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Mitochondrial DNA Sequence Variation and Phylogenetic Relationships among Michigan Brown Trout (*Salmo trutta*) Strains

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MITOCHONDRIAL DNA SEQUENCE VARIATION AND PHYLOGENETIC RELATIONSHIPS AMONG MICHIGAN BROWN TROUT (*Salmo trutta*) STRAINS

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By

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To

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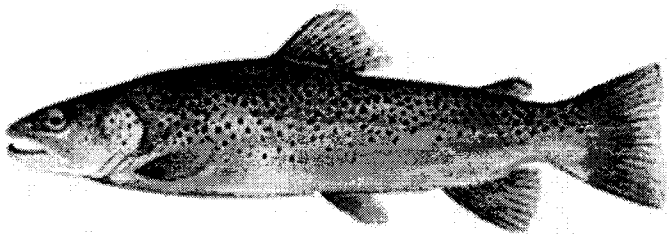

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ABSTRACT

Despite the extensive use of mitochondrial DNA (mtDNA) in studies of European brown trout (*Salmo trutta*) populations, little information is available regarding the genetic composition or phylogenetic relationships among strains of Michigan brown trout. The objective of this study was to quantify the amount of polymorphism among three strains of Michigan brown trout at the mtDNA level and to infer genetic relatedness among representative individuals of these strains. This was accomplished by sequencing the ND1 region of mtDNA and constructing phylogenetic trees based on detected sequence variation. A total of 23 single nucleotide polymorphisms (SNPs) were found in the ND1 region with two additional SNPs found in the 16S rRNA region directly preceding ND1. All but two fish examined displayed synonymous substitutions at all 23 ND1 SNP sites. Two fish belonging to a mtDNA haplotype found in the Seeforellen strain showed nonsynonymous substitutions at two of the sites. Further study would be necessary to determine if these amino acid substitutions have any functional significance.

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CHAPTER I

INTRODUCTION

Brown trout (*Salmo trutta*) is a member of the family Salmonidae which consists of three subfamilies; Coregoninae (whitefish and ciscoes), Thymallinae (grayling), and Salmoninae (char, trout, and salmon). The subfamily Salmoninae has the largest number of species and includes five genera. Brown trout and Atlantic salmon (*Salmo salar*) comprise the *Salmo* genus (Crespi and Fulton 2004).

Within the species of brown trout there is considerable variation in life history (Bernatchez et al. 1992). Brown trout includes three forms; the sea-run or migratory trout (anadromous), lake dwelling, and stream resident forms, that differ mainly in their migratory behavior (Laikre 1999, Pakkasmaa and Piironen 2001). A population of brown trout may split into both anadromous and resident forms where anadromous migrate to the sea and residents stay in fresh water. The migrant and resident adult forms can spawn together with the decision to migrate being influenced by genetic factors, such as metabolic rate, as well as environmental factors (Cucherousset et al. 2005).

Brown trout also exhibit significant variation in morphology. For example, in Finland some brown trout may have silvery or dark coloring while others are more brownish. In addition, certain resident brown trout may display red spots only as juveniles while others maintain the spots throughout their lives (Pakkasmaa and Piironen 2001). In early studies of brown trout, populations were often characterized on the basis of their morphology and life history tactics leading to the proposal of numerous species names for the various forms (Laikre 1999). However, differences observed between

the forms of brown trout are now generally attributed to environmental and phenotypic plasticity and, therefore, delineation of populations for taxonomic as well as for conservation and management purposes should be based on genetic differences (Laikre 1999).

Genetic polymorphism at the nucleotide level of mitochondrial DNA (mtDNA) has been used in population studies of salmonid fishes (Ferguson et al. 1995, Billington and Hebert 1991). Recently, mtDNA has been used to study the genetic relatedness of brown trout within natural and hatchery-reared populations (Ruzzante et al. 2004, Apostolidis et al. 1996, 1997, Hanson and Loesche 1996). The relatively smaller size of mtDNA compared to nuclear DNA, its maternal inheritance, and its general lack of recombination make this molecular marker an attractive tool in population studies (Ingman and Gyllensten 2001). Compared to nuclear DNA the substitution rate of mtDNA is five to ten times higher allowing it to retain a history of evolutionary events such as bottlenecks that have occurred in the recent past (Ingman and Gyllensten 2001). In addition, the maternal inheritance and lack of recombination allow for detection of lineage-specific genetic differences (Hynes et al. 1989).

The wide range of adaptations observed in brown trout implies that there may be a high degree of genetic variation in this species. In fact, brown trout is considered to be one of the most genetically diverse vertebrate species known (Ferguson 1989). A large amount of genetic variability has been found within and among brown trout populations in Europe. Ferguson et al. (1995) found that geographically separate brown trout populations are usually genetically distinct. However, in a study by Apostolidis et al.

(1996), genetic diversity among 13 brown trout populations was very high but genetic diversity within most populations was low. In contrast, analysis of two brown trout populations in Scotland revealed no genetic variation among individuals or between the populations (Prodohl et al. 1997).

Various environmental factors can contribute to further genetic subdivision within the brown trout species. For example, population genetic studies have found that physical barriers such as dams and waterfalls influence the genetic structure of brown trout populations. Carlsson et al. (1999) found significant differences in allele frequencies for brown trout populations in a stream divided by impassable waterfalls as well as in mainstream trout populations compared to tributary populations. Brown trout populations from different tributaries of the Karup River in Denmark also showed significant differences in mtDNA haplotype frequencies (Hanson and Loeschcke 1996).

Phylogenetic relationships of brown trout populations have been determined by analyzing trout from 174 populations from Europe, the Middle East and North Africa. These relationships were determined using sequence analysis of a portion of the mtDNA control region and PCR-RFLP analysis of the remaining portion of the control region as well as the mitochondrial ND5/6 and cytochrome b oxidase regions. Bernatchez (2001) found that there were five major evolutionary lineages from which subsequent populations of brown trout have developed. Each lineage was shown to have a different evolutionary history with each evolving in geographic isolation and developing unique population genetic structures. For example, the Mediterranean lineage present in the Balkans was found to have the most diversity among populations despite its smaller size

presumably due to isolation over a long period of time in differing environments (Bernatchez 2001). Apostolidis et al. (1997) found that in the southern Balkans there were four phylogenetic groups indicating much subdivision of the ancestral lineage. These groups were not obvious based on geographic characteristics, suggesting that there may have been a long period of isolation combined with bottlenecks and genetic drift.

