

# Range expansion by invasion: genetic characterization of invasion of the greenside darter (*Etheostoma blennioides*) at the northern edge of its distribution

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**Abstract** Species introductions in freshwater ecosystems are often complex processes, yet an understanding of the nature of the introduction can inform management and conservation actions. The greenside darter (*Etheostoma blennioides*), until recently a species of special concern, expanded its Canadian range and is now common and widespread in the Grand River watershed (GRW). This is despite there being no evidence of greenside darter in the GRW prior to 1990. The goal of this study was to genetically characterize the GRW greenside darter introduction. Greenside darter were sampled in the GRW, the three known native watersheds in Canada, and one site from Ohio. We measured genetic diversity and population

structure, and tested for population bottlenecks using eight microsatellite loci. Genotype assignment was used to identify possible introduction sources. Populations in the GRW showed similar genetic diversity to native watershed populations with no evidence for recent or historical population bottlenecks. Genotype assignment showed that one of the Canadian watersheds and the Ohio site were not potential sources of the GRW greenside darter, whereas the Thames River watershed was the most likely source. Substantial population genetic structure exists among the sample sites in the GRW. Clearly, the current widespread and abundant distribution of the greenside darter in the GRW is not the result of recent expansion of an existing native population, but rather multiple introductions into at least three sites in the GRW, followed by rapid population growth. Although the GRW *E. blennioides* is introduced, it harbours considerable genetic diversity and represents an important northern range extension for this species.

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## Introduction

It is widely accepted that the introduction of a non-native species can impact the receiving ecosystem in

unanticipated and potentially detrimental ways (Carlson 2008; Kerr et al. 2005; Strayer et al. 2006). In many cases, the identification of an introduced species is straightforward, for example, when the introduction occurs far from the native range (e.g. between continents). The characterization of a putative introduction that is near, or within, the native species range, as either (1) a previously unknown native population, (2) natural range expansion, or (3) a human-mediated introduction, is not straightforward (Gopurenko et al. 2003; Carlton 1996; Templeton 1998). However, such a distinction is important for effective resource management. Ideally, the origin of such occurrences would be verified using historical data. In cases where such historical data are absent or equivocal, population genetic techniques can be used to clarify whether newly detected populations constitute a native population, recent range expansions or anthropogenic introductions (Brown and Stepien 2009; Carlton 1996; Gopurenko et al. 2003). For example, microsatellite markers and mitochondrial sequence variation have been used to demonstrate recent population range expansion in both freshwater (Hrbek et al. 2005; Reeves and Bermingham 2006) and marine species (D'Amato 2006; Gopurenko et al. 2003; Sotka et al. 2005). The management and conservation implications of natural range expansion versus human-mediated introductions are subtle, but important. Natural northward range expansions are expected in North American fish species due to ongoing climate change (Chu et al. 2005; Magnuson et al. 1997; Mandrak 1989). However, such range expansions would likely involve not just a single species introduction, but rather a gradual change in ecosystem composition over decades. Single species introductions, on the other hand, can result in dramatic changes in species interactions and foodweb function in only a few years (Carlson 2008).

The greenside darter (*Etheostoma blennioides*) is a benthic, freshwater fish with a history of recent range expansion (Beneteau et al. 2009; Neely and George 2006). It has been found in drainages where its population status (introduced or native) is controversial (Jenkins and Burkhead 1994; Starnes 2002). The greenside darter is native to three Laurentian Great Lake watersheds in Ontario (Ausable, Sydenham, and Thames rivers), and has recently been discovered in two others (Grand and Maitland rivers) (COSEWIC 2006; Beneteau et al. 2009). These populations

