frontiers | Frontiers in Ecology and Evolution

TYPE Original Research
PUBLISHED 27 February 2025
DOI 10.3389/fevo.2025.1546874

OPEN ACCESS

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RECEIVED 17 December 2024
ACCEPTED 10 February 2025
PUBLISHED 27 February 2025

CITATION
Adhikari D, Karlsen BO, Jørgensen TE, Johansen SD, Nordeide JT and Moum TB (2025) The genomics of postglacial vicariance and freshwater adaptations in European subarctic threespine sticklebacks. *Front. Ecol. Evol.* 13:1546874. doi: 10.3389/fevo.2025.1546874

The genomics of postglacial vicariance and freshwater adaptations in European subarctic threespine sticklebacks

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Marine threespine sticklebacks (*Gasterosteus aculeatus*) have colonized numerous freshwater lakes since the last ice age. The loss of body armor, such as reduced pelvic spines and lateral plate numbers, is a recurrent feature upon freshwater colonization and is attributed to parallel evolution. This study examines genome-wide genetic diversity and differentiation among sticklebacks from a marine site and two freshwater lakes of the same watercourse in subarctic Europe. The upper lake is unique in that it harbors a population of polymorphic sticklebacks, some with fully developed and others with reduced pelvic structures. Our results based on deep sequencing of pooled population samples showed common signatures of selection for freshwater sticklebacks in certain parts of the genome, such as the *Eda* containing region, but also evidence of differential selection, and the presence of large chromosomal inversions that seem to play an essential role in stickleback evolution. Pelvic reduction in sticklebacks has previously been linked to deletions in the enhancers of the pituitary homeobox transcription factor gene (*Pitx*1). While the genetics of *Pitx*1 seem unable to fully explain pelvic spine polymorphism in this population, we found differentiation between spined and spineless sticklebacks in several genomic regions, which harbor genes that might be involved in pelvic development. Most significantly, genetic differentiation between spined and spineless sticklebacks was noted in a region of chromosome 9 where the gene *Hand*2, previously implicated in limb development, is located. Our findings suggest that pelvic reduction in these sticklebacks involves multiple genetic factors, indicating parallel evolution through polygenic influences.

KEYWORDS

stickleback, spineless, parallel evolution, pooled population samples, genome-wide, *Pitx*1, *Hand*2

1 Introduction

The threespine stickleback (*Gasterosteus aculeatus*) is a small teleost fish species (typically 30-60 mm in length) that inhabits coastal and inland waters in the Northern Hemisphere. Marine sticklebacks are anadromous; they live in marine habitats, but migrate to breed in brackish or fresh waters. Being tolerant to changes in salinity, marine threespine sticklebacks commonly colonize freshwater bodies and establish novel populations, some of which, upon restricted gene flow, form freshwater ecotypes that complete their entire life cycle in freshwater. The evolutionary trajectory from marine to freshwater ecotypes in threespine sticklebacks involves a range of changes in life history, physiology, behavior, and morphology, among which the reduction of external body armor is a common and recurrent feature. Marine sticklebacks invariably possess strong armor, including lateral bony plates, three dorsal spines, and two pelvic spines, which serve as protection against gape-limited predators such as birds and fishes (Wootton, 1976). In contrast, freshwater sticklebacks typically exhibit fewer bony plates and, less commonly, reduced pelvic structures (Figure 1). These reductions in body armor are thought to be due to changes in selective regime that sticklebacks experience upon transitioning to freshwater habitats, which often feature lower calcium availability and relaxed predation

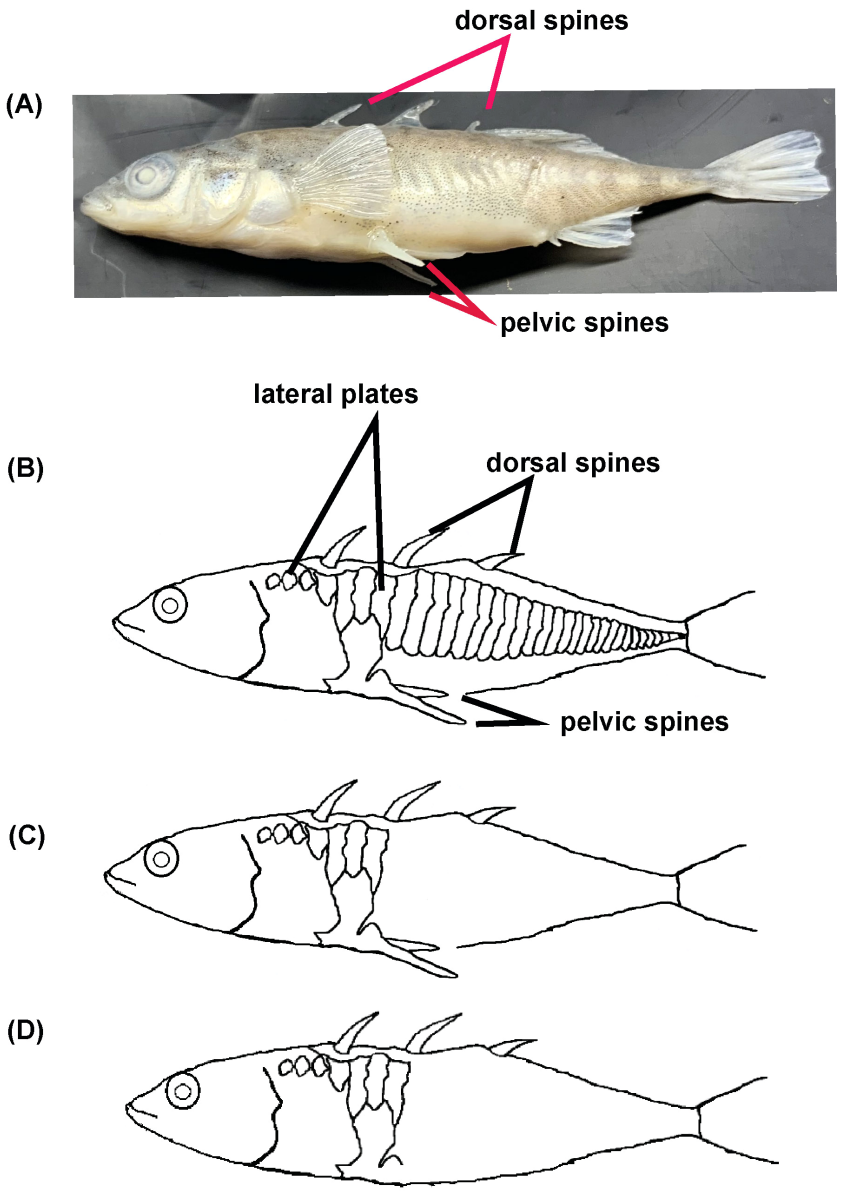

FIGURE 1
Variation in morphological features in threespine sticklebacks. **(A)** A photographic image of a marine threespine stickleback showing dorsal spines and pelvic spines. Schematic diagrams of **(B)** marine stickleback with well-developed lateral plates, dorsal spines, and pelvic spines; **(C)** freshwater stickleback with reduced lateral plates and well developed pelvic spines; **(D)** freshwater stickleback with a complete reduction of pelvic spines and reduced lateral plates.

pressure compared to marine environments (Bell et al., 1993; Hagen and Gilbertson, 1973; Klepaker, 1995; Moodie and Reimchen, 1976; Reimchen, 1983).

In principle, the evolution of adaptive traits in organisms colonizing new habitats could arise either from selection on standing genetic variation (SGV) in the ancestral population or from *de novo* mutations in the founder population (reviewed by Schluter and Conte, 2009). SGV refers to the pre-existing genetic variation, or the presence of multiple alleles at particular loci, within a population. Several authors have argued that alleles from SGV are more commonly involved than *de novo* mutations for several reasons: (*i*) they are usually present in higher frequencies, (*ii*) they are immediately available in the new habitat, and (*iii*) they have already been tested in past environments (Barrett and Schluter, 2008; Innan and Kim, 2004; Schluter et al., 2004). Supporting this view, there are indications of the reuse of ancestral genetic variants for freshwater adaptation in sticklebacks, distributed across multiple chromosomes and genomic regions (Hohenlohe et al., 2010; Jones et al., 2012; Liu et al., 2018). Some of these genetic variants, including some that reside within chromosomal inversions, appear to be ancient, dating back several million years (Nelson and Cresko, 2018). However, it has also been shown that the evolution of similar phenotypic traits in subspecies or populations of the same species could arise either from *de novo* mutations in genes known to be involved, or from the combined effects of several loci, as the traits in question are often quantitative and polygenic (reviewed by Arendt and Reznick, 2008; Hoekstra et al., 2006).

The number of ancestral alleles within the SGV of sticklebacks that are adapted to freshwater environments could be reduced due to genetic drift and selection against these alleles during range expansions through the marine environment. Thus, we expect the amount of shared genetic variants for freshwater adaptation to be associated with colonization history and geographic distance among a given collection of populations. Fang et al. (2018) reconstructed the worldwide phylogenetic relationships and colonization history of threespine sticklebacks, inferring that current populations originated in the Pacific Ocean during the late Pleistocene. Sticklebacks then colonized the Atlantic Ocean through the Arctic approximately 40-50 thousand years ago, initially forming a southern European clade and later a derived trans-Atlantic clade that includes stickleback populations in eastern North America and northern Europe. In compliance with this, shared ancestral polymorphisms appear to be more common among Eastern Pacific freshwater locations than on a global scale (DeFaveri et al., 2011; Fang et al., 2020; Jones et al., 2012). Also, while the majority of earlier studies were based on limited geographic sampling and focused on the Eastern Pacific region, recent studies have suggested the presence of previously unexplored large-scale geographic heterogeneity in the genomic basis of parallel evolution among sticklebacks (Fang et al., 2020; Terekhanova et al., 2019).

The loss of lateral bony plates, a feature most typical of marine-freshwater transitions in sticklebacks, can evolve within a few decades (Bell, 1994; Klepaker, 1993; Roberts Kingman et al., 2021). The ectodysplasin gene (*Eda*) on chromosome 4, which is regulated by a *cis*-regulatory element, appears to be responsible for the majority of variation in bony plates (Colosimo et al., 2004; Cresko et al., 2004; O'Brown et al., 2015). However, several loci of minor importance, mapping to other linkage groups, have also been implicated (Colosimo et al., 2004; Cresko et al., 2004; Peichel et al., 2001). The reduction of lateral bony plates in freshwater populations is commonly attributed to selection on SGV (Colosimo et al., 2005; Schluter and Conte, 2009).