The native range of brown trout is primarily Europe and parts of Asia and North Africa. Artificial introductions to countries throughout the world, including the USA, Canada, and Australia, occurred in the late 1800s and early 1900s. Brown trout was first imported to the USA in 1883 from Germany and stocked in the Pere Marquette River, Michigan, by the U.S. Fish Commission (Westerman 1977). Brown trout have subsequently been introduced into nearly every state (MacCrimmon 1968). Because these introductions occurred before the genetic consequences of such introductions were understood, the effect on the genetic composition of the species was unknown and the ecological consequences generally were not recognized (Laikre 1999).

The state of Michigan currently maintains and stocks three hatchery strains of brown trout; Wild Rose, Gilchrist and Seeforellen (Michigan Department of Natural Resources (MDNR) 2007). Although much phenotypic and life history information is available for these strains, very little genetic information is currently available. Previous analysis of the ND-1 and ND5/6 regions of mtDNA of brown trout from the Oden State Fish Hatchery, the Muskegon and Rogue Rivers, and Lake Michigan using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques revealed two haplotypes within the Gilchrist strain, two within the Wild Rose strain, and

two unknown haplotypes in the Rogue River (Tiano 2005). An additional haplotype was found in three Lake Michigan trout which were determined to be Seeforellen based on Michigan DNR stocking records (Tiano 2005). The Wild Rose and Gilchrist strains differed by four single nucleotide polymorphisms (SNPs) with two fish found in the Rogue River having additional variation not present in either the Wild Rose or the Gilchrist stocks. Because these strains differ substantially in survival and growth (Wills 2006), further study of their genetic composition may help to better understand the differences in life history of Michigan brown trout strains and to better guide their management. The objective of this study was to identify all polymorphism in the NADH dehydrogenase subunit 1 (ND1) region of Michigan brown trout mtDNA by sequencing the ND1 region in representatives of each strain and to use this information to: 1) provide additional markers to separate the Gilchrist from the Wild Rose strains and establish markers to identify the Seeforellen strain beyond RFLP; 2) analyze the polymorphism for the site discrimination; and 3) determine phylogenetic relationships among these strains based on ND1 polymorphism. The information obtained by sequencing the ND1 segment will thus provide a more complete understanding of the genetic composition and relatedness of Michigan brown trout strains.

CHAPTER II

METHODS

Sample Collection

We used previously obtained samples from Wild Rose and Gilchrist hatchery and broodstock brown trout from the Oden State Fish Hatchery, as well as samples from the Muskegon River, Rogue River, and Lake Michigan for our analysis (Tiano 2005). Additionally, samples from Seeforellen strain broodstocks were obtained from the Oden State Fish Hatchery in 2006. The number of each fish analyzed is shown in Table 1. A total of 48 brown trout samples have been sequenced.

PCR-RFLP techniques were previously used to analyze samples designated as Wild Rose or Gilchrist from the hatchery and this information was used to assign fish from the Muskegon and Rogue Rivers to a hatchery strain (Tiano 2005). A subset of these samples was selected from each strain and location.

Table 1. Number of brown trout sequenced from each location.

	Hatchery	Broodstock	Rogue River	Muskegon River	Lake Michigan	n
Wild Rose	2	2	2	3	2	11
Gilchrist (1)	7	3	3	3	2	18
Gilchrist (2)	1	2	3			6
Gilchrist (3)	1					1
Gilchrist (4)				1		1
Seeforellen (1)		1	1		2	4
Seeforellen (2)		3				3
Seeforellen (3)		2				2
Seeforellen (4)					1	1
Rogue River 26			1			1
total	11	13	10	7	7	48

DNA Amplification and Sequencing

Total DNA was extracted from fin clips with the Qiagen DNeasy Tissue Kit with final DNA elution in 60ul of distilled water. The ND1 mtDNA segment, along with a portion of the 16S rRNA region directly preceding ND1, was amplified using PCR with previously published NADH-dehydrogenase 1 forward and reverse primers (Nielsen et al. 1998). PCR reactions were performed in 50 µl total volume with 1X Thermopol Buffer from New England Biolabs©, 100 pmoles of each primer, 2.5 U *Taq* DNA Polymerase, and 2 µl DNA template. Eppendorf Master Cycler was used to perform PCR reactions. The thermal cycles consisted of 5 minutes at 94°C followed by 30 cycles of denaturation for 30 seconds at 94°C, annealing for 45 seconds at 62.4°C, and extension for 2 minutes 30 seconds at 72°C, ending with a termination step of 7 minutes at 72°C (Tiano 2005). PCR products were run on a 1% agarose gel with ethidium bromide staining to verify amplification, purified with the Qiagen MinElute PCR Purification Kit, and sent to the University of Michigan Sequencing Core for sequencing.

The sequencing was initially done using the forward PCR primer, ND1F. A sequence of approximately 1000 base pairs was obtained using this primer. Based on the initial sequence data, additional primers were designed for further sequencing close to the 3' end of the initial sequence using the Primo 3.4 Sequencing Primer Design algorithm from Chang Bioscience (http://www.changbioscience.com/primo/primoseq.html). New internal sequencing primers are listed in Table 2; the segment of mtDNA amplified by each primer is shown in Figure 1. After sequencing samples with each new sequencing primer, it was determined that the Pol F primer resulted in the greatest amount of accurate

sequence so this was used for subsequent analyses. Representative trout for each haplotype were also sequenced using the ND1R primer to sequence the last portion of the ND1 region. Thus, approximately 1100 base pairs were analyzed for each sample, or about 6% of the entire mtDNA genome.

Table 2. Primer sequences for mtDNA analysis of Michigan brown trout. ND1 primers were previously published (Nielsen et al. 1998); all others were designed for this study.

