Fisheries and Oceans Canada | Pêches et Océans Canada
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Canadian Science Advisory Secretariat (CSAS)

Research Document 2020/051

Central and Arctic Region

River Darter (*Percina shumardi*) mitochondrial DNA haplotype diversity across COSEWIC National Freshwater Biogeographic Zones

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September 2020

Canada

Foreword

This series documents the scientific basis for the evaluation of aquatic resources and ecosystems in Canada. As such, it addresses the issues of the day in the time frames required and the documents it contains are not intended as definitive statements on the subjects addressed but rather as progress reports on ongoing investigations.

Published by:

Fisheries and Oceans Canada
Canadian Science Advisory Secretariat
200 Kent Street
Ottawa ON K1A 0E6

http://www.dfo-mpo.gc.ca/csas-sccs/
csas-sccs@dfo-mpo.gc.ca

ISSN 1919-5044

Correct citation for this publication:

Reid, S. M., Haxton, T., and McCarthy, A. 2020. River Darter (*Percina shumardi*) mitochondrial DNA haplotype diversity across COSEWIC National Freshwater Biogeographic Zones. DFO Can. Sci. Advis. Sec. Res. Doc. 2020/051. v + 15 p.

Aussi disponible en français :

*Reid, S. M., Haxton, T., et McCarthy, A. 2020. Diversité des haplotypes d'ADN mitochondrial du dard de rivière (*Percina shumardi*) dans les zones biogéographiques nationales d'eau douce du COSEPAC. Secr. can. de consult. sci. du MPO. Doc. de rech. 2020/051. v + 15 p.*

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ABSTRACT

River Darter *(Percina shumardi*) is a small, benthic fish that is a member of the perch family (Percidae). In Canada, the species has a continuous distribution through most of Manitoba into northwestern Ontario in the Saskatchewan-Nelson drainage as well as the Hudson Bay drainage west of James Bay. River Darter is also found in Lake St. Clair and its tributaries in southwestern Ontario. Based on evidence of discreteness and evolutionary significance, groups of populations can be assessed by COSEWIC as separate Designatable Units (DUs). For freshwater fishes, the delineation of DUs has been informed using COSEWIC National Freshwater Biogeographic Zones (NFBZ) and population genetic structure. Based on a three DU structure, COSEWIC assessed the status of the River Darter as Not at Risk in the Saskatchewan–Nelson River (DU1) and Southern Hudson Bay–James Bay (DU2) zones and Endangered in the Great Lakes – Upper St. Lawrence (DU3) zone. In this study, haplotype data from two mitochondrial DNA genes (cytochrome-b [cyt-b] and cytochrome oxidase subunit 1 [CO1]) were used to assess whether River Darter population genetic structure corresponds with the three NFBZ. The assessment was based on: (i) the distribution of private and shared haplotypes; (ii) phylogenetic relationships among haplotypes; and, (iii) distance- and ordination-based tests of haplotype structure. One hundred-forty-nine sequences from both mitochondrial DNA genes were used in the analysis, representing River Darter from 14 waterbodies. Overall, 29 cyt-b haplotypes and eleven CO1 haplotypes were identified. Based on private haplotypes, the cyt-b minimum spanning network, and Principal Coordinate Analysis (PCoA) of cyt-b and CO1 haplotype data, differentiation among River Darter populations was greatest between DU3 and the two western DUs. Private haplotype data and PCoA (cyt-b only) provide some evidence of differentiation between DU1 and DU2. These interpretations are largely influenced by samples from two waterbodies: Lake Badesdawa (DU2) and the Thames River (DU3). Samples from additional populations within DU2 are required for more robust support of the existing DU structure.

INTRODUCTION

River Darter (*Percina shumardi*) is a small, elongate, benthic fish that is a member of the perch family (Percidae). In Canada, the species has a continuous distribution through most of Manitoba into northwestern Ontario in the Saskatchewan-Nelson drainage as well as the Hudson Bay drainage west of James Bay. River Darter is also found in Lake St. Clair and its tributaries in southwestern Ontario (Scott and Crossman 1973, Stewart and Watkinson 2004, Holm et al. 2009). Collections of the species have mainly been from medium to large rivers that typically have moderate currents and deeper water, or from along the shorelines of larger lakes. The most current information on the diet, life history characteristics, habitat, distribution and relative abundance of Canadian populations is provided by COSEWIC (2016) and Pratt et al. (2016).

In 2016, the status of Canadian River Darter populations was assessed by COSEWIC. Based on evidence of discreteness and evolutionary significance, groups of populations can be assessed as separate Designatable Units (DUs) (COSEWIC 2015). For freshwater fishes, the delineation of DUs has been informed using COSEWIC National Freshwater Biogeographic Zones and population genetic structure (e.g. Lake Sturgeon - COSEWIC 2017). The status of River Darter was assessed using three separate DUs, aligning with National Freshwater Biogeographic Zones (NFBZ). NFBZ across Canada were identified based on similarities of fish assemblages across watershed boundaries (COSEWIC 2015). Each NFBZ represents a distinct biogeographic history associated with biogeographic provinces of differing ecological properties. Movement of River Darter between the three NFBZ is restricted except for the Albany River in the Southern Hudson Bay - James Bay NFBZ, where a portion of the outflow of Lake St. Joseph has been diverted since 1957 into the Winnipeg River drainage via Lac Seul (COSEWIC 2016). Based on a three DU structure, the status of the River Darter was assessed as Not at Risk in the Saskatchewan – Nelson River (DU1) and Southern Hudson Bay – James Bay (DU2) zones, and Endangered in the Great Lakes – Upper St. Lawrence (DU3) zone.

