. AFLP analysis has been demonstrated to have a greater efficiency in detecting polymorphisms than other commonly used high resolution analyses, such as random amplified polymorphic domains (RAPDs) and restriction fragment length polymorphisms (RFLPs) [Russell et al. 1997; Garcia et al. 2004]. However, sample size was limited in this initial study, limiting utility by management agencies. For example, few individuals were analyzed from the South Fork (n = 5) and none from Beech Fork.

Management plans for *E. moorei* can be enhanced with a better understanding of the genetic diversity of and gene flow between populations. Breeding programs such as for the yellowcheek darter must maximize the genetic diversity of their brood stock. The above findings and questions are the basis of this study which addressed the objectives below.

Objectives of the study

1. Better characterize the genetic structure of *E. moorei* populations of the Middle, South and Beech forks.
2. Estimate the gene flow within the Middle and South forks.
3. Identify the genetic structure of sample sites to assist breeding program decisions.

METHODS AND MATERIALS

Data Collection Techniques

Soft dorsal fins from individual adult *E. moorei* (n = 55) were collected at several sites from the Middle (MFB, MFC and MFD), South (SFA and SFC) and Beech (BF) forks of the Little Red River in north central Arkansas from August 29, 2003 through September 17, 2004 (Figure 1; Table 1). Darters were collected in riffles using the kick seine technique similar to the one used by Robison and Harp (1981), McDaniel (1984), Mitchell (1999), and Wine et al. (2001). Darters were anesthetized with MS-222 prior to handling and marking. Individual riffles within the MFC site were recorded so as to determine within-site genetic variation.

Table 1. Locations of *Etheostoma moorei* study sites providing genetic samples in the Little Red River basin, Arkansas.

Site	Township and Range	GPS location (lat. and long)	Sample Size
SFA	T 11N, R 15W, Sec. 33, SE¼	N 35° 32.913', W 092° 35.109'	17
SFC	T 13N, R 13W, Sec. 26, NE¼	N 35° 34.878', W 092° 27.741'	16
MF B	T13N, R14W, Sec. 29, SE¼	N 35° 44.087', W 092°23.387'	11
MFC	T 13N, R 13W, Sec. 26, NE¼	N 35° 44.525', W 092° 20.006'	40
MFD	T 12N, R 13W, Sec. 25, NE¼	N 35° 39.122', W 092° 19.163'	39
BF	T 12N, R10W, Sec. 14, SE¼	N 35°40.561', W 092°01.359'	17

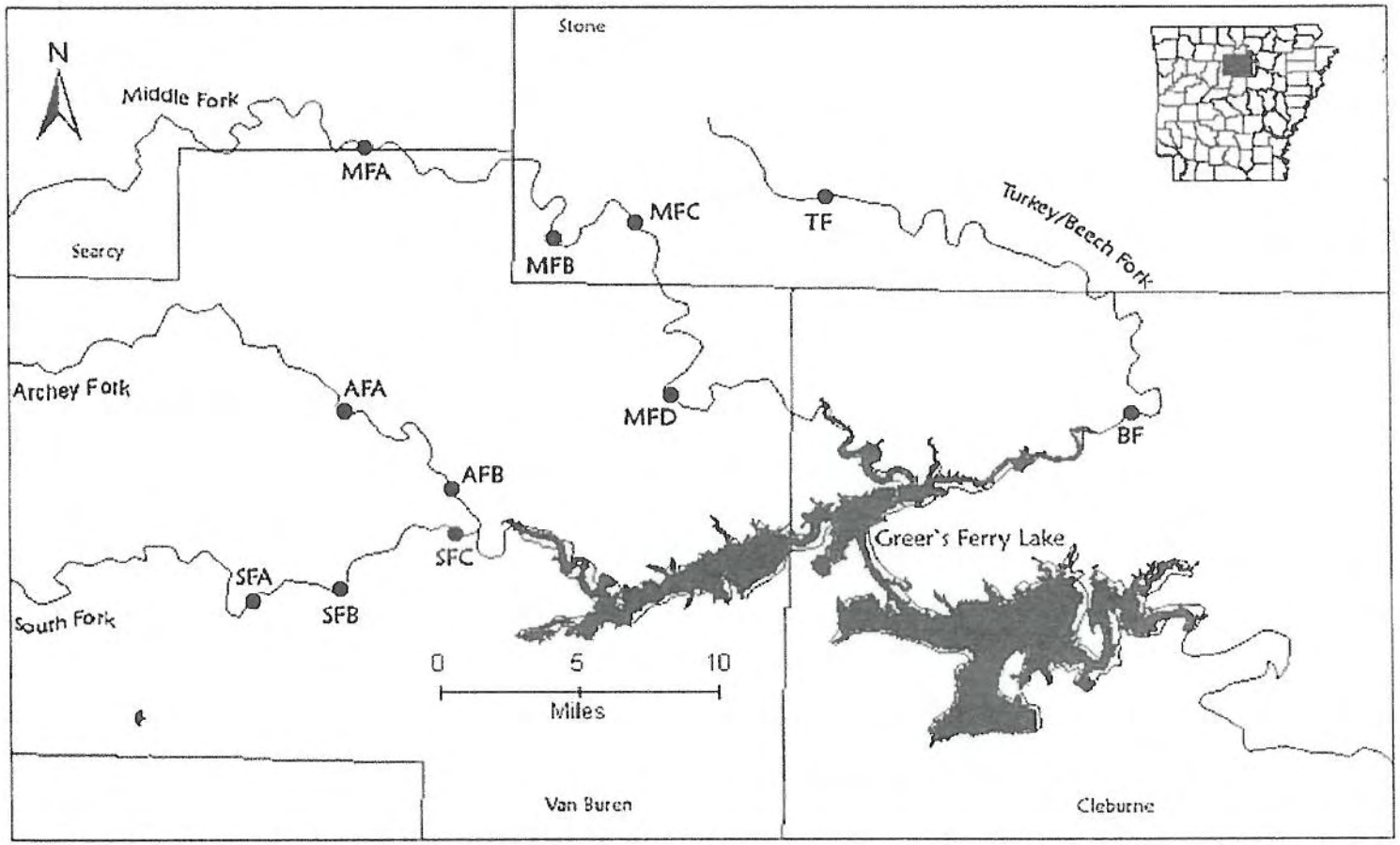


Figure 1. *Etheostoma moorei* study sites (present and historic) on tributaries of the Little Red River in Arkansas.

