Genomics of freshwater adaptation in threespine stickleback from eastern Canada

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Significance statement

Threespine stickleback are a model for parallel evolution, yet most research has focused on Pacific and European populations. We studied overlooked Atlantic Canadian populations and found distinct genomic patterns of freshwater adaptation. Unlike the classic armour plate changes, adaptation in this region involves divergence near dopamine receptor genes, pointing to behavioural and hormonal pathways. These results highlight how evolution can take different genetic routes to similar ecological outcomes, underscoring the value of studying diverse geographic lineages.

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 Europe. Here, we use restriction site-associated DNA sequencing ('RAD-seq') 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 the degree of genomic differentiation between marine and freshwater populations. 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 suggests that changes in neurological phenotypes could play an important role in adaptation to freshwater environments.

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 have 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 can be 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 (the '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 (Hagen and Moodie 1982), largely confined to western North America (eastern Pacific, e.g., Colosimo et al. 2005) and parts of Europe (e.g., Klepaker 1995). For instance, considering the armour phenotype, in several regions such as eastern 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. 2023), although some exhibit distinct morphological variation (Scott et al. 2023). Despite these observations, genomic studies in this region remain limited (but see Fang et al. 2020a).

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. 2020b). As a result of this range expansion, Atlantic populations exhibit lower genetic diversity compared to their Pacific counterparts (Fang et al. 2020a). Notably, most Atlantic populations examined to date have been from 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 have been observed in freshwater habitats (Hagen and Moodie 1982; Haines et al. 2023). 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.

Results

Data sets and genomic differentiation among populations

After processing the raw RAD-seq data, we identified 18,582 SNPs shared across all nine populations and present in 36 pairwise population comparisons, including those within and between habitat types (M-M, FW-FW, and M-FW). Median genome-wide F_{ST} varied widely, ranging from 0.0118 to 0.0824 (Table S2; Figure 2). We observed considerable genomic heterogeneity among M-FW and FW-FW comparisons (Figure 3; Figure S1, S2). 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).

Excluding comparisons between populations from the same habitat type (M-M and FW-FW), we obtained 25,236 SNPs across the 20 possible M-FW comparisons within and across provinces. We used this SNP set to search for genomic regions potentially

involved in parallel freshwater adaptation (Figure 4), identifying 2,100 SNPs as F_{ST} outliers in two or more M-FW comparisons within Nova Scotia (19 SNPs across all six comparisons). In Newfoundland, we identified 1,306 SNPs as F_{ST} outliers in two or more comparisons. 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 4). 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 that 180 of 279 F_{ST} outliers map within 5 kb of 133 genes (Table S3), with 68 genes containing at least one outlier (Table S4). 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 4). The latter SNP is located 920 bp downstream from a dopamine receptor gene, *Drd2l* (XX: 14002115-14008728). The armour-plating locus (IV:12811481) was not sampled in our RAD-seq dataset, with the closest SNP mapping 72.3 Kb downstream (IV:12883820). The FET analysis identified 18 SNPs with significant allele frequency differences (FDR < 0.10) across the 10 intraprovincial M-FW comparisons, each located within 5 kb of different genes (Table S5). Three of these genes also overlap with F_{ST} outlier genes (*dmap1*, *hnrnpm*, and *drd2l*).

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 S6). Enriched molecular functions included neurotrophin binding and dopamine neurotransmitter receptor activity (Table S7).

Clustering of marine and freshwater populations

Hierarchical clustering using the WPGMA method grouped the freshwater populations into distinct clades: Lake Ainslie and Black River formed one sister group, while Pomquet Lake and Pinchgut Lake clustered together in a separate clade nested within the marine populations. Blue Pond appeared as a more genetically divergent freshwater population (Figure 5). In contrast, marine populations showed low bootstrap support at internal branches, suggesting weak genetic structure consistent with high connectivity and ongoing gene flow.

