Multiple genetic routes to pelvic spine loss in brook stickleback

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ABSTRACT

Pelvic spine loss has evolved repeatedly in stickleback species and represents an intriguing opportunity to study genetically parallel adaptation. While previous work has identified key loci associated with this trait, the extent to which different populations rely on the same genetic mechanisms remains unclear. The genetic basis of pelvic spine loss in *Culaea inconstans* (Kirtland 1840) has only been investigated in three populations. In two of these populations, the trait is associated with a mutation in *lmbr1*. In the third population, the trait is associated with a mutation in *tbx4*. In this study, we investigated the genetic basis of pelvic spine loss across seven additional populations. We used Sanger sequencing to identify if either previously described mutation in *lmbr1* or *tbx4* was present and associated with the trait in these populations. A mutation in *lmbr1* was found to be associated in two populations, while a mutation in *tbx4* was found to be associated in one population. Four populations showed no evidence that either *lmbr1* or *tbx4* was associated with pelvic spine loss, potentially implicating alternative mutations. These findings highlight the complexity of the evolutionary processes underlying repeated adaptation, including the extent of non-parallelism in the genetic basis of adaptive traits.

KEYWORDS

Culaea inconstans, repeated adaptation, pelvic spine loss, genetic parallelism

INTRODUCTION

Repeated evolution provides an excellent opportunity to study the mechanisms behind adaptation. When the same traits evolve repeatedly in independent instances of adaptation to similar environments, the genes or mutations that underlie those evolutionary changes may or may not be the same. Genetic parallelism occurs when repeated instances of adaptation are driven by

the same mutation, gene, or biological pathway (James et al. 2023) and is more likely to occur in closely related species (Bohutinska and Peichel 2023). A shared mutational basis for the same adaptive change could be due to shared ancestral variation, gene flow, or even independent de novo mutations (Cerca 2023). Genetic non parallelism should be more likely when there is redundancy in the genetic basis for a trait, allowing different mutations to become fixed in different populations that are evolving in parallel (Mee et al. 2025). The extent of genetic (non) parallelism should also depend on population size if adaptation is constrained by the limited availability of standing genetic variation (Mee et al. 2025) or by the limited availability of new mutations (James et al. 2023). Additionally, different mutations may have very similar (i.e. parallel) effects on a given adaptive phenotype, but these mutations may differ in pleiotropic effects and, hence, vary in fitness costs/benefits across different environments (Yeaman et al. 2018). The extent to which the cumulative effects of genetic redundancy, genetic drift, and pleiotropy influence genetic (non) parallelism is unresolved, and studying cases where these factors all have the potential to influence the course of adaptive evolution will help resolve our understanding of the repeatability of evolution.

The stickleback family (Gasterosteidae) consists of five genera and approximately eight species worldwide (Nelson and Paetz 1992) that shared a common ancestor approximately twenty-seven million years ago (Varadharajan et al., 2019; Jeffries et al. 2022). Sticklebacks have become model organisms for studying parallel adaptation. Following the last glacial retreat approximately 20,000 years ago, threespine stickleback, *Gasterosteus acculeatus* (Linnaeus 1758), colonized many isolated lakes throughout the Northern Hemisphere (Keivany and Nelson 2004), and adaptation to freshwater in these populations commonly involved the loss or reduction of pelvic spines (Klepaker et al. 2013). In fact, freshwater populations of brook stickleback, *Culaea inconstans* (Kirtland 1840*)*, and ninespine stickleback, *Pungitius pungitius* (Linnaeus 1758), have

also independently evolved extensive variation in external spines (Nelson 1969; Nelson 1971). The ancestral stickleback pelvic phenotype consists of a pelvic girdle and bilateral fin spines (Bell et al. 1993). The evolution of pelvic spine reduction or loss across stickleback species is thought to be influenced by habitat-specific selection pressures such as predation (Blouw and Hagen 1984) and the energetic cost of growing spines in low-calcium environments (Smith et al. 2014). Gape-limited predators such as small pike and perch (Hoogland et al. 1956) and the red-necked grebe (Godfrey 1966) have been found to preferentially prey on stickleback lacking pelvic spines, suggesting that spines can provide protection against predation. In fact, (Reimchen et al. 1994) suggests that three decades of stickleback research indicates that across most populations of stickleback, the presence of predatory fish is associated with increased spines. However, the fitness of pelvic spines across populations may be dependent on which predators are present. Reist (1980) found that large pike and diving beetles consumed more brook stickleback with pelvic spines and Marchinko (2009) demonstrated that macroinvertebrates, such as dragonfly naiads, preferentially prey upon juvenile threespine stickleback with pelvic spines. Meanwhile, Smith et al. (2014) found that in North Uist, threespine stickleback with reduced pelvic spines were only observed in low calcium environments, despite variation in trout abundance, suggesting that a minimum calcium concentration is required for pelvic spine development. The presence of multiple predators across variable habitats, combined with variable calcium availability, likely drives balancing selection maintaining pelvic spine polymorphism is several stickleback populations (Klepaker et al. 2013; Lowey et al. 2020).

