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Mitochondrial RFLP Analysis of Montana Sculpins

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Abstract

The genus *Cottus* includes several species of sculpins. The shorthead sculpin, *C. confusus*, was recognized as a distinct species in 1963 and bears close resemblance to the mottled sculpin, *C. bairdi*. On the basis of allozyme analysis, it has been proposed that *C. confusus* resides in a watershed in northern Montana, Tepee Creek of the Flathead River, beyond what is considered the present range of the fish. In the current study, five populations were analyzed, originating from Clearwater River, Tepee Creek, Yellowstone River, and Missouri River at Dearborn and at Craig. An outgroup of a known species, the slimy sculpin , *C. cognatus*, collected from Grant Creek was also analyzed. DNA was isolated and mitochondrial DNA sequences were amplified using PCR. Restriction enzyme digestions using sixteen enzymes were performed on the resulting PCR product. Restriction fragment length polymorphisms (RFLPs) were subsequently analyzed and the results compiled to produce a table of haplotypes and estimated phylogenetic trees. Analysis of the trees showed the slimy sculpin to be a separate clade but did not support the existence of *C. confusus* in the Montana watersheds studied. The few exceptions observed were attributed to individual genetic variation. Future analysis includes collecting samples from locations in which *C. confusus* is known to exist and from watersheds where its presence is questionable. DNA sequencing of *C. confusus, C. bairdi,* and *C. cognatus* will also be performed in order to better convert RFLP data into restriction site data.

Materials and Methods

Sixty samples of fin tissue were received from six drainages in the state of Montana and ten from a drainage in Kentucky (Figure 1). These sites and the individual

samples studied are listed in Table 1. DNA isolation, amplification of mitochondrial DNA, and restriction enzyme digestions were performed on the samples. Tissue samples were used in the isolation of DNA following the protocol of the Qiagen DNA isolation kit. Isolated DNA samples were kept frozen until DNA amplification could be completed using the Polymer Chain Reaction (PCR). Amplification of mitochondrial sequences used a primer that binds in the histidine tRNA gene (5' CTCCCTTTATATGTTCC 3') and one that binds in the glycine tRNA gene (5' GATAATGAACCTAGTT 3'). The map of the mitochondrial genome in Figure 2 indicates the amplified region. PCR reactions contained 4µl of DNA template (approx. 100 ng), 20 pmol each primer, 25 mM KCl, 0.05% Tween 20, 200µM dNTPs, 1 unit of Taq DNA polymerase, 1 mM Mg2+, and water to a final volume of 25 µl. The thermal cycler was then programmed with the following set of operating perameters for the PCR:

1) One cycle at four minutes at 94 degrees centigrade for an initial denature.
2) Thirty cycles of one minute at 94 degrees centigrade followed by a one minute annealing followed by a 74 degree extension.
3) A ten minute cycle of 74 degrees centigrade for a final extension.
4) A final hold cycle at 4 degrees centigrade.

The PCR products were stored in refrigeration until a restriction enzyme digest was ready to be performed. Eighteen restriction enzymes were used in separate digests. The formula for the restriction digests used was: 2 L o f 10 X reaction buffer, 0.2 L of restriction enzyme (1 unit), 12.8 L of dH2O, 5 L o f the PCR product (approx. 1 g). The digests were placed in a 37° water bath for over two hours but not exceeding 12 hours. Gel electrophoresis was used to observe restriction fragment length polymorphisms (RFLPs). The resulting data were used in combination with DNA sequence information to construct a presence/absence matrix of restriction sites. This matrix was imported into PAUP where it was used for the construction of estimated phylogenetic trees.