Genetic support for the River Darter DU structure was based on a preliminary examination of mitochondrial DNA (mtDNA) haplotypes from 16 populations distributed across the three NFBZ (DFO 2015, Figure 1). In contrast to microsatellite DNA markers, among-group population structure identified with slower-evolving mtDNA markers are thought to reflect relatively deep intraspecific phylogenetic divergence (COSEWIC 2015). Haplotype data were generated by sequencing two mtDNA regions: the barcoding region of cytochrome oxidase subunit 1 (CO1), and cytochrome-b (cyt-b). Based on the distribution of shared and unique haplotypes, there was evidence of partitioning of haplotype diversity across at least two of the biogeographic zones (Saskatchewan – Nelson River and Great Lakes – Upper St. Lawrence). However, no statistical tests were done to determine if mtDNA haplotype variation among Canadian populations was congruent with DU structure.

Prior to the 2016 COSEWIC status assessment, quantitative analysis of the genetic structure of River Darter populations was deferred until more samples could be obtained. Sequencing of more individuals and populations from DU2 and DU3 was recommended during the 2014 pre-COSEWIC assessment meeting of the species (DFO 2015). The objectives for additional sequencing were to: (i) provide a more robust assessment of the proposed DU structure, and (ii) better understand whether the Lake St. Joseph water diversion (into the Winnipeg River drainage) has resulted in genetic exchange between DU1 and DU2 populations.

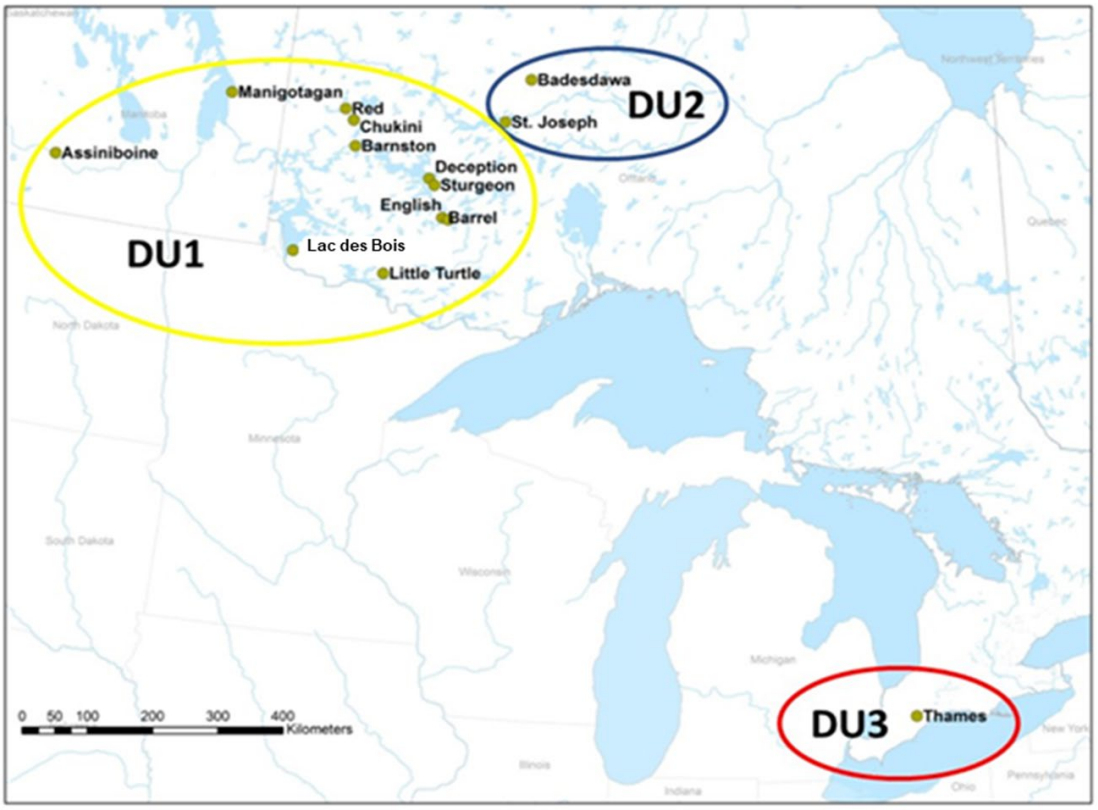

Figure 1. Distribution of waterbodies used in River Darter mtDNA analysis. The general locations of each DU are identified on the map.

For this study, the objectives were to:

1. update the mtDNA haplotype dataset with additional 2015 and 2016 Sydenham and Thames rivers (DU3) samples;
2. characterize mtDNA haplotype diversity within populations and DUs; and,
3. test whether the mtDNA-based genetic structure of River Darter populations corresponds with the three NFBZ used in the COSEWIC status assessment.

Results provide a more robust support for the DU structure used by COSEWIC and will inform evaluations of the feasibility of River Darter population enhancement and reintroductions as recovery actions (Mandrak 2018).

