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Genetic population structure constrains local adaptation in sticklebacks

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Abstract

Repeated and independent adaptation to specific environmental conditions from standing genetic variation is common. However, if genetic variation is limited, the evolution of similar locally adapted traits may be restricted to genetically different and potentially less optimal solutions or prevented from happening altogether. Using a quantitative trait locus (QTL) mapping approach, we identified the genomic regions responsible for the repeated pelvic reduction (PR) in three crosses between nine-spined stickleback populations expressing full and reduced pelvic structures. In one cross, PR mapped to linkage group 7 (LG7) containing the gene *Pitx1*, known to control pelvic reduction also in the three-spined stickleback. In the two other crosses, PR was polygenic and attributed to 10 novel QTL, of which 90% were unique to specific crosses. When screening the genomes from 27 different populations for deletions in the *Pitx1* regulatory element, these were only found in the population in which PR mapped to LG7, even though the morphological data indicated large-effect QTL for PR in several other populations as well. Consistent with the available theory and simulations parameterized on empirical data, we hypothesize that the observed variability in genetic architecture of PR is due to heterogeneity in the spatial distribution of standing genetic variation caused by >2× stronger population structuring among freshwater populations and >10× stronger genetic isolation by distance in the sea in nine-spined sticklebacks as compared to three-spined sticklebacks.

KEYWORDS

convergent evolution, epistasis, local adaptation, pelvic reduction, *Pitx1*, *Pungitius pungitius*

1 | INTRODUCTION

Failure to evolve in response to changing environmental conditions may lead to extirpation or even extinction (Orr & Unckless,

2008). If heritable variation necessary for adapting to environmental change already exists in the form of standing genetic variation, genetic adaptation may proceed swiftly, at least compared to the time it would take for populations to adapt from novel mutations

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(Barrett & Schluter, 2008; Orr & Unckless, 2008; Thompson et al., 2019). Furthermore, when exposed to novel yet similar environments, populations derived from the same ancestral population—hence carrying the same pool of alleles—can often be expected to respond to similar selection pressures in a similar fashion, leading to parallel phenotypic evolution (Arendt & Reznick, 2007; Bolnick et al., 2018; Conte et al., 2015; Elmer & Meyer, 2011; Hermisson & Pennings, 2017; Schluter & Conte, 2009; Stern, 2013). However, in genetically highly structured species, potentially advantageous rare alleles may be lost due to founder events and random genetic drift, thus preventing adaptation. Alternatively, due to heterogeneity in the distribution of standing genetic variation, adaptation to given selection pressures could more likely be acquired with phylogenetically independent alleles (rather than alleles that are identical by descent) at the same or different loci influencing the same trait, even if they may differ significantly in their fitness effects (Cohan, 1984; Merilä, 2013, 2014; Rosenblum et al., 2014). Epistatic interactions and the order at which beneficial alleles enter and become established in a population also play important roles in determining the genetic architecture of the trait in question (Orr, 1998). Thus, the demographic history of populations probably plays a central role in determining the likelihood of local adaptation, and hence also parallel phenotypic evolution.

In recent years, the nine-spined stickleback (*Pungitius pungitius*) has been emerging as a model system for studying adaptive evolution in the wild (Merilä, 2013) alongside the more widely studied three-spined stickleback (*Gasterosteus aculeatus*; Bell & Foster, 1994; Gibson, 2005). The latter species' ability to rapidly adapt to local environmental conditions has often been shown to stem from a global pool of ancestral standing genetic variation (Jones et al., 2012; Schluter & Conte, 2009; Terekhanova et al., 2014; Terekhanova et al., 2019). The nine-spined stickleback differs from the three-spined stickleback by having smaller effective population sizes (N_e), reduced gene flow in the sea and a tendency to occur in small landlocked ponds in complete isolation from other populations (DeFaveri et al., 2012; Merilä, 2013; Shikano, Shimada, et al., 2010; this study). Given their contrasting population demographic characteristics, three- and nine-spined sticklebacks can thus be expected to respond differently with respect to local adaption to newly colonized freshwater habitats.

Regressive evolution of the pelvic complex has occurred in freshwater populations of three (namely *Gasterosteus*, *Pungitius* and *Culaea*) of the five recognized stickleback genera since the last glacial period (reviewed in Klepaker et al., 2013). While marine populations of three- and nine-spined sticklebacks usually have complete pelvic structures with fully developed pelvic girdles and lateral pelvic spines, partial or even complete pelvic reduction is common in freshwater populations (Blouw & Boyd, 1992; Herczeg et al., 2010; Klepaker et al., 2013; Shapiro et al., 2004, 2006). Several factors may contribute to this, including the absence of gape-limited predatory fish and limited calcium availability, as well as the presence of certain insect predators (Bell et al., 1993; Chan et al., 2010; Giles, 1983; Karhunen et al.,

2013; Reimchen, 1983; Reist, 1980) in freshwater environments. Collectively, sticklebacks provide an important model system to study the genetic mechanisms underlying the adaptive parallel pelvic reduction at both intra- and interspecific levels, under a wide range of population demographic settings. However, studies of parallel patterns of marine–freshwater divergence in nine-spined sticklebacks are still scarce (Herczeg et al., 2010; Shikano, Ramadevi, et al., 2010; Wang et al., 2020), especially at the genetic level, precluding any comprehensive and conclusive comparison of the two species.

The genetic basis of pelvic reduction in the three-spined stickleback is well understood. Quantitative trait locus (QTL) mapping studies have identified a single chromosomal region containing the gene *Pituitary homeobox transcription factor 1* (*Pitx1*) that explains more than two thirds of the variance in pelvic size in crosses between individuals with complete pelvic girdles and spines, and pelvic-reduced individuals (Coyle et al., 2007; Cresko et al., 2004; Shapiro et al., 2004). Pelvic loss in the marine–freshwater three-spined stickleback model system (as well as in benthic–limnetic pairs of three-spined sticklebacks; Peichel et al., 2001) is predominantly caused by expression changes of the *Pitx1* gene due to recurrent deletions of the pelvic enhancer (*Pel*) upstream of *Pitx1* (Chan et al., 2010; Xie et al., 2019; in lake–stream pairs of three-spined sticklebacks, the genetic architecture of pelvic reduction is more diversified; Peichel & Marques, 2017; Deagle et al., 2012; Stuart et al., 2017; Rennison et al., 2019). While pelvic reduction in freshwater is much less common in nine- than in three-spined sticklebacks (Klepaker et al., 2013; Figure 1), two independent QTL studies also identified *Pitx1* in linkage group (LG) 7 as a major cause for pelvic reduction in a Canadian (Shapiro et al., 2006) and a Finnish (Shikano et al., 2013) population. Another large-effect region in LG4 was found to be associated with pelvic reduction in an Alaskan population (Shapiro et al., 2009). Similar to the three-spined stickleback, pelvic spine and pelvic girdle sizes are strongly correlated in the population from the Finnish pond Ryttilampi, as *Pitx1* controls both phenotypes (Shikano et al., 2013; Figure 1; Table S1). In contrast, a considerable amount of phenotypic variation with respect to these traits and their intercorrelations exists among different freshwater pond populations in northern Europe (Herczeg et al., 2010; Karhunen et al., 2013, 2014; Figure 1). Given their high heritability (Blouw & Boyd, 1992; Leinonen et al., 2011), the lack of correlation between spine and girdle lengths suggests that they can be independently controlled by different QTL. Thus, the genetic underpinnings of pelvic reduction in nine-spined sticklebacks (when it occurs) appear to be more diversified than those in the marine–freshwater three-spined stickleback model system (Merilä, 2013, 2014).