Results and Conclusions

Figure 1 shows the sites of sample collection in Montana. DNA was isolated from fin clips of the individuals listed in Table 1. The arrows shown in Figure 2 indicate the sites of primer binding for DNA amplification. The amplification products of approximately 2000 bp were digested with a total of sixteen restriction enzymes in order to detect variations. Figure 3 shows the results of RFLP analysis of the six populations studied. As described in Materials and Methods, the amplified mitochondrial DNA sequences were digested with the enzymes indicated. Table 2 shows the haplotypes detected by the RFLP analysis. The majority of individuals from Clearwater, Tepee, Missouri River at Dearborn and Craig, and Yellowstone River had the same haplotype for each of the 16 enzymes studied. Individuals from Grant Creek had a different haplotype from those above for all enzymes that revealed polymorphisms. Six enzymes clearly showed Grant Creek individuals to have a different haplotype from the other five Montana populations. This indicates that the individuals from Grant Creek are genetically distinct from the other five Montana populations, an idea which is consistent with their identification as slimy sculpins.

Figures 4 and 5 are neighbor joining and UPGMA trees constructed using the mitochondrial RFLP data. For these trees, the haplotypes listed in Table 2 were used. Since five of the populations had the same haplotype, they were combined in the analysis as a group called Montana on the trees. The group labeled Kentucky contains mottled sculpins and the one labeled Niangua contains Ozark sculpins.

It has been suggested from allozyme analysis that shorthead sculpins exist in Tepee creek. It also also been suggested that they may exist in the Flathead River. Our data do not support these ideas and suggest that all of the five populations studied contain mottled sculpins.

Table 3 lists all the exceptions to the pattern in which all individuals in Clearwater, Tepee, Yellowstone, and Missouri River at Dearborn and Craig are the same

haplotype. For the sixteen enzymes studied for 5 individuals in each of those locations, there are a total of 400 haplotypes determined. Of these there are only 14 exceptions to the idea stated above. The Grant Creek individuals had the same haplotype as each other and a different haplotype from the other Montana site for the majority of enzymes studied. For the five individuals and 16 enzymes studied, there are 80 haplotypes and only 13 of these were exceptions to the idea above. The level of exceptions in each of these case can be attributed to individual genetic variation.

This preliminary study has demonstrated that methods are available for the genetic analysis of sculpin populations in Montana. Important questions remain, however, as to the presence of theshorthead sculpin in watersheds outside its established range. Further analysis should be conducted and will include the collection and analysis of sample from locations where the shorthead sculpin is known to exist and from those where it is suspected to exist. Continuation of this study will also include the cloning and DNA sequencing of samples already studied and of samples to be collected. Conversion of the RFLP fragment data into restriction site data will require DNA sequences of the amplified mitochondrial genome from slimy, mottled, and shorthead sculpins.

Table 1

Site Sampled	Individuals Tested
Missouri River at Craig	#32, 33, 34, 38, 40
Missouri River at Dearborn	#22, 23, 24, 29, 30
Yellowstone River, So. of Livingston	#42, 43, 44, 49, 50
Tepee Creek. North Fork of Flathead River	#12, 13, 14, 19, 20
Grant Creek, Clark Fork	#52, 53, 54, 57, 60
Clearwater River, Blackfoot Drainage	#2, 3, 4, 9, 10

Table 2
Haplotypes of mitochondrial sequences studied.

Sites	Enzymes															
	A	F	V	C	D	P	E	H	I	F	M	R	S	O	T	Q
Montana	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Grant	B	A	B	A	B	A	B	A	B	A	B	A	B	A	A	A
Kentucky	A	B	C	B	C	B	C	A	B	B	C	B	B	B	A	B
Niangua	A	A	A	N	D	C	N	A	C	C	D	C	N	N	N	C

A = AccI	F = AflII	V = AvaII	C = ClaI	D = DdeI	P = DpnII
E = HaeIII	H = HhaI	I = HindIII	F = HinfI	M = MseI	R = RsaI
S = SmaI	O = EcoRI	T = PstI	Q = TaqI		

N = not determined

Table 3

Mitochondrial RFLP Data Exceptions

The following are exceptions to the pattern in which all individuals in Clearwater, Tepee, Yellowstone, and Missouri River at Dearborn and Craig are the same while individuals in Grant Creek are either the same as those above or are all different for the 16 enzymes studied.