METHODS

SAMPLE COLLECTION

Three collections provided samples for genetic analysis:

1. 2012 to 2014 Fisheries and Oceans Canada targeted surveys for River Darter in Manitoba and Ontario (Pratt et al. 2016);

2. 2015 and 2016 Fisheries and Oceans Canada fishes-at-risk surveys along the Sydenham and Thames rivers; and,
3. archived Sydenham and Thames river samples from the Royal Ontario Museum (Accession numbers: 5824, 6520 and 7499).

River Darter were collected using a variety of sampling gears: bag seine, boat electrofisher, and fine-mesh bottom trawls. In 2015 and 2016, targeted sampling along the Sydenham and Thames rivers using a fine-mesh bottom trawl was undertaken by DFO, and along the North Sydenham River using a bag seine by the Ministry of Natural Resources (MNRF). MNRF crews sampling the fish community along the Attawapiskat River (DU2) were also instructed to keep tissue samples from any River Darter collected. In total, samples were collected from 15 waterbodies (Table 1). Samples from populations outside of Canada were not included in the analysis.

Lab Analysis

Between 1 and 25 individuals from each population (Table 1) were sequenced for the mtDNA regions CO1 (524 base pairs) and cyt-b (926 base pairs) (Song et al. 1998, Ward et al. 2005). The two mtDNA regions have been widely applied to develop fish species identification markers, and to investigate the evolutionary lineages of freshwater fishes. The mean number of individuals sequenced was 9.9 per population (Tables 1 and 2).

MtDNA was extracted using a simple isopropanol precipitation (Kyle and Wilson 2007, Wozney et al. 2011). DNA quantity and quality were assessed by horizontal electrophoresis on 1.5% agarose gels alongside molecular mass ladders. Working solutions of DNA template were made using a 1:30 dilution of stock DNA with ddH_2O.

Polymerase chain reactions (PCR) were performed in 10 µL reactions containing 2 µL of DNA template (6-12 ng). The cyt-b PCR cocktail also included 2 µL 5x PCR buffer, 1 µL BSA (0.2 µg/mL), 0.2 µL $MgCl_2$ (25 mM), 0.2 µL dNTPs (10 mM), 0.2 mM of each primer (10 µM), and 0.05 µL Taq polymerase (0.25 U). The CO1 cocktail also included 2 µL 5x PCR buffer, 0.2 µL $MgCl_2$ (25 mM), 0.2 µL dNTPs (10 mM), 0.2 mM of each primer (10 µM), and 0.05 µL Taq polymerase (0.25 U). The amplification reaction used an initial denaturation of 94°C (10 min), followed by 35 cycles of 94°C (45 sec), 50°C (cyt-b) / 54°C (CO1) (60 sec), 72°C (60 sec), with a final extension of 72°C (20 min). Amplified DNA quantity and quality was assessed by horizontal electrophoresis on 1.5% agarose gels alongside molecular mass ladders. ExoSAP cleanup (New England Biolabs, Pickering, Ontario) was completed on all amplified products to remove excess primers: 0.9µL Antarctic Phosphatase Buffer, 0.1 µL Antarctic Phosphatase and 0.03 µL Exonuclease I was added to each 8 µL of amplified product, and incubated at 37°C for 15 minutes, 80°C for 15 minutes and then cooled to 10°C.

Clean amplified products were sequenced in both directions following the ABI BigDye v3.1 terminator cycle sequencing kit (Applied Biosystems, Foster City, California). Sequenced product was cleaned by ethanol (EtOH) precipitation. For each 12 µL of sequenced product, 1.2 µL 5M Sodium Acetate and 37 µL of 95% EtOH was added, mixed gently, and spun for 45 min at 6200 rpm. Supernatant was poured off and 150 µL of 70% EtOH was added and the product was spun for a further 45 min at 6200 rpm. Supernatant was removed and residual EtOH allowed to evaporate. Clean and sequenced product was eluted in 10µL HiDi formamide (Applied Biosystems, Foster City, California). Amplified product was visualized on an AB 3730 sequencer. Sequences were analyzed using Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, Michigan) and confirmed by manual proofreading.

Data Analysis

Genetic support for the COSEWIC River Darter DU structure was evaluated by:

1. examining the frequency and distribution of haplotypes unique to each DU (private haplotypes), and haplotypes found among multiple DUs (shared haplotypes);
2. constructing minimum spanning networks to examine phylogenetic relationships among haplotypes; and,
3. using distance- and ordination-based tests of haplotype structure.

All analyses were conducted separately for CO1 and cyt-b sequences.

Haplotype Analysis v10.5 (Eliades and Eliades 2009) was used to summarize haplotype data including effective number of haplotypes (Ne) and genetic diversity (He). Other genetic analyses were conducted in R (R Core Team 2014) using the package '*haplotypes*' (last accessed November 6, 2018). Haplotypes were identified from DNA sequences using the function '*haplotype*'. Putative DUs and waterbodies were assigned to each sample. Summaries of haplotypes were produced by waterbody and by putative DU. Genetic diversity was calculated for populations with at least five sequenced individuals. Haplotype richness (H_R) was defined as the number of haplotypes sequenced from a population. Richness estimates using rarefraction-based methods were not successful.

