Genomics of freshwater adaptation in threespine stickleback from eastern Canada

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Abstract

The threespine stickleback (*Gasterosteus aculeatus*) is a key species for studying parallel evolution. However, most evidence for parallel evolution at the genetic and phenotypic levels comes from the west coast of North America and parts of southern Europe. Here, we use RAD-sequencing of pooled samples to examine marine-freshwater differentiation in stickleback populations from Atlantic Canada and to test for signatures of parallel evolution. Our results reveal substantial heterogeneity in genomic differentiation among populations, with some freshwater populations showing a high degree of allele frequency divergence relative to marine ones. The strongest candidate loci were two SNPs near dopamine receptor genes (*Drd4a* and *Drd2l*), which were repeatedly differentiated between marine and freshwater habitats. Gene ontology (GO) analysis of candidate genes showed enrichment for functions related to nervous system development and dopamine receptor activity. These findings suggest a more complex evolutionary history for Atlantic stickleback populations than previously recognized, potentially involving multiple colonization events or ongoing gene flow. The repeated differentiation of genomic regions linked to dopaminergic signalling points to adaptation to freshwater environments through changes in neurological phenotypes.

Introduction

Knowledge of the genetic basis of adaptation is central to understanding the mechanisms driving the diversification of species. Threespine stickleback fish (*Gasterosteus aculeatus*) are native to the oceans of the northern hemisphere and has been an important species in studies of ecology and evolution over the last century (Bertin 1925; Heuts 1947a, b; Moodie and Reimchen 1976). Following the last glacial maximum (~21 kya), retreating glaciers created new freshwater lakes and rivers, into which marine stickleback populations dispersed and became isolated. Many of the nascent freshwater populations evolved similar low-plated morph phenotypes, particularly in western North America (Baumgartner and Bell 1984; Colosimo et al. 2005; Hohenlohe et al. 2010). Alleles that confer higher fitness in freshwater are found at low frequencies in marine populations. Bidirectional gene flow is thought to maintain the genetic variation needed to seed new freshwater habitats where similar phenotypes evolve independently ('transporter hypothesis', Schluter and Conte 2009). Today, the threespine stickleback is considered an exemplar of parallel evolution.

However, even in the early stages of establishing stickleback as a model species, researchers noted that the striking parallel evolution observed among freshwater populations might be a geographically restricted phenomenon, largely confined to western North America (i.e., the eastern Pacific) and parts of southern Europe (Hagen and Moodie 1982). For instance, considering the armour phenotype, in several regions such as North America, Northern Europe, the Baltic Sea, and eastern Asia, the completely plated morph—typical of marine environments—also predominates in local freshwater lakes (Münzing 1963; Penczak 1965; Hagen and Moodie 1982; Mäkinen et al. 2008;

Raeymaekers et al. 2014; Ferchaud and Hansen 2016; Yamasaki et al. 2019). This limited phenotypic divergence between marine and freshwater populations has also been observed at the genetic level using allozymes (Rafiński et al. 1989). More recently, a large-scale genomic study by Fang et al. (2020a) confirmed that high levels of marine–freshwater (M–FW) genomic differentiation are primarily restricted to the eastern Pacific, whereas Atlantic populations show relatively low differentiation. In contrast to the strong divergence seen in British Columbia populations, many freshwater populations in Eastern Canada are dominated by the fully plated ecotype (Hagen and Moodie 1982; Haines et al. 2022), although some exhibit distinct morphological variation (Scott et al. 2023). Despite these observations, genomic studies in this region remain limited (e.g., Fang et al. 2020).

One potential explanation for the reduced extent of parallel evolution and M–FW genomic differentiation outside the eastern Pacific is the stochastic loss of freshwater-adapted alleles. Threespine stickleback originated in the Pacific Ocean, where they have persisted for approximately 26 million years (Matschiner et al. 2011; Betancur-R et al. 2015). It was only much more recently, during the late Pleistocene (36.9–346.5 thousand years ago), that they expanded their range into the western Pacific and the Atlantic basin (Orti et al. 1994; Fang et al. 2018; Fang et al. 2020). As a result of this range expansion, Atlantic populations exhibit lower genetic diversity compared to their Pacific counterparts (Fang et al. 2020). Notably, most Atlantic populations examined to date have been from northern Europe, leaving other regions, such as Atlantic Canada, relatively understudied.

Here, we investigate marine and freshwater threespine stickleback from eastern Canada, specifically from the provinces of Nova Scotia and Newfoundland, where fully plated phenotypes are commonly observed in freshwater habitats (Hagen and Moodie 1982). Using genome-wide SNP data, we characterize M–FW genomic differentiation and test for signatures of parallel natural selection. Our goal is to gain insight into the evolutionary history and genetic basis of freshwater adaptation in stickleback populations from eastern North America.

Methods

Field sampling and DNA extraction

We used minnow traps and beach seines to collect 30 adult stickleback (> 30 mm in length) from nine populations in Nova Scotia and Newfoundland, Canada (Figure 1). In Nova Scotia, freshwater samples were collected from two lakes (Pomquet Lake and Lake Ainslie) and one stream (Black River), while marine samples were obtained from Antigonish Landing and Porper Pond. In Newfoundland, freshwater populations were sampled from Pinchgut Lake and Blue Pond, and marine populations from Cooks Brook and Humber Arm. Genomic DNA was extracted from all individuals using a standard phenol–chloroform protocol.

DNA sequencing of pooled samples

For DNA quantification, we used a Picogreen® ds DNA assay (Thermo Fisher Scientific, Waltham, USA) and an Infinite® 200 Nanoquant (Tecan Group Ltd. Männedorf, Switzer-

land). We then normalized the concentration of dsDNA and pooled according to sampling location. Thus, we created nine pools consisting of 30 individuals each. RAD-seq libraries were prepared and sent for sequencing the McGill University and Genome Quebec Innovation Center, Montreal, Canada. Sequencing was carried out on one lane of Illumina HiSeq2500 which produced 125bp paired-end reads.

Bioinformatics

We used the *trim-fastq.pl* script of *Popoolation* (Kofler et al. 2011a) to process raw reads, filtering based on read quality (--quality-threshold 20) and length (--min-length 50). We then mapped the processed reads to the stickleback reference genome (BROADS S1) with the program *Bowtie2* (Langmead and Salzberg 2012), using the --end-to-end mapping option. We then used *SAMtools* (Li et al. 2009) to convert the output SAM files to BAM format and subsequently removed reads with mapping quality below 20 (samtools view -q 20). We generated an mpileup file (samtools mpilep -B) and converted the mpileup file to the synchronized (sync) format using *Popoolation2* (Kofler et al. 2011b), which was used for downstream analyses.

Estimates allele frequency differentiation

We estimated population differentiation using F_{ST}, as formulated by Hartl and Clark (1997), using the *fst-sliding.pl* script of *Popoolation2*. We applied the following parameters: --min-count 2, --min-coverage 5, --max-coverage 500, --min-covered-fraction 0, --window-size 1, --step-size 1, --pool-size 30:30:30:30:30:30:30:30:30, --suppress-noninformative. We only analyzed genomic regions assembled at the chromosome level (i.e.,

scaffolds excluded). We categorized F_{ST} outliers as SNPS in the top 5% of the F_{ST} distribution of a given M-FW comparison. We then looked for overlap of F_{ST} outliers among M-FW population pairs. We quantified F_{ST} outlier overlap among M-FW population pairs using a custom bash script. Loci showing relatively large changes in allele frequency (top 5%) in two or more M-FW comparisons within provinces and across Nova Scotia and Newfoundland were taken as three subsets. We then considered 'F_{ST} parallel outliers' SNPs that overlapped among the three sets.

Identification of candidate genes and analysis of molecular function

Genes potentially under parallel selection are expected to show outlier loci or, more commonly, be located near them, given that we used a reduced representation approach. To identify such genes, we applied a custom bash script to map outlier loci to or near protein-coding genes in the reference genome. Our search was limited to 14,252 protein-coding gene annotations, filtered by the attributes "ID=gene" and "biotype=protein_coding." To investigate the traits under selection, we analyzed the candidate genes for enrichment of molecular functions using ShinyGO 0.77 (Ge et al. 2020), a graphical web application that links gene lists to functional categories, such as gene ontology (GO), based on annotation databases from Ensembl and STRING-db. We assessed enrichment for GO terms in the categories of biological process and molecular function, using a false discovery rate (FDR) threshold of 10% and reporting the 20 most significant results. Given that our data is based on a reduced representation of the genome, there may be cases where the causal mutation is not directly sampled but rather a SNP in linkage disequilibrium with it. Therefore, we also examined GO term enrichment for genes associated with outlier loci or located within 5 Kb of one. Finally, we compared

our list of candidate genes to the 14,252 gene set from which they were sampled, using the ‘best matching species’ option for further analysis.