We used subcutaneous injections of Visible Implant Fluorescent Elastomer® (VIFE) to give adult darters a unique mark. The fin clip combined with the VIFE allowed us to distinguish recaptures and prevent pseudoreplication. The soft dorsal fin of each marked darter was stored in 95% ethanol, placed on dry ice and stored at –70 °C until the DNA could be isolated and analyzed.

DNA Isolation and Analysis

DNA isolation was performed utilizing the Qiagen® DNeasy ™ extraction kit. Briefly, the dorsal fins were rinsed with distilled water, minced, and placed in lysis buffer and Proteinase K at 55° C for 3 h. The homogenate was purified with several washes, ethanol precipitated and eluted in buffer. The elutant was stored at –70 °C until the DNA was further processed.

DNA concentration within the elutant was determined by uv visible spectrophotometry. DNA fragments for electrophoretic analysis were generated using the AFLP® Analysis System I/ AFLP Starter Primer Kit (Invitrogen). 250 ng of darter DNA was digested at 37 °C for 7 h by *Eco*RI and *Mse*I restriction endonucleases (2.5 units each). *Eco*R1 and *Mse*I adapters (50 pM each) were then ligated to digest at room temperature for 2 h. A 1/10 dilution of the ligation reaction was performed with TE buffer.

Preamplification of the ligated DNA was achieved through standard PCR protocol and *Eco*RI and *Mse*I preamplification primers (Table 2). Twenty cycles were performed under the following conditions: 94 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min. A 1/50th preamplification dilution in TE was then selectively amplified with one of the five primer sets chosen (Table 2). Primer sets were first tested for suitable amplification and high levels of variability. The five most polymorphic primer sets were then used in the selective amplification.

Table 2. Primers and primer sets for preamplification and amplification reactions. Amplification sequence includes preamplification sequence in addition to three nucleotides listed.

Preamplification Primers	**Sequence**
*Eco*RI	5'- GAC TGC GTA CCA ATT C -3'
*Mse*I	5'- CGA TGA GTC CTG ACC G -3'
Amplification Primer Sets	**Sequences**
Primer Set A	E-AAC; M-CAA
Primer Set B	E-AAG; M-CAA
Primer Set C	E-ACC; M-CAA
Primer Set D	E-ACT; M-CAC
Primer Set E	E-ACG;M-CAC

Three primary modifications of the AFLP® Analysis System I protocol include an increase in the concentrations of nucleotides, an increase in the number of amplification cycles and the use of SyBr® green stain as a non-radioisotopic means of detection. SyBr® green is not as sensitive a stain as by using radioisotopic means, necessitating an increase in both the number of cycles and available nucleotides and primers. Changes from the AFLP reagent protocol included unlabeled *Eco*RI primer solution, 2.5 mM $MgCl_2$ (versus 1.5 mM) and the addition of 0.34 µl 40 mM NTPs per reaction.

Cycling parameters included a first cycle of 94 °C for 30 s, 65 °C for 1 min and 72 °C for 1 min, followed by a successive touch down phase of 13 cycles, with each annealing temperature 0.7 °C lower than the preceding cycle. The touch down phase was followed by an additional 40 cycles (versus 23) at a final annealing temperature of 56 °C. The amplified products were denatured with formamide and heat (90 °C for 3 min). Each sample was electrophoresed in a

vertical 6.5% polyacrilamide gel (LongRanger™) and stained in SyBr® green. Stained fragments were documented and number of base estimated by way of the UVP Bioimaging System®. Most individual samples were duplicated and electrophoresed in different gel combinations to verify repeatability and to best determine band homology.

Statistical Analysis

Individual bands (loci) were aligned for homology using the LecPCR program of the DistAFLP® package (Mougel et al. 2002). Individual bands (loci) were converted into a 0/1-matrix (0 for absence, 1 for presence of a specific DNA marker; Nei 1987). Bands were conservatively evaluated (minimal banding intensity of 5% of maximum intensity within lanes) to ensure successful amplification comparisons among groups (Bensch and Akesson 2005). Pearson product correlation analysis of fragment size and allele frequencies was utilized to determine band homoplasy (Vekemans et al. 2002), which was not present ($r = -0.0816$; $p = 0.376$), and to optimize size ranges used for further analysis. Band sizes chosen for comparison ranged from 75-600 base pairs in length (e.g., David et al. 2001; Arias et al. 2004; Riberon et al. 2004). Nei's genetic distance (D; Nei 1978) was used to compare the relationships of sample groups studied, with data bootstrapped ($n = 1000$) using PHYLIP (Felsenstein 1995). Allele frequencies were estimated by the square root method of Nei (1987), where data was treated as diallelic (present/absent), and the frequency of the null or absent allele at each locus was estimated as the square root of the proportion of individuals in the sample that lack the AFLP fragment. Heterozygosity was estimated using an assumption of Hardy-Weinberg equilibrium, as AFLP loci are neutral (Lynch and Milligan 1994). F_{ST} values were calculated with 100 permutations using Lynch's analog of Wright's F_{ST} (1991). A dendrogram was constructed by the unweighted pair group method (UPGMA; Sokal and Sneath 1963) using matrices of distance

values with PHYLIP. A cladogram was also constructed for all individuals and sites using DOLLO parsimony (PHYLIP) using presence/absence data. To test an isolation-by-distance model genetic distance values were compared to geographic distance in river km using a Mantel test with 1000 randomizations (Mantel 1967). I predicted a significantly positive correlation to support isolation-by-distance.

Results and Discussion

Band Size Structure and Characteristics

Each of the primer combinations used (A-E) generated a high number of fragments possessing a wide range of sizes (Figure 2). Fragments ranged in size from a low of -600 (negative values due to software limitations for very small fragments) for primer set A to a high of 4,714 for primer set B. Number of fragments generated ranged from a high of 68 for primer set B to a low of 52 for primer set E. As stated in the methods, the size range of fragments analyzed was pared to 75-600 bp to optimize analysis, representing a far lower number of bands actually analyzed (n = 127, range of 25 – 35 per primer set).