Starting with a survey of previously published phenotypic data on pelvic reduction in the wild, we aimed to investigate the possible genetic heterogeneity underlying pelvic reduction in different nine-spined stickleback populations by mapping QTL for pelvic reduction in three independently colonized ponds. One was the previously studied Ryttilampi (earlier analysed only

FIGURE 1 Summary of previously published nine-spined stickleback pelvic phenotypes from the wild and common garden experiments. Scatterplot depicts relative (to standard body length) pelvic spine and girdle lengths coloured according to population and habitat type and with size indicating Pearson's product moment correlation (r^2) between girdle and spine lengths for a given population. Populations with complete spine and/or girdle reductions (BYN, RYT and MAS) are here given $r^2 = 0$. Further details can be found in Figure S3 and Table S1. Phenotypic data for F_2 individuals from the current study are also presented (HEL × RYT, HEL × BYN, and HEL × PYÖ) for comparison. Details of sample locations and data collection can be found from Herczeg et al. (2010) and Karhunen et al. (2013). Data from common garden experiments (CG) is indicated by the suffix “_CG” in the population labels

with microsatellites, Shikano et al., 2013), now re-analysed along with two new populations (Bynästjärnen and Pyöreälampi) using >75,000 single nucleotide polymorphisms (SNPs). These data were subjected to a cutting-edge mapping approach (Li et al., 2017, 2018) that can provide more information on the source of the QTL effects than has been previously possible. To further assess the extent to which *Pel* could be responsible for pelvic reduction in the nine-spined stickleback, we screened the whole genomes of individuals from 27 wild populations for deletions in the genomic region spanning the *Pel* element and the *Pitx1* gene. Finally, utilizing more comprehensive geographical sampling than in previous studies (DeFaveri et al., 2012; Merilä, 2013; Shikano, Shimada, et al., 2010), along with high-quality SNP data, we re-assessed the differences in population structuring and genetic isolation by distance (IBD) between nine- and three-spined sticklebacks. We hypothesized that, in contrast to three-spined sticklebacks, both the scarcity and the variability in the genetic architecture of pelvic reduction in nine-spined sticklebacks is due to heterogeneity in the spatial patterns of standing genetic variation caused by strong population structuring—both among adjacent pond populations as well as in the ancestral sea population(s). Building on previous theoretical work, this hypothesis was tested using simulated data parameterized on population demographic parameters obtained from the empirical data.

2 | MATERIALS AND METHODS

2.1 | Fish collection, crossing and rearing

For the QTL crosses, three different marine F_0 generation females from the Baltic Sea (Helsinki, Finland; 60°13'N, 25°11'E) were crossed with a freshwater F_0 generation male from either Bynästjärnen (64°27'N, 19°26'E), Pyöreälampi (66°15'N, 29°26'E) or Rytilampi (66°23'N, 29°19'E) ponds. Fish crossing, rearing and sampling followed the experimental protocol used in the earlier study of the Rytilampi population (Laine et al., 2013; Shikano et al., 2013). For Rytilampi, the F_0 generation fish were artificially mated in the laboratory during the early breeding season of 2006 (Shikano et al., 2013), and the resulting full-sib F_1 offspring were group-reared in aquaria until mature, as explained in Shikano et al. (2013). Two randomly chosen F_1 individuals were mated repeatedly (seven different clutches) to produce the F_2 generation between September and October 2008. The offspring were reared in separate aquaria. The same procedure was followed for Pyöreälampi (F_0 mating: June 2011; F_1 mating: July–September 2012; F_2 rearing: July 2012–April 2013; eight different clutches) and Bynästjärnen (F_0 mating: June 2011; F_1 mating: November 2013–January 2014, F_2 rearing: November 2013–August 2014; six different clutches). The F_2 fish were

euthanized at 187, 238 and 238 days post-hatch for Rytilampi, Pyöreälampi and Bynästjärnen, respectively. At this point, the fish were on average 52.3 mm in standard length (Rytilampi = 48.6 mm; Pyöreälampi = 52.3 mm; Bynästjärnen = 53.6 mm), and weighed on average 1.34 g (wet weight; Rytilampi = 1.07 g; Pyöreälampi = 1.49 g; Bynästjärnen = 1.44 g). In total, 308, 283 and 279 F_2 offspring were available for analyses from Helsinki $\times$ Bynästjärnen (HEL $\times$ BYN), Helsinki $\times$ Pyöreälampi (HEL $\times$ PYÖ) and Helsinki $\times$ Rytilampi (HEL $\times$ RYT) crosses, respectively.

The experiments were conducted under licences from the Finnish National Animal Experiment Board (#STH379A and #STH223A). Experimental protocols were approved by the ethics committee of the University of Helsinki, and all experiments were performed in accordance with relevant guidelines and regulations.

2.2 | Morphological data

To visualize bony elements, all of the F_2 progeny were stained with Alizarin Red S following Pritchard and Schluter (2001). Spine lengths was measured from its distal point to the basal point and the length of branch and girdle was measured at its maximum linear length with digital callipers to the nearest 0.01 mm (both sides of the body). Landmark definitions with illustrations depicting them can be found from Herczeg et al. (2010) and Shapiro et al. (2004). Although it is known that the left-right asymmetry of the pelvic girdle is heritable in sticklebacks (Bell et al., 2007; Blouw & Boyd, 1992; Coyle et al., 2007), we did not specifically study this. To reduce the number of tests, the mean of the left- and right-side measurements was used (analyses conducted for the two sides separately always yielded qualitatively similar results as the tests conducted with the averages; results not shown). All measurements were made by the same person twice with repeatability >0.97 for all traits (R; Becker, 1984). The QTL analyses were performed on both absolute and relative (scaled to the total body length) trait values, but for all of the analyses that compared phenotypic data between populations (which also differ in body sizes), only relative trait values were used. Previously published phenotypic data from 19 wild populations were obtained from Herczeg et al. (2010) and Karhunen et al. (2013). These included data on pelvic spine and girdle lengths of wild-collected individuals from 10 pond populations (Abbortjärn = ABB, Bolotjone = BOL, Karhulampi = KAR, Kirkasvetinen lampi = KRK, Mashinnoje = MAS, Lil-Navartjärn = NAV, Hansmytjärn = HAN, Rytilampi = RYT, Bynästjärnen = BYN), four lake populations (Iso Porontima = POR, Riikojärvi = RII, Joortilajärvi = JOR, Västreskavträsket = SKA) and five marine populations (Fiskebäckskil = FIS, Trelleborg = TRE, Bölesviken = BÖL, Helsinki = HEL, Levin Navolä = LEV), as well as data on common garden-reared F_1 generation individuals from two marine (HEL and LEV) and two pond (BYN and PYÖ) populations. Visibly broken spines were treated as missing data. A map showing the geographical location of these populations is provided in Figure S1.

2.3 | Genotyping and linkage map construction

The RAD sequencing protocol used to obtain the SNP data was the same as in Yang et al. (2016) and Li et al. (2017). In short, genomic DNA was extracted from ethanol-preserved fin clips using the phenol-chloroform method (Taggart et al., 1992). DNA was fragmented with the *Pst*I restriction enzyme and the resulting 300–500-bp-long fragments were gel-purified. Illumina sequencing adaptors and library-specific barcodes were ligated to the digested DNA fragments, and the barcoded RAD samples were pooled and sequenced on 24 lanes of the Illumina HiSeq2000 platform with 45-bp single-end strategy. RAD library construction and sequencing were conducted by BGI Hongkong. After eliminating adapters and barcodes from reads, a quality check was done using FASTQC (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>).

