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Population genetic structure and temporal stability at the northern range boundary of the Greenside Darter (*Etheostoma blennioides*).

Courtney Lynn Beneteau
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POPULATION GENETIC STRUCTURE AND TEMPORAL STABILITY AT THE
NORTHERN RANGE BOUNDARY OF THE GREENSIDE DARTER
(*ETHEOSTOMA BLENNIOIDES*)

By

Courtney Lynn Beneteau

A Thesis Submitted to the Faculty of Graduate Studies and Research
through Environmental Science
In Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
University of Windsor

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ABSTRACT

The greenside darter occurs in four major tributaries in Canada, which represent the northern boundary of the species distribution. This system provides the opportunity to observe population and temporal dynamics at the range-edge of a species through connectivity and gene flow estimation among, and within-watersheds. To address these questions, I developed novel microsatellite DNA markers for the species. Using these markers, I found very little among-watershed migration, and identified the Ausable watershed as isolated and having reduced genetic diversity relative to the other watersheds, likely due to bottleneck effects. I found significant levels of temporal variation over a single year. The dramatic population abundance increase and distribution expansion in the Grand River greenside darter populations are hypothesized to be the result of multiple introductions based on high genetic diversity and anomalous population structure. This work demonstrates how genetic data provides unique insights into poorly understood population and conservation biology issues.

CO-AUTHORSHIP STATEMENT

This thesis includes materials reprinted from co-authored and submitted articles. In all cases the contribution of co-authors was primarily in an advisory capacity or through the provision of facilities and materials to complete the research. The primary contributions, experimental designs, data collection and interpretation as well as the preparation of all manuscripts were performed by the author except in the case of samples collected from Sugar Creek, Ohio for Chapter 4, which was done by Amanda Haponski.

1- Beneteau, CL, Mandrak NE, Heath DD (2007) Characterization of eight polymorphic microsatellite DNA markers for the greenside darter, *Etheostoma blennioides* (Percidae) Molecular Ecology Notes (OnlineEarly Articles). doi:10.1111/j.1471-8286.2006.01661.x

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1.0 GENERAL INTRODUCTION

There are no ubiquitous species. Hence, the distribution of species on Earth is not random, but rather structured and often predictable. We tend to think of species distributions in the form of habitable ranges. The boundary of these species ranges often coincides with discontinuities in habitat, for example, a mountain range or dam on a river (Kirkpatrick & Barton 1997). However, in some instances this boundary is the result of physiological tolerances of the organism, or the result of interactions with other organisms (e.g. competition for resources). Genetically, it can be said that the range limit of a species is determined by an evolutionary constraint, where the species lacks the appropriate mutations to adapt to greater environmental extremes (Kirkpatrick & Barton 1997). For these reasons, populations at the edge of the species range typically occur in sub-optimal habitat, and are subject to different environmental factors than those in the more central and continuous populations. These unfavorable conditions include stronger selective pressure, which can speed genetic divergence, but also puts smaller, fragmented populations at risk (Bunnell 2004). This potential for increased genetic divergence is the reason populations at the boundary of a species' range may play an important role in evolution (Mayr 1963; Templeton 1981). Rapid evolution can occur when the peripheral populations become isolated from gene flow, causing them to evolve rapidly to their local ecological optimum (Garcia-Ramos & Kirkpatrick 1997). Alternatively, if the boundary populations do not become isolated, and continue to receive gene flow from large, central populations this might inhibit the necessary adaptation to the periphery environment and prevent further range extension (Mayr 1963). Suggesting that gene flow, while introducing genetic diversity, may also be the force maintaining limitations in species' distributional ranges.

The largest events to shape the distribution of species in North America, particularly freshwater species, were the glaciations of the current ice age. Although controversy surrounds the role of the last ice age in vertebrate speciation (Klicka & Zink 1997; Avise *et al.* 1998), it is clear that the glaciers formed what has become the current distribution of the species of northern North America (Hewitt 1996; Hewitt 2000; Bernatchez & Wilson 1998). Canada in particular, was almost completely covered in ice at several times during the last Ice Age (Pielou 1991), wiping a clean slate for future founding populations of species. As the glaciers retreated northward, the glacial melt-water filled the trenches carved in the earth, leaving behind large bodies of water. As a result, many freshwater fishes were able to colonize the newly forming habitat. Bernatchez & Wilson (1998) described the founding routes for several freshwater fish species and the refuges they inhabited during the Pleistocene era of freezing, retreat and refreezing. In contrast to the changes in species ranges due to the glaciations, the distributions of species may be changing now as a result of global climate change.

It has been suggested that populations at the edges of geographic ranges may be important in surviving long-term environmental changes (Hunter 1991; Quinn & Karr 1992). Confronted with rapidly changing environments, leading-edge peripheral populations (the advancing front of a species' distribution) are likely to fare much better than lagging-edge ones and be key to founding new populations as ranges shift (Davis & Shaw 2001). In the northern hemisphere, for example, range extensions are occurring along the northern boundaries of species ranges, and extinctions along the southern boundaries (Arnell *et al.* 1996; Magnuson *et al.* 1997). In some cold-water aquatic species, global warming represents potential habitat

loss (Keleher *et al.* 1996) as fish distributions are simulated to move pole-ward across North America, forecasting a shrinking range for coldwater species (Lehtonen 1996). However, in warm water species, this increase in temperature may lead to an increase in available habitat. In this case, peripheral species at the northern limit may be those most suited to establishing themselves in the new habitats created by warming temperatures (Fraser 2000). So populations at the northern boundary of their species' range may possess the genetic requirements for adaptation and long-term survival because they are more capable than southern populations to expand.

Species range expansions typically do occur in populations located at the limits of the distribution, therefore, increasing the area occupied by the species. Although many organisms are regarded as relatively sedentary and specialized in marginal parts of their geographical distribution (Thomas *et al.* 2001), several species (Spivak *et al.* 1991, Hill *et al.* 1999) develop more dispersive forms at range fronts, which increases the rate of range expansion (Andersen *et al.* 2004). Range expansions can happen when a population is recovering from a bottleneck, or following a successful founder/introduction event.

A genetic bottleneck occurs when a population dramatically decreases in size, such that the remaining individuals carry only a fraction of the total genetic variation found within the original source population (Figure 1.1). Bottlenecks can range in severity and duration (England *et al.* 2003) and are common in nature, and in addition to founder events, can be the result of habitat loss, disease outbreaks and environmental catastrophes (Frankham *et al.* 2002). A newly founded population will have gone through a genetic bottleneck, where only few of the individuals from the original population have colonized a new habitat, essentially

starting a new population with less genetic variety (Mayr 1963). The most critical factor affecting the severity of such an event is population size. If populations are founded by a small number of individuals, the new populations will have reduced genetic diversity relative to the source population (Nei 1975).

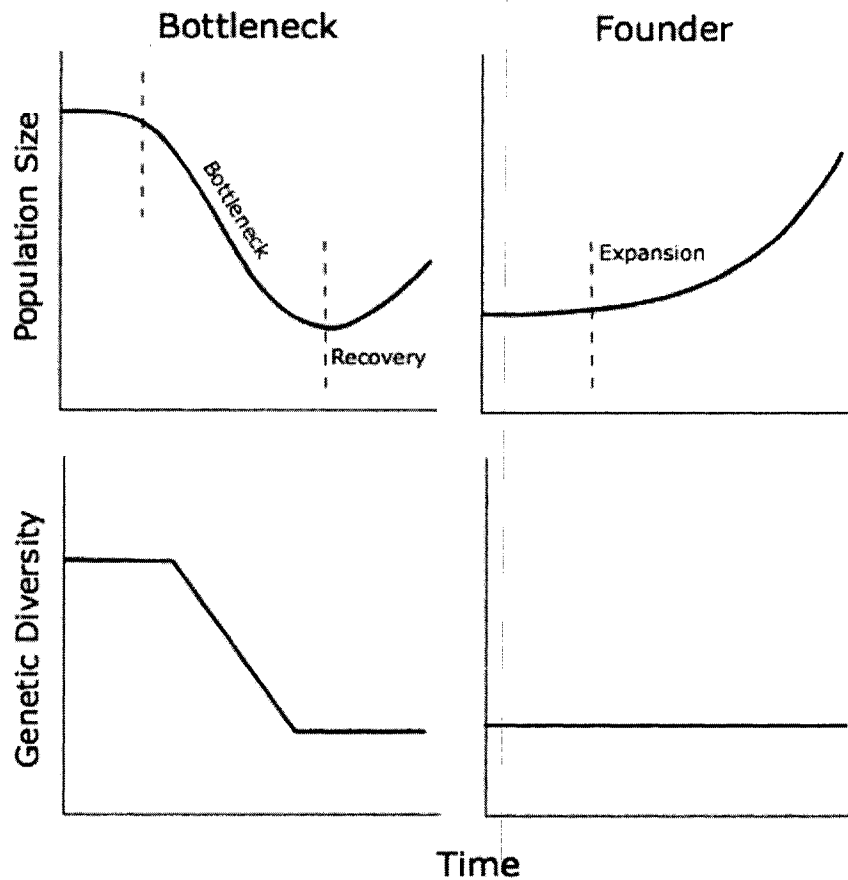


Figure 1.1 Illustration of the difference between bottleneck and founder effects on population size and the resulting changes in genetic diversity.

However, not all bottlenecked and introduced populations have reduced genetic variation due to genetic drift. This has been shown during a rapid population expansion, where much of the

genetic diversity was retained among the founder populations as a result of limited genetic drift (because of the decreased amount of time) and increased gene flow (Friar *et al.* 2000; Zenger *et al.* 2003). Another way that the deleterious effects typical of a founder event can be limited in a population is through multiple introductions. Kolbe *et al.* (2004) showed that genetic diversity in introduced populations was actually increased when multiple introductions were made from different genetic sources. Even if multiple introductions are made from the same source, this can still curb the founder effects in the population simply by increasing the size of the founding population. Many introduced species that have experienced founder effects during initial introductions manage to persist, evolve rapidly, and expand their ranges (Kolbe *et al.* 2004).

The detection of range expansion events has proven controversial (Templeton 1998) and typically requires supplemental historical data. Determining the difference between recovery from a population bottleneck and an introduction event, both followed by a range expansion, can prove difficult in natural populations. Stream fishes are excellent models in which to study population expansion because most are habitat specific and all are dependant on water routes for dispersal, essentially decreasing their dispersal range to one dimension (Strange & Burr 1997; Gaston 2003). The greenside darter is a freshwater fish species with its most northern boundary in Canada (Miller 1968), and a history of recent range expansion (Neely & George 2004).

The greenside darter (*Etheostoma blennioides*), is a benthic, stream dwelling fish with quite an extensive species distribution in the United States, extending east from Arkansas to

Georgia in the south and Michigan to New York in the north (Page & Burr 1991). The northern boundary of the species range lies just inside of southern Ontario, Canada. These Canadian populations consist of the subspecies *E. blennioides blennioides* as described by Miller (1968). About 10 000 years ago, at the time of the Wisconsin deglaciation, this subspecies migrated north from the Mississippian refugium into the glacial Lake Maumee (ancestor of the present-day Lake Erie) (Mandrak & Crossman 1992). This Mississippian population expanded its range west by spreading through the Michigan basin and managed to colonize only a few watersheds in southern Ontario. The historic distribution of the species in Canada consists of: the Thames River watershed, with records dating back to the 1800s; the Sydenham River watershed, with greenside darters first discovered in 1927 (Dextrase 2001); the Ausable River watershed, with greenside darters first discovered in 1974; and the Grand River watershed, with greenside darters first discovered in 1990 (N. Mandrak, pers. comm. 2006). Greenside darters have also been collected in the Detroit River, Lake St. Clair, Big Creek and Big Otter Creek.

The greenside darter is the largest species of the *Etheostoma* genus, reaching an average length in Canada of 76 mm. They achieve 60% of their total growth during the first year (Fahy 1954) and live an average of three years (Bunt *et al.* 1998), so the generation time for the species is estimated at two years. Greenside darters spawn in the spring when water temperatures reach above 10.6 °C (Fahy 1954), and as a result, spawning is initiated later (April), and extends later (June) in the northern part of their range (Winn 1958). Bunt *et al.* (1998) found that greenside darters in the Grand River (Ontario) had a significantly lower fecundity than those more southern United States populations studied by Winn (1958), and

suggested that the longer colder winters in southern Canada might limit the energy available for egg production (COSEWIC 2006). Females lay 1.8 mm eggs in several batches, over four to five weeks, on filamentous algae or aquatic moss that is attached to cobble - boulder size rocks (Fahy 1954; Trautman 1981). Although there is no direct parental care, males defend spawning territories around a rock with algae (Winn 1958). Eggs hatch in 18 – 20 days and newly hatched larvae are 6.8 – 7.5 mm long (Fahy 1954; Winn 1958). The larvae develop to a length of about 20 mm after which they transform into the juvenile stage (Baker 1979). The juvenile stage is relatively short lived as fish mature in the spring following hatching (COSEWIC 2006). Greenside darters are benthic insectivores that feed primarily on the larvae of midges, blackflies and mayflies (COSEWIC 2006).

In Canada, in 1991, greenside darters were listed as a Special Concern species by COSEWIC, due in part to increased turbidity in the Ausable watershed such that the species was thought to be extirpated (COSEWIC 2006). However in 2006, the species was re-assessed and determined to be increasing in numbers and expanding its range to the extent that it was no longer considered a species at risk (COSEWIC 2006). This was partly due to the result of this species being first discovered in the Grand River watershed in Ontario in 1990, and subsequently rapidly increasing its range so that it now is present throughout most of the watershed. There are currently no known distinctions among the Canadian populations of the greenside darter that warrant conservation designations below the species level (Dalton 1991). To date, there have been no genetic studies done to assess the connectivity or isolation of these peripheral Canadian populations.

1.1 THESIS OBJECTIVE

The overall aim of this study was to determine the population structure of a riverine fish at its range boundary, and to determine how recent range expansions have affected the population dynamics using the greenside darter populations in Canada as a model system.

1.2 CHAPTER 2 OBJECTIVE

Microsatellites are a powerful genetic tool enabling population geneticists and molecular ecologists to resolve fine-scale ecological questions (Selkoe & Toonen 2006). To characterize the population structure of *Etheostoma blennioides* in Canada and describe the migration and gene flow throughout the system, selectively neutral markers that are Mendelianally inherited were required.

The purpose of this work was to isolate microsatellite loci in the *E. blennioides* genome, then design and optimize working primers to genotype this, and other related, darter species, so that populations studies could be executed.

1.3 CHAPTER 3 OBJECTIVE

Species ranges have been studied for over a hundred years (Grinnell 1917), but only for a few selected species. Although riverine fishes present an excellent model to study range-edge populations, few studies have been done. Peripheral populations are predicted to be temporally and genetically unstable, primarily due to their use of sub-optimal habitats (Hoffman & Blows 1994) and limited connectivity to the central species distribution populations (Kyle & Strobeck 2002). The greenside darter in Ontario represent a good model

for observing range boundary dynamics and temporal stability because they inhabit several distinct watersheds with varying connectivity, some of which may have been isolated since their foundation after the last glacier melt at the northern boundary of the species.

The purpose of this study was to determine the Canadian distribution of the greenside darter, and using microsatellites, test for within-watershed barrier effects on gene flow patterns as well as within-watershed migration, temporal stability and potential gene flow among watersheds to provide a better understanding of the dynamics of isolated and fragmented populations of riverine fish at their northern range-edge.

1.4 CHAPTER 4 OBJECTIVE

In the early 1990s, greenside darters were identified in the Grand River watershed in southern Ontario for the first time. By the mid-1990s, the species was found at several other sites, above and below dams, and in different tributaries. Now, the populations have expanded almost throughout the entire watershed, and in great abundance (COSEWIC 2006). The mechanism and date of initial colonization of the species in this watershed is unknown and under debate. Utilizing molecular markers to genotype the Grand watershed populations and to compare them to the other Canadian populations would allow me to determine whether the species is native to the system, or if it was accidentally introduced.

The purpose of this study was to analyze genetic diversity and gene flow in order to determine the native or introduced status of the greenside darter in the Grand River

watershed. If the populations were determined to be introduced, the introduction source could be identified, provided it was from Canada.