While the loss of lateral bony plates is common in freshwater populations, the partial or complete loss of pelvic spines is less frequent, except in certain freshwater populations such as those in Cook Inlet, Alaska (Bell et al., 1993; Bell and Ortí, 1994; reviewed by Klepaker et al., 2013). A few other populations with reduced pelvic structures are found in lakes across British Columbia, Iceland, Scotland, and Norway (Chan et al., 2010; Coyle et al., 2007; McPhail, 1992; Peichel et al., 2001; Shapiro et al., 2004; reviewed by Klepaker et al., 2013). In Norway, pelvic reduction is reported in only eight out of more than 200 examined populations (Klepaker and Østbye, 2008; Klepaker et al., 2013). A major determinant of pelvic development in threespine sticklebacks is the pituitary homeobox transcription factor gene *Pitx*1, located on chromosome 7 (Chan et al., 2010; Coyle et al., 2007; Cresko et al., 2004; Shapiro et al., 2004). The upstream enhancer *Pel*A of the *Pitx*1 is crucial for regulating its expression, and the presence of multiple TG-repeats in *Pel*A makes it prone to mutation, providing a molecular mechanism for recurrent pelvic reductions (Chan et al., 2010). Another enhancer of *Pitx*1, the *Pel*B, and additional loci on chromosomes 2, 4, and 8 are also suggested to be involved in the development of pelvic spines (Peichel et al., 2001; Shapiro et al., 2004; Thompson et al., 2018).

In this study, we examined the genomics of polymorphic sticklebacks from two lakes within the same watercourse, along with specimens from a nearby marine location in subarctic Norway, Northern Europe. These sticklebacks are presumably among the most distantly related to ancestral Pacific populations (Fang et al., 2018). Sticklebacks from both lakes exhibit the typical freshwater phenotypic feature of having fewer lateral plates than marine conspecifics. Additionally, sticklebacks from the upper lake of these two lakes exhibit polymorphism in their pelvic structures, ranging from fully spined to asymmetrically spined and spineless. Previously, we showed that the number of TG-repeats in *Pel*A differed between the two lakes with *Pel*A being consistently shorter in individuals from the upper lake. However, no clear association was observed between *Pel*A enhancer variants and pelvic status among individuals from the upper lake. These findings suggest that additional loci may be involved in the variable manifestation of pelvic spines in this species (Adhikari et al., 2023).

We sequenced pooled DNA samples of sticklebacks from each of the three study locations to provide a first assessment of genome-wide diversity and differentiation, including chromosomal inversions, among marine and vicariant freshwater sticklebacks in the European subarctic. We sought to identify genomic regions displaying signatures of selection potentially involved in the adaptation of sticklebacks to freshwater environments. Further, we conducted a bulk segregant analysis of spined and spineless sticklebacks from the same lake, specifically aiming to identify

differences in the *Pitx*1 locus and genomic signatures of differential selection between the two groups, and to indicate candidate genes that could be involved in the evolution of pelvic reduction. Two individual sticklebacks, one spined and one spineless specimen, were further subjected to long-read sequencing, aiming to resolve their *Pitx*1 genotypes.

2 Materials and methods

2.1 Sampling and data collection

A total of 426 threespine sticklebacks were collected from two freshwater lakes - Lake Storvatnet (68°46′49″N, 15°9′36″E; 80 m altitude), and Lake Gjerdhaugvatnet (68° 46'17"N, 15° 9'2"E; 20 m altitude), which are part of the same watercourse (Figure 2) in June 2017, 2019 and 2020. Additional samples were taken from a nearby marine location at the tidal mouth of a small river at Sandstrand (68°44′45″N, 15°20′42″E), Langøya Island in Northern Norway in June 2020 (Figure 2). Several waterfalls between the two lakes and between the lower Lake Gjerdhaugvatnet and the sea, prevent gene flow between the three stickleback populations. The sticklebacks from the marine site were collected at the outlet of a small river, where they can move freely between water of varying salinity, which is dynamic due to the tidal cycle. The total length of each specimen was measured, and only specimens ≥ 3 cm long were included. The specimens were euthanized with tricaine methanesulfonate (MS222), and care was taken to preserve their pelvic structures. The caudal fin was cut off and discarded, and 5 mm posterior fin-muscle samples were collected, homogenized by bead beating using a Dremel 8220 rotary tool (MP Biomedicals) and 0.5 ml DNA/RNA Shield solution (Zymo Research), and then kept at low temperatures for subsequent analysis. Morphological traits, such as lateral plate number and pelvic score (PS), were assessed using a 20x magnifying stereomicroscope. Each side of the fish (left and right) was assigned a PS between 0 to 4 (as defined by Bell's five-graded scale; Bell,

FIGURE 2
Sampling sites in Norway. Map showing the sampling sites at Langøya island: the upper Lake Storvatnet (68° 46'49''N, 15° 9'36''E), the lower Lake Gjerdhaugvatnet (68° 46'17''N, 15° 9'2''E) in the watercourse, and the marine sampling site (68° 44'45''N, 15° 20'42''E) located about 8 km from the two lakes.

1987). The combined pelvic scores (CPS), ranging from 0 to 8, were then computed (Adhikari et al., 2023) (see Supplementary S1). Based on lateral plate morphology, specimens were categorized as "completely plated", "partially plated", and "low plated" (Klepaker et al., 2012; O'Brown et al., 2015; Schröder et al., 2023; Wootton, 1976) (see Supplementary S2).

2.2 Preparation of samples for pooled DNA sequencing

DNA was extracted from 20 µl of stored homogenized samples with a Monarch genomic DNA purification kit (New England Biolabs), and quality checked with Qubit 4 fluorometer (Invitrogen, Thermo Fisher Scientific). DNA samples with concentrations ≥20 ng/µl were included.

Four DNA pools were prepared from three locations, with each pool containing 40 individuals (Karlsen et al., 2024). The four DNA pools were: *i)* "Spined-Storvatnet", featuring specimens from Lake Storvatnet with fully developed symmetric pelvic spines on both sides (CPS = 4 + 4), *ii)* "Spineless-Storvatnet", also from Lake Storvatnet, including specimens with reduced pelvic spines or pelvic apparatus on both sides (spineless – defined by CPS= 2-4; see Table 1), *iii)* "Gjerdhaugvatnet", and *iv)* "Marine". Thus, although symmetric-spined, spineless, and asymmetric-spined sticklebacks were sampled in Lake Storvatnet, only DNA pools consisting of spined and spineless sticklebacks were prepared for genomic comparison of the two morphotypes through bulk segregant analysis. No sticklebacks with reduced pelvic spines were found in Lake Gjerdhaugvatnet or the marine site; all specimens from these locations were fully spined (CPS = 4 + 4) (Adhikari et al., 2023).

First, DNA samples with concentrations above 20 ng/µl were diluted to 20 ng/µl. Then 5 µl (100 ng) aliquots from each of 40 DNA samples were mixed to prepare a 200 µl (4000 ng) DNA pool in a 1.5 ml Eppendorf tube (Sham et al., 2002), using the elution buffer provided with the kit as a diluent. The concentration of each DNA pool was verified using both Nanodrop and a Qubit 4 fluorometer, and sample quality was assessed by agarose gel electrophoresis and visual inspection.

2.3 Sequencing

2.3.1 Library preparation and sequencing

The four DNA pools were sent to the Norwegian Sequencing Center (NSC) at the University of Oslo (UiO) for whole genome sequencing (WGS). From each of the four DNA pools, 250 ng genomic DNA was used as input to the Illumina DNA Prep protocol. This protocol applies the tagmentation procedure to fragment DNA and add adapters (formerly known as Nextera Flex; Illumina, San Diego, CA, USA). Libraries were prepared according to the manufacturer's procedures, using six cycles of PCR amplification and incorporating unique-dual-indexes. Sequencing was performed on a NovaSeq instrument (Illumina) using a ¼ S4 flow-cell, with the XP workflow, employing 150 bp paired-end reads following the manufacturer's recommendations and running RTA v3.4.4. Demultiplexing was carried out using bcl2fastq v2.20.0.422, and the data were received as raw reads from NSC.

2.3.2 Bioinformatic analysis

Before further processing, the raw data for the pooled population samples with higher read numbers were downsized to approximately the same number of reads as the "Spined-Storvatnet" pool using the reformat tool of BBMap (version 39.01) (https://sourceforge.net/projects/bbmap/). Raw reads were trimmed from adapters and low-quality bases using the Fastp program (version 0.20.0) and data quality was checked using the FastQC program (version 0.11.9). Sequence alignment was performed with the BWA-mem alignment algorithm (version 0.7.17-r1188), producing SAM mapping files. During alignment, the data were mapped to the recent version of the reference genome of threespine stickleback, GAculeatus_UGA_version5 (GeneBank assembly accession: GCA_016920845.1; RefSeq assembly accession: GCF_01692084). The SAM files were converted into sorted BAM files using Samtools version 1.13. PCR duplicates from reads were removed with Picard.jar (version 2.27.2). The statistics, coverage, and depth of the BAM files were checked with Samtools. Subsequently, Mpileup and Synchronization (sync) files were generated from the BAM files, and these files were used with the PoPoolation tool (version 1.2.2 and version 2_1201) (Kofler et al., 2011a, b) to analyze population genetics parameters for the stickleback population samples (see Supplementary S3).

2.4 Estimation of population genetic parameters among freshwater and marine sticklebacks

We used the PoPoolation toolbox (version 1.2.2) (Kofler et al., 2011a) to estimate (i) nucleotide diversity (π), which represents the average number of nucleotide differences per site between DNA sequences in a population sample (Nei and Li, 1979), and (ii) Tajima's D (T_D), a test statistic for assessing neutrality by comparing the mean pairwise difference (π) between sequences in a population sample to the number of polymorphic sites (s) (Tajima, 1989) across all chromosomes for the three population samples. It should be noted that the "Spined-Storvatnet" pool was used to represent stickleback specimens from Lake Storvatnet, as the other two populations contained only spined sticklebacks. We employed a sliding window approach with a window size of 4000 bp and a step size of 2000 bp (see Supplementary S3; Kofler et al., 2011a).

Genetic differentiation, quantified using F_{ST}, measures the genetic variance between two or more populations (Gregorius, 1987) based on allele frequencies. In this study, pairwise F_{ST} values were calculated between the three population samples using the PoPoolation2 tool (version 2_1201) (Kofler et al., 2011b). F_{ST} values were computed across all chromosomes using a sliding window approach with a window size of 4000 bp and a step

size of 2000 bp, consistent with the parameters used for analyzing nucleotide diversity and T_D (see Supplementary S3).