A linkage map based on the three crosses was constructed using LEP-MAP3 (LM3; Rastas, 2017), as described in detail in Li et al. (2018). LM3 can infer the parental/grandparental phase based on dense SNP data, which allowed us to utilize the four-way cross QTL mapping method described below. Input data were generated by first mapping individual fastq files to the nine-spined stickleback reference genome using BWA MEM (Li, 2013) and SAMTOOLS (mpileup; Li et al., 2009), followed by pileupParser.awk and pileup2posterior.awk scripts from the LM3 pipeline using default parameters (see File S1 for details).

2.4 | Dimensionality reduction by linkage disequilibrium network clustering

It is essential in QTL mapping to correct for multiple testing in order to reduce the rate of false positives. Moreover, in large genomic data sets, physically adjacent SNPs—particularly those from experimental crosses—are often in linkage disequilibrium (LD; i.e., correlated). Since a group of SNPs in high LD explains similar amounts of genetic variation in a given trait, it is reasonable to apply a dimensionality reduction procedure before QTL-mapping to exclude the redundant information from the data. Here we used LD network clustering (LDn-clustering) and PC regression as dimensionality reduction tools prior to single-locus QTL mapping (Li et al., 2018). The first step of this approach involves an extension of a method developed by Kemppainen et al. (2015), which uses LD network analysis for grouping loci connected by high LD. The second step involves principal component analysis (PCA) as a method for dimensionality reduction in each cluster of loci connected by high LD (LD-clusters). This was achieved by the function LDnClustering in the R-package LDNA (version 0.64; Li et al., 2018). The method used here differs slightly from the original method described in Li et al. (2018) to increase efficiency in both computational speed and complexity reduction (see File S2 for details).

2.5 | QTL mapping: four-way crosses model

In some circumstances, such as a four-way cross (Xu, 1996), F_1 hybrids of two heterozygous parents (Van Ooijen, 2009), and an

outbred F_2 design (Xu, 2013a), it is possible that up to four different alleles—A and B from the dam, and C and D from the sire—segregate in the population. In such cases, a linear regression model for QTL analysis of the outbred F_2 data (Li et al., 2018) is defined by:

$$y_i = \beta_0 + x_{dij}\beta_{dj} + x_{sij}\beta_{sj} + z_{ij}\gamma_j + \varepsilon_i, \varepsilon_i \sim N(0, \sigma^2), \quad (1)$$

where y_i is the phenotype value of individual i ($i = 1, \dots, n$), and x_{dij} , x_{sij} and z_{ij} are genotypes coded as

$$\begin{array}{|l|l} +1+1+1 & \text{for genotype AC,} \\ +1-1-1 & \text{for AD,} \\ -1+1-1 & \text{for BC,} \\ -1-1+1 & \text{for BD.} \end{array}, \quad (2)$$

(Xu, 2013b), β_0 is the parameter of the population mean, β_{dj} is the substitution effect of alleles A and B of the dam (i.e., the grandfather in F_0) at the locus j ($j = 1, \dots, p$; p is the total number of SNPs), β_{sj} is the substitution effect of alleles C and D of the sire (i.e., the grandmother in F_0), γ_j is the dominance effect, and ε_i is the residual error term mutually following an independent normal distribution.

The model (1) requires knowledge of the grandparental phase (produced by LM3) with the benefit that the source (namely F_0 female or F_0 male) of the observed QTL effect can be inferred, as described in more detail in File S3. A multiple correction on the basis of permutation tests was further conducted to control for false positives due to multiplicity (Li et al., 2017) with 1×10^5 replicates.

2.6 | Estimating the proportion of phenotypic variance explained by SNPs

The overall proportion of phenotypic variance (PVE) explained jointly by all SNPs (an approximation of the narrow sense heritability) was obtained using LASSO regression (Tibshirani, 1996), which incorporates all the SNPs into a multilocus model:

$$\frac{1}{2n} \sum_{i=1}^n (y_i - \beta_0 - x_{dij}\beta_{dj} - x_{sij}\beta_{sj} - z_{ij}\gamma_j) + \lambda \sum_{j=1}^p (|\beta_{dj}| + |\beta_{sj}| + |\gamma_j|), \quad (3)$$

where the l_1 penalty term $\lambda \sum_{j=1}^p (|\beta_{dj}| + |\beta_{sj}| + |\gamma_j|)$ ($\lambda > 0$) shrinks the regression parameters towards zero; all other symbols are defined in the same way as in Equation (1).

Following Sillanpää (2011), the PVE can be estimated by the formula:

$$\text{PVE}_{\text{total}} = \frac{\text{var}(\sum_{j=1}^p x_{ij}\hat{\beta}_j)}{\text{var}(y)} \approx \frac{\text{var}(y) - \sigma_0^2}{\text{var}(y)}, \quad (4)$$

where $\hat{\beta}_j$ is the effect of the SNPs, and σ_0^2 is the LASSO residual variance estimated by a cross-validation-based estimator introduced by Reid et al. (2016). The PVE explained by each linkage group was

estimated on the basis of the LASSO estimates using the following formula:

$$\text{PVE}_{\text{LG}} \approx \text{PVE}_{\text{total}} - \frac{\text{var}(\sum x_{ij}\hat{\beta}_j)}{\text{var}(y)}, \quad (5)$$

where $\hat{\beta}_j$ ($j \notin G$) represents all the effects estimated by the LASSO of the SNPs that do not belong to a set of SNPs (e.g., to a chromosome/linkage group). A similar approach was used to estimate the contribution of grandparental alleles and to evaluate the dominance component by treating the coding systems $[x_{dij}, x_{sij}, z_{ij}]$ (2) as different groups of SNPs.

2.7 | Scanning for *Pel* deletions in full-genome sequence data

A minimum of 20 samples from populations RYT, MAS, BOL, BYN and PYÖ, and 10–31 samples from an additional 22 populations (from Northern Europe and USA; Figure S1 and Table S2) were sequenced to 10× coverage by BGI Hongkong and DNA Sequencing and Genomics Laboratory, University of Helsinki, Finland. Reads were mapped to the nine-spined stickleback reference genome (Varadharajan et al., 2019) with BWA MEM (Li, 2013), and site-wise sequencing coverages were computed with SAMTOOLS (depth; Li et al., 2009). Relative sequence depths across the *H2afy-Pitx1* intergenic region were estimated for the five focal populations by first computing the median depths for 1,000-bp sliding windows, and then normalizing these by the median depth for the full intergenic region. The *Pel*-2.5kb^{SALR} region was extracted from the original BAC contig (GenBank accession no. GU130433.1) and mapped to the nine-spined stickleback intergenic region with MINIMAP2 (Li, 2018). The sequencing depths for *Pel*-2.5kb^{SALR} were normalized by dividing the mean depths of the *Pel* region by the mean depths of the full intergenic region. Gene annotations and relative sequencing depths (average and confidence intervals) were computed and visualized using the R-package *gviz* (Hahne & Ivanek, 2016). Lastly, we scanned the literature for genes that are known to affect pelvic and hind limb development, and searched for potential matches in the nine-spined stickleback genome (Varadharajan et al., 2019) in regions where significant QTL were found.