Location	Individual Sample No.	Enzyme
MR C	32	DdeI
MR C	32	AvaII
MR C	40	MseI

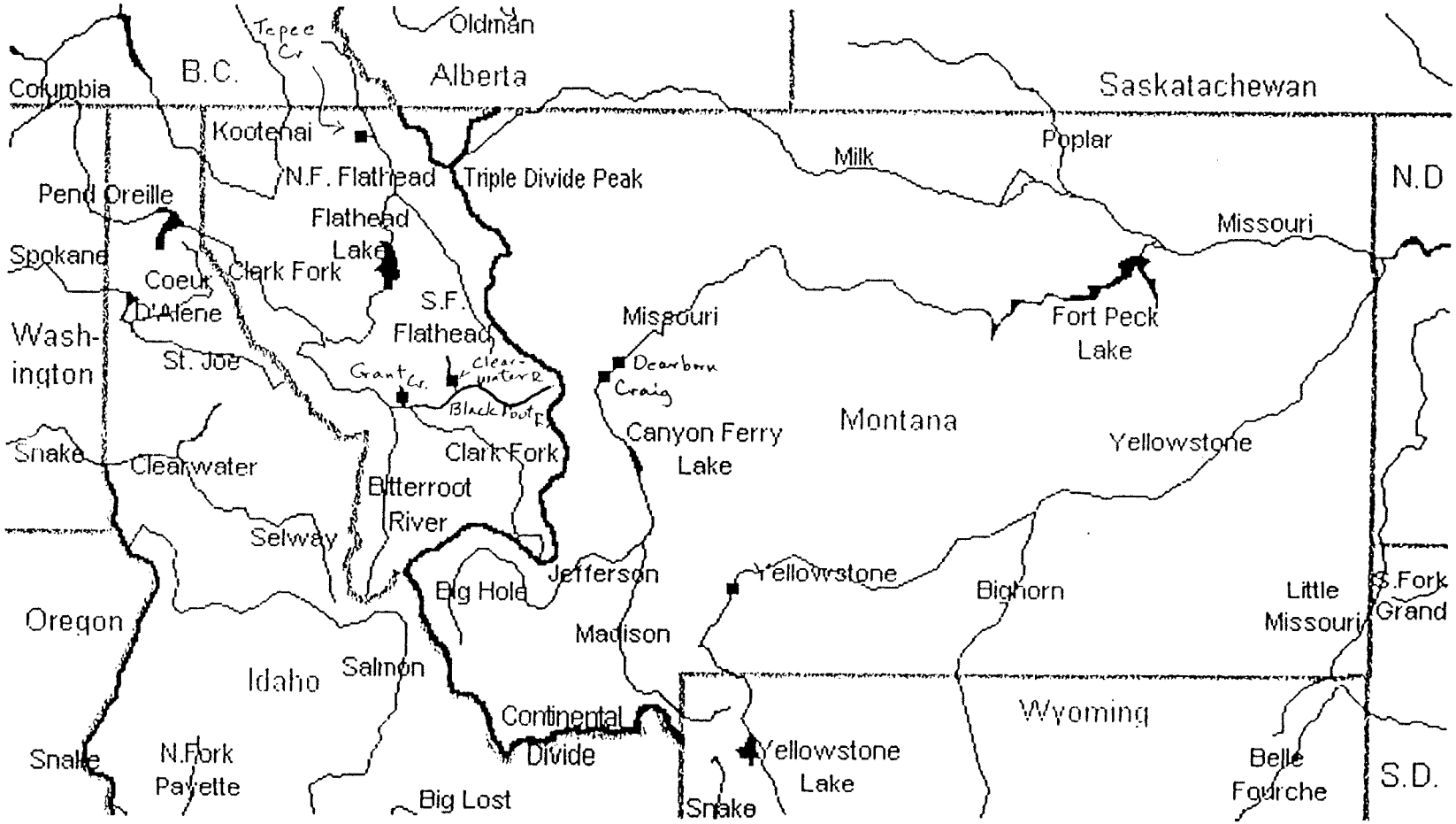
MR D	23	MseI
YR	42	DdeI
YR	42	AvaII
YR	44	MseI
YR	50	AvaII
YR	50	DdeI
C	2	DdeI
T	14	MseI
T	12	MseI
T	12	DdeI
T	13	MseI
T	20	MseI
G	52	DdeI
G	52	MseI
G	53	DdeI
G	53	MseI
G	54	DdeI
G	54	MseI
G	57	HinfI
G	57	HaeIII
G	57	HindIII
G	57	RsaI
G	57	DdeI
G	60	MseI
G	60	DdeI