The resulting nucleotide diversity, T_D, and F_{ST} values were then imported into R (version 4.2.0, R studio v 1.4.1717) for downstream analysis. Our aim was to identify genomic regions displaying structural variation such as inversions, and signatures of selection indicating local adaptation. We also identified loci potentially involved in limb or bone development, residing within genomic regions that were highly differentiated between sites. We specifically focused on examining key loci, including the *Eda* locus and the *Pitx*1 locus, to understand their role in the variation of bony armor structures observed across the studied stickleback populations.

2.5 Bulk segregant analysis of spined and spineless sticklebacks from Lake Storvatnet

2.5.1 Whole genome analysis

The genetic basis for pelvic spine reduction in sticklebacks from Lake Storvatnet is not well understood (Adhikari et al., 2023). We calculated nucleotide diversity and T_D along the genome for spined and spineless groups using "Spined-Storvatnet" and "Spineless-Storvatnet" DNA pools, respectively, as previously described. To identify genomic loci associated with pelvic spine reduction, we examined genome-wide differentiation (F_{ST}) between the spined and spineless groups, applying the same parameters as outlined above. Fisher's Exact test (with the same parameters as described above) was then employed to estimate statistically significant differences in SNP frequencies between the two groups, with a significance level set to $-\log_{10}(p) < 5x10^{-8}$ (Kofler et al., 2011b) (see Supplementary S3).

2.5.2 *Pitx*1 (BAC clone) analysis

Unfortunately, the latest reference genome assembly against which we mapped the PoolSeq reads does not include the *Pitx*1 locus. Previous reference genome assemblies also lack this locus. The *Pitx*1 gene is located within a repetitive region at the sub-telomeric end of chromosome 7, which poses challenges for assembling it as a linked contig. Much of the work on the complete sequence of this region is based on Sanger-sequenced BAC libraries (GenBank: GU130435.1) (see Chan et al., 2010). Therefore, to examine the *Pitx*1 locus, we mapped the PoolSeq data from spined and spineless specimens from Lake Storvatnet against the BAC sequences. As controls, we used PoolSeq data from downstream Lake Gjerdhaugvatnet and the marine site. Coverage depths at the *Pitx*1 locus were calculated from the mapped BAM files for both spined and spineless sticklebacks from Lake Storvatnet, as well as the samples from Lake Gjerdhaugvatnet and the marine site. Following this, we estimated nucleotide diversity, T_D, and genetic differentiation using the previously described parameters.

2.5.3 Nanopore long-read sequence analysis of the *Pitx*1 locus

We performed Nanopore long-read sequencing of one spined (CPS=4 + 4 = 8) and one spineless stickleback (CPS=1 + 1 = 2) from Lake Storvatnet to further inspect the *Pitx*1 locus. The Oxford Nanopore Technologies (ONT) library was prepared using the Native Barcoding Kit 24 V14 (SQK-NBD114.24), following the manufacturer's standard protocol. Total DNA for ONT sequencing was extracted using the Monarch HMW DNA Extraction Kit (New England Biolabs). Duplex sequencing was performed on the PromethION P2 Solo platform, following the recommended procedures for priming and loading the flow cell.

The raw sequencing data were processed using the Dorado basecaller (v. 0.5.0) to convert raw reads into unaligned BAM format. These unaligned BAM files were then converted into FASTQ format using Samtools (version 1.13). The Porechop (version 0.2.4) was used to trim adapters and chimeric reads present in the FASTQ sequences, and NanoFilt (version 2.6.0) was used to remove low quality reads. The trimmed FASTQ files were mapped with the BAC libraries (GenBank GU130435.1) to inspect changes within *Pitx*1 locus with the help of the minimap2 (version 2.24-r1122) program to create SAM files. SAM files were then converted to sorted BAM files with the help of Samtools. Further, coverage depth of the BAM files was compared between the two individual (spined S01 and spineless S27) sticklebacks to identify differences along the *Pitx*1 locus.

2.6 Animal welfare

The study was carried out according to ethical guidelines stated by the Norwegian Ministry of Agriculture and Food through the Animal Welfare Act. According to these guidelines, we were not required to, and therefore do not have, a specific approval or approval number.

3 Results

Of the 304 sticklebacks from Lake Storvatnet, 37% were fully spined, 33% had asymmetric spines (mostly left-biased asymmetric), and 30% were spineless (Table 1). In contrast, all specimens from Lake Gjerdhaugvatnet (N=73) and the marine site (N=50) had fully developed right and left pelvic spines, proportionate to their body size (Figure 3). For this study, only fully spined and spineless specimens from Lake Storvatnet were analyzed. All examined sticklebacks in both Lake Storvatnet and Lake Gjerdhaugvatnet were low plated. In contrast, the marine pooled sample consisted of 32 completely plated, 5 partially plated, and 3 low plated specimens (see Supplementary S1, S2).

3.1 Genome coverage and sequencing depth of pooled DNA samples

Overall sequencing depth (raw data) for each DNA pool ranged from 507x to 728x. Approximately 660 million (M) reads per population sample were successfully mapped against the threespine stickleback reference genome. Of these, about 94%

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TABLE 1 Morphological measurements of threespine sticklebacks from the three examined populations.

Site	Frequency	Morphology	Pelvic score		CPS	Spine length (cm) (mean ± sd)	Body length (cm) (mean ± sd)	Lateral plate
			Left	Right				
Lake Storvatnet (Total = 304)	**113** (37%)	Spined	4	4	8	0.26 ± 0.100	4.7 ± 0.60	LP
	92 (30%)	Spineless						
	32		3	3	6			
	1		1	3	4			
	51		1	1	2			
	8		2	2	4			
	99 (33%)	Asymmetric						
	35		4	4 (short)	8			
	22		4 (short)	4	8			
	7		3	4	7			
	29		4	3	7			
	1		4	2	6			
	5		4	1	5			
Lake Gjerdhaugvatnet	73 (100%)	Spined	4	4	8	0.37 ± 0.070	4.1 ± 0.60	LP
Marine site	50 (100%)	Spined	4	4	8	0.55 ± 0.100	4.8 ± 0.70	CP+PP+LP

LP-Low-plated (Lateral plate consisted of few anterior plates only); PP, Partially-plated (higher number of plates (and keel at the posterior portion) than LP but not all plates are present; CP, Completely-plated.

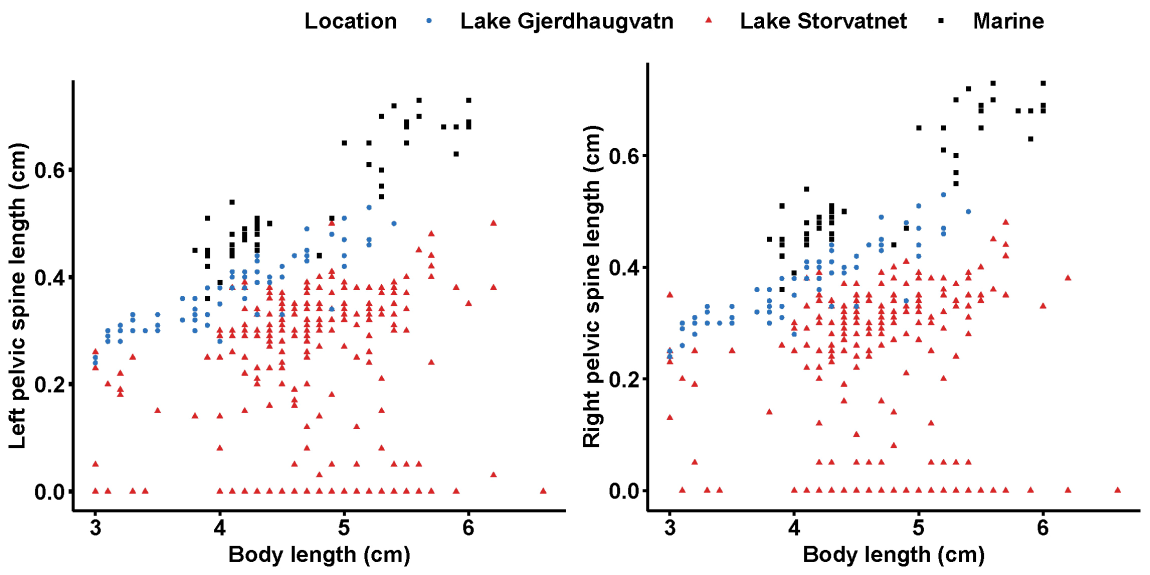

FIGURE 3
Scatter plot presenting the distribution of specimens among three locations (Lake Storvatnet, Lake Gjerdhaugvatnet, and the marine site) based on the total length of the body and the length of left and right pelvic spines. In general, specimens from the marine site have longer pelvic spines relative to their body size compared to those from the lakes. Specimens from Lake Storvatnet exhibit the shortest or most reduced spines in proportion to body size.

were properly paired, yielding an average mapping quality of 35.7 for each population sample (see Supplementary S4). Across the chromosomes, sequencing coverage ranged from 96% to 99%, with a depth of 159x to 225x, except for the Y chromosome (chr). The Y chromosome showed lower coverage depth in all samples compared to other chromosomes and was therefore excluded from the subsequent calculations of population genetic parameters (see Supplementary S5).

3.2 Genome-wide patterns of genetic diversity and differentiation among populations

The assessment of genome-wide diversity and differentiation among sticklebacks from a marine population and two freshwater lakes in Northern Norway revealed demographic patterns and evidence of selection, shedding light on the evolutionary processes shaping these populations. The marine population exhibited an average nucleotide (genetic) diversity (π) of 0.33% (0.0033), while sticklebacks from Lake Gjerdhaugvatnet and Lake Storvatnet showed lower average π values of 0.21% and 0.23%, respectively (see Supplementary S6, S10). Additionally, the marine population had a more negative average Tajima's D (T_D) of -1.10, indicating a possible excess of rare alleles (purifying selection), compared to the less negative averages of -0.73 in Lake Gjerdhaugvatnet and -0.78 in Lake Storvatnet (see Supplementary S6, S11). The average genetic differentiation (F_{ST}) value between sticklebacks from the two freshwater populations was relatively high (0.21), reflecting pronounced genetic separation between these populations. Furthermore, marine sticklebacks exhibited slightly greater genetic differentiation from sticklebacks in Lake Storvatnet (average F_{ST} = 0.15) compared to those in Lake Gjerdhaugvatnet (average F_{ST} = 0.14) (Figure 4; see Supplementary S7, S12). Notably, all of the population genetic parameters F_{ST}, π, and T_D varied widely among chromosomes and chromosomal segments (see Supplementary S6, S7, S10-S12).