2.8 | Simulations

The repeated local adaptation in independently colonized freshwater stickleback populations is widely considered to be due to selection on standing genetic variation available in the sea, which in turn is maintained by recurrent gene flow from previously colonized freshwater populations (the “transporter hypothesis”; Schluter & Conte, 2009). The effects of standing genetic variation (assuming a panmictic ancestral population) on the probability of parallel local adaptation have already been studied (Galloway et al., 2019;

Lee & Coop, 2017; MacPherson & Nuismer, 2016; Ralph & Coop, 2015a), along with the effects of population structure on adaptation in continuous landscapes (Hermisson & Pennings, 2005; Ralph & Coop, 2010, 2015b). However, no simulations to date have explicitly considered the effects of heterogeneous patterns in the distribution of standing genetic variation on local adaptation/parallel evolution. Thus, we used forward-in-time simulations to explore the effects of the differences in population demographic parameters between nine- and three-spined sticklebacks on the probability of local adaptation, as described in File S4. These simulations were parameterized using empirical estimates of population structure and IBD in the sea for the two species (File S5).

In short, two freshwater populations were connected to different marine populations at the ends of a stepping-stone chain of marine populations with the carrying capacity (K) and migration (M) both in the sea, and between the founding (marine) and founder (freshwater) population (Figure S2), thus generating comparable patterns of population structuring and IBD in the sea as in the empirical data (see Section 3). The two freshwater populations were colonized once and allowed to become locally adapted for 10,000 years (20,000 generations). All freshwater adaptation was due to standing genetic variation in the sea that was continually replenished from a “refuge” freshwater population (situated at an equal distance from the two focal freshwater populations), as well as from the two focal freshwater populations once they were colonized. Three different genetic architectures were studied. One (architecture A) corresponds to a single large-effect QTL (allowing 100% local adaptation, but with minor-effect QTL also affecting the trait), another (architecture B) to a situation where at least two loci are needed to allow local adaptation (allowing 40% or 60% local adaptation), and the third (architecture C) corresponds to the same scenario as A, except that the large-effect locus is recessive. Mutation rate was fixed at $1e^{-8}$ and all loci were polymorphic at the beginning of the simulations. More details are given in File S4.

3 | RESULTS

3.1 | Heterogeneity of pelvic reduction in the wild

Re-analysis of previously published phenotypic data from the wild confirmed a high degree of variation among different populations with respect to pelvic spine and girdle lengths and their intercorrelations (Figure 1; Table S1 and Figure S3). For instance, while spines were absent and pelvic girdles strongly reduced (but not completely absent) in RYT, spines and girdles were completely lacking in the MAS population (Figure 1; Table S1). Furthermore, in the BYN population, spines were absent but pelvic girdles were only partially reduced; in BOL (a population adjacent to MAS), large variation in both spine ($SD = 0.037$) and girdle ($SD = 0.047$) lengths was observed, although these two traits were strongly correlated ($r^2 = 0.61$, Figure 1; Table S1 and Figure S3). This suggests that a large-effect locus affecting both pelvic spines and girdles segregated in this population

at the time of sampling. In six pond populations (ABB, KAR, KRK, NAV, HAN and PYÖ; Figure 1), relative spine (mean = 0.079) and girdle (mean = 0.15) lengths were only slightly smaller relative to the marine populations (0.11 and 0.16 for spine and girdle lengths, respectively; Table S1). A lack of pelvic reduction (relative to the marine populations) was observed only in one pond population (KAR; Figure 1; Table S1 and Figure S3).

3.2 | QTL mapping of pelvic reduction in the Helsinki × Ryttilampi cross

After LD-network-based complexity reduction, all QTL mapping analyses were performed on 241 PCs (six sex-linked PCs were removed). Re-analyses of the 279 F_2 progeny from the HEL × RYT cross confirmed a single QTL region on LG7 for both pelvic spine and girdle lengths in the single-locus analyses (Figure 2a,b and Table 1). In the multilocus analyses (absolute trait values), LG7 explained 74%–86% of the PVE for both spine and girdle lengths, while all other chromosomes individually explained <3% of the phenotypic variance (Table 2). An approximately equal amount of the phenotypic variance was explained by alleles inherited from the F_0 male (pond individual) and the F_0 female (marine individual; ~30% of the total variance for all traits; Table 2), respectively, with 15%–21% of the phenotypic variance also explained by dominance effects. This is the expected outcome for a recessive QTL when the F_0 individuals are fixed for different large-effect causal alleles, and when no additional smaller effect loci affect the trait (Klug & Cummings, 2016). This is also consistent with a strong correlation between relative pelvic spine and girdle lengths ($r^2 = 0.85$, Figure 1; Figure S3 and Table S1).

3.3 | QTL mapping of pelvic reduction in the Helsinki × Bynästjärnen cross

Among the 308 F_2 progeny of the HEL × BYN cross, single-locus mapping analyses of pelvic spine lengths detected two significant QTL on LG15 (PVE = 8.9%) and LG16 (PVE = 13.7%; Figure 2c, Tables 1 and 2) for alleles deriving from the F_0 male (pond individual). Thus, the causal alleles for these QTL segregated in the F_0 pond male (as explained in File S3). No dominance effects were detected for these QTL, suggesting that the allelic effects were additive. One QTL on LG6 with an allelic effect deriving only from the F_0 female was also detected (Figure 2c and Table 1). The QTL significance profiles, in particular for LG15 and LG16, spanned large genomic regions with no obvious peaks (in contrast to LG7 in the HEL × RYT cross; Figure 2a,b). This remained true when analysing all SNPs individually (fine-mapping; Figure S4). In addition, phenotypes of the F_2 individuals from the HEL × RYT cross were not normally distributed and the individual and multilocus phenotypic effects on spine lengths for the QTL on LGs 6, 15 and 16 indicated epistatic interactions (File S6). One significant male

FIGURE 2 Quantitative trait locus mapping of pelvic reduction in three independent stickleback crosses. Single-mapping four-way analyses of four morphological traits associated with pelvic reduction in (a,b) HEL × RYT cross, (c,d) HEL × BYN cross, and (e,f) HEL × PYÖ cross. QTL for pelvic spine length and girdle length are shown, with the x-axis indicating position in centi Morgans (cM). Results are based on permutations, and the dotted vertical line indicates genome-wide significance at $\alpha = .05$. Results are shown separately for alleles inherited from the male F_1 (M), the female F_1 (F), together with the dominance effect (D) according to the legend. Absence of a dominance effect indicates that the trait inheritance is additive, whereas a peak only for M or F indicates that the allelic effect was segregating in the F_0 male or the F_0 female, respectively (see File S3 for details). Candidate genes involved in pelvic development are indicated with black text representing genes that affect expression of *Pitx1*, and red text indicates genes that affect pelvic development downstream of *Pitx1* expression. Results for analyses based on relative trait values can be found in Figure S5

QTL on LG4 for girdle length was also detected (Figure 2d and Table 1). Results for relative trait values were highly similar to the absolute trait values, except for an additional significant male and female QTL on LG1, as well as another significant male QTL on LG14 (Figure S5, Table 1; Table S3).

