Genetic Architecture of Parallel Pelvic Reduction in Ninespine Sticklebacks

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ABSTRACT Teleost fish genomes are known to be evolving faster than those of other vertebrate taxa. Thus, fish are suited to address the extent to which the same vs. different genes are responsible for similar phenotypic changes in rapidly evolving genomes of evolutionary independent lineages. To gain insights into the genetic basis and evolutionary processes behind parallel phenotypic changes within and between species, we identified the genomic regions involved in pelvic reduction in Northern European ninespine sticklebacks (Pungitius pungitius) and compared them to those of North American ninespine and threespine sticklebacks (Gasterosteus aculeatus). To this end, we conducted quantitative trait locus (QTL) mapping using 283 F₂ progeny from an interpopulation cross. Phenotypic analyses indicated that pelvic reduction is a recessive trait and is inherited in a simple Mendelian fashion. Significant QTL for pelvic spine and girdle lengths were identified in the region of the Pituitary homeobox transcription factor 1 (Pitx1) gene, also responsible for pelvic reduction in threespine sticklebacks. The fact that no QTL was observed in the region identified in the mapping study of North American ninespine sticklebacks suggests that an alternative QTL for pelvic reduction has emerged in this species within the past 1.6 million years after the split between Northern European and North American populations. In general, our study provides empirical support for the view that alternative genetic mechanisms that lead to similar phenotypes can evolve over short evolutionary time scales.

Understanding the genetic basis of the evolution and diversity of phenotypic traits is a central topic in evolutionary biology. Similar phenotypes often evolve independently across multiple populations or closely related species facing similar environmental selection pressures (Barrett and Schluter 2008; Nadeau and Jiggins 2010). Such parallel phenotypic changes have been observed in a wide variety of organisms (Jones et al. 1992; Losos et al. 1998; Huey et al. 2000; Colosimo et al. 2005; Rogers and Bernatchez 2007), strongly suggesting that the observed phenotypic parallelism is shaped by natural selection (Endler 1986; Schluter 2000). Parallel phenotypic evolution can be based on utilization of the same or different genetic mechanisms to acquire the same phenotypes (Arendt and Reznick 2008; Elmer and Meyer 2011). Although several empirical studies have provided examples in which similar phenotypes are produced by the repeated selection of the same pool of standing variation (Barrett and Schluter 2008; Nadeau and Jiggins 2010), the relative importance of standing variation and new mutations in parallel phenotypic evolution is as yet poorly understood (Elmer and Meyer 2011; Conte et al. 2012).

Comparative genome mapping provides a framework to assess the similarities and dissimilarities in the genetic bases of phenotypic evolution both at intraspecific and interspecific levels, and also offers an opportunity to gain insights into the evolutionary history of genomes. Recent studies have discovered that the rate of genome evolution differs among taxonomic groups (Kohn et al. 2006; Ferguson-Smith and Trifonov 2007; Hufton et al. 2007; Huttley et al. 2007; Hufton et al. 2008). For instance, teleost fish genomes exhibit a higher incidence of chromosomal rearrangements and a faster evolution of protein coding...
sequences than other vertebrate taxa, implying that their genomes are evolving rapidly (Jaillon et al. 2004; Naruse et al. 2004; Woods et al. 2005; Sémon and Wolfe 2007; Hufston et al. 2008). Thus, as seen in frequent turnover and rearrangements of fish sex chromosomes (Devlin and Nagahama 2002; Mank and Avise 2009; Cioffi et al. 2012; Kitano and Peichel 2012), fish genomes are considered plastic. The rapid evolution of fish genomes is associated with the high morphological, ecological, and physiological diversity in this taxon (Ravi and Venkatesh 2008), and parallel phenotypic evolution has been observed in several fish species (Bell and Foster 1994; Reznick et al. 1996; Landry et al. 2007; Jeffery 2009; Chan et al. 2010). Therefore, teleost fish are well-suited for studying the evolutionary dynamics and processes of genome rearrangements, as well as the genetic basis of parallel phenotypic evolution. In particular, they are useful to address the extent to which the same genes can be responsible for similar phenotypic changes in rapidly evolving genomes.