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 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 between marine and freshwater populations (Di Poi et al. 2016). Our results suggest that, even in the likely absence of key alleles for reduced armour in eastern Canadian 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. 2020b). 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. 2020a). 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, Figure S2). 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 dif-

fer substantially in their genetic divergence from marine populations (Figure 3). 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) and Europe (Mäkinen et al. 2006). The clustering of freshwater populations is consistent with at least three colonization events: one in Blue Pond, another in Lake Ainslie and Black River, and a more recent expansion into Pomquet and Pinchgut lakes. Pomquet Lake is a few kilometres (< 4 km) from the ocean and likely experiences ongoing gene-flow via Taylor Creek. An alternative scenario, though one that is less likely given the small geographic scale, involves different lineages (e.g., Blue Pond and Lake Ainslie) diverging 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 freshwater Eda allele, we did not find signs of molecular evolution near the Eda locus in our populations (Hagen and Moodie 1982; Fang et al. 2020a), although our nearest marker was more than 70 Kb away. Despite overall low differentiation, we identified signals of parallel evolution at 1.1% of SNPs, which showed repeated marine–freshwater divergence both within and across Nova Scotia and Newfoundland. Notably, this included SNPs located near dopamine receptor genes *Drd4a* and *Drd2l*, and candidate genes were enriched for functions related to dopamine receptor activity and nervous system development. These results suggest that freshwater adaptation in eastern Canadian stickleback may involve behavioural changes such as increased aggression (Di Poi et al. 2014).

While individual variation in endocrine function remains less studied than other physiological traits in evolutionary biology, it holds strong potential for linking genetics, physiology, and ecology (Williams 2008). In zebrafish (*Danio rerio*), expression levels of dopamine receptors *Drd2c* and *Drd3* have been associated with individual aggression, particularly in dominant versus subordinate males (Filby et al. 2010). In stickleback, exposure to predators has been shown to reduce *Drd4a* expression (Abbey-Lee et al. 2018), and marine and freshwater populations exhibit marked differences in behaviours such as sociability, aggression, and activity (Di Poi et al. 2014). At the population level, Di Poi et al (2016) showed evolutionary changes in physiological regulatory networks often target receptor genes—such as *Drd2*—rather than their ligands or neurotransmitters. In a separate study using stickleback from a Nova Scotia lineage (Blouw and Ha-

gen 1990), *Drd2* expression was associated with male behavioural trade-offs between courtship and territorial defence (Barbasch et al. 2023).

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. 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 repeated adaptation of behavioural and hormone-mediated phenotypes.

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 over the months of June and July in 2014 (Figure 1; Table S1). 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 (Table S1). 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, Switzerland). We then normalized the concentration of dsDNA and pooled according to sampling location. Thus, we created nine pools consisting of 30 individuals each. We applied the RAD-seq protocol used in Paccard et al. (2020). Briefly, pooled samples were digested with NlaIII and MluCI, ligated to unique barcoded adapters, size-selected

for ~400 bp fragments, enriched for biotin-tagged fragments using streptavidin beads, and PCR-amplified. Cleanup steps were performed using AMPure XP beads. RAD-seq libraries were sent for sequencing at the McGill University and Genome Quebec Innovation Center in 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 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 of allele frequency differentiation

We estimated population differentiation using F_{ST}, as defined by Hartl and Clark (1997), with the *fst-sliding.pl* script from PoPoolation2 (Kofler et al. 2011b). Because low coverage can bias FS estimates due to sampling effects, we also used a Fisher's Exact Test (FET) via *fisher-test.pl* to assess allele frequency differences. The following parameters

were applied: --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, and --suppress-noninformative. Only genomic regions assembled at the chromosome level were analyzed (i.e., scaffolds excluded).

For each marine–freshwater (M-FW) population comparison, we identified SNPs in the top 5% of the F_{ST} distribution. To identify loci with consistent differentiation (i.e., putative parallelism), we defined three sets of SNPs in the top 5% across at least two M-FW comparisons: within Nova Scotia, within Newfoundland, and across both provinces. SNPs shared among all three sets were designated as "F_{ST} outliers."