3.2.1 Genomic segments showing signatures of selection

Prominent signatures of selection acting on specific parts of several autosomes (chromosomes 1, 3, 4, 5, 6, 7, 8, 9, 11, 12, 14, 17, 20, and 21) were observed based on patterns in π, T_D and F_{ST} values along the genome (see Supplementary S10-S12). Typically, we observed evidence of directional selection in specific parts of the genome, characterized by highly negative T_D values (~ -2.0 to -4), lower π values, and high genetic differentiation between one or both of the freshwater populations and the marine (exemplified in Figure 5). In specific, on chr 9, regions spanning 12.1-12.3 Mb, and 12.6-12.8 Mb (Figure 5A) show indications of directional selection in Lake Storvatnet. Similarly, in Lake Gjerdhaugvatnet, patterns indicative of directional selection were detected in a region spanning 10.1-10.8 Mb on the same chromosome (Figure 5B). These observations underscore directional selection as an important evolutionary force shaping the genetic landscape of these subarctic freshwater stickleback populations.

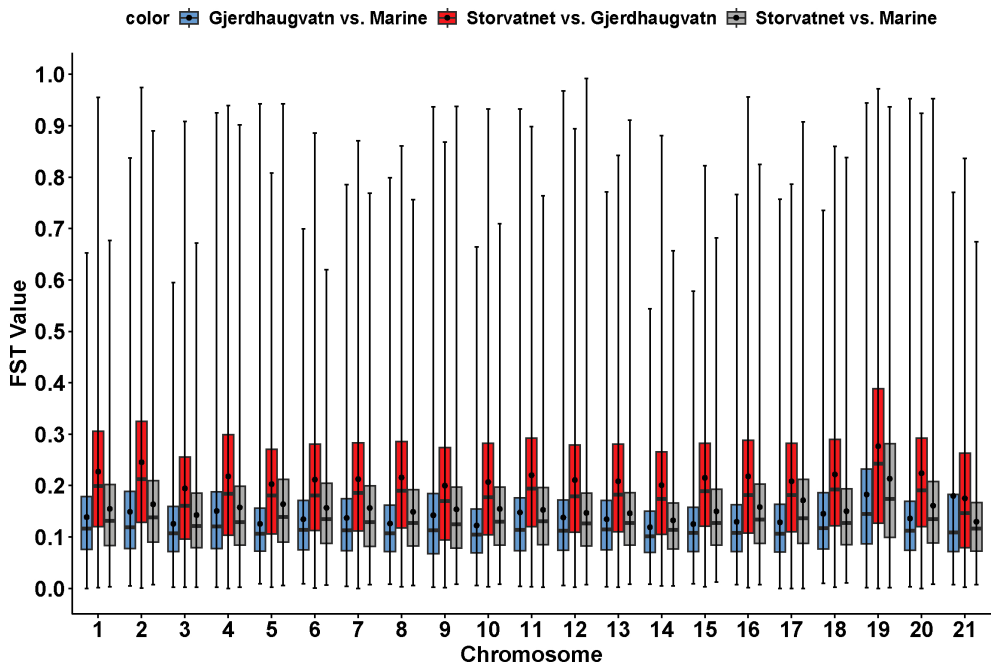

FIGURE 4
Boxplots presenting F_{ST} values among three populations across 21 chromosomes. F_{ST} values between Lake Gjerdhaugvatnet and the marine populations, and between Lake Storvatnet and the marine populations, are shown in blue and grey boxes, respectively, while F_{ST} values between the two freshwater populations (Storvatnet and Gjerdhaugvatnet) are shown in red boxes. Black points within each box indicate the average F_{ST} values for each chromosome. Notably, the average F_{ST} values between the two freshwater populations are higher compared to the other two pairs (marine and freshwater specimens) along each chromosome, with the exception of chromosome 21.

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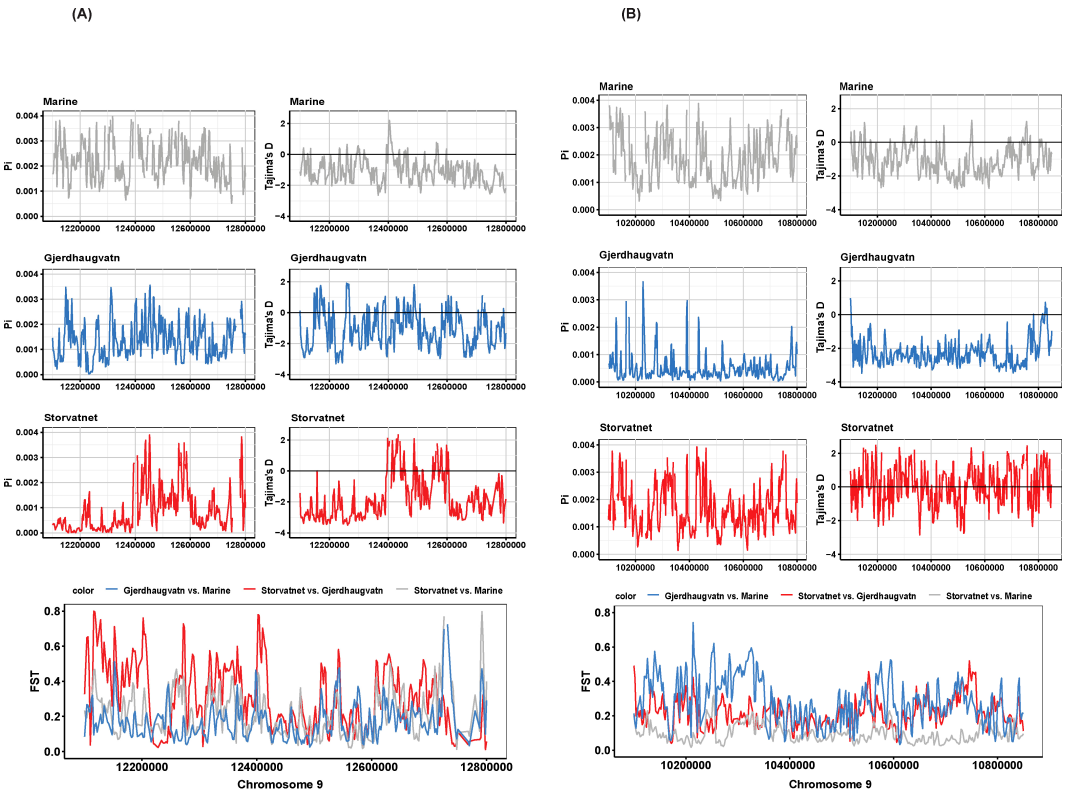

FIGURE 5
Population genetic parameters of selected regions at chromosome 9 indicating signatures of selection. **(A)** In Lake Storvatnet genomic regions on chr 9 (from 12.1 to12.3 Mb, and 12.6 to 12.8 Mb) characterized by highly negative T_D values (~ -2.0 to -4), lower π values, and higher genetic differentiation compared to other populations. Similarly, **(B)** in Lake Gjerdhaugvatnet, regions on chr 9 (from 10.1 Mb to 10.8 Mb) are characterized by highly negative T_D values, lower π values, and higher genetic differentiation compared to other populations.

3.2.2 Identification of genes potentially involved in bone and limb development

Based on high genetic differentiation of specific genomic regions between sites, we identified several loci that may be associated with limb or bone development and phenotypic variation. We found highly differentiated regions between the two freshwater populations containing the T-box transcription factor 4 (chr 1; *Tbx*4; 18.98 to 19.01 Mb), fibroblast growth factor 8a (chr 6; *Fgf*8a; 27.36 to 27.41 Mb), POU class 1 homeobox 1(chr 16; *Pou1f*1; 13.13 to 13.14 Mb), and ALX homeobox 1 (chr 19; *Alx*1; 13.56 to 13.57 Mb). In Lake Storvatnet sticklebacks, regions on chromosome 14, encompassing astrotactin-2-like (*Astn*2/LOC120831669; 5.32 to 5.48 Mb), bone morphogenetic protein/retinoic acid inducible neural-specific 1 (*Brinp*1; 5.12 to 5.21 Mb), and LIM homeobox 3 (*Lhx*3; 4.99 Mb to 5.0 Mb), were found to be highly differentiated compared to the two other sites. Additionally, the 3.0 to 3.4 Mb region on chromosome 5, including the growth/differentiation factor 10-like gene (*Gdf*10), bone morphogenic protein 2-like gene (LOC120819618/*Bmp*2), and transcription factor Sox-9-A-like gene (*Sox*9a), as well as two regions (11.0 to 12.0 Mb and 13.0 to 14.0 Mb) on chromosome 7, containing LOC120822309 (histone H2A.Z: *H2az*), MLLT3 super elongation complex subunit (*Mllt*3), LOC120822009 (HMG box transcription factor BBX: *Bbx*), brain-specific homeobox (*Bsx*), and LOC120822868 (histone H2A: *H2a*), exhibited elevated F_{ST} values between marine and Lake Storvatnet sticklebacks (see Supplementary S14).

3.2.3 Genomic divergence at the *Eda* locus on chromosome 4

We observed lower genetic diversity (π) across the *Eda* locus in freshwater populations (Figure 6A). Further, we also observed an elevated F_{ST} peak at the *Eda* locus between freshwater and marine specimens, supported by coverage depth assessments of the *Eda* locus from pooled sequences (Figures 6B, C). Notably, there was a lack of freshwater sequences aligning to specific intronic regions near exon 2, where a possible deletion of ~350 bp in freshwater sticklebacks may contribute to the observed genetic differentiation in this area (Figures 6B, C). Furthermore, we identified nine SNPs within exons 1 and 8 that were fixed for the reference allele in freshwater sticklebacks, while marine sticklebacks were polymorphic, with the reference allele occurring as the minor allele. Similarly, in the intergenic region between *Eda* and *TNFSF13*B, three SNPs (Ref/Alt: T/C, G/T, and G/A) were fixed for the reference allele in freshwater sticklebacks, whereas marine sticklebacks were polymorphic (Table 2, Figure 6D).