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2.0 CHARACTERIZATION OF EIGHT POLYMORPHIC MICROSATELLITE DNA MARKERS FOR THE GREENSIDE DARTER, *ETHEOSTOMA BLENNIOIDES* (PERCIDAE)*

2.1 INTRODUCTION

The greenside darter (*E. blennioides*) is the largest member of the *Etheostoma* genus, reaching lengths of up to 170mm (Page & Burr 1991). This benthic fish is found primarily in creeks and small to medium rivers with fast moving water and low sediment load (Smith 1979; Lee *et al.* 1980). In the United States, the native range of this fish is quite extensive, extending east from Arkansas to Georgia in the south and Michigan to New York in the north (Page & Burr 1991). Southern Ontario represents the extreme northern range of this species. The greenside darter is native to several tributaries to southern Lake Huron, Lake St. Clair, and Lake Erie, has been introduced into one Lake Erie tributary, in southwestern Ontario (Dalton 1991). It was regarded by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) as a species of Special Concern until November of 2006, when it was deemed no longer a species at risk (www.cosewic.gc.ca).

Population genetic studies in American darter populations using allozymes have found that the extent of gene flow, if any, greatly varies among populations (Heithaus & Laushman 1997; Turner & Trexler 1998; Faber & White 2000). There are currently no known distinctions among the Canadian populations of the greenside darter that warrant conservation designations below the species level (Dalton 1991). It is suspected that this

* Beneteau, CL, Mandrak NE, Heath DD (2007) Characterization of eight polymorphic microsatellite DNA markers for the greenside darter, *Etheostoma blennioides* (Percidae) Molecular Ecology Notes (OnlineEarly Articles). doi:10.1111/j.1471-8286.2006.01661.x

species may have very complex genetic structure, even within a single drainage due to strong habitat preference. To test this hypothesis we developed and characterized eight polymorphic microsatellite loci for the greenside darter to investigate population genetic structure and diversity. These markers can also be used in future assessments of conservation units for this and other related species.

2.2 MATERIALS AND METHODS

Genomic DNA for library construction was extracted from fin clips stored in 95% ethanol using Promega® Wizard kits. Extracted DNA was enriched for microsatellites according to a protocol adapted from Fischer & Bachman (1998). Genomic DNA from a single fish was digested with *RsaI* and the blunt-ended fragments were ligated to *MluI* adapter-primer complexes consisting of a 21-mer (5' TAGTCCACGCGTAAGCAAGAGCACA 3') and a phosphorylated 25-mer (5' P-TCCACGCGTAAGCAAGAGCACA 3'). The resulting segments were hybridized with biotinylated oligo (GACA)₄ probes and captured with streptavidin beads (Roche, Indianapolis, USA). After washing away the unbound DNA, the enriched fragments were cloned into TOPO vectors and then used to transform One Shot® competent *Escherichia coli* cells (Invitrogen, Burlington, Canada).

Inserts from approximately 150 recombinant clones were amplified using M13 forward and reverse primers and sequenced at the Genome Quebec Innovation Centre (McGill University, QC). To check for redundancy, clone sequences were aligned using OMIGA 1.1 (Oxford Molecular Ltd.). Primers were designed for 38 clones that contained >8 uninterrupted di- or

tetra-nucleotide repeats using PRIMER 3 (Rozen & Skaletsky 2000) and NETPRIMER (Premier Biosoft International) software.

Initial screening for microsatellite amplification and polymorphism was performed in 25 μ L PCR reactions with an Eppendorf epgradient S Mastercycler (Brinkmann Instruments, Inc.). Each reaction included approximately 50 ng template DNA, 32 μ M dye-labelled forward primer, 0.5 μ M reverse primer, 200 μ M of each dNTP, 2.5 mM MgCl₂, and 0.5 U *Taq* DNA polymerase (Applied Biosystems, Foster City, USA) in a 1X PCR buffer. Primers were tested on 60 *E. blennioides* individuals from the Sydenham River population using the following PCR cycling protocol: 2 min at 95 °C; 30 cycles of 15 s at 95 °C, 15 s at 55 °C, 30 s at 72 °C, followed by a 2 min extension at 72 °C and a 4 °C hold. Optimal annealing temperatures and suitability of loci were determined using a LiCor 4300 DNA Analyzer and the software GENE IMAGIR 4.05 (Scanlytics, Inc.) to score the genotypes. Adherence to Hardy-Weinberg equilibrium was calculated with 10,000 permutations using TOOLS FOR POPULATION GENETIC ANALYSIS (TFPGA) v1.3 (Miller 1997) and corrected for multiple tests using the Bonferroni method (Rice 1989).

These eight markers were also tested on seven related species: *Ammocrypta pellucida* (eastern sand darter), *Etheostoma caeruleum* (rainbow darter), *Etheostoma flabellare* (fantail darter), *Etheostoma microperca* (least darter), *Etheostoma nigrum* (johnny darter), *Percina caprodes* (logperch), and *Percina maculata* (blackside darter).

2.3 RESULTS AND DISCUSSION

Eight loci were chosen for polymorphism and ease of scoring. The number of alleles ranged from 4 to 42 and the observed and expected heterozygosity ranged from 0.14 - 0.82 and 0.13 – 0.93 respectively (Table 2.1). Six of the eight loci tested amplified appropriate products in at least one of these related species (Table 2.2).

Microsatellite markers are powerful genetic tools enabling population geneticists and molecular ecologists to resolve fine-scale population questions. The greenside darter microsatellite markers described here will enable researchers and managers to determine population structure for populations of specific interest. This will assist in future assessments of conservation units and critical habitat for the greenside darter and related species, and will provide tools for the genetic analysis of the introduction of this species into other rivers.

Table 2.1 Polymorphic microsatellite loci developed for *Etheostoma blennioides*. Genbank accession numbers are listed for each primer pair. H_o is the observed proportion of heterozygotes and H_e is the expected heterozygosity.

Locus	Accession No.	Primer Sequence (5' - 3')	Repeat Motif	No. of Alleles	Allelic Range (bp)	H_o	H_e
Ebl1	DQ868321	F: CCCTTTCGTAACCCTTTTCA R: GGGACCAGATGCTGTGAGAT	(CA) ₁₂	4	247-261	0.14*	0.13
Ebl2	DQ868322	F: TGGTGC GACTGAACAAGAAC R: TACCACAACCACCTGCATTC	(AC) ₂₈	11	150-182	0.75	0.86
Ebl3	DQ868323	F: CTGCTCTAAAGGATGAGTAACTGG R: CCCTCACCGAAACACTTCTG	(GT) ₁₂ N ₈ (GT) ₇	9	81-115	0.60	0.53
Ebl4	DQ868324	F: TGTGACTGATATTTTGCTGCTG R: TGCATATCAAGATTCCCATTG	(TATC) ₇ GT(TCTA) ₇	15	146-210	0.82	0.87
Ebl5	DQ868325	F: TTGTTACACACGCACATAGGC R: CCATCCCTCCGACATCTCTA	(AC) ₄₆	23	86-188	0.57*	0.69
Ebl6	DQ868326	F: TATCATCCCATCGTCTGTCTG R: TGGCCCAAACAACAAGCTG	(GT) ₂₂	21	283-353	0.68	0.93
Ebl7	DQ868327	F: CACTCTCGGTTGTCAAGCTG R: ACAGGTATTAGGGCATTTAGCA	(GT) ₄₄	42	183-313	0.77	0.89
Ebl8	DQ868328	F: ACTGACAAGTTTTGGCACA R: CGTTCAAGTGGCATCAGAGA	(CA) ₇ CG(CA) ₃ CG(CA) ₅	5	150-168	0.52*	0.48

*significant departure from Hardy Weinberg equilibrium ($P < 0.05$)

Table 2.2 PCR amplified allele sizes of seven related Percidae species using eight microsatellite primer pairs designed for *Etheostoma blennioides*. Four individuals were used per species. A single number followed by a dash indicates one amplified band, likely a homozygote at that allelic size, two numbers indicate two amplified bands, likely a heterozygote with corresponding allelic sizes, 'ns' indicates non-specific amplification, and '∅' indicates no amplification.

Species and No.	Locus							
	Ebl1	Ebl2	Ebl3	Ebl4	Ebl5	Ebl6	Ebl7	Ebl8
<i>A. pellucida</i> 1	261/-	ns	121/115	∅	ns	257/253	212/-	ns
<i>A. pellucida</i> 2	261/-	ns	121/115	∅	ns	257/253	212/-	ns
<i>A. pellucida</i> 3	261/-	ns	121/115	∅	ns	257/253	230/-	ns
<i>A. pellucida</i> 4	261/-	ns	121/115	∅	ns	∅	230/-	ns
<i>E. caeruleum</i> 1	242/-	ns	85/-	201/193	385/-	ns	207/-	ns
<i>E. caeruleum</i> 2	242/-	ns	85/-	201/193	385/-	ns	205/199	ns
<i>E. caeruleum</i> 3	242/-	ns	85/-	193/-	385/-	ns	199/-	ns
<i>E. caeruleum</i> 4	242/-	ns	85/-	201/-	385/-	ns	207/199	ns
<i>E. flabellare</i> 1	255/-	ns	87/-	210/-	ns	272/268	183/177	ns
<i>E. flabellare</i> 2	255/-	ns	85/-	194/178	ns	272/268	205/183	ns
<i>E. flabellare</i> 3	255/-	ns	85/-	210/202	ns	272/268	205/199	ns
<i>E. flabellare</i> 4	255/-	ns	85/-	210/202	ns	272/268	205/183	ns
<i>E. microperca</i> 1	261/-	ns	ns	∅	ns	ns	∅	ns
<i>E. microperca</i> 2	255/-	ns	∅	∅	ns	268/-	∅	ns
<i>E. microperca</i> 3	∅	ns	∅	∅	ns	∅	∅	ns
<i>E. microperca</i> 4	∅	ns	∅	∅	ns	∅	∅	ns
<i>E. nigrum</i> 1	242/238	ns	87/-	186/180	ns	240/-	219/205	ns
<i>E. nigrum</i> 2	242/-	ns	89/-	166/166	ns	278/-	ns	ns
<i>E. nigrum</i> 3	242/238	ns	103/89	186/180	ns	ns	215/-	ns
<i>E. nigrum</i> 4	242/238	ns	87/-	184/180	ns	280/-	215/205	ns
<i>P. caprodes</i> 1	261/-	ns	∅	∅	∅	ns	∅	∅
<i>P. caprodes</i> 2	261/-	ns	81/-	300/300	∅	ns	∅	∅
<i>P. caprodes</i> 3	∅	ns	∅	232/232	∅	ns	∅	∅
<i>P. caprodes</i> 4	261/-	ns	∅	∅	∅	ns	ns	∅
<i>P. maculata</i> 1	261/-	ns	81/-	∅	ns	244/-	∅	ns
<i>P. maculata</i> 2	∅	ns	81/-	151/151	ns	244/-	∅	ns
<i>P. maculata</i> 3	261/-	ns	81/-	147/139	ns	244/-	∅	ns
<i>P. maculata</i> 4	ns	ns	115/109	185/175	ns	280/-	196/-	ns

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3.0 POPULATION GENETIC STRUCTURE AND TEMPORAL STABILITY AT THE NORTHERN RANGE BOUNDARY OF THE GREENSIDE DARTER*

3.1 INTRODUCTION

Most species have spatially limited distributions, and hence, multiple range-edges exist. Species range-edges have been studied for over a hundred years but only in a restricted set of species (Grinnell 1917; Allee 1923; Wardle 1968; Garner *et al.* 2004). Populations at the boundary of a species range likely play an important role in evolution (Mayr 1963; Templeton 1981). Populations at the edge of a species range, isolated by distance if nothing else, will diverge over time from the central populations. Mayr (1954) reported that the periphery or margin of a species range is the most active region of speciation. Now that loss of biodiversity, spread of invasive species, and global climate change are major environmental concerns facing all ecosystems, processes driving species boundary dynamics play an increasingly important role in conservation and management. This is because endangered species typically have a restricted dispersal range and restricted habitat through which they can disperse, and individuals at the edge of these ranges may possess critical phenotypic or genetic traits; consideration of this is important for management and conservation decisions (Fahrig & Merriam 1994). Alternatively, the area of greatest concern regarding invasive species is commonly the 'invasion front', which is actually the moving edge of the expanding species distribution, where the populations are adapting to new habitat.

* Beneteau, CL, Mandrak NE, Heath DD (2007) Population genetic structure and temporal stability at the northern range boundary of the greenside darter (*Etheostoma blennioides*) (Manuscript submitted to *Conservation Genetics*: June 2007).

Lastly, it has been suggested that populations at the edges of geographic ranges may be important in surviving long term environmental change (Hunter 1991; Quinn & Karr 1993). In the northern hemisphere, range extensions are occurring along the northern boundaries of species ranges, and extinctions are occurring along the southern boundaries (Arnell & Reynard 1996; Magnuson *et al.* 1997). Therefore peripheral populations at northern boundaries may be those most suited to establishing themselves in the new habitats created by warming temperatures (Fraser 2000). Understanding the distribution limitations of native and invasive species is growing increasingly important in view of these pressing environmental concerns.

There are two principal explanations for limited species distributions, and hence, species distribution boundaries. One is that the species is in the process of dispersing and colonizing (range expansion) and thus, the current distribution boundary is a temporary artifact of a non-equilibrium process, for example, a newly introduced invasive species exploiting a novel habitat (Ricciardi 2003). The second possibility is that the species distribution is at equilibrium, and the organisms at the range-edge are at their acclimation/adaptation limit and cannot successfully colonize past that boundary. In either scenario, population dynamics tend to differ at the periphery of the distribution relative to the range-center populations.

Generally, if a species distribution is at equilibrium, populations located near the periphery of the range are more likely to be imperiled than central populations because they tend to occur in less suitable environments and are often isolated from the central populations (Hoffman & Blows 1994; Lesica & Allendorf 1995). As the distance from the center of the species distribution increases, fewer migrants and lower gene flow result, thereby facilitating genetic

differentiation and loss of genetic diversity by genetic drift (Bunell *et al.* 2004). This has been quantified by Hutchison (2003), where peripheral populations of the eastern collared lizard were estimated to have at least a ten-fold increase in genetic divergence compared to central populations. Many other studies have shown similar population dynamics in a variety of species (Lammi *et al.* 1999; Kyle & Strobeck 2002 ; Bouzat & Johnson 2004) however, few exist for freshwater fishes of North America. Freshwater fish, riverine species in particular, are good models to study gene flow, migration and isolation because their dispersal range is essentially limited to one dimension (Gaston 2003) thus simplifying the study of species distributions in equilibrium that typically extend into marginal habitat near the range-edge.

The greenside darter, *Etheostoma blennioides*, is a stream dwelling fish species that has a continuous distribution and high abundance in the eastern United States, with its most northern boundary in southern Ontario. These Canadian populations consist of the subspecies *E. blennioides blennioides* as described by Miller (1968). About 10 000 years ago, at the time of the Wisconsin deglaciation, this subspecies migrated north from the Mississippian refugium into the glacial Lake Maumee (ancestor of the present-day Lake Erie) (Mandrak & Crossman 1992). This Mississippian population expanded its range west by spreading through the Michigan basin and managed to colonize only a few watersheds in southern Ontario. In the 1991, greenside darters were listed as a Special Concern species by the Committee On the Status of Endangered Wildlife In Canada (COSEWIC) (Dalton 1991). In 2006, the species was re-evaluated and determined to be increasing in numbers and expanding its range such that it was no longer considered a species at risk (COSEWIC).

Also, in the early 1990s this species was first collected in the Grand River watershed in Ontario, adding to the three watersheds (Thames, Sydenham, and Ausable Rivers) known to have greenside darters in Canada. Though no longer a species at risk, the greenside darter in Canada exemplifies the contentious issue of the protection status of species on the edge of their range when that range crosses a political boundary (Lesica & Allendorf 1995; Fraser 2000; Bunnell *et al.* 2004). Allozyme electrophoresis and mtDNA sequence data on American populations of greenside darters showed that gene flow varies among regions studied (Heithaus & Laushman 1997; Turner & Trexler 1998). Faber & White (2000) found greenside darter populations in two Ohio River tributaries to have θ values not significantly different from zero. Turner (2001) also determined moderate gene flow ($N_m = 7.33 \pm 2.50$) between populations in the Spring River, Arkansas. Although allozyme and mtDNA data are appropriate for detecting lineages of central populations that have been diverging for tens of thousands of years (before the Wisconsin glaciation), they are not suitable to detect the genetic structure of the peripheral, newly diverging Canadian populations.

