Temporal stability of Killifish hybrid clonal lineages

By

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ABSTRACT

The majority of vertebrates reproduce sexually, but a small percentage can reproduce clonally (asexual reproduction). The Common killifish (*Fundulus heteroclitus*) and Banded killifish (*Fundulus diaphanus*) are found along the Atlantic coast of Canada and sympatric populations are able to hybridize and produce female clones. These clonal lineages have independently arisen multiple times throughout the Maritimes, and Porter's Lake, Nova Scotia, is the home of the best-studied population. Ten years earlier, ten clonal lineages were found, and one major clone was most prevalent in Porter's Lake. As well, three out of 138 hybrids had genotypes consistent with sexual reproduction, and all clonal F1 hybrids had *F. diaphanus* mothers. The goals of my study were to: i) determine if the same major clone is still the most prevalent and if the same clonal lineages still persist at Porter's Lake ten years later, ii) search for further sexually-reproducing hybrids, and iii) test if any hybrids have *F. heteroclitus* mothers. To do so, we used a species-specific mitochondrial DNA restriction fragment length polymorphism assay to assess maternal lineages and four nuclear microsatellite loci that have species-specific alleles to assign clonal lineages and test for sexually reproducing hybrids. Five new clonal lineages were detected as well as a new major clone, indicating that the clonal lineages are not temporally stable. Additionally, six potentially all-female sexual hybrids were identified out of the 51 hybrids found in 2018 and I found one F1 hybrid with a *F. heteroclitus* mother.

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1. INTRODUCTION

1.1 The evolutionary costs and benefits of sexual and asexual reproduction

Animals can reproduce via two main methods: sexual and asexual reproduction. In sexual reproduction, both parents provide genetic material (egg and sperm) to produce genetically unique offspring via recombination during gamete formation and the merging of DNA from two different individuals. Asexual reproduction, on the other hand, involves only one parent and the offspring produced is genetically identical to the single parent because neither chromosomal recombination nor fertilization take place (reviewed by Otto, 2008). Current evolutionary theory suggests that sexual reproduction is a better long-term strategy to adopt than asexual reproduction (Sharp and Otto, 2016, Crouch, 2017). This is because the recombination of parental chromosomes that occurs during meiosis in sexual reproduction varies the combinations of alleles present in each parental gamete, and the genetic contribution from two individuals can also increase genetic variation among offspring, which may allow populations to persist in changing environments (Lehtonen et al., 2012, Sharp and Otto, 2016). Furthermore, asexual lineages tend to accumulate detrimental mutations because they cannot purge deleterious mutations via recombination, which breaks up genetic linkage. Thus, asexual lineages may be more prone to the accumulation of deleterious mutations and to parasitic infection due to the lower genetic diversity in clonal lineages, slowing the rate of host adaptation to parasitic infections (The Red Queen hypothesis, Van Valen, 1973, Lively et al., 1990). In the long run, these factors are predicted to make asexual lineages short-lived on an evolutionary timescale (Muller, 1964, Lynch et al., 1995).

However, in the 1970's, John Maynard Smith identified a cost of sexual reproduction which he termed the "twofold cost" of sex (Smith, 1971). Assuming that both asexual and sexual females are equally fecund, and the survival rate of the offspring is also equal, the number of offspring produced per individual in an asexual population would be twice that of

a sexual population (Gibson et al., 2017). Despite having numerous predicted benefits, sexually reproducing organisms face costs that asexual species tend to avoid (Lehtonen et al., 2012). Beyond the 'two fold cost of sex' (Smith, 1971, Lehtonen et al. 2012), to reproduce sexually, individuals need to find and attract potential mates, which requires time and energy that could instead be used to look for resources (Otto, 2009,Otto, 2008). Additionally, while mating, individuals take the risks of being exposed to predation, contracting sexually transmitted diseases and being harmed by their mate (Otto, 2009, Otto, 2008). Favorable allele combinations could also be broken during sexual reproduction (Otto, 2009, Otto, 2008).

Based upon the prevalence of sex in vertebrates, it seems that the overall benefits of sex outweigh the costs; however, the specific reasons why so many organisms reproduce sexually given these high costs is still a mystery because of the difficulties in experimentally testing these hypotheses (Crouch, 2017). Overall, the question of why sex has evolved in so many species and the debate about asexual reproduction being an "evolutionary dead-end" (White, 1978), compared to sexual reproduction, still divides in the evolutionary biology community (Lehtonen et al., 2012, Sharp and Otto, 2016, Crouch, 2017).

1.2 Asexual reproduction in animals

Sexual reproduction predominates in vertebrates, and asexual reproduction occurs most commonly in microbes, unicellular eukaryotes, plants and invertebrates (Avise, 2015, Warren et al. 2018). Asexual reproduction was long thought to be impossible for vertebrates; however, in the past few decades, numerous taxa of fish, amphibians and reptiles have been found to be able to reproduce asexually, producing all-female clonal lineages (Neaves and Baumann, 2011, Avise, 2015). The Amazon molly fish (*Poecilia formosa*), which arose from interspecific hybridization between the Atlantic molly (*Poecilia Mexicana*) and Sailfin molly (*Poecilia latipinna*), was the first unisexual vertebrate to be reported (Hubbs and Hubbs, 1932), followed by the discovery of 50 other species of fishes, amphibians and reptiles

(Avise, 2015). The discovery of these asexual lineages leads to the ultimate questions of why, and how, some organisms have evolved asexual reproduction.

Even though asexual reproduction has drawbacks (see section 1.1), there are numerous asexual lineages that have survived for many generations and seem to have adapted to their environments. For example, asexual lineages of mole salamanders from the genus *Ambystoma* are close to 5 million years old and are considered the oldest unisexual vertebrates in existence (Hedges et al., 1992, Spolsky et al., 1992). An analysis of the age estimates of several asexual lineages has also revealed that more than half of those assessed are at least 500,000 years old (Neiman et al., 2009). Moreover, researchers have estimated that the first hybridization event of the Amazon molly fish occurred approximately 280,000 years ago (Lampert and Scharlt, 2008). The Amazon Molly can produce three generations per year (Hubbs and Hubbs, 1932), so this asexual species has been able to prevail for 840,000 generations without sexual recombination (Stock et al., 2010). While some asexual lineages are able to survive for thousands of years and colonize numerous environments (Loewe and Lamastch, 2008, Stock et al., 2010, Tucker et al., 2013, Warren et al., 2018), others cannot. For example, whole genome analysis of asexual genotypes of waterflea indicated that they have high rates of gene conversion and gene deletions, which uncover deleterious recessive alleles due to loss of heterozygosity; this is assumed to be a more considerable cause of genetic disintegration in asexual lineages than the accumulation of new mutations (Tucker et al., 2013).

Approximately 100 species of vertebrates are known to produce all-female clonal lineages with daughters genetically identical to the mothers and to each other (Avise, 2015), hence the term unisexual “species”, or “biotype”. These unisexual biotypes are nearly all products of interspecific hybridization which makes the clonal lineages produced heterozygous to a certain extent (Vrijenhoek, 1989). Hence, these individuals could be as

genetically diverse as any sexual individual. The unisexual lineages reproduce via special types of asexual reproduction: parthenogenesis, gynogenesis or hybridogenesis (Avise, 2015). In parthenogenesis, also known as 'virgin birth', the female produces chromosomally unreduced eggs which divide by mitosis to form identical daughter cells (Avise, 2015). Parthenogenesis does not require sperm or paternal gametes to initiate clonal reproduction and species in the order Squamata (scaled reptiles) are known practitioners of this process (Avise, 2015). On the contrary, both gynogenesis and hybridogenesis require sperm to either stimulate mitosis in an unreduced egg (gynogenesis) or be expressed in a reduced egg (hybridogenesis) (Avise, 2015). However, any paternal chromosomes are later discarded to produce an offspring with chromosomes coming only from the mother (Avise, 2015).