Multilocus analyses (for absolute trait values) identified 11 LGs that accounted for at least 3% of the phenotypic variance in pelvic spine or girdle lengths in the HEL × BYN cross (Table 2). The largest of these effects were found on LG15 and LG16, which explained 9% and 12% of the variation in pelvic spine lengths, respectively (Table 2). For pelvic spine and girdle lengths, 39% and 32% of the PVE, respectively, were accounted for by all SNPs in the data set, which equates to the narrow sense heritability (h^2) also accounting for dominance (but not epistatic interaction) effects. For spine lengths, 24% of the total PVE was attributed to alleles deriving from the F_0 male, and 17% was attributed to alleles deriving from the F_0 female; only 1% was attributed to the dominance effect (Table 2). For girdle lengths, the corresponding numbers were 16%, 6% and 13% (Table 2).

Compared to the HEL × RYT cross, the correlation between relative pelvic spine and girdle lengths was much weaker in the HEL × BYN cross ($r^2 = .11$, Figure 1; Figure S3 and Table S1) consistent with pelvic spine and girdle reductions being independently controlled by different QTL. Furthermore, of the 306 F_2 individuals, only four displayed complete lack of spines (and the spine was

absent in the F_0 male), consistent with spine length being controlled by multiple additive loci.

3.4 | QTL mapping of pelvic reduction in the Helsinki × Pyöreälampi cross

In the HEL × PYÖ cross (283 F_2 individuals), one QTL for spine length was found on LG9, which was explained by alleles inherited from the F_0 male. Two significant QTL for girdle length were found, one on LG19 (explained by alleles inherited from the F_0 male) and the other on LG4 (explained by alleles inherited from the F_0 female; Figure 2f and Table 1). In the multilocus analyses, the PVE for different LGs mirrored these results; the LGs that contain significant QTL explain most of the PVE, while PVE for all other LGs were <4% (Table 2). Overall, the multilocus analyses revealed that PVE for pelvic traits was lower than in the HEL × BYN cross: 14% and 16% for spine length and girdle length, respectively, with <2% PVE due to dominance effects (Table 2). When analysing relative trait values, one additional female QTL peak was found for both girdle and spine lengths on LG1 (Table 1; Figure S5 and Table S3). Due to the large size of the identified QTL regions, it is not possible to know whether this QTL and that on LG1 detected in the HEL × BYN cross are due to the same or different underlying causal mutations (we consider this as a single QTL region).

TABLE 1 Summary of QTL-mapping analyses

Cross	Trait	Coding	LG	QTL	Std.	<i>p</i>	<i>p</i> _{COR}	PVE _{TOT}	β
HEL × RYT	Spine	Male	7	1	No	1.31e-04	.019	0.285	1.654
HEL × RYT	Spine	Female	7	1	No	1.26e-04	.026	0.311	1.573
HEL × RYT	Girdle	Male	7	1	No	3.66e-04	.04	0.311	2.041
HEL × RYT	Girdle	Female	7	1	No	1.08e-04	.024	0.263	1.711
HEL × RYT	Spine	Dom	7	1	No	1.08e-07	<.001	0.213	1.432
HEL × RYT	Girdle	Dom	7	1	No	1.35e-04	.028	0.154	1.463
HEL × BYN	Spine	Male	15	2	No	6.12e-06	.001	0.095	0.54
HEL × BYN	Spine	Male	16	3	No	1.16e-04	.01	0.086	0.493
HEL × BYN	Spine	Male	21	4	No	3.36e-04	.031	0.037	0.36
HEL × BYN	Spine	Female	6	5	No	1.37e-04	.018	0.07	0.5
HEL × BYN	Girdle	Male	14	6	No	2.49e-04	.024	0.039	0.307
HEL × PYÖ	Spine	Male	9	7	No	8.96e-11	<.001	0.108	0.352
HEL × PYÖ	Girdle	Male	19	8	No	1.56e-05	.001	0.056	0.325
HEL × PYÖ	Girdle	Female	4	9	No	1.32e-04	.02	0.045	0.301
HEL × BYN	Spine	Female	16	10	Yes	2.53e-04	.03	0.047	0.007
HEL × BYN	Girdle	Female	1	11	Yes	2.13e-05	.002	0.077	0.006
HEL × BYN	Girdle	Male	1	11	Yes	1.41e-04	.016	0.026	0.005
HEL × PYÖ	Spine	Male	1	13	Yes	4.75e-06	<.001	0.075	0.005
HEL × PYÖ	Girdle	Male	1	13	Yes	9.69e-05	.016	0.044	0.005

Note: Each row corresponds to a significant (arbitrarily numbered) QTL region, with the proportion of variance explained (PVE), jointly estimated for all PCs (one for each significant LD cluster) extracted from such regions. Coding indicates whether the QTL was significant for the alleles inherited from the F_1 female (φ), the F_1 male (σ) or the dominance effect (D). Effect size (β) is based on the first PC extracted from all SNPs from each significant QTL region. "Std." indicates whether the trait was standardized (Yes) or not (No). *p* and *p*_{COR} represent nominal and corrected *p*-values from single-mapping four-way analyses, respectively. Results for standardized trait values are only shown for QTL that were not also significant for absolute trait values.

3.5 | Scanning for *Pel* deletions in the full-genome sequence data

In the full-genome resequencing data of wild-collected individuals, a large deletion upstream of *Pitx1* was fixed for all 21 individuals from Ryttilampi, where pelvic reduction maps to this region (Figure 3). The deletion is around 3.5 kb (LG7: 17015109–17018744; Figure S6) in size and fully encompasses the *Pel*-2.5kb^{SALR} region of the three-spined stickleback (Chan et al., 2010). No comparable deletion was found in any other individuals in the data set (Figure 3b). This included the two White Sea populations, in which either complete reduction of both the pelvic spines and girdles was observed (MAS), or a putative large-effect locus affecting both spine and girdle length was found to be segregating (BOL; Figure 1; Figure S3 and Table S1).

3.6 | Candidate genes

Seven candidate genes or regulatory elements for pelvic reduction identified from the literature (Table S4) were found in the LGs with significant QTL (Figure 2). *Hif1a* (Mudie et al., 2014), *Pel* (Chan et al., 2010) and *Pou1f1* (Kelberman et al., 2009) are known to regulate the expression of *Pitx1*, whereas four genes (*Fgf8*, *Wnt8c*, *Wnt8b* and *Hoxd9*) are involved in the pelvic fin/hind limb

development downstream of *Pitx1* (Don et al., 2012; Tanaka et al., 2005). However, aside from *Pel* (LG7), only three—*Wnt8c* (LG6), *Hif1a* (LG15) and *Pou1f1* (LG16)—were clearly within the significant QTL regions (Figure 2). One candidate locus, *Hif1a*, is also on LG1 where significant QTL peaks were found when analysing relative (but not absolute) trait values (Table 1; Figure S5).