Despite substantial phenotypic parallelism in pelvic spine loss or reduction, and likely similar fitness effects of pelvic spine presence-absence across stickleback species, genetic parallelism is not necessarily expected if there is genetic redundancy involved in the loss of pelvic spines. Multiple genes are known to influence pelvic spine development, providing several potential

genetic routes to a similarly reduced pelvic phenotype. Mutations in genes that regulate *shh* signaling during hindlimb development, including *pitx1*, *lmbr1, tbx4*, and *fgf10,* all have the potential to influence pelvic girdle and spine formation (Lettice et al. 2003; Duboc et al. 2021). In threespine stickleback, pelvic reduction has been found in a multitude of both extant and fossil populations (Bell 1987). Despite these populations evolving independently of one another, pelvic spine loss in threespine stickleback has been universally attributed to a deletion in the pel1 enhancer of the *pitx1* gene (Chan et al. 2010). Although the specific deletions can differ across populations, each occurs within the same regulatory region, indicating a high degree of within-species genetic parallelism for pelvic spine loss in *Gasterosetus* sp. *Pitx1* was also found to be involved in the pelvic reduction of Canadian ninespine stickleback populations (Shapiro et al. 2006) but not in Alaskan ninespine stickleback (Shapiro et al. 2009).

Across Alberta and Saskatchewan, forty-six populations of brook stickleback have been found to vary in pelvic spine morphology (Klepaker et al. 2013). Mee et al. (2025) found that in three of these populations in Alberta, two independent gene mutations were associated with pelvic spine reduction. In Muir Lake and Astotin Lake, a Single Nucleotide Polymorphism (SNP) with the highest association with pelvic spine loss was identified at position 4,425,247 (Mee et al. 2025) in the fifth intron of *lmbr1*, which contains an enhancer for the expression of *shh* in the developing limb bud (Lettice et al. 2003). In Shunda Lake, one single SNP associated with pelvic spine loss was identified at position 18,698,851 (Mee et al. 2025) in an exon of *tbx4*, an upstream regulator of *shh* (Duboc et al. 2021). Hence, the genetic basis of pelvic spine reduction shows non-parallelism both within brook stickleback and relative to threespine stickleback. In this study, we expand on the previously sampled brook stickleback populations in Alberta to determine if the same mutations found by Mee et al. (2025) are associated with pelvic spine loss in additional populations across Alberta and Saskatchewan. We hypothesize that the same SNP positions in

lmbr1 and *tbx4* found by Mee et al. (2025) will be re-used due to recent or ongoing gene flow or due to shared ancestry among geographically proximate populations. The extent of gene re-use across brook stickleback populations with pelvic spine reduction will lead to further insights into the factors influencing the genetic and ecological basis of adaptive phenotypes.

MATERIALS AND METHODS

SAMPLE COLLECTION

In 2025, we visited twelve waterbodies (Figure 1; Table 1) to sample populations of brook stickleback. Although predator abundance and composition are important ecological factors influencing selection on pelvic spines (see introduction), predator communities have not been characterized for the populations included in this study. Nevertheless, these populations have been previously reported to include pelvic-reduced phenotypes (Nelson, 1971; Nelson 1977; Reist 1981). We were able to sample brook stickleback from six populations in Alberta and Saskatchewan in 2025 (Table 1). To collect brook stickleback, we used 0.5 cm mesh minnow traps (n=24) set within 5 meters of the shore and retrieved within 24 hours. Any additional non-stickleback fish caught in traps were immediately released on capture. Brook stickleback were anesthetized and euthanized in a mixture of eugenol and lake water. Caudal fins were preserved in 95% ethanol in 1.5 ml Eppendorf microcentrifuge tubes and whole fish in 75% ethanol in 15-50 ml Falcon tubes. Preserved specimens are stored in a fireproof cabinet at Mount Royal University. Sampling permits were granted by The Government of Saskatchewan (25AR005fp), The Government of Alberta (25-1001 RL) and Alberta Parks (25-267). Fish handling protocols were approved by the Animal Care Committee at Mount Royal University (MRU ACC Protocol #102491). We also used samples from two additional populations (Spring Lake and Goldeye Lake) collected in 2012, 2023, and 2024 (Table 1). The sampling protocols for 2023 and 2024 were