constitute northern range edge of the species (Beneteau et al. 2009). In 1991, the greenside darter was assessed as Special Concern by the Committee On the Status of Endangered Wildlife in Canada (Dalton 1991). In 2006, with greenside darter numbers in Canada increasing, the species was reassessed as Not at Risk (COSEWIC 2006). This decision was, in part, due to the 1990 discovery of the greenside darter in the Eramosa River in the Grand River watershed (GRW) in Ontario. The greenside darter in the GRW is now known to be present above and below barriers in all four major tributaries (Nith, Speed, Conestogo, and Eramosa rivers; Fig. 1). Although the mechanism of the dramatic increase in distribution and abundance of the GRW greenside darter is unknown, it is believed to have resulted from an introduction into the Eramosa River via bait bucket release or in association with the stocking of game fishes (COSEWIC 2006). Alternatively, the greenside darter may have been native to the GRW but, as a result of very low numbers, went undetected until 1990. However, the greenside darter is an unmistakable bright green fish, which has been regularly identified in the Thames River watershed for over a 100 years, thus the possibility that fisheries biologists have consistently overlooked this fish in the GRW seems remote. In particular, extensive fish inventories at dozens of sites in both watersheds conducted by the Ontario Ministry of Natural Resources in the 1980s found greenside darter throughout the Thames watershed, but none in the Grand River watershed. Nevertheless, if the greenside darter is native to the GRW, population genetic theory would predict that, due to population persistence over many generations at very low numbers, the GRW greenside darter should exhibit reduced genetic diversity and evidence for moderate to severe bottleneck effects (Nyström et al. 2006). Small isolated native populations are also expected to show substantial genetic population structure due to elevated genetic drift effects. If the GRW greenside darter is the result of recent introduction followed by rapid population expansion, we would also expect to see low genetic diversity (depending on the size of the introduction), but little or no population genetic structure. Thus, a population genetic analysis will verify whether the GRW greenside darter is native or introduced, and characterize the nature of the introduction process if it is not native.

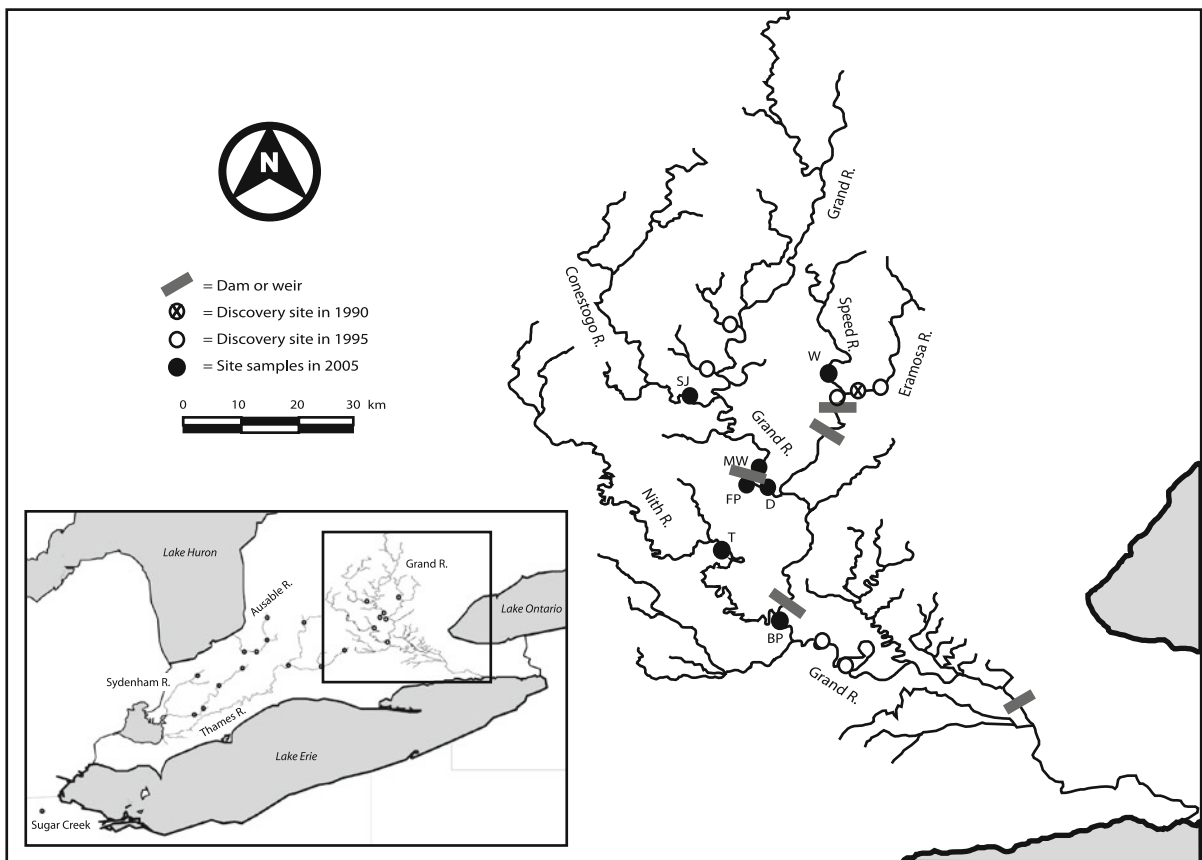
The goal of this study was to determine the origin of the greenside darter in the GRW, and to genetically characterize the remarkable population expansion in the watershed over the last 20 years. Fish were collected from the known native Canadian watersheds, one population from Ohio, and from seven sites in the GRW. We measured genetic variation, genetic population bottleneck effects, and population genetic structure to characterize the genetic status of the GRW greenside darter. We also used genotype assignment to identify the putative source(s) of introduction of the GRW greenside darter. We use the genetic data to reject the hypothesis that the GRW *E. blennioides* are native, and argue that they likely result from multiple large introductions into three separate areas of the watershed.