3.7 | Simulations

In the empirical data from the marine populations, IBD (the slope of the regression line between linearized F_{ST} and geographical distance point distance [km]) was $2.13 \cdot 10^{-7}$ (95% of the 10^6 bootstrap replicates were between $2.05 \cdot 10^{-7}$ and $2.2 \cdot 10^{-7}$) for nine-spined sticklebacks, and $1.53 \cdot 10^{-8}$ (95% of the bootstrap replicates were between $1.10 \cdot 10^{-8}$ and $1.96 \cdot 10^{-8}$) for three-spined sticklebacks. Thus, the slope of the IBD regression line in this data set was 13.9 times higher for nine-spined sticklebacks compared to three-spined sticklebacks (Figure 4a). The geographical distance (which is on an arbitrary scale) in the simulated data was scaled based on the observed levels of IBD in the empirical data. This resulted in the distance between the two simulated marine populations furthest away from each other (i.e., the marine populations from which the focal freshwater populations were founded) corresponding to 264 km and 352 km for

TABLE 2 Proportion of the phenotypic variance explained (PVE) in pelvic traits

LG	HEL × RYT		HEL × BYN		HEL × PYÖ	
	Spine	Girdle	Spine	Girdle	Spine	Girdle
1	—	0.01	0.03	0.05	—	—
2	—	—	0.01	0.03	—	0.02
3	—	—	—	0.01	—	—
4	—	0.02	0.01	0.01	—	0.03
5	—	—	—	—	—	—
6	—	—	0.03	—	0.02	—
7	0.85	0.82	0.02	0.02	—	—
8	—	—	0.01	0.01	—	—
9	—	—	—	0.02	0.11	—
10	—	—	—	0.06	—	—
11	—	—	0.02	—	—	—
12	0.02	0.02	0.03	0.01	0.01	—
13	—	—	—	0.01	—	0.02
14	—	—	—	0.05	0.01	0.02
15	—	—	0.09	0.01	—	—
16	—	—	0.12	0.01	—	—
17	—	—	0.01	0.01	—	—
18	—	—	—	0.04	—	0.01
19	—	—	0.02	0.06	—	0.05
20	—	—	—	0.01	—	—
21	—	—	0.03	—	0.01	—
Tot	0.8	0.76	0.39	0.32	0.14	0.16
♂	0.3	0.38	0.24	0.16	0.14	0.08
♀	0.29	0.27	0.17	0.06	—	0.09
D	0.23	0.17	0.01	0.13	0.01	—

Note: Percentages of total phenotypic variance explained by different linkage groups (LG), by all SNPs (Tot), by loci inherited from females (♀) and males (♂), as well as the dominance effect (Dom). Results are shown for each cross and trait separately, and for absolute trait values.

three- and nine-spined sticklebacks, respectively (Figure 4a). Thus, with comparable levels of IBD as in the empirical data, our simulations mimic the levels of parallel evolution that can be expected in three- and nine-spined sticklebacks at relatively short geographical scales (<400 km). The difference in IBD between the two species is also close to that in the empirical data (264/352 km = 0.75). In the empirical data, genetic differentiation between freshwater habitats for populations <400 km from each other (mean F_{ST} = 0.19 and 0.49 for three- and nine-spined sticklebacks, respectively) was also on par with the simulations (mean [across simulation replicates] F_{ST} = 0.21 and 0.58, respectively). Notably, F_{ST} was >0.8 (linearized F_{ST} > 4) between Pyöreälampi (no pelvic reduction) and Ryttilampi (pelvic reduction controlled by *Pitx1/Pel*), although these ponds are situated only 15 km from each other (Figure 4a; maximum and median F_{ST} = 0.96 and 0.62, respectively, for all pairwise freshwater-freshwater comparisons). This is higher than the F_{ST} between any

pair of three-spined stickleback populations in the data (maximum = 0.78 and median = 0.26 for all pairwise freshwater-freshwater comparisons).

3.8 | Do population demographic parameters influence local adaptation?

In the simulations (File S4), the relationship between marine-freshwater F_{ST} and freshwater-freshwater F_{ST} depended on both the species and genetic architecture (Figure 4b). For instance, when the genetic architecture included one additive large-effect locus (architecture A; homozygotes for this locus were 100% locally adapted to freshwater), this locus was often involved in parallel evolution when population structure was parameterized based on three-spined sticklebacks (65% of replicates, n = 100), but not when population structure was parameterized based on nine-spined sticklebacks (3% of the replicates). In 20% of the replicates for both species, the freshwater allele for this locus was fixed in only one of the focal freshwater populations (i.e., local adaptation, but not parallel evolution). When the trait under selection was controlled by several medium-effect loci (architecture B), parallel evolution was more common in both stickleback scenarios, particularly for the locus with the largest allelic value (20% and 80% for nine- and three-spined sticklebacks, respectively; Figure 4b,c; homozygotes for this locus were 60% locally adapted to freshwater). Local adaptation also occurred in 57% of the replicates in the nine-spine stickleback-like scenario, and in 18% in the nine-spine stickleback (Figure 4b,c). For the locus with the second highest allelic value in architecture B (homozygotes for this locus were 40% locally adapted to freshwater), the cases of both parallel evolution and local adaptation collectively dropped to 38% and 43% for three- and nine-spined like stickleback scenarios, respectively. With one nonadditive large-effect locus with recessive alleles locally adapted to freshwater (architecture C), this locus was less likely to be involved in parallel evolution in the three-spined stickleback-like scenario (48%), compared to architecture A. However, results for the nine-spined stickleback-like scenario were similar to architecture A (6% parallel evolution; Figure 4b,c). Thus, at relatively short (<400 km) geographical distances, parallel evolution is an expected outcome in three- but not nine-spined stickleback-like scenarios, particularly when a single additive large-effect locus is responsible for freshwater adaptation. Results addressing the questions of how local adaptation depend on ancestral allele frequency are presented in File S7 and Figure S7, and QTL use in parallel evolution as a function of allelic effect size is shown in Figure S8.

4 | DISCUSSION

The results demonstrate that pelvic reduction in nine-spined sticklebacks is not nearly as common as in three-spined sticklebacks, and when it does exist, the genetic basis is more variable. This is consistent with the re-analyses of neutral population genomic data showing that both IBD in the sea and the genetic structure among marine

(a) | Sequence depths for H2afy-Pitx1 region

(b) | Sequence depths Pel region

FIGURE 3 Sequence depths for *H2afy-Pitx1* region. (a) Relative sequencing depth across the *H2afy-Pitx1* intergenic region for 10 individuals from five different populations. Blue line and shading indicate population average and 95% confidence intervals, respectively. The 3.5-kb deletion in RYT, seen as a dip in sequencing depth, fully encloses the Pel-2.5kb^{SALR} of the three-spined stickleback indicated by the red box. (b) Normalized sequencing depths for the Pel-2.5kb^{SALR} region for 563 samples from 27 populations, showing that the complete deletion of the *Pel* region is unique to RYT. Phenotypic data in the Russian populations MAS and BOL suggest that a large-effect locus is responsible for pelvic reductions, and BYN and PYÖ (analysed here) are populations in which pelvic reduction does not map to LG7

populations are >10 times higher in nine-spined sticklebacks, causing heterogeneity in the distribution of standing genetic variation that is not present to the same extent in the marine three-spined stickleback populations. Thus, the level and distribution of ancestral variation available for local adaptation (and also for parallel phenotypic evolution) is probably a function of population demographic parameters, with local adaptation being less likely in poorly connected species, as suggested by Merilä (2013, 2014) and corroborated by our simulations. However, other non mutually exclusive factors, such as the genetic architecture (i.e., dominance effects, heritability, mutation rates and number of causal loci involved), as well as non-parallelism in phenotypic selection optima are also likely to play roles. In the following, we discuss the possible causes of the discrepancy in pelvic structure development between nine- and three-spined sticklebacks, as well as their implications to our understanding of adaptive evolution in the wild.

4.1 | Can the genetic architecture of pelvic reduction be explained by population demographic parameters?