Networks representing phylogenetic relationships among haplotypes were estimated from sequence data using statistical parsimony with the function '*parsimnet*', indel coding method equal 'sic', and a probability estimate of 0.95. Using haplotype data, River Darter population structure was characterized using distance and ordination-based methods. Nei's distance, which provides the mean number of pairwise Nei's (D) differences between populations, was calculated using the function '*pairnei*'. Finally, principal coordinate analysis (PCoA) was conducted based on DU and then waterbody using the function '*pcoa*' in the R package '*ape*' (Paradis and Schliep 2019).

RESULTS

The total number of sequences obtained for both cyt-b and CO1 was 149 (Tables 1 and 2). River Darter cyt-b haplotype diversity was greater than CO1 haplotype diversity. Overall, 29 cyt-b haplotypes were identified and grouped by DU (Table 3), and waterbody (Table 4). Eleven CO1 haplotypes were identified and grouped by DU (Table 5), and waterbody (Table 6). Mean number of haplotypes per population was 3.4 for cyt-b and 1.9 for CO1; mean number of effective haplotypes was 2.1 for Cyt-b and 1.2 for CO1; and mean genetic diversity was 0.6 for cyt-b and 0.2 for CO1 (Tables 1 and 2). River Darter haplotype richness differed among DUs, with the highest number of cyt-b and CO1 haplotypes obtained from DU1 samples. With the lowest number of individuals sequenced, DU3 samples produced the fewest haplotypes (Tables 3 and 5). Sydenham River samples were not successfully sequenced and were therefore not included in the analysis.

Table 1. Number of samples (N), haplotypes detected (H_R), private haplotypes (H_P), effective number of haplotypes (Ne), and genetic diversity (He) of Cyt-b genes for each population.

DU	Population	N	H_R	H_P	N_e	He
1	Assiniboine River	16	4	1	2.37	0.62
1	Barnston Lake	1	1	0	1.00	-
1	Barrel Lake	1	1	0	1.00	-
1	Chukini Lake	5	2	0	1.47	0.40
1	Deception Bay	1	1	0	1.00	-
1	English River	14	7	5	3.38	0.76
1	Lake of the Woods	10	6	2	4.17	0.84
1	Little Turtle Lake	1	1	1	1.00	-
1	Manigotagan River	25	7	2	4.31	0.80
1	Red Lake	1	1	1	1.00	-
1	Sturgeon River	17	3	1	1.44	0.32
2	Badesdawa Lake	24	6	4	2.44	0.62
2	Lake St. Joseph	23	3	1	1.19	0.17
3	Thames River	9	5	4	3.86	0.83

Table 2. Number of samples (N), haplotypes detected (H_R), private haplotypes (H_P), effective number of haplotypes (Ne), and genetic diversity (He) of CO1 genes for each population.

DU	Population	N	H_R	H_P	N_e	He
1	Assiniboine River	16	2	1	1.13	0.13
1	Barnston Lake	1	1	0	1	-
1	Barrel Lake	1	1	0	1	-
1	Chukini Lake	5	1	0	1	0
1	Deception Bay	1	1	0	1	-
1	English River	14	1	0	1	0
1	Lake of the Woods	10	2	0	1.22	0.20
1	Little Turtle Lake	1	1	0	1	-
1	Manigotagan River	25	6	4	1.86	0.48
1	Red Lake	1	1	0	1	-
1	Sturgeon River	18	1	0	1	0
2	Badesdawa Lake	24	1	0	1	0
2	Lake St. Joseph	23	4	3	1.31	0.25
3	Thames River	9	3	1	2.79	0.72

For each River Darter DU, 4 to 17 private cyt-b haplotypes and 1 to 4 private CO1 haplotypes were identified (Tables 3 and 5). Private cyt-b and CO1 haplotypes were associated with 20% of individuals sequenced, and 5% of individuals sequenced, respectively. Multiple private cyt-b haplotypes were identified within five populations: Badesdawa Lake (DU2), English River (DU1), Lake of the Woods (DU1), Manigotagan River, MB (DU1) and Thames River (DU3). Multiple private CO1 haplotypes were identified within two populations: Manigotagan River (DU1) and Lake St. Joseph, ON (DU2) (Tables 4 and 6). Four cyt-b haplotypes and two CO1 haplotypes were shared across all three River Darter DUs (Tables 3 and 5). Three cyt-b haplotypes were present in both DU1 and DU2 samples.

Table 3. Distribution of Cyt-b haplotypes across the three River Darter designatable units (DU). Numbers of individuals with each haplotype are presented.

Haplotype	DU		
	1	2	3
1	42	21	3
2	20	0	0
3	5	0	0
4	1	0	0
5	4	1	0
6	1	0	0
7	4	1	0
8	1	0	0
9	0	6	0
10	0	1	0
11	1	14	0
12	0	1	0
13	0	1	0
14	1	0	0
15	1	0	0
16	1	0	0
17	1	0	0
18	1	0	0
19	1	0	0
20	2	0	0
21	1	0	0
22	0	1	0
23	1	0	0
24	1	0	0
25	2	0	0
26	0	0	3
27	0	0	1
28	0	0	1
29	0	0	1

Table 4. Distribution of Cyt-b haplotypes across the 14 waterbodies with River Darter. Numbers of individuals with each haplotype are presented.