MR C = Missouri River at Craig
MR D = Missouri River at Dearborn
YR = Yellowstone River
T = Tepee Creek
G = Grant Creek
C = Clearwater River

Figure 1

Environ Mt

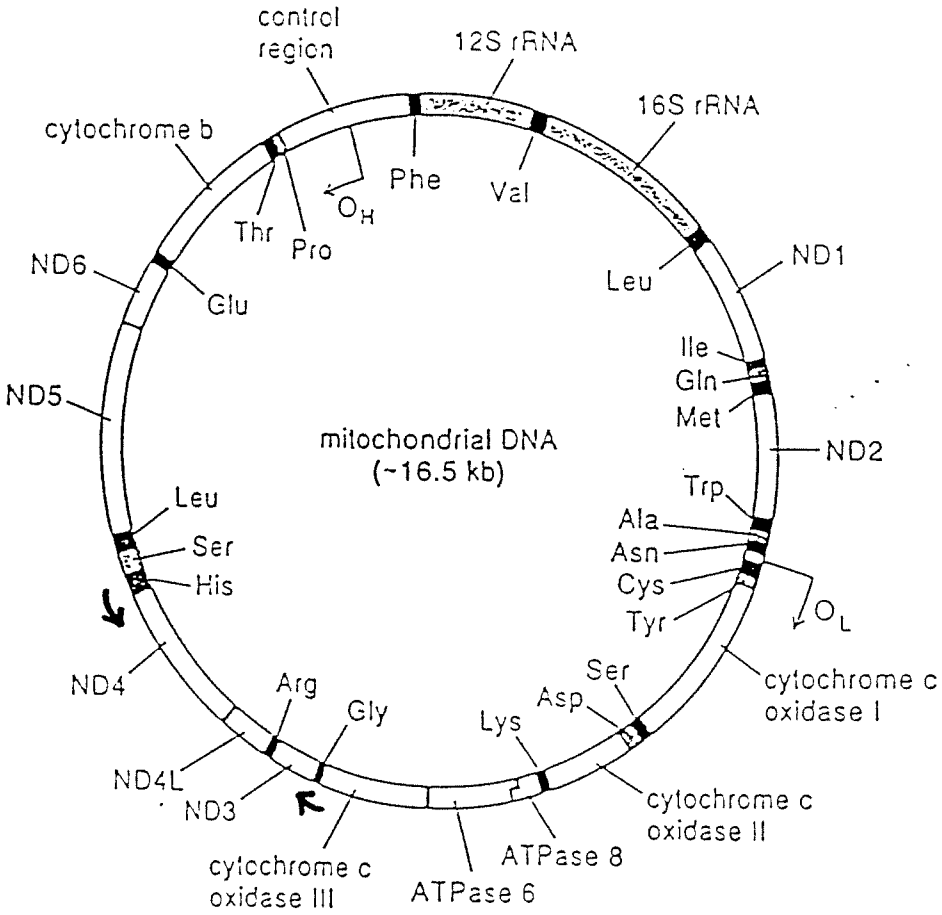
Montana Rivers



Major Rivers of Montana

Montana is unique in the United States as the source of water for three distant ocean destinations. Two river divides are found in the state, the Continental Divide and the Hudson Bay Divide. The three great watersheds meet at a mountain in Glacier Park called Triple Divide Peak.