To complement the F_{ST} approach, we performed a genome-wide FET scan across 10 intraprovincial M-FW comparisons to identify SNPs showing consistent allele frequency shifts. To control for multiple testing, raw p-values were converted to q-values using the qvalue package (Storey et al. 2003), and SNPs with a false discovery rate (FDR) below 10% were retained. This FDR threshold reflects the expected proportion of false positives among significant results.

Identification of candidate genes and analysis of molecular function

Outlier SNPs are expected to be located within genes potentially under parallel selection 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 "bio-

type=protein_coding." Given that our data represent a reduced portion of the genome, the causal mutation is unlikely to be directly sampled and is more likely in linkage disequilibrium (LD) with a nearby SNP. To account for this, we examined genes either associated with outlier loci or located within 5 kb of one (e.g., Nichols et al. 2016; Chen et al. 2018). To investigate the traits putatively under selection, we analyzed the candidate genes for enrichment of molecular functions using ShinyGO 0.77 (Ge et al. 2020), which 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. We compared our list of candidate genes to a background set of 14,252 genes from which outliers were sampled, using the 'best matching species' option for further analysis.

Phylogram of marine and freshwater populations

To visualize genetic relationships among marine and freshwater populations, we constructed a phylogram using the WPGMA clustering method based on median pairwise F_{ST} values. A distance matrix was generated from genome-wide SNP data, and clustering was performed using the hclust(method = "mcquitty") function in R. The resulting dendrogram was visualized as an unrooted phylogram using phangorn::plotBS(), with branch lengths scaled to genetic distance and bootstrap support values added from 10,000 replicates. Only support values ≥10% are shown. The tree was explicitly unrooted to avoid assumptions about ancestral directionality.

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Data availability

All sequencing data generated in this study will be deposited in the NCBI Sequence Read Archive (SRA) under a BioProject accession number that will be provided upon acceptance.

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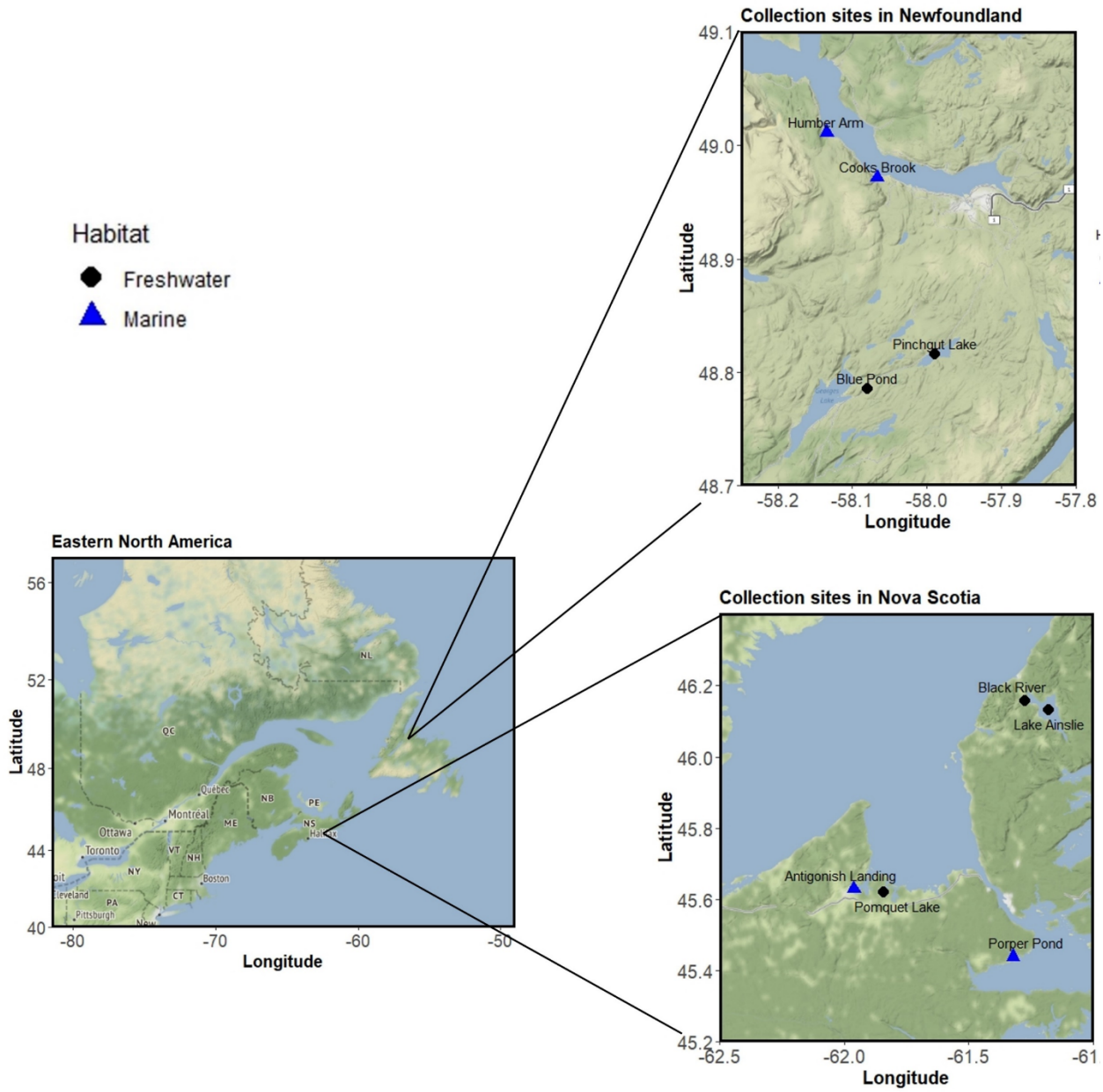