## Methods

### Study site and sampling

The greenside darter currently inhabits at least five watersheds at the northeastern edge of its range in southwestern Ontario, Canada (Ausable, Sydenham, Thames, Maitland, and Grand rivers; Fig. 1). The GRW has four major tributaries: the Nith, Conestogo, Speed, and Eramosa rivers (Fig. 1). Draining 6,965 square kilometers, the GRW is the largest watershed in southern Ontario. There are several large dams and weirs that restrict fish movement throughout the watershed (Fig. 1).

In October 2005, we sampled greenside darter at seven sites in four of the five major tributaries in the GRW (Fig. 1; Table S1). For genetic comparison,



**Fig. 1** The Grand River watershed marked with greenside darter original discovery sites, capture sites from this study, and known dams and weirs. The inset silhouette map of Ontario, Canada, shows the location of the expanded map.

Sampled site abbreviations are as follows: Bean Park (BP); St. Jacobs (SJ); Trussler (T); Woodlawn (W); Doon (D); Freeport (F) and Mannheim Weir (MW)

and to identify possible sources of introduction, samples were also collected from three to four sites in each of the native watersheds of southwestern Ontario: the Ausable and Sydenham rivers in October 2005; and, the Thames River in June 2006 (Fig. 1; Table S1). Some of these collections were for a related study that examined genetic structure and gene flow in the greenside darter (Beneteau et al. 2009). One Lake Erie tributary site in Ohio (Sugar Creek) was sampled as a geographically distant out-group (Fig. 1; Table S1). Caudal fin clips were taken from all specimens and stored in 95% ethanol.

### Genetic analyses

DNA was extracted from fin clips using a column-based plate extraction method (Elphinstone et al. 2003). Eight microsatellite markers (four tetra-nucleotide and four di-nucleotide repeat motifs) were used to genotype all individuals (as described elsewhere; Beneteau et al. 2007; Tonnis 2006). Allele sizes were scored using a LiCor 4300 DNA Analyzer and the software GENE IMAGIR version 4.05 (Scanalytics, Inc.).

Microsatellite loci were tested for adherence to Hardy–Weinberg equilibrium with 100,000 permutations using ARLEQUIN version 3.0 (Excoffier et al. 1992). Significance was corrected for multiple simultaneous comparisons using the sequential Bonferroni method (Rice 1989). All loci were tested for linkage disequilibrium using ARLEQUIN at each sampled site.

To determine if greenside darter populations in the GRW exhibited reduced genetic diversity relative to known native populations, mean observed heterozygosity (ARLEQUIN), mean allelic richness across all loci (corrected for sample size), and the number of private alleles (FSTAT version 2.9.3; Goudet 2001) were calculated for each of the sample sites.

To test for evidence of population bottlenecks at each sampled site, three different analytical methods were employed. First, historic population bottlenecks for each site were identified by calculating the mean ratio (across loci) of the number of alleles to the range in allele size ( $M$ ; Garza and Williamson 2001), which decreases with the severity and duration of the bottleneck. Second, we graphed allele frequency distributions across all loci for each GRW population to identify allele frequency distortions expected due to population bottlenecks (Luikart et al. 1998). Finally, BOTTLENECK version 1.2.02 (Cornuet and

Luikart 1996; Piry et al. 1999) was used to test for significant bottleneck-related genotype biases at the population level, with the Wilcoxon test for heterozygosity excess under the two-phase mutation model.