Haplotype	DU1											DU2		DU3
	Assiniboine	Barnston	Barrel	Chukini	Deception	English	Lake of the Woods	Little Turtle	Manigotagan	Red Lake	Sturgeon	Badesdawa	St Joseph	Thames River
1	5	0	0	4	1	7	2	0	9	0	14	0	21	3
2	9	1	0	0	0	0	4	0	6	0	0	0	0	0
3	1	0	0	0	0	0	0	0	4	0	0	0	0	0
4	1	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	1	0	3	0	0	0	1	0
6	0	0	0	0	0	0	0	0	1	0	0	0	0	0
7	0	0	1	0	0	2	0	0	1	0	0	1	0	0
8	0	0	0	0	0	0	0	0	1	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	6	0	0
10	0	0	0	0	0	0	0	0	0	0	0	1	0	0
11	0	0	0	1	0	0	0	0	0	0	0	14	0	0
12	0	0	0	0	0	0	0	0	0	0	0	1	0	0
13	0	0	0	0	0	0	0	0	0	0	0	1	0	0
14	0	0	0	0	0	1	0	0	0	0	0	0	0	0
15	0	0	0	0	0	1	0	0	0	0	0	0	0	0
16	0	0	0	0	0	1	0	0	0	0	0	0	0	0
17	0	0	0	0	0	1	0	0	0	0	0	0	0	0
18	0	0	0	0	0	1	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	1	0	0	0	0	0	0	0
20	0	0	0	0	0	0	1	0	0	0	1	0	0	0
21	0	0	0	0	0	0	1	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0	0	1	0
23	0	0	0	0	0	0	0	1	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	1	0	0	0	0
25	0	0	0	0	0	0	0	0	0	0	2	0	0	0
26	0	0	0	0	0	0	0	0	0	0	0	0	0	3
27	0	0	0	0	0	0	0	0	0	0	0	0	0	1
28	0	0	0	0	0	0	0	0	0	0	0	0	0	1
29	0	0	0	0	0	0	0	0	0	0	0	0	0	1

Table 5. Distribution of CO1 haplotypes across the three River Darter Designatable Units (DU). Numbers of individuals with each haplotype are presented.

Haplotype	DU		
	1	2	3
1	83	44	4
2	1	0	0
3	4	0	3
4	1	0	0
5	1	0	0
6	1	0	0
7	1	0	0
8	0	1	0
9	0	1	0
10	0	1	0
11	0	0	2

Table 6. Distribution of CO1 haplotypes across the 14 waterbodies with River Darter. Numbers of individuals with each haplotype are presented.

Haplotype	DU1											DU2		DU3
	Assiniboine	Barnston	Barrel	Chukini	Deception	English	Lake of the Woods	Little Turtle	Manigotagan	Red Lake	Sturgeon	Badesdawa	St. Joseph	Thames River
1	15	1	1	5	1	14	9	1	18	1	17	24	20	4
2	1	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	1	0	3	0	0	0	0	3
4	0	0	0	0	0	0	0	0	1	0	0	0	0	0
5	0	0	0	0	0	0	0	0	1	0	0	0	0	0
6	0	0	0	0	0	0	0	0	1	0	0	0	0	0
7	0	0	0	0	0	0	0	0	1	0	0	0	0	0
8	0	0	0	0	0	0	1	0	0	0	0	0	1	0
9	0	0	0	0	0	0	0	0	0	0	0	0	1	0
10	0	0	0	0	0	0	0	0	0	0	0	0	1	0
11	0	0	0	0	0	0	0	0	0	0	0	0	0	2

Table 7. Pairwise Nei's distance measures for River Darter DUs, based on cyt-b (lower diagonal) and CO1 (upper diagonal) haplotypes.

	DU1	DU2	DU3
DU1	-	0.24	0.97
DU2	1.92	-	0.97
DU3	3.27	3.83	-

Haplotype networks were constructed for each mtDNA region (Figures 2 and 3). The cyt-b network was more complex than the CO1 network, with a higher maximum number of connection steps (mutations or DNA substitutions) separating individual haplotypes (cyt-b = 47; CO1 = 12). Some of the more developed nodes in the cyt-b network reflect the geographic location of individual River Darter haplotypes. Haplotypes 6 and 8 were only sequenced from Manigotagan River samples (DU1 waterbody in Manitoba). Haplotypes 10, 11, 12, and 13 were almost entirely sequenced from Badesdawa Lake samples (DU2 waterbody in northwestern Ontario). Lastly, haplotypes 26, 27, 28, and 29 were only sequenced from southwestern Ontario samples (DU3). No pattern related to waterbodies and haplotypes was evident from the CO1 network.