Figure 1. Collection sites in Eastern Canada.

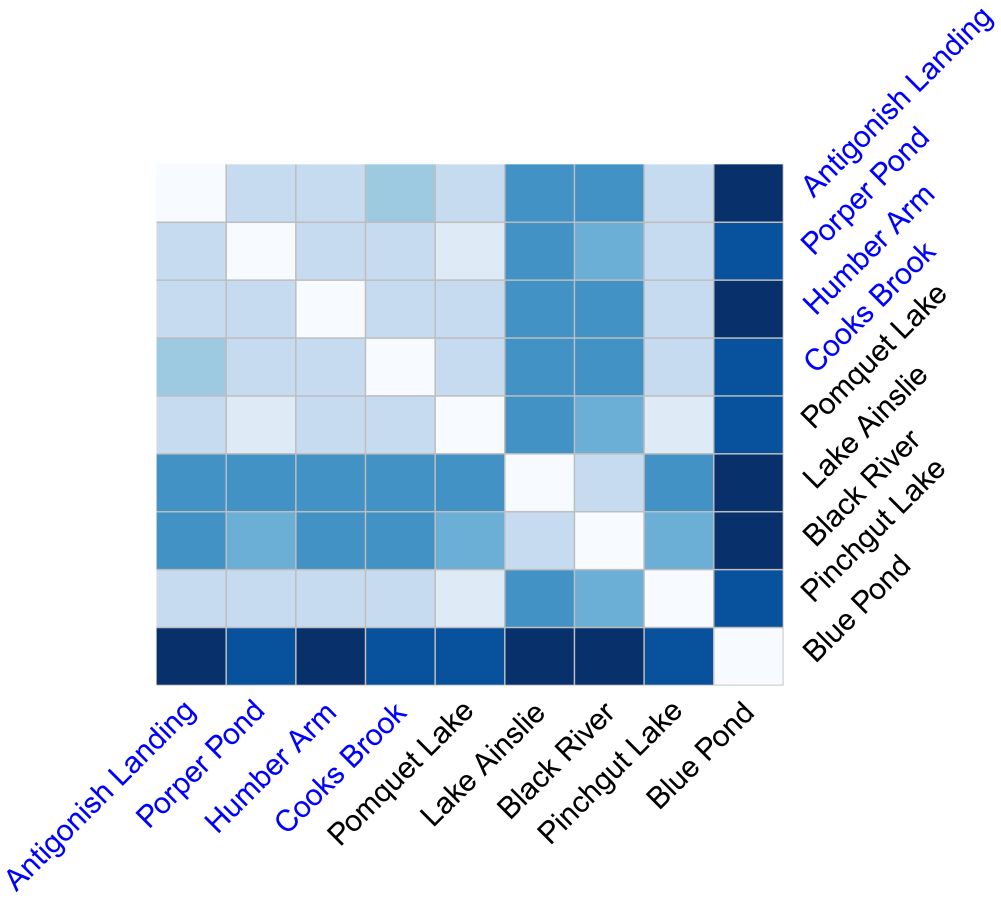

Figure 2. Heat plot depicting average (median) pair-wise F_{ST}. Freshwater sites are shown in black and marine in blue.