Among asexual vertebrates, fish are more prone to hybridization, largely due to the fact that they use external fertilization for reproduction, increasing the chances of sperm and egg of different species coming into contact (Allendorf and Waples, 1996). The example of the Amazon Molly is just one of many asexual, clonal lineages created via hybridization. In Nova Scotia, Canada, hybrids between the Banded killifish (*Fundulus diaphanus)* and Common killifish (*Fundulus heteroclitus)* are found in several places (Fritz and Garside, 1974, Dawley, 1992). The Common killifish (*Fundulus heteroclitus*) is one of the best-studied species of fish as a result of its high stress tolerance, prevalence, and extensive genomic resources (Burnett et al. 2007, Reid et al. 2016), so this hybrid lineage has the potential to become an excellent model system for the study of clonal reproduction.

1.3 Killifish as a model organism for clonal reproduction

The Common killifish (*Fundulus heteroclitus*) is a model species for numerous biological disciplines including physiology, population biology, and toxicology as well as biochemistry (Powers et al., 1993, Wood and Marshal, 1994, Oleksiak et al., 2002, McMillan et al., 2006, Burnett et al. 2007). Over 35 species of *Fundulus* are found in North America

inhabiting both freshwater and marine environments (Wiley, 1986, Bernardi, 1997). The high tolerance to a number of environmental stressors, diversity among populations in physiology, and availability of this species has contributed to its widespread popularity and uses throughout the biological sciences (Burnett et al. 2007).

Two species, namely, the Banded killifish (*Fundulus diaphanus*) and Common killifish (*Fundulus heteroclitus*) are found along the Atlantic coast of Canada and the United States (Scott and Crossman, 1973, Hernandez-Chavez and Turgeon, 2007). While *Fundulus heteroclitus* has received great attention from researchers, *Fundulus diaphanus* has not been as extensively studied (April and Turgeon, 2006). Both species occupy a range of habitats; however, their differing salinity preferences (Fritz and Garside, 1974) leads to differences in their distribution. *Fundulus heteroclitus* is mainly found in the brackish water of estuaries and salt marshes while *Fundulus diaphanus* is inclined towards freshwater habitats and therefore is found in lakes and streams (Scott and Crossman, 1973). Different habitat preferences represent an ecological barrier and therefore it was assumed that *F. heteroclitus* and *F. diaphanus* were allopatric species. However, as a result of their broad osmoregulatory capabilities, they can both tolerate a wide range of salinities (Fritz and Garside, 1974, Griffin, 1974) and sympatric populations have been found at several locations in Nova Scotia, including Porter's Lake (Fritz and Garside, 1974), the Saint Mary's River (Dawley, 1992, Hernandez-Chavez and Turgeon, 2007) and Lawrencetown lake (LeBlanc, 1987).

Apart from differences in habitat preferences, these species also differ in their morphology. Lateral line scale count and the ratio of the distance between the dorsal fin to the caudal peduncle and peduncle height are two distinct traits that can differentiate between the two species since *F. diaphanus* have a greater scale count and a lower morphological ratio than *F. heteroclitus* (Scott and Crossman, 1973, Fritz and Garside, 1974, Hernandez-Chavez and Turgeon, 2007).

1.4 Hybridization of *F. diaphanus* and *F. heteroclitus*

Killifish species living in sympatry have undergone interspecific hybridization and hybrids have been reported at several locations in Canada and the United States (Fritz and Gardside, 1974, Hernandez-Chavez and Turgeon, 2007, Merette et al., 2009). A single female hybrid was reported in Lake of Shining Waters in Prince Edward Island (Hubs et al., 1943), as was one in New Haven, Connecticut (Chen and Ruddle, 1970). Fritz and Garside (1974) were the first to report a significant population of 170 hybrids in Porter's Lake, Nova Scotia. They indicated that the hybrids were all female and appeared to be an intermediate in morphology between *F. heteroclitus* and *F. diaphanus* (Fitz and Garside, 1974, Dawley, 1992).

Dawley (1992) first discovered that these hybrids were asexual clones. Following this discovery, Dawley et al., (1999, 2000) used allozyme variation and skin grafts to test for histocompatibility of immune responsive loci to determine that populations in Porter's Lake and the Saint Mary's River, Nova Scotia each had one major asexual clone along with some other minor clones (Dawley et al., 1999, 2000). Furthermore, with the use of nuclear DNA microsatellite markers, 11 different clonal genotypes were identified in Porter's Lake with one major clone comprising of approximately 90% of all the hybrids (Hernandez-Chavez and Turgeon, 2007). The population at Saint Mary's River was also found to consist of different clonal types, and a different major clone that was present in Porter's Lake (Hernandez-Chavez and Turgeon, 2007). These data suggest that, unlike the Amazon molly, which arose from a single hybridization event (Stock et al., 2010), *Fundulus* hybrids either emerged many times independently in Nova Scotia or that clonal lineages have an extremely high rate of mutation. These clonal lineages are also thought to be quite young, as Fritz and Garside (1974) suggest that *F. heteroclitus* and *F. diaphanus* only started hybridizing in Porter's Lake the 1950's.

Merette et al. (2009) resurveyed hybrids in Porter's Lake a couple of years after Hernandez-Chavez and Turgeon (2007) and found that the same major clone was still prevalent (Merette et al., 2009). These observations suggest that there may be stability in the asexual lineages of killifish hybrids over the past 70 years, despite the fact that asexual hybrids have arisen multiple times independently. However, these populations have not been surveyed over the past decade, so the current status of these clonal lineages is unknown.

The direction of hybridization among species is also biased. Hernandez-Chavez and Turgeon (2007) and Merette et al. (2009) used mitochondrial genetic markers to identify the maternal lineage of the hybrids and found that all hybrids had the mitochondrial DNA of *F. diaphanus*, suggesting that *F. heteroclitus* females did not successfully mate with *F. diaphanus* males to produce hybrids. However, reciprocal hybrid crosses produced in the lab using both *F. diaphanus* and *F. heteroclitus* mothers did produce viable offspring (Fritz and Garside 1974). At present, the potential pre- and post-zygotic factors leading to this bias in hybridization are not known. A further survey of clonal lineages would help determine how widespread this species-specific bias in female hybridization is by increasing population sampling. In addition, Hernandez- Chavez and Turgeon (2007) found evidence for a small number of sexually reproducing hybrids at a number of locations in the Maritimes. Further surveys of these hybrids are required to determine how prevalent sexual reproduction in hybrids may be.

1.5 Thesis Goals

The goals of this study were to: 1) test if the same clonal lineage still dominates in Porter's Lake in 2018 as in 2004-2007 and determine if the clonal lineages of asexual *F. diaphanus* x *F. heteroclitus* hybrids surveyed over a decade ago still persist in Porter's Lake; 2) check for the presence of sexually-reproducing hybrids; and 3) analyse hybrids to further test for the presence of *F. heteroclitus* mothers.

To accomplish these goals, I identified and analyzed the *Fundulus* hybrids using a combination of nuclear and mitochondrial markers. Three different nuclear microsatellite loci (FhCA-1, FhCA-21 and Fhe57) with species-specific alleles (Merette et al. 2009) were analyzed to first identify hybrids. Then, a fourth, highly variable, microsatellite (FhATG-B103) was studied to determine which clonal lineage hybrids belong to, following the methods of Merette et al. (2009). These microsatellite loci (FhCA-1, FhCA-21 and Fhe57) were specifically chosen because all hybrids were found to be heterozygous at these loci for species-specific alleles (except for locus FhATG-B103 which is variable among the hybrids), with one allele coming from *Fundulus heteroclitus* and the other one from *Fundulus diaphanus* (Hernandez-Chavez and Turgeon, 2007). To test for the presence of sexually reproducing hybrids, I compared the species-specific alleles at the four loci to look for evidence of backcrossing (goal #2).