Allele frequencies for individual loci were highly variant (Table 3A-E). Some alleles were infrequent (e.g., 0.16 for E21; 0.17 for E23; 0.18 for C24), whereas others were quite common (e.g., 0.82 for E35 and C28; 0.78 for A18). Average allele frequencies ranged from 0.41 for primer set E to 0.63 for primer set A. Allele frequencies among sites were also highly variable, with SFA having the greatest number of fixed loci (15) and MFD having no fixed loci (Table 3).

Figure 2. Representative polyacrylamide gel, demonstrating banding patterns from four different primer sets. Top lane is standard (right to left 200 bp, 400 bp, 600 bp, 800 bp, 1000 bp, 1500 bp, 2000 bp), primers set D, blank lane, primer set A four lanes, standard, 2 blank lanes, primer set B five lanes, blank lane, primer set C, standard.

Table 3A. Allele frequencies for bands analyzed (75 - 600 bp) for the yellowcheek darter of the Little Red River, Arkansas, from primer set A. Total fragments analyzed and average allele frequencies at bottom of table.

ALLELE	BF	MFB	MFC	MFD	SFA	SFC	AVG
1	0.9167	0.6667	1.0000	0.8000	0.3333	0.8750	0.7659
2	0.0909	0.6667	0.4286	0.8500	1.0000	1.0000	0.6714
3	0.2727	0.6667	1.0000	0.8571	0.8571	0.7500	0.7478
4	0.1667	1.0000	0.5000	0.7727	0.6250	0.8000	0.6263
5	0.1538	0.6667	0.6667	0.8261	0.6667	0.6154	0.6101
6	0.2308	0.9000	0.8333	0.7826	0.5833	0.8462	0.6854
7	0.3846	0.8000	0.6667	0.5652	0.7857	0.6923	0.6364
8	0.3077	0.8000	0.7692	0.7826	0.6429	0.6923	0.6658
9	0.3077	0.7000	0.6154	0.8696	0.5000	0.7692	0.6308
10	0.3077	0.6000	0.8824	0.7826	0.6667	0.6154	0.6566
11	0.5000	0.8000	0.6471	0.4783	0.6875	0.7692	0.6250
12	0.4286	0.8000	0.9412	0.8261	0.5000	0.9231	0.7310
13	0.4286	0.6667	0.7059	0.3043	0.3750	0.7692	0.5123
14	0.3333	0.7778	0.4118	0.6957	0.5625	0.7692	0.5796
15	0.1333	0.5556	0.5294	0.6087	0.1875	0.6154	0.4340
16	0.5625	0.3333	0.5294	0.5652	0.6250	0.8462	0.5776
17	0.5000	0.6667	0.7647	0.5652	0.7222	0.9231	0.6759
18	0.6875	1.0000	0.9412	0.7273	0.7222	0.6154	0.7758
19	0.6471	0.6667	0.8824	0.7273	0.6875	0.6923	0.7226
20	0.7647	0.8000	0.8235	0.8000	0.6250	0.7692	0.7631
21	0.2143	0.6250	0.7333	0.6667	0.5625	0.6154	0.5723
22	0.2000	0.7500	0.8000	0.5333	0.5000	0.8462	0.5848
23	0.4000	0.6250	0.2222	0.4667	0.2143	0.6250	0.4061
24	0.1000	0.8750	0.8000	0.8667	0.8571	0.6250	0.6939
25	0.4000	0.1250	0.8571	0.7143	0.2143	0.5000	0.5001
MEAN							0.6340

Table 3B. Allele frequencies for bands analyzed (75 - 600 bp) for the yellowcheek darter of the Little Red River, Arkansas, from primer set B. Total fragments analyzed and average allele frequencies at bottom of table.

ALLELE	BF	MFB	MFC	MFD	SFA	SFC	AVG
1	0.2500	0.6667	0.8000	0.6429	0.6667	1.0000	0.6694
2	0.5000	0.5000	0.7000	0.8214	0.3333	0.5000	0.6989
3	0.5000	0.0000	0.5000	0.5357	0.3333	0.5000	0.4405
4	0.2500	0.4000	0.6364	0.5172	0.0000	1.0000	0.5051
5	0.2500	0.2000	0.2727	0.4138	0.3333	0.0000	0.3251
6	0.0000	0.2000	0.3846	0.6207	0.6667	0.0000	0.4499
7	0.0000	0.4000	0.1667	0.4333	0.3333	0.0000	0.3209
8	0.5000	0.4000	0.4167	0.6667	1.0000	1.0000	0.5788
9	0.2500	0.4000	0.1818	0.6765	1.0000	0.5000	0.4976
10	0.5000	0.2000	0.2727	0.4857	0.6667	0.5000	0.4018
11	0.2500	0.3333	0.2500	0.6000	0.3333	0.5000	0.4419
12	0.0000	0.1818	0.0833	0.6000	1.0000	1.0000	0.4024
13	0.0000	0.4000	0.4167	0.7429	0.3333	1.0000	0.5583
14	0.4000	0.0000	0.4615	0.5143	0.6667	0.5000	0.4271
15	0.6000	0.1667	0.2308	0.5143	0.6667	1.0000	0.4200
16	0.0000	0.3333	0.3846	0.6286	0.6667	1.0000	0.4982
17	0.4000	0.0000	0.3846	0.5429	0.6667	0.5000	0.4223
18	0.2000	0.1667	0.2308	0.6111	0.6667	1.0000	0.4415
19	0.4000	0.0000	0.3846	0.6216	1.0000	0.5000	0.4735
20	0.4000	0.0000	0.4545	0.4000	0.3333	1.0000	0.3697
21	0.2000	0.5000	0.4545	0.4571	0.6667	0.5000	0.4552
22	0.4000	0.2000	0.4167	0.4286	0.6667	0.5000	0.4026
23	0.2000	0.2000	0.4167	0.6000	1.0000	0.5000	0.4861
24	0.2000	0.0000	0.5000	0.6216	0.6667	0.5000	0.4759
25	0.2000	0.2000	0.3333	0.7027	1.0000	0.5000	0.5158
26	0.4000	0.2000	0.3333	0.5294	0.5000	0.5000	0.4250
27	0.4000	0.0000	0.1818	0.5000	1.0000	0.5000	0.3648
28	0.2000	0.0000	0.3636	0.5000	0.5000	0.5000	0.3770
29	0.8000	0.2500	0.4000	0.5938	0.5000	1.0000	0.5181
30	0.0000	0.2500	0.4000	0.3871	1.0000	0.5000	0.3726
31	0.0000	0.2500	0.6000	0.7200	1.0000	0.5000	0.5835
32	0.6667	0.0000	0.6000	0.7619	0.5000	0.5000	0.5921
33	0.0000	0.2500	0.4000	0.6667	1.0000	0.5000	0.5088
34	1.0000	0.0000	0.8000	0.7619	1.0000	1.0000	0.6943
MEAN							0.4740

Table 3C. Allele frequencies for bands analyzed (75 - 600 bp) for the yellowcheek darter of the Little Red River, Arkansas, from primer set C. Total fragments analyzed and average allele frequencies at bottom of table.