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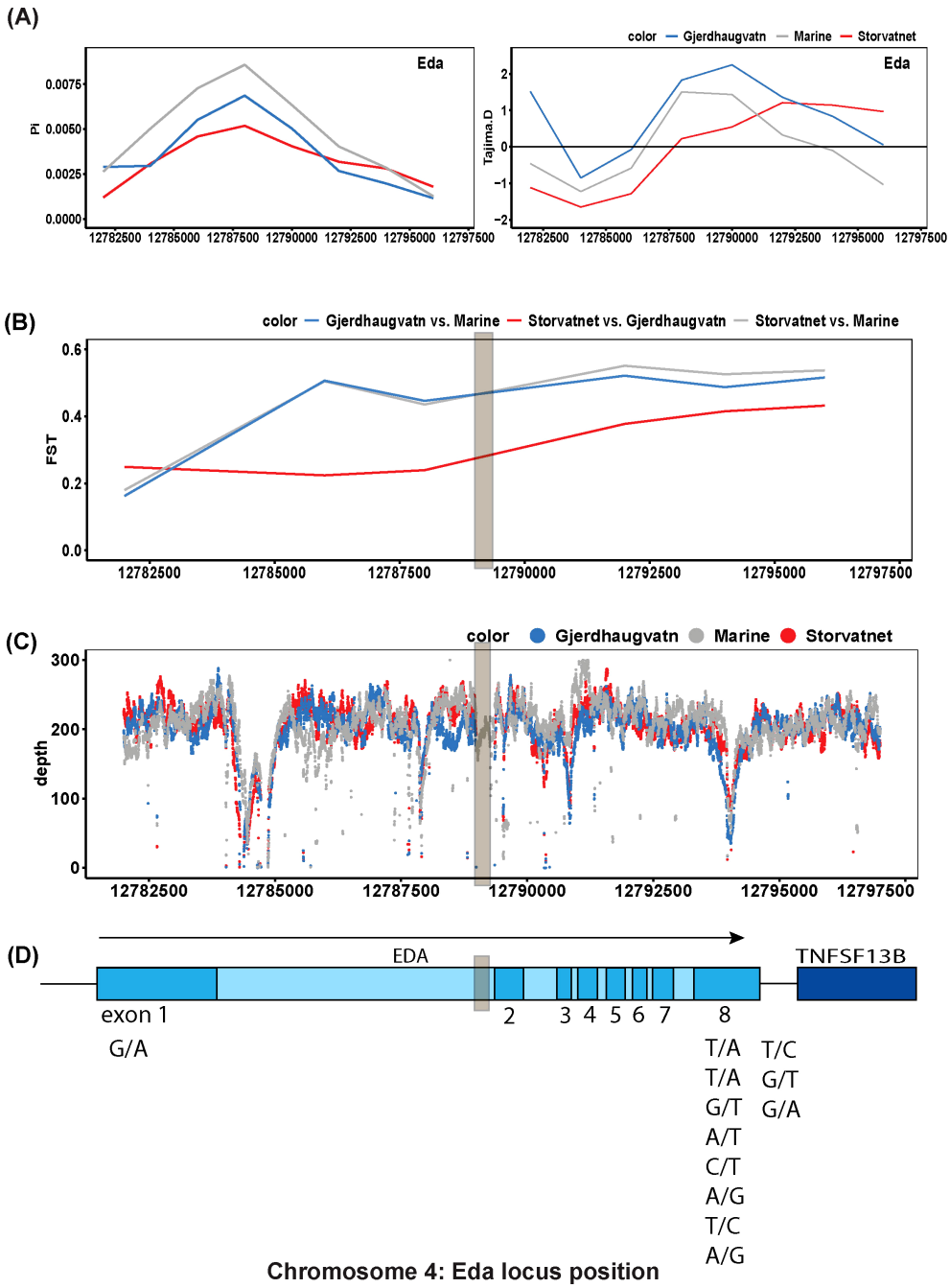

FIGURE 6
Illustration of the *Eda* locus between freshwater and marine sticklebacks. **(A)** Nucleotide diversity (π) and Tajima's D plots for the *Eda* locus. The red line represents the Lake Storvatnet population which shows lower genetic diversity and Tajima's D values compared to other populations. **(B)** F_{ST} plot showing genetic differentiation across the *Eda* locus on Chromosome 4 among sticklebacks from Lake Gjerdhaugvatnet, Lake Storvatnet, and a marine site. The blue line represents F_{ST} values for the Gjerdhaugvatnet versus marine comparison, while the red and gray lines represent Storvatnet versus Gjerdhaugvatnet and Storvatnet versus marine, respectively. Higher genetic differentiation was observed between the marine and freshwater sticklebacks at this locus. **(C)** Coverage depth plot for the same region as shown in A, indicating sequencing depth for each population. The region highlighted in grey in both B and C near to the exon 2 of *Eda* represents an area with sequence coverage for marine sticklebacks only. **(D)** Gene structure of the *Eda* locus, indicating differentiation at a number of nucleotide positions between freshwater and marine sticklebacks. Freshwater sticklebacks were fixed for the reference nucleotide (left) at these positions, while the alternate allele (right) occurred as the major allele in marine sticklebacks (see Table 2).

3.2.4 Assessment of genome wide diversity and differentiation suggests chromosomal rearrangements

Our genome-wide analysis revealed potential chromosomal rearrangements, such as inversions, which have been reported in other stickleback populations (Jones et al., 2012). In chr 1, elevated π values between 26.0 to 26.5 Mb were observed in the marine population but were absent in the lake populations (Figure 7A), suggesting a clear differentiation between marine and freshwater sticklebacks. This, combined with high F_{ST} values between marine and lake populations (see Supplementary S12) and elevated T_D values in the marine sticklebacks (Figure 7B, and see also Supplementary S11), points to either balancing selection or a marine-specific inversion, at least in a subset of marine samples. In chr 11, a region spanning 6.2-6.6 Mb, harboring 23 genes, shows strong peaks in both π and T_D in Lake Storvatnet (Figures 7C, D), indicating the likely presence of both inverted and non-inverted variants, suggestive of balancing selection in this population. Similarly, in chr 21, we identified a potential inversion spanning 9.8-11.7 Mb in both lake populations. The patterns of π (Figure 7E) and T_D (Figure 7F) in this region suggest the presence of both inverted and non-inverted variants, with T_D values in Lake Storvatnet particularly emphasizing the inversion, while F_{ST} comparisons suggest a more balanced mix of these variants in Lake Storvatnet compared to Lake Gjerdhaugvatnet (Figures 7E–G).

3.3 Bulk segregant analysis of spined and spineless sticklebacks from Lake Storvatnet

3.3.1 Whole genome analysis

In our comparative analysis of spined and spineless sticklebacks from Lake Storvatnet, we observed consistent patterns in π and T_D values between the two groups, which is expected given that both groups belong to the same population (see Supplementary S6, S10, S11). The overall genetic differentiation between the groups was low, as reflected by a mean F_{ST} value of 0.0099, which aligns with the notion of minimal divergence across most of the genome (see Supplementary S7, S13).

We identified 37 SNPs exceeding the genome-wide significance threshold ($-\log_{10}(p)$ value $5x10^{-8}$) (Figure 7A). A notable discovery was a highly differentiated region on chromosome 9 (spanning 4.0-4.4 Mb), which we refer to as an "F_{ST}-island" (Figures 8A, B). This island contains 31 genes (see Supplementary S8), including a gene of particular interest, *Hand2*, which is known for its role in hind limb development and its potential involvement in pelvic spine formation. Additionally, the spineless group exhibits higher π and elevated T_D values compared to the spined group within the "F_{ST}-island" (Figure 8C), suggesting that balancing selection may be maintaining heterozygous alleles at higher frequencies in the spineless group.

3.3.2 The *Pitx*1 locus

Approximately 11.41 M, 10.87 M, 11.17 M, and 11.46 M paired-end reads were successfully mapped for Spined-Storvatnet, Spineless-Storvatnet, Gjerdhaugvatnet, and marine PoolSeq data, respectively, against the *Pitx*1 BAC library of the threespine stickleback (GenBank: GU130435.1), with an average mapping quality of 35.6 across all population samples. The median sequencing depth across the *Pitx*1 locus was 225x.

While analyzing the PoolSeq coverage depth along the BAC *Pitx*1 locus, we identified a notable reduction in depth between positions 129973 to 130025 (~52 bp), located adjacent to the TG-III repeats within the *Pel*A region, in samples from Lake Storvatnet (both spined and spineless) compared to those from Lake Gjerdhaugvatnet and the marine site. In the Storvatnet specimens, this reduction in coverage depth indicates the presence of two alleles, with one allele carrying the deletion (~52 bp) and the other without. Interestingly, the coverage depth in the PoolSeq data for the spineless group is not zero, but remains lower than in the spined group, suggesting that a higher proportion of individuals in the spineless group carry the 52 bp deletion in both alleles. This pattern points to the deletion being more prevalent among the spineless specimens (Figures 9A–C).

Additionally, individual nanopore duplex sequencing of a spined and a spineless stickleback from Lake Storvatnet produced 191,722, and 144,670 mapped reads, respectively, with an average mapping quality of 40 for each individual. The median nanopore-sequencing-coverage-depths for the spined and spineless specimens were 18x and 11x, respectively. Nanopore sequencing of the spineless individual showed zero coverage depth in the 52 bp region, consistent with a deletion at both alleles, while the spined individual exhibited higher coverage depth in this region, consistent with the pooled sequencing data (Figures 9A–C). These findings suggest that the 52 bp deletion is present in the spineless individual, most likely in the homozygous state, whereas in the spined individual, the deletion is either absent or it occurs in a heterozygous state.

When assessing $-\log_{10}(p)$ values between spined and spineless groups along this region, we observed elevated peaks, suggesting some degree of genetic differentiation. Additionally, the analysis of nucleotide diversity and T_D revealed that the spined group has slightly higher nucleotide diversity and more negative but higher T_D values, which is indicative of balancing selection (Figures 9D, E). These data suggest that the spined group contains more heterozygous individuals carrying both the deleted and non-deleted alleles, while the spineless group has a higher proportion of homozygous individuals with the deletion in both alleles.

4 Discussion

Genomic analysis of marine and freshwater sticklebacks revealed high genetic diversity among marine sticklebacks while two freshwater populations that are located close to each other in the same watercourse are genetically more different from each other than they are from their marine conspecifics. We identified genomic regions with signatures of selection, contributing to our understanding of evolutionary forces shaping these populations. We also identified variation in *Pitx*1 enhancer regions and other genomics regions that might contribute to pelvic spine reduction.