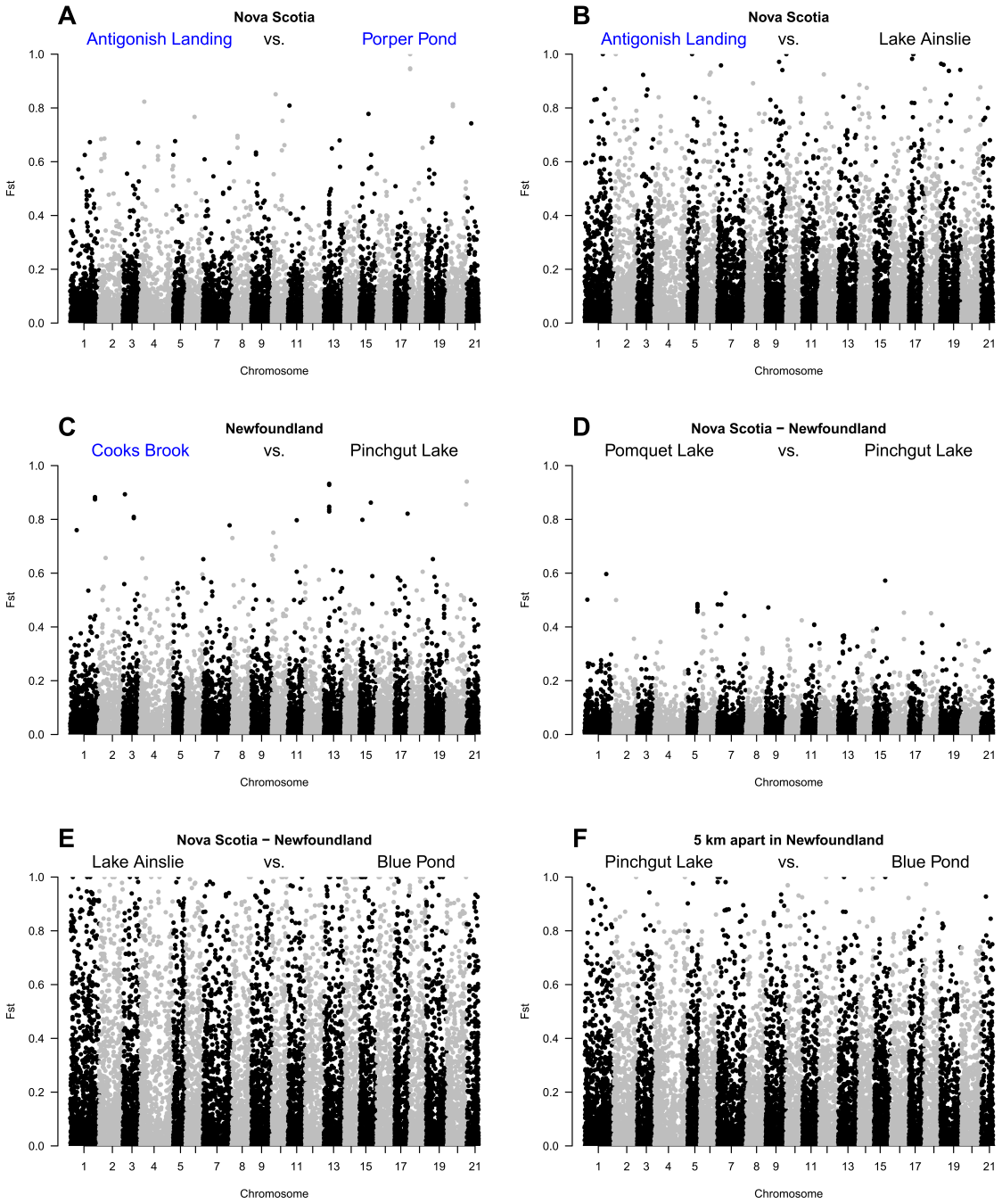

Figure 3. Manhattan plots of F_{ST} values showing heterogeneity among threespine stickleback populations from eastern Canada. Freshwater populations are shown in black and marine in blue.