ALLELE	BF	MFB	MFC	MFD	SFA	SFC	AVG
1	1.0000	0.0000	0.7619	0.5000	0.5000	0.4444	0.5726
2	0.5000	1.0000	0.6667	0.6667	0.8000	0.8889	0.7322
3	0.0000	0.2500	0.5862	0.5714	0.4000	0.2222	0.4665
4	1.0000	0.2500	0.4857	0.7500	0.5000	0.2500	0.5228
5	1.0000	1.0000	0.7143	0.6000	0.9091	0.6923	0.7275
6	1.0000	1.0000	0.4286	0.2500	0.4545	0.0769	0.3755
7	0.0000	0.2000	0.5556	0.5882	0.5833	0.5000	0.5234
8	1.0000	0.2000	0.2973	0.4000	0.5000	0.4167	0.3830
9	0.0000	0.4000	0.4324	0.4091	0.5833	0.3333	0.4148
10	1.0000	0.2000	0.3243	0.3478	0.5833	0.3333	0.3756
11	0.0000	0.4000	0.4054	0.4348	0.2727	0.3333	0.3741
12	1.0000	0.2000	0.4595	0.2400	0.2727	0.1667	0.3314
13	1.0000	0.4000	0.5000	0.5600	0.5000	0.6000	0.5406
14	1.0000	0.2000	0.6000	0.2000	0.4000	0.1429	0.3834
15	1.0000	0.4000	0.2727	0.4000	0.4000	0.2143	0.3409
16	1.0000	0.0000	0.3636	0.1538	0.1000	0.0000	0.2144
17	1.0000	0.4000	0.3636	0.4615	0.3000	0.2143	0.3786
18	1.0000	0.0000	0.6364	0.5385	0.4000	0.6667	0.5604
19	A	0.8000	0.2424	0.4444	0.4000	0.3333	0.3655
20	A	0.2000	0.3939	0.2963	0.2000	0.5714	0.3603
21	A	0.1667	0.2121	0.4643	0.0000	0.0833	0.2322
22	A	0.3333	0.2121	0.3103	0.1667	0.3333	0.2606
23	A	0.8333	0.6970	0.4483	0.8333	0.8333	0.6748
24	A	0.4000	0.2581	0.1071	0.0000	0.2000	0.1824
25	A	0.4000	0.4000	0.2963	0.7500	0.6000	0.4475
26	A	0.8000	0.4000	0.4800	0.2500	0.3000	0.4103
27	A	0.2000	0.2500	0.2727	0.0000	0.1000	0.1977
28	A	0.6000	0.9310	0.6471	1.0000	0.8000	0.8200
29	A	0.4000	0.2414	0.4118	0.0000	0.1000	0.2448
MEAN							0.4281

Table 3D. Allele frequencies for bands analyzed (75 - 600 bp) for the yellowcheek darter of the Little Red River, Arkansas, from primer set D. Total fragments analyzed and average allele frequencies at bottom of table.

ALLELE	BF	MFB	MFC	MFD	SFA	SFC	AVG
1	0.8261	1.0000	0.7083	0.6923	0.8750	0.9000	0.7022
2	0.6087	0.6364	0.8750	0.6923	1.0000	0.5000	0.6862
3	0.4583	0.5385	0.4138	0.5000	0.6000	0.9000	0.4364
4	0.4583	0.3846	0.3103	0.2353	0.5000	0.4000	0.3173
5	0.4400	0.3846	0.4063	0.3143	0.5455	0.8182	0.3662
6	0.4400	0.3846	0.5455	0.3235	0.2727	0.4545	0.3728
7	0.5000	0.2857	0.4118	0.4118	0.7692	0.6364	0.4190
8	0.6154	0.5000	0.5294	0.5556	0.5333	0.4545	0.5077
9	0.5769	0.6429	0.4706	0.5405	0.8125	0.9091	0.5237
10	0.4615	0.3571	0.3529	0.2162	0.4375	0.5455	0.3107
11	0.6538	0.4286	0.5882	0.2703	0.6250	0.4545	0.4469
12	0.6538	0.7857	0.3824	0.5946	0.5000	0.5333	0.5093
13	0.4615	0.3846	0.4706	0.4054	0.6250	0.6000	0.4217
14	0.5769	0.4615	0.4865	0.4146	0.5000	0.3333	0.4361
15	0.6154	0.3846	0.5263	0.5610	0.6875	0.8667	0.5137
16	0.4615	0.2308	0.4737	0.4571	0.5625	0.2000	0.4084
17	0.4444	0.2308	0.2703	0.2941	0.4375	0.6000	0.2950
18	0.4444	0.2308	0.2432	0.3529	0.3750	0.5333	0.3061
19	0.5926	0.5714	0.5263	0.2647	0.6250	0.6667	0.4425
20	0.5556	0.3571	0.4474	0.5000	0.3750	0.6000	0.4227
21	0.5185	0.6429	0.5405	0.3714	0.6875	0.6000	0.4644
22	0.5769	0.3571	0.4571	0.3824	0.6250	0.4000	0.4330
23	0.6154	0.6429	0.5714	0.8387	0.6667	0.6000	0.6139
24	0.7308	0.5714	0.4706	0.4828	0.6000	0.5000	0.5113
25	0.8077	0.2857	0.5758	0.6087	0.5333	0.5714	0.5396
26	0.3462	0.5000	0.4545	0.5000	0.6667	0.4286	0.4305
27	0.6538	0.5000	0.5313	0.5455	0.6000	0.7857	0.5105
28	0.6800	0.6667	0.7000	0.6667	0.5000	0.6923	0.5982
29	0.5294	0.5455	0.5833	0.2727	0.5000	0.3333	0.4228
30	0.5714	0.5000	0.5789	0.3333	0.5385	0.6667	0.4451
31	0.3846	0.2000	0.5625	0.7500	0.6923	0.2500	0.5077
32	0.6667	0.6667	0.7857	0.6000	0.7273	0.5833	0.6233
33	0.6364	0.5556	1.0000	0.6000	0.7273	0.2500	0.6641
34	0.9091	0.8889	1.0000	0.2000	0.8000	0.7500	0.6427
MEAN							0.4780

Table 3E. Allele frequencies for bands analyzed (75 - 600 bp) for the yellowcheek darter of the Little Red River, Arkansas, from primer set E. Total fragments analyzed and average allele frequencies at bottom of table.