There are currently no known distinctions among the northern populations of the greenside darter that warrant conservation designation below the species level (Dalton 1991); however, there have been no genetic studies done on these populations. The greenside darters in Ontario represent a good model for observing range-edge dynamics because they inhabit several distinct watersheds, with varying connectivity, some of which may have been isolated since their initial colonization following the Wisconsinian ice age.

The present study used variation at nine microsatellite loci to determine population structure and dynamics among all extant greenside darter populations in Canada, both within- and among-watersheds. Using molecular markers that evolve on an ecological timescale, I explicitly tested for temporal stability and measured population genetic structure and dispersal. I also tested for within-watershed barrier effects on gene flow patterns as well as within-watershed migration and potential gene flow among watersheds. My analyses provide a better understanding of the dynamics of isolated and fragmented populations of riverine fishes at their northern range-edge. The results of this study identify isolated rivers and watersheds as potential distinct populations for conservation, and more generally, it provides new insight into the gene flow connecting populations within a watershed, as well as migration and invasion among watersheds. This study will also provide managers with information on the temporal stability of riverine fish populations at their distribution edge.

3.2 MATERIAL AND METHODS

Survey Sampling

I sampled the Canadian distribution of greenside darters in 2005 and 2006 to determine the current distribution of greenside darters in Southern Ontario, and to collect fin clips for genetic analyses. To determine the current species distribution in Canada, rivers where greenside darters were known to occur were targeted as well as the streams, rivers and lake shores immediately surrounding the known distribution. Sites to be sampled were selected based on the preferred habitat of greenside darter: moderate flow, gravel to boulder substrate, low turbidity and presence of riffles. Where appropriate, sites were sampled by either electrofishing or beach seining. Most sites were single-passed electroshocked, both

systematically and in likely habitats, using a Smith-Root LR-24 backpack electrofisher, with an average effort of 1800 shocking seconds per site. Electrofisher settings ranged from 140 to 180 volts and 30 to 85 watts depending on the site. Where electrofishing would not be effective (i.e. shores of lakes and large rivers), sites were seined by hand 3-10 times with a 6 m beach seine with 2.5 mm mesh. Where greenside darters were not collected within the first 1000 shocking seconds or within three passes of the beach seine, the sites were deemed to have no greenside darters.

Sample collection

In October 2005, greenside darters were collected from 14 different sites in the Ausable, Grand and Sydenham rivers in southwestern Ontario. In June 2006, greenside darters were collected at six additional sites: one new site in each of the Ausable and Sydenham rivers, and four new sites in the Thames River (Figure 3.1). To evaluate the temporal stability of the genetic structure of the greenside darter populations, six sites, three sites in each of the Ausable and Sydenham rivers, were sampled in 2005 and re-sampled in 2006. A small caudal fin clip was taken from each captured fish and stored in 95% ethanol for later DNA extraction.

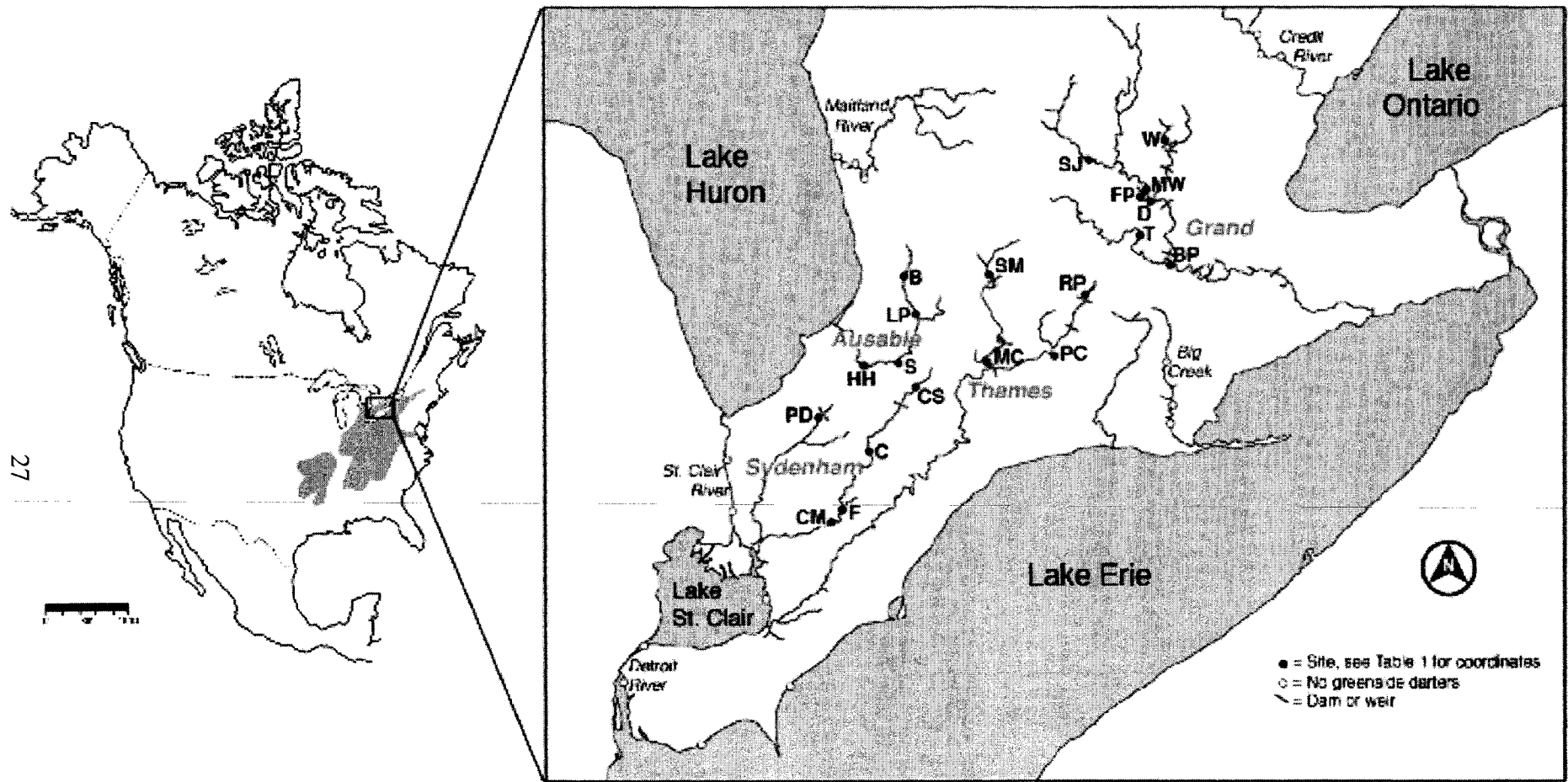


Figure 3.1 North American distribution of *Etheostoma blennioides*, indicated by grey shading. Close-up: Canadian distribution and study sites. Major watersheds sampled indicated by grey type. Potential barriers indicated by grey bars.

DNA extraction and genotyping

DNA was extracted from 1352 greenside darter fin clips using either Promega® Wizard kit salt-based extraction, or column-based plate extraction (Elphinstone *et al.* 2003) methods. To ensure allele sizes and resolution were unaffected by the extraction method, 64 fin clips were extracted using both methods, and no genotype anomalies resulted. Six *E. blennioides* microsatellite markers (one tetra-nucleotide and five di-nucleotide repeat markers; Ebl4, Ebl1, Ebl3, Ebl5, Ebl7 and Ebl8) from Beneteau *et al.* (2006), and three tetra-nucleotide microsatellite markers designed for *Etheostoma caeruleum* (Eca10, Eca11 and Eca48) from Tonnis (2006) were used to genotype all individuals. Genotyping was performed in 10 μ L PCR reactions with an Eppendorf epgradient S Mastercycler (Brinkmann Instruments, Westbury, USA). Each reaction included approximately 50 ng template DNA, 32 μ M dye-labelled forward primer, 0.5 μ M reverse primer, 200 μ M of each dNTP, 2.5 mM MgCl₂, and 0.5 U *Taq* DNA polymerase (Applied Biosystems, Foster City, USA) in a 1X PCR buffer supplied by the manufacturer. Genotyping at all loci was performed using the following thermal cycling protocol: 2 min at 95 °C; 30 cycles of 15 s at 95 °C, 15 s at 56 °C, 30 s at 72 °C, followed by a 2 min extension at 72 °C and a 4 °C hold. Allele sizes were scored using a LiCor 4300 DNA Analyzer and the software GENE IMAGIR 4.05 (Scanlytics, Inc.).

Statistical analyses

Microsatellite loci were tested for adherence to Hardy-Weinberg equilibrium using 100,000 permutations in ARLEQUIN v3.0 (Schneider *et al.* 2000), with significance adjusted for multiple tests using the Bonferroni method (Rice 1989). The microsatellite loci were also tested for linkage disequilibrium using ARLEQUIN v3.0 (Schneider *et al.* 2000) at each

sampling site. I tested for the presence of null alleles, large allele drop-out, etc., using MICROCHECKER v2.2.3 (Van Oosterhout *et al.* 2004). Allelic richness at each sampling site was calculated using FSTAT v2.9.3 (Goudet 2001).

Exact tests of allele frequency distribution differences were calculated for all pairwise watershed comparisons, as well as all within watershed pairwise sampling site comparisons with 10 000 permutations (Raymond & Rousset 1995) using TFPGA v1.3 (Miller 1997). Genetic divergence was estimated by calculating pairwise F_{ST} estimates (Weir & Cockerham 1984) between watersheds as well as between sampling sites within watersheds in ARLEQUIN v3.0 (Schneider *et al.* 2000). I also calculated genetic chord distance (D_C) (Cavalli-Sforza & Edwards 1967) between sampling sites and performed neighbor-joining cluster analyses with 10 000 bootstraps over loci in POPULATIONS v1.2.28 (Langella 2002). Cavalli-Sforza & Edwards' (1967) genetic chord distance has been shown to provide accurate tree topology for closely related populations (Angers & Bernatchez 1998). Trees were prepared with TREEVIEW (Win32) (Page 2001). A nested hierarchical analysis of molecular variance with 10 000 permutations (AMOVA; ARLEQUIN v3.0; Schneider *et al.* 2000) was performed among sample sites nested within watersheds to quantify the molecular variance explained by among-watershed versus within-watershed variation.

Mantel tests were performed to test an isolation-by-distance model of population genetic differentiation, and to identify potential barriers to gene flow among sampling sites within each watershed separately (Mantel 1967). The shortest water distance, to represent natural migration of the fish, among sampled sites were measured using Google Earth and were

plotted against Cavalli-Sforza and Edward's chord distance (D_C), and the coefficient of determination (r^2) and linear relationship significance (p-value) was calculated using GENALEX 6.0 with 9999 permutations (Peakall & Smouse 2006). To test for isolation-by-distance within watersheds (excluding among watershed comparisons), a correlation analysis between D_C and water distance between sites, within all watersheds, was demonstrated by a linear regression line and significance was tested by Spearman's rank order correlation analysis in SYSTAT v7.0.1.

To determine dispersal magnitude and direction at large and small scales, I performed rank-based genotype assignment across all individuals (watershed scale), as well as within watersheds using the Bayesian method of Rannala & Mountain (1997) with a 95% assignment threshold with GENECLASS 2.0 (Piry *et al.* 2004). With the resulting likelihood data, I determined migrants among sites within watersheds using the criterion that the ratio of the highest likelihood score of assignment to the second most likely score must exceed four for the individual to be successfully assigned to the most likely reference population. All identified migrants were scored as upstream or downstream dispersal events, and I tested for a direction bias using χ^2 . To determine among watershed movement (or transfer) I performed the same genotype assignment method as above, but combined all individuals within each watershed as a single potential source. Among-watershed migration was determined more conservatively, as among-watershed migration is less likely, on the basis of 90% or greater confidence of assignment to a watershed where they were not caught (a likelihood ratio of 9:1).

Temporal Stability

All tests of temporal stability were performed with the temporally replicated (2005 and 2006) sample sites in the Ausable (LP, B and HH) and Sydenham (F, C and PD) watersheds. The temporal variation of genetic diversity was examined by comparing the following indices: expected heterozygosity (H_E); and, allelic richness (A) of the two temporal samples of a given site. Two-tailed t-tests were performed in Excel to determine if there was a significant increase or decrease in H_E or A from 2005 to 2006. Exact tests were used to compare allele frequency similarity of each sampling site in 2005 versus 2006 (TFPGA v1.3; Miller 1997). Estimates of F_{ST} (Weir & Cockerham 1984) between years within each site were generated using ARLEQUIN v3.0 (Schneider *et al.* 2000). A nested hierarchical analysis of molecular variance with 10 000 permutations (AMOVA; ARLEQUIN v3.0; Schneider *et al.* 2000) was performed among years (2005 and 2006) nested within sampled sites to quantify the molecular variance explained by among-site versus among-year variation.

3.3 RESULTS

Survey sampling

Sampling to determine the Canadian distribution of *Etheostoma blennioides* in the streams, rivers and lake shores immediately surrounding the known distribution resulted in no additional locations with greenside darters (see Figure 3.1), compared to the most recent published distribution map (COSEWIC 2007).

Genotyping

All nine microsatellite loci were highly variable (7 to 70 alleles per locus; Table 3.1). Locus Ebl1 was fixed in Ausable sites sampled in 2005 and 2006 with one exception (Table 3.1). Population-level tests for adherence to Hardy-Weinberg equilibrium resulted in three sites rejecting the null hypothesis at one locus, following Bonferroni correction (Table 3.1). Corrected site-level tests of linkage disequilibrium failed to detect any instances of significant linkage among the nine microsatellite markers. In the three populations out of Hardy-Weinberg equilibrium, no null allele effects, band calling errors, or large allele drop out were evident, suggesting that Hardy-Weinberg equilibrium departures at those sites are likely due to true non-equilibrium effects.

Table 3.1 Site name, letter code (see Figure 3.1) and coordinates, as well as the number of *E. blennioides* caught at each site separated by sampling year. Observed heterozygosity (H_O), expected heterozygosity (H_E), and number of alleles (N_A) calculated for all sampled sites in 2005 and 2006 at each of nine microsatellite loci. Populations out of Hardy-Weinberg equilibrium are indicated with the H_O in bold type.