A mitochondrial marker (restriction fragment length polymorphism of the D-loop) was used to identify which maternal species is contributing to hybrid formation (goal #3). The D-loop region of the mitochondrial DNA is genetically differentiated between the two species of killifish and digestion of an amplified region of the D-loop with a restriction enzyme (Hph1) allows us to differentiate the *F. diaphanus* from *F. heteroclitus* mitochondrial genomes using restriction fragment length polymorphism analysis (Hernandez-Chavez and Turgeon, 2007).

Finally, the combined nuclear and mitochondrial data were used to help determine whether the major clone in 2004-2009 (Clone A; Hernandez-Chavez and Turgeon, 2007, Merette et al., 2009), is still the most prevalent in 2017-2018 and test if the other clonal lineages still persist in Porter's lake (goal #1).

2. MATERIALS AND METHODS

2.1 Collection of killifish (*F. diaphanus, F. heteroclitus* and hybrids)

Fish were collected from Porter's Lake, Nova Scotia (44.7433°N, 63.2972°W, Figure 1, Figure 2) from May to October of 2018. The fish were caught using a variety of collection methods, including dip netting, seine netting and Gee minnow traps. Traps were left overnight, but for no longer than 12 hours. All collection methods followed those approved by the Saint Mary's University Animal Care Committee protocols (SMU ACC AUPF 17-18) and Department of Fisheries and Oceans permit (Licence #343930) held by Dr. Anne Dalziel. The fish were then brought back to Saint Mary's University Aquarium facilities where they were used to study salinity tolerance evolution as part of Lauren Jonah's M.Sc. thesis in Applied Science and were euthanized at the end of that experiment (SMU ACC AUPF 17-17). Morphological measures of all fish were taken to assign them to species following the methods of Merette (2009), which was found to have a 91.5% success rate. After euthanization, fin clips were collected and stored in 95% ethanol to be used for DNA extraction.

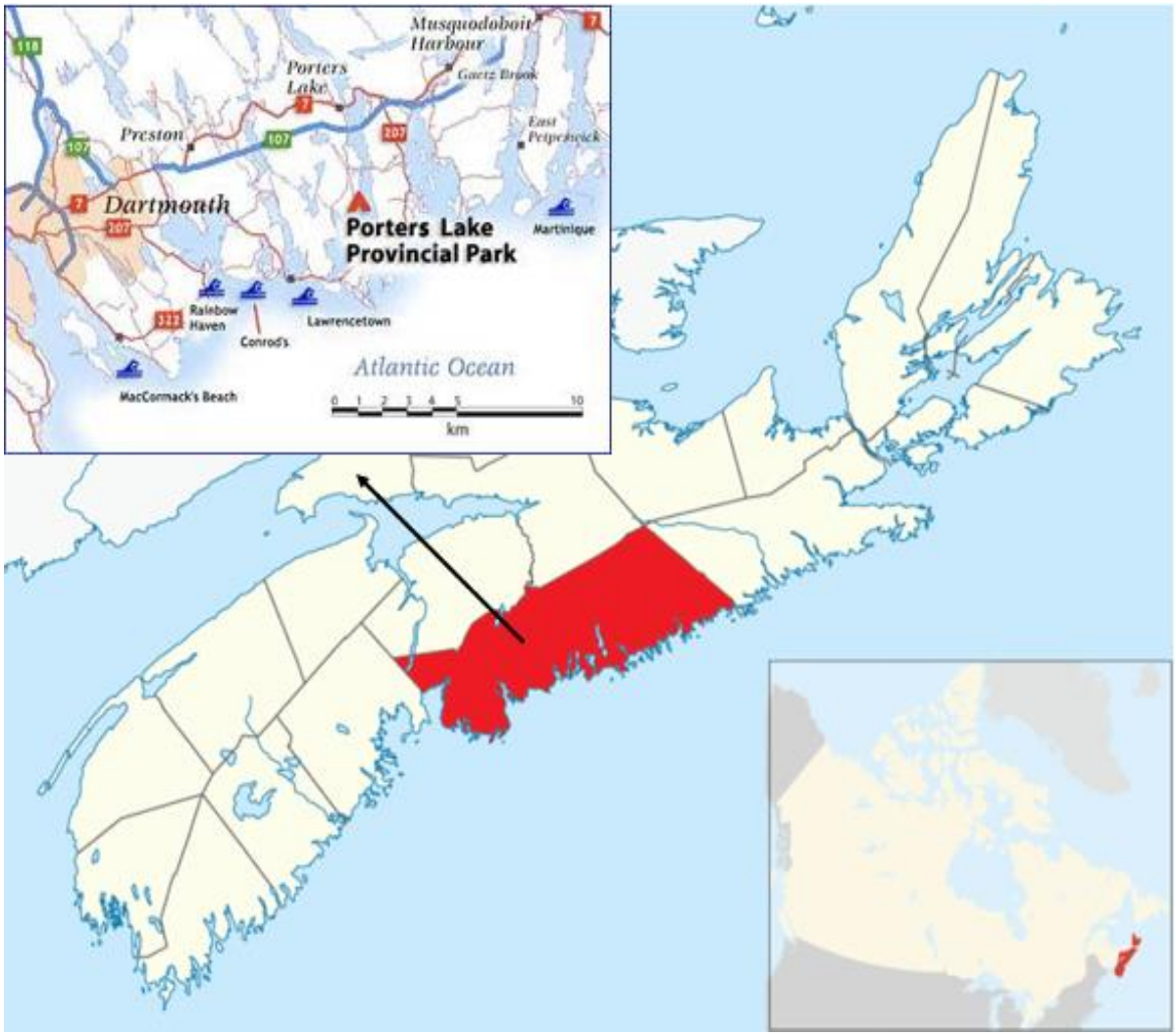

Figure 1 : Map indicating the location of Porter's Lake in Nova Scotia, Canada. (Modified picture from Wikimedia Commons and Seaside Tourism and Business Association)

A) B)

