GENETIC COMPARISONS OF POPULATIONS AND MORPHOTYPES OF *POTAMILUS CAPAX*

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Summary of major findings

1) St. Francis and Ohio river collections of *P. capax* appear genetically differentiated

2) Morphotypes found in the Ohio river were part of the same population suggesting differences in phenotypes were due to phenotypic plasticity of the organism

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Introduction

Freshwater mussels of North America are currently facing catastrophic declines (Williams (Williams et al., 1993). One such mussel species, *Potamilus capax* (Green 1832), is distributed in the St. Francis, Mississippi and Wabash (Ohio drainage) rivers (Ahlstedt and Jenkinson, 1991). Due to a variety of perceived factors (e.g. habitat loss, pollution, and over-harvest), *P. capax* was listed as endangered in the mid 1970s (USFWS, 1976). To assist in the recovery of this species the USFWS Conservation Genetics Lab (CGL) assessed the population structure of *P. capax* in the St. Francis and Ohio rivers. CGL also attempted to resolve the putative taxonomic status of this species. Specifically, recent *P. capax* collections made in the Ohio River have shown that some individuals possess a thinner shell, a rounder shape and a slightly more inflated umbo, as well as, possessing rays on the outer shell (P. Hartfield, USFWS, pers. comm.; Appendix). The objectives of this study were to estimate standard population genetic parameters to assess the genetic diversity within and among sampled populations and putative morphotypes.

Methods

CGL was provided mantle clips from *P. capax* (*n* = 54) that were previously collected by state and private agencies. Samples sizes for the St. Francis River (Arkansas) and Ohio River (Kentucky) were 24 and 30, respectively. DNA was extracted from ethanol preserved mantle tissue using the DNeasy® Blood and Tissue kit (QIAGEN, Inc., Valencia, California) protocol.

Twelve microsatellite loci (Table 1) were PCR amplified following the protocol of Diaz-Fergusen et al (Molecular Ecology Resources Primer Development et al., 2011). Specifically, PCR amplifications were performed in 5 µL reactions using the following reaction components: 1× *Taq* reaction buffer (Applied Biosystems Inc., Foster City, California), 3.75 mM $MgCl_2$, 0.423 mM of each dNTP, 0.25 µM of each primer, and 0.08 U *Taq* polymerase (Applied Biosystems, Inc.). PCR conditions were an initial

denaturation at 94 ºC (10 min), followed by a touchdown procedure involving 33 cycles and consisting of denaturing (94 ºC), annealing, and extension (74 ºC) cycles, where the initial annealing temperature was initiated at 56 ºC and decreased by 0.2 ºC/cycle.

Prior to electrophoresis, 2 µL of a 1:100 dilution of PCR product was mixed with a 8 µL solution containing 97% formamide and 3% Genescan™ LIZ® 500 size standard (Applied Biosystems, Inc.). Microsatellite reactions were visualized with an ABI 3130 genetic analyzer (Applied Biosystems, Inc.) using fluorescently labeled forward primers and analyzed using GeneMapper® software v3.7 (Applied Biosystems, Inc.).

Locus conformance to Hardy–Weinberg equilibrium (HWE) was assessed for each sampling locality (i.e., St. Francis and Ohio rivers) using exact tests implemented in GENEPOP v4.0.10 (Raymond and Rousset, 1995) with specified Markov chain parameters of 5000 dememorization steps followed by 500 batches of 2000 iterations per batch. Tests for gametic disequilibrium (all pairs of loci in each sample) were also performed using GENEPOP v4.0.10. Significance levels for all multiple comparisons were adjusted using a sequential Bonferroni correction (Rice, 1989). Estimates of genetic diversity in the form of number of alleles, observed heterozygosity, and expected heterozygosity per population were made using GenAIEx v6.4 (Goudet, 2001). Allelic richness was calculated using HP-RARE (Kalinowski 2005).