Results

Data sets and Genomic differentiation among populations

After processing the raw RAD-seq data, we identified 18,582 SNPs across 36 population comparisons. Median genome-wide F_{ST} varied widely, ranging from 0.0118 to 0.0824 (Table S1; Figure 2). We observed considerable genomic heterogeneity both among M-FW comparisons (Figure 3) and between populations from similar environments (Figure 4). Notably, both the highest and lowest levels of genomic differentiation were observed among freshwater populations. The least differentiated populations were Pomquet Lake (Nova Scotia) and Pinchgut Lake (Newfoundland), while the most differentiated populations were Lake Ainslie (Nova Scotia) and Blue Pond (Newfoundland; Figure S1, Table S1). Excluding M-M and FW-FW comparisons, we obtained 25,236 informative SNPs across 20 M-FW comparisons. We used this SNP set to search for genomic regions potentially involved in parallel freshwater adaptation, identifying 2,100 SNPs as F_{ST} outliers in two or more M-FW comparisons within Nova Scotia (19 SNPs across all six comparisons; Figure S2a). In Newfoundland, we identified 1,306 SNPs as F_{ST} outliers in two or more comparisons (Figure S2b). Comparing interprovincial differentiation among 10 M-FW population comparisons revealed 3,422 SNPs as F_{ST} outliers in at least two comparisons, with three SNPs appearing in nine of the 10 comparisons (Figure S2c). In the top 5% of the F_{ST} distribution, we identified 279 SNPs (referred to

as 'F_{ST} outliers'), which were outliers in at least 6/20 M-FW comparisons, representing 1.1% of the SNP dataset. We found 133 genes associated with these SNPs (Table S2), with 68 genes containing at least one outlier (Table S3). Notably, two SNPs (VI: 16449327 and XX: 14009648) were in the top 5% of F_{ST} values in 19/20 M-FW comparisons (6/6 in Nova Scotia, 4/4 in Newfoundland, and 9/10 interprovincial comparisons; Figure S2). The latter SNP is located 920 bp downstream from the gene for the dopamine receptor *Drd2l* (XX: 14002115-14008728). The low-plated locus (IV:12811481) was not sampled in our RAD-seq dataset, with the closest SNP mapping 2.3 Mbp upstream (IV:10156713).

Gene Ontology

Genes within 5 kb of F_{ST} outliers were enriched for biological processes related to adenylate cyclase-inhibiting dopamine receptor signalling and nervous system development (Table S4). Enriched molecular functions included neurotrophin binding and dopamine neurotransmitter receptor activity (Table S5).

Clustering of marine and freshwater populations

Phylogenetic clustering of populations using WPGMA placed freshwater populations together, with Lake Ainslie and Black River forming one sister clade, Pomquet Lake and Pinchgut Lake forming another, and Blue Pond as an out-group (Figure 5). Low bootstrap values corresponded to marine population branches, indicating weak phylogenetic structure and suggesting panmixia or ongoing gene flow among them.

Discussion

In this study, we investigated marine and freshwater populations of threespine stickleback along Canada's east coast to gain insights into their evolutionary history and the potential genetic basis of freshwater adaptation. Contrary to expectations of low genome-wide differentiation between marine and freshwater populations in the western Atlantic (Fang et al. 2020a), we found that differentiation among M-FW populations varied widely along the east coast of North America. This suggests a more complex evolutionary history, likely involving multiple colonization events, distinct glacial refugia, or ongoing gene flow. Evidence of parallel evolution was observed as repeated allele frequency differences at SNP loci across several M-FW population comparisons. Notably, we identified a consistent signal of differentiation at one SNP near a dopamine receptor previously found to be differentially expressed in freshwater populations (Di Poi et al. 2016). Our results suggest that, even in the likely absence of key alleles for reduced armour in eastern populations, behavioural or endocrine adaptations may have facilitated freshwater persistence.

Heterogeneous marine-freshwater differentiation in Eastern Canada

Current evidence suggests that threespine stickleback colonized the Atlantic during the late Pleistocene, as recently as ~36.9 thousand years ago (Fang et al. 2018; Fang et al. 2020). Repeated bottlenecks during this expansion may have led to the stochastic loss of some freshwater-adaptive alleles, such as the low-plated Eda allele (Fang et al. 2020). Loci under selection in freshwater are expected to differentiate rapidly following colonization. Thus, the low M-FW differentiation observed in many Atlantic populations

may reflect a lack of such adaptive alleles in their standing genetic variation, compared to Pacific populations.

Here, we show that while genome-wide M-FW differentiation is indeed low in some eastern North American comparisons, other comparisons show levels of differentiation comparable to those seen on the Pacific coast (Figure 3). The highest levels of differentiation were observed between different freshwater populations. For example, two lakes in Newfoundland—Pinchgut Lake and Blue Pond—are only 5 km apart but differ substantially in their genetic divergence from marine populations (Figure 4). Blue Pond is a small, rain-fed lake without input or output streams, making gene flow from neighbouring populations unlikely. Such barriers to gene flow likely promoted divergence through genetic drift. Our findings may also reflect multiple colonization events in eastern Canada, similar to the patterns observed in the Japanese Archipelago (Kakioka et al. 2020). The clustering of freshwater populations is consistent with at least three colonization events: one leading to Blue Pond, another to Lake Ainslie and Black River, and a more recent expansion into Pomquet and Pinchgut lakes. However, our results also align with a scenario in which freshwater populations evolved independently in separate glacial refugia during the last ice age (Mäkinen and Merilä 2008).

Evidence of selection on dopamine receptors

Adaptation to similar environments via re-use of standing genetic variation is considered a primary mechanism underlying parallel evolution (Schlötterer 2023). The low-plated Eda allele, though present at low frequencies in marine populations, confers a fitness advantage in freshwater (Marchinko and Schluter 2007; Barrett et al. 2008; Rouzic et al. 2011). Its widespread loss in the Atlantic gene pool may explain the absence of parallel

evolution for the low-plated phenotype in eastern Canadian lakes. However, Eda is not the only gene involved in freshwater adaptation; genes involved in osmoregulation, such as Na^+ /K^+ -ATPase and Na^+ /K^+ /2Cl^- cotransporters, have been repeatedly implicated across global stickleback populations (Deagle et al. 2013; Divino et al. 2016; McCairns and Bernatchez 2010).

Consistent with previous observations and the predicted loss of the Eda allele, we did not find signs of molecular evolution near the Eda locus in our populations (Hagen and Moddie 1982; Fang et al. 2020). Nonetheless, we detected evidence of parallel evolution in 1.1% of SNPs, which showed repeated differentiation in M-FW comparisons within and between Nova Scotia and Newfoundland. Among these, we identified repeated M-FW differentiation near dopamine receptor genes *Drd4a* and *Drd2l*, with gene function enrichment related to dopamine receptor activity and nervous system development. These findings suggest that eastern Canadian stickleback may have adapted to freshwater through behavioural changes, such as increased aggression.

Individual variation in endocrine function remains less explored in evolutionary biology, despite its potential to connect physiology, ecology, and genetics (Williams 2008). In zebrafish (*Danio rerio*), expression of dopamine receptors *Drd2c* and *Drd3* correlates with individual aggression, particularly among dominant and subordinate males (Filby et al. 2010). In stickleback, predator exposure reduces *Drd4a* expression (Abbey-Lee et al. 2018), and marine and freshwater populations differ significantly in behavioural traits like sociability, aggression, and activity levels (Di Poi et al. 2014). Furthermore, population-level differences in physiological regulatory networks indicate that divergence is

more often associated with evolutionary changes in receptors (e.g., *Drd2*) than in ligands or neurotransmitters.

The dopaminergic system also plays a key role in osmoregulation through inhibition of prolactin, a hormone critical for ion retention and water balance in freshwater environments (Liu et al. 2006; Yamamoto and Vernier 2011; Mancera and McCormick 2019). Prolactin mRNA levels correlate with local ion concentrations in natural populations of black-chinned tilapia (*Sarotherodon melanotheron*) adapted to different salinities (Tine et al. 2007). In the euryhaline fish *Scatophagus argus*, renal dopaminergic signalling via *Drd1* inhibits Na^+ /K^+ -ATPase under hypo-osmotic stress (Su et al. 2016). In stickleback, prolactin is associated with freshwater migration and salinity tolerance (Lam and Hoar 1967; Ishikawa et al. 2016; Pavlova et al. 2020; Taugbøl et al. 2022). Thus, the repeated differentiation near dopamine receptor genes observed in our study may reflect adaptive responses involving behavioural, endocrine, or osmoregulatory pathways.

Conclusion

Using genome-wide SNPs, our study revealed heterogeneous genomic differentiation between marine and freshwater threespine stickleback populations in eastern Canada. We detected evidence of parallel evolution near dopamine receptor genes, suggesting that behavioural traits may have been under selection during the colonization of freshwater lakes. Alternatively, individuals carrying alleles associated with particular behavioural profiles may have been predisposed to expand into freshwater habitats. Genetic

changes linked to dopamine regulation may also play a role in osmoregulatory adaptation. Taken together, our results suggest that parallel evolution in eastern Canada may be expressed through the adaptation of behavioural and hormone-mediated phenotypes.