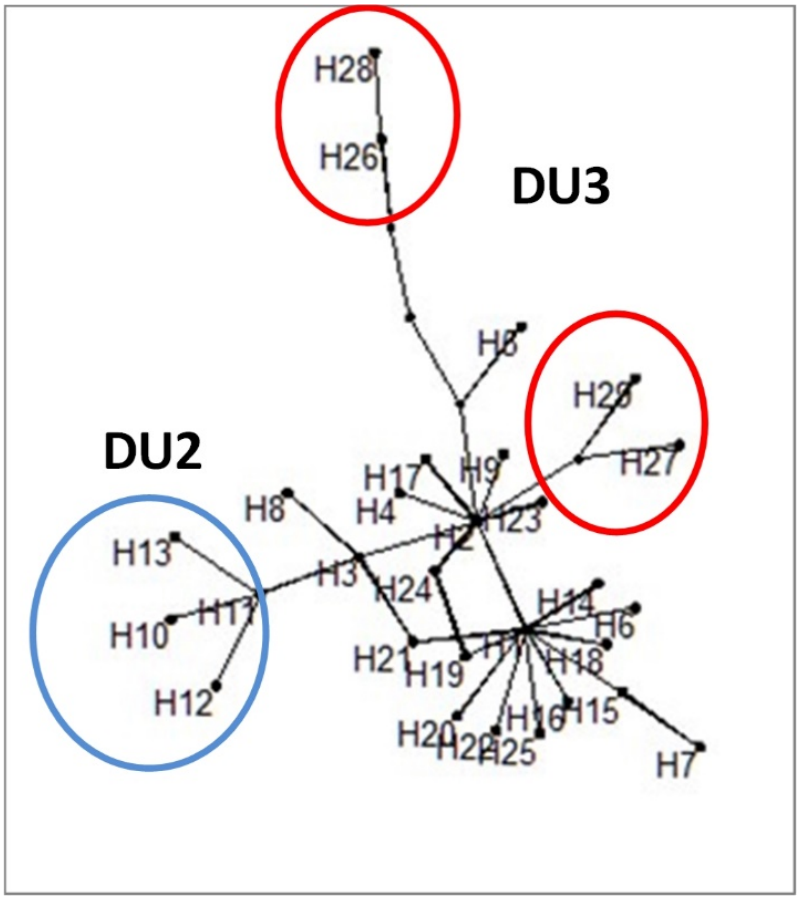

Figure 2. Minimum spanning network for cytochrome b sequences identifying each haplotype. Haplotype codes correspond with results provided in Tables 3 and 5.

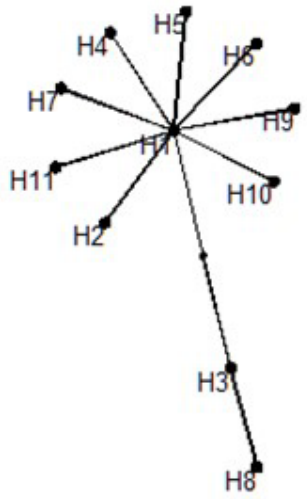

Figure 3. Minimum-spanning network for CO1 sequences identifying each haplotype. Haplotype codes correspond with results provided in Tables 4 and 6.

DU3 was the most dissimilar River Darter DU based on Nei's distance estimates for both cyt-b and CO1 regions (Table 7). When samples were grouped by DU, PCoA bi-plots indicate that DUs are distinct (Figures 4 and 5); with the separation of DU3 from the other two DUs evident along the first axis, and the separation of DU1 and DU2 evident along the second axis. When samples were grouped by waterbody, Thames River (DU3) samples were different from other waterbodies. However, separation between groups of DU1 and DU2 waterbodies was not apparent (Figures 6 and 7). Within DU1, separation from other waterbodies was limited to Barrel Lake (along the Axis 1 for cyt-b region) and for Manigotagan River (along Axis 2 for CO1 region). Based on cyt-b haplotypes, Badesdawa Lake was differentiated from other waterbodies along the second axis (Figure 6).

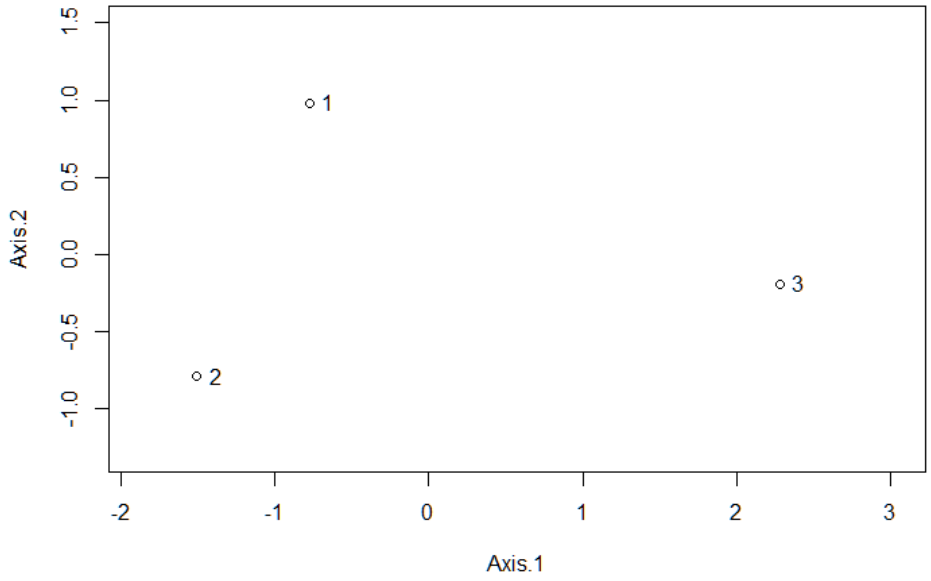

Figure 4. Bi-plot of Principal Coordinate Analysis (PCoA) scores calculated for each River Darter DU using cyt-b haplotypes and Nei's distance measures. Axis 1 represents 85% of the variation among DUs, and Axis 2 represents 15% of the variation.