To assess the degree of potential population substructure of *P. capax*, we first compared allele frequency distributions between sampling localities to assess the null hypothesis of panmixia among *P. capax* from the St Francis and Ohio rivers using the genic differentiation option in GENEPOP v4.0.10 (default options). We also calculated an F_{ST} value (a measure of population differentiation based on genetic polymorphism data) between sampling sites using the program MSA v4.05 (Dieringer and Schlotterer, 2003). Significance of the F_{ST} value was assessed via 10,000 genotype permutations.

Analysis of population structure was also performed using a Bayesian-based clustering algorithm implemented in the program STRUCTURE v2.3.3 (Pritchard et al., 2000; Falush et al., 2003). STRUCTURE does not assume *a priori* sampling information; rather, individuals are probalistically assigned to groups in such a way as to achieve Hardy-Weinberg and linkage equilibriums. Essentially, STRUCTURE assesses the question: how many significant groups (*K*) are there given the data? STRUCTURE was run with five independent replicates for *K* (i.e., distinct populations or gene pools), with *K* set from one to four. The burn-in period was 20,000 replicates followed by 500,000 Monte Carlo simulations run under a model that assumed no admixture and independent allele frequencies. The mean and standard deviation of likelihood estimates (i.e., Pr[*X*/*K*], which equals the posterior probability of the data given *K* populations) among runs at each value of *K* and estimates of delta *K* (Evanno et al., 2005), were used to determine the most likely value of *K*.

The Ohio River samples comprised two morphotypes – one of which could be a different species. To assess whether two genetically distinct groups existed in the Ohio River samples, we reran STRUCTURE using only the Ohio River samples. STRUCTURE analysis was as describe above except *K* was constrained to a value of two (only one replicate was run).

Results

All loci conformed to HWE for the St. Francis and Ohio river sampling sites after adjusting for multiple comparisons (all p >0.02). Four (*PcA10, PcC112, PcC131,* and *PcC111*) of twelve loci were monomorphic for the St Francis samples and two (*PcA10* and *PcC112*) were monomorphic for the Ohio River samples. Gametic disequilibrium tests between all pairs of loci showed no significant disequilibrium and all loci were considered to be independently segregated. Observed heterozygosity ranged from 0.425 to 0.513 for the St. Francis and Ohio River samples, respectively (Table 2). Expected

heterozygosity values for the microsatellite loci ranged from 0.433 (St Francis) to 0.518 (Ohio). Mean allelic richness estimates across all loci ranged from 4.43 (St. Francis) to 5.22 (Ohio; Table 2).

The test of genic differentiation between sampling sites was significant ($P \leq 0.00001$) indicating that *P. capax* sampling sites do not represent a panmictic population. Significant differences in allele frequency distributions were observed among sites at 6 of 10 loci (all $p < 0.01$; two additional loci were monomorphic for both populations and for which the statistic could not be calculated). The estimated F_{ST} value between the two sites was 0.06 and significantly (p=0.0001) different from zero, further supporting the hypothesis that *P. capax* sampling sites do not represent one panmictic population. Analysis of data using STRUCTURE was congruent with genic and F_{ST} tests.

STRUCTURE analysis identified a *K*-value of two as the most likely number of distinct gene pools in the total dataset using the delta *K* criterion (Figure 1). St. Francis samples clustered together but separate from Ohio River samples (Table 3). The proportion of individual membership at a *K* of two was high (>70%) for most specimens; however, there were several samples (USFWS 4692, 13004, 13005, 13009, 13017) with low assignment success (Table 3), which could be intraspecific hybrids but more loci are necessary to confidently determine this observation. STRUCTURE analysis identified a *K*-value of one when only Ohio River samples were analyzed. The portion assigned to each group (when *K* was set to two) was symmetrical (i.e., 50%; Table 4) indicating that the Ohio River samples comprised a single group.