The pattern and magnitude of population genetic structure reflects invasion history; therefore, we characterized the genetic differentiation among the seven sample sites in the GRW. First we used pairwise exact tests of allele frequency distribution differences (100,000 permutations; Raymond and Rousset 1995) implemented in TFPGA. Next, we calculated pairwise  $F_{ST}$  (Weir and Cockerham 1984) between all sites within the GRW using ARLEQUIN. Genetic clustering among sampling sites can reflect native subpopulation structure or multiple introduction events (Brown and Stepien 2009), thus principal coordinates analyses (PCoA, covariance-standardized) were performed on GRW pairwise  $F_{ST}$  and Nei's standard genetic distance ( $D_S$ ; 1972) matrices separately in GENALEX version 6.0 (Peakall and Smouse 2006). Finally, a Mantel test (Mantel 1967) was performed to test for an isolation-by-distance model of population genetic differentiation, a pattern expected to result from the equilibrium between dispersal, mutation and drift and, hence, likely associated with a relatively long-standing distribution. The shortest pairwise water distance between sample sites within the GRW was measured using Google Earth (<http://earth.google.com/>). These distances were then correlated with Cavalli-Sforza and Edward's chord distance ( $D_C$ ) to calculate the coefficient of determination ( $r^2$ ) and linear relationship significance ( $P$  value) using the Mantel test in GENALEX.

We used Bayesian genotype assignment to determine source-sites relationships for the GRW greenside darter in GENECLASS version 2.0 (Piry et al. 2004; Rannala and Mountain 1997). We performed an exclusion analysis, where GRW samples are treated as 'individuals of unknown origin', whereby GRW individuals are either *excluded* ( $P < 0.05$ ) or *assigned* ( $P > 0.05$ ) to potential source populations (sites) within the Sydenham, Ausable, Thames, and Ohio watersheds. The probability of assignment was computed using 10,000 simulated individuals using the algorithm of Paetkau et al. (2004). We also identified high probability assignment to a particular source watershed using the threshold probability of  $P > 0.90$ .

## Results

### Marker assessment

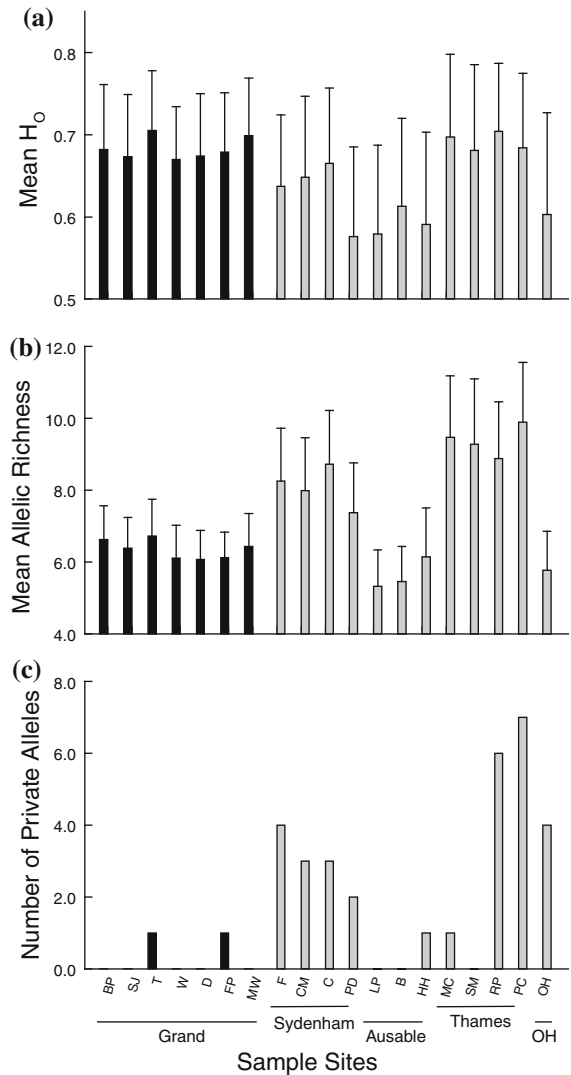
All eight microsatellite loci were highly variable (7–70 alleles per locus). Population-level tests for adherence to Hardy–Weinberg equilibrium resulted in one locus at one site within the Ausable watershed significantly departing from Hardy–Weinberg equilibrium after the Bonferroni significance correction (i.e., 1 out of 152 comparisons). No significant linkage disequilibrium was detected at the site-level for any of the eight microsatellite markers.

### Genetic diversity

The GRW sites showed little evidence of reduced genetic diversity relative to the historic native populations (Fig. 2; Table S1). Mean observed heterozygosity of GRW populations was not substantially different from the other populations, while allelic richness was lower than the native populations in the Sydenham and Thames watersheds, but was not different from the Ausable watershed populations (Fig. 2a, b; Table S1). Only two GRW populations showed private alleles, one each (Fig. 2c; Table S1).