Together with earlier QTL studies (Shapiro et al., 2006, 2009; Shikano et al., 2013), we show that multiple genomic regions (11 QTL, 10 of which are novel to this study) are associated with pelvic reduction in nine-spined sticklebacks across their distribution range. Only one small-effect QTL region (LG1; Table 1; Figure S5) was shared between any two crosses (HEL × BYN and HEL × PYÖ)

in our study, but even here it is not certain whether the underlying causal mutations are the same. Although the high frequency of *Pel* deletions (disrupting pelvic armour development) means that most of the deletions associated with pelvic reduction in three-spined sticklebacks are independently derived (Xie et al., 2019), *Pitx1/Pel* is nevertheless predominantly responsible for pelvic reduction in three-spined sticklebacks (Chan et al., 2010; Xie et al., 2019). In contrast, the major-effect and recessive *Pitx1/Pel* allele in nine-spined sticklebacks is known to be responsible for pelvic reduction in only one Canadian population and Ryttilampi. In both Bynästjärnen and Pyöreälampi, pelvic reduction is less heritable, polygenic and additive. In addition, based on morphological data, it seems likely that major-effect loci not associated with *Pel* deletions (Figure 3) control pelvic reduction in two Russian ponds, with one additional Alaskan population being controlled by a large-effect additive locus mapping to LG4 (Shapiro et al., 2009). We do not yet know the mutation rate of the *Pel* deletions in nine-spined sticklebacks. Thus, the lack of *Pel* deletions in nine-spined sticklebacks could be simply due to lower mutation rates compared to three-spined sticklebacks. Regardless, pelvic reduction in the nine-spined stickleback is much less common than in three-spined sticklebacks in general (Klepaker et al., 2013), and when it occurs, the QTL that control pelvic reduction are much more variable with respect to heritability, effect size distribution and dominance relationships. Assuming that selection for pelvic reduction in nine-spined stickleback freshwater populations is universal (see below), such a pattern is consistent with poor connectivity, causing heterogeneity in the patterns of standing genetic variation. This would restrict local adaptation, and hence, also parallel phenotypic

FIGURE 4 Simulation results. (a) Linearized F_{ST} against geographical distance (IBD) for empirical and simulated data, with slope and squared Pearson's product moment correlation coefficient indicated. Geographic distance for simulated data (IBD in the sea) is scaled to match the slope for the IBD-plot in the sea in the empirical data. (b) Freshwater–freshwater F_{ST} against marine–freshwater F_{ST} for all QTL from all simulated data ($n = 100$), with allelic effect sizes indicated as shown in the key (phenotypic optima in marine and freshwater habitats are 0 and 20, respectively). Loci in the upper left quadrant are classified as being involved in parallel evolution, and loci in the upper right quadrant are loci that are involved in local adaptation in only one freshwater population. The data are summarized in (c) focusing on the four largest effect loci, with genetic architecture and effect sizes indicated by the figure titles

evolution. However, the focus of this study is on the role of population demographic parameters on local adaptation from standing genetic variation, regardless of whether the alleles that confer freshwater adaptations are identical by descent (e.g., EDA) or independently derived (e.g., *Pel* deletions in three-spined sticklebacks), and only secondarily on parallelism (i.e., when the same trait is locally adapted in multiple independently colonized populations, regardless of genetic architecture).

It has been shown that multiple independent alleles for the same adaptation can temporarily co-exist in structured populations, causing heterogeneity in standing genetic variation across the distribution range of a species. This can be true when considering the dynamics of novel mutations (Ralph & Coop, 2010) or neutral alleles segregating in the population before they become adaptive (Ralph & Coop, 2015a). However, these studies assume uniform selection across single continuous populations and thus do not consider the scenario where locally adapted freshwater alleles—which can be different in different parts of the distribution range—are continually introduced to the sea. Furthermore, simulations showing that the probability of local parallel evolution from standing genetic variation depends on the migration rate from the sea (Galloway et al., 2019) or from other populations of the same habitat (Ralph & Coop,

2015a) implicitly assume a homogeneous pool of ancestral standing genetic variation. Our simulations show that local adaptation is also a function of the allele frequency in the founding marine population; with stronger IBD in the sea, standing genetic variation in the ancestral marine population was a stronger bottleneck for parameters that resulted in nine-spined stickleback-like population structure (9-sp) than for parameters that resulted in three-spined stickleback-like population structure (3-sp; Figures S7 and S8). Particularly for smaller effect loci, there was a stronger dependence between the allele frequency in the ancestral marine population and local adaptation in 9- compared to 3-sp, indicating that smaller effect QTL have a stronger influence on local adaptation in 9- compared to 3-sp (Figures S7 and S8). This resulted in more polygenic and/or less complete local adaptation in 9- compared to 3-sp (Figures S7 and S8), indicating that population demography is sufficient to preclude local adaptation.

While our simulation results match closely with estimates of population structure and IBD in the empirical data, these simulations explore only a small proportion of the possible parameter space with respect to selection intensity, effective population sizes and migration rates (detailed in File S4). We also assume that all parallel evolution is due to standing genetic variation of alleles that are identical

by descent (since mutation rate is low), whereas it is known that pelvic reduction in three-spined sticklebacks is, to a large extent, due to independently derived *Pel* deletions attributable to high mutation rates (Xie et al., 2019). Nevertheless, most three-spined stickleback studies indicate that freshwater adaptation is indeed chiefly due to alleles identical by descent that have segregated in the population for millions of years (e.g., Jones et al., 2012; Nelson & Cresko, 2018), making *Pitx1* an exception. In the case of nine-spined sticklebacks, *Pitx1* is with certainty associated with pelvic reduction in only two of all studied populations, suggesting a minor role for recurrent mutation in determining pelvic reduction in this species. While our simulations demonstrate that heterogeneous patterns of standing genetic variation is sufficient for limiting local adaptation in species characterized by low connectivity, further simulations with a wider parameter space could be helpful in advancing our understanding of the limits of adaptation in small and structured populations/species.

Interestingly, in the HEL × BYN cross complete spine reduction probably occurred when the allele responsible for spine reduction for the LG6 QTL was combined with at least one allele causing spine reduction from the LG15 and LG16 QTL, indicating epistatic interactions (File S6). If a threshold number of alleles are needed for complete pelvic reduction, this could also explain how standing genetic variation in the sea is maintained, as the necessary multilocus genotypes that cause suboptimal phenotypes in the sea are rarely formed, due to overall lower frequencies of spine-reducing alleles in the sea. This is analogous to “epistatic shielding” that can contribute to the persistence of disease alleles in populations (Phillips, 2008; Phillips & Johnson, 1998). Consistent with this possibility, LG6 of the F_0 female of the HEL × BYN cross (from the sea) was polymorphic for the pelvic spine QTL effect—evidently, a single pelvic-reducing allele alone in this female was not enough to cause any pelvic reduction at all (this female had a complete pelvis).

4.2 | Geographical heterogeneity in selection optima

The high heterogeneity in the genetic architecture of pelvic reduction in the nine-spined stickleback can alternatively be attributed to within-habitat environmental variation resulting in different selection optima in different pond populations (cf. Stuart et al., 2017; Thompson et al., 2019). For example, the small differences between pelvic reduced phenotypes in our study (e.g., in BYN and MAS/BOL spines are completely absent, while in RYT they are only strongly reduced; Figure 1; Table S1 and Figure S3) could in fact indicate different selection optima in the different populations (Stuart et al., 2017; Thompson et al., 2019). It is possible to use available phenotypic data to estimate the phenotypic optima of pelvic morphology (hypersphere) in each of the populations using Fisher's geometric model (Stuart et al., 2017; Thompson et al., 2019), where a strong overlap would suggest a higher probability of genetic parallelism (Thompson et al., 2019). However, this assumes that the populations have access to exactly the same ancestral variation and are free to evolve and

reach their optima, which is at odds with the results presented here. Without further detailed environmental data or direct estimates of strength of selection on pelvic phenotypes, disentangling the effects of gene flow and within-habitat environmental variation (assuming this leads to non-parallel angles of selection) is not possible (Stuart et al., 2017).