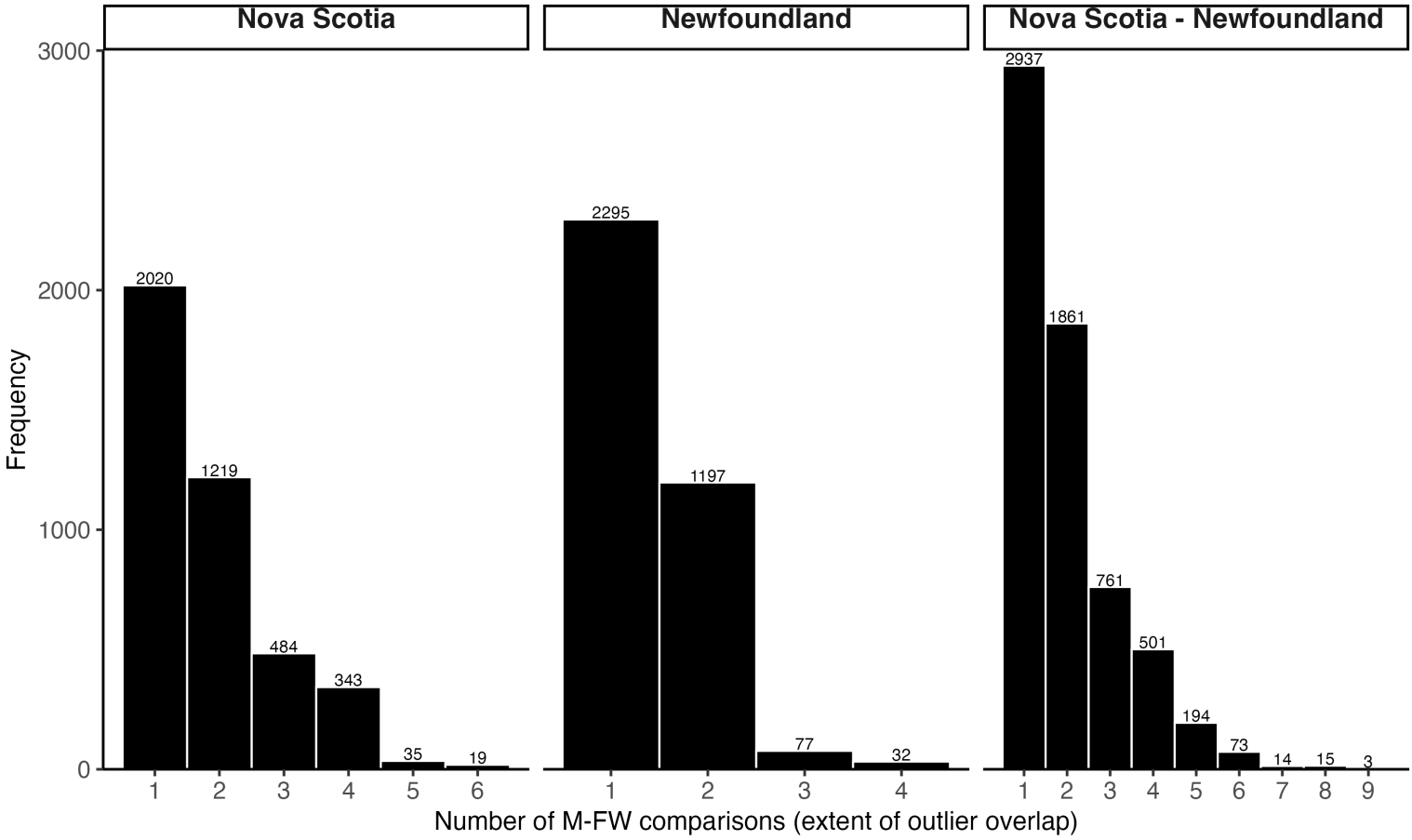

Figure 4. Overlap of F_{ST} outliers from M-FW comparisons in Nova Scotia, Newfoundland, and between Nova Scotia and Newfoundland. No SNPs were in the