Discussion

The major objective of this study was to assess whether putative morphotypes found in the Ohio River comprise a single unit; alternatively, were there two groups – with each group being comprised of differing morphotypess. Three lines of evidence indicated that the observed Ohio River morphotypes were phenotypic variants of Ohio River *P. capax*. We would expect that if the Ohio River

samples comprised two distinct groups, but were analyzed as if they were one sample then 1) a large portion of the loci should exhibit 1) a significant heterozygote deficiency (i.e., the observed heterozygosity should be significantly less than expected for numerous loci), and 2) a significant deviation from gametic disequilibrium (in theory, these deviations from expectations are termed a Wahlund effect). Both expectations were not observed after analysis of the Ohio river samples; conversely, all loci conformed to expectations indicating one panmictic population. STRUCTURE analyses corroborated these analyses by indicated that the most likely number of groups comprising the Ohio River sample was a value of one. Thus the observed morphological variation is suggestive of phenotypic plasticity and not characteristic of a cryptic species.

Phenotypic plasticity in freshwater mussel shells is well-documented, occurring in both bivalves and gastropods (Minton et al., 2011). In both groups, shell shape and sculpture tend to exhibit a unidirectional transition from thinner, less inflated, smoother shells in shallow headwater reaches to more inflated, thicker, sculptured shells in deeper main stem areas (Ortmann, 1920). A similar morphological association has been seen in *P. capax* morphotypes – some individuals possess a thinner shell, a rounder shape and a slightly more inflated umbo. It is not clear why both morphotypes are present in the same environment; however, the occurrence of both morphotypes might be explained by passive downstream displacement during flood events (Hastie et al., 2001).

Potamilus capax was listed as federally endangered in the U.S. in 1976 and a recovery plan (USFWS, 1989) created. Recovery objectives stipulated protection for the existing population in the St. Francis Floodway and tributaries and establishment and protection of two other viable populations within the historic range of the species. Genetic analyses confirmed that samples from Ohio and St. Francis rivers comprised two genetically distinct groups. Tests of genic differentiation were significant for 60% of the polymorphic loci and the estimate of F_{ST} between populations was 0.06 indicating

moderate population differentiation (Hartl, 1988).

In conclusion, our data indicate that St. Francis and Ohio populations do not represent one large panmictic population; rather significant genetic differences were found between these two populations. Finally, morphotypes seen in the Ohio River were not genetically distinct suggesting that the observed morphological variation among samples of *P. capax* in the Ohio River may be attributed to environmental factors. Our data also serve as a baseline for future genetic monitoring of this species.

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Table 1. *Potamilus capax* microsatellite loci and summary information. Accession no. is the Genbank deposit number. All loci were successfully amplified using a 2.00 mM $MgCl_2$ concentration.

Locus	Repeat motif	Primer sequence (5'-3')	Size range)	Accession no.
PCA10	$(TTGT)_5$ $(AACA)_2$	F: ATATGGCCTCCAGAGTAATTG R: TTCCCAGTTTCTTCTTCCAC	108	*HM991151*
PCD110	$(TAGA)_{17}$	F-TGCAGTTTACAGTGGCAGTA R-GCAGACGGAGTTGAGATG	254-294	*HM991153*
PCC111	$(GATG)_8$	F-GGAGCGAACAGAACCAGA R-TCAAAAGACCATCCATTAGTTG	108	*HM991154*
PCC112	$(CCAT)_{18}$	F-AATCCATCCATCCATCATTC R-TTCCCCCATCAAGATAAAAG	183-195	*HM991155*
PCD118	$(TATC)_{15}$ $(AATG)_9$	F-ATGTTGTCTGTCTGTCTGTCTG R-TTGGTGTGAGTACCTACATTG	161-193	*HM991156*
PCC122	$(TATC)_{27}$	F: ATCGGATCAATTTTAGCAGTA G R: TCCCGTATTGTGCATAGATAC	167-200	*HM991157*
PCD123	$(TAGA)_{28}$	F-ACAGAAGGACAGCCACACATAC R-GTCCGACACCAATTTTATCTGA	201-215	*HM991158*
PCD124	$(ATCT)_{18}$	F-AATGTGAACGACTACGTGTGA R-CCACTTTGTCATCAACATACAG	201-323	*HM991159*
PCC127	$(TCCA)_9$ $(TATC)_{13}$	F-GGAATCGGGATAAGGATAAC R-AAGATGGATAAGATGCGTCTAC	159-183	*HM991160*
PCC131	$(ATGG)_{10}$	F-AGTGAAAGTGAGATGGACACA R-TCGGACTAGGAGTACATCATTC	276	*HM991161*
PCD132	$(CTAT)_{31}$	F-ACAGCACAACATACCTGACTCC R-CTACGGCAGTGATGCTAGTCTC	154-175	*HM991162*
PCC135	$(TGGA)_{17}$	F-TTCTGGTAGTCCTACTGACTGG R-GCCGCAATGTTAAAGTGTATAT	199-215	*HM991163*