### Genetic bottlenecks

Population bottlenecks were indicated by low  $M$  values in the OH population and many populations in Canadian watersheds (Table S1). The Ausable River watershed ( $M = 0.49–0.55$ ), followed by the Ohio population ( $M = 0.58$ ) showed the lowest  $M$  values (Table S1), indicating these populations had undergone either a very recent, very severe, or prolonged population size contraction. On average, populations in the GRW had lower  $M$  values ( $M = 0.59–0.68$ ) than the Thames River ( $M = 0.61–0.81$ ) and Sydenham River populations ( $M = 0.66–0.83$ ; Table S1), indicating that the GRW fish had suffered more of a population bottleneck than in some of the historic native watersheds. Recent population bottlenecks are known to distort allele frequency distribution by shifting the distribution to the right (i.e. fewer rare alleles; Luikart et al. 1998). There was no evidence for recent population bottlenecks based on allele frequency distributions in any GRW populations



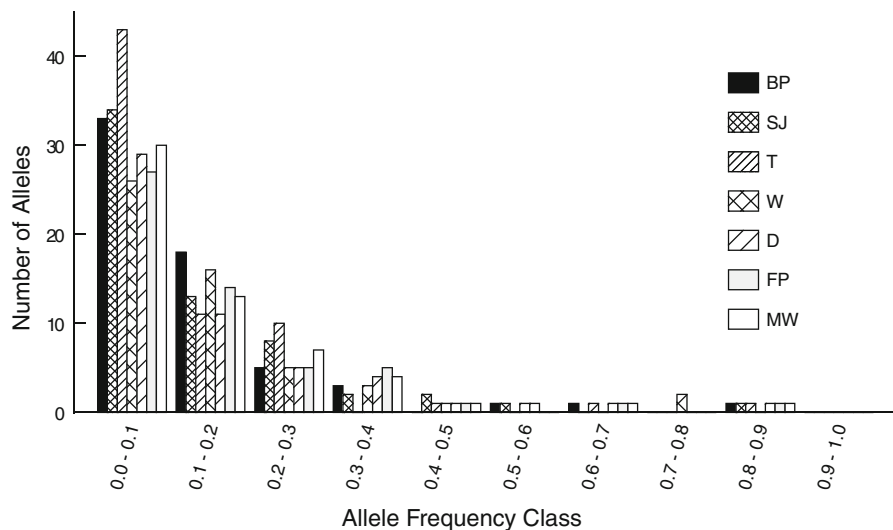
**Fig. 2** Greenside darter genetic diversity indices for GRW sites (black) and other watershed sites (grey) averaged over eight microsatellite loci, error bars indicate one standard error: Panel a: mean observed heterozygosity, Panel b: mean allelic richness (corrected for sample size), Panel c: the total number of private alleles at each site

(Fig. 3) or other watershed (data not shown). Finally, none of the sampled sites, either within the GRW or elsewhere, showed significant evidence for bottlenecks based on the Wilcoxon test under the two-phase mutation model in BOTTLENECK.

### Genetic structure within the GRW

Exact tests showed 18 out of 21 pairwise comparisons within the GRW had significantly different allele

**Fig. 3** Microsatellite allele frequency distributions at eight loci for GRW greenside darter at each sampled site plotted to identify recent population bottlenecks. In the event of a bottleneck, the least frequent alleles are not the most abundant. In this case, all distributions suggest the populations have not undergone recent genetic bottlenecks



frequency distributions, following Bonferroni correction (Table 1). Twelve out of 21 pairwise  $F_{ST}$  estimates within the GRW were statistically different from zero, with BP and D showing the highest level of divergence and W showing the least divergence (Table 1). Global  $F_{ST}$  estimates for the four sampled watersheds were: GRW = 0.012; Thames = 0.007; Sydenham = 0.029; and Ausable = 0.008.

The results of both PCoAs, based on  $F_{ST}$  and  $D_S$  matrices, partitioned the GRW sites into three groups (Fig. 4). Only one site (FP) changed group membership between the two analyses (Fig. 4). The  $D_S$  PCoA grouped FP with the downstream sites (D, T and BP), while the  $F_{ST}$  PCoA grouped the FP site with the sites upstream and over the Mannheim Weir (MW and SJ). Bunt et al. (1998) reported movement of greenside darter through fishways across the Mannheim Weir,

thus the inconsistent clustering of the FP site (just below the weir) may reflect either mixing of the two groups, or perhaps hybrid fish resulting from across-weir gene flow. Nevertheless, the three clusters reflect the geographical pattern of greenside darter discovery in the GRW from 1990 to 1995 (Fig. 1). The pattern of genetic divergence among sample sites in the GRW did not conform to an isolation-by-distance model (Mantel test,  $P > 0.05$ ).