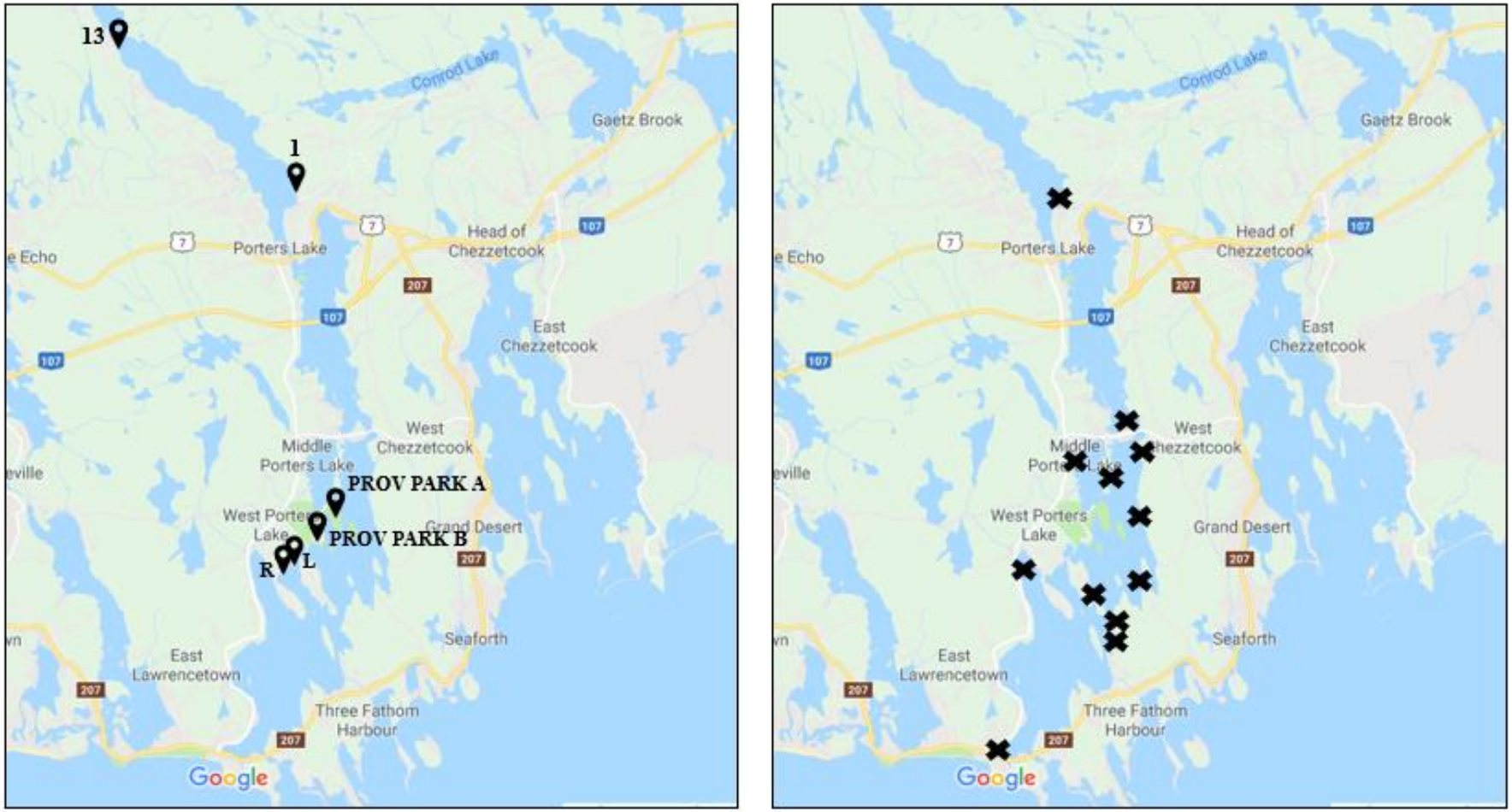

Figure 2 : Map of Porter's Lake, Nova Scotia indicating the locations where fish were collected (A) in the summer of 2018 for this study and, B) by Merette et al. (2009) in 2007

2.2 DNA Extraction and Quantification

DNA was extracted from fin clips taken from the 176 collected fish using the Omega-Biotek kit (D3396-02, 200 preps) following the manufacturer's instructions for DNA extractions from tissue samples. Prior to extractions, 30 mg of each fin clip were minced in individual micro centrifuge tubes using forceps and scissors that were cleaned with bleach between each sample to prevent DNA cross-contamination. After extractions, the concentration of DNA in each sample was also estimated using a spectrophotometer (Spectromax M3 M series Microplate reader, Molecular devices, using Softmax Pro7 software or Thermoscientific nanodrop 1000). DNA was then stored at -20°C for later use in polymerase chain reactions (PCRs).

2.3. Microsatellite Amplification and Analysis

Based on previous studies by Hernandez-Chavez and Turgeon (2007) and Merette et al. (2009), alleles at four autosomal microsatellite loci (Table 1) were amplified to identify hybrids and assign hybrids to a particular clonal lineage. The first three microsatellites (FhCA-1, FhCA-21 and Fhe-57) have species-specific alleles that differ between *F. diaphanus* and *F. heteroclitus* (Hernandez-Chavez and Turgeon, 2007). So, these loci were solely used for hybrid identification, as first generation clonal hybrids should be heterozygous at all three of these loci. Lastly, the microsatellite FhATG-B103 was used to determine clonal genotype since this locus is highly variable in the parental species and all previously identified hybrid clonal lineages had different alleles at this specific locus (Hernandez-Chavez and Turgeon, 2007).

Table 1: Selected microsatellite loci and corresponding primers for the genetic identification of *Fundulus* species and hybrids.

Locus	Primer sequence (fluorochrome 5'-3')	Size range (bp)	Designed by
FhCA-1	5'-6FAM-GTCCATGCAATGTCGTTCAC-3' 5'-GAGGCCAGAAACGCATACAT-3'	142-153	Adams et al. (2005)
FhCA-21	5'-TAMN-GGTCATTATGGAAAACAGCAACAGATC-3' 5'-GCTCACTGACACACTGGATTTGGTAGA-3'	144-206	Adams et al. (2005)
Fhe57	5'-HEX-CTAACTGAACCGCTCACAAGG-3' 5'-ACTGGTCCACTCTGGCTTC-3'	131-243	Hernandez-Chavez and Turgeon (2007)
FhATG-B103	5'-PET-CGGAGCATTGTGATTGTGTTGTTTT-3' 5'-CCGGGGGACACTTATATGAAATCAGA-3'	298-436	Adams et al. (2005)
D-loop	5'-TTCCACCTCTAACTCCCAAAGCTAG-3' 5'-CCTGAAGTAGGAACCAGATG-3 '	441	Lee et al. (1995)
Fundulus specific D-loop *	5'- TTAACCCCCACCCCTAGCTC -3' 5'- GCACTGTGAAATGTCAACTGAA -3'	660	Present study

*New D-loop primers were designed to improve amplification efficiencies after finding that primers designed by Lee et al. (1995) had numerous mismatches to *Fundulus diaphanus* and *Fundulus heteroclitus* D-loop sequences. The new *Fundulus* specific D-loop primers amplify a longer region which has 2 restriction sites for *Fundulus diaphanus* (bands at 211, 166 and 215 base pairs) and 1 restriction site for *Fundulus heteroclitus* (bands at 211 and 381 base pairs).

2.3.1 Multiplex PCR amplification of microsatellite markers

Different combinations of the microsatellite primers were tested, including all four primer pairs multiplexed together, to make sure that the primers were working well, and the correct PCR product sizes were obtained. We finally opted to use two duplex PCR amplification reactions combining primers for loci FhCA-1 and FhCA-21 (Duplex 1), and Fhe57 and FhATG-B103 (Duplex 2). Before amplification, the concentration of DNA of each sample was normalized to 50 ng/µL.

For each reaction, final PCR concentrations were as follows: 1X PCR buffer (Colourless GoTaq® Flexi buffer), 2 mM of $MgCl_2$, 0.1 µM – 0.15 µM of each primer (0.1 µM for primers amplifying loci FhCA-1, FhCA-21 and Fhe57, 0.15 µM for primers amplifying locus FhATG-B103), 0.4 mM of deoxynucleotides triphosphates (dNTPs) and 0.025 U/µL of GoTaq® DNA polymerase. Forty nanograms of DNA (0.8 µL) was used for each reaction and distilled water was added to obtain a final volume of 10 µL. Amplification conditions followed those outlined by Hernandez-Chavez and Turgeon (2007) which were as follows: initial denaturation of 3 minutes at 98°C, followed by 8 cycles of 45 seconds at 95°C, 40 seconds at 60°C and 40 seconds at 72°C, followed 22 cycles of 95°C for 45 seconds, 40 seconds at 55°C, 40 seconds at 72°C, and a final extension of 45 seconds at 72°C.

The multiplex PCR products from primers FhCA-1 and FhCA-21 were diluted by 1/15 and the products from primers Fhe57 and FhATG-B103 were diluted by 1/5 to optimize fluorescent signal output. Samples were then prepared in 12.25 µL volumes consisting of 10 µL of HiDi™ Formamide (Life technologies), 0.25 µL of GeneScan™ 600 Liz™ dye Size Standard v2.0 (Applied Biosystems) and 2 µL of diluted samples. The samples were size-separated and visualized on an Applied Biosystems 3500xL Genetic Analyser. Amplified products were then visualized using the program GeneMarker® version 2.7.4.