Site			Locus								
			2005	Ebl 1	Ebl 3	Ebl 4	Ebl 5	Ebl 7	Ebl 8	Eca10	Eca 11
Florence 42°38'59" N 82°00'30" W	F N=59	H_O	0.1429	0.5763	0.8136	0.5690	0.7797	0.5085	0.8621	0.8814	0.7414
		H_E	0.1350	0.5143	0.8790	0.7018	0.8995	0.4794	0.8372	0.9237	0.8510
		N_A	3	8	14	19	27	3	16	18	11
Cider Mills 42°36'16" N 82°04'30" W	CM N=68	H_O	0.1061	0.5075	0.8657	0.6618	0.7656	0.4478	0.8529	0.8824	0.8636
		H_E	0.1280	0.6145	0.9042	0.6309	0.8743	0.4494	0.8729	0.9151	0.8761
		N_A	2	8	16	12	35	3	15	16	12
Carolinian 42°46'43" N 81°50'08" W	C N=64	H_O	0.2258	0.6667	0.9531	0.4762	0.7097	0.4194	0.8226	0.9524	0.7705
		H_E	0.2019	0.6156	0.8935	0.7912	0.8998	0.5847	0.8573	0.9159	0.8970
		N_A	3	9	16	14	28	4	15	17	14
Petrolia Dam 42°52'46" N 82°08'20" W	PD N=27	H_O	0.0741	0.3333	0.6923	0.3704	1.0000	0.4815	0.8846	0.9167	0.6667
		H_E	0.1398	0.4137	0.8703	0.4864	0.9486	0.5723	0.8808	0.8679	0.8414
		N_A	2	6	13	5	25	4	13	9	7
Lyon's Park 43°08'44" N 81°32'39" W	LP N=46	H_O	fixed	0.2955	0.7174	0.5556	0.6444	0.5652	0.8696	0.9333	0.6889
		H_E	0	0.3289	0.7778	0.6789	0.7598	0.5342	0.8672	0.8564	0.8607
		N_A	1	3	6	6	9	3	9	10	8
Brinsley 43°12'29" N 81°31'05" W	B N=90	H_O	fixed	0.3708	0.7978	0.7333	0.7317	0.5056	0.8876	0.8444	0.7865
		H_E	0	0.3926	0.7554	0.6837	0.7495	0.5461	0.8826	0.8119	0.8471
		N_A	1	3	8	8	9	4	10	9	9
Hungry Hollow 43°04'38" N 81°47'39" W	HH N=52	H_O	fixed	0.1961	0.7647	0.7885	0.7843	0.6000	0.8200	0.8269	0.8163
		H_E	0	0.4599	0.7670	0.6977	0.8144	0.6168	0.9115	0.8182	0.8940
		N_A	1	3	7	8	9	4	17	9	11

Bean Park 43°10'53" N 80°22'13" W	BP	H ₀	0.2703	0.4167	0.8472	0.7945	0.4865	0.6487	0.8219	0.7826	0.7838
	N=74	H _E	0.2698	0.4912	0.8364	0.8086	0.6158	0.6709	0.7903	0.8571	0.8476
		N _A	3	4	8	13	13	6	10	9	9
St Jacob's 43°32'25" N 80°33'03" W	SJ	H ₀	0.1961	0.5700	0.8247	0.8020	0.7327	0.6400	0.7800	0.8317	0.7790
	N=102	H _E	0.1980	0.5486	0.8321	0.7833	0.8273	0.7067	0.8065	0.8410	0.8393
		N _A	3	4	8	13	19	5	9	9	10
Trussler 43°17'47" N 80°28'60" W	T	H ₀	0.2857	0.5263	0.8839	0.7864	0.6395	0.6903	0.7586	0.8761	0.8496
	N=116	H _E	0.2737	0.5130	0.8290	0.8411	0.7409	0.6793	0.8037	0.8531	0.8507
		N _A	3	4	9	18	15	5	9	10	9
Woodlawn 43°34'10" N 80°16'18" W	W	H ₀	0.4219	0.3692	0.8769	0.6923	0.6094	0.6774	0.7846	0.7231	0.8065
	N=65	H _E	0.4143	0.3760	0.8340	0.7601	0.7030	0.6311	0.8190	0.7971	0.8340
		N _A	2	3	8	10	11	5	8	9	9
Doon 43°23'45" N 80°23'44" W	D	H ₀	0.2619	0.4286	0.7750	0.6905	0.8571	0.7317	0.8571	0.8049	0.7805
	N=42	H _E	0.3233	0.4880	0.8218	0.7063	0.8127	0.6055	0.7837	0.8443	0.8052
		N _A	3	3	7	9	11	5	8	9	8
Freepport 43°24'14" N 80°26'60" W	FP	H ₀	0.3220	0.4237	0.7368	0.8167	0.6897	0.6379	0.8333	0.8833	0.7288
	N=60	H _E	0.3304	0.5002	0.7853	0.7884	0.7130	0.6940	0.8329	0.8611	0.8511
		N _A	4	4	8	8	14	5	9	8	11
Mannheim Weir 43°25'00" N 80°25'00" W	MW	H ₀	0.2750	0.5500	0.7180	0.8000	0.5500	0.7500	0.8750	0.8500	0.8250
	N=40	H _E	0.2491	0.5085	0.8065	0.7854	0.7949	0.7054	0.8142	0.8487	0.8997
		N _A	3	4	9	13	13	5	7	8	14
2006											
Florence 42°38'59" N 82°00'30" W	F	H ₀	0.0400	0.7778	0.9200	0.4468	0.8125	0.4400	0.9400	0.9575	0.6600
	N=50	H _E	0.0396	0.6652	0.9022	0.6161	0.8871	0.4099	0.8699	0.9140	0.8588
		N _A	2	7	16	12	33	3	14	14	11

Carolinian 42°46'43" N 81°50'08" W	C N=49	Ho	0.1225	0.4286	0.8776	0.6667	0.8776	0.4694	0.8571	0.9268	0.8776
		He	0.1515	0.4305	0.8912	0.6173	0.9203	0.4040	0.9106	0.9238	0.8698
		NA	2	6	13	10	34	3	15	16	14
Petrolia Dam 42°52'46" N 82°08'20" W	PD N=19	Ho	0.1111	0.5263	0.8421	0.6316	0.8421	0.5263	0.7647	0.8947	0.7647
		He	0.2984	0.5477	0.8407	0.6060	0.9502	0.5363	0.8913	0.9033	0.8538
		NA	3	5	13	5	20	4	12	10	8
Coldstream 43°00'55" N 81°30'04" W	C N=31	Ho	0.1290	0.7333	0.8065	0.7097	0.7419	0.6452	0.9032	0.8710	0.8065
		He	0.1227	0.5836	0.8525	0.7335	0.9069	0.5685	0.8879	0.9149	0.8937
		NA	2	6	9	13	20	3	15	14	12
Lyon's Park 43°08'44" N 81°32'39" W	LP N=46	Ho	0.0217	0.2826	0.6667	0.7111	0.3556	0.5556	0.7955	0.8261	0.8478
		He	0.0432	0.2917	0.7685	0.7071	0.7109	0.5371	0.8668	0.8598	0.8306
		NA	3	4	6	7	6	3	9	9	8
Brinsley 43°12'29" N 81°31'05" W	B N=28	Ho	fixed	0.1786	0.7857	0.6429	0.7143	0.6071	0.6667	0.7857	0.7857
		He	0	0.2033	0.7597	0.6669	0.7351	0.5896	0.8714	0.8487	0.8903
		NA	1	5	6	5	7	3	9	10	9
Hungry Hollow 43°04'38" N 81°47'39" W	HH N=64	Ho	fixed	0.2656	0.7188	0.6191	0.4828	0.5938	0.8438	0.8750	0.6825
		He	0	0.2750	0.7662	0.7237	0.7544	0.6173	0.8564	0.8492	0.8542
		NA	1	3	7	10	7	3	11	9	9
Springbank 43°03'46" N 81°41'20" W	S N=27	Ho	fixed	0.3704	0.7037	0.7600	0.5000	0.3913	0.8889	0.7407	0.8889
		He	0	0.3746	0.7827	0.7094	0.7112	0.5807	0.8721	0.8113	0.8477
		NA	1	3	7	7	5	3	9	9	8
St. Mary's 43°17'57" N 81°09'60" W	SM N=22	Ho	0.1364	0.5000	1.0000	0.8095	0.9091	0.4546	0.9546	0.8636	0.7273
		He	0.1300	0.5032	0.9083	0.8177	0.9324	0.5909	0.9260	0.9123	0.8626
		NA	2	4	11	13	23	5	17	13	10
Roth Park 43°08'55" N 80°45'26" W	RP N=52	Ho	0.1923	0.6923	0.8846	0.6800	0.8163	0.5882	0.8864	0.8431	0.8628
		He	0.2061	0.5590	0.9115	0.7040	0.8797	0.7001	0.8605	0.8835	0.8930
		NA	2	5	17	19	30	7	16	12	15

Among watersheds

Exact tests of allelic frequency distribution differences among watersheds were significant for all pairwise comparisons ($P < 0.0001$). F_{ST} values among watersheds indicated moderate to high levels of divergence and all values were highly significant ($p < 0.0001$; Table 3.2). The two least divergent watersheds were the Thames and Sydenham ($F_{ST} = 0.026$; Table 3.2), suggesting possible gene flow occurring through Lake St. Clair. Interestingly, despite the geographic distance, the Grand and Thames watersheds exhibit only slightly higher divergence than the Sydenham and Thames watershed ($F_{ST} = 0.027$; Table 3.2), suggesting historic headwater connections or artificial transfer. The watershed most diverged from all others was the Ausable, possibly due to its geographical isolation on Lake Huron (Table 3.2).

Table 3.2 Watershed-level pairwise comparison of Cavalli-Sfoza and Edwards chord distance (D_C ; above diagonal) and Weir and Cockerham's measure of genetic divergence (F_{ST} ; below diagonal). All F_{ST} values were significant following Bonferroni correction.

	Sydenham	Ausable	Grand	Thames
Sydenham	***	0.3746	0.3434	0.2324
Ausable	0.0919	***	0.3819	0.3275
Grand	0.0453	0.0870	***	0.3070
Thames	0.0262	0.0553	0.0272	***

The nested hierarchical AMOVA performed with all individuals grouped by sites within watersheds identified that variation among individuals, within-sites explained most of the genetic variance (93.2%, $P < 0.0001$), while 5.4% ($P < 0.0001$) of the variation was attributed to among-watersheds, and 1.4% ($P < 0.0001$) to among-sites within watersheds. A chord distance phylogeographical neighbor-joining tree, prepared for all sites (in 2005 and 2006) in all watersheds combined, resolves four distinct clades with moderate bootstrap

support (Figure 3.2). These clades clearly correspond to the watersheds, more closely grouping the Thames and Sydenham watersheds, and suggest further geographical-related structure within each watershed.

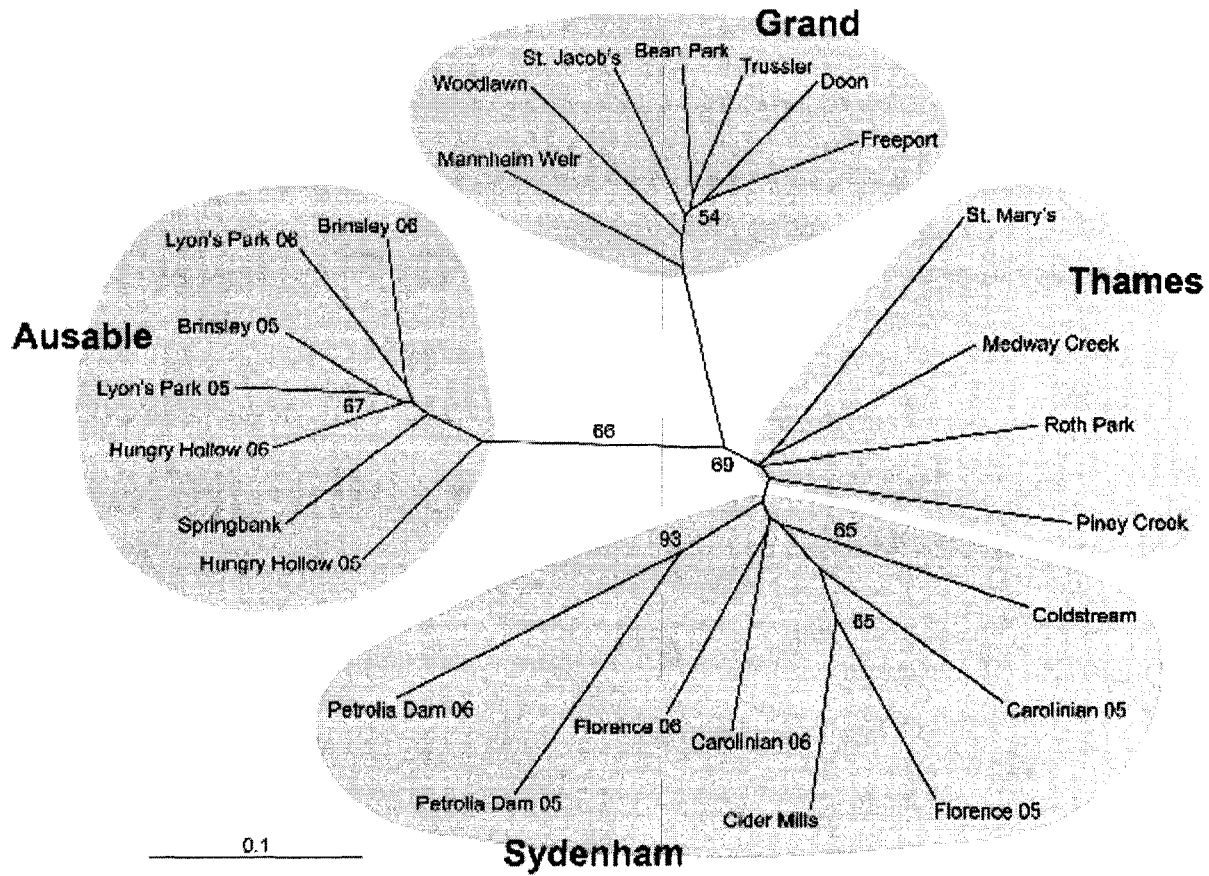


Figure 3.2 *E. blennioides* phylogeographic chord distance (D_C ; Cavalli-Sforza & Edwards 1967) un-rooted neighbor-joining tree, based on nine microsatellite loci, using all sampled sites, in both years combined. Numbers indicate replicated bootstrap values out of 10 000. Watershed associations indicated by grey shading.

Genotype assignment used to estimate the direction and magnitude of dispersal showed that among watersheds, the largest number of migrants was shared between the Thames and Sydenham watersheds. Sixteen individuals caught in the Sydenham held the genetic

signature of the Thames with over 90% confidence, while 12 individuals caught in the Thames assigned back to the Sydenham. The Ausable watershed was determined to be isolated from migration or immigration from both the Sydenham and Grand watersheds, sharing only one migrant with the Thames. The Grand watershed shared migrants with only the Sydenham with less than four individuals between them.

Within watersheds

I analyzed within-watershed genetic structure for each of the four watersheds separately by sampling year (2005 and 2006). Overall, exact tests of allelic frequency distribution differences among sampled sites were significant for most pairwise comparisons within watersheds, with close proximity between sites appearing to be the main exception from this trend (Table 3.3). However, in 2006 in the Ausable watershed, only one of six pairwise site comparisons showed significant allele frequency differences, suggesting a high level of gene flow throughout the watershed. In 2006, the Sydenham watershed showed a change from none (in 2005) to two of six site comparisons with non-significant allele frequency distribution differences. The pattern of site divergence in the Sydenham indicated a split between the upper and lower reaches of the watershed (Table 3.3).

Table 3.3 Within watershed sampling site comparison of Cavalli-Sforza and Edwards chord distance (D_C ; above diagonals) and Weir and Cockerham's measure of genetic divergence (F_{ST} ; below diagonal). Non-significant allele frequency exact tests indicated by D_C value in bold type, non-significant values of F_{ST} indicated by bold type. Temporal sites are differentiated by year and denoted as 05 or 06 after the site name.

SYDENHAM	F 05	F 06	C 05	C 06	PD 05	PD 06	CM	CS
F 05	***	0.2727	0.2373	0.2718	0.3748	0.3843	0.1974	0.2771
F 06	0.0547	***	0.254	0.2052	0.313	0.3141	0.2469	0.2365
C 05	0.0117	0.0218	***	0.2515	0.3436	0.3528	0.2317	0.2646
C 06	0.0533	0.0025	0.0237	***	0.2895	0.3093	0.2581	0.2518
PD 05	0.0426	0.007	0.0115	0.0115	***	0.2977	0.3721	0.3424
PD 06	0.0669	0.0152	0.0302	0.008	0.0232	***	0.3816	0.3391
CM	0.0049	0.0533	0.0172	0.0581	0.0506	0.0743	***	0.2855
CS	0.0708	0.0155	0.0365	0.0125	0.027	0.0052	0.0794	***
AUSABLE	LP 05	LP 06	B 05	B 06	HH 05	HH 06	S	
LP 05	***	0.1849	0.1254	0.184	0.2049	0.1487	0.2044	
LP 06	0.003	***	0.1521	0.1587	0.2263	0.1526	0.1694	
B 05	0.0073	0.0063	***	0.1598	0.179	0.1287	0.1646	
B 06	0.0101	0.0061	0.0047	***	0.2357	0.1553	0.2053	
HH 05	0.0125	0.0127	0.0037	0.0123	***	0.1919	0.1876	
HH 06	0.0076	0.0077	0.0063	0.0061	0.0051	***	0.1501	
S	0.0044	0.0023	-0.0048	-0.0026	-0.0006	-0.0055	***	
GRAND 2005	BP	SJ	T	W	D	FP	MW	
BP	***	0.1549	0.1283	0.1645	0.1482	0.1656	0.1971	
SJ	0.0037	***	0.1613	0.2105	0.1621	0.1754	0.1586	
T	0.0019	0.0091	***	0.1696	0.1584	0.1579	0.1993	
W	0.0115	0.0238	0.0137	***	0.176	0.1965	0.2393	
D	0.0002	0.0041	0.0045	0.0069	***	0.1582	0.2098	
FP	0.0037	0.0035	0.0067	0.0164	0.0038	***	0.1888	
MW	0.0084	0.0029	0.0113	0.0224	0.0126	0.0006	***	

THAMES 2006	SM	RP	PC	MC
SM	***	0.2904	0.3078	0.2644
RP	0.0082	***	0.2786	0.2537
PC	0.0048	0.0034	***	0.2754
MC	0.0045	0.0069	-0.0053	***

Patterns of genetic divergence (F_{ST}) estimates among sites in each watershed varied by watershed and by year, although the same general trends evident with the exact tests of allele frequency differences emerged (Table 3.3). In the Ausable watershed, sites within the watershed were highly differentiated in 2005, but analogous to the exact test results, pairwise F_{ST} estimates were much lower in 2006 (Table 3.3). In 2005, all sites but one in the Ausable were significantly diverged (i.e. significant F_{ST} estimates) from each other, while in 2006, all sites but one *lacked* significant genetic divergence. In 2005 and 2006, pairwise F_{ST} measures in the Sydenham were comparable: F_{ST} values were generally low within the upper and lower reaches, but F_{ST} values between the reaches were generally significant (Table 3.3). The patterns of divergence within the Thames (2006) and Grand (2005) watersheds were similar, both showing one site in the upper watershed significantly diverged from every other site, and non-significant F_{ST} values, indicating gene flow, between sites of close geographic proximity (Table 3.3).