#### Genotype assignment

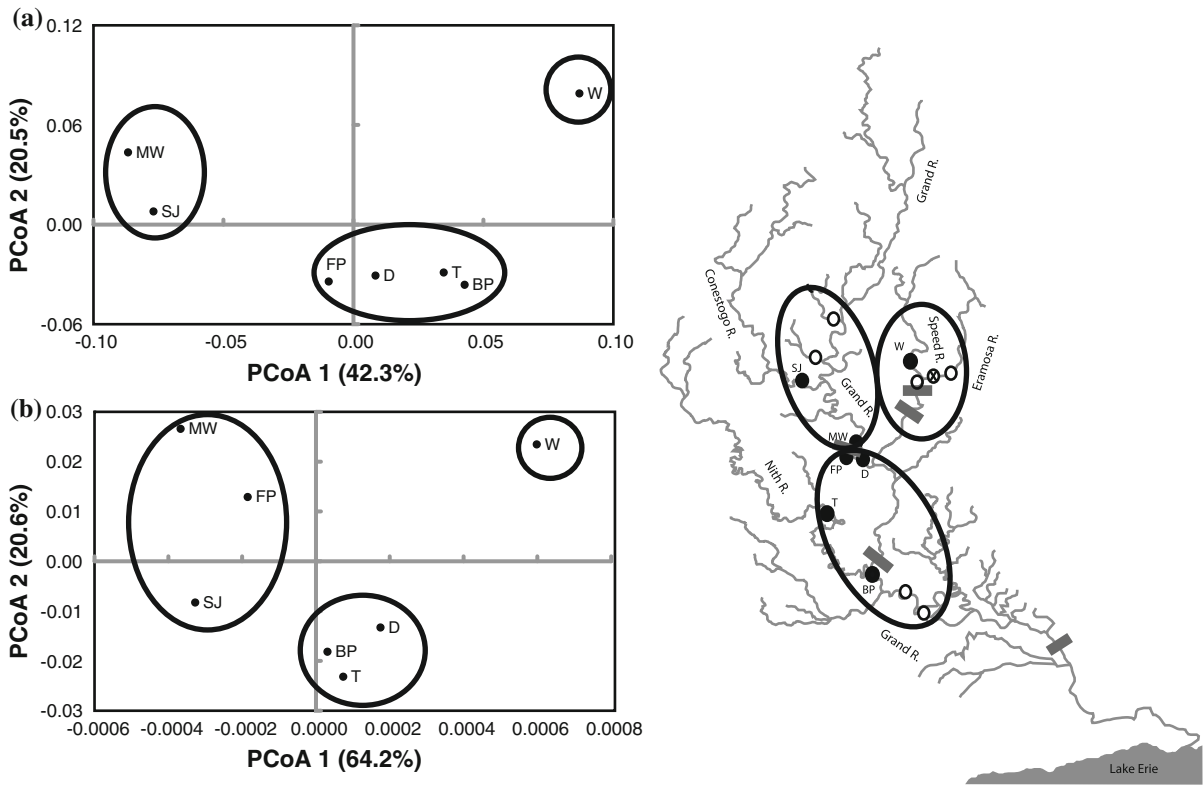
Bayesian genotype assignment revealed sites within the Thames and Sydenham watersheds as potential sources for individuals in the GRW (Fig. 5). The Thames was identified as the most likely potential source watershed as close to 90% of GRW individuals

**Table 1** Matrix of pairwise Nei's standard genetic distance ( $D_S$ ) (above diagonal) and  $F_{ST}$  (below diagonal) for all GRW sampled site populations of greenside darter

	BP	SJ	T	W	D	F	MW
BP	***	<b>0.051</b>	<b>0.017</b>	<b>0.046</b>	0.031	<b>0.031</b>	<b>0.064</b>
SJ	0.0037	***	<b>0.047</b>	<b>0.084</b>	<b>0.037</b>	<b>0.045</b>	0.020
T	0.0019	<b>0.0091</b>	***	<b>0.046</b>	<b>0.031</b>	<b>0.032</b>	<b>0.058</b>
W	<b>0.012</b>	<b>0.024</b>	<b>0.014</b>	***	<b>0.056</b>	<b>0.065</b>	<b>0.082</b>
D	0.0002	0.0041	0.0045	<b>0.0069</b>	***	0.035	<b>0.057</b>
F	0.0037	0.0035	<b>0.0067</b>	<b>0.016</b>	0.0038	***	<b>0.042</b>
MW	0.0084	0.0029	<b>0.011</b>	<b>0.022</b>	0.013	0.0006	***