Stickback fishes have served as important model organisms in evolutionary biology (Wootton 1976; Bell and Foster 1994; Kingsley and Peichel 2007; Östlund-Nilsson and Mayer 2007; Merilä 2013). In particular, threespine (Gasterosteus aculeatus) and ninespine sticklebacks (Pungitius pungitius) exhibit diverse phenotypic and ecological characteristics across their global distributions (Bell and Foster 1994; Merilä 2013). Although these species diverged from a common ancestor approximately 13 million years ago (Bell et al. 2009), they share similar morphological features, including bony lateral plates and pelvic apparatus. Thus, they are well-suited for studying the genetic basis of phenotypic evolution at both intraspecific and interspecific levels. Parallel phenotypic evolution has been widely observed and extensively studied in threespine sticklebacks (Bell 1976; Bell and Foster 1994; Walker and Bell 2000). Specifically, although ancestral marine threespine sticklebacks have a full row of lateral armor plates, long pelvic spines, and well-developed pelvic girdles, all of these traits have become reduced or even lost in independently and repeatedly colonized freshwater populations in which predation pressure by piscine predators is reduced. Genetic analyses have demonstrated that the repeated reduction in the number of lateral bony plates is governed by the Ectodysplasin (Eda) gene in globally distributed populations (Colosimo et al. 2004, 2005; Cresko et al. 2004). Similarly, the Pituitary homeobox transcription factor 1 (Pitx1) gene has been shown to be responsible for pelvic reduction in both Pacific and Atlantic populations of threespine sticklebacks (Cole et al. 2003; Cresko et al. 2004; Shapiro et al. 2004; Coyle et al. 2007; Chan et al. 2010). Although fewer genetic studies have been conducted using ninespine sticklebacks, phenotypic analyses of F1 hybrids between North American ninespine and threespine sticklebacks suggested that pelvic reduction is controlled by the same gene in these species (Shapiro et al. 2006). Nevertheless, a quantitative trait locus (QTL) mapping study of North American ninespine sticklebacks indicated that a major genomic region influencing pelvic reduction is distinct from the Pitx1 locus (Shapiro et al. 2009). Thus, genetic variation for pelvic reduction is likely to exist at multiple loci in this species. Phylogenetic analyses have uncovered the presence of several distinct lineages in ninespine sticklebacks across the Northern Hemisphere (Haglund et al. 1992a; Takahashi and Goto 2001; Aldenhoven et al. 2010; Shikano et al. 2010a; Teacher et al. 2011). Although threespine sticklebacks have undergone rapid morphological transitions after freshwater colonization, mainly after the last ice age (Bell and Foster 1994), morphological diversification might have occurred through different genetic changes in distinct lineages—and in different time scales—in ninespine sticklebacks because of their more heterogeneous evolutionary history. Therefore, comparative analyses of these two stickleback species can provide important insight into the evolutionary processes and genetic underpinnings that have led to similar phenotypes.

The main aim of this study was to improve our understanding of the genetic basis of parallel pelvic reduction in ninespine sticklebacks and to ask whether this reduction is likely to have occurred through similar or different genetic mechanisms in different lineages. In particular, we were interested in exploring the origin and evolution of genetic variation for pelvic reduction in this species. To this end, we identified the genomic regions involved in pelvic reduction in Northern European ninespine sticklebacks—with the aid of QTL mapping with 283 F2 segregating progeny of the cross between populations with and without pelvic spines—and compared them to those of North American ninespine and threespine sticklebacks (Cole et al. 2003; Cresko et al. 2004; Shapiro et al. 2004, 2006, 2009; Coyle et al. 2007; Chan et al. 2010). In addition, intraspecific and interspecific comparative genomic analyses were conducted to assess possible chromosomal rearrangements by comparing our linkage map with that of North American ninespine sticklebacks (Shapiro et al. 2009) and the threespine stickleback genome (Jones et al. 2012). Given that the stickleback sex chromosomes are known to evolve in rapid pace (Ross et al. 2009), we also mapped the sex-determining locus in the European lineage of ninespine sticklebacks to see whether it corresponds to that found in the North American lineage of this species.