ALLELE	BF	MFB	MFC	MFD	SFA	SFC	AVG
1	1.0000	1.0000	0.7200	0.5000	1.0000	0.8571	0.7406
2	0.0000	1.0000	0.6154	0.5263	0.6667	0.7143	0.5260
3	0.8000	1.0000	0.4516	0.4000	0.5455	0.5714	0.5327
4	0.7778	0.0000	0.4688	0.2273	0.5000	0.1429	0.3903
5	0.2500	0.2500	0.3125	0.2174	0.2500	0.5714	0.2808
6	0.2222	0.4000	0.2813	0.3462	0.5000	0.7143	0.3523
7	0.4000	0.0000	0.3636	0.3214	0.2857	0.4286	0.3332
8	0.2000	0.3333	0.2353	0.3667	0.2857	0.5714	0.3075
9	0.7273	0.5000	0.3056	0.2333	0.6429	0.6250	0.4104
10	0.4615	0.5000	0.2432	0.5526	0.5000	0.7500	0.4528
11	0.2857	0.3333	0.2432	0.5349	0.3571	0.1111	0.3525
12	0.2000	0.3333	0.2564	0.4222	0.2143	0.1111	0.2924
13	0.4706	0.0000	0.3590	0.3696	0.6429	0.1111	0.3704
14	0.3529	0.3333	0.2703	0.1522	0.4286	0.6667	0.2905
15	0.4737	0.3333	0.4211	0.3478	0.2857	0.2222	0.3727
16	0.3684	0.8333	0.3684	0.7174	0.6429	0.7000	0.5586
17	0.4444	0.4286	0.4359	0.1667	0.4286	0.7000	0.3658
18	0.6316	0.4286	0.4878	0.5000	0.3571	0.3000	0.4841
19	0.4737	0.4286	0.2250	0.6800	0.3333	0.3000	0.4393
20	0.5789	0.2500	0.2308	0.3061	0.3571	0.1818	0.3175
21	0.0526	0.1429	0.1579	0.2083	0.0714	0.2727	0.1579
22	0.3684	0.0000	0.2105	0.1458	0.5000	0.1000	0.2220
23	0.2632	0.1429	0.1622	0.1458	0.2143	0.1000	0.1715
24	0.7500	0.4286	0.1944	0.3830	0.7143	0.3000	0.4102
25	0.3500	0.4286	0.2973	0.4043	0.4286	0.3000	0.3604
26	0.5000	0.5000	0.3243	0.4468	0.5714	0.7778	0.4567
27	0.4000	0.3333	0.4444	0.0426	0.7143	0.7778	0.3490
28	0.7000	0.6000	0.5625	0.2174	0.7857	0.5556	0.4919
29	0.5000	0.8000	0.5313	0.1522	0.5714	0.0000	0.3810
30	0.5000	0.0000	0.2759	0.5435	0.6154	0.5000	0.4327
31	0.5294	0.2500	0.2692	0.3902	0.6154	0.6250	0.4063
32	0.7500	0.3333	0.5200	0.2162	0.4545	0.2857	0.4209
33	0.6000	1.0000	0.7619	0.5278	0.7000	0.7143	0.6631
34	0.5333	0.6667	0.6471	0.1250	0.4286	0.2857	0.4111
35	0.8750	0.6667	0.7647	0.9583	0.5714	0.7143	0.8178
MEAN							0.4092

Number of polymorphic loci and polymorphism was directly tied to sample size (Table 4). The greatest number of individuals analyzed were from sites MFC and MFD and these also represented the greatest polymorphisms (96.9 and 100% of loci polymorphic, respectively). This relationship of polymorphism values to sample size is to be expected, as the more individuals sampled, the greater number of opportunities for identifying within-locus variation (Nei 1987). Nonetheless, polymorphism was high for all site samples ($\bar{x}$ = 92.7). Estimated heterozygosity was high for all site samples ($\bar{x}$ = 0.496 ± 0.007 SE; Table 4), consistent among sites.

Previous allozyme analysis also revealed the genetic diversity of yellowcheek darters to be high relative to other darters and freshwater fishes in general despite numerical decline and range contraction. Average allozyme diversity values (direct count H = 0.063; P = 27.7%) for all sites in the current study were significantly higher (t-test; $p < 0.01$) than averages for other darter species studied to date, including other *Etheostoma* species (H range of 0.000 to 0.079; P = 2.9 to 31.6 % for 27 species; Buth et al. 1980; Karlin and Rickett 1990; Wood 1996; Faber and White 2000). For example, 15 other *Etheostoma* species studied by Wood (1996) further supports this relatively high genetic diversity for *E. moorei*, with average allozyme values of 1.1 alleles per locus, H of 0.024 and P of 11.6 for those other species. These high allozymic diversity values for the yellowcheek darter were attributed in part by Mitchell et al. (2002) to the geologic stability of their habitat (reduced impact of glaciation) relative to that of more northern darter species (Bermingham and Avise 1986).

AFLP genetic diversity values (H and P) were consistently greater than those values identified by allozyme analysis by Mitchell et al. (2002) [Table 4]. This increase would be predictable due to mutations resulting in AFLP polymorphisms being largely neutral in nature, compared to mutations in allozymes representing the potential disruption of functional enzyme products. AFLP analysis has also been demonstrated to have a greater efficiency in detecting

Table 4. Comparison of standard genetic variation measures for polymorphism (P) and estimated heterozygosity (H) based upon allele frequencies for AFLP (n = 127 loci) and allozyme data (n = 17 loci; Mitchell et al. 2002) of *E. moorei* within the Little Red River drainage system, Arkansas. Allozyme data for Beech Fork originated from the Turkey Fork upstream.