Significant allele frequency differences are indicated by  $D_S$  values in bold type as are significant values of  $F_{ST}$ . All tests for significance are Bonferroni corrected. Sample site abbreviations are as defined in Table S1, location shown in Fig. 1

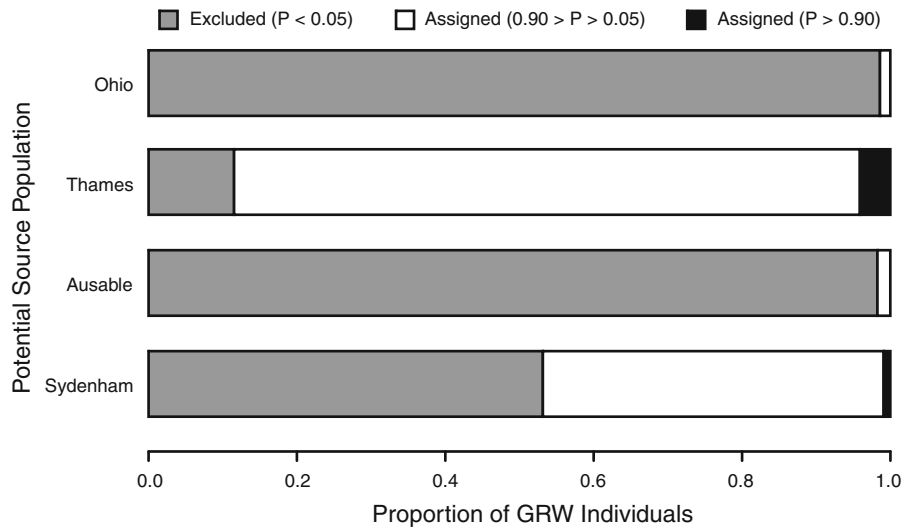




**Fig. 4** Principal coordinates analysis (PCoA) of genetic divergence among the seven greenside darter sample sites in the GRW. Panel *a*: PCoA based on Nei's standard genetic distances across eight loci ( $D_8$ ; 1972), Panel *b*: PCoA based on

$F_{ST}$  (Weir and Cockerham 1984). Percentage of variation explained by each axis (PCo) is indicated in parentheses on the axis. *Right*: a map of the GRW with PCoA results circled, groups correspond with areas of discovery

**Fig. 5** Genotype assignment results for GRW greenside darter assigned to the other sampled populations. For each sampled watershed, the proportion of GRW greenside darter that were excluded as having come from that watershed as well as those that are likely to have come that watershed are shown



were assigned to the Thames watershed, and 4.1% were assigned with a high probability ( $P > 0.90$ ; Fig. 5). Both the Ohio and Ausable watersheds were

excluded as potential source populations as 99 and 98% of GRW individuals were excluded from these populations ( $P < 0.05$ ), respectively (Fig. 5).

## Discussion

The absence of the greenside darter in the GRW prior to 1990, its first collection in the GRW in 1990, and its subsequent dramatic increase in both distribution and abundance is generally consistent with an introduction shortly prior to 1990. The alternative hypothesis that the species was historically present in the GRW, but was at undetectably low numbers, is highly improbable. If greenside darter was native to the GRW and it had persisted for many generations at such low levels, subpopulations should exhibit limited genetic diversity and severe bottleneck effects (Eckert et al. 1996; Nyström et al. 2006; Tsutsui et al. 2000). However, we found limited evidence for either reduced genetic diversity or a population bottleneck in the GRW populations. Indeed, other studies of microsatellite genetic diversity (allele number and heterozygosity) in *Etheostoma* spp. from populations located in central parts of their range report diversity values similar to that reported here for the GRW *E. blennioides* (Fluker et al. 2010; Gabel et al. 2008; Haponski et al. 2009; Switzer et al. 2008). If the greenside darter is native to the GRW, we would also expect elevated genetic differentiation among GRW subpopulations relative to other Canadian watersheds due to increased genetic drift associated with their very low population sizes, and that they follow an isolation-by-distance model of genetic divergence (Beneteau et al. 2009; Driscoll 1999). Neither elevated genetic differentiation, nor isolation-by-distance divergence was found for the GRW greenside darter. We thus conclude that the GRW greenside darter must have been introduced sometime prior to 1990.