In a recent simulation study, Thompson et al. (2019) showed that genetic parallelism from standing genetic variation rapidly declines as selection changes from fully parallel (optima angle of 0°) to divergent (optima angle of 180°), especially when the trait is polygenic. However, although selection was fully parallel in our simulations, we did not observe strong genetic parallelism for smaller effect loci (with allelic effects <6) in both the three- and nine-spined stickleback-like scenarios (Figure 4b,c). This suggests that the effects of the underlying genetic architecture on parallelism (in conjunction with some IBD and population structuring) can be independent of the angle of optimal phenotypes between two habitats. Thus, overlapping selection optima are necessary but nevertheless not sufficient for parallel evolution to take place and it is important not to disregard the population demographic setting as a factor that could severely restrict heritability for adaptation and/or constrain adaptation to less optimal solutions. Evolutionary studies of species with population demographic parameters comparable to those typical for vulnerable or endangered species/populations, such as the nine-spined stickleback, would be valuable to gain a better understanding of how such species may respond to environmental changes and urbanization (Thompson et al., 2018).

4.3 | Pelvic reduction outside marine–freshwater study systems

While the evidence for genetic parallelism on large geographical scales in the marine–freshwater stickleback model system is extensive (Colosimo et al., 2005; Jones et al., 2012; Terekhanova et al., 2014; Terekhanova et al., 2019; Nelson & Cresko, 2018; Fang et al., 2019), the level of parallelism in lake–stream and pelagic–benthic ecotype pairs of three-spined sticklebacks is much more diverse (Conte et al., 2012; Conte et al., 2015; Peichel et al., 2001; Peichel & Marques, 2017; Rennison et al., 2019; Stuart et al., 2017). For instance, Conte et al. (2015) found that among benthic–limnetic three-spined stickleback pairs from Paxton and Priest lakes (Vancouver Island, BC, Canada), 76% of 42 phenotypic traits diverged in the same direction, whereas only 49% of the underlying QTL evolved in parallel in both lakes. For highly parallel traits in two other pairs of benthic–limnetic sticklebacks, only 32% of the underlying QTL were shared (Conte et al., 2012). Similarly, Stuart et al. (2017) found that among 11 evolutionary independent replicate pairs of lake–stream three-spined stickleback populations (Vancouver Island, BC, Canada), both within-habitat variation and constraints to gene flow contributed to the observed variation in levels of phenotypic parallelism. Different lakes and streams probably do not have similar access to the same global pool of ancestral variation as pairs of

marine–freshwater three-spined sticklebacks, where gene flow in the sea is high. This is consistent with the notion that more heterogeneous access to ancestral variation can indeed limit genetic parallelism. This is also true for one example of marine–freshwater three-spined stickleback divergence among isolated insular freshwater populations in the Haida Gwaii archipelago off the northern Pacific coast of Canada (Deagle et al., 2013). Here, similarly to the nine-spined sticklebacks in this study, several freshwater populations did not display any reduction in pelvic armour. However, those populations that were fully plated were also genetically more similar to adjacent marine individuals, suggesting that recent marine–freshwater admixture and/or selection favouring plated freshwater individuals could explain this pattern. Thus, with respect to access to ancestral variation available for freshwater adaptation, nine-spined sticklebacks are probably closer to the three-spined stickleback lake–stream and benthic–limnetic study systems than to the three-spined stickleback marine–freshwater study system. The only notable exception is the lake–stream three-spined sticklebacks mentioned above, where genetic structuring also is high.

In a small geographical region in Belgium, Raeymaekers et al. (2017) compared the strength and nature of neutral and adaptive divergence along a salinity gradient in nine- and three-spined sticklebacks. Since the lake and pond populations in this study were connected by creeks (allowing contemporary gene flow), in contrast to our study—where a much larger geographical area is considered—population structuring among the nine-spined stickleback population was not stronger than in the three-spined sticklebacks. Despite gene flow, both phenotypic and genetic parallelism was still lower in nine- compared to three-spined sticklebacks. We suspect that lack of parallelism in nine-spined sticklebacks in Raeymaekers et al. (2017) simply lies in lack of ancestral variation—it does not matter how much gene flow there is if the necessary ancestral variation for local adaptation is lacking. Based on our findings it thus seems likely that ancestral variation for local adaptation in nine- but not three-spined sticklebacks was already lacking in the marine ancestral populations from which the Raeymaekers et al. (2017) study area was colonized.

4.4 | Candidate genes

While the QTL peak for the *Pitx1/Pel* region in the HEL × RYT cross was narrow, this was not the case for the other QTL we detected. Hence, due to the large QTL regions detected by four-way single-mapping analyses, it was not meaningful to perform gene ontology enrichment (GO) analyses—the QTL regions would have contained possibly thousands of genes. Instead, we searched the literature for known candidate genes related to pelvic reduction and found three (excluding *Pitx1/Pel*) that were clearly contained within the identified QTL regions (Figure 2; Table S4). Due to the aforementioned large QTL regions, these can only be considered as highly putative candidate genes for pelvic reduction and will not be discussed further. However, further studies of pelvic reduction might find these candidates worthy of attention.

4.5 | Conclusions

Our results show that the repeated parallel reduction in pelvic structures in freshwater populations of nine-spined sticklebacks is due to a diverse set of genetic changes: only one small-effect QTL for pelvic reduction was shared between the three experimental crosses in this study. In one cross, pelvic reduction was mapped to the previously identified *Pitx1/Pel* regions, but in the other two crosses, the genetic basis of pelvic reduction was polygenic, and mapped to many different chromosomes. In addition to these, yet another large-effect QTL different from the *Pitx1/Pel* locus probably segregates in one nine-spined stickleback population, which is yet to be identified. The results also shed light on the possible drivers of the observed genetic heterogeneity underlying pelvic reduction; as shown by simulations, heterogeneous genetic architectures are more likely to emerge when access to ancestral variation is limited by strong IBD and population structuring. This reinforces the role of the nine-spined sticklebacks as a useful model system, alongside the three-spined stickleback, to study adaptive evolution in the wild. Furthermore, because the population demographic characteristics of nine-spined sticklebacks are similar to small and endangered species/populations, it is also likely to be a well-suited model to study the genetics of adaptation in populations of conservation concern.

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AUTHOR CONTRIBUTION

T.S., J.Y. and B.G. performed linkage crossing experiments and measured phenotypic traits. P.R. performed linkage map analyses and Z.L. developed and performed QTL mapping analyses with contributions from P.K. P.K. performed simulations and epistatic interaction analyses. A.L. performed *Pel*-deletion analyses. J.M. was responsible for the original idea and supervised this project. P.K., J.M. and Z.L. wrote the manuscript with contributions from all other authors.

DATA AVAILABILITY STATEMENT

The data and code for analyses that support the findings of this study are openly available in Dryad at <https://doi.org/10.5061/dryad.76hdr7str> (Kemppainen et al., 2020). Raw sequence reads

have been submitted to NCBI's short-read archives with accession nos.: PRJNA673430 and PRJNA672863.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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