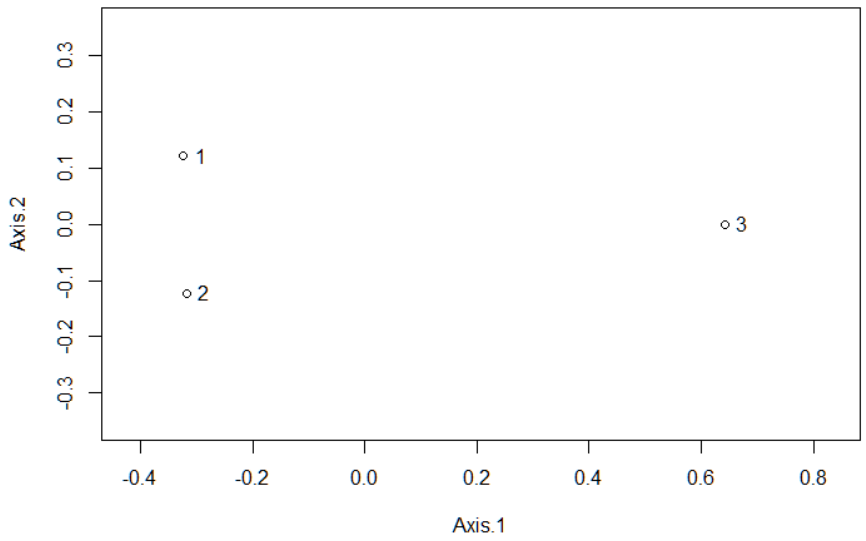

Figure 5. Bi-plot of Principal Coordinate Analysis (PCoA) scores calculated for each River Darter DU using CO1 haplotypes and Nei's distance measures. Axis 1 represents 93% of the variation among DUs, and Axis 2 represents 6% of the variation.

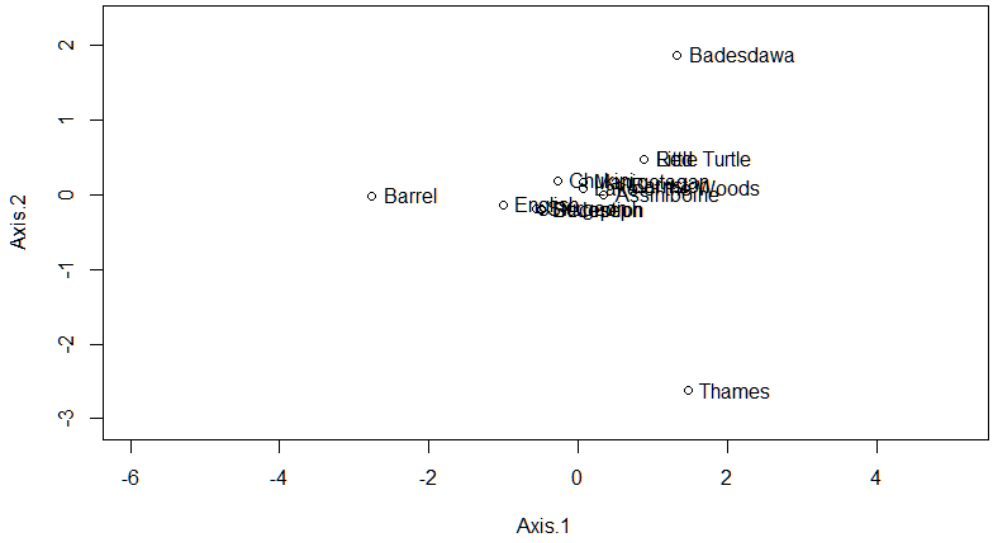

Figure 6. Bi-plot of Principal Coordinate Analysis (PCoA) scores calculated for each River Darter waterbody using cyt-b haplotypes and Nei's distance measures. Axis 1 represents 45% of the variation among DUs, and Axis 2 represents 32% of the variation.