**MATERIALS AND METHODS**

**Fish**

The grandparental fish (F0) were collected from the Baltic Sea (Helsinki, Finland; 60°13’N, 23°11’E) and a pond (Ryttilampi, Finland; 66°23’N, 29°19’E) in northeastern Finland in 2006. Pelvic reduction was observed in the pond population, whereas no pelvic reduction was found in the marine population (Herczeg et al. 2010). A female from the marine population was artificially crossed with a male from the pond population in July 2006, and the F1 offspring were reared in an aquarium at approximately 15°C. They were fed brine shrimp (Artemia sp.) nauplii in larval and juvenile stages, and, later, frozen bloodworms (Chironomidae sp.). After an artificial hibernation at 6°C without light, fish were maintained at 17°C under permanent light to facilitate reproduction. One female and one male were randomly chosen and mated in an aquarium. Seven successive clutches were obtained in September and October 2008. These clutches were reared in 1.4-liter tanks in a zebrafish rack system equipped with physical, biological, and UV filters (Aquaneering Inc., San Diego, CA). The F2 offspring were placed individually in 1.4-liter tanks in the zebrafish rack systems 6 d after hatching. White plastic sheets were placed between the tanks to block visibility between the fish. The fish were reared at 17°C under the 14-hr light and 10-hr dark photoperiod and fed twice per day. Food was, at first, brine shrimp nauplii. After 4 wk, it was changed to brine shrimp nauplii and frozen Cyclops. After another 4 wk, it was changed to frozen Cyclops and bloodworms. Finally, after another 4 wk, it was changed to frozen bloodworms. At 187 days posthatching, the fish were anesthetized with MS-222 (tricaine methanesulfonate) and photographed with a digital camera. The specimens were fixed in 4% formalin for morphological measurements. Fin clips were preserved in ethanol for DNA analyses. In total, 283 fish (56, 44, 38, 37, 40, 31, and 27 from seven clutches) were used for analyses. The experiments were conducted under the license from the Finnish National Animal Experiment Board (#STH379A).
Morphological measurements

The fish were stained with Alizarin Red S, following the study of Pritchard and Schluter (2001). Pelvic spine and girdle lengths were measured with a digital caliper to the nearest 0.01 mm. Both left and right pelvic girdles and spines were measured twice by the same person, and the averaged values were used for analyses. As a size proxy, we calculated centroid size, which is the square root of the summed squared distances from the landmarks to the centroid (Bookstein 1991). We used the same landmarks on the digital photographs as outlined by Herczeg et al. (2010), and we calculated centroid size using tpsRelw 1.46 (Rohlf 2006). Gender was determined by checking the gonads.

DNA extraction and genotyping

Total genomic DNA was extracted from ethanol-preserved fin clips using a silica fine-based purification method (Elphinstone et al. 2003) after proteinase K digestion. All the F2 offspring (N = 283) and their parents and grandparents (N = 4) were genotyped for 235 microsatellite markers (Largiadèr et al. 1999; Peichel et al. 2001; Heckel et al. 2002; Colosimo et al. 2004; Miller et al. 2007; Mäkinen et al. 2008; Shapiro et al. 2009; Shikano et al. 2010b; Shimada et al. 2011; Laine et al. 2012; Supporting Information, Table S1). Out of the 235 markers, 46 were developed for specific genes with known biological functions in fish (Shapiro et al. 2009; Shikano et al. 2010b; Shimada et al. 2011; Laine et al. 2012). Polymerase chain reactions (PCRs) for all markers except Ppbig were performed in a 10-μl volume containing 1× Qiagen Multiplex PCR Master Mix (Qiagen), 0.5× Q-Solution, 2 pmol of each primer, and 10 ng of template DNA. One of each primer pair was labeled with FAM, HEX, or TET fluorescent dye. PCR cycling started with an initial activation step at 95°C for 15 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 90 sec, and 72°C for 60 sec, and completed with a final extension at 60°C for 5 min. PCRs for Ppbig markers were conducted according to the methods of Laine et al. (2012). All PCR products were diluted 1:500 with Milli-Q water and genotyped using a MegaBACE 1000 automated sequencer (Amersham Biosciences) with ET-ROX 400 size standard (Amersham Biosciences). Alleles were scored using Fragment Profiler 1.2 program (Amersham Biosciences) and edited by eye. To ensure consistency in genotyping, all alleles were read by the same person.