2.4 Restriction Fragment Length Polymorphism

PCR was used to amplify the D-loop region of mitochondrial DNA of each sample, in 25 µL reactions. We used D-loop primers 'd-loopA' and 'd-loopE' (Table 1) designed by Lee et al. (1995) using the conditions outlined by Hernandez-Chavez and Turgeon (2007). However, because of a mutation site in the D-loop region of *Fundulus heteroclitus,* we had difficulties identifying the mitochondrial DNA of this species. New consensus primers were then designed (Table 1; D-loop*) that amplify a longer segment of the D-loop region. Final concentrations of the PCR cocktail consisted of 1X PCR buffer (Green GoTaq® Flexi buffer), 2 mM of $MgCl_2$, 0.4 µM of each primer (forward and reverse), 0.4 mM of $dNTP_s$ and 0.025 U/µL of GoTaq® DNA polymerase . For each reaction, 2 µL of DNA was used and nuclease-free deionized distilled water (ddH_2O) was added to obtain a final volume of 25 µL. PCR cycling conditions followed those used by Hernandez-Chavez and Turgeon (2007) and were as follows: initial denaturation for 3 minutes at 95°C, followed by 40 cycles of 45 seconds at 95°C, 45 seconds at 50°C and 1 min at 72°C, followed by a final extension of 5 minutes at 72°C. After amplification, we obtained a final product that was 441 (D-loop, Table 1) or 660 (*Fundulus* specific D-loop, Table 1) base pairs long depending on which primers were used.

After amplification of the D-loop region, restriction enzyme digestion was performed to identify the maternal species contributing to the formation of the hybrids. Hernandez-Chavez and Turgeon (2007) discovered a diagnostic Hph1 restriction site which is only present in the D-loop of *F. diaphanus*. Mitochondrial DNA of *F. diaphanus* (two cut sites) and *F. heteroclitus* (one cut site) thus differ in the number of restriction sites present in the D-loop region, and digestion of the amplified PCR product from the Lee et al. (1995) D-loop primers yields bands at 275 and 166 base pairs in *F. heteroclitus*, while *F. diaphanus* shows bands at 211, 64 and 166 base pairs. Individual samples that did not amplify well with the

Lee et al. (1995) D-loop primers were re-amplified with our *Fundulus* specific D-loop primers (Table 1). The product sizes are 211, 166 and 215 base pairs for *F. diaphanus* and 211 and 381 base pairs for *F. heteroclitus.* Restriction enzyme digestion was performed by setting up reactions containing 2X Concentrated CutSmart® buffer and 250 U/ml Hph1 enzyme. Distilled water was then added to obtain a final volume of 5 µL. Twenty-five microliters of amplified PCR product was added to the tubes which were then incubated at 37°C for 2 hours. The products were then run at 100V for 2 hours on a 3% agarose gel stained with ethidium bromide and visualized.

2.5 Data Analysis

The hybrids were identified by looking at the alleles at three loci (FhCA-1, FhCA-21 and Fhe57; Table 1) and were scored as hybrids if they had one allele from each parental species at all of the three loci. For individuals where only partial data were obtained, we only scored an individual as a hybrid if we had data for at least two of the three loci. Sexual hybrids were identified by looking for evidence of backcrossing at the three loci. For example, instead of having one allele from each species, sexual hybrids might have two alleles from the same species at a given locus. To identify the clonal lineage, we looked at loci FhATG-B103, which Merette et al. (2009) found to be highly variable and used to identify clonal lineages. To determine the species identity of the mother of each clonal lineage, we compared the number of bands obtained after PCR-RFLP with the expected size of products for *F. diaphanus* and *F. heteroclitus*. Lastly, I compared my data with that of Merette et al. (2009) to answer the three main questions of this thesis.

3. RESULTS

3.1 Parental species' allele distributions at the four nuclear microsatellite loci

At locus FhCA-1, three alleles were found in *F.diaphanus* (163, 171 and 175 bp), with allele 171 bp having the highest frequency, and four alleles in *F.heteroclitus* (134, 141, 161 and 177 bp), with allele 141 bp having the highest frequency. At locus FhCA-21, both parental species were found to consist of the two alleles: 147 and 149 bp in *F.diaphanus* with allele 147 having the highest frequency and, alleles 169 and 181 bp in *F.heteroclitus*, with allele 169 having the highest frequency. Eight alleles were found in *F.diaphanus* at locus Fhe57 with allele 175 bp having the highest frequency. Allele 143 was found to have the highest frequency among the other alleles found in *F.heteroclitus* for locus Fhe57. Lastly, both *F.diaphanus* and *F.heteroclitus* had a wide range of alleles at locus FhATG-B103 (Figure 3). All these allele distributions are in agreement with that other studies have also found (Hernandez-Chavez and Turgeon, 2007, Merette et al., 2009).

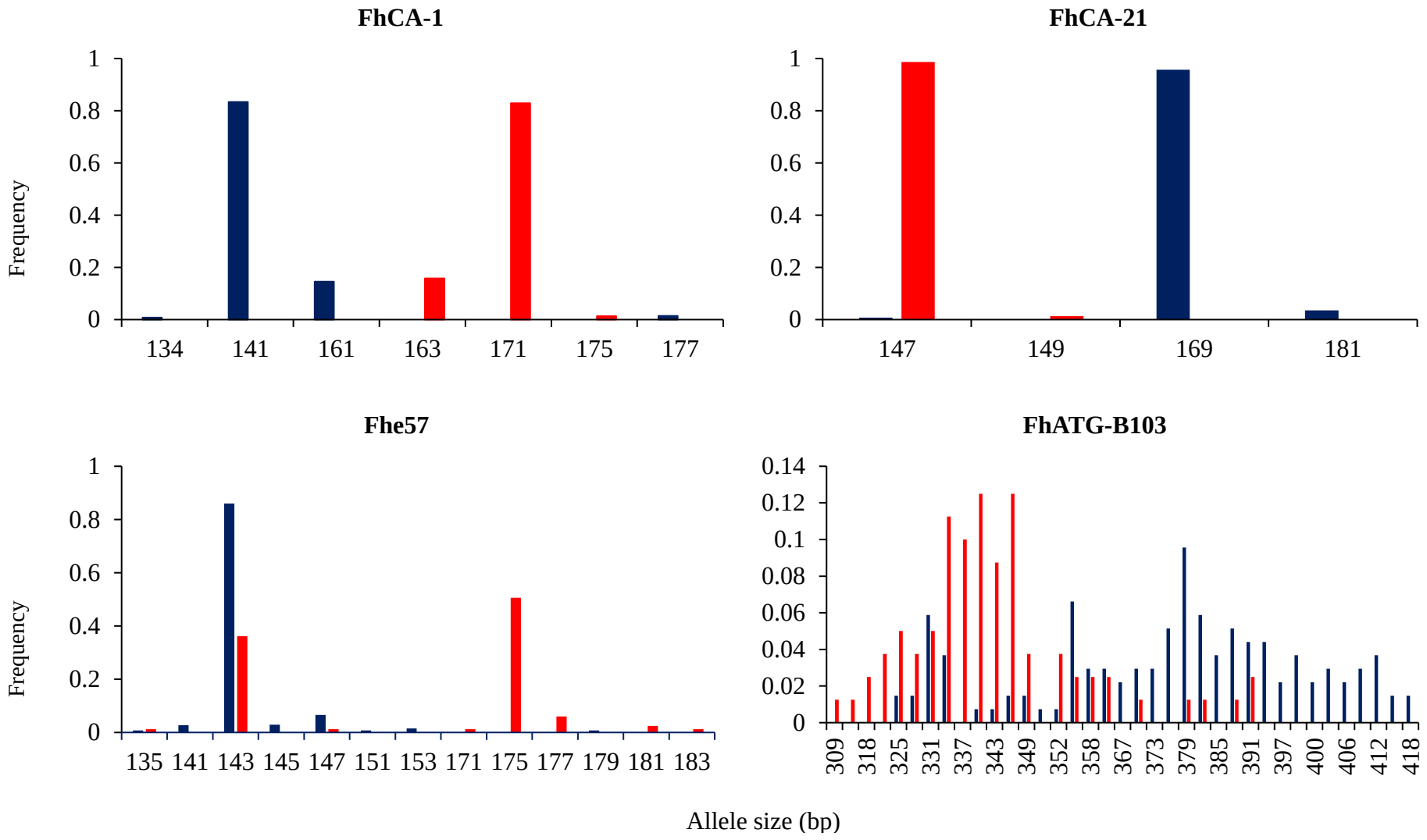

Figure 3 : Allele distribution at the four microsatellite loci for the parental species. Red and blue bars represent *F.diaphanus* and *F.heteroclitus*, respectively.