identical to those described above (and in Mee et al. 2025). Sampling protocols (including ACC and provincial permitting) for 2012 collections are described in Lowey et al. (2020).

Using visual inspection of preserved samples with fine-tipped tweezers under a dissecting microscope, we classified pelvic phenotypes into three categories (following Klepaker et al. 2013): 1) fully spined (or "normal") pelvis, with both spines present, including all bony elements of the pelvic girdle; 2) intermediate (or "vestigial") pelvis, with at least one spine or pelvic element missing; 3) unspined (or "lost pelvis"), with both spines and all pelvic elements missing.

LMBR1 AND TBX4 GENOTYPING

Genomic DNA was extracted from the caudal fins of each specimen using a Qiagen DNEasy Blood and Tissue Kit (Qiagen, Germany). Primers were designed using the *Pungitius pungitius* fPunPun2.1 (GCF_949316345.1) reference genome (Varadharajan et al. 2019) to amplify regions flanking the target locus in *tbx4* and *lmbr1* (Table 2). This is the same reference genome that was used by Mee et al. (2025) to guide the identification of SNPs associated with pelvic spine loss. Targeted regions were amplified by polymerase chain reaction (PCR) using Platinum^{TM} Taq DNA Polymerase (Thermo Fisher Scientific, United States of America) following the manufacturer's protocol, with an annealing temperature of 55 degrees Celsius. We used gel electrophoresis run on 2% agarose gel to confirm successful amplification. A single primer pair successfully amplified the *tbx4* region in all populations analyzed in this study. No single primer pair successfully amplified the *lmbr1* region across all populations. Amplification of *lmbr1* required multiple primer pairs, with primer choice varying among populations and among samples within some populations (Table 2). As a result, sample sizes differed between loci because some samples could not be

161 successfully genotyped[1], despite multiple attempts at PCR. PCR fragments were purified using the
162 QIAquick PCR Purification Kit (Qiagen, Germany) and DNA concentration was quantified using
163 the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, United States of America). Samples
164 were submitted for forward-direction Sanger sequencing (University of Calgary Centre for Health
165 Genomics and Informatics, Calgary AB, Canada) using an Applied Biosystems 3730xl (96
166 capillary) Genetic Analyzer.

167 ANALYSIS

168 Sequence identity was confirmed using BLAST (Altschul et al. 1990) and sequences were
169 subsequently aligned to each other and to the reference genome using Clustal Omega (Sievers et
170 al. 2011). Multiple sequence alignments were visualized in Jalview (Waterhouse et al. 2009) to
171 identify the target locus positions in *lmbr1* and *tbx4* previously reported by Mee et al. (2025). The
172 *lmbr1* and *tbx4* genotype (i.e. homozygosity or heterozygosity) was determined by visual
173 inspection of sequencing chromatograms at the polymorphic locus. Chromatograms were visually
174 inspected to assess overall sequence quality including peak clarity, background noise, and the
175 presence of unambiguous base calls surrounding the polymorphic locus. Sequences with poor
176 chromatogram quality or ambiguous reads were not used for genotype assignment. Heterozygous
177 loci were identified based on the presence of two distinct peaks at the polymorphic site; if the peak-
178 height ratio was below 30%, the locus was not considered heterozygous (Applied Biosystems
179 2013). Additionally, because the same alternative allele was observed at the same site across
180 multiple independent samples, it is unlikely that these variants arose independently due to random
181 sequencing or PCR errors. For each population, Fisher's exact tests were performed in R (R Core
182 Team 2024) to evaluate the association between genotype at each locus and pelvic spine