Primer	Primer Sequence (5' to 3')
NADH-dehydrogenase 1 F	GCCTCGCCTGTTTACCAAAAACAT
NADH-dehydrogenase 1 R	GGTATGGGCCCGAAAGCTTA
Pol F	AGAAGGGGCCCATGCTTAAGG
Pol R	GGACAAGAGCTAGTGTTAAAGG
704 F	CCAAAATGGCCCAAAAGAACGG
830 F	TGTTAACCCACTCGCATACATC
694 F	GCCTTCCCCGAATTAACAGCC
863 F	TAGCGCTTCCAACCGCAACAG

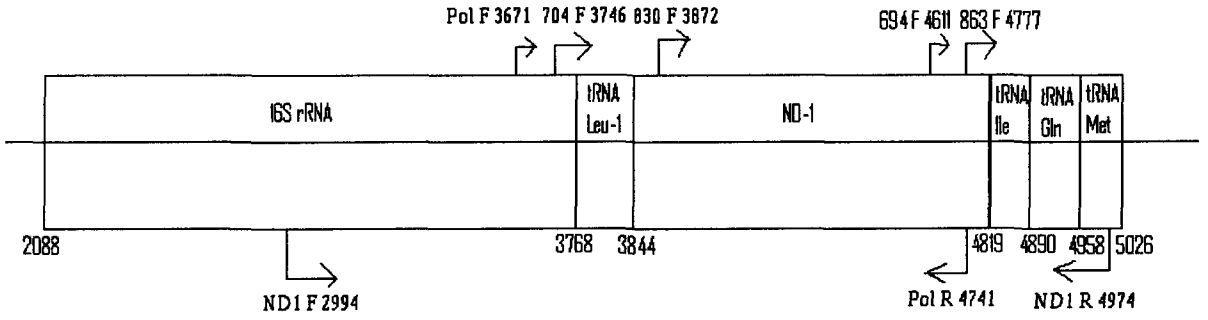

Figure 1. Location of primers and portion of mtDNA amplified based on NCBI NC_001717 rainbow trout sequence.

Sequence Analysis

Once sequence data was obtained, a database with the nucleotide sequence for each fish was created. DNAstar Lasergene sequence analysis software was used for sequence alignment and identification of SNPs. The sequences for each strain were compared to known mtDNA sequences for rainbow trout (*Oncorhyncus mykiss*), brook char (*Salvelinus fontinalis*), arctic char (*Salvelinus alpinus*), and Atlantic salmon *(Salmo salar*) from the National Center for Biotechnology Information (NCBI) database as shown in Table 3. Reference sequences for each of these four salmonids, as well as for six brown trout available on the NCBI database, were used to investigate phylogenetic relationships among Michigan brown trout strains.

Phylogenetic trees were constructed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) (Sokal and Sneath 1973) algorithm in the CLC Free Workbench for Macintosh, version 3.2.1. Three trees were constructed using the UPGMA algorithm to show relationships among Michigan brown trout sampled in this study, relationships among Michigan brown trout and available brown trout sequences from the NCBI database, and representative Michigan brown trout to additional salmonid species.

Table 3. Identification of polymorphic sites and site discrimination in representative sequenced Michigan brown trout and nucleotide positions at polymorphic sites identified in Michigan brown trout from known salmonid and brown trout sequences from the NCBI database. Nucleotide positions are based on rainbow trout (*Oncorhyncus mykiss*) NC_001717. Variable nucleotides within the codon are underlined. Nonsynonymous substitutions are in bold.

Species/strain	n	16S rRNA	16S rRNA	ND1	ND1	ND1	ND1	ND1	ND1
	SNP position:	3739	3747	3879	3922	4038	4101	4116	4146
	Codon position:			3	1	3	3	3	3
Wild Rose	11	C	A	CT**C** - L	**T**TA - L	CC**G** - P	CT**C** - L	TG**A** - W	AC**G** - T
Gilchrist (1)	18	C	A	CT**C** - L	**T**TA - L	CC**G** - P	CT**C** - L	TG**A** - W	AC**A** - T
Gilchrist (2)	6	T	A	CT**C** - L	**T**TA - L	CC**G** - P	CT**C** - L	TG**G** - W	AC**A** - T
Gilchrist (3)	1	C	A	CT**C** - L	**T**TA - L	CC**G** - P	CT**C** - L	TG**A** - W	AC**A** - T
Gilchrist (4)	1	C	A	CT**C** - L	**T**TA - L	CC**G** - P	CT**C** - L	TG**G** - W	AC**A** - T
Seeforellen (1)	4	C	A	CT**C** - L	**T**TA - L	CC**A** - P	CT**C** - L	TG**A** - W	AC**G** - T
Seeforellen (2)	3	C	A	CT**C** - L	**T**TA - L	CC**G** - P	CT**C** - L	TG**A** - W	AC**A** - T
Seeforellen (3)	2	C	G	CT**T** - L	**C**TA - L	CC**A** - P	CT**T** - L	TG**A** - W	AC**G** - T
Seeforellen (4)	1	T	A	CT**C** - L	**T**TA - L	CC**A** - P	CT**C** - L	TG**A** - W	AC**G** - T
Rogue River 26	1	C	A	CT**C** - L	**T**TA - L	CC**G** - P	CT**C** - L	TG**G** - W	AC**C** - T

ND1 4161 3	ND1 4167 3	ND1 4311 3	ND1 43124353 1	ND1 3	ND1 4410 3	ND1 4419 3	ND1 4425 3	ND1 4431 3	ND1 4467 3	ND1 4584 3	ND1 4593 3	ND1 4710 3	ND1 4713 3
GGG - G	CTG - L	CTG - L	AGC - S	TTC - F	ATA - M	ATC - I	ACC - T	GCT - A	GGA - G	TTT - F	GCG - A	CAG - Q	CTT - L
GGA - G	CTG - L	CTC - L	AGC - S	TTC - F	ATA - M	ATC - I	ACC - T	GCC - A	GGA - G	TTT - F	GCG - A	CAG - Q	CTC - L
GGA - G	CTG - L	CTC - L	AGC - S	TTC - F	ATA - M	ATC - I	ACC - T	GCC - A	GGA - G	TTT - F	GCG - A	CAG - Q	CTC - L
GGA - G	CTA - L	CTC - L	AGC - S	TTC - F	ATA - M	ATC - I	ACC - T	GCC - A	GGA - G	TTT - F	GCG - A	CAG - Q	CTC - L
GGA - G	CTG - L	CTC - L	AGC - S	TTC - F	ATA - M	ATC - I	ACC - T	GCC - A	GGA - G	TTT - F	GCG - A	CAG - Q	CTC - L
GGG - G	CTG - L	CTC - L	AGC - S	TTC - F	ATA - M	ATC - I	ACC - T	GCT - A	GGA - G	TTT - F	GCG - A	CAG - Q	CTC - L
GGA - G	CTG - L	CTC - L	AGC - S	TTC - F	ATA - M	ATC - I	ACC - T	GCC - A	GGA - G	TTT - F	GCG - A	CAA - Q	CTC - L
GGA - G	CTA - L	CTC - L	**GGC - G**	TTT - F	ATG - M	ATT - I	ACT - T	GCT - A	GGG - G	TTC - F	GCA - A	CAG - Q	CTC - L
GGG - G	CTG - L	CTC - L	AGC - S	TTC - F	ATA - M	ATC - I	ACC - T	GCT - A	GGA - G	TTT - F	GCG - A	CAG - Q	CTC - L
GGG - G	CTG - L	CTC - L	AGC - S	TTC - F	ATA - M	ATC - I	ACC - T	GCT - A	GGA - G	TTT - F	GCG - A	CAG - Q	CTC - L