3.2 Comparison of morphological and genetic methods to identify species

Morphological identification using a three-trait combination defined by Merette et al. (2009) was used as a preliminary method to identify each killifish species in the field (Lauren Jonah, MSc Thesis, in progress). However, because this method only has a success rate of 91.5% (Merette et al., 2009), nuclear microsatellites were also used to confirm the species of each sample, based on the same methods and criteria as Hernandez-Chavez and Turgeon (2007).We compared species identities using these two methods in Table 2.

Table 2: Number and percentage of individuals of each species correctly identified using morphological identification, as determined by nuclear microsatellite genotypes.

Morphological ID	Nuclear DNA ID		
	F.diaphanus	Hybrid	*F.heteroclitus*
F.diaphanus (n=38)	38 (100%)	0 (0%)	0 (0%)
Hybrid (n=51)	0 (0%)	45 (88.2%)	6 (11.8%)
F.heteroclitus (n=75)	0 (0%)	8 (10.7%)	67 (89.3%)

Table 2 clearly indicates that the three-trait combination method correctly identifies any *F. diaphanus* individuals with a success rate of 100%. However, this method was only about 89% successful at differentiating between *F. heteroclitus* individuals and hybrids.

3.3 Clonal lineages present in Porter's Lake

We identified the clonal lineages by analyzing the combination of alleles present at the four microsatellites with a focus on locus FhATG-B103 because it is the most variable. All the hybrids analyzed had the same alleles at loci FhCA-1, FhCA-21 and Fhe57, thus the number of clonal lineages were based on the genotype at locus FhATG-B103. Hybrids having the same genotype at the locus FhATG-B103 were grouped together and were considered as one clonal lineage (Table 3).

Figure 1A shows that approximately 10 years ago, 11 different clonal lineages (A-K) were present in Porter's Lake (Merette et al. 2009) with distinct alleles at locus FhATG-B103 (Table 3). Clone A was most prevalent based on the genotypes of the loci analyzed, with alleles 343, 349 at locus FhATG-B103 (Table 3) and consisted of approximately 75% of the population along with some minor and less prevalent clones (Figure 3A).

After all the hybrids were grouped based on the genotype at locus FhATG-B103, I compared the alleles obtained at this locus with that of Merette et al. (2009) to see if the hybrids of 2018 belonged to the same clonal lineages that Merette et al. (2009) found. In 2018, I found evidence for five new clonal lineages (L-P). Table 3 shows the genotypes of the new lineages at the four microsatellite loci, and Figure 3 shows the distribution of allele frequencies for loci FhATG-B103 in the hybrids from 2004 and 2018. I found no evidence of the clonal types that were present in 2007. Instead, Clone L consists of approximately 78% of the hybrid population and is the most prevalent clone in Porter's Lake based on the hybrid population we surveyed (Figure 1B).

It is important to note that we are limited by what we can say about clonal turnover because we did not run the samples used by Merette et al. (2009) on the same machine using the same technique to calibrate the size of the alleles. Thus, it is possible that the alleles we

called as 340 bp, might actually be equivalent to alleles Merette et al.'s (2009) classified as larger or smaller (e.g. of size 334, 337, 343 or 346). Our finding that our major 2018 genotype as FhATG-B103 (340/340) was homozygous, while the major clone in 2007 was heterozygous (343/349) does suggest we detected a new clone, but there is also the possibility that our major clone was a heterozygote, but with a 'null allele' that did not amplify well in our hands. However, alleles frequencies at locus FhATG-B103 of the hybrids in 2007 and 2018 (Figure 4), is consistent with the possibility that the shift in alleles from 2007 to 2018 might be real, as shifts in allele sizes across methods are normally within 6 bp (e.g. Pasqualotto et al., 2006, DeValk et al., 2009).

Table 3: Multilocus genotypes of confirmed hybrids from Porter's Lake in 2007 (from Merette et al (2009)), and in 2018. Diagnostic alleles of *Fundulus heteroclitus* and *Fundulus diaphanus* are shown in blue and red, respectively. Note that some allele sizes from Merette et al (2009) were corrected to match microsatellite mutation patterns for dinucleotide repeats for FhCA-1 and FhCA-21.

Clone	n 2007	n 2018	Locus							
			FhATG-B103		FhCA-1		Fhe57		FhCA-21	
A	76	0	343	349	**141**	**179**	**143**	**181**	**147**	**169**
B	2	0	343	352	**141**	**179**	**143**	**181**	**147**	**169**
C	3	0	349	349	**141**	**179**	**143**	**181**	**147**	**169**
D	4	0	343	343	**141**	**179**	**143**	**181**	**147**	**169**
E	3	0	340	349	**141**	**179**	**143**	**181**	**147**	**169**
F	4	0	346	349	**141**	**179**	**143**	**181**	**147**	**169**
G	2	0	334	349	**141**	**179**	**143**	**181**	**147**	**169**
H	2	0	343	349	**141**	**179**	**143**	**181**	**147**	**169**
I	6	0	337	349	**141**	**179**	**143**	**181**	**147**	**169**
J	0	0	346	346	**141**	**179**	**143**	**181**	n/a	n/a
K	1	0	343	355	**141**	**179**	**143**	**181**	**147**	**169**
L	0	36	340	340	**141**	**177**	**143**	**179**	**147**	**169**
M	0	5	334	334	**141**	**177**	**143**	**179**	**147**	**169**
N	0	1	331	331	**141**	**177**	**143**	**179**	**147**	**169**
O	0	1	328	328	**141**	**177**	**143**	**179**	**147**	**169**
P	0	2	337	337	**141**	**177**	**143**	**179**	**147**	**169**
Total	105	46								

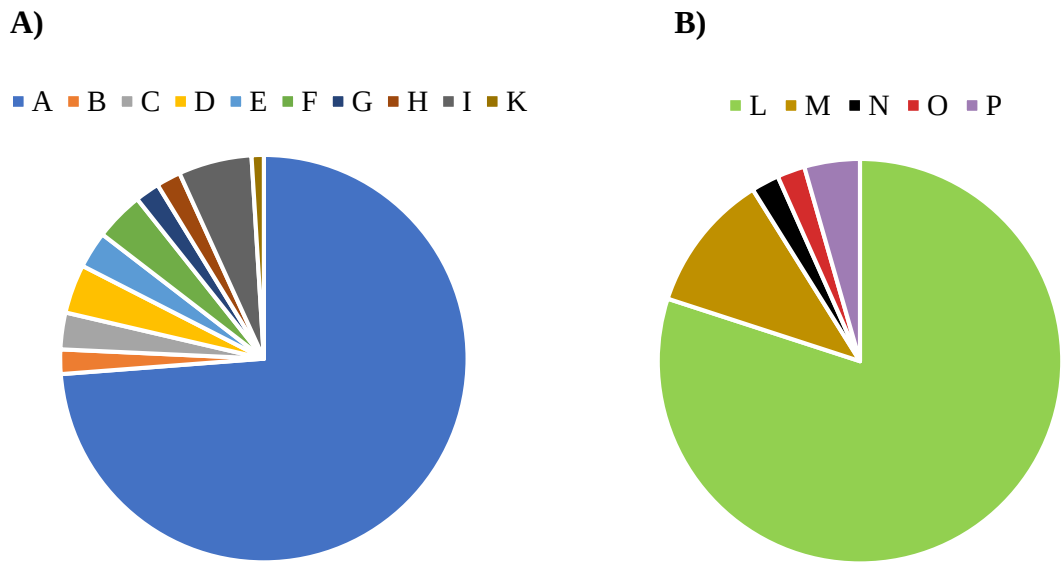

Figure 4 : Percentage of each clonal types found in 2007 (Fig. A) by Merette et al. (2009) and in 2018 (Fig. B).