[1] See Supplementary Material Table 1

phenotype. For each population with a Fisher's exact p-value <1, a Cramer's V test was performed in R to determine the effect size of the association. Pairwise nucleotide diversity (π) (Equation 1) was calculated following the method described by Nei and Li (1979) for the sequenced fragments of *tbx4* and *lmbr1* in Biopython version 3.14.0 (Cock et al. 2009) after aligned sequences were trimmed using trimAI (Capella-Gutiérrez et al. 2009).

$$\boldsymbol{\pi} = \frac{\left(\sum_{i<j} \boldsymbol{\pi}_{ij}\right)}{\mathbf{(n/2)}} \qquad (1)$$

RESULTS

Of the twelve waterbodies where sampling was attempted in 2025 (Table 1), no fish were captured in six, despite equivalent sampling effort in all waterbodies (typically 24 traps set at each site for 24-48 hours). Notably, the six waterbodies in which brook stickleback were captured were all lakes, and no brook stickleback were captured in rivers, creeks, or other flowing water (Table 1). Sample sizes were different across sampling sites, reflecting variation in capture success across locations. All the brook stickleback captured from Cold Lake were spined, while the other seven lakes were polymorphic for pelvic spine phenotypes.

One primer pair was successful in amplifying the genomic region flanking the *tbx4* SNP, while three different primer pairs were used to amplify the region flanking the *lmbr1* SNP, because not all primers pairs successfully amplified a fragment in every population (Table 2). For populations that had any unsuccessful amplification of *lmbr1,* success/failure was compared between spined and reduced-spined individuals (unspined and intermediate) to determine whether PCR success was associated with pelvic spine phenotype. The Fisher's exact tests were not statistically significant ($p>0.05$) for four populations with varying sample numbers (LPG, VTR,

206 SPL, VVL) and one population could not be tested by a Fisher's exact test due to a lack of the
207 spined phenotype[2].

208 Nucleotide diversity (π) was estimated for each gene fragment within and across all sampled
209 populations (Table 3). Across all populations, nucleotide diversity was higher in *lmbr1* (π=0.12)
210 than in *tbx4* (π=0.005). Within individual populations, nucleotide diversity ranged from 0.0004
211 to 0.005 for *tbx4* and from 0.009 to 0.36 for *lmbr1* (Table 3). Nucleotide diversity estimates for
212 the *lmbr1* region were higher than expected, suggesting that the observed variation may be
213 associated with an underlying genomic feature, such as a copy number variant. The basis of this
214 elevated diversity remains to be determined in future studies.

215 To determine if the SNP genotype at the *lmbr1* or *tbx4* locus identified in Mee et al. (2025)
216 was associated with pelvic spine loss, a Fisher's exact test was used to evaluate the association
217 for both SNPs for each study population (Table 4 and Table 5). Four of the sampled populations
218 with pelvic polymorphism, hereafter referred to by site codes (UPG, GDL, LPG and VTR; Table
219 1), were monomorphic at the *lmbr1* locus (Fisher's exact test p-value = 1). One population with
220 pelvic polymorphism, VVL, was polymorphic at the *lmbr1* locus, but there was no association
221 with pelvic spine phenotype (Fisher's exact test p-value = 1). A single nucleotide change from a
222 thymine to a cytosine at the *lmbr1* position tended to be associated with the unspined phenotype
223 in two polymorphic populations: WKL and SPL. In WKL, this association was moderate
224 (Cramer's V = 0.47) but not significant (Fisher's exact p-value = 0.06). In SPL, this association
225 was strong (Cramer's V = 0.85) and was statistically significant (Fisher's exact p-value = 0.004)
226 (Table 4). Although these findings agree with the nucleotide change reported at the *lmbr1* position
227 in Mee et al. (2025), this study is constrained by limited sample sizes and unequal representation

[2] See Supplementary Material Table 1

of spined and unspined samples within populations. Future analyses using larger sample sizes and a more balanced representation of phenotypes will provide additional support for evaluating the robustness of these findings.