ND1	ND1	ND1
47334764		4767
2	3	3
AGC - S	CTA - L	TGA - W
AGC - S	CTA - L	TGA - W
AGC - S	CTA - L	TGA - W
AGC - S	CTA - L	TGA - W
AGC - S	CTA - L	TGA - W
AGC - S	CTA - L	TGA - W
AGC - S	CTA - L	TGA - W
AAC - N	CTG - L	TGG - W
AGC - S	CTA - L	TGA - W
AGC - S	CTA - L	TGA - W

Oncorhynchus mykiss NC_001717	Rainbow trout	C	G	CTA - L CTA - L CCG - P CTT - L TGA - W ACA - T
Salvelinus alpinus NC_000861	Arctic char	C	G	CTT - L CTA - L CCA - P CTT - L TGA - W ACA - T
Salvelinus fontinalis NC_00860	Brook char	C	A	CTT - L CTA - L CCG - P CTT - L TGA - W ACA - T
Salmo salar NC_001960	Atlantic salmon	C	G	CTC - L TTA - L CCA - P CTT - L TGA - W ACA - T
Salmo trutta:				
Denmark Gudenaa 2 AF117716		C	A	CTC - L TTA - L CCA - P CTC - L TGA - W ACG - T
Denmark Gudenaa 4 AF117719		C	A	CTC - L TTA - L CCG - P CTC - L TGA - W ACA - T
Denmark Gudenaa 7 AF117718		C	A	CTC - L TTA - L CCG - P CTC - L TGG - W ACC - T
Denmark Gudenaa 10 AF117717		C	A	CTC - L TTA - L CCG - P CTC - L TGA - W ACA - T
Denmark Romania isolate D1 AF117720				CTT - L CTA - L CCA - P CTT - L TGA - W ACG - T
Denmark Romania isolate D2 AF117721				CTC - L CTA - L CCA - P CTC - L TGA - W ACG - T
Spain haplotype 1 NADH1 DQ257411				
Spain haplotype 2 NADH1 DQ257412				
Spain haplotype 3 NADH1 DQ257413				
Spain haplotype 4 NADH1 DQ257414				

GGA - G CTA - L CTC - L AGC - S TTC - F ATA - M ATT - I ACC - T GCG - A GGA - G TTT - F GCA - A CAA - Q CTC - L

GGA - G CTG - L CTT - L AGC - S TTC - F ATA - M ATT - I ACC - T GCC - A GGA - G TTT - F GCA - A CAA - Q CTT - L

GGA - G CTG - L CTT - L AGC - S TTT - F ATA - M ATT - I ACC - T GCT - A GGG - G TTT - F GCA - A CAA - Q CTT - L

GGG - G CTA - L CTC - L AGC - S TTC - F ATA - M ATC - I ACT - T GCT - A GGA - G TTT - F GCA - A CAA - Q CTC - L

GGG - G CTG - L CTC - L AGC - S TTC - F ATA - M ATC - I ACC - T GCT - A GGA - G TTT - F GCG - A CAG - Q CTC - L
GGA - G CTG - L CTC - L AGC - S TTC - F ATA - M ATC - I ACC - T GCC - A GGA - G TTT - F GCG - A CAG - Q CTC - L
GGG - G CTG - L CTC - L AGC - S TTC - F ATA - M ATC - I ACC - T GCT - A GGA - G TTT - F GCG - A CAG - Q CTC - L
GGG - G CTG - L CTC - L AGC - S TTC - F ATA - M ATC - I ACC - T GCT - A GGA - G TTT - F GCA - A CAG - Q CTC - L
GGA - G CTA - L CTC - L **GGC - G** TTT - F ATA - M ATT - I ACT - T GCT - A GGG - G TTC - F GCA - A CAG - Q CTC - L
GGA - G CTA - L CTC - L **GGC - G** TTT - F ATA - M ATT - I ACT - T GCT - A GGA - G TTC - F GCA - A CAG - Q CTC - L
TTT - F GCA - A CAA - Q CTC - L
TTT - F GCA - A CAA - Q CTC - L
TTT - F GCA - A CAA - Q CTC - L
TTT - F GCG - A CAG - Q CTC - L

AGC - S CTA - L TGA - W

AGC - S CTA - L TGA - W

AGC - S CTA - L TGA - W

AGC - S CTA - L TGA - W

AGC - S CTA - L TGA - W
AGC - S CTA - L TGA - W
AGC - S CTA - L TGA - W
AGC - S CTA - L TGA - W
AAC - N CTG - L TGG - W
AAC - N CTG - L TGG - W
AGC - S CTA - L TGA - W
AGC - S CTA - L TGA - W
AGC - S CTG - L TGA - W
AGC - S CTA - L TGA - W

CHAPTER III

RESULTS

Within the Wild Rose, Gilchrist, and Seeforellen strains a total of 23 variable nucleotide positions were found in the ND1 region with two additional polymorphic sites located in the 16S rRNA region (Table 3). Based on these sequences, both the Wild Rose and Gilchrist haplotype one were found in the Wild Rose strain. Four haplotypes with a total of three nucleotide differences were identified in the Gilchrist strain. Four haplotypes were identified in the Seeforellen strain. Ten SNPs were detected among all fish sequenced, excluding the two samples that belonged to Seeforellen haplotype three. Seeforellen haplotype three contained 15 unique SNPs. One previously unidentified Rogue River sample displayed a haplotype that could not be assigned to any of the hatchery strains (Table 3), although it was nearly identical to a Danish brown trout sample (NCBI AF117718).

Analysis of the site discrimination revealed that the majority of polymorphic sites within the ND1 region were synonymous and located in the third nucleotide position of the codon triplet. Of the synonymous sites, only SNP 3922 was not located in the third codon position, but rather was in the first. There were two nonsynonymous polymorphic sites, both of which were found exclusively in Seeforellen haplotype three. Polymorphic site SNP 4312 was located in the first codon position and SNP 4733 was located in the second codon position (Table 3). The substitution at position 4312 replaced a serine with a glycine while position 4733 replaced a serine with an asparagine.