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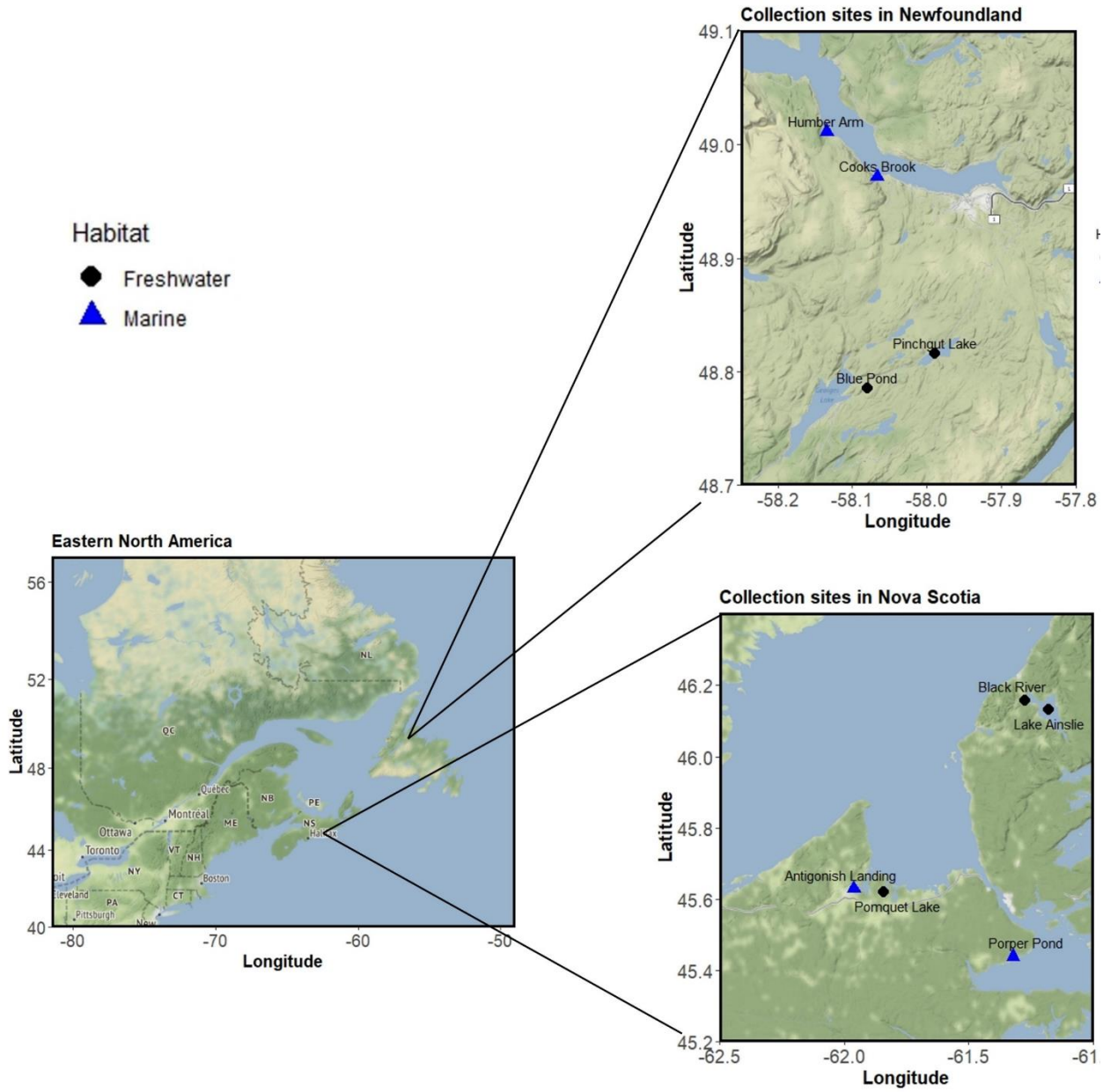


Figure 1. Collection sites in Eastern Canada.

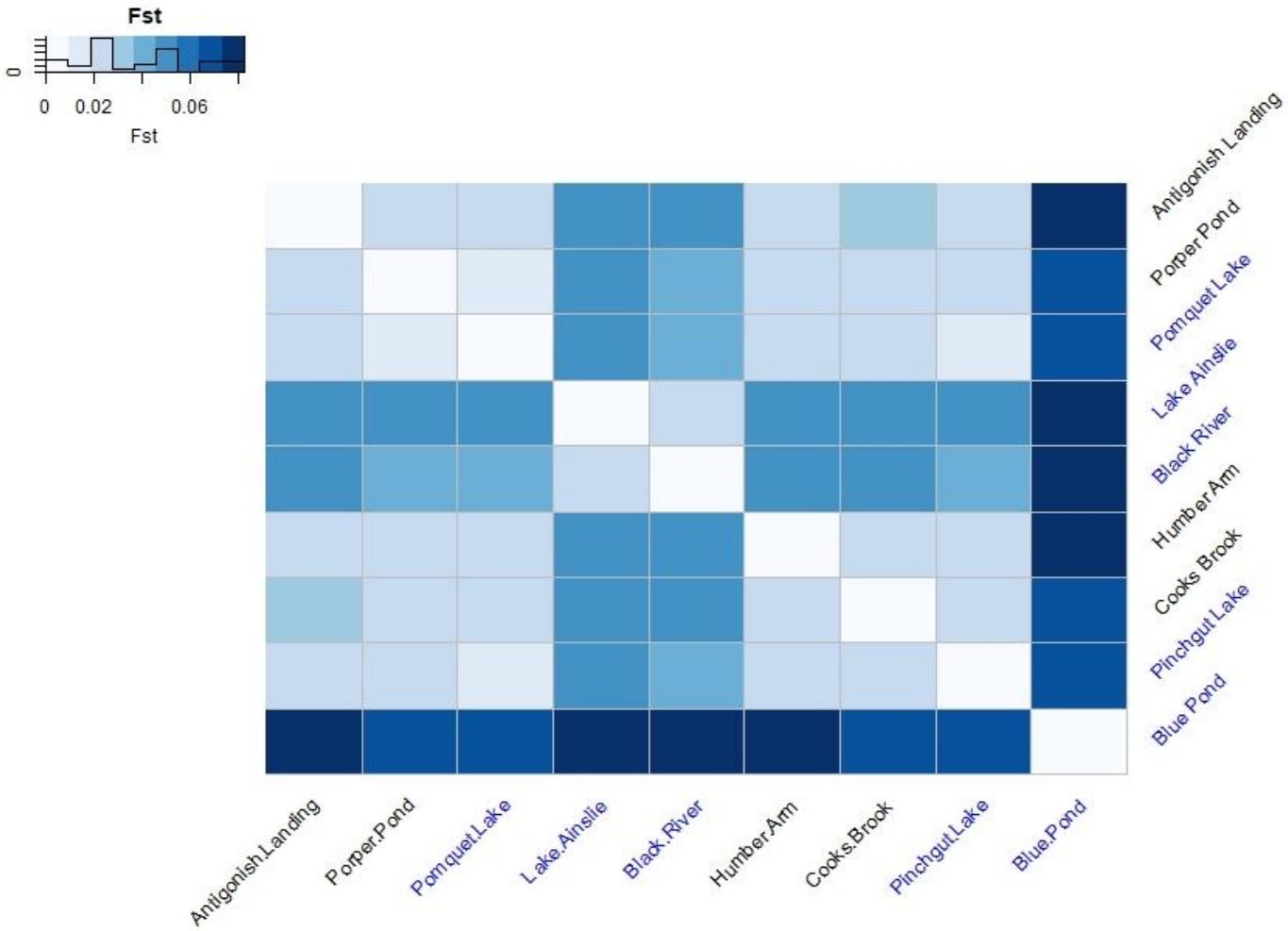
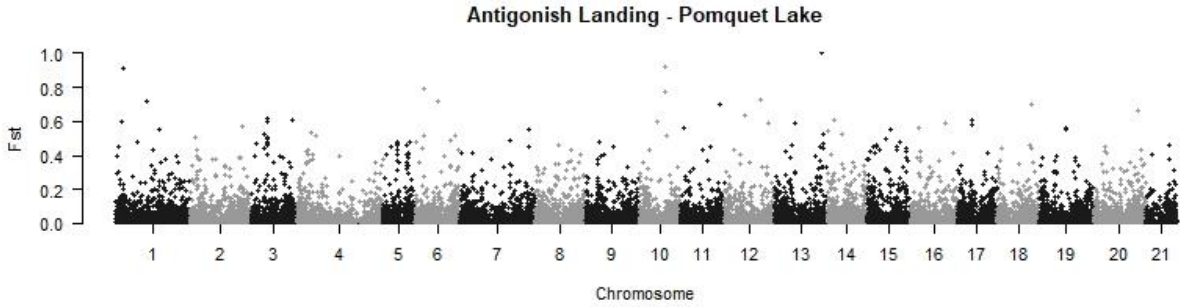
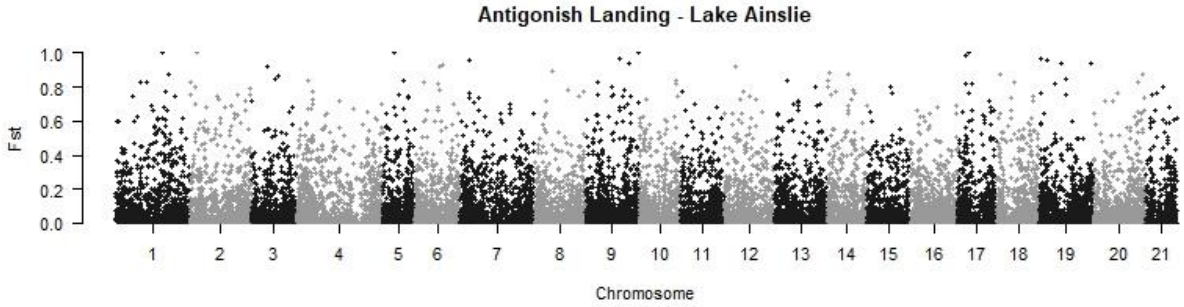