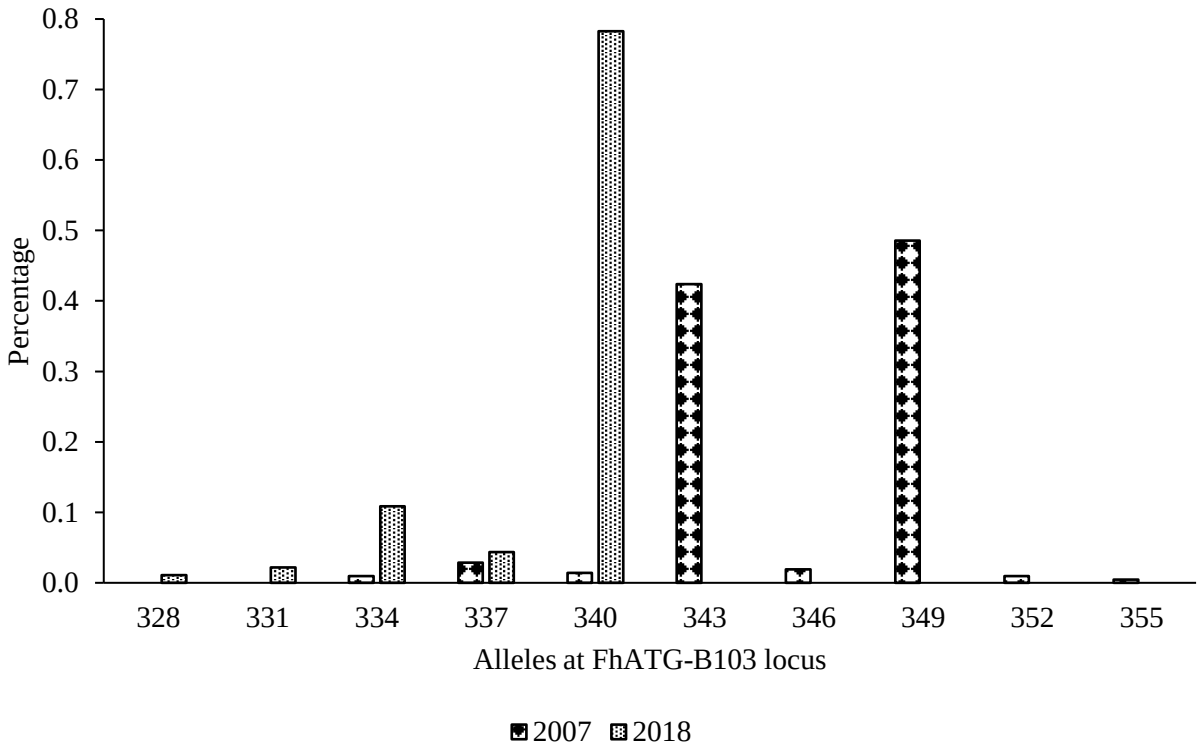

Figure 5 : Allele frequencies in hybrids at the trinucleotide microsatellite locus FhATG-B103 in 2007 and 2018.

3.4 Evidence of sexual clones

First generation hybrid clones should have one allele from each parental species and should not show evidence of backcrossing with either parental species. We classified hybrids as sexual if an individual fish had a combination of genotypes that was not consistent with either pure parental genotypes or F1 hybrids at the three microsatellite loci (FhCA-1, FhCA-21 and Fhe57) with species-specific alleles. This indicates that there has been mixing of alleles that are mostly fixed for both parental species and that this individual is most likely not a first generation hybrid and is the product of sexual parents. For example, if two alleles at a given locus came from *F. heteroclitus*, but the other two loci had alleles from both parental species, this was viewed as evidence for potential backcrossing of a first generation hybrid with an *F. heteroclitus*, indicating they must be sexual. Of the 51 hybrids examined, six were found to match the pattern expected for sexual clones and had two *F. heteroclitus* alleles (143,143) at locus Fhe57, but one allele from each species at the other two loci (FhCA-1 and FhCA-21), indicating backcrossing with *Fundulus heteroclitus*.

3.5 Maternal species contributing to hybrid formation

When the hybrids at Porter's Lake were surveyed in 2004, all had a maternally-inherited mitochondrial genome of *Fundulus diaphanus*, making this species the mother of all the hybrids. Of the 51 hybrids analyzed in 2018, only one had mitochondrial DNA from *Fundulus heteroclitus* (Figure 5).

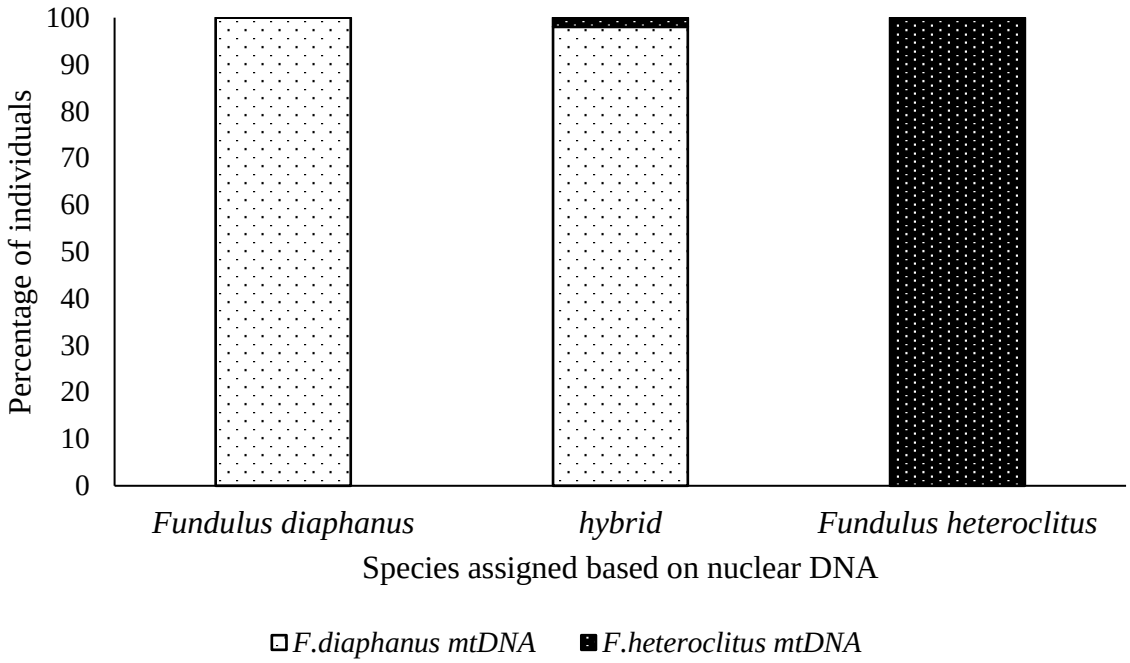

Figure 6: Percentage of individuals classified as each 'species' by nuclear microsatellite markers, having either *F. diaphanus* or *F. heteroclitus* mitochondrial DNA. White and black represent the percentage of individuals with either *F. diaphanus* or *F.heteroclitus* mitochondrial DNA, respectively.