	AFLP Data			Allozyme Data		
Site	**n**	**P**	**H (SE)**	**n**	**P**	**H (SE)**
SFA	17	88.2	0.496 (0.021)	13	35.3	0.111 (0.041)
SFC	16	90.6	0.494 (0.011)	14	29.4	0.128 (0.050)
MFB	11	92.1	0.491 (0.022)	6	5.9	0.016 (0.016)
MFC	40	96.9	0.499 (0.017)	15	29.4	0.067 (0.030)
MFD	39	100.0	0.494 (0.016)	23	17.6	0.044 (0.028)
BF	17	88.1	0.493 (0.23)	4	58.8	0.241 (0.055)
Mean	140	92.7	0.496 (0.007)	75	25.9	0.083 (0.022)

polymorphisms than other commonly used high resolution analyses, such as RAPDs and RFLPs (Garcia-Mas et al. 2000). High AFLP polymorphisms comparable to that obtained here have also been identified in other closely related populations (e.g., 85.5% in sweet potatoes, Tseng et al. 2002; 95.2% in pit vipers, Giannasi et al. 2001). The high number of polymorphic loci identified using AFLPs also offsets the loss of information revealed within dominant marker systems (versus codominance through the use of allozymes) [Gerber et al. 2000]. AFLP analyses also generate more loci for analysis than do RAPD, RFLP or microsatellite analyses (Tseng et al. 2002). Many of these loci can be generated within a single lane (for example, see Figure 2.). For this study, 127 loci were studied using AFLP analysis as compared to 17 loci in our previous allozyme study (Mitchell et al. 2002; Johnson et al. 2006), an eight-fold increase in data.

Genetic Relationships of Sample Sites

Nei's genetic distance values were concordant with previously determined allozyme data (Table 5). A correlation coefficient performed between AFLP and allozyme data demonstrated AFLP and allozyme distance data to be highly correlated ($r = 0.806$; $p < 0.001$). A Spearman rank correlation analysis showed similar results ($r_s = 0.682$; $p < 0.01$). Each was significant, and equal to or greater than most correlations found for AFLP versus other techniques, such as with microsatellites, RAPDs and RFLPs (range of technique correlations from 0.33 to 0.87; Russell et al. 1997; Garcia et al. 2004).

Genetic distances were much greater using AFLP versus allozyme data. Non-selective loci are "free" to mutate without selective constraints. Genetic distances ranged from 0.0987 to 0.2944 for AFLP data, whereas genetic distances calculated from allozyme analysis ranging from 0.001 to 0.188.

Table 5. Pairwise Nei's genetic distance values for *E. moorei* between sample sites within the Little Red River, Arkansas. AFLP distance values are above the diagonal whereas allozyme data (Mitchell et al. 2002) are below the diagonal.

	BF	MFB	MFC	MFD	SFA	SFC
BF	**0.0000**	0.2522	0.1824	0.2436	0.2610	0.2944
MFB	0.182	**0.0000**	0.0987	0.1978	0.1939	0.1757
MFC	0.172	0.006	**0.0000**	0.1296	0.1370	0.1400
MFD	0.188	0.001	0.011	**0.0000**	0.1387	0.1394
SFA	0.185	0.017	0.025	0.015	**0.0000**	0.1287
SFC	0.159	0.027	0.039	0.027	0.014	**0.0000**

Previous allozyme analysis revealed the Turkey Fork individuals to be markedly divergent from both Middle and South Fork populations (D = 0.19 - 0.22; Mitchell et al. 2002), concordant with previously identified ecological variables (McDaniel 1984). This unique population may be permanently lost. Repeated recent sampling efforts have failed to produce individuals (Weston 2006; M. Wine, USF&WS, personal communication), although downstream a small population remains.

Both the cladogram (Figure 3) and UPGMA dendrogram (Figure 4) demonstrated the Beech Fork population to be divergent from the other populations. Similar to distance data, AFLP-derived phenograms are consistent with that from allozyme data (Figure 5; Mitchell et al. 2002). One anomaly in both AFLP-derived phenograms (UPGMA and DOLLO parsimony) from that predicted is the grouping of the MFD samples with the South Fork samples. The MFB and MFC sites are geographically and genetically closer than are other sites and their samples (6.7 km; D = 0.0987).

Figure 3. Cladogram of yellowcheek darter genetic relationships of the Little Red River, Arkansas, as determined from DOLLO parsimony analysis of AFLP data.

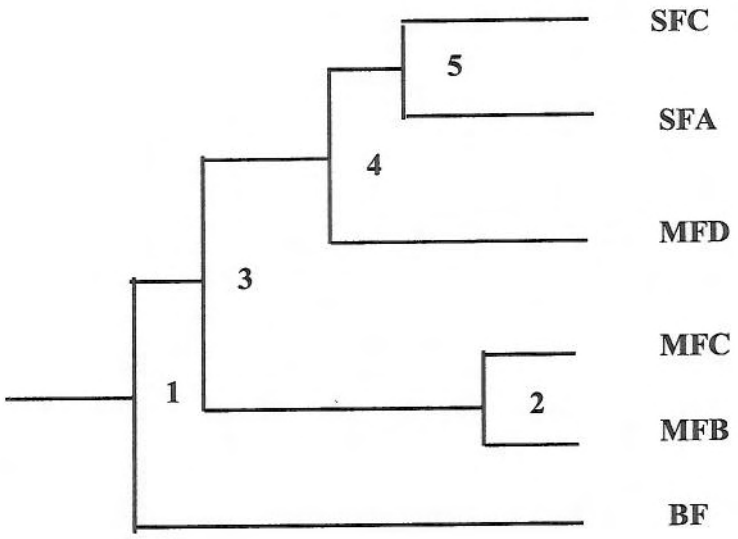


Figure 4. UPGMA dendrogram of yellowcheek darter genetic relationships of the Little Red River, Arkansas, as determined from DNA distance matrix of AFLP data.