Figure 2. Heatplot depicting average (median) pair-wise F_{ST} with histogram of F_{ST} values. Freshwater sites are labelled blue.


Antigonish Landing - Pomquet Lake
Fst
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Antigonish Landing - Lake Ainslie
Fst
1.0
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0.6
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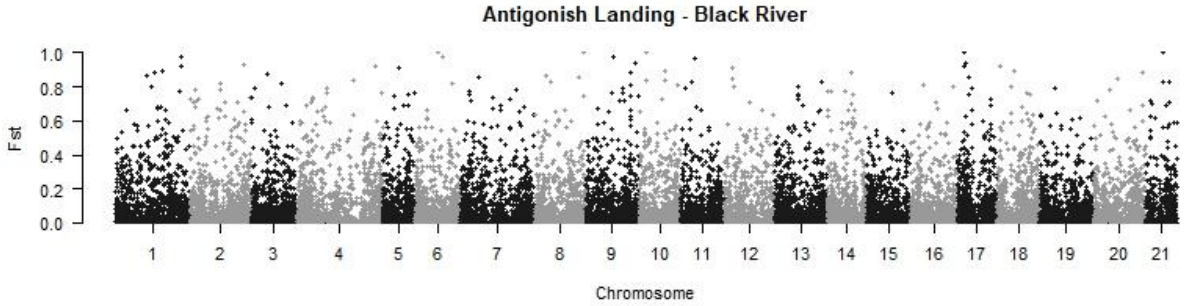
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Antigonish Landing - Black River
Fst
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0.2
0.0
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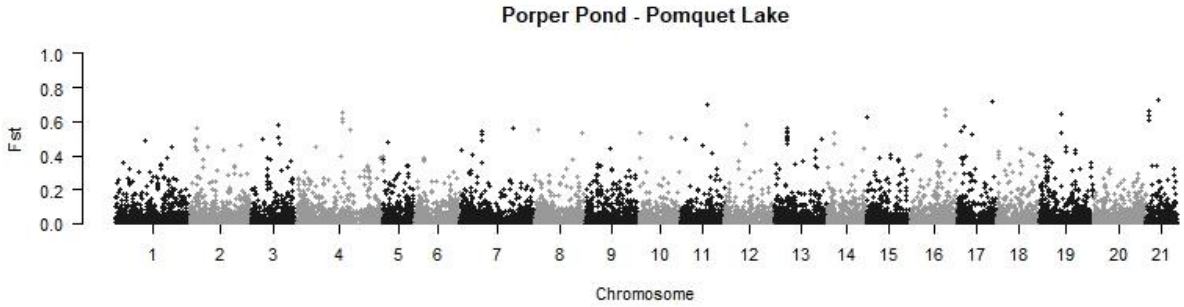
Chromosome


Porper Pond - Pomquet Lake
Fst
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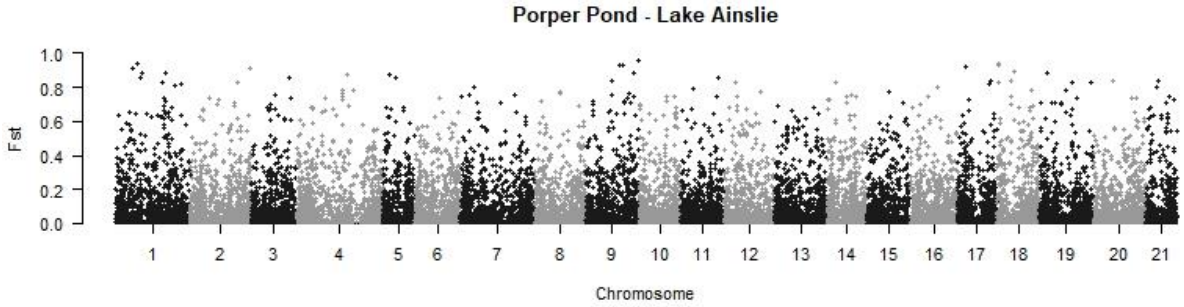
Chromosome


Porper Pond - Lake Ainslie
Fst
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0.2
0.0
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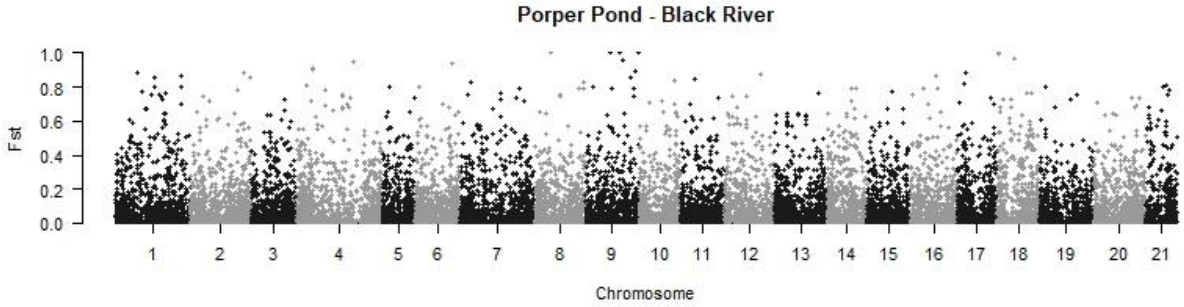
Chromosome


Porper Pond - Black River
Fst
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0.4
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0.0
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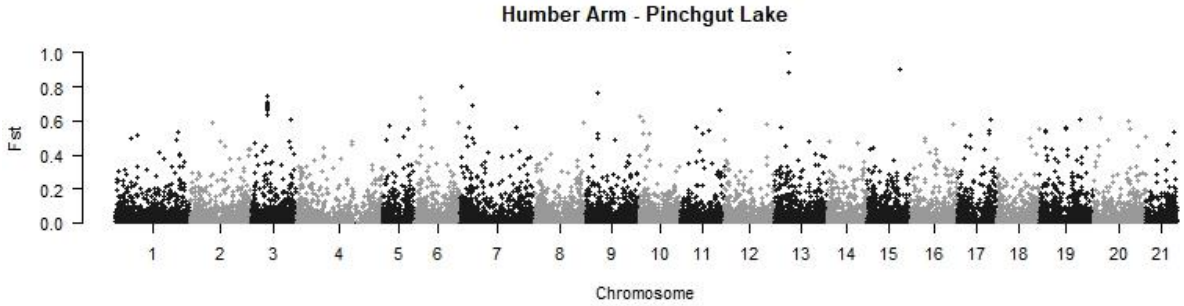
Chromosome


Humber Arm - Pinchgut Lake
Fst
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0.2
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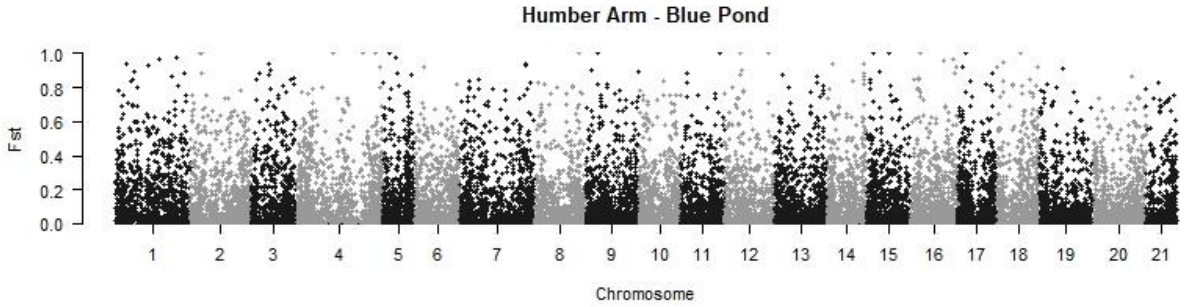
Chromosome