TABLE 2 Selected regions along the *Eda* locus (chr 4) showing differentiation at single nucleotide positions between freshwater stickleback populations (Lake Storvatnet and Lake Gjerdhaugvatnet) and marine sticklebacks.

Chr	Position	Eda region	Ref (Alt)	Storvatnet allele count	Gjerdhaugvatnet allele count	Marine allele count	Minor allele frequency in marine sticklebacks
4	12783996	Exon 1	G (A)	175	183	35 (146)	0.19
	12794065	Exon 8	T (A)	88	47	20 (64)	0.24
	12794105	Exon 8	T (A)	121	89	31 (83)	0.27
	12794180	Exon 8	G (T)	135	119	52 (104)	0.33
	12794319	Exon 8	A (T)	132	161	43 (115)	0.27
	12794386	Exon 8	C (T)	143	152	42 (119)	0.26
	12794401	Exon 8	A (G)	149	139	34 (113)	0.23
	12794415	Exon 8	T (C)	154	147	40 (103)	0.28
	12794454	Exon 8	A (G)	159	152	36 (104)	0.26
	12794938	IR	T (C)	178	185	50 (113)	0.31
	12794969	IR	G (T)	174	187	67 (94)	0.42
	12794973	IR	G (A)	171	187	58 (104)	0.36

Freshwater sticklebacks were fixed for the reference allele at these positions, while marine sticklebacks were polymorphic, with the reference allele occurring as the minor allele.
IR refers to intergenic region between *Eda* and *TNFSF*13 genes. Alternative bases (Alt) of each reference base are shown in the brackets. Allele counts for each population were extracted from synchronization files. Both freshwater populations have same allele as the reference allele (Ref), whereas the marine population exhibits a biallelic pattern at each position listed above. In all cases, the alternate allele is more frequent than the reference allele in the marine population.

4.1 Technical implications of pooled DNA sequencing

Although the PoolSeq method has limitations, such as the loss of information on haplotypes, heterozygosity, and linkage disequilibrium (Cutler and Jensen, 2010), it remains a cost-effective strategy for collecting representative data on population samples (Anand et al., 2016). Typically, DNA pools with ≥30 individual DNA samples offer a reliable estimate of allele frequencies (Gautier et al., 2013; Rode et al., 2018). We used a sample size of 40 individuals per DNA pool coupled with high-coverage and deep sequencing to improve allele frequency estimation and decrease the risk of false positives (Cutler and Jensen, 2010; Ferretti et al., 2013; Gautier et al., 2013; Rode et al., 2018). To further validate our findings, we supplemented the PoolSeq data with nanopore long-read sequencing of one spined and one spineless stickleback from Lake Storvatnet, both of which were included in the PoolSeq samples. Nanopore sequencing, known for its ability to read long DNA fragments, typically exceeding 10 kb, is particularly effective at resolving repetitive DNA sequences. This approach enabled a more accurate reconstruction of complex genomic regions, providing clearer and more comprehensive insights into the DNA sequence (Amarasinghe et al., 2020).

4.2 Genetic diversity and differentiation between populations

The upper Lake Storvatnet (altitude 80 m) and Lake Gjerdhaugvatnet (altitude 20 m) exhibit different levels of genetic diversity compared to marine sticklebacks, consistent with Klepaker et al. (2012). Recolonization of Northern Europe by threespine sticklebacks began approximately 17.1 – 37.3 thousand years ago (Fang et al., 2018). As the freshwater lakes like Lake Gjerdhaugvatnet and Lake Storvatnet were likely colonized by small subsets of the marine stickleback populations, this have resulted in a founder effect, leading to reduced genetic diversity in these lakes. Genetic diversity is influenced by the interplay of mutations, gene flow, genetic drift and natural selection. While mutations are rare and selection targets specific genetic loci, overall diversity is largely determined by the long-term effective population size (N_e), which is conversely proportional to genetic drift. Marine sticklebacks, with their moderate dispersal capabilities, possibly reinforced by site fidelity, likely exhibit some degree of population structuring. However, their large census population sizes (N) and the lack of impassable barriers to gene flow in the marine environment likely result in high effective population sizes, translating into less genetic drift and higher overall genetic diversity compared to freshwater populations. Since only a single marine site, a river outlet, was sampled in this study, the full extent of marine genetic diversity remains uncertain.

Sticklebacks in Lake Storvatnet and Lake Gjerdhaugvatnet are highly differentiated, a finding supported by the signatures of selection observed in specific genomic regions of several autosomes. The presence of both spined and pelvic reduced sticklebacks in Lake Storvatnet further underscores this differentiation. Firstly, the colonization history of these lakes is unclear, but given that "the marine limit" (the maximum altitude of the sea surface relative to today's sea level since the last ice-age; Geological Survey of Norway) in this area is approximately 35 m above sea level, [Available online at: https://geo.ngu.no/kart/losmasse_mobil/?lang=nor&map=9 (Accessed February 20, 2025)]

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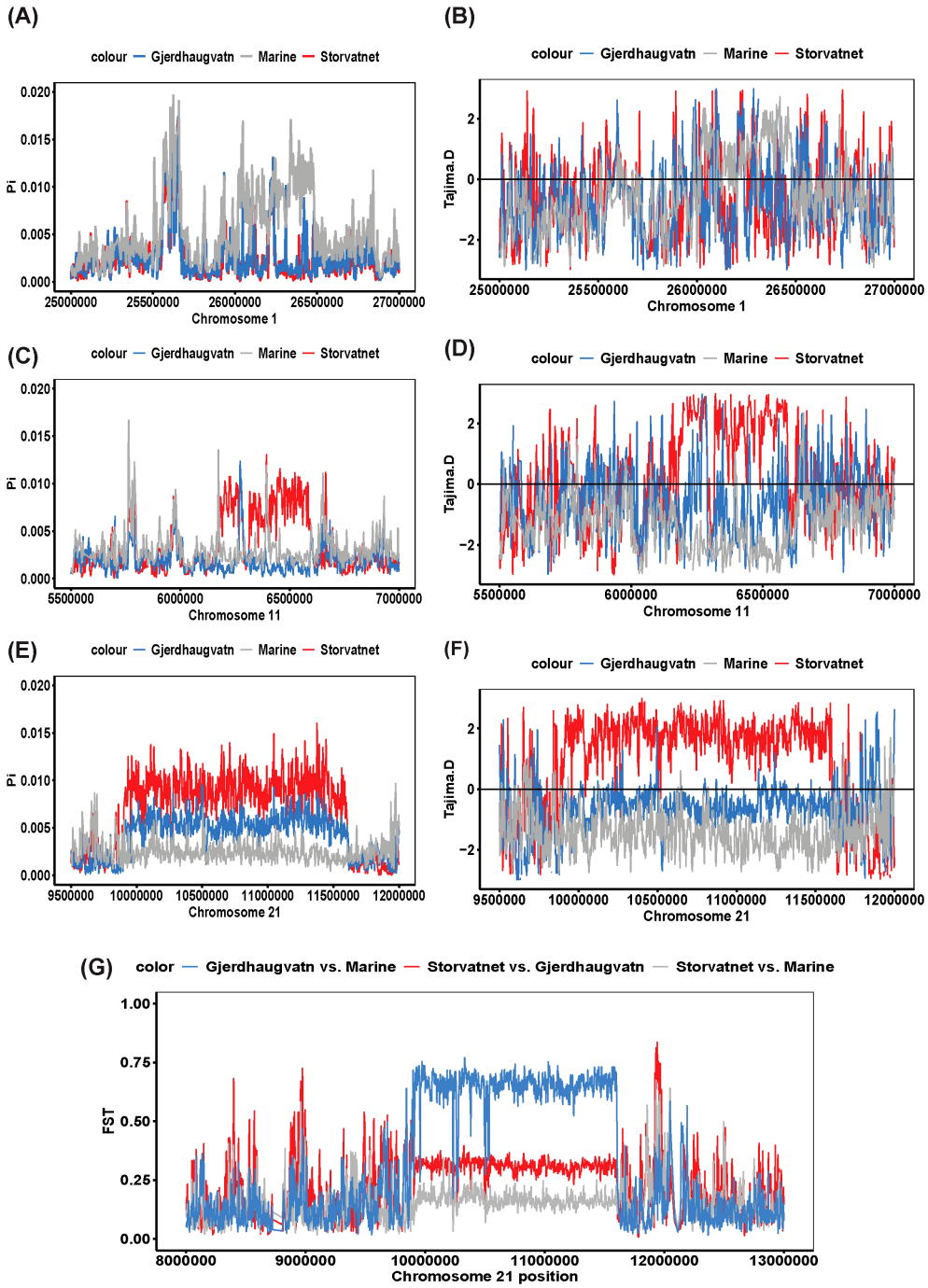

FIGURE 7
Population genetic parameters of selected regions at chromosomes 1, 11 and 21 indicating putative chromosomal inversions. Chromosome 1 (26 to 26.5 Mb); based on **(A)** nucleotide diversity pi, and **(B)** Tajima's **(D)** Chromosome 11 (6.2 to 6.6 Mb); based on **(C)** nucleotide diversity pi, and **(D)** Tajima's **(D)** Chromosome 21 (9.8 to 11.7 Mb); based on **(E)** nucleotide diversity pi, **(F)** Tajima's D, and **(G)** pairwise F_{ST}.

sticklebacks could have reached Lake Storvatnet (80 m altitude) only through non-typical means, such as transport by humans or birds, natural disasters such as tsunami, or via ancient streams that are no longer present. In contrast, Lake Gjerdhaugvatnet, at 20 m, sits below the marine limit, and may have experienced more frequent and prolonged gene flow from nearby marine stickleback populations, influencing its genetic composition. Secondly, genetic drift may have a greater impact on the population in Lake Gjerdhaugvatnet due to its smaller size and potential for population fluctuations caused by environmental factors like weather. Although population size estimates are unavailable, it is reasonable to assume that Lake Storvatnet, being 8–10 times larger, supports a higher effective population size (Ne), which would make it less susceptible to drift. In contrast, the smaller Lake Gjerdhaugvatnet may experience more pronounced effects of drift, leading to reduced genetic diversity. Thirdly, if these