Within-watershed dispersal seemed limited only by geographic distance, with no significant difference in the number of upstream versus downstream dispersal in any watershed ($p > 0.05$ for each watershed, for actual dispersal values see Table 3.4). In all watersheds, dispersal among sites was highest at lower distances, with the exception of the Grand, which showed the highest number of migrants between sites 121 – 140 km apart (Figure 3.3). The total number of individuals moving among sites at any distance was considerably lower in the Ausable watershed than all other watersheds for both years.

Table 3.4 Number of *E. blennioides* migrants within each watershed, separated by years, dispersing either down- or upstream, and across a known barrier (dam or weir) or not.

Migrant Dispersal Type	Sydenham 2005	Sydenham 2006	Ausable 2005	Ausable 2006	Grand	Thames
Downstream	28	23	13	17	20	37
Upstream	25	24	9	8	33	28
No barrier	53	30	22	25	18	0
Across barrier	0	17	0	0	35	65

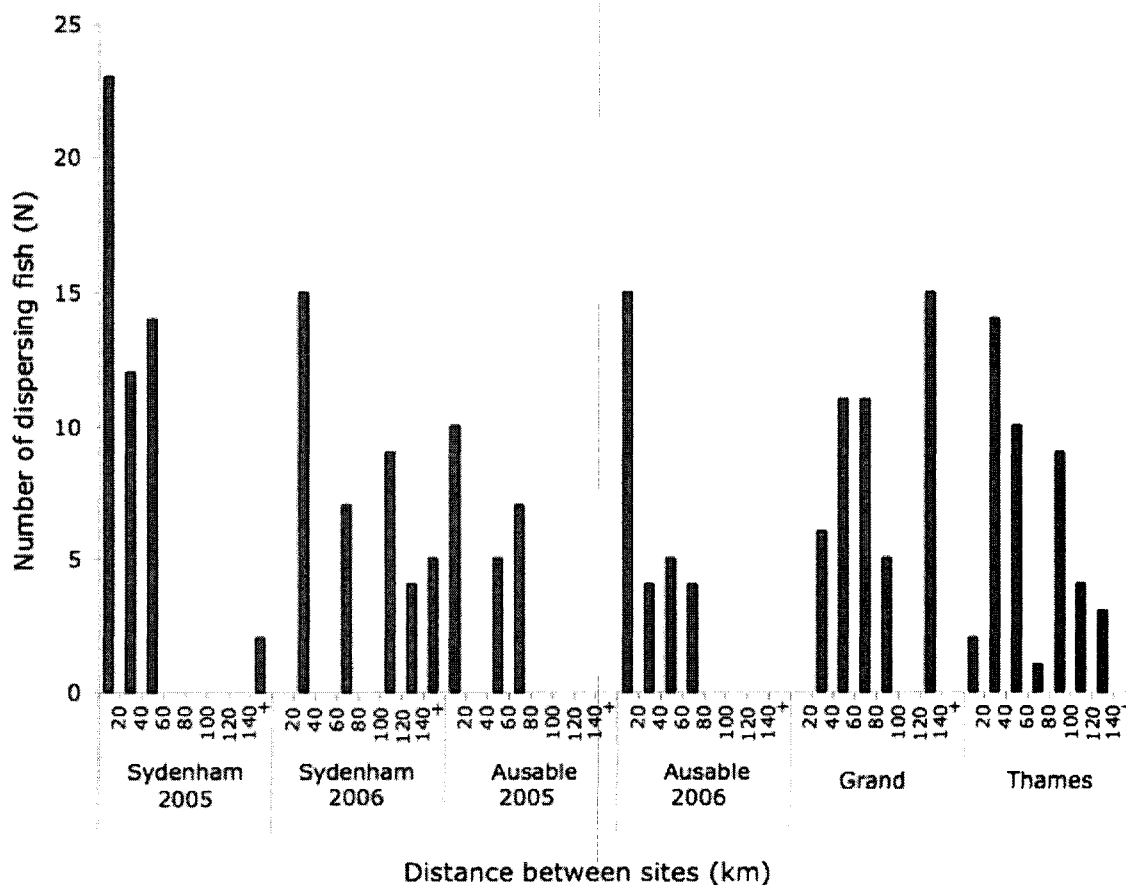


Figure 3.3 Frequency distribution of *E. blennioides* within-watershed dispersal among sampled sites within each watershed by shortest water distance traveled, determined by genotype assignment.

Isolation by distance

Mantel tests of isolation-by-distance for each watershed resulted in no significant correlation between geographic and genetic distance, and may be due to the sensitivity of the Mantel test to low sample size (i.e. the Ausable 2005 had only three comparisons). The correlation analysis over all watersheds however, resulted in a significant linear regression ($p < 0.0001$; $r = 0.6179$, Figure 3.4), and Spearman rank correlation analysis confirmed the significance ($p < 0.005$, $r_s = 0.4264$). To determine if a pattern of genetic isolation existed across known barriers (dams and weirs), those pairwise comparisons were identified on the correlation scatter plot, and no relationship involving genetic distance was evident (Figure 3.4).

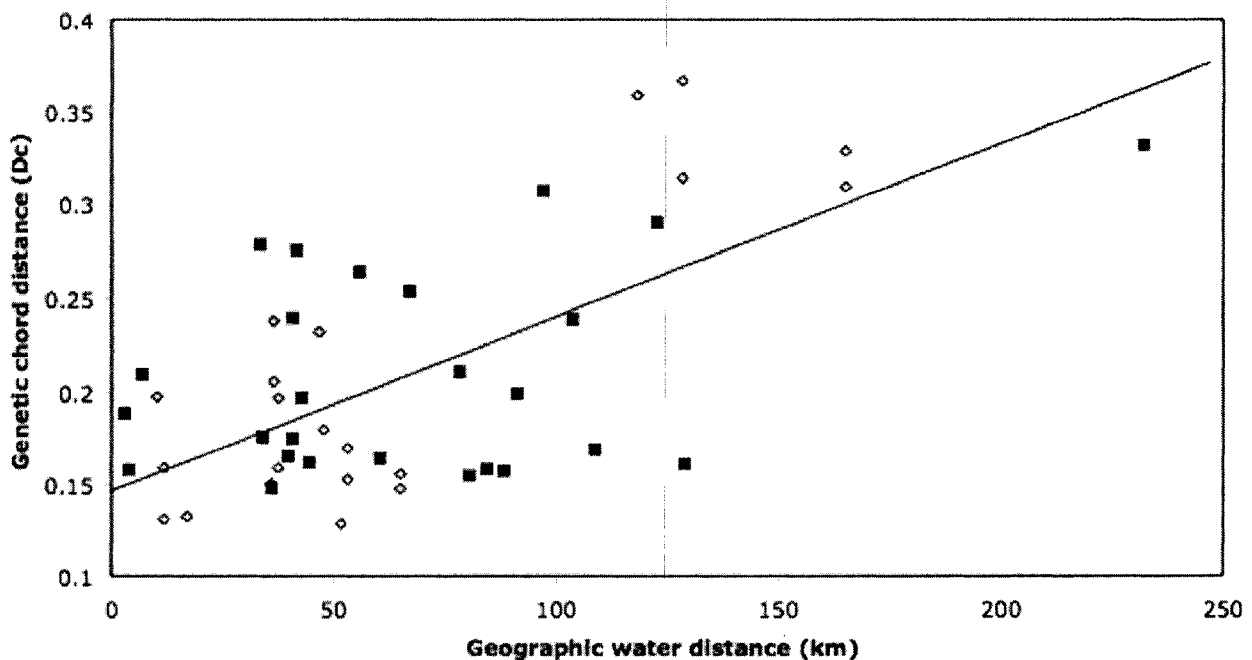


Figure 3.4 Linear regression of *E. blennioides* genetic chord distance (D_C ; Cavalli-Sforza & Edwards 1967) with geographic water distance among sites in each watershed ($p < 0.0001$; $r = 0.6179$). Open diamonds represent no barrier between site comparison, closed squares represent a dam or weir between site comparison.

Temporal stability

Allele frequency distributions for each of the six sites sampled in both 2005 and 2006 were tested for differences over time using exact tests and five of six sites showed significant differences between the two years, the exception being PD in the North Sydenham River (Table 3.3). Temporal changes in population genetic diversity were examined by comparing expected heterozygosity (H_E) and allelic richness (A) of the two temporal samples at a given site. In all instances, H_E and A were not significantly different from 2005 to 2006. Generally, F_{ST} values across the two sampling years grouped into two categories; the more geographically isolated sites (the North Sydenham River site; PD, and the Ausable watershed sites) which showed non-significant F_{ST} values, and the two sites in the main East Sydenham River (F and C), which had highly significant F_{ST} values (Table 3.3; Figure 3.5). In both watersheds (Ausable and Sydenham), total dispersal increased from 2005 to 2006 (Figure 3.3), and an increase in long-distance dispersal was also observed in the Sydenham from 2005 to 2006.

The hierarchical AMOVA between- years nested within each sample site identified that variation among individuals explained most of the genetic variance (93.14%, $P < 0.0001$), while 4.83% ($P = 0.0127$) of the variation was attributed to among-sites, and 2.04% ($P < 0.0001$) to between-years within sites.

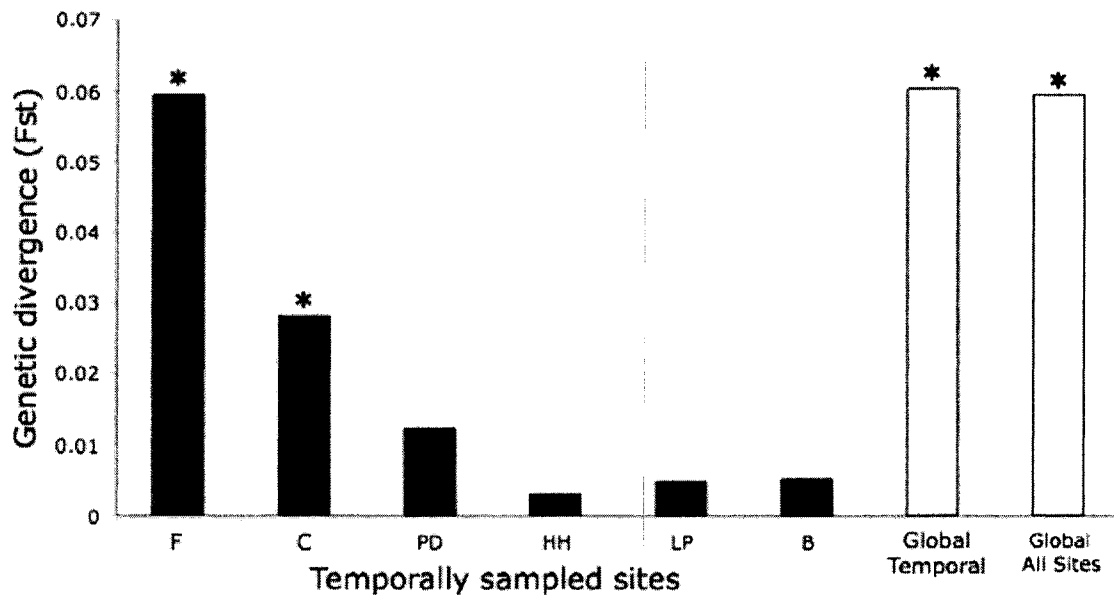


Figure 3.5 Genetic divergence (F_{ST}) of *E. blennioides* between temporal replicates at six sites sampled in 2005 and 2006. Global F_{ST} for all temporally sampled sites and for all data combined are presented as white bars. Significant F_{ST} values indicated with an asterisk.

3.4 DISCUSSION

Populations at the boundary of their species range typically share traits associated with being isolated from more central and continuous populations of the species (Lesica & Allendorf 1995). Greenside darter populations in Canada are situated at the most northern range-edge and my results support some of the population dynamics expected in peripheral populations. Generally, all four of the Canadian watersheds containing greenside darters showed distinct allele frequencies, had significant and high pairwise F_{ST} values, and shared few migrants, indicating substantial genetic divergence and hence meaningful reproductive barriers among these populations at the watershed level. These results indicate that the populations have been separated for many years, in some instances perhaps since the species re-colonized the region following the last ice age

10 000 years ago. These northern range greenside darter populations may represent distinct genetic resources, important for the long-term survival of the species.

The Ausable watershed populations, in particular, exemplify the expectation for population attributes at the northern boundary of a species range. These populations are highly genetically isolated based on their high F_{ST} values, and lack of immigration and dispersal from all other watersheds. Extensive sampling in the Maitland River, the watershed adjacent and to the north of the Ausable, identified several other darter species present in the system, but no greenside darters. This implies that, while there is suitable darter habitat further north than the Ausable, there is some factor limiting the northern dispersal of the species. Either the greenside darters are incapable of dispersing to the Maitland (i.e. they cannot tolerate the contemporary dispersal route through Lake Huron), or they are able to disperse to the new habitat but are unable to successfully survive or colonize. The limited genetic variation in the Ausable watershed is likely due to the combined effect of isolation and a severe population bottleneck in the early 1990s when greenside darters were thought to have been extirpated from the system (Dalton 1991). Although the greenside darters have now recovered, the Ausable populations may be fundamentally unstable; however, I do not see this as an impediment to their potential colonization into other watersheds draining into Lake Huron. It would then seem more likely that competitive or physiological barriers limit the northward expansion of greenside darters from the Ausable. However, greenside darters are the largest in the *Etheostoma* genus and have been shown to frequently coexist with other darters, particularly rainbow (*Etheostoma caeruleum*) and fantail (*Etheostoma flabellare*, the dominant darter species in the Maitland) and should, therefore, adequately compete for

resources (Page 1983; Heithaus & Laushman 1997). The greenside darters also have a lower critical thermal maximum temperature relative to other darters whose ranges do extend further north (Hlohowskyj & Wissing 1986), suggesting water temperature tolerance is not the limiting factor.

Whereas the Ausable greenside darters are genetically isolated, the Thames and Sydenham watersheds proved to have weak isolation as shown by their close grouping on the phylogeographic tree, low pairwise F_{ST} value and high watershed-level migration between them. This suggests on-going gene flow, possibly occurring through Lake St. Clair, as greenside darters have been reported along the shore between the two river mouths (Dalton 1991, COSEWIC 2007). Another possible source of gene flow could be historic headwater connections, since I found low genetic divergence among sites in the upper watersheds separated by short land distance but considerable water distance. I found weaker evidence for gene flow between the Thames and Grand watersheds. However, in this case, natural migration can be ruled out since the water distance for natural dispersal is very large (over 890 km), greenside darters are relatively weak swimmers (Layher & Ralston 1997), and they have never been reported in intermediate waters. Thus, the relatively low genetic divergence between these watersheds must be result of historic headwater migration, or of human mediated movement.