Phylogenetic analysis of Michigan brown trout resulted in the formation of three

distinct clusters (Figure 2). One branch contained all Gilchrist and one of the Seeforellen haplotypes, a second branch contained Wild Rose, two Seeforellen haplotypes, and the unknown Rogue River haplotype whereas the third branch contained only the Seeforellen haplotype 3.

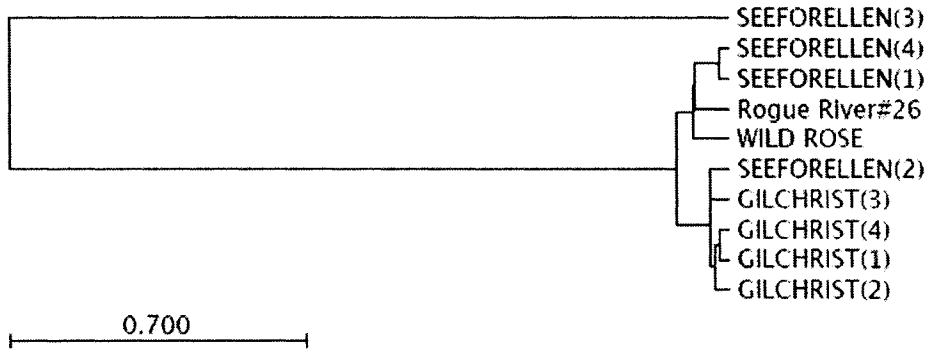

Figure 2. Unrooted phylogenetic tree of Michigan brown trout, using the UPGMA algorithm. Bar represents percent sequence divergence.

Although few sequences were available for comparison of the mitochondrial ND1 gene in other brown trout, one study with four Danish haplotypes from the Gudenaa stream as well as two Romanian haplotypes were available (NCBI AF117716-21). When brown trout sequences available on the NCBI database were included in a phylogenetic tree, similar results were found with the formation of three distinct clusters (Figure 3). One branch contained all of the Gilchrist and one Seeforellen haplotype along with one Danish haplotype. A second branch contained Wild Rose, two Seeforellen haplotypes, the unknown Rogue River haplotype, and three Danish haplotypes. The third branch contained Seeforellen haplotype 3 and two Romanian haplotypes from the NCBI database.

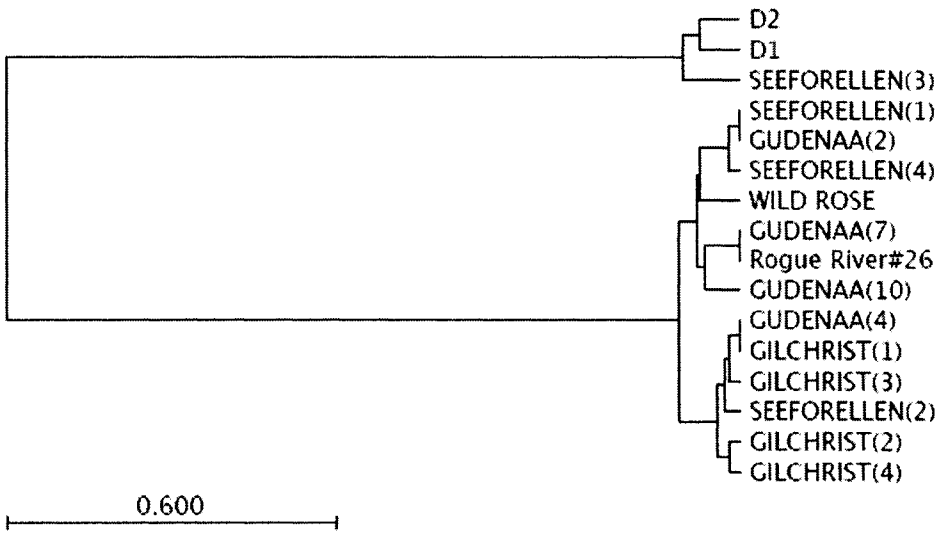

Figure 3. Unrooted phylogenetic tree of Michigan brown trout and European brown trout from the NCBI database, using the UPGMA algorithm. Bar represents percent sequence divergence.

Further analysis of the NCBI database produced sequence similarity at polymorphic sites between Seeforellen haplotype three and other salmonids. Thus, we analyzed relationships between Seeforellen haplotype three, other Michigan brown trout, and other salmonids. A phylogenetic tree containing Seeforellen haplotype three, representatives of the Gilchrist and Wild Rose strains, rainbow trout, brook char, arctic char, and Atlantic salmon was constructed to further investigate their genetic relatedness (Figure 4). This tree grouped all salmonids in accordance with their conventional phylogeny; the genus *Salvelinus* (arctic char and brook char) formed one branch and the genus *Salmo* (Atlantic salmon and Wild Rose and Gilchrist strains) formed another branch. Rainbow trout formed a separate branch as expected by conventional phylogeny.

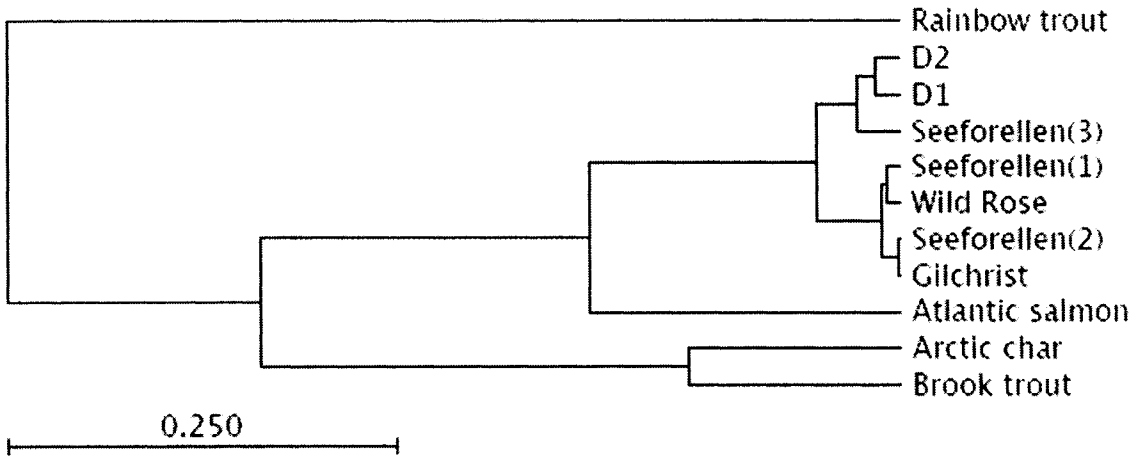

Figure 4. Unrooted phylogenetic tree of representative Michigan brown trout and other selected salmonids, using the UPGMA algorithm. Bar represents percent sequence divergence.