Five of the sampled populations with pelvic polymorphism, SPL, LPG, VVL, UPG, and WKL (Table 1), were monomorphic at the *tbx4* locus (Fisher's exact test p-value = 1). One population with pelvic polymorphism, VTR, was polymorphic at the *tbx4* locus but there was no association with spine phenotype (Fisher's exact p-value = 1). A single nucleotide change from a cytosine to a thymine at the *tbx4* position in GDL was significantly associated with the unspined phenotype (Fisher's exact test p-value <0.001), and the association was strong (Cramer's V = 0.66) (Table 5). These findings contrast with those of Mee et al. (2025), who identified a thymine to a cytosine nucleotide substitution at the *tbx4* locus. It is unclear why the findings of this study differ to that of Mee et al. (2025), but the discrepancy may be due to the fact that Mee et al. (2025) used a *Pungitius pungitius* reference genome (fPunPun2.1, GCF_949316345.1; Varadharajan et al. 2019) for their SNP analysis, whereas we are directly analyzing *C. inconstans* genomes. Indeed, a reanalysis of the whole-genome sequence data used in Mee et al. (2025) using a newly available *C. inconstans* (unpublished) reference genome revealed that the *tbx4* SNP associated with spine loss involved a cytosine to thymine change (unpublished results), which is consistent with the Sanger sequencing results reported in the present paper. It is also possible that the thymine to cytosine substitution reported in Mee et al. (2025) was the result of a typographical error at some stage of the analysis (e.g. causing a reversal of phenotype coding) – we are investigating this latter possibility and will suggest publication of an erratum if such an error is discovered.

Overall, two populations showed an association between the *lmbr1* SNP genotype and pelvic spine phenotype, while one population showed an association between the *tbx4* locus genotype

and pelvic spine phenotype. The pelvic polymorphism was not associated with either the *lmbr1* or the *tbx4* SNP in four populations: UPG, LPG, VTR, and VVL. These results highlight variation in the genetic mechanisms underlying pelvic spine loss across the sampled populations, providing a basis to explore the ecological and genetic factors that may drive these differences.

DISCUSSION

The purpose of this study was to determine if the *tbx4* and *lmbr1* mutations previously implicated in pelvic spine reduction in three brook stickleback populations in Alberta (Mee et al. 2025) are also associated with pelvic spine reduction in other, more geographically widespread, brook stickleback populations in Alberta and Saskatchewan. We found two populations (WKL and SPL) where pelvic spine reduction was associated with the same SNP in *lmbr1* that was discovered in Muir Lake and Astotin Lake, and one population (GDL) where pelvic spine reduction was associated with the same *tbx4* SNP discovered in Shunda Lake (Mee et al. 2025). We found four populations (VVL, UPG, LPG, VTR) where the pelvic polymorphism was not associated with either of the mutations identified in Mee et al. (2025). These findings suggest that the genetic basis of pelvic spine loss in brook stickleback is largely non-parallel. The mechanisms causing the apparent re-use of the same *lmbr1* and *tbx4* mutations for pelvic reduction across some populations, but not others, remain undescribed.

During the last glacial maximum (~20, 000 years ago), much of North American was covered by the Laurentide Ice Sheet (Dyke et al. 2002), causing large portions of Canada and northern United States to be uninhabitable by freshwater fish. As a result, many aquatic species persisted in southern refugia, including the Mississippi River Basin (Ward and McLennan 2009). As glaciers retreated and new waterbodies became available, freshwater fish such as the brook stickleback began expanding their geographic range (Shafer et al. 2010). It is possible that both

mutations underlying pelvic spine loss arose within the same ancestral population or existed as standing variation, and as populations diverged into new ecological environments, selection may have favored the mutation conferring higher fitness in each environment. The populations using the *tbx4* SNP for pelvic reduction (Shunda Lake and GDL) border the Montane Cordillera ecozone, characterized by old growth coniferous forests and mountain ranges with groundwater-fed, neutral/alkaline lakes (Turk and Spahr 1989) with a high concentration of dissolved minerals (Roy and Hayashi 2008). In contrast, populations using the *lmbr1* mutation for pelvic reduction (Astotin Lake, Muir Lake, SPL, WKL) occur in the Boreal Plains ecozone, characterized by mature conifer forests, peatlands and typically smaller waterbodies (Roy and Hayashi 2008) with lower pH and mineral concentrations (Ireson et al. 2015). Although ecological variation among lakes was not directly examined in this study, local adaptation, driven by differences between these ecozones, may influence which mutation is favored if different mutations have pleiotropic effects on traits that confer different fitness consequences depending on the local environment. In threespine stickleback, the *eda* locus influences both bony lateral plating and growth rate, with the fitness effects of alleles differing between freshwater and marine environments (Barrett et al. 2009), illustrating how pleiotropy acting within an environment shapes fitness. If the alternate alleles at *lmbr1* and *tbx4* affect additional aspects of development, behavior or physiology, selection may favor different genetic mechanisms for pelvic reduction depending on the environmental context.