Table 2. *Potamilus capax* population genetic summary statistics. Abbreviations are as follows: *N* = sample size, *A* = number of alleles, *Ar* = allelic richness, *Ho* = observed heterozygosity, *He* = expected heterozyosity.

Population	Locus	*N*	*A*	*Ar*	*Ho*	*He*
St Francis						
	PcA10	25	1.000	1.000	0.000	0.000
	PcC122	20	8.000	7.452	0.750	0.817
	PcD110	17	10.000	8.000	0.941	0.875
	PcC112	25	1.000	1.000	0.000	0.000
	PcC127	22	5.000	4.373	0.591	0.552
	PcC135	24	3.000	1.611	0.125	0.121
	PcC131	24	1.000	1.000	0.000	0.000
	PcD123	20	13.000	11.145	1.000	0.924
	PcD118	24	7.000	5.055	0.542	0.604
	PcC111	25	1.000	1.000	0.000	0.000
	PcD124	21	10.000	7.953	0.714	0.861
	PcD132	23	5.000	3.607	0.435	0.438
	Average	22.5	5.416	4.433	0.424	0.432
Ohio River						
	PcA10	30	1.000	1.000	0.000	0.000
	PcC122	22	8.000	7.619	0.818	0.823
	PcD110	28	10.000	9.034	0.857	0.878
	PcC112	30	1.000	1.000	0.000	0.000
	PcC127	28	6.000	4.871	0.679	0.705
	PcC135	27	2.000	2.225	0.000	0.073
	PcC131	28	3.000	2.695	0.393	0.458
	PcD123	28	12.000	10.56	0.929	0.900
	PcD118	29	8.000	5.661	0.690	0.684
	PcC111	22	2.000	1.635	0.091	0.089
	PcD124	28	15.000	11.628	0.929	0.931
	PcD132	30	5.000	4.722	0.767	0.679
	Average	27.5	6.083	5.221	0.512	0.518

Table 3. STRUCTURE analysis results for *Potamilus capax* samples collected from the St. Francis and Ohio rivers. Sample population refers to where the sample was collected and Inferred population refers to the population assignment based on STRUCTURE analyses. The last two columns represent the proportion of time STRUCTURE assigned that individual to its respective population (i.e., St. Francis or Ohio). We called any individual with an assignment value less than 90% a intraspecific hybrid or backcross. This number was arbitrary and should be subjected to a more rigorous evaluation.