Contrary to previous studies on greenside darter dispersal (Winn 1958; Bunt *et al.* 1998), I found no evidence of direction-biased (upstream vs. downstream) movement within any of the sampled watersheds in either year. Surprisingly, dispersal generally did not appear to be

affected by the presence of dams or weirs. This could be a reflection of efficient fish-ladder use, or could be an artifact of the assignment method. In most cases, dispersal within each watershed seemed to be driven by unknown factors, although simple distance does appear to explain part of the variation in genetic divergence among sample sites. While known barriers don't seem to be a problem, there is an unknown impediment to gene flow among the Canadian populations and until we better understand what is limiting or promoting dispersal within these watersheds, further conservation of this native species is recommended.

Populations on the periphery of a species distribution are expected to be temporally and genetically unstable, primarily due to their use of sub-optimal habitats (Hoffman & Blows 1994) and limited connectivity to the populations in the centre of the species range (Kyle & Strobeck 2002). I found that the temporally sampled greenside darter populations generally showed remarkable levels of temporal instability with five of six of the populations exhibiting significant changes in allele frequencies and the AMOVA attributing over 40% of the total among-site variance to among-year differences. I found two sites had significant temporal F_{ST} values, and furthermore, those F_{ST} estimates were comparable to the global F_{ST} across all the sites in all the watersheds. This means that the populations in 2006 were so different from the fish caught at the same site in 2005 that they (hypothetically) could have originated from any watershed within the Canadian greenside darter range. Such a level of population genetic structure change over a single year is difficult to attribute to drift or other genetic effects, rather it is more likely due to movement of the fish. Those differences may be better explained by seasonal migration or dispersal effects, since sites were sampled in October 2005, and in June 2006. The sample sites exhibited lower levels of dispersal and

reduced genetic divergence between neighboring sites in 2006 (June) relative to 2005 (October). Since spawning in northern greenside darters is initiated in April and continues into June (Fahy 1954; Winn 1958), I may have identified spawning-related movement in the June 2006 sampling. Thus, greenside darters may not be as sedentary as once believed, and may disperse long distances or occupy larger home ranges in the early summer or during the spawning months. There are many reasons why darters may exhibit this seasonal dispersal behavior: to minimize metabolic requirements; escape environmental extremes; or maximize foraging or breeding opportunities (Schlosser and Toth 1984; Cunjak 1988; Labbe and Fausch 2000). Further sampling would be required to determine if the temporal instability documented is the result of the unstable nature of the peripheral Canadian populations, or an artifact of seasonal dispersal/migration.

This is the first genetic study to use ecological-scale genetic markers to address the population structure of greenside darters, as well as the first to address such questions in the range-edge Canadian populations. Although I found no genetic distinctions among the Canadian populations that warrant conservation designation below the species level (Dalton 1991), there is substantial genetic structure, especially among watersheds. Special concern should be given to the Ausable watershed populations, which have undergone severe bottlenecks and exhibit substantially reduced genetic diversity relative to other Canadian populations. My work highlights the complex interactions among the Canadian populations of the greenside darter and indicates that those populations represent an important study system for range-edge effects.

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4.0 GREENSIDE DARTERS AND THE GRAND RIVER PARADOX: EXPLOSIVE ABUNDANCE AND RANGE EXPANSION IN TEN GENERATIONS

4.1 INTRODUCTION

Recently introduced populations usually consist of a small number of individuals, especially if the introduction is accidental. Mayr (1963) described the effect of few founder individuals, carrying only a fraction of the total genetic variation found within their original source population. Such population “bottlenecks” can have major consequences for a population, including inbreeding (loss of fitness, inbreeding depression), loss of genetic variation (increased homozygosity, reduced additive genetic variation), and increased likelihood of population extinction due to chance events. Bottlenecks are common in nature, and in addition to founder events, can result from habitat loss, disease outbreaks and environmental catastrophes (Frankham *et al.* 2002). However, not all introduced populations are negatively affected by bottleneck effects, many introduced species that have experienced founder effects during initial introductions manage to persist, expand their ranges and evolve rapidly (Kolbe *et al.* 2004).

There are mechanisms by which populations experience limited detrimental effects of small founder population size. For example, founder effects can be reduced or eliminated in non-indigenous species through multiple introductions. Multiple introductions buffer founder effects in two ways; first, multiple introductions will simply increase the population size, increasing effective population size and overriding the effects of drift; and introductions from multiple genetically diverse sources would eliminate even the initial reduction in genetic variation. Both effects of multiple introductions will mask the expected genetic

characteristics of an introduced population making differentiating between an invading versus a native population difficult, given no historical data (Kolbe *et al.* 2004). However, even multiple introduced non-native populations generally have distinctive genetic signatures. For example, Kreiser *et al.* (2000) used allozymes and mitochondrial DNA sequencing to compare the genetic structure of introduced populations of plains killifish (*Fundulus zebrinus*) with native populations and determined single versus multiple sources of introduction.

Although the initial bottleneck associated with founder events reduces genetic diversity, further loss of variation can occur during subsequent range expansions (Hewitt 1999; Nichols *et al.* 2001). However, during a rapid population expansion, genetic diversity will be retained among multiple introduced populations as a result of reduced genetic drift (fast population growth limits the severity of the bottleneck) and increased gene flow among the populations (Friar *et al.* 2000; Zenger *et al.* 2003). Although some animals are relatively sedentary and specialized in marginal parts of their geographical distribution (Thomas *et al.* 2001), several species (Spivak *et al.* 1991, Hill *et al.* 1999) develop more dispersive forms at range fronts, which increases the rate of range expansion as well as gene flow among sub-populations (Anderson *et al.* 2004).

The detection of range expansion events has proven both logistically difficult, and controversial (Templeton 1998), and typically requires supplemental historical data. However, using molecular conservation genetics to detect population bottlenecks and expansion events with little or no *a priori* information can prove a successful strategy. For

example, Okello *et al.* (2005) used mitochondrial DNA sequence variation to demonstrate recent population expansion in the common hippopotamus (*Hippopotamus amphibius*). Using microsatellites, Ayllon *et al.* (2006) showed that, in as little as 20 years, brown trout populations from two known introduction events dispersed and rapidly differentiated into four distinct genetic groups among rivers on Kerguelen Island in the Subantarctic Ocean. Population bottlenecks are, at least in theory, relatively easily identified using rapidly evolving molecular genetic markers, since allelic diversity, expected heterozygosity and temporal allelic variance (F_C) all demonstrated a strong correlation with bottleneck severity ($R = 0.914$; $R = 0.905$; $R = -0.981$, respectively; Spencer *et al.* 2000). However, in practice, determining the difference between recovery from a population bottleneck and an introduction event, both followed by a rapid population size increase, can prove difficult.

The greenside darter (*Etheostoma blennioides*), is a freshwater fish species with a history of recent range expansion (Neely & George 2004). The greenside darter also occurs in drainages where the population status (introduced or native) has been debated (Jenkins & Burkhead 1994; Starnes 2002). This benthic fish is believed to be native to three, and introduced into one, Great Lakes watersheds in Canada, constituting the northern edge of the species range (Miller 1968). In the 1991, the greenside darter was listed as a Special Concern species by the Committee On the Status of Endangered Wildlife In Canada (COSEWIC) (Dalton 1991). In 2006, the species was re-assessed and determined to be increasing in numbers and expanding its range such that it was no longer considered at risk (COSEWIC 2006). This decision was, in part, due to the discovery of the greenside darter in the Grand River watershed (Eramosa River) in Ontario, for the first time early in the 1990s. The

greenside darter in the Grand River have been increasing their range so that they are now present in all four major Grand tributaries (Nith, Speed, Conestogo, and Eramosa) and their drainages. If this species was introduced into the Grand River watershed, the vector of introduction remains unknown, however, although anecdotal evidence suggests they may have been introduced into the Eramosa River via bait bucket release or in association with the intentional release of game fish. In either case, such an introduction would necessarily include only a small number of individuals and hence, should lead to severe founder effects. Here, I evaluate an alternative hypothesis that greenside darters are native to the Grand River watershed and were undetected until 1990 due to very low numbers.

In this study, I use nine polymorphic microsatellite markers to genotype greenside darters from each of the native Canadian watersheds, one population from Ohio, and from the Grand River watershed. My goal is to determine if the species is native or introduced to the Grand River watershed, and to genetically characterize the remarkable population explosion in the watershed over the last 17 years. My analyses include genotype assignment analyses, so that if the greenside darter populations in the Grand River watershed are introduced, I should be able to identify the source(s) of introduction. Alternatively, if the Grand River greenside darters are native, they must be experiencing a major increase in abundance as well as a substantial range expansion. In either case, the transition of the Grand River from being apparently free of greenside darters to having the species in great abundance spread throughout the watershed in a matter of 17 years is not easily explained, and the repercussions of which are still unknown. It is in this context I evaluate the “Grand River paradox”.

4.2 MATERIAL AND METHODS

Study site

The greenside darter currently inhabits at least four major watersheds in southwestern Ontario, Canada; it is native to the Ausable River, Sydenham River and Thames River watersheds, and the putatively introduced into the Grand River watershed. The Grand River has four major tributaries: the Nith; Conestogo; Speed; and Eramosa Rivers (Figure 4.1). Altogether the watershed drains 6,965 square kilometers, making it the largest in southern Ontario, and is a designated Canadian Heritage River worthy of special management and conservation. There are several large dams and weirs that may restrict fish movement throughout the watershed, including the Dunville, Caledonia, and Wilkes dams on the lower-main Grand River channel; the Cherry-Taylor and American Standard dams on the downstream branch of the Speed River; and to a lesser extent, the Mannhiem Weir on the Grand River just above the mouth of the Speed River (Figure 4.1).

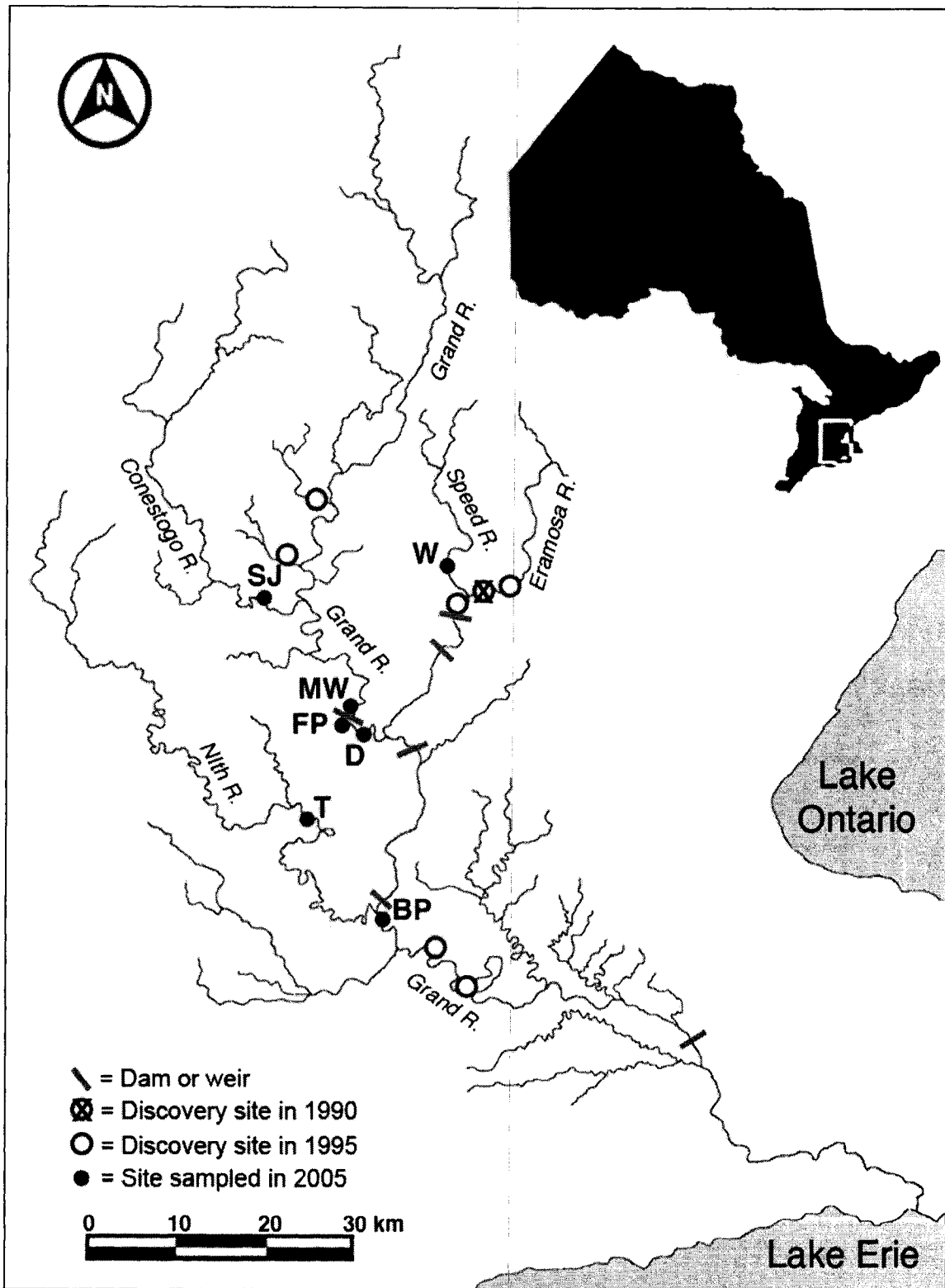


Figure 4.1 The Grand River watershed marked with greenside darter original discovery sites, capture sites in 2005 used in this study, and known dams and weirs. Inset black map of Ontario with location of expanded map indicated.

Sample collection

In October 2005, I sampled four of the five major tributaries to the Grand River for greenside darter, collecting specimens at seven sites. For genetic comparison and to identify possible sources of introduction, samples were also collected from three to four sites in each of the known native watersheds of southwestern Ontario: the Ausable, and Sydenham rivers in October 2005; and the Thames River in June 2006. One American Lake Erie tributary site in Ohio (Sugar Creek) was sampled as a geographically distant out-group. Most sites were single-pass electroshocked, both systematically and around potential habitats, using a Smith-Root LR-24 backpack electrofisher with an average effort of 1800 shocking seconds per site. Electrofisher settings ranged from 140 to 180 volts and 30 to 85 watts depending on the site. Where electrofishing was not effective (i.e. shores of lakes and large rivers), sites were seined by hand 3-10 times with a 6 m beach seine with 2.5 mm mesh. Caudal fin clips were taken from all specimens and stored in 95% ethanol.

Microsatellite genotyping

Microsatellite genotype variation was examined in 1068 specimens of greenside darter from four watersheds in southwestern Ontario and one creek in Ohio. DNA was extracted from the fin clips using a column-based plate extraction method (Elphinstone *et al.* 2003). Six novel microsatellite markers (one tetra-nucleotide and five di-nucleotide repeat motifs) characterized for *E. blennioides* (Beneteau *et al.* 2006), were used to genotype all individuals. An additional three tetra-nucleotide microsatellites designed for *Etheostoma caeruleum* (Eca10, Eca11 and Eca48) by Tonnis (2006) were optimized for *E. blennioides* and also used to genotype all individuals. Genotyping was performed in 10 μ L PCR reactions

with an Eppendorf epgradient S Mastercycler (Brinkmann Instruments, Westbury, USA). Each reaction included approximately 50 ng template DNA, 32 μM dye-labelled forward primer, 0.5 μM reverse primer, 200 μM of each dNTP, 2.5 mM MgCl_2 , and 0.5 U *Taq* DNA polymerase (Applied Biosystems, Foster City, USA) in a 1X PCR buffer supplied by the manufacturer. All genotyping was performed using the following PCR cycling protocol: 2 min at 95 °C; 30 cycles of 15 s at 95 °C, 15 s at 56 °C, 30 s at 72 °C, followed by a 2 min extension at 72 °C and a 4 °C hold. Allele sizes were scored using a LiCor 4300 DNA Analyzer and the software GENE IMAGIR 4.05 (Scanlytics, Inc.).