CHAPTER IV

DISCUSSION

As a salmonid species, brown trout (*Salmo trutta*) is economically and ecologically significant. Its ability to readily adapt to a wide range of environmental conditions has made it one of the most widely distributed freshwater fishes (Bernatchez 2001). It has been introduced to countries throughout the world where hatcheries and stocking programs have been established to supplement naturally reproducing populations. Within the species there is much variation in life history, morphology, and intraspecific variability which has complicated efforts to identify distinct brown trout populations and thus to implement effective conservation strategies (Bernatchez 1992, Carlsson et al. 1999).

In response to increasing demand for brown trout and lack of naturally reproducing populations, stocking has become a frequently used practice in the management of brown trout populations (Laikre 1999). One approach to stocking is to use hatchery trout bred for commercial purposes that are not related to native populations (Hansen et al. 1995). In such "put and take" stocking, large numbers of trout are stocked to create fishing opportunities where environmental conditions may not support establishment of wild populations. In this case, altering the genetic composition of brown trout populations would not be a concern; however, exogenous trout are also stocked to enhance fisheries in areas where naturally reproducing populations occur (Laikre 1999). This practice is often considered to be a beneficial management tool for enhancing local brown trout populations (Laikre 1999). Although environmental

degradation is the main cause of brown trout extinction, stocking with hatchery strains of brown trout may alter the genetic composition of wild populations leading to decreased genetic variability and eventually to the extinction of wild brown trout populations (Laikre 1999).

Incorporation of stocked brown trout DNA from hatcheries into native populations has been a major concern in studies of brown trout genetic variation. Rates of incorporating exogenous DNA are increasing due to habitat destruction and introduction of nonnative species (Almodovar 2006). Despite the widespread use of stocking practices, the consequences of stocking can be unpredictable. Many studies have demonstrated introgression of hatchery stocks into natural populations with negative genetic effects including reduced fitness. However, there can also be no genetic effects or complete replacement leading to the extinction of natural populations (Ryman et al. 2006, Caputo et al. 2004, Heggenes et al. 2002, Hansen et al. 1995). Allelic frequencies in hatchery stocks may differ from those of wild fish, therefore stocking without taking into account the genetic composition of wild populations may lead to alterations in the genetic structure of native populations.

Consequences of using hatchery-reared exogenous trout for stocking are evident throughout European brown trout populations. Analysis of 11 brown trout populations in Italy, which were subject to stockings, found haplotypes identical or similar to Danish brown trout populations, the source of the stocked brown trout. Even though the populations had not been subject to stocking in the previous ten years, there was no significant genetic difference between them based on PCR-RFLP analysis of two mtDNA

segments and a nuclear locus. This indicates substantial gene flow between wild and introduced brown trout (Caputo et al. 2004). In Spain, reduction or complete loss of natural brown trout populations has been documented (Machordom et al. 2000). Natural populations in several rivers have nearly been eliminated after stocking with hatchery brown trout presumably originating from Germany and Italy (Marchordom et al. 2000, Garcia-Marin et al. 1991). Apostolidis et al. (1996) found that Greek brown trout populations had high levels of genetic diversity. Populations possessed distinct mtDNA genotypes and therefore should be considered individual gene pools for conservation purposes.

Programs where offspring of indigenous brown trout populations are used for stocking, or supportive breeding, is another approach to stocking (Laikre 1999). The objective of this approach is to increase the population size without causing negative genetic effects associated with introducing non-local genes into wild populations (Palm et al. 2003). Although this method of stocking avoids some of the problems caused by other methods, it still has the potential to negatively impact wild populations of brown trout. For example, since a small portion of indigenous populations is used for artificial reproduction within the hatchery, the rate of inbreeding may increase while at the same time genetic variability may decrease (Laikre 1999). In order to preserve the genetic variation present in wild populations, the most desirable method of fishery management would be to improve environmental conditions and impose angling restrictions so that naturally reproducing populations of brown trout would be able to survive and proliferate. Since this may not always be feasible, however, supportive breeding is

generally considered preferable to other stocking methods (Laikre 1999).

In the late 1800s and early 1900s, practices such as logging and over harvesting led to the decline or loss of many fish species in Michigan waters, including the Great Lakes (MDNR 2007). To prevent further loss of species, the first hatchery was opened in 1901 and to date six hatcheries are located in Michigan (MDNR 2007). Fish stocking continues to be the primary method used in fishery management. Stocked fish provide about 40% of all recreational fishing in Michigan with state fish hatcheries producing approximately 13 million trout and salmon annually (MDNR 2007). Brown trout for stocking are currently reared at three facilities; Oden State Fish Hatchery, Harrietta State Fish Hatchery, and Thompson State Fish Hatchery. Only the Oden State Fish Hatchery maintains brown trout broodstock in Michigan (MDNR 2007). Fisheries generate a significant amount of revenue for the state. In 2003-2004 about 49 million dollars were generated from hunting and fishing licenses, making effective fisheries management a priority within the MDNR. In addition to licenses, the purchase of equipment and other fishing related goods and services by licensed anglers contributes an estimated 2 billion dollars annually to the Michigan economy (MDNR 2007).

Michigan waters are currently stocked with one wild strain of brown trout and two domesticated strains. The strains are nearly identical in appearance and, unless identified with a fin clipping, difficult to differentiate (Tiano 2005). Wild Rose and Seeforellen strains are considered domestic strains. They have been in Michigan hatcheries for nearly two decades and are used in other hatcheries across America (Wills 2006). The Seeforellen strain was originally brought to Michigan in 1989 as eggs from

the Caledonia State Fish Hatchery in New York (Dan Sampson, MDNR, personal communication). Offspring of this original shipment were used to establish the Oden State Fish Hatchery Seeforellen broodstock from which all subsequent Seeforellen broodstock lots have been developed (D. Sampson, personal communication). The broodstock at Caledonia were progeny of eggs transferred from the Catskill State Fish Hatchery which originally received eggs directly from Germany in 1985. In Germany the Seeforellen strain was reported to survive well and grow up to 60 pounds in large lake environments, although at the Oden State Fish Hatchery they have not readily taken to artificial feeds and have had slower initial growth rates than the other two strains (D. Sampson, personal communication). Seeforellen is generally considered to be a lake strain and is primarily used to stock the Great Lakes (MDNR 2007).