USFWS#	Sampled population	Inferred population	Ohio assignment	St Francis assignment
4672	St. Francis	St. Francis	0.158	0.842
4673	St. Francis	St. Francis	0.097	0.903
4675	St. Francis	St. Francis	0.082	0.918
4676	St. Francis	St. Francis	0.19	0.81
4677	St. Francis	St. Francis	0.201	0.799
4678	St. Francis	St. Francis	0.067	0.933
4679	St. Francis	St. Francis	0.101	0.899
4680	St. Francis	St. Francis	0.197	0.803
4681	St. Francis	St. Francis	0.151	0.849
4682	St. Francis	St. Francis	0.182	0.818
4683	St. Francis	St. Francis	0.049	0.951
4684	St. Francis	St. Francis	0.026	0.974
4685	St. Francis	St. Francis	0.032	0.968
4686	St. Francis	St. Francis	0.218	0.782
4687	St. Francis	St. Francis	0.069	0.931
4692	St. Francis	St. Francis	0.529	0.471
4700	St. Francis	St. Francis	0.168	0.832
4701	St. Francis	St. Francis	0.056	0.944
10243	St. Francis	St. Francis	0.058	0.942
10244	St. Francis	St. Francis	0.033	0.967
10245	St. Francis	St. Francis	0.052	0.948
10246	St. Francis	St. Francis	0.222	0.778
10247	St. Francis	St. Francis	0.157	0.843
10248	St. Francis	St. Francis	0.06	0.94
12990	Ohio	Ohio	0.845	0.155
12991	Ohio	Ohio	0.792	0.208
12992	Ohio	Ohio	0.641	0.359
12993	Ohio	Ohio	0.949	0.051
12994	Ohio	Ohio	0.791	0.209
12995	Ohio	Ohio	0.974	0.026

12996	Ohio	Ohio	0.953	0.047
12997	Ohio	Ohio	0.939	0.061
12998	Ohio	Ohio	0.9	0.1
12999	Ohio	Ohio	0.798	0.202
13000	Ohio	Ohio	0.972	0.028
13001	Ohio	Ohio	0.962	0.038
13002	Ohio	Ohio	0.978	0.022
13003	Ohio	Ohio	0.741	0.259
13004	Ohio	Ohio	0.478	0.522
13005	Ohio	Ohio	0.459	0.541
13006	Ohio	Ohio	0.965	0.035
13007	Ohio	Ohio	0.963	0.037
13008	Ohio	Ohio	0.967	0.033
13009	Ohio	Ohio	0.541	0.459
13010	Ohio	Ohio	0.965	0.035
13011	Ohio	Ohio	0.956	0.044
13012	Ohio	Ohio	0.949	0.051
13013	Ohio	Ohio	0.955	0.045
13014	Ohio	Ohio	0.905	0.095
13015	Ohio	Ohio	0.896	0.104
13016	Ohio	Ohio	0.95	0.05
13017	Ohio	Ohio	0.548	0.452
13018	Ohio	Ohio	0.886	0.114
13019	Ohio	Ohio	0.962	0.038

Table 4. Assignment success of STRUCTURE analysis for Ohio River specimens with an inferred number of groups set to 2. Morph1 and Morph2 are arbitrary and simply indicate the possibility of two distinct morphotypes. A value of 0.5 in each group indicates that the individual is equally probable of being placed into each group. See Appendix for morphologies.

	Inferred population	
USFWS #	Morph1	Morph2
12990	0.500	0.500
12991	0.500	0.500
12992	0.499	0.501
12993	0.500	0.500
12994	0.498	0.502
12995	0.501	0.499
12996	0.500	0.500
12997	0.500	0.500
12998	0.501	0.499
12999	0.502	0.498
13000	0.500	0.500
13001	0.499	0.501
13002	0.500	0.500
13003	0.501	0.499
13004	0.500	0.500
13005	0.499	0.501
13006	0.497	0.503
13007	0.502	0.498
13008	0.499	0.501
13009	0.499	0.501
13010	0.498	0.502
13011	0.503	0.497
13012	0.499	0.501
13013	0.498	0.502
13014	0.500	0.500
13015	0.499	0.501
13016	0.500	0.500
13017	0.500	0.500
13018	0.500	0.500
13019	0.495	0.505