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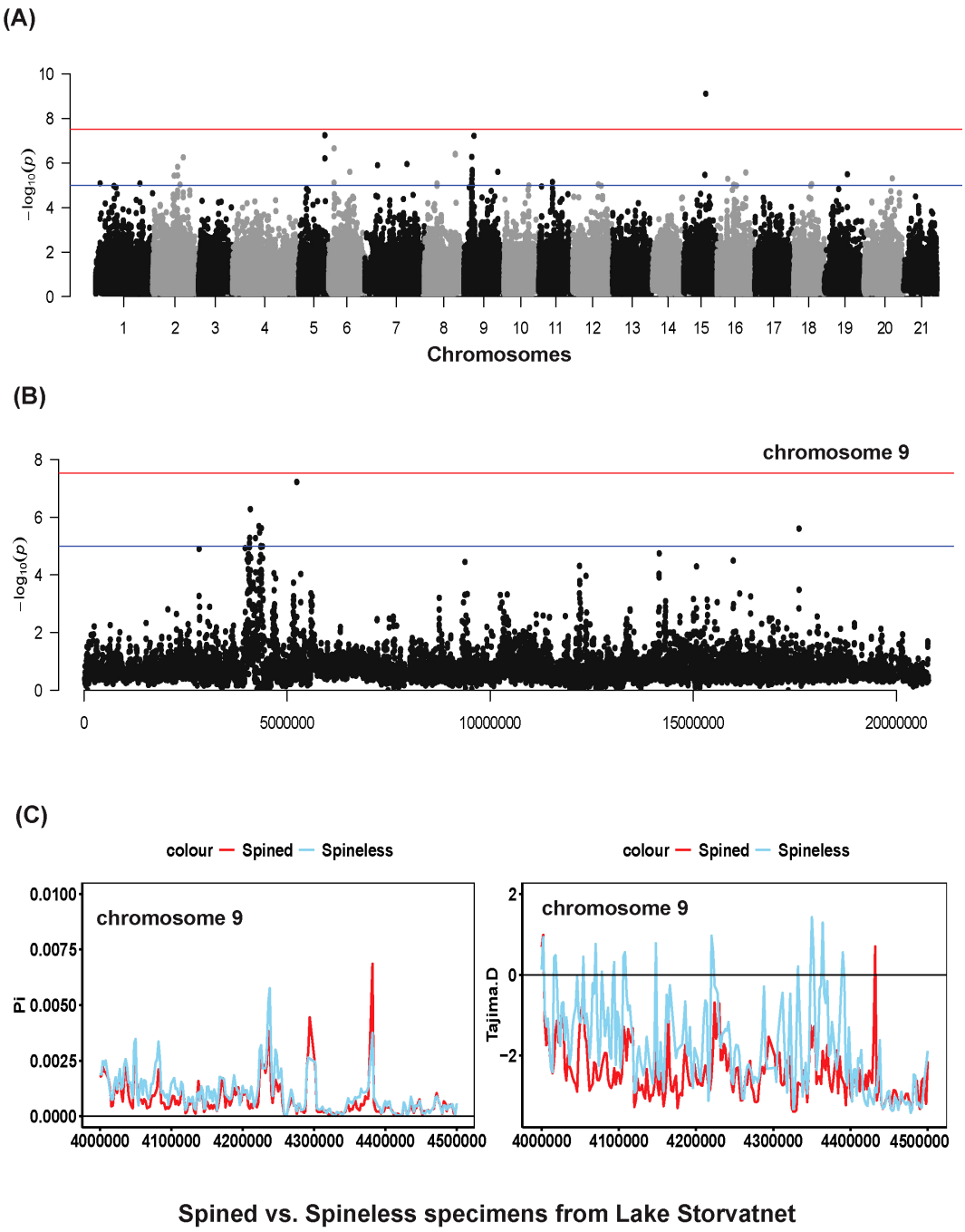

FIGURE 8
Manhattan Plots and nucleotide diversity analysis between spined and spineless groups from Lake Storvatnet. **(A)** Manhattan plot visualizing -log 10 (p) values derived from Fisher's exact test, identifying significant differences in allele frequencies between the two groups. Thirty-seven SNPs surpass the significance threshold of -log10(p) = $5x10^{-8}$ (blue line), indicating that these loci are associated with spinelessness. **(B)** Manhattan plot showing genetic differentiation on chromosome 9 between spined and spineless groups from Lake Storvatnet, using a sliding window genome-wide Fisher's exact test. A highly differentiated "F_{ST}-island" located between 4 and 4.4 Mb. **(C)** Nucleotide diversity (π) and Tajima's D plots for the 4 to 4.4 Mb region. The sky-blue line represents the spineless group, which shows higher genetic diversity than spined group (red line) in this region.

differences in population size and drift hold, it could render natural selection on adaptive traits, such as pelvic spine morphology, more effective in Lake Storvatnet. The genomic evidence, particularly the distinct signals of directional selection in Lake Storvatnet (e.g., regions on Chr 1, 7, 9, and 17), underscores the role of selection as an important evolutionary force shaping this subarctic freshwater stickleback population.

4.3 Genes potentially involved in bone or limb development

Based on population genetic differentiation among sites we identified several genes that might contribute to the adaptive structuring of stickleback populations. Genes such as *Tbx4* and *Fgf8a* are known to be crucial in pelvic fin and limb development,

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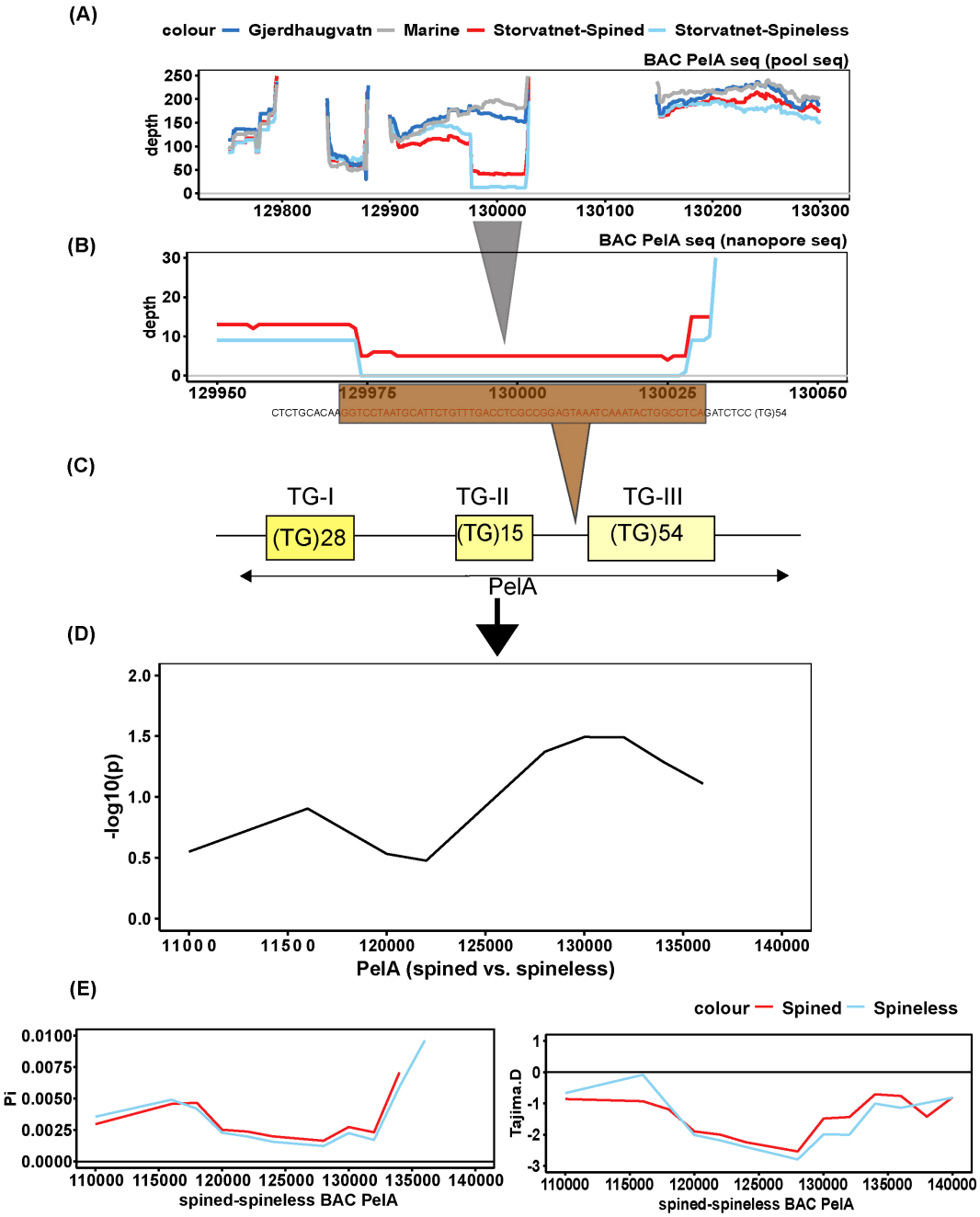

FIGURE 9
Analysis of the *Pel*A region across stickleback populations using data from 40 pooled individuals and from nanopore sequencing. **(A)** Coverage depth plots for the *Pel*A region (BAC sequence) using pooled sequencing, comparing populations from Lake Gjerdhaugvatnet (blue), the marine environment (gray), spined individuals from Lake Storvatnet (red), and spineless individuals from Lake Storvatnet (light blue). Differences in sequencing coverage indicate structural variation, with a deletion observed in the spineless group near position 130,000 bp. **(B)** Nanopore sequencing depth for the *Pel*A region from one specimen, highlighting the absence of aligned sequences near position 130,000 bp in the spineless group, supporting that a deletion has occurred in this region. One spined and one spineless specimen from Lake Storvatnet were subject to Nanopore sequencing. **(C)** DNA sequence of the *Pel*A enhancer region, showing the location of TG repeats. Red sequences inside highlighted area indicate the deleted sequences (52 bp) adjacent to the TG-III repeats in spineless groups **(D)** F_{ST} plot across the *Pel*A region between spined and spineless groups from Lake Storvatnet, showing a differentiation peak around position 130,000 bp, where deletion is suggested. **(E)** Nucleotide diversity (π) and Tajima's D plots showing differences between spined and spineless individuals in the *Pel*A region.

with their reduced expression linked to pelvic reduction in some fish species (Cole et al., 2003; Petit et al., 2017). Similarly, *Pou1f1* and *Alx* homeobox genes play essential roles in growth regulation, pituitary organogenesis, and limb and craniofacial development (Cole et al., 2003; Işık and Bilgen, 2019; McGonnell et al., 2011; Petit et al., 2017). *Astn2*, *Brinp*, *Eng*, and *Lhx3* are involved in embryonic development, neuronal identity, and brain development in vertebrates (Berkowicz et al., 2016; Srivastava et al., 2010). Genes

*Gdf*10, *Bmp*2, and *Sox*9a play vital roles in interdigital webbing, limb morphogenesis, dorsoventral patterning, inhibition of osteoblast differentiation, and cartilage formation (Cheng et al., 2016; Cresko et al., 2003; Kishimoto et al., 1997; Lai et al., 2006). Similarly, genes like *H2az*, *Mllt*3, *Bbx*, *Bsx*, and *H2a* have broad implications in chromatin structure, hematopoietic stem cell maintenance, central nervous system development, and brain-specific functions (Chen et al., 2014; Cremona et al., 2004; Germano et al., 2022; Giaimo et al., 2019).