Humber Arm - Blue Pond
Fst
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0.0
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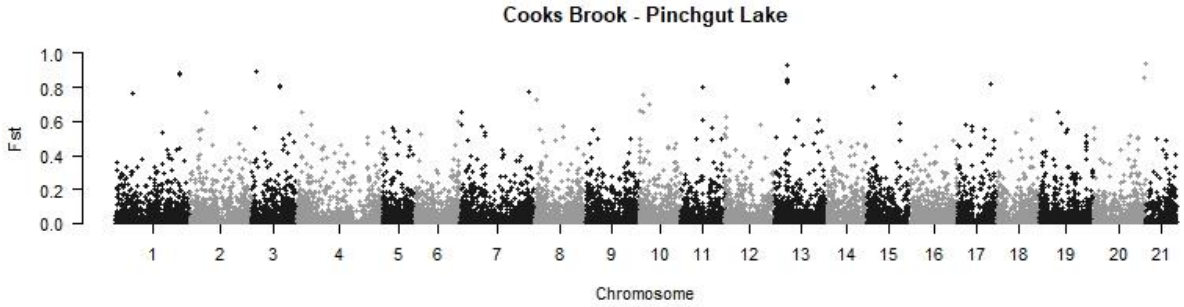
Chromosome


Cooks Brook - Pinchgut Lake
Fst
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0.4
0.2
0.0
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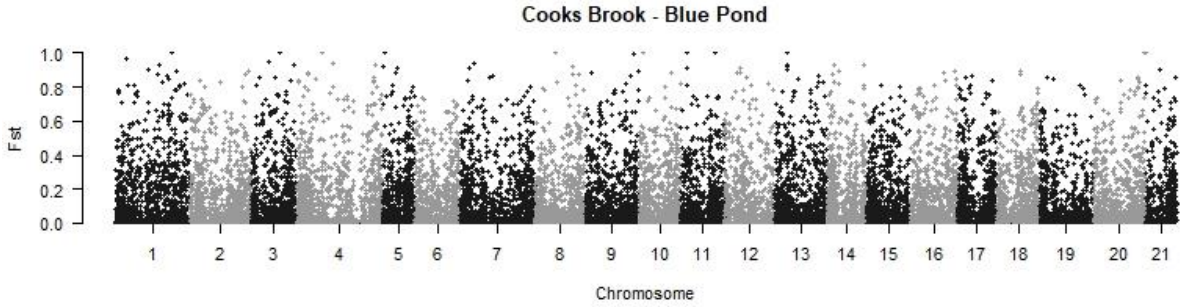
Chromosome


Cooks Brook - Blue Pond
Fst
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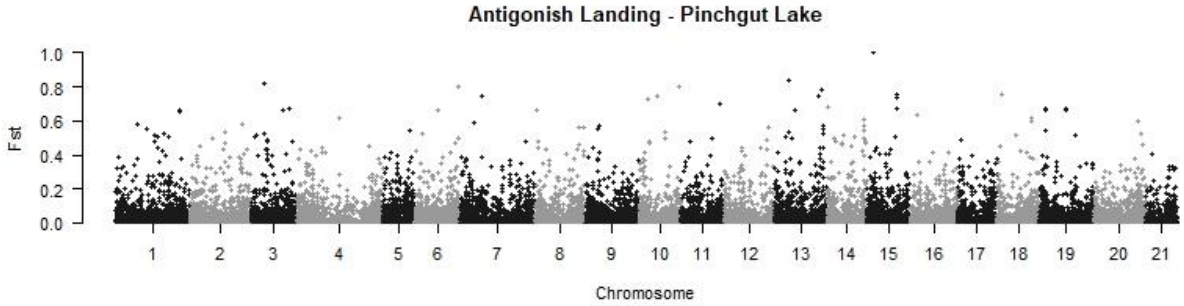
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Antigonish Landing - Pinchgut Lake
Fst
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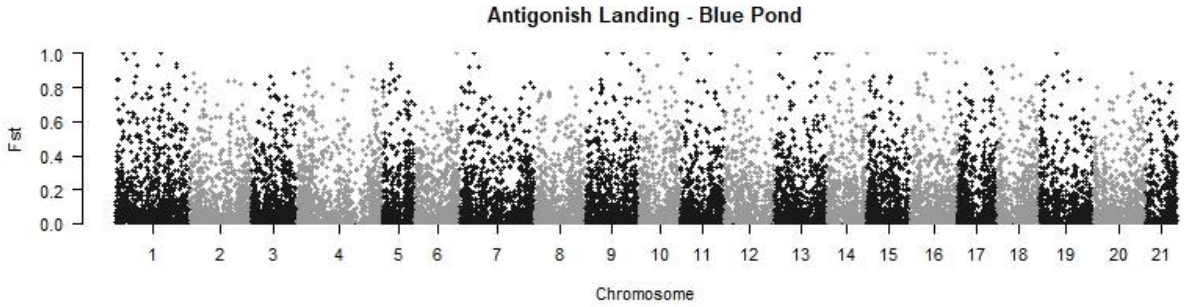
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Antigonish Landing - Blue Pond
Fst
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0.6
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0.2
0.0
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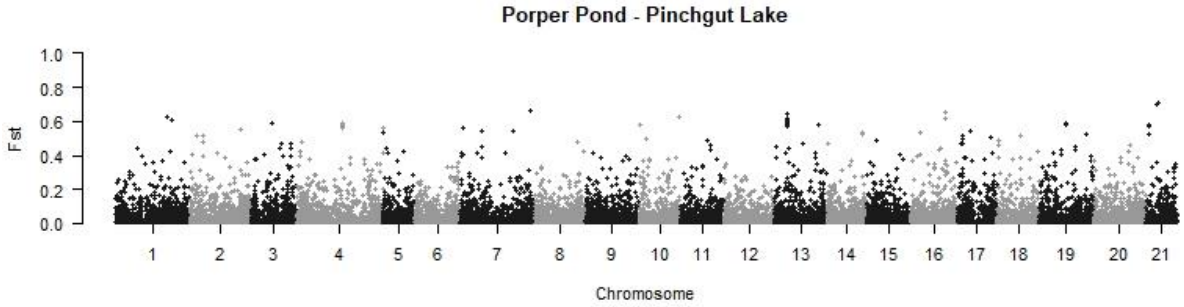
Chromosome


Porper Pond - Pinchgut Lake
Fst
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0.6
0.4
0.2
0.0
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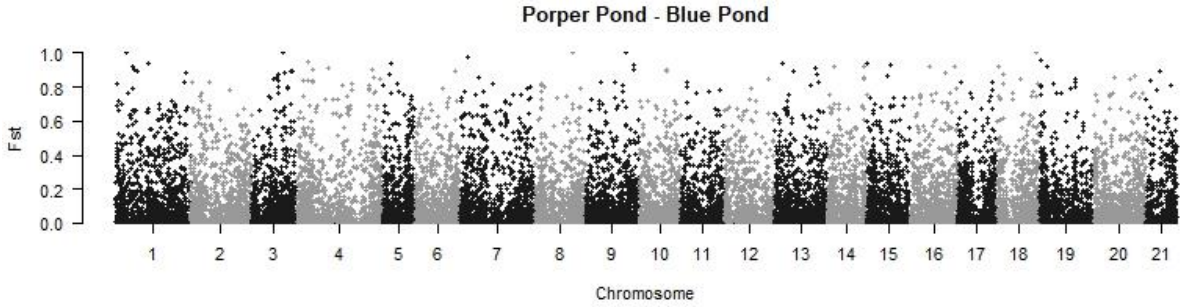
Chromosome