Figure 2



→ = primer binding site

Figure 4

Neighbor Joining

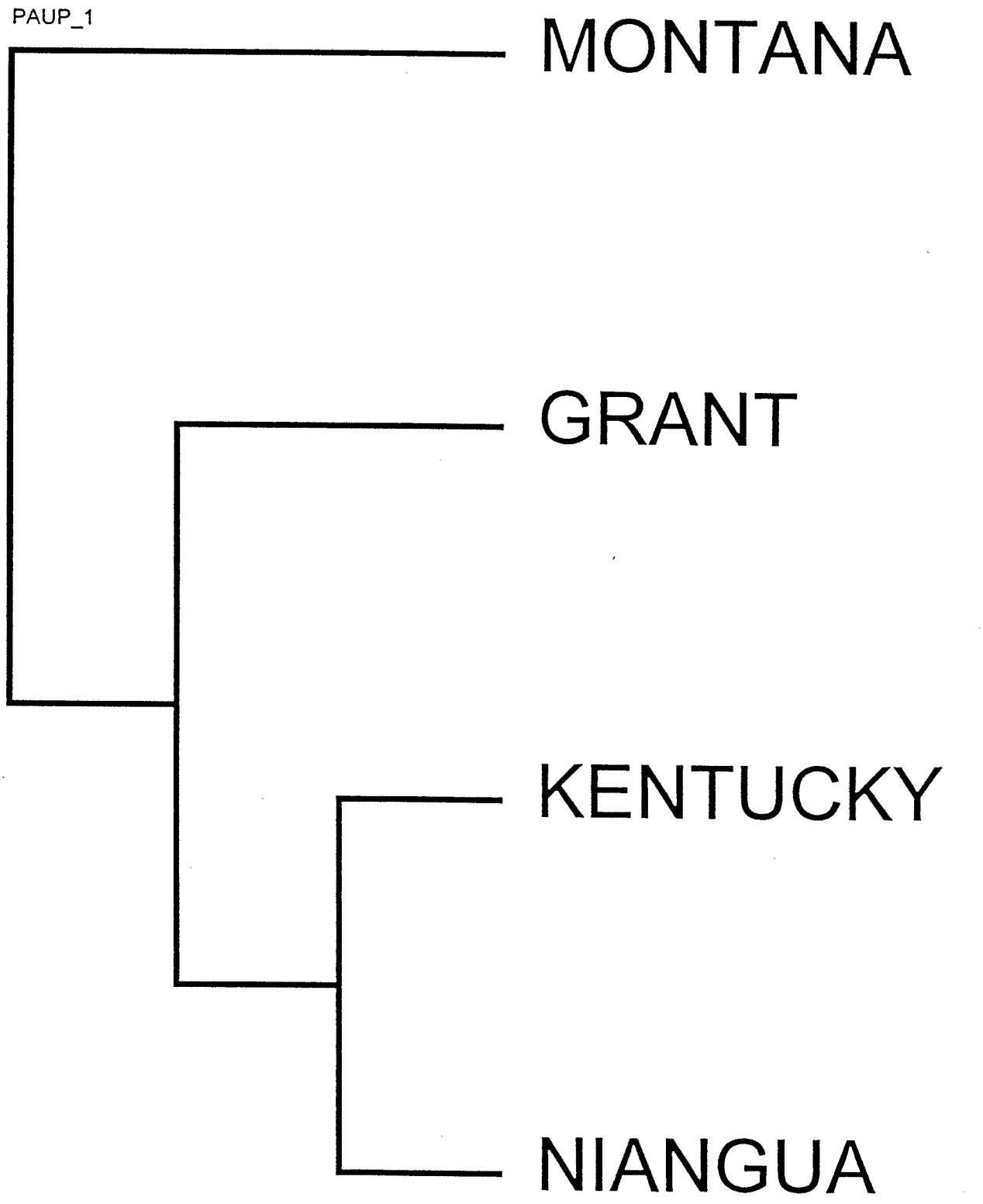


Figure 5

UPGMA

PAUP_1