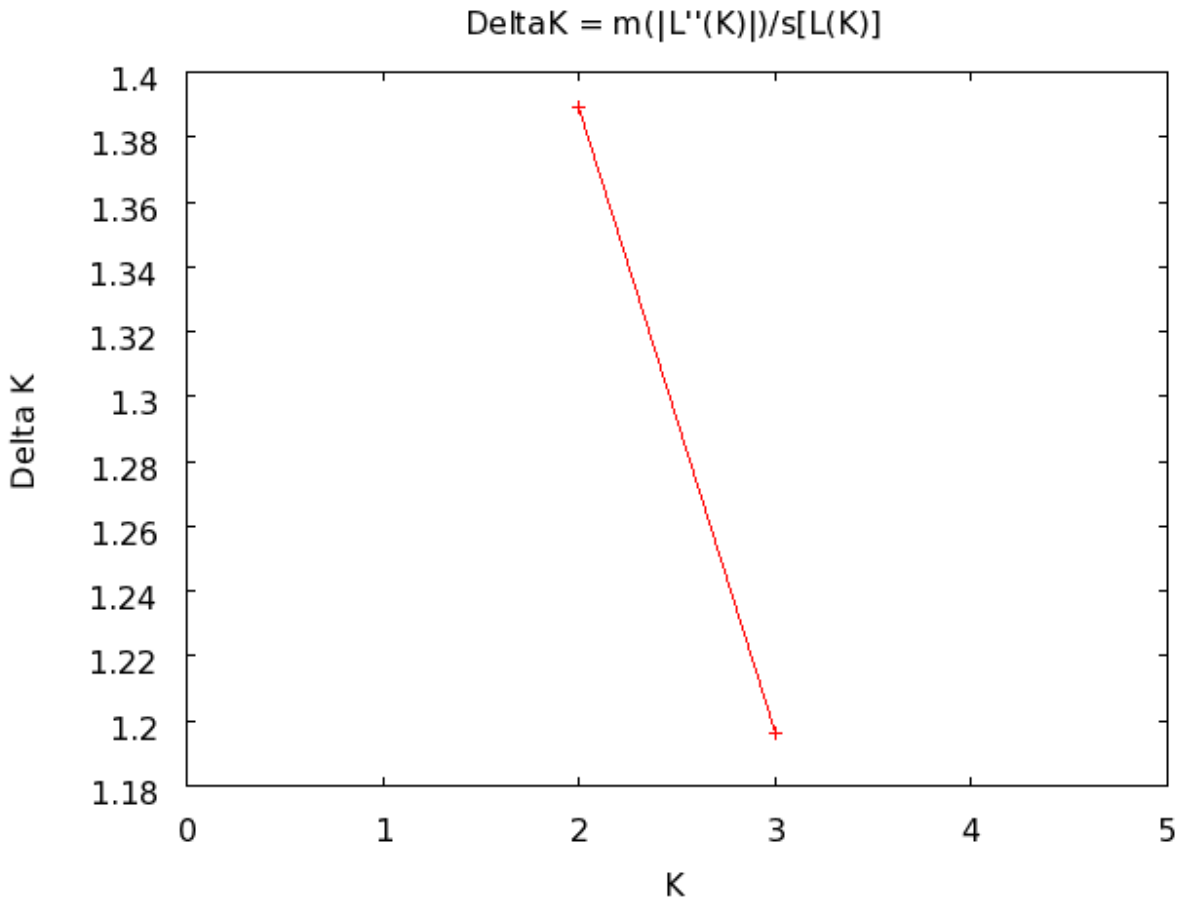

Figure 1. Delta K averaged across five replicate simulations with K-values of 1-4. Simulation results indicated that the most plausible value for the number of groups (K) represented by sampled *P. capax* from the St. Francis and Ohio rivers was two as observed by the distinct reduction in delta K from K of 2 to K of 3.

Appendix. Ohio River photo vouchers of *Potamilus capax* used in this study. Photos taken by Lewis Environmental Consulting, LLC.

USFWS # 12990

USFWS # 12991

USFWS # 12992

USFWS # 12993

USFWS # 12994

USFWS # 12995

USFWS # 12996

USFWS # 12997

USFWS # 12998

USFWS # 12999

USFWS # 13000

USFWS # 13001

USFWS # 13002

USFWS # 13003

USFWS # 13004

USFWS # 13005

USFWS # 13006

USFWS # 13007

USFWS # 13008

USFWS # 13009

USFWS # 13010

USFWS # 13011

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