Porper Pond - Blue Pond
Fst
1.0
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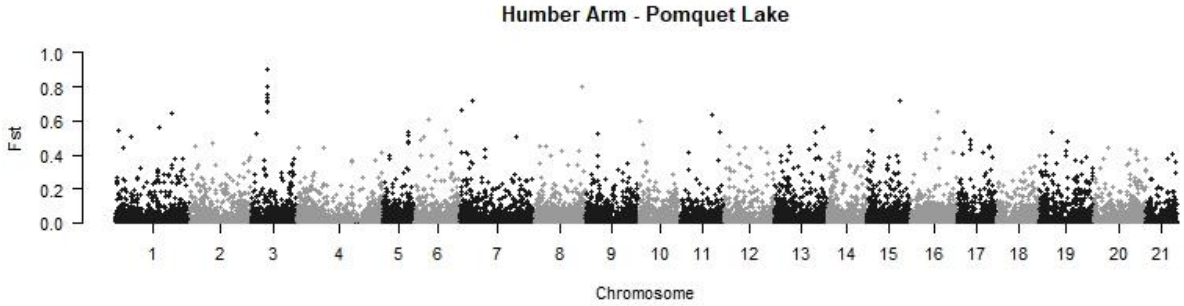
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Humber Arm - Pomquet Lake
Fst
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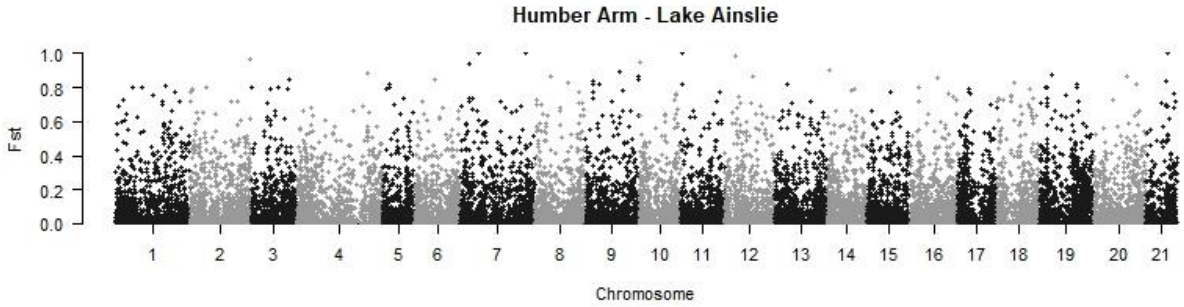
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Humber Arm - Lake Ainslie
Fst
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0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome

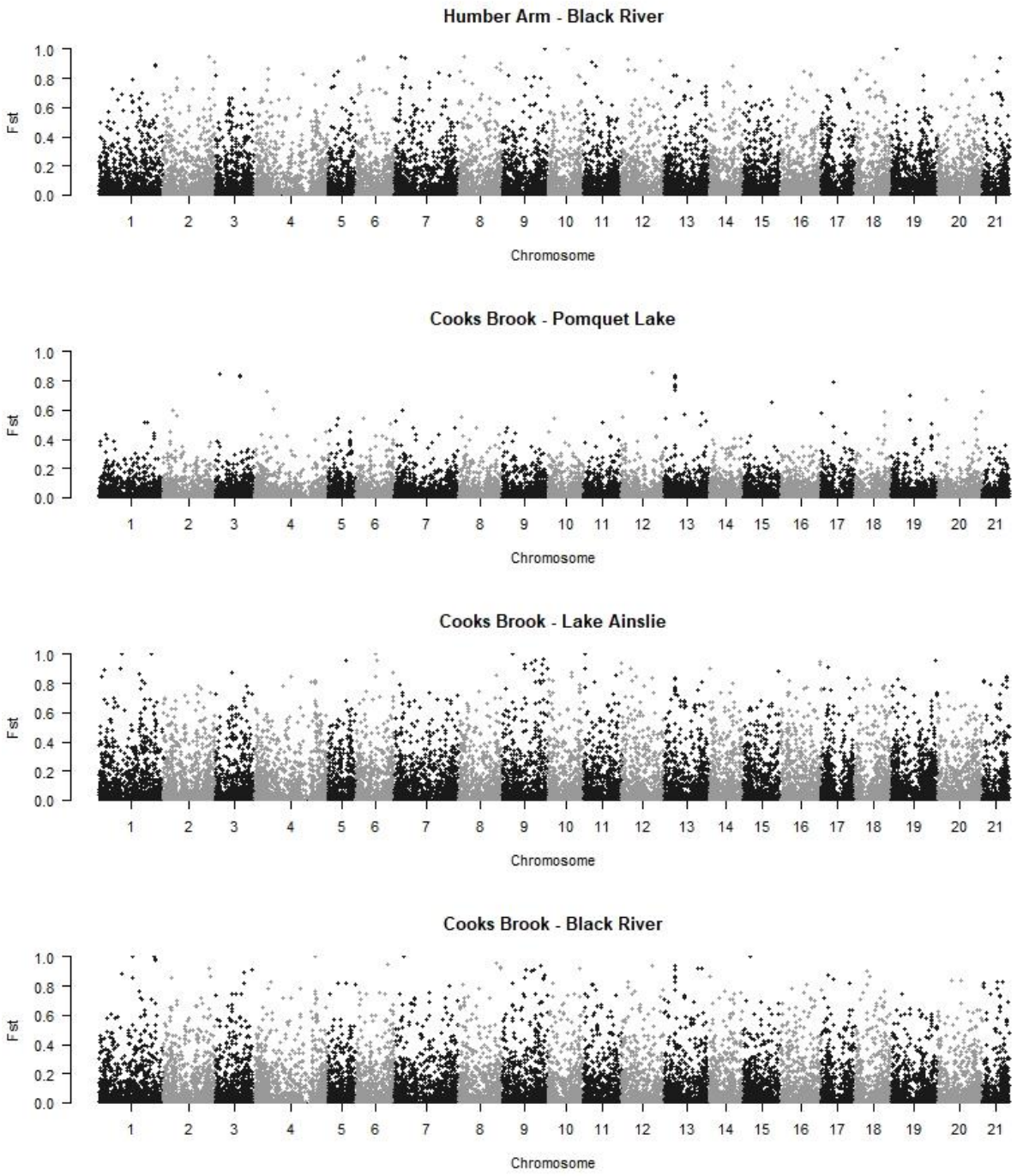
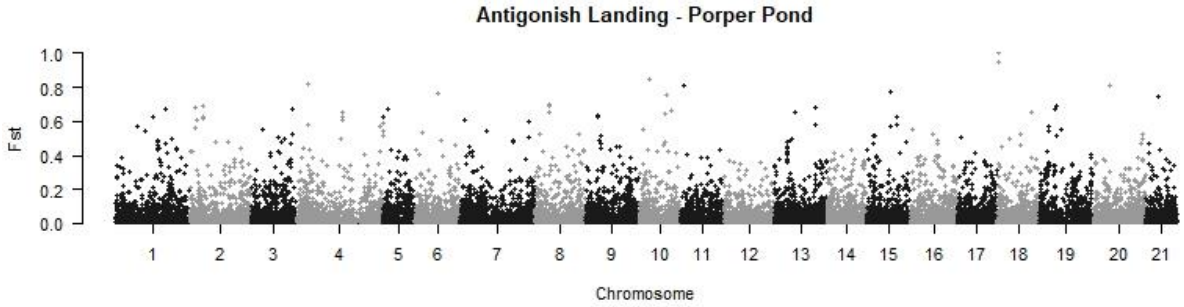
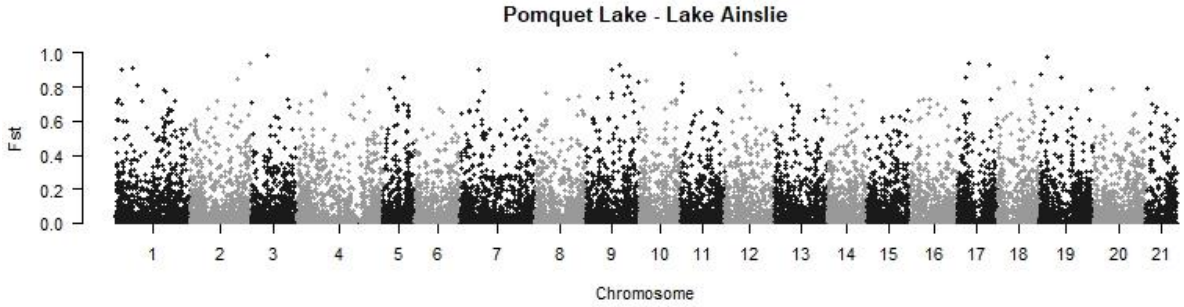


Figure 3. Manhattan plot of F_{ST} values showing heterogeneity in 20 marine-freshwater (M-FW) comparisons.