Alternatively, it is possible that the two mutations arose independently in different populations after they diverged from an ancestral refugium that lacked pelvic spine polymorphism. During the last glacial maximum, available habitats were repeatedly fragmented due to freeze-thaw cycles (Shafer et al. 2010) and may have led to multiple isolated refugial

297 populations. Independent refugial lineages may have accumulated these distinct genetic mutations
298 (*lmbr1* or *tbx4*), and the subsequent spread of these lineages into additional waterbodies could
299 have carried these mutations along with them. Additionally, gene flow between recently diverged
300 populations could help maintain the presence of each mutation among geographically proximate
301 populations. In this scenario, the different mutations observed across Alberta may reflect which
302 mutation happened to arise in distinct refugium rather than which mutation confers the highest
303 fitness. Future studies aimed at clarifying the origin of these mutations could integrate analyses
304 of population structure with genome-wide population data to estimate when and where they arose.
305 Despite evidence of genetic parallelism across the study populations, some populations
306 did not carry the mutations identified by Mee et al. (2025), suggesting that additional genetic
307 mechanisms may contribute to pelvic spine loss. In four of the populations analyzed in the
308 present study (VTR, UPG, LPG and VVL), pelvic spine reduction was not associated with either
309 the *lmbr1* or *tbx4* SNP identified by Mee et al. (2025). These findings suggest that pelvic spine
310 loss in these populations could be associated with either a different gene, or a different mutation
311 in *lmbr1* or *tbx4* not genotyped in the present study (or identified by Mee et al. 2025). In
312 threespine stickleback, a mutation in *pitx1* is consistently associated with pelvic spine loss across
313 their distribution (Chan et al. 2010), and has also been implicated in pelvic spine loss in
314 Canadian ninespine stickleback (Shapiro et al. 2006). As *pitx1, tbx4* and *lmbr1* all play some role
315 in the regulation of *shh* in hindlimb development (Lettice et al. 2003; Chan et al. 2010; Duboc et
316 al. 2021) , and because a mutation in *pitx1* is known to underlie pelvic spine loss in at least two
317 other species of stickleback (Chan et al. 2010), it is plausible that this mutation could have
318 occurred in brook stickleback as well. Another possibility is that a different mutation in *lmbr1* or
319 *tbx4* outside of the regions sequenced in this study is involved. While the results of the genome-

wide association studies conducted by Mee et al. (2025) show only one SNP in *tbx4* in Shunda Lake that is strongly associated with pelvic spine loss, the results for the *lmbr1* populations (Astotin and Muir Lakes) showed multiple SNPs that could be associated with the phenotype, and the one SNP with the strongest association was shared between the two lakes. It is possible that this one strongly associated *lmbr1* SNP identified by Mee et al. (2025), and investigated in the present paper, is not the causal lesion, and that its strong association with pelvic spine loss is due to linkage disequilibrium to a nearby causal lesion (e.g. a deletion, rearrangement, or uncharacterized substitution). It is also possible that pelvic spine loss may arise from substitutions at multiple loci within *lmbr1*.

To identify the specific mutations associated with pelvic spine loss in these populations, follow up studies could conduct genome-wide association studies to detect SNPs associated with pelvic phenotype in VTR, UPG, LPG and VVL, while quantitative trait locus (QTL) mapping in controlled crosses could help narrow down genomic regions influencing the trait. While these findings highlight the potential for additional mutations contributing to pelvic spine loss in some populations, the functional consequences of the known *lmbr1* and *tbx4* mutations remain an important area for further investigation.

In populations where *lmbr1* and *tbx4* mutations have been identified, differences in their known biological functions provide insight into potential mechanisms underlying pelvic spine loss. *Tbx4* is a highly conserved T-box transcription factor involved in lung and limb development across many species (Karolak et al. 2023), consistent with the strong conservation of the *tbx4* region found in this study. In contrast, the sequenced region of *lmbr1* exhibits greater genetic variability and may contribute to pelvic spine loss through a broader range of genetic mechanisms. *Lmbr1* encodes a membrane protein (Luan et al. 2024) and its 5th intron contains the functionally

conserved ZRS enhancer, a regulatory element that controls *shh* expression during limb development (Lettice 2003) across many vertebrate (Letelier et al. 2018). Notably, this same intron contains the SNP previously associated with pelvic spine loss in brook stickleback (Mee et al. 2025) and identified in WKL and SPL in this study. The ZRS regulates both forelimb and hindlimb (Letelier et al. 2018) development; however, the effects of the known *lmbr1* mutation on pectoral fins have not been investigated. Examining these effects in future studies may help identify if the ZRS is involved in pelvic spine loss in brook stickleback.