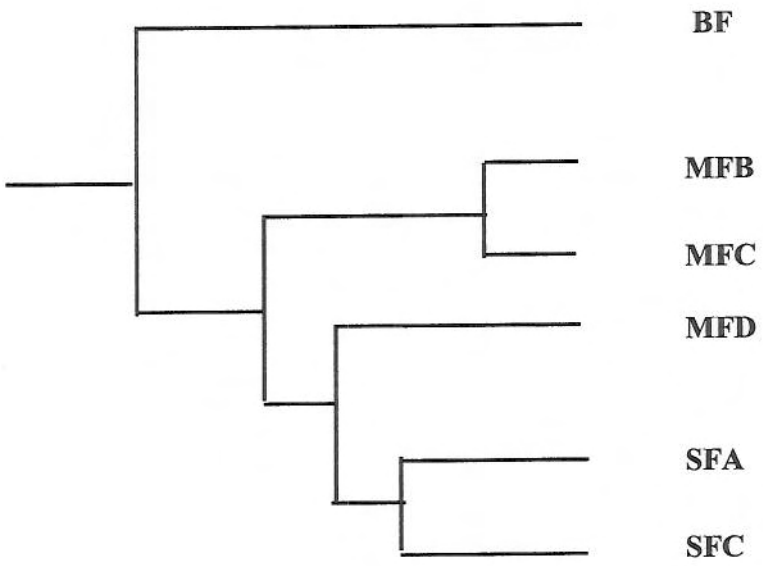
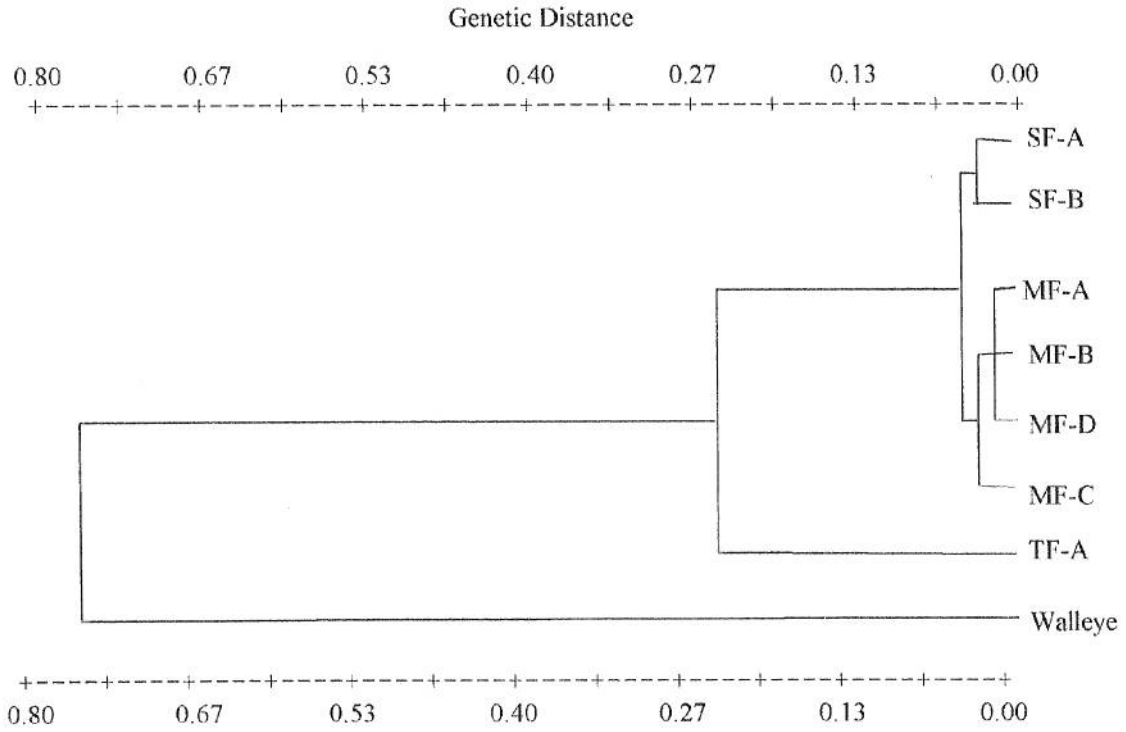


Figure 5. Phenogram showing Nei's unbiased genetic distance (1978) between seven sample locations of *E. moorei* within the South (SF), Middle (MF), and Turkey (TF) Forks of the upper Little Red River drainage. Cophenetic correlation coefficient = 0.997.