The above-mentioned genes were identified mainly based on population genetic differentiation of the genomic region in which they reside, which is influenced by demographic factors such as founder effects and genetic drift as well. Also, the present study lacks the resolution, including data on recombination frequencies, needed to conclude on the effect of specific loci. Thus, the evidence of candidate genes remains inconclusive, nevertheless, several genes such as *H2az* and *Mllt3* on chromosome 7 (see Supplementary S14), are located within genomic regions displaying patterns in line with selection, potentially reflecting selective pressures acting on these regions and linked to functions such as limb or bone development.

4.4 Parallel evolution of chromosome 4 in freshwaters

Chromosome 4 shows notable genetic differentiation between freshwater and marine stickleback populations, underscoring its importance in adaptation to freshwater environments. Several regions on this chromosome, including the *Eda* locus, exhibit pronounced genetic differentiation, suggesting strong selection pressures driving divergence between marine and freshwater populations. In our study, the absence of coverage-depth in intronic regions near exon 2 of the *Eda* locus in freshwater populations, along with SNPs showing high allele frequency differences between freshwater and marine populations, suggests an interplay between structural variation and allelic diversity contributing to the genetic divergence between these populations. The presence of fixed SNPs in intergenic regions, including a fixed G base at position 12,794,969 (Table 2) between the *Eda* and *Tnfsf13b* loci in freshwater sticklebacks and a polymorphic (G/T) base at the same position in marine sticklebacks, aligns with previous findings (Jones et al., 2012; Laurentino et al., 2022; O'Brown et al., 2015; Rodríguez-Ramírez et al., 2023) that differentiate low-plated from fully plated sticklebacks. These patterns support the concept of parallel local adaptation, where similar selective forces have repeatedly shaped genetic variation in freshwater sticklebacks, potentially influenced by standing genetic variation (SGV) present in this chromosomal region. Moreover, the absence of coverage-depth in intronic regions near exon 2 of the *Eda* locus in freshwater populations may indicate structural modifications at this locus, which could play a role in shaping the evolutionary trajectory of chromosome 4 and contribute to the distinct adaptations observed in these subarctic freshwater populations.

4.5 Role of chromosomal inversion in local adaptation

We detected three major putative chromosomal inversions of sizes 0.5 Mb, 0.4 Mb, and 2 Mb on chromosomes 1, 11, and 21, respectively. These inversions are likely to be associated with freshwater adaptations. Similar genetic adaptations have been reported in stickleback populations from North- America and Russia (Jones et al., 2012; Terekhanova et al., 2014). Jones et al. (2012) also provide evidence for the role of chromosomal inversions in divergent selection of sticklebacks in marine and freshwater environments. Hence, these repeated genome changes seem to be examples of the reuse of shared SGV at a global scale (Jones et al., 2012).

In recent years inversions have gained attention for their role in protecting large chromosomal regions containing hundreds of genes from meiotic recombination, thus facilitating local adaptation. Genomic studies have demonstrated that inversions are taxonomically diverse, and often large in size, ranging from 130 Kb to 100 Mb across plants and animals (reviewed by Wellenreuther and Bernatchez, 2018). Inversions are often old and maintained through balancing selection, which indicate their role in maintaining genetic diversity (Wellenreuther, 2017). They also have broad biological impacts, including phenotype–genotype associations, mating behavior, and environmental adaptation. Moreover, the varying proportions of inversions between our two freshwater lakes might reflect reproductive isolation and genetic drift, as previously suggested (Klepaker et al., 2012).

4.6 Divergence between spined and spineless sticklebacks within Lake Storvatnet

4.6.1 Chromosome 9

The *Hand*2 gene, located within the "F_{ST}-island" on chr 9, is known to be involved in pathways related to pelvis development in vertebrates (reviewed in Swank et al., 2021). The *Hand*2 also plays a role in diverse cellular processes related to heart and forelimb development in zebrafish and mouse (Osterwalder et al., 2014; Yelon et al., 2000). Along with its upstream enhancer, *Hand*2 collaborates with *Hoxd*13 to activate the Sonic Hedgehog (SHH) pathway during limb bud development (Galli et al., 2010). This SHH pathway is crucial for limb patterning, as it inhibits the GLI3 repressor (GLI3R), preventing it from suppressing *Hand*2 activity (see Supplementary S9) (Osterwalder et al., 2014; Zuniga, 2015). Disruptions in this pathway can lead to a loss of limb polarity and defects in limb development (Galli et al., 2010).

In addition, the spineless group exhibits higher π and elevated T_D values compared to the spined group within the "F_{ST}-island" on chromosome 9, suggesting that balancing selection may be maintaining heterozygous alleles at higher frequencies in the spineless group. The persistence of heterozygous alleles in these regions could contribute to the spineless phenotype by enabling the population to maintain genetic variation essential for this trait.

Given these insights, our study opens up avenues for further research into the role of *Hand*2 in the development of pelvic spines in threespine sticklebacks, especially since there are currently no reported studies demonstrating the involvement of *Hand*2 in pelvic spine reduction.

4.6.2 Pitx1

The *Pitx*1 locus, situated at the sub-telomeric end of chromosome 7, contains repetitive DNA characterized by TG dinucleotide repeats, which makes sequence assembly difficult (Chan et al., 2010). This inherent complexity likely contributes to its absence from the latest reference genome assemblies. Previous studies (Chan et al., 2010; Thompson et al., 2018) have linked indels at the *Pel*A and *Pel*B enhancers of the *Pitx*1 locus to reduced pelvic spine length in North American sticklebacks. In our prior work, we identified deletions adjacent to TG-III repeats within the *Pel*A enhancer in both spined and spineless sticklebacks from Lake Storvatnet, which were absent in marine and Lake Gjerdhaugvatnet populations, but found no clear association with pelvic spine reduction (Adhikari et al., 2023).

Expanding on this, our current study suggests that deletions near the TG-III repeats are more prevalent in spineless sticklebacks from Lake Storvatnet, where they predominantly occur as homozygous deletions. This homozygous state means that both copies of the gene have the deletion, likely acting as a recessive allele favoring the spineless phenotype. In contrast, the spined group appears to exhibit a higher frequency of the heterozygous genotype, where one copy of the gene has the deletion and the other does not. This heterozygote state suggests a dominance of non-deleted allele, possibly favoring pelvic spine development. This pattern was consistently observed in both PoolSeq and nanopore sequence data analyses. Further, F_{ST} values indicated some degree of genetic differentiation between spined and spineless groups in this region. The slightly higher nucleotide diversity and more negative, but higher T_D values in the spined group compared to the spineless group, suggest that it likely contains more heterozygous individuals, indicating balancing selection. In contrast, the spineless group showed a higher proportion of homozygous individuals, potentially linking this genetic pattern to the spineless phenotype by influencing pelvic spine development.

These observations are in line with the concept that recessive alleles, such as those leading to pelvic reduction, may be carried at low frequencies in the spined individuals (heterozygous state), while the spineless phenotype is more likely to be expressed in individuals that are homozygous for the deletion. Thus, the presence of both homozygous and heterozygous states in these populations underscores the role of recessive and dominant genetic mechanisms in driving phenotypic evolution in sticklebacks (Bell et al., 2007).

As the *Pel*A deletion observed in our study was smaller than those documented in North American sticklebacks, we speculate that these deletions might be at a tipping point for transcriptional activity in Lake Storvatnet sticklebacks. It is therefore likely that loci other than *Pel*A and *Pel*B, such as *Hand*2 and others, contribute to the observed pelvic spine reduction, pointing to a more complex genetic architecture than previously thought. Such findings suggest a case of parallel evolution, where similar phenotypic traits arise independently through different genetic mutations. A similar phenomenon has been observed in *Peromyscus polionotus* populations in Florida, where variations in fur color arose through different genetic changes (Hoekstra et al., 2006). Indeed, as noted by Poore et al. (2022), it remains uncertain how frequently genetic parallelism, i.e. the use of the same genetic changes, underlies phenotypic parallelism in sticklebacks.

5 Conclusion

In this study, we present evidence of genomic differentiation between marine and freshwater sticklebacks in the European subarctic, and the genomic signatures of local adaptation in two closely located freshwater stickleback populations with differing pelvic morphologies. The study revealed genetic differences between spined and spineless sticklebacks within the Lake Storvatnet. Our previous study using Sanger sequencing found no clear association between deletions in *Pitx*1 enhancers and pelvic reduction in Lake Storvatnet. The present study, based on deep sequencing of pooled population samples and long-read sequencing of two specimens suggests that homozygous deletions adjacent to TG-III repeats within the *Pel*A region may contribute to this trait. Validation with a larger sample of individual Nanopore long-read sequences of spined and spineless individuals could confirm or disprove these findings. Additionally, we identified a differentiated "F_{ST}-island" on chromosome 9 between the spined and spineless groups, which includes *Hand*2, a gene known to be involved in limb development and potentially playing a supportive role in pelvic spine reduction in the Lake Storvatnet population. Together, these findings indicate that pelvic reduction in these sticklebacks is caused by the interplay of several genes, suggesting a case of parallel evolution shaped by polygenic effects rather than a single-gene effect.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, Bioproject PRJNA1073316.

Ethics statement

The animal study was approved by Norwegian Ministry of Agriculture and Food. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

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Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by general grants from Nord University.

Acknowledgments

We thank the genomic facility at Nord University for general support. We also thank Karoline Lauritzen, a former MSc student at Nord University, for helping with the Nanopore sequencing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2025.1546874/full#supplementary-material

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Available online at: https://geo.ngu.no/kart/losmasse_mobil/?lang=nor&map=9 (Accessed February 20, 2025).

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