4. Discussion

4.1 Have the clonal lineages present in Porter's Lake remained stable over time?

Analysis of the four microsatellite markers suggest that the most prevalent clone found in 2007 by Merette et al. (2009) might no longer be present in Porter's Lake. Instead, we find evidence that a new clone (clone L; Table 2) might now be the major clone and consists of approximately 78% of the surveyed hybrid population (Figure 3B). None of the other clonal lineages found in 2009 were found in 2018, and we also found evidence of five new clonal lineages (Table 2, Figure 3B) that did not exist when Merette et al. (2009) analyzed the hybrids in 2004.

If correct, our finding that new clonal lineages might have formed since the last survey in 2007 indicate that unlike the Amazon Molly, which is believed to have originated from one single hybridization event (Stock et al., 2010), the killifish hybrids are being formed constantly by multiple hybridization events. This is in agreement with Hernandez-Chavez and Turgeon's (2007) findings of different clonal lineages in many locations across the Maritimes. Furthermore, the presence of new clonal lineages in just a decade indicates that the lineages are constantly forming and dying, which may explain why we did not find any clone from the same lineages as in 2007 (Table 3), and hence indicate that the clonal lineages of killifish hybrids are not temporally stable.

However, it is also important to note that there is lab to lab variation in allele sizes of microsatellites (e.g. Pasqualotto et al., 2006). Past studies where same samples were analyzed at different laboratories identified that there can be mismatches up to 6 base pairs between the actual size and size reported by capillary electrophoresis due to differences in equipment used (Pasqualotto et al., 2006, DeValk et al., 2009). Our data collection was done in a different laboratory, using different equipment than Merette et al. (2009). Thus, it is possible that the

alleles we found were classified as a different size by Merette et al. (2009), and we cannot be sure that new clones have become dominant, without directly measuring allele sizes of the fish collected in 2007 using the same method as for 2018.

There is also a possibility that we might have missed one of Merette et al.'s (2009) clones due to our small sample size (n=51), but also because of the limited number of locations where fish were collected in Porter's Lake. The fish were collected at six different locations in Porter's Lake (Figure 5A) and because it is difficult to sample all locations and every hybrid, it is quite likely that we did miss some clonal lineages. Furthermore, Merette et al. (2009) had a higher sample size than our study (n=105 versus n=51) and also covered a larger geographical region of Porter's Lake (Figure 5B) for fish collection than we did. Additionally, Hernandez-Chavez and Turgeon (2007) conducted an analysis of the total number of clones sampled and number of different clones discovered and found that at least 200 hybrids need to be analyzed using nuclear DNA microsatellite markers to identify all the possible clonal lineages present in Porter's Lake, which is about four times larger than our sample size.

Lastly, we had trouble scoring the alleles for some individuals due to the presence of three alleles at some loci (FhATG-B103, FhCA-1) in some species. Furthermore, all the clonal lineages that were detected in 2018 were homozygous at locus FhATG-B103 compared to 2007 where hybrids were mostly heterozygous at this locus (Table 3). Thus, this is good evidence that the clonal lineages found in 2018 are new, and not simply the same clonal lineages as 2007 but with genotyping errors, but we must first further assess the possibility of a null allele at FhATG-B103 in our 2018 samples.

If a new clone has come to dominate, we do not know if the presence of a new major clone after 10 years is because this new clone has a higher fitness than clone A (dominant in

2007) in the current environment, or if random fluctuations in population demographics have led to variations in the clonal composition of Porter's Lake. One possibility could be because of the Red-Queen hypothesis suggested by Gibson et al., 2016, in which parasites usually select against the common clonal genotypes. Furthermore, Koskella and Lively (2009) also found that when *P. antipodarum* clones are infected by trematode *Microphallus*, the frequency of the clones drops over time. This provides good evidence that parasite-mediated frequency-dependent selection gives an advantage to sexually-reproducing populations over asexual populations. Clonal *Fundulus* hybrids in the wild were found to be very parasitized (King, 2009), which might explain why the clonal lineages in Porter's Lake have short life span since they are very susceptible to parasites, which can adapt to better infect a common clone.

4.2 Is there any evidence of sexual hybrids?

Of the 51 hybrids that were analyzed, only six were sexual hybrids. They all had the same genotype at FHCA-1 (***144***,**177**), FhCA-21 (**147**,***180***), and Fhe 57 (***143***,***143***) (Blue italics and red bold represent *F. heteroclitus* and *F. diaphanus* alleles, respectively) and they were also all females. While alleles at the first two loci were from both parental species, locus Fhe 57 had two *F. heteroclitus* alleles. These data indicate that these individuals may be the progeny of first generation hybrids that backcrossed with *F. heteroclitus*. However, a potential drawback when using microsatellite markers is the presence of null alleles which are found across all taxa to some extent (Dakin and Avise, 2004). Null alleles are caused by mutations in the primer-binding region that affect microsatellite amplification (Pemberton et al., 1995). Thus, alleles (143,143) at locus Fhe57 might be due to null alleles and not because this individual is the product of sexual reproduction. There are multiple statistical analyses, for example, Microchecker software, that can be used to detect null alleles (Van Oosterhout et al., 2004) which was not done in this study. It is also possible that allele frequencies for

Fhe57 changed in *F. diaphanus*, but further analysis such as re-analyzing the alleles at locus Fhe57 in *F. diaphanus*, will need to be done to confirm this.

The reason why backcrossing only occurred with *F. heteroclitus* and not *F. diaphanus* may be due to differences in mate preference. During hybridization events, a female *F. diaphanus* normally mates with a male *F. heteroclitus*. Male *F. heteroclitus* are larger and more colourful than male *F. diaphanus*, and females of many fish species prefer to mate with larger males (Bakker and Mundwiler, 1994). Therefore, female *F. diaphanus* may be more attracted to male *F. heteroclitus* than *F. heteroclitus* females over the smaller and more drably coloured male *F. diaphanus*. This hypothesis may explain why mating occurs more frequently between female *F. diaphanus* and male *F. heteroclitus* than female *F. heteroclitus* and male *F. diaphanus*, but remains to be tested. Additionally, reciprocal hybrids can be easily made in a laboratory by crossing any combination of parental species and produce both males and females, which may be sexual (Fritz and Garside, 1974), so the reason why male hybrids or sexual hybrids do not happen more frequently in nature still remains unknown.

4.3 Is there any evidence of hybrids with *F. heteroclitus* mothers?

Analysis of the hybrid's mitochondrial DNA revealed that 98% of the hybrids had the mitochondrial genome of *F. diaphanus*, indicating that the mother of most hybrids is usually a *F. diaphanus*. Earlier survey of the hybrids in Porter's Lake indicated that no hybrids had *F. heteroclitus* mothers, however, in this study we did found a very small proportion of hybrids (1 out of 51 hybrids) with *F. heteroclitus* mothers. The reason for this *F. diaphanus* maternal bias might also be explained by the differences in mate preference among species, as described for the sexual hybrids (see section 4.2).

4.4 Future directions

To test if difference in equipment used might have an effect on the alleles we scored at the microsatellites loci, we will re-analyze the hybrids samples used by Merette et al. (2009) using the same equipment as we used in this study to identify any potential difference in scoring the alleles. The next step required to better estimate the stability of the clones in Porter's Lake would be to increase our sample size by collecting more hybrids in Porter's Lake; this would give us a better representation of the different clones present. The mate preference hypothesis (female *F.diaphanus* preference to mate with male *F.heteroclitus*) will also be directly tested in the lab to study mate preferences in the killifish species.

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