Statistical analyses

Scoring errors, null alleles, and large allele drop-out were checked for using MICROCHECKER v2.2.3 (Van Oosterhout *et al.* 2004). Microsatellite loci were tested for adherence to Hardy-Weinberg equilibrium using observed and expected heterozygosities of microsatellites calculated per site with 100,000 permutations using ARLEQUIN v3.0 (Schneider *et al.* 2000), with significance corrected for multiple tests using the Bonferroni method (Rice 1989). All loci were also tested for linkage disequilibrium using ARLEQUIN v3.0 at each sampled site.

Comparison of Grand to known native watersheds

To determine whether the Grand River watershed greenside darter populations exhibited reduced genetic diversity relative to known native populations, several genetic indices were calculated: mean observed heterozygosities calculated using ARLEQUIN v3.0; mean allelic richness across all loci; and the number and frequency of private alleles, using FSTAT v2.9.3 (Goudet 2001). All of these calculations were at the watershed, rather than population, level.

Exact tests of allele frequency distribution differences were calculated for all pairwise watershed and Ohio out-group comparisons with 100 000 permutations (Raymond & Rousset 1995) using TFPGA v1.3 (Miller 1997). Genetic divergence was quantified using pairwise F_{ST} estimates (Weir & Cockerham 1984) among watersheds, as well as with the out-group, calculated in ARLEQUIN v3.0 (Schneider *et al.* 2000). Allele frequencies were used to calculate genetic chord distance (D_C ; Cavalli-Sforza & Edwards 1978) among watersheds and out-group, and neighbor-joining cluster analyses with 10 000 bootstraps were performed in POPULATIONS v3.0 (Langella 2002). Phylogeographical neighbor-joining trees were prepared with TREEVIEW (Win32) (Page 2001).

To identify possible historic or recent population bottlenecks in each of the watersheds and out-group, three different methods were employed. Historic population bottlenecks for each watershed were identified by calculating the mean ratio (across loci) of the number of alleles to the range in allele size (“ M ”; Garza & Williamson 2001), which decreases with population size and is positively correlated to the severity and duration of the reduction. Bottlenecks within the past few dozen generations were identified by grouping the allele frequencies in each population for all loci into classes and graphing the distribution to determine allele frequency distortions (Luikart *et al.* 1998). Finally, I also used the program BOTTLENECK v1.2.02 (Cornuet & Luikart 1996; Cornuet *et al.* 1999) to test for significant bottleneck-related genotype biases with the Wilcoxon test for heterozygosity excess under the two-phase model (TPM).

To determine if the Grand watershed populations were recently seeded (introduced) from another Canadian watershed, or from a more ancestral American population, I used rank-based genotype assignment to assign all Grand River fish to all sampled watersheds and creeks (i.e. Grand, Ausable, Thames, Sydenham, and Ohio out-group). I used the Bayesian method (Rannala & Mountain 1997) with a 95% assignment threshold in GENECLASS v2.0 (Piry *et al.* 2004). I used the resulting likelihood data to determine migrants among sites within watersheds by dividing the reference population with the highest likelihood score of assignment by the second highest likelihood score. The individual was assigned to the most likely reference population only if that ratio was higher than nine, indicating the most likely population was nine times as likely as any other. If the ratio was less than nine, the individual was unassigned.

Structure within the Grand River watershed

Exact tests of allele frequency distribution differences were calculated for sites within the Grand River watershed with 100 000 permutations (Raymond & Rousset 1995) using TFGA v1.3 (Miller 1997). Genetic divergence among all pairwise site comparisons within the Grand watershed was estimated with Weir & Cockerham's F_{ST} (1984) calculated in ARLEQUIN v3.0 (Schneider *et al.* 2000). Allele frequencies were used to calculate Nei's standard genetic distance (D_S ; 1972) among sites within the Grand watershed. Principal coordinates analyses, using covariance matrix with standardized F_{ST} and D_S data separately, were performed using GENALEX v6.0 (Peakall & Smouse 2006) to determine groups within the Grand River sampling sites based on genetic divergence and genetic distance variation.

A Mantel test was performed to test an isolation-by-distance model of population genetic differentiation, and to identify potential barriers to gene flow among sampling sites within the Grand watershed (Mantel 1967). The shortest water distance among sample sites was measured using Google Earth. These distances were then plotted against Cavalli-Sforza and Edward's chord distance (D_C) to calculate the coefficient of determination (r^2) and linear relationship significance (p-value) using GENALEX v6.0 (Peakall & Smouse 2006).

4.3 RESULTS

Genotyping

All nine microsatellite loci were determined to be highly variable (7 to 70 alleles per locus). Population-level tests for adherence to Hardy-Weinberg equilibrium resulted in one site within the Ausable watershed significantly departing from Hardy-Weinberg equilibrium at one locus, following Bonferroni correction. No significant linkage disequilibrium was detected at the site-level for any of the nine microsatellite markers. In the one population out of Hardy-Weinberg equilibrium, no scoring errors, null allele effects, or large allele drop out were evident, suggesting that Hardy-Weinberg equilibrium departures at those sites are likely due to true non-equilibrium effects.

Comparison of Grand to known native watersheds

The Grand River watershed population showed no evidence of consistent loss of heterozygosity compared to the known native watersheds (Figure 4.2). The allelic richness of the Grand populations appeared to be lower than the native populations in the Sydenham and Thames watersheds, but was not obviously different in allelic richness from the Ausable

watershed population (Figure 4.2). The Grand watershed populations showed private alleles in three of the nine microsatellite loci, with very low frequency (0.001 – 0.051) within the populations (Figure 4.2).

All watersheds had significantly different allelic frequency distributions ($p < 0.0001$) and had significant F_{ST} values in all pairwise comparisons (Table 4.1). The Grand watershed population was most genetically diverged from the Sugar Creek, Ohio population ($F_{ST} = 0.1305$), and least diverged from the Sydenham and Thames watersheds ($F_{ST} = 0.0326$ and 0.0279 , respectively) suggesting the Grand watershed populations were not likely introduced from an American source. The Sydenham and Thames watersheds are connected via Lake St. Clair and have been shown to have migration between them (Chapter 3). Interestingly, the Grand population was more closely related to the Thames River greenside darters than to the Sydenham population ($F_{ST} = 0.0326$; Table 4.1).

Table 4.1 Matrix of pairwise Cavalli-Sforza & Edwards chord distance (D_C) (above diagonal) and F_{ST} (below diagonal) for all Canadian watershed and Sugar Creek, Ohio populations of *E. blennioides*. All pairwise comparisons were significantly different from zero following Bonferroni correction.

	Sydenham	Ausable	Grand	Thames	Ohio
Sydenham	***	0.3945	0.3543	0.2411	0.4357
Ausable	0.1015	***	0.4085	0.3376	0.4873
Grand	0.0523	0.0903	***	0.3139	0.5064
Thames	0.0326	0.0560	0.0279	***	0.4049
Ohio	0.1102	0.1669	0.1305	0.0823	***

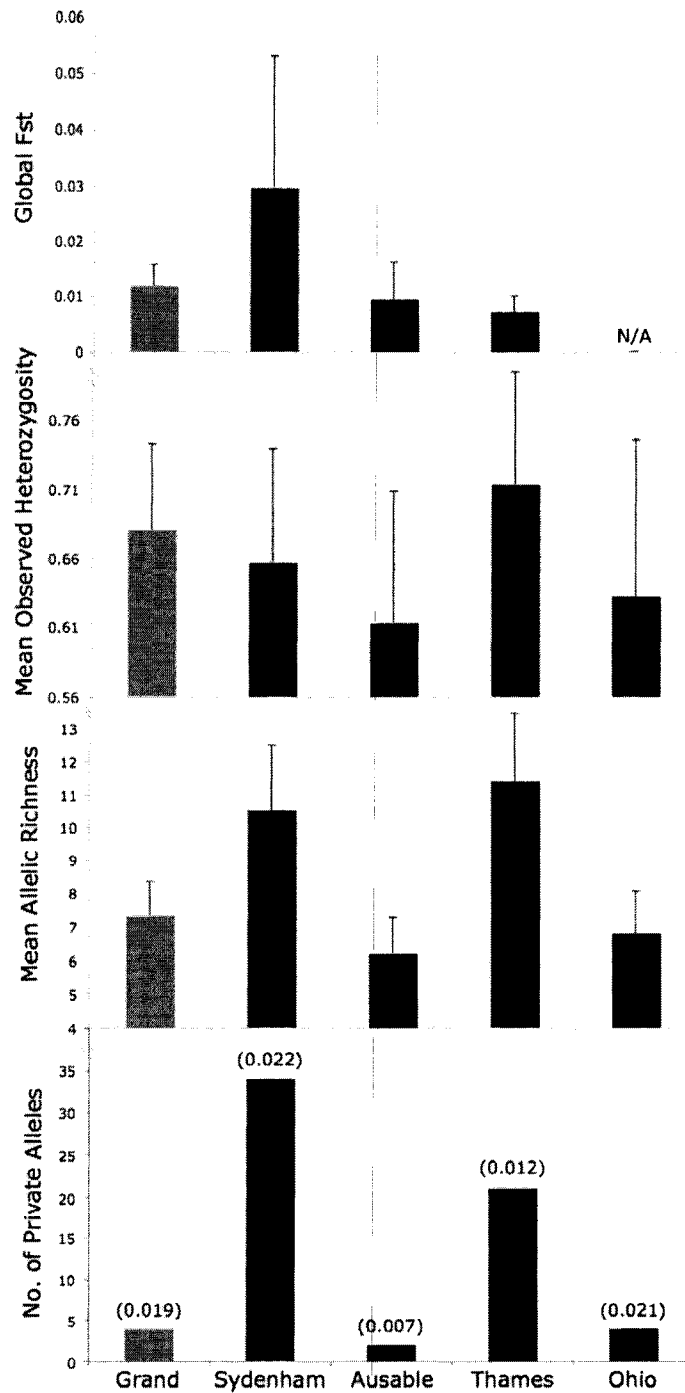


Figure 4.2 Genetic diversity indices for each watershed averaged over nine microsatellite loci, error bars indicate standard error: global F_{ST} (not applicable for Ohio as this is only one site); mean observed heterozygosity; mean allelic richness across nine microsatellite loci; and the total number of watershed private alleles with the mean allele frequency indicated in parentheses, for *E. blennioides* separated by watershed.

A chord distance phylogeographical neighbor-joining tree, rooted with the Ohio population and prepared for all watersheds, grouped the native watersheds (Sydenham and Thames) more closely, as well as the more isolated watersheds (Ausable and Ohio) with moderate bootstrap support (Figure 4.3). This gives no indication of a possible introduction source (evident in a closer grouping) for any of the Grand sites in particular, and suggests this population is not directly related to any of the sampled sites.

Historic population bottlenecks were indicated by low M values in all Canadian watersheds, with the exception of the Sydenham, and the Ohio population (Table 4.2). The Ohio population, followed by the Ausable watershed, showed the lowest M value, which indicates these populations have undergone either a very recent, very severe, or prolonged population size contraction (Table 4.2). The Sydenham watershed had a value of M consistent with a stable population ($M = 0.918$; Table 4.2). Surprisingly, the populations in the Grand watershed had a larger M value than the Thames population, suggesting the Grand population had suffered less of a population size reduction than one of the native watersheds (Table 4.2).

Table 4.2 Mean ratio of the number of alleles to the range in allele size (M) for *E. blennioides* over nine microsatellite loci described for each watershed. No. of alleles and sample size are mean values over loci.

	M	No. of alleles	Sample size
Grand	0.720	11.22	499
Sydenham	0.918	19.11	218
Ausable	0.638	8.67	188
Thames	0.674	18.56	133
Ohio	0.604	7.00	30

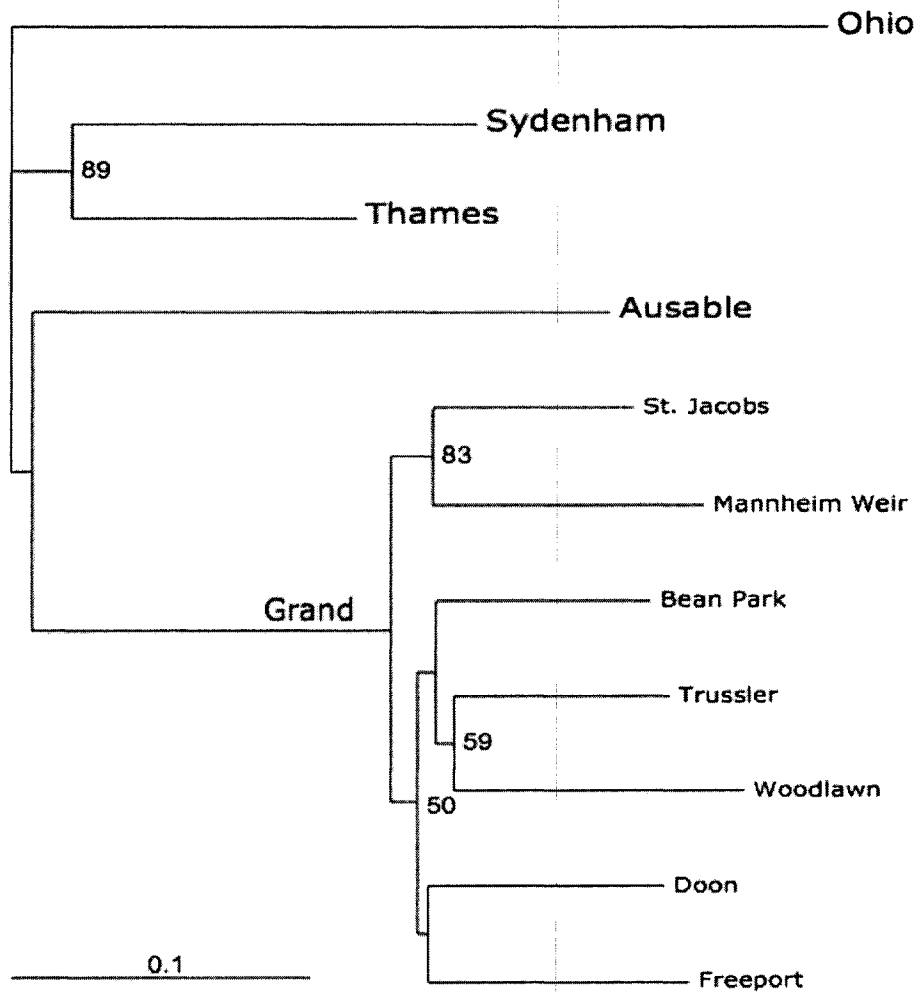


Figure 4.3 *E. blennioides* phylogeographic chord distance (D_C ; Cavalli-Sforza & Edwards 1967) neighbor-joining tree rooted with the Ohio population, based on nine microsatellite loci, with all watersheds. Numbers indicate replicated bootstrap values out of 10 000 (values under 50% not shown). Names following the Grand watershed branch refer to sites sampled within the Grand watershed.

However, no recent population bottlenecks were suggested by distorted allele frequency distributions of the Luikart *et al.* (1998) method for any population or watershed (Figure 4.4). Also, none of the populations showed significant bottlenecks based on the Wilcoxon test under the two-phase mutation model.

Genotype assignment results gave no indication that the Grand watershed had been recently seeded from any of the Canadian watersheds, or the Ohio out-group population (Table 4.3). Migration among watersheds, particularly with the Grand, was very low (Table 4.3). Self-assignment (assignment of an individual to the watershed in which it was captured) was exceptionally high in the Grand, Ausable and Ohio watersheds (100 – 98%; Table 4.3). Only one individual in the Grand watershed was successfully assigned to a different watershed (the Thames; Table 4.3).

Table 4.3 Results of a genotype assignment analysis on *E. blennioides*, grouped by watershed, using GENECLASS software. Correct assignment based on 9:1 likelihood ratio. No. dispersers refers to the number of individuals from that watershed assigned to a different watershed with over 90% confidence.