The genetic signature of the GRW greenside darter is not consistent with that expected from a single introduction of a small number of fish (Yonekura et al. 2007). First, there is no evidence for founder effect loss of genetic diversity. However, a rapid population expansion following introduction will minimize founder effects on genetic diversity (Friar et al. 2000; Nei et al. 1975; Roman and Darling 2007; Zenger et al. 2003). Founder effects can be further reduced, or eliminated, through multiple introductions. Multiple introductions buffer founder effects in two ways: (1) they increase the number of introduced organisms, increasing effective population size and overriding the effects of drift; and (2) introductions from genetically diverse sources, followed by hybridization, can lead to

elevated genetic diversity relative to the source populations (Kolbe et al. 2004; Roman and Darling 2007). However, multiple introductions generate populations that have distinctive genetic signatures (Brown and Stepien 2009; Darling et al. 2008; Fonseca et al. 2006; Kreiser et al. 2000).

The high genetic diversity and the unusual genetic structure of the GRW greenside darter are consistent with three introduction events into different parts of the watershed. Interestingly, the three genetic groups roughly correspond to the areas of first discovery in the GRW between 1990 and 1995. The similarities in spatial and genetic patterns suggest that the three introduction events were from genetically distinct sources. This may have been the result of accidental stocking with game fishes, or of releases of unused baitfishes by anglers. Although the release of baitfishes is a common practice, darters are not popular bait (Litvak and Mandrak 1993), making it unlikely that baitfish release was the sole source of the GRW greenside darter.

Our genotype assignment analysis identified the Thames River watershed as the most likely source of the GRW greenside darter, although the Sydenham River watershed cannot be excluded. On the other hand, the Ohio and the Ausable River watershed populations can be unambiguously excluded as possible sources for the GRW greenside darter introduction. Interestingly, the GRW greenside darter exhibit lower allelic diversity than the Thames, perhaps indicating some loss of diversity during the introduction process. The relatively low proportion of GRW fish that were assigned with high confidence to any source site in the Thames is unexpected. However, Beneteau et al. (2009) demonstrated substantial genetic divergence among greenside darter sampling sites within Canadian watersheds. It is thus possible that the GRW greenside darter may have been introduced from the Thames River watershed, but from sites that were not sampled for this study. Extensive sampling of potential source populations is important for accurate identification of the source of an introduced population, but if source population divergence is correlated to geographic distance, as is the case in greenside darter (Beneteau et al. 2009), then regional assignment is possible (Colautti et al. 2005; Kelly et al. 2006; Muirhead et al. 2007). An alternative possibility is that the GRW greenside darter may have experienced introgression among fish introduced from multiple



sites—such hybrid fish would be expected to harbour novel genotypes that would not strongly assign to any of the native populations. Our genotype assignment analysis assumes that alleles common to multiple watersheds are identical by descent and, although this may not be true for all, it is likely the case for the majority of the GRW greenside darter alleles, since the introduction of this species is recent.

Determining the invasion status of populations thought to be introduced can be difficult, even when historical data are available (Beebee et al. 2005). For example, although historical sampling records and preserved specimens have been used successfully to determine range expansion and dispersal in greenside darter of the Atlantic Slope, there are other populations in that same area that are of unknown invasion status due to a paucity of suitable data (Jenkins and Burkhead 1994; Neely and George 2006; Starnes 2002). Furthermore, the characterization of introductions far from the native range (e.g. between continents) can be relatively straightforward using historic records; however, introductions near the native range are more problematic and may contribute to “cryptic” invasion. Differentiating between native and introduced populations of species within, or near, the species’ native range is an emerging management need, as cryptic introductions are likely to become increasingly more common in aquatic systems as the result of baitfish transfers (Litvak and Mandrak 1993), illegal stocking of game fishes (Jackson and Mandrak 2002), inter-basin transfers of water (Mills et al. 1993), and range expansion due to climate warming (Mandrak 1989).

Regardless of the source of greenside darter in the GRW, its dramatic increase in population abundance and range throughout the watershed in less than 10 generations is truly remarkable. The greenside darter in the GRW is now a significant part of the aquatic community, represents a substantial northeastern range extension in the species and, despite its non-native origin, represents an important contribution to the genetic biodiversity of this species in Canada. Nevertheless, future ecological studies should focus on the effects of this species on other fishes utilizing similar habitat in the GRW.

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