Wild Rose is the second domestic strain of brown trout used to stock Michigan waters. The Wild Rose strain was originally brought to Michigan in 1987 as eggs from the Wisconsin Wild Rose State Fish Hatchery. Origins of this strain in Wisconsin are unknown. Although they have been at the Wild Rose State Fish Hatchery since the 1950s, they are believed to have been established at the hatchery earlier (Randal Larson, Wisconsin Department of Natural Resources, personal communication). This strain has been found to perform very well in the Oden State Fish Hatchery where they grow quickly and readily take to different tanks and artificial diets (D. Sampson, personal communication).

The only wild brown trout strain used to stock Michigan waters is the Gilchrist strain. This strain was developed from brown trout captured from Gilchrist Creek in

Michigan's Montmorency County. Broodstock for the Gilchrist strain were first established at Oden State Fish Hatchery in 1995 from captive wild fish that had been held at the Hunt Creek Fisheries Research Station since being taken directly from the Gilchrist Creek in 1991 (Wills 2005). How brown trout were initially established in Gilchrist Creek is unknown, although they probably were from unrecorded plantings of European brown trout earlier in the 20th century (Wills 2005). In the Oden State Fish Hatchery the Gilchrist strain has maintained its wild character as they avoid overhead movement and do not do well with hand feeding. They also have somewhat slower growth rates in the hatchery than the Wild Rose strain (D. Sampson, personal communication).

Michigan strains of brown trout have been shown to differ substantially in growth rates and survival in streams after stocking. Wills (2005, 2006) compared the post-stocking performance of paired plantings of all three brown trout strains in seven Michigan rivers from 1997 to 2000. Stocked brown trout from the wild Gilchrist strain were initially smaller but were shown to have higher survival and growth rates than the other two strains. Gilchrist strain had significantly greater survival to age two than the Wild Rose or Seeforellen strains. About 20% of the Gilchrist survived to year two while only 5% of the Wild Rose and 2 % of the Seeforellen survived (Wills 2006). In addition, several Gilchrist brown trout three and four years old were captured while fewer Wild Rose or Seeforellen survived past age two. Within one year of stocking, Gilchrist survival was more than one hundred times greater than Seeforellen and six times greater than Wild Rose (Wills 2006). The study also suggested that natural reproduction of brown trout was occurring in six out of the seven rivers sampled. Although improving

environmental conditions and imposing angling restrictions to allow these naturally reproducing populations to proliferate would seem to be the best strategy in terms of conserving natural genetic variation, this may not be practical. At least within Michigan rivers, if stocking is necessary then the Gilchrist strain of brown trout would be preferable as they displayed much greater potential for post-stocking survival than the Wild Rose or Seeforellen strains (Wills 2006).

Increased survival of the Gilchrist strain may have a genetic basis. The results of the current study uncovered a significant amount of nucleotide polymorphism in the hatchery Gilchrist strain. It would be important to examine whether or not there is a correlation between any of the three Gilchrist haplotypes and survival after stocking. This knowledge could improve brown trout management in Michigan. Since the Wild Rose and Seeforellen strains have been in Michigan hatcheries for nearly two decades, they have been subject to artificial selection due to hatchery conditions (Wills 2006). In the wild, brown trout would adapt to local environmental conditions which presumably would enhance survival and reproduction, but in the hatchery trout are maintained in holding tanks and undergo artificial breeding. Ruzzante et al. (2004) attributed increased introgression of stocked salmonids with wild populations to the lower survival of stocked fish compared to wild populations which would be better adapted to migratory spawning. Ruzzante et al. (2004) attributed this to lower survival of stocked fish compared to wild populations which would be better adapted to migratory spawning. According to population genetic theory, over several generations, hatchery brown trout would lose

some of their genetic variability and when placed in the wild there would not be enough variation available for selection and adaptation to occur (Laikre 1999).

Despite the selective advantage wild populations of brown trout may possess, if enough domestic or hatchery-reared brown trout are released it may result in the extinction of local populations (Laikre 1999). Hansen et al. (1995) refer to this as the immigration-selection balance. If a sufficient number of trout are stocked, then the selective advantage of wild populations will not be enough to overcome the massive immigration imposed by stocking (Hansen et al. 1995). If selection is strong, however, then wild populations may be found even in bodies of water that have been heavily stocked (Hansen et al. 1995).

The highly variable life history of brown trout may be a result of the substantial interpopulation diversity in this species. For example, brown trout may migrate from salt water to spawn in fresh water, spawn in a river, or spawn at the bottom of a lake (Bernatchez 2001). The atypical lack of genetic variability found in some studies could be explained by the life history characteristics of the particular populations studied. Brown trout in two populations studied by Prodohl et al. (1997) had limited spawning and nursery conditions in their environment compared to other populations which could lead to low recruitment. Atypical recruitment resulting in low effective population sizes or repeated bottlenecks could lead to a loss of genetic variability (Prodohl et al. 1997). However, isolation combined with a population bottleneck and genetic drift could also result in significant genetic differentiation among brown trout populations (Apostolidis et al. 1996).

Previous analysis of brown trout from the Oden State Fish Hatchery, the Muskegon and Rogue Rivers, and Lake Michigan using PCR-RFLP techniques indicated that there were at least four polymorphic sites within the ND1 region and at least two polymorphic sites in the ND5/6 region of brown trout mtDNA (Tiano 2005). Furthermore, it was found that a single RFLP is sufficient to differentiate between the most common Wild Rose haplotype and the most common Gilchrist haplotye (Tiano 2005). In the current study, by sequencing the entire segment the extent of total polymorphism detected by RFLP techniques was determined. Our study uncovered eight SNPs in the ND1 region among the fish studied in the aforementioned report, indicating that RFLP analysis detected 50% of the total genetic variation present. While RFLP techniques may be of value for fisheries management to quickly and inexpensively determine the strain to which a particular fish belongs, sequencing techniques allow for the detection of a greater number of nucleotide sequence variations and therefore more in-depth comparison of the genetic composition of different strains.

The results of current investigation demonstrate that the Wild Rose strain has lost much of its genetic variability, at least in the mtDNA region studied, while the Gilchrist and Seeforellen strains have retained a significant amount of genetic variation within each strain. The Seeforellen strain has retained significant genetic variation despite being considered a domesticated strain. Hatchery records indicate that Seeforellen is difficult to manage and that it displays distinct patterns of behavior. In addition, the Seeforellen strain displays significantly reduced growth and survival in Michigan streams after stocking which is consistent with an assumption of the existence of a genetic basis for

these differences (Wills 2006).