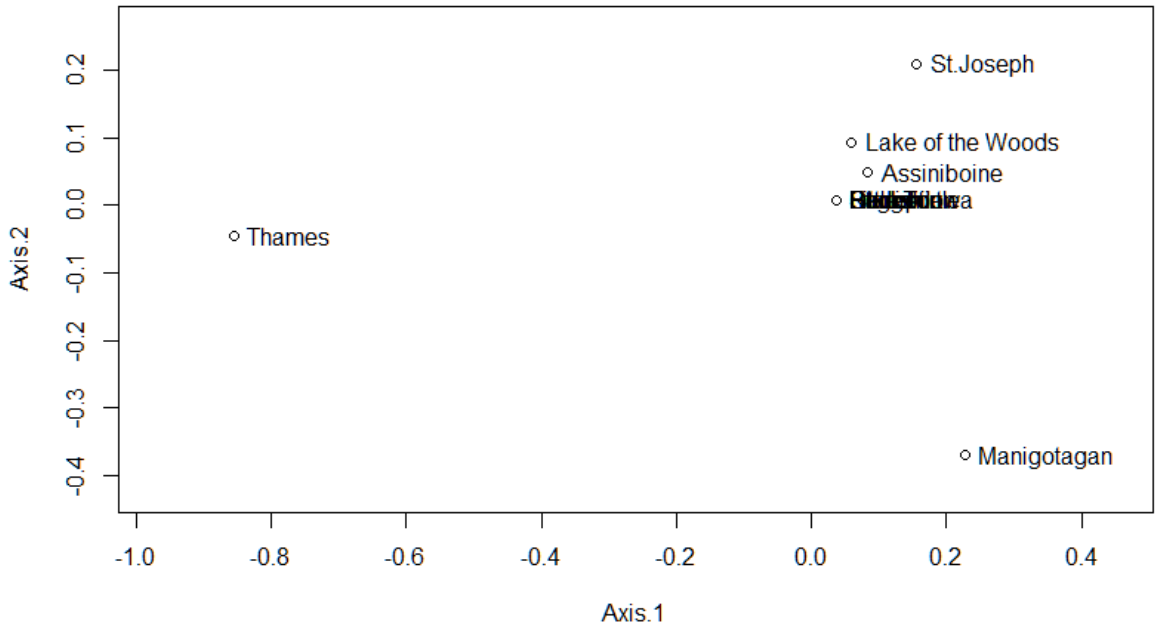

Figure 7. Bi-plot of Principal Coordinate Analysis (PCoA) scores calculated for each River Darter waterbody using CO1 haplotypes and Nei's distance measures. Axis 1 represents 79% of the variation among DUs, and Axis 2 represents 18% of the variation.

DISCUSSION

The primary objective of our study was to characterize River Darter mtDNA haplotype diversity across its Canadian distribution. Across North America, the genetic structure of darter (Percidae) populations has been found to vary over both local and regional spatial scales. Landscape features such as barriers (dams and waterfalls), drainage patterns (different catchments), and glacial refugia and post-glacial colonization routes influence genetic variation among populations (Piller et al. 2008, Beneteau et al. 2009, Haponski et al. 2009, Ginson et al. 2015, Argentina et al. 2018, Euclide and Marsden 2018). Such variation can be evidence of discreteness and evolutionary significance which is required when identifying multiple DUs (COSEWIC 2015). For two darter species (Channel Darter and Eastern Sand Darter), recent genetic-based research has provided support for a multiple-DU structure within the Great Lakes – Upper St. Lawrence FWBZ (Reid et al. 2013 unpublished report prepared for COSEWIC Freshwater Fishes Subcommittee, Ginson et al. 2015).

Our study provides evidence of partitioning of River Darter mtDNA haplotype diversity across Canada; with support for at least 2 DU. Differentiation among River Darter populations was greatest between DU3 (Great Lakes – Upper St. Lawrence FWBZ) and the two western DUs (found in the Saskatchewan – Nelson River and Southern Hudson Bay – James Bay FWBZ). This interpretation was informed by the distribution of private haplotypes among DUs, the cyt-b genealogy network, and PCoA ordinations of cyt-b and CO1 haplotype data. Private haplotype data and PCoA (cyt-b only) provide some evidence of differentiation between DU1 and DU2. Both interpretations are heavily influenced by samples from two waterbodies: Lake Badesdawa (DU2) and the Thames River (DU3).

Crossman and McAllister (1986) proposed a single Mississippian refugial origin for River Darter across the Hudson Bay drainage (DU1 and DU2); with the species' dispersal eastward via the Barlow-Ojibway connection to Hudson Bay and James Bay rivers in Ontario. Similarly, Mandrak

and Crossman (1992) proposed a single Mississippian refugial origin for Ontario populations of River Darter; with two dispersal routes into northwestern Ontario and one dispersal route into southwestern-Ontario. Lake Sturgeon (*Acipenser fulvescens*) is one of the few COSEWIC-assessed freshwater fish with a distribution encompassing the 3 FWBZ where River Darter are found. Based on microsatellite DNA data, the ancestry of Lake Sturgeon populations in Great Lakes – Upper St. Lawrence FWBZ was interpreted to be from a Mississippian refugial origin, and from a Missourian refugial origin for populations in the Saskatchewan–Nelson River FWBZ and Southern Hudson Bay – James Bay FWBZ (COSEWIC 2017). For another percid fish (Walleye, *Sander vitreus*), a Missourian ancestry for Ontario populations north and west of Lake Superior has been proposed based on genetic data (Stepien et al. 2009, Walter et al. 2012). However, given the absence of River Darter from western North America (unlike Lake Sturgeon or Walleye), a Missourian ancestry is unlikely. Instead, Canadian River Darter populations probably represent multiple lineages associated with several Mississippian refugia; as seen for other freshwater fishes with central North American distributions such as Muskellunge (Miller et al. 2017) and Redside Dace (Serrao et al. 2018).

An important objective of this study was to collect and sequence additional samples from River Darter populations in DU2 and DU3. However, supplemental sampling only provided 6 additional sequences from the Thames River (July 2016) and, therefore, only a minor improvement to the original dataset. No additional interpretation can be provided at this time regarding the discreteness of DU1 and DU2 populations, or the genetic structure of other DU2 populations. Very small sample sizes were associated with five waterbodies in DU1. Accordingly, results based on the distribution of private haplotype across DUs, and waterbody-based PCoA ordinations of haplotype data may be biased. Therefore, the targeted collection and sequencing of additional samples from all three DUs remains an important task.

ACKNOWLEDGEMENTS

We acknowledge Fisheries and Oceans Canada staff (Jason Barnucz, Bill Gardner, Robin Gespardy, Tom Pratt, and Doug Watkinson) and the Royal Ontario Museum (Erling Holm) for providing tissue samples. Caleigh Smith (MNRF) assisted with sequencing and data compilation. The report was improved by comments provided by Chris Wilson, Tom Pratt, and Doug Watkinson.

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