top 5% across all 10 M-FW comparisons between Nova Scotia and Newfoundland.

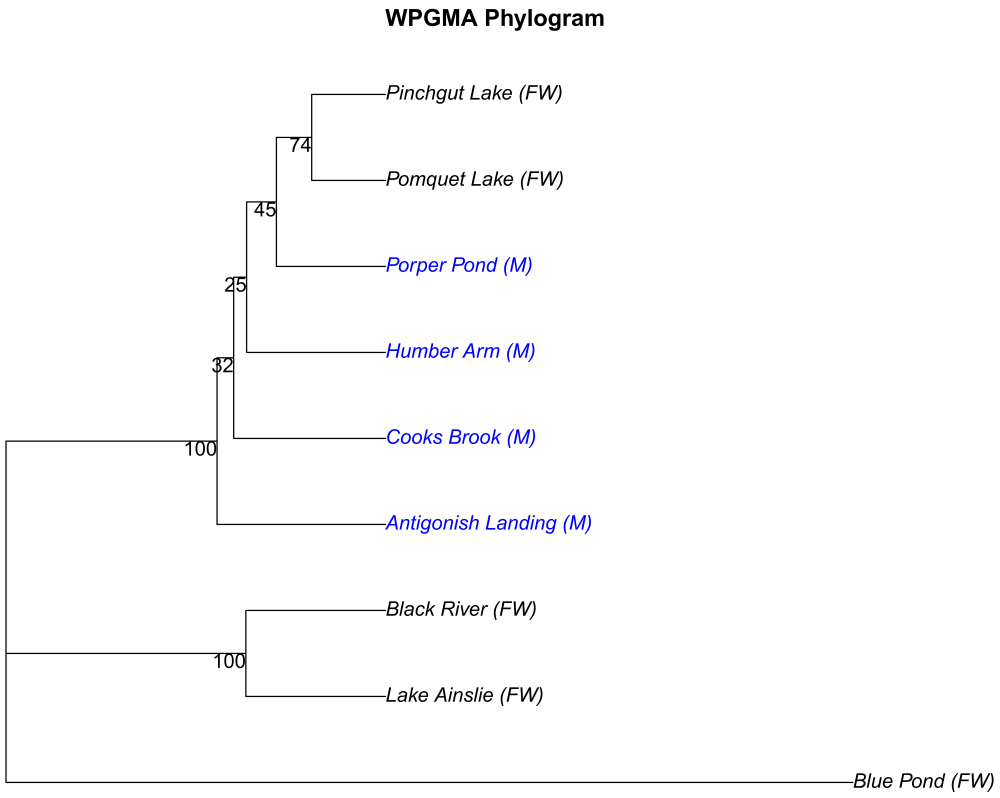

Figure 5. Phylogenetic tree of marine (M) and freshwater (FW) populations.