Our sequencing results identified one of the two Rogue River fish from the Tiano (2005) study that did not match any of the RFLP types to be Seeforellen haplotype one. According to the Fish Stocking Database maintained by the MDNR, the Seeforellen strain was not stocked into this river until 2006, while the Rogue River samples in the Tiano (2005) study were collected in 2003. The presence of this fish may indicate that the Seeforellen haplotype one had been present in either the Wild Rose or Gilchrist stocks at some point before 2003, or that this fish is a survivor of pre-Wild Rose and Gilchrist stocking events of the Rogue by other brown trout strains, of which the Seeforellen haplotype one may have been a part. This haplotype was also the most prevalent within the Seeforellen strain with fish displaying this haplotype being found in Lake Michigan and the hatchery in addition to the Rogue River fish. Therefore it seems that brown trout possessing this haplotype may be better able to grow and survive in Michigan waters compared to other haplotypes found within the Seeforellen strain.

The Seeforellen strain of brown trout exhibited a highly variable composition. When a phylogenetic tree was constructed, Seeforellen haplotype three formed a distinct branch, Seeforellen haplotype two formed a branch with the Gilchrist strain, and Seeforellen haplotypes one and four formed a branch with Wild Rose and the unknown Rogue River haplotype from Tiano (2005) (Figure 2). Within this strain the majority of polymorphic sites were identified, including both nonsynonymous substitutions. The significantly different patterns of polymorphism observed within the Seeforellen strain suggest that it may not constitute a strain in the traditional sense as haplotypes within the

strain are more closely related to those of other strains. In terms of fisheries management, this may account for the low survival of Seeforellen when stocked into Michigan bodies of water.

Michigan brown trout were closely related to northern European brown trout (Figure 3). Although few brown trout studies have sequenced the ND1 region, a Danish study analyzing four Danish haplotypes as well as two Romanian haplotypes was available on the NCBI database. When a phylogenetic tree was constructed using these sequences along with Michigan brown trout, a similar clustering pattern to that of Michigan-specific brown trout was observed. One branch contained all of the Gilchrist and one Seeforellen haplotype along with Danish haplotype four. A second branch contained Wild Rose, Seeforellen haplotypes one and four, the unknown Rogue River haplotype, and Danish haplotypes two, seven and ten. The third branch contained Seeforellen haplotype three and Romanian haplotypes D1 and D2. Interestingly, both of these Romanian haplotypes are nearly identical to the Seeforellen haplotype three. They are also the only other salmonid that exhibited the two nonsynonmous substitutions found in Seeforellen haplotype three. This suggests that the Seeforellen strain and the Romanian brown trout may have a common ancestral origin in Europe.

While the higher mutation rate of mtDNA relative to nuclear DNA makes it a useful tool in population genetics, the ND1 region of mtDNA would be expected to display conservatism as it is a protein coding region. Phylogenetic trees of expected topology have been constructed using this gene (Nilsson et al. 2001, Corneli and Ward 2000). Relatively constant rates of mutation have also been observed for this gene

among different salmonid species (Doiron et al. 2002). In Michigan brown trout the majority of polymorphic sites were synonymous and therefore selectively neutral. This supports the idea that stabilizing selection may be acting on the mitochondrial genome.

Nonsynonymous substitutions detected in the Seeforellen haplotype three fish resulting in changes of amino acids may have some functional significance for brown trout populations. The ND-1 region codes for genes of the mitochondrial respiratory chain which could affect energy production. If these amino acid substitutions confer some adaptive advantage, such as allowing the trout to live in colder water, then selection may be acting to maintain the observed amino acid changes. For example, introgression of the mtDNA genome of arctic char into brook char populations has been implicated in allowing brook char to live in a more northerly distribution (Doiron et al. 2002).

Functional significance of the amino acid substitutions in the ND1 region is difficult to determine as no studies are available regarding the structural composition of this gene in salmonids. A model of the ND1 subunit has, however, been developed by Valentino et al. (2004) based on human mitochondrial DNA. This model proposes that the ND1 subunit consists of five transmembrane helices with several extramembrane loops. According to this model both brown trout nonsynonymous substitutions would be located in the extramembrane loop portions of the ND1 gene, with position 4733 being located near the C-terminal end. The substitution at position 4312 replaces a serine with glycine while position 4733 replaces a serine with asparagine.

Further study would be necessary to determine whether or not these amino acid substitutions confer an adaptive advantage. Since the ND1 gene is important in

metabolic functioning, if these changes were detrimental they likely would have been eliminated from the population. Because these nonsynonymous substitutions have been maintained one could speculate that they may not significantly affect the functioning of the organism and therefore a phenotypic alteration may be difficult to detect. If there is an adaptive advantage, however, it seems likely that these substitutions would manifest in the form of altered energy production. Analysis of wild brown trout populations may provide some insight. If brown trout displaying the Seeforellen haplotype three were found predominantly at a different temperature or were found to be active for a longer period of time relative to other brown trout, this would suggest that these amino acid substitutions may have functional significance.

Several Michigan lakes and streams are stocked with the Wild Rose, Gilchrist, and Seeforellen strains of brown trout. The genetic composition of these strains was unknown. The results of this study indicate that there is little genetic variation within the ND1 region for the Wild Rose strain, with considerably more variation in the Gilchrist and Seeforellen strains. Michigan strains of brown trout have been shown to differ substantially in growth rates and survival after stocking and the extremely variable genetic composition observed indicates that hatcheries need to be more conscientious in the selection and maintenance of their hatchery stocks in order to preserve the genetic identity of brown trout strains.

CHAPTER V

CONCLUSIONS

Although brown trout populations have been studied throughout Europe using both PCR-RFLP and sequencing techniques, this is the first study to address in detail the genetic composition and phylogenetic relationships among Michigan brown trout strains. Since the Wild Rose, Gilchrist, and Seeforellen strains of brown trout have been shown to differ substantially in both growth and survival in Michigan streams after stocking, this study may have practical implications for fisheries management. A total of 23 polymorphic sites were found in the ND1 region with two additional SNPs in the 16S rRNA region. Of the 23 ND1 SNPs, two were found to be nonsynonymous. Further investigation would be necessary to determine whether the amino acid substitutions observed in Seeforellen haplotype three have any functional significance. Phylogenetic relationships were in accordance with expected salmonid topology, with Michigan brown trout being closely related to northern European brown trout. Results indicated that more stringent measures need to be taken in the selection of Michigan brown trout strains and in the monitoring of their genetic composition.

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