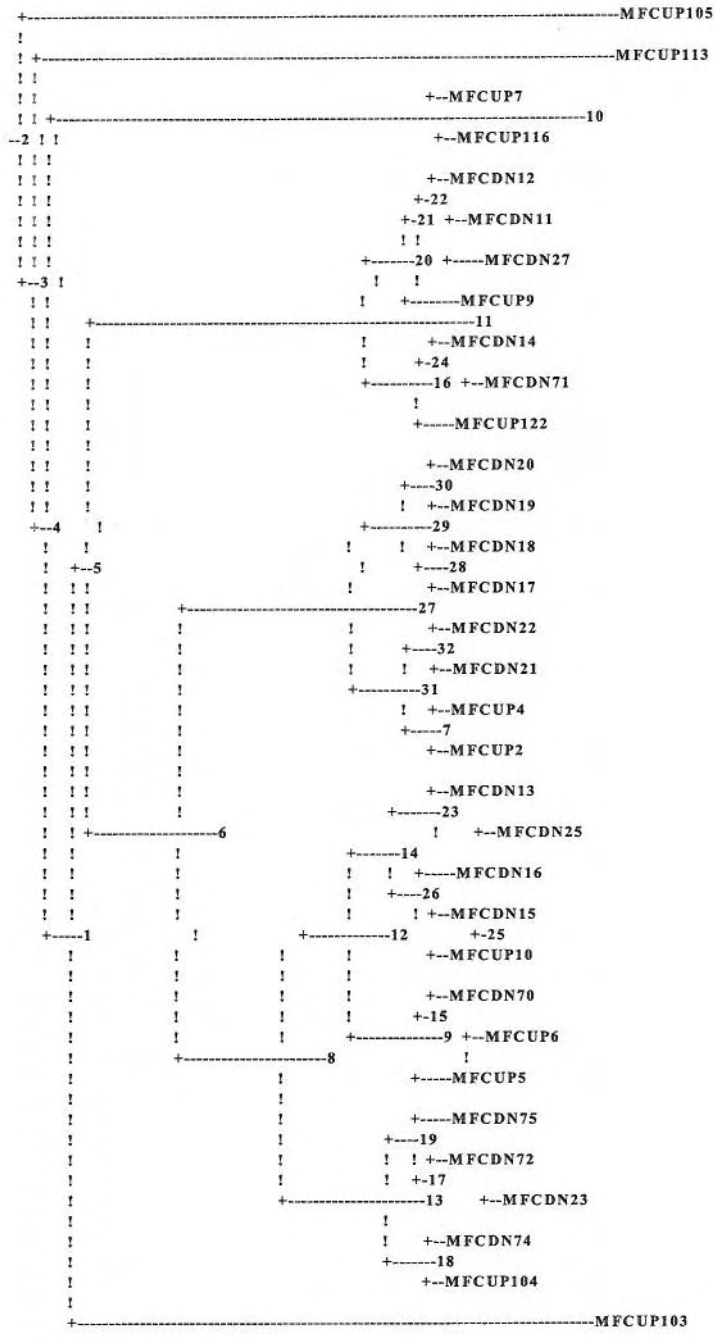


Population Structure and Gene Flow

F-statistics were used to analyze the genetic interactions of these sample sites (Table 7). As *E. moorei* have been identified as being sexually mature at Age 1 (McDaniel 1984), there have been over 40 generations with effectively no gene flow between streams due to the isolating barrier of Greers Ferry Lake. To date, no specimens of *E. moorei* have been identified within the reservoir (G. Harp, Arkansas State University, personal communication). Therefore, the most realistic approach to studying gene flow was to compare within stream values. F_{ST} values within the Middle (F_{ST} = 0.003) and South (0.010) forks showed very little differentiation among populations. These values are inconsistent with F_{ST} values derived from allozyme data (F_{ST} = 0.059; Johnson et al. 2006), which showed some genetic structuring among populations. Several of the populations surveyed previously with allozymes were not in Hardy-Weinberg equilibrium, potentially inflating those F_{ST} values. Repeated recolonization of previously extirpated sites (founder effect) may have resulted in this genetic disequilibrium (Mitchell et al. 2002).

Further cladistic analysis was performed on yellowcheek darters from two differing riffles at MFC (Figure 6). These riffles are separated by a low water bridge and several hundred meters. Cladistic analyses showed no partitioning among riffle groups, with individuals showing high levels of interaction. This is inconsistent with mark-recapture data, which showed little movement of adults among even proximal riffles during periods of normal flow (Weston 2006). However, little migration is required to genetically homogenize subpopulations (Slatkin 1985). Still to be studied is the migration patterns of yellowcheek darters during low flow and riffle drying.

Figure 6. Cladogram of yellowcheek darters at upstream (MFCUP) and downstream (MFCDN) riffles of Middle Fork C of the Little Red River, Arkansas, as determined by DOLLO parsimony analysis of AFLP data.



Despite the low F_{ST} values, genetic structuring was evident among subpopulations. Results of the Mantel test supported isolation-by-distance ($r = 0.608$; $p = 0.03$), consistent with darter species sharing similar evolutionary histories (Turner and Trexler 1998). The life history characteristics of *E. moorei* are typical of fish species having low levels of gene flow: small clutch size and larger lecithotrophic eggs, which have reduced larval drift compared to planktotrophic eggs (Paine 1984; Turner and Trexler 1998; Faber and White 2000). Turner and Trexler (1998) also found that darter species inhabiting headwater streams have high levels of genetic structuring relative to other darters. However, the common extirpation and recolonization events in the headwater streams of the Little Red River necessitate the movement of individuals, and reduce the opportunities for divergence within streams (Ruttledge et al. 1990). Increased rainfall the past several years has restored previously dry sections of these headwater streams; densities of *E. moorei* are greater than noted by Wine et al. (2001) and recolonization of most upper stream sections has occurred (Weston and Johnson 2005). Nonetheless, the Turkey Fork, Archey Fork and MFA populations to this date have apparently not been re-colonized, and total numbers estimated by Weston (2005; $n = 28{,}000$) are roughly one-half that of previous estimates of Robison and Harp (1981; $n = 60{,}000$).

Habitat loss from drought appears to be the greatest threat to the yellowcheek darter. The range for this species has been narrowed dramatically upstream and downstream by the formation of Greers Ferry Lake and low water conditions, respectively. *Etheostoma moorei* is an obligatory riffle species (Raney and Suttkuss 1964; Robison and Harp 1981; McDaniel 1984), with suitable habitat widely separated in these headwater streams. Whereas other riffle species have been collected from refugial pools during periods of drought in these streams (e.g., rainbow darter *(E. caeruleum)* and the central stoneroller *(Campostoma anomalum)*), the yellowcheek darter has not (Wine et al. 2001). In an extensive study of several other *Etheostoma* species,

genetic diversity was significantly less for species having highly specialized niche requirements (e.g., *E. moorei* obligatory to high gradient riffles) and for populations in degraded water quality (e.g., lack of water or siltation events) [Heithouse and Laushman 1997].

The genetic aspects of small populations must be considered at the onset of management programs in order to maximize the probability of their long-term survival and continued adaptability (Meffe 1986). Efforts should be made to enhance within-site genetic variation while simultaneously maintaining the genetic integrity of differing populations. Outbreeding depression can result from the introduction of exogenous alleles into existing populations (Philipp et al. 2003).

The genetic separation of the Turkey Fork population from those of the other two streams by way of allozyme analysis and the Middle and South fork populations by AFLP analysis suggests that each should be treated as distinct management units. Moritz (1994) elaborated on the distinction of evolutionary significant units (ESUs) and management units (MUs) on the basis of the historical framework of a species. Whereas classification of populations as ESUs requires interpretation of the historical relationship of the populations in question, the MU concept focuses on the current genetic and population dynamics. Not enough is known at present to appropriately place these stream populations as ESUs. Nonetheless, loss of any group as has apparently occurred within the Archey and Turkey forks would result in a decline in the genetic diversity of *E. moorei* and subsequent hindrance of the long term stability of the species. A captive-breeding program such as is being investigated by Conservation Fisheries Incorporated should contain stock from both the remaining stream systems both to increase numbers and to serve as artificial refuges during drought conditions. Captive breeding and refugee programs have been developed with some success for other endangered freshwater fishes

such as the Colorado squawfish (*Ptychocheilus lucius*), humpback chub (*Gila cypha*) and the desert pupfish (*Cyprinodon* spp.) (Hamman 1981; 1982; Turner 1984).

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