Antigonish Landing - Porper Pond
Fst
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Pomquet Lake - Lake Ainslie
Fst
1.0
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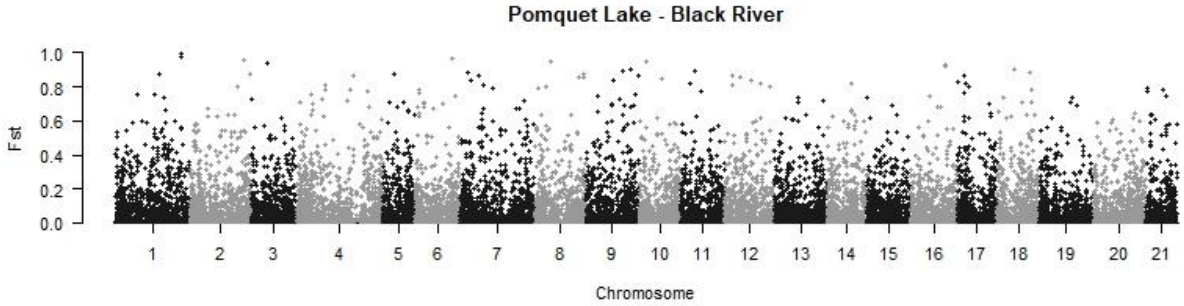
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Pomquet Lake - Black River
Fst
1.0
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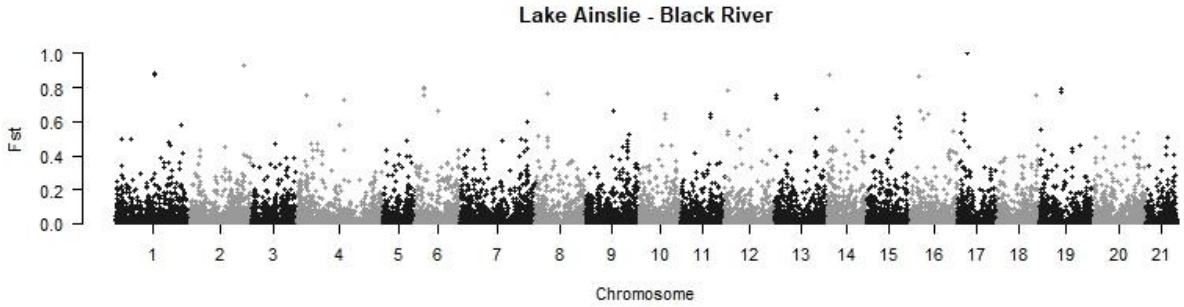
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Lake Ainslie - Black River
Fst
1.0
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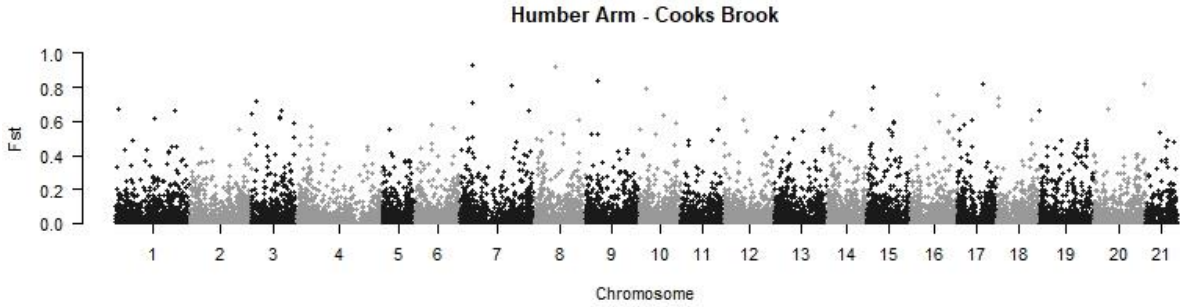
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Humber Arm - Cooks Brook
Fst
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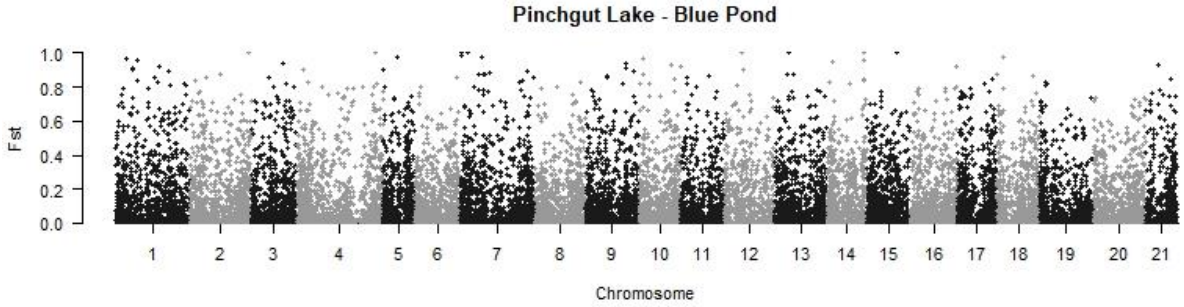
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Pinchgut Lake - Blue Pond
Fst
1.0
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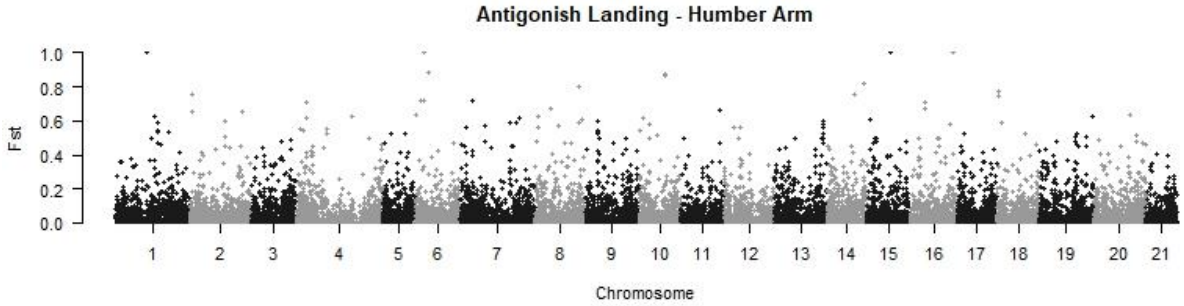
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Antigonish Landing - Humber Arm
Fst
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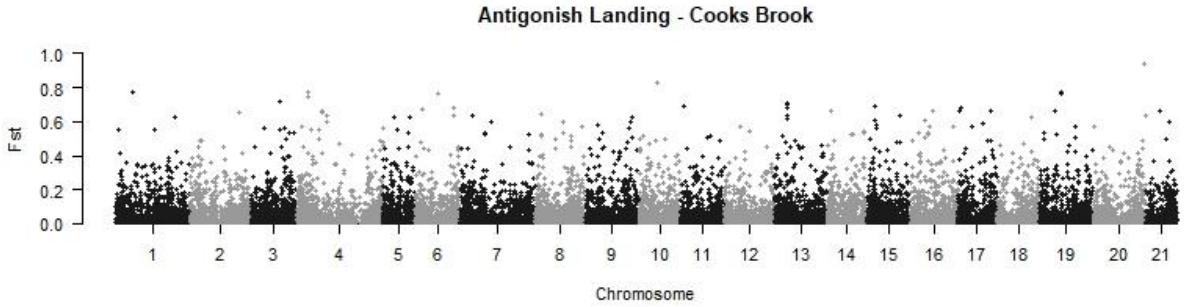
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Antigonish Landing - Cooks Brook
Fst
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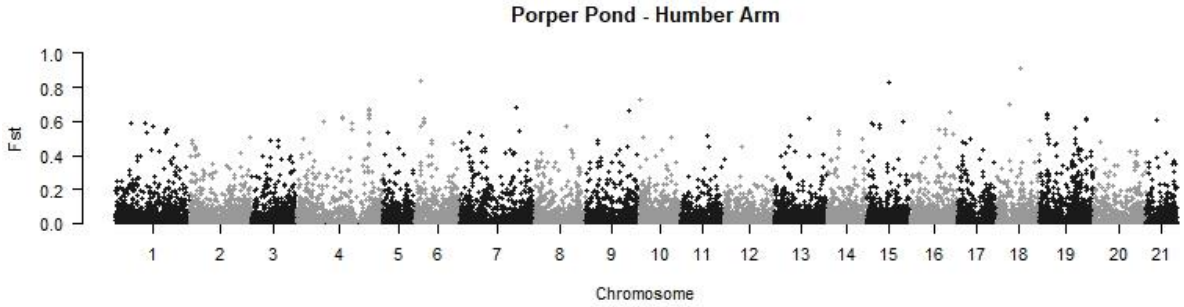
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Porper Pond - Humber Arm
Fst
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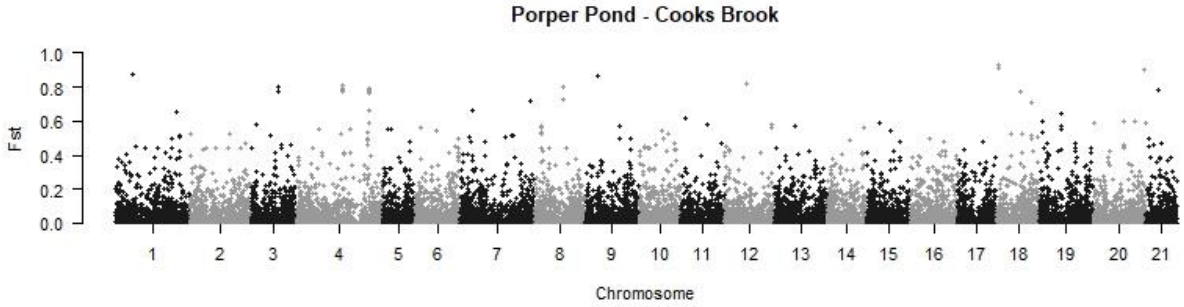
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Porper Pond - Cooks Brook
Fst
1.0
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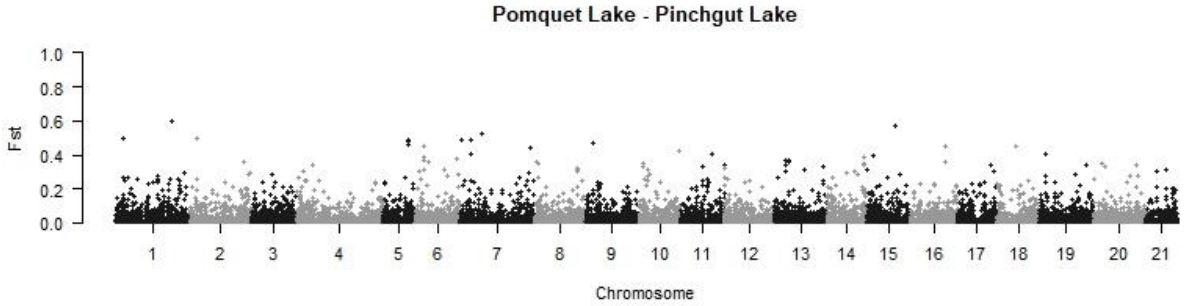
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Pomquet Lake - Pinchgut Lake
Fst
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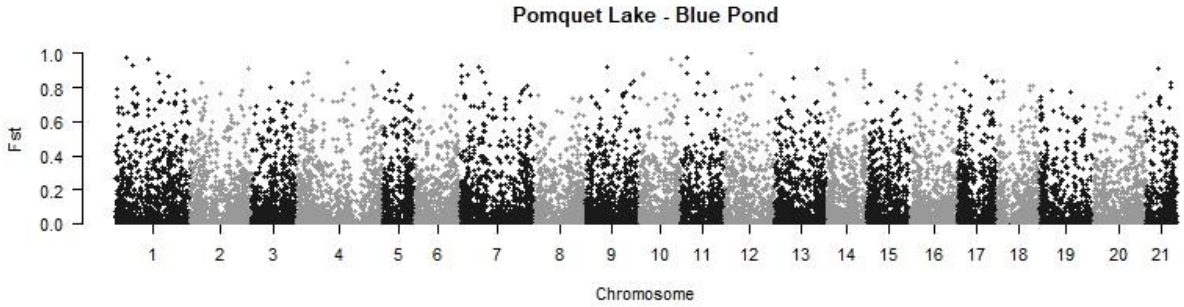
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome


Pomquet Lake - Blue Pond
Fst
1.0
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0.6
0.4
0.2
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome

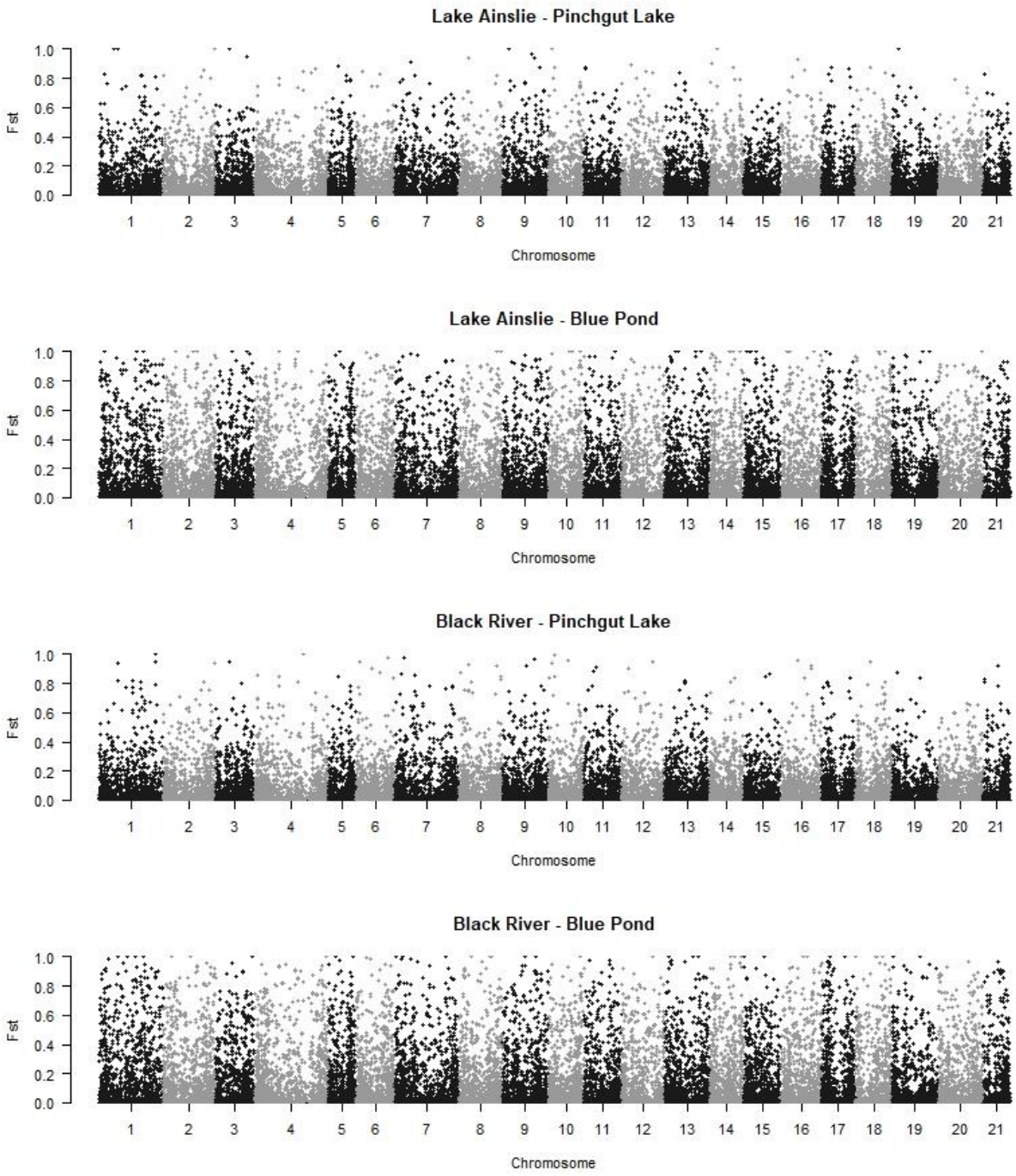

Figure 4. Manhattan plots of F_{ST} between populations from similar environments (M-M and FW-FW comparisons).

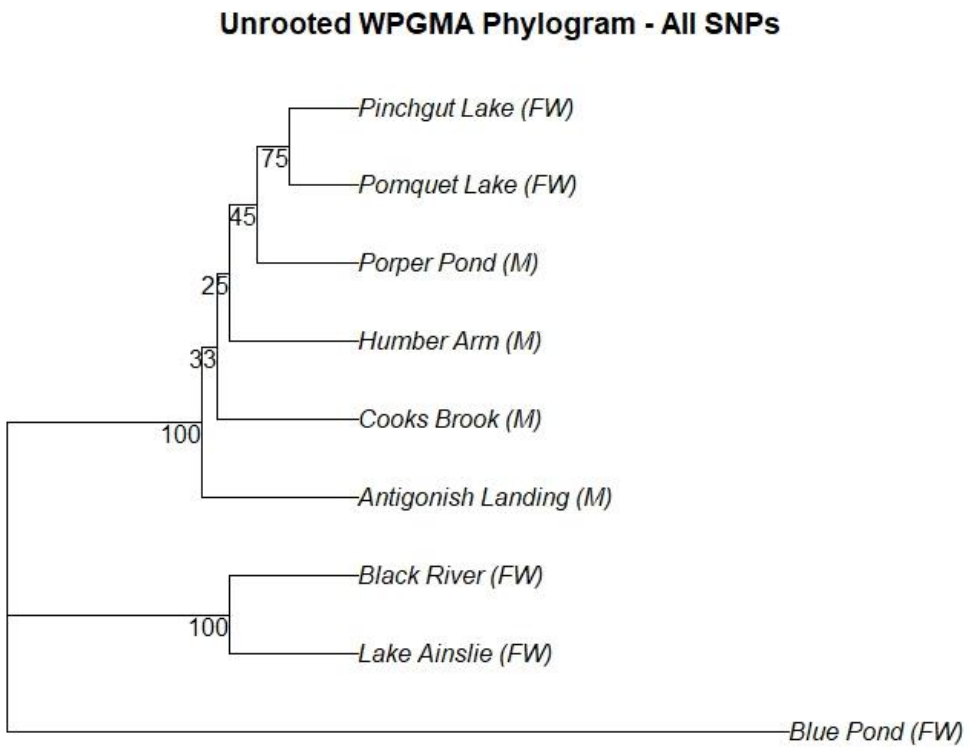

Figure 5. Phylogenetic tree of marine (M) and freshwater (FW) populations.