The differences in the genetic basis of pelvic spine loss observed across the study populations emphasize that parallel phenotypic adaptation does not necessarily require parallel genetic change. While some populations were found to use the same genetic mutations in *lmbr1* and *tbx4* that had been previously found in Alberta, other populations appear to rely on alternative mutations (or perhaps different genes), suggesting that more than two genetic routes can underlie pelvic spine loss in brook stickleback. In cases where the genetic basis of pelvic spine loss is known, the ecological and evolutionary mechanism driving this gene re-use and the functional consequence of each mutation remain unknown.

COMPETING INTERESTS STATEMENT

The authors declare there are no competing interests

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Figures and Tables

Figure 1. Map of the sites sampled. Black dots represent waterbodies included in this study. The map is divided into ecozones: Montane Cordillera (blue), Boreal Plain (green) and Prairies (pink).

551 Table 1. Sampling locations and sample sizes collected with consistent trapping effort across all
552 sites (24 minnow traps set for 24-48 hours), with “0” indicating absence of *Culaea inconstans*,
553 despite sampling effort.

Sampling location	Ecozone	Population Code	Latitude/Longitude	Year	Total Samples	Spined	Intermediate	Unspined
Upper Pierre Greys Lake, AB	Boreal Plain	UPG	53°54'29.4"N 118°36'34.5"W	2025	40	1	0	39
Lower Pierre Greys Lake, AB	Boreal Plain	LPG	53°54'04.2"N 118°35'03.9"W	2025	35	1	0	34
Victor Lake, AB	Montane Cordillera	VTR	53°53'11.7"N 119°05'15.0"W	2025	36	14	0	22
Wakomao Lake, AB	Boreal Plain	WKL	54°08'41.6"N 113°31'44.9"W	2025	17	16	0	1
Spring Lake, AB	Boreal Plain	SPL	53°30'45.8" N 114°08'34.0" W	2012	49	4	4	41
Goldeye Lake, AB	Boreal Plain	GDL	52°26'50"N 116°11'27"W	2023 2024	48	0	13	35
Cold Lake, AB	Boreal Plain	CDL	54°27'42.8"N 110°08'19.4"W	2025	41	41	0	0
Vivian Lake, SK	Boreal Plain	VVL	54°24'42.0"N 108°54'31.3"W	2025	23	1	0	22
Whitemud Creek, AB	Prairie	WMC	53°29'32.3"N 113°33'38.3"W	2025	0	0	0	0
Redwater River, AB	Prairie	RWR	53°53'27.8"N 112°58'14.0"W	2025	0	0	0	0
Fairydell Creek, AB	Boreal Plain	FDC	54°06'02.6"N 113°29'08.4"W	2025	0	0	0	0
Bonnie Lake, AB	Boreal Plain	BNL	54°08'44.3"N 111°52'44.7"W	2025	0	0	0	0
Matheson Lake, SK	Boreal Plain	MSL	54°25'00.3"N 108°55'14.0"W	2025	0	0	0	0
Punk Creek Tribunary, AB	Boreal Plain	PCT	54°32'43.57"N 111°12' 28.68"W	2025	0	0	0	0

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555 Table 2. Table 2. Sequencing primers used to amplify regions of *tbx4* and *lmbr1*

Primer ID	**Forward Sequence**	**Reverse Sequence**	**Used in populations**
TBX4	5'-CAG GTA CTC ACA TGC CCA AA-3	5'-ATG TGG TGA CAG GGA CAA ATA G-3'	UPG, LPG, VTR, WKL, SPL, GDL, VVL
LMBR1-3	5'-TGA ACA GGT AGA GGA CTT TA-3'	5'-CCT TAA TGC CCT GAC AGA AGA G-3'	UPG, LPG, VTR, SPL
LMBR1-3A	5'-TGA ACA GGT AGA GGA GGA CTT TA-3'	5'-CCT TAA TGC CCT GAC AGA AGA G-3'	LPG, VTR, WKL,
LMBR1-SB	5'-GGA GGA CTT TAT TAA CCA CTC AGC-3'	5'-GCT ACT GTC TGC GAT CAA GAG-3'	VVL, VTR, GDL

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570 Table 3. Nucleotide diversity for *lmbr1* and *tbx4* across individual populations and all populations
571 combined.