	Total no. individuals	Successful (9:1) assignment	Self assigned	% Self assignment	No. and watershed origin of migrants
Sydenham	218	182	172	0.945	10, Thames
Ausable	188	173	170	0.983	3, Thames
Grand	499	454	453	0.998	1, Thames
Thames	133	85	80	0.941	3, Sydenham; 2, Ausable
Ohio	30	29	29	1.000	none

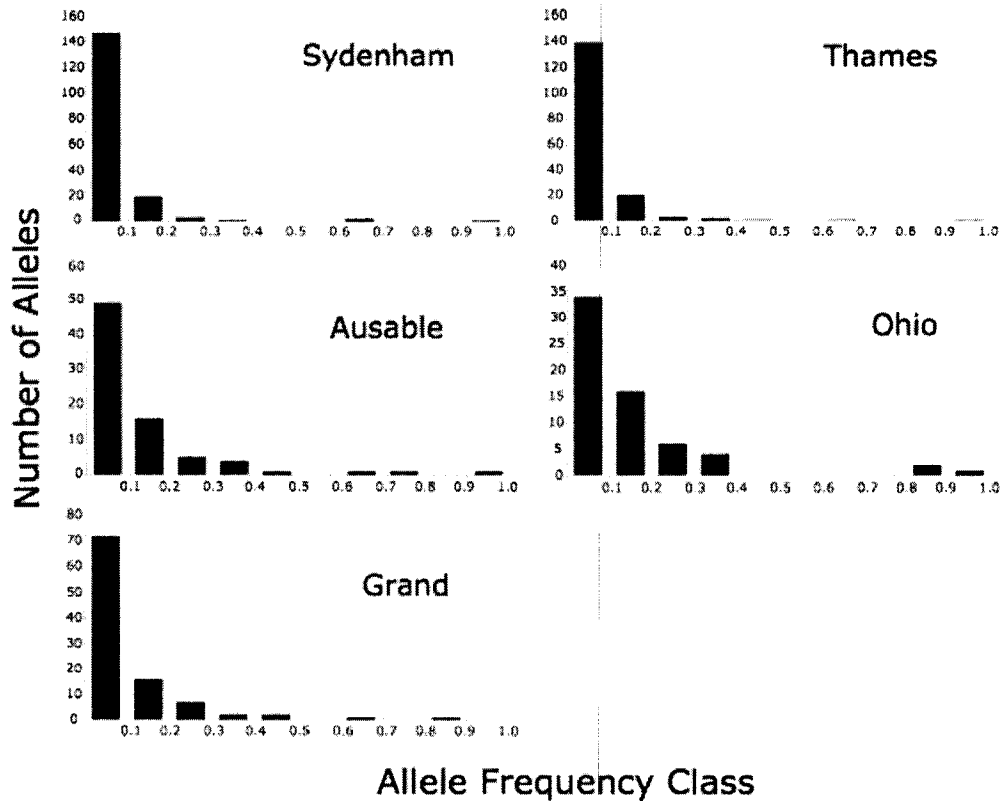


Figure 4.4 Allele frequency distributions of *E. blennioides* across nine microsatellite loci, by watershed, to determine bottlenecked populations.

Structure within the Grand River watershed

Exact tests showed three pairwise comparisons within the Grand watershed population having non-significantly different allele frequency distributions following Bonferroni correction (Table 4.4). They were the two sites upstream of the Mannheim Weir (SJ and MW), the two sites of closest proximity (FP and D), as well as BP and D (Figure 4.1; Table 4.4).

Table 4.4 Matrix of pairwise Nei's standard genetic distance (D_S) (above diagonal) and F_{ST} (below diagonal) for all Grand River watershed sampled site populations of *E. blennioides*. Non-significant allele frequency exact indicated by D_S value in bold type, non-significant values of F_{ST} indicated by bold type. All tests for significance following Bonferroni correction.

	BP	SJ	T	W	D	F	MW
BP	***	0.0509	0.0171	0.0457	0.0306	0.0313	0.0642
SJ	0.0037	***	0.0468	0.0844	0.0372	0.0448	0.0200
T	0.0019	0.0091	***	0.0458	0.0307	0.0321	0.0584
W	0.0115	0.0238	0.0137	***	0.0561	0.0648	0.0822
D	0.0002	0.0041	0.0045	0.0069	***	0.0353	0.0568
F	0.0037	0.0035	0.0067	0.0164	0.0038	***	0.0416
MW	0.0084	0.0029	0.0113	0.0224	0.0126	0.0006	***

Both the principal coordinates analyses (PCA), using F_{ST} and D_S matrices, partitioned the Grand watershed sites into three groups (Figure 4.5). Only one site differed in group membership between the two PCAs (Figure 4.5). The D_S PCA grouped FP with the downstream sites (D, T and BP); however, the F_{ST} PCA grouped the FP site with the sites upstream and over the Mannheim Weir (MW and SJ). The three distinct groups reflect the pattern of greenside darter discovery in the Grand River watershed from 1990 to 1995 (Figure 4.1). The pattern of genetic divergence among sample sites in the Grand River watershed did not conform to an isolation by distance model as evident by a non-significant Mantel test.

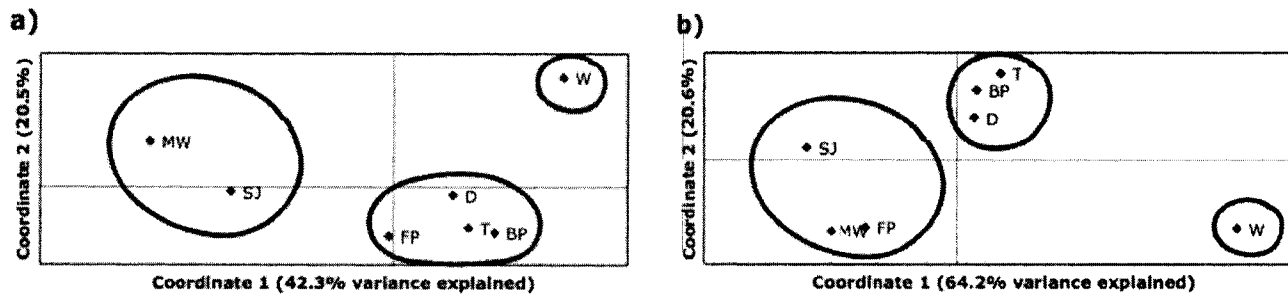


Figure 4.5 Principal coordinates analysis of a) Nei's standard genetic distances across nine loci (D_S ; 1972) and b) F_{ST} for nine loci (Weir & Cockerham 1984) among sampled sites within the Grand watershed for *E. blennioides*. Percentage of variation explained by each axis indicated in parentheses beside coordinate.

4.4 DISCUSSION

Determining the invasion status of populations under suspicion of being introduced can be difficult, even with historical data (Beebee *et al.* 2005). Population genetic data can aid in identifying introduced populations in circumstances when historic data does not exist (Okello *et al.* 2005); however, the detection of an introduced population native to the general area (an 'introduced native') can still be problematic. Although the range expansion, rapid dispersal and long-range movement capabilities of the greenside darter have been previously documented using historical sampling records and preserved specimens (Neely & George 2006), there are other populations in this same Atlantic slope area that are of unknown invasion status (Jenkins & Burkhead 1994; Starnes 2002). The greenside darters of the Grand River watershed in Canada are another example of uncertain invasion status.

The sudden appearance of greenside darters in the Grand River watershed is difficult to explain. Despite regular and extensive government sampling, greenside darters were not detected in the Grand watershed until 1990. Their abundance and distribution have since

increased dramatically. Two possibilities could explain this phenomenon: these populations were native to the watershed and have recently increased in numbers due to some favorable change in the environment (recovery from a long term bottleneck); or, these populations were accidentally introduced and have succeeded in dramatically expanding throughout the system (founder effect). In either situation, these populations should have limited genetic diversity (Eckert et al. 1996; Tsutsui et al. 2000; Nyström 2006), but that was not the case.

If the Grand populations were introduced, they should assign to a source population, however, the individuals caught in the Grand watershed self-assigned in 99.8% of the successful assignments, suggesting the populations were not introduced. Also, in an introduction, the newly founded populations should lack genetic structure, as they could not have diverged in only ten generations due to drift and mutation alone (even at a rate of 10^{-3} to 10^{-4} per locus per generation; Ellegren 2000), but at least three genetically distinct groups emerged. On the other hand, these three groups as indicated by the PCAs roughly correspond to the areas of greenside darter discovery from 1990 to 1995 (see Figure 4.1), and could be indicative of three introduction events.

If the Grand River populations were native, they would have gone through an extended genetic bottleneck, as not to have been discovered until 1990, however, analyses for bottleneck signatures detected none, suggesting the populations are not native. Also, if the Grand River greenside darters were native, one would expect high genetic differentiation among all subpopulations via drift (since greenside darters do not disperse much; Chapter 3), and the structure should follow an isolation by distance model. Neither was found to be true,

indicating that the populations are likely not native. Furthermore, how could these bright green, almost unmistakable fish have gone unnoticed by fisheries workers for so long if the species has been identified in the Thames watershed for over a hundred years? Thus the Grand River paradox is defined by genetic comparisons of the Grand River with known native populations of greenside darters at the watershed-level that show the Grand populations are not introduced, while structure within the Grand River watershed and historical abundance data that indicate the populations are not native.

The solution to this apparent paradox lies in multiple (at least three) introductions of large numbers of greenside darters into the Grand River watershed from genetically differentiated sources. The multiple sources and multiple introduction sites explain the genetic divergence patterns among sites in the Grand, while the large numbers of founders explain the lack of any detectable bottleneck effects. A similar scenario has occurred in the nassariid gastropod (*Cyclope neritea*), where the range is expanding north and human-mediated introductions are suspected, and the same pattern of high genetic diversity and low genetic structure among the introduced populations was shown (Simon-Bouhet et al. 2007). Genton *et al.* (2005) showed that multiple introductions of the common ragweed (*Ambrosia artemisiifolia*) in France resulted in the overall genetic variability of the introduced populations being similar to that of the native, no isolation by distance in the introduced, and assignment failure to identify a single population of origin. This is further evidence of the buffering capacity of multiple introductions on the detrimental founder effects in a population. Genetic diversity retention in the Grand populations could have also been influenced by the rapid expansion following introduction, where the reduction in average heterozygosity is rather small as a result of

reduced genetic drift (Nei *et al.* 1975; Friar *et al.* 2000; Zenger *et al.* 2003). Clearly, multiple, large-scale introductions of genetically diverse greenside darters does serve to explain the observed patterns of genetic diversity in the Grand River watershed. However, it is difficult to imagine a reasonable scenario whereby at least three large-scale introductions, from three independent sources, into widely geographically separated regions could be attributed to accidental introductions. Even if intentional multiple introductions of many greenside darters occurred in the Grand River watershed, it is still astounding how the population abundance and range exploded throughout the watershed in such a short time.

Regardless of how the greenside darters arrived in the Grand River watershed, they are now a large, and influential part of the aquatic community, and are extending their range northward. This situation exemplifies how animals from peripheral populations at the northern species boundary can be key in founding new populations as a result of rapidly changing climates and environments (Davis & Shaw 2001). Historic range limitations that, in the past, excluded greenside darters from the Grand River watershed have now been changed, and made the relatively recently inhospitable northern watersheds exploitable by the greenside darter. This is a trend common across the whole northern hemisphere, where range extensions are occurring along the northern boundaries of species ranges, and extirpations along the southern boundaries (Arnell *et al.* 1996; Magnuson *et al.* 1997). These Canadian populations of greenside darter may be those most suited to establishing themselves in the new habitats created by warming temperatures (Fraser 1999) and may be important for the species to survive long term environmental changes (Hunter 1991; Quinn & Karr 1992). Greenside darters and the Grand River paradox clearly demonstrate how genetic data can provide

unique insights into dynamic and poorly understood population and conservation biology issues.

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5.0 GENERAL DISCUSSION

This thesis addresses several ecological concepts pertinent to current environmental, conservation and evolutionary issues, such as population structure and stability, introduced populations, and rapid range expansion at the northern boundary of a species, using the greenside darter system in Canada as a model. These concepts are particularly relevant now that loss of biodiversity, spread of invasive species, and global climate change are major environmental concerns largely affecting the world today.

To address the above concepts, which rely on fine-scale population inspection, molecular markers were needed. Chapter 2 of this thesis details the development of microsatellite markers designed for the greenside darter, which were selected because they evolve on an ecological-scale, allowing us to examine population dynamics even in a system with high connectivity. Chapters 3 and 4 made use of the resolving power of these markers to characterize the population structure of the greenside darters in Canada, make inferences as to their temporal stability, and explain the introduction mechanism and expansion in the Grand watershed.

The Canadian populations of greenside darter are characterized by distinct allele frequencies, substantial genetic diversity, and limited gene flow among watersheds. The Thames and Sydenham watersheds showed weak divergence and high levels of among-watershed migration, suggesting on-going gene flow. Furthermore, the low divergence among upper watershed sites also suggests historic headwater connections. The Ausable watershed was identified as genetically isolated from other all other populations within Canada. This, in

combination with the fact that greenside darters in this watershed went through a severe population bottleneck such that the species was thought to be extirpated from the system (Dalton 1991), resulted in a marked loss of allele diversity and heterozygosity (Chapter 3).

Conversely, the Grand watershed greenside darter populations showed high genetic diversity, comparable to the native Sydenham and Thames populations at the watershed-level, suggesting multiple introductions and rapid expansion allowed the retention of genetic variation. Within the watershed, there was limited genetic structure, except for three distinct groups that correspond with discovery sites of greenside darter in 1990 through to 1995. Although no putative origin of introduction was identified, I am confident greenside darters were introduced into this watershed in at least three different areas, and from different genetic sources. There was no evidence of a population bottleneck in the Grand watershed, which implies that the introductions must have included substantial numbers of fish, suggesting the occurrence of greenside darters in the Grand River may not have been entirely accidental.

The greenside darters in Canada represent the most northern range-edge populations of the species and provided an excellent model with which to study peripheral population dynamics, expansion and stability in a fragmented riverine system. These populations are very fragmented at the watershed-level, however, within each river system dispersal, and subsequent potential gene flow is more common. They also show remarkable instability from one year to the next, and despite being at the edge of their range, can be very invasive as evident by the explosive range expansion in the Grand watershed.

Further research is required to fully characterize the range-edge effects on peripheral populations by making direct comparisons of the Canadian greenside darters with more central populations, and this could be done more readily now that the molecular markers have been developed for the species. Within the Canadian populations, further sampling in both the fall and summer would help to characterize the temporal instability outlined in this work, and to determine if this documented instability is actually due to unstable peripheral population environments, or if seasonal dispersal is occurring.

5.1 MANAGEMENT

Although no genetic distinctions among the Canadian populations of greenside darter that warrant conservation designation below the species level were found, a high degree of genetic structure exists, especially among watersheds. Fragmentation within watersheds, such as dams and weirs, appeared to have little effect on the dispersal of the organisms but this is not say that in time these may represent more substantial reproductive barriers. Due to the recent downlisting of the greenside darter in Canada, special concern is no longer required when making management decisions regarding areas they inhabit. This is clearly due, in part, to the rapid colonization and expansion of the Grand watershed. Other watersheds, such as the Ausable, are not faring as well. The Ausable populations were determined to have undergone severe bottlenecks in recent history and in combination with the lack of immigration and isolation from other watersheds, exhibit substantially reduced genetic diversity relative to other Canadian populations. These populations appear to increasing in abundance and may recover over time.

Rapid expansion of greenside darter populations throughout the Grand watershed may have both beneficial and detrimental effects on the system. The greenside darter is an important forage fish for smallmouth bass (*Micropterus dolomieu*) and rock bass (*Ambloplites rupestris*) (Cooper 1983), and has been found in the gut contents of stonecat (*Noturus flavus*) (Bunt *et al.* 1998). On the other hand, sympatric fish species that use similar habitat, such as the rainbow darter (*Etheostoma caeruleum*), may be negatively affected by an increase in greenside darter abundance by increased interspecific competition (despite the habitat partitioning shown among most darters; Welsh & Perry 1998). Monitoring, via more widespread sampling and analysis, of the explosive abundance increase and on-going range expansion in the Grand River watershed is recommended.

5.2 FINAL NOTE

This is the first genetic study to use ecological-scale genetic markers to address the population structure of greenside darter, as well as the first to address such questions in range-edge Canadian populations. The data presented in this thesis highlight the interactions among the Canadian populations of the greenside darter and indicate that those populations represent an important study system for range-edge population dynamics. This work further demonstrates how genetic data can provide unique insights into poorly understood population and conservation biology issues.

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