Population Code	**π (*tbx4*)**	**π (*lmbr1*)**
All	0.0054	0.1229
UPG	0.0010	0.0101
LPG	0.0026	0.0682
VTR	0.0004	0.1941
GDL	0.0049	0.3574
VVL	0.0013	0.0091
SPL	0.0018	0.0106
WKL	0.0004	0.0286

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Table 4. Phenotype and *lmbr1* genotype count across study lakes with Fishers exact tests reported. The phenotypes are 1: spined, 2: intermediate, 3: unspined. Samples included in this analysis reflect successful PCR amplification and samples from Table 1 that failed PCR amplification were excluded.

UPG

	CC	*CT*	*TT*
1	0	0	1
2			
3	0	0	0
	0	0	19

Fishers exact test p-value: 1

LPG

	CC	*CT*	*TT*
1	0	0	2
2			
3	0	0	0
	0	0	10

Fishers exact test p-value: 1

VTR

	CC	*CT*	*TT*
1	0	0	6
2			
3	0	0	0
	0	0	9

Fishers exact test p-value: 1

GDL

	CC	*CT*	*TT*
1	0	0	0
2			
3	0	0	2
	0	0	8

Fishers exact test p-value: 1

WKL

	CC	*CT*	*TT*
1	0	0	16
2			
3	0	0	0
	1	0	0

Fishers exact test p-value: 0.05882
Cramer's V: 0.4688

SPL

	CC	*CT*	*TT*
1	0	0	2
2			
3	0	1	0
	10	1	0

Fishers exact test p-value: 0.00366
Cramer's V: 0.8528

VVL

	CC	*CT*	*TT*
1	0	0	1
2			
3	0	0	0
	0	1	11

Fishers exact test p-value: 1

Table 5. Phenotype and *tbx4* genotype count from sequenced samples across study lakes. The phenotypes are 1: spined, 2: intermediate, 3: unspined. Samples included in this analysis reflect successful PCR amplification and samples from Table 1 that failed PCR amplification were excluded.

UPG

	CC	*CT*	*TT*
1	1	0	0
2			
3	0	0	0
	19	0	0

Fishers exact test p-value: 1

LPG

	CC	*CT*	*TT*
1	2	0	0
2			
3	0	0	0
	22	0	0

Fishers exact test p-value: 1

VTR

	CC	*CT*	*TT*
1	3	3	0
2			
3	0	0	0
	4	6	0

Fishers exact test p-value: 1

GDL

	CC	*CT*	*TT*
1	0	0	0
2			
3	5	7	3
	1	3	25

Fishers exact test p-value: $2.78*10^{-5}$
Cramer's V: 0.6575

WKL

	CC	*CT*	*TT*
1	16	0	0
2			
3	0	0	0
	1	0	0

Fishers exact test p-value: 1

SPL

	CC	*CT*	*TT*
1	2	0	0
2			
3	2	0	0
	12	0	0

Fishers exact test p-value: 1

VVL

	CC	*CT*	*TT*
1	1	0	0
2			
3	0	0	0
	17	0	0

Fishers exact test p-value: 1

Supplementary Material

Table 1. The association between successful PCR amplification of *lmbr1* and pelvic spine phenotype. Reduced-spines refers to both unspined and intermediate phenotypes. The Fisher's exact test could not be performed for GDL as it has no values in the 'spined' phenotype.

LPG

PCR SUCCESS	SPINED	REDUCED-SPINES
YES	2	10
NO	0	12

Fisher's exact p-value = 0.4783

VTR

PCR SUCCESS	SPINED	REDUCED-SPINES
YES	6	9
NO	0	1

Fisher's exact p-value = 1

GDL

PCR SUCCESS	SPINED	REDUCED-SPINES
YES	-	10
NO	-	34

SPL

PCR SUCCESS	SPINED	REDUCED-SPINES
YES	2	12
NO	0	2

Fisher's exact p-value = 1

VVL

PCR SUCCESS	SPINED	REDUCED-SPINES
YES	1	12
NO	0	5

Fisher's exact p-value = 1