Linkage map

A linkage map was constructed using improved CRI-MAP 2.5 (Green et al. 1990). The logarithm of the odds (LOD) scores for all pairs of markers were obtained using the TWOPOINT option. LOD score threshold of 3.0 was used as a significant criterion for linkage. The linkage map of North American ninespine sticklebacks (Shapiro et al. 2009) and the threespine stickleback genome sequence (Ensembl, database v. 66.1) were used as a reference for the initial linkage group (LG) building. For each LG, the best order of the markers was determined using the BUILD option by beginning with the most informative marker pair. Markers that could not be fitted straight with BUILD and with LOD score ≥4.0 were fitted manually. The FLIPS option (N = 3–5) was used to evaluate the statistical significance of the obtained order. After the best order was determined within each LG, double recombination events were detected using the CHROMPIC option. Individuals with more than four recombinations were removed and a second CRIMAP analysis round was conducted. Out of the 235 markers used for linkage analyses, nine with low polymorphism were discarded because of low LOD scores in TWOPOINT. Linkage maps were drawn using MAPCHART 2.2 (Voorrips 2002).

Genomic synteny was investigated by comparing our linkage map with the threespine stickleback genome (Jones et al. 2012) and the linkage map of North American ninespine sticklebacks (Shapiro et al. 2009). For the markers developed specifically for ninespine sticklebacks, microsatellite flanking sequences were subject to BLASTN searches against the threespine stickleback genome to identify homologous genomic regions. BLAST hits were considered significant at a threshold of E ≤ 10−5. Out of 226 informative markers in our linkage map, 110 were used for the linkage map of North American ninespine sticklebacks (Shapiro et al. 2009).

QTL mapping

QTL analyses were conducted with 226 markers, each of which had genotyping success of the F2 offspring between 74% and 99%. Sex-averaged linkage map distances were used for QTL mapping. The analyses were performed with GridQTL (Seaton et al. 2006) by using the BCF2 portlet and fitting both additive and dominance effects at 1-cM intervals (File S1, File S2, and File S3). Sex was included in the models as a fixed effect, and centroid size was included as a covariate. Experiment-wide and chromosome-wide significance levels of QTL were determined based on 10,000 permutations. QTL was considered significant when the F-value was more than the 5% experiment-wide threshold, and was considered suggestive when the F-value was more than the 5% chromosome-wide threshold. Confidence intervals were estimated with 10,000 bootstrap iterations. The proportion of the phenotypic variance explained by the QTL was calculated according to the methods of Zhou et al. (2006).

RESULTS

Linkage and comparative mapping

The genetic linkage map constructed with 226 informative markers consisted of 21 LGs (Figure S1), in accord with chromosome number based on cytogenetic analysis (Ocaliewicz et al. 2008). The sex-averaged linkage map spanned 1,632.7 cM, with an average intermarker distance of 7.2 cM. The number of loci per LG ranged from 5 to 21, and the size of the LGs spanned from 31.1 to 121.3 cM (Table S2). The female map covered 2178.7 cM and the male map covered 1211.6 cM (Table S2). Out of the 226 markers mapped, 217 had significant similarity to sequences of the threespine stickleback genome (Table S1). All of the 217 markers except one were assigned to specific LGs of the threespine stickleback genome.

In the linkage map, 206 markers were located in LGs in accordance with those of the threespine stickleback genome (Table 1). Likewise, in a comparison with the linkage map of North American ninespine sticklebacks (Shapiro et al. 2009), 105 out of the 110 markers used in both studies were mapped to the same LGs (Table 1). Although 12 markers were located on LG7 in the threespine stickleback genome, six of them were mapped to LG12, together with a set of 15 markers belonging to LG12 in the threespine stickleback genome (Figure 1). In the 21 markers mapped to LG12, pairwise LOD scores between the markers belonging to threespine stickleback LG7 and LG12 were significant (≥3.0) in 30 out of 90 combinations. However, the six markers belonging to threespine stickleback LG7 showed no significant LOD scores in any of 48 pairwise comparisons with the markers mapped to LG7 (Table S3). In addition to the interchromosomal discordance, the marker order within each LG was consistently inverted between the threespine stickleback genome and the linkage map of Northern European ninespine sticklebacks in partial regions of several chromosomes, including LG1, LG4, LG5, LG8, LG9, LG11, and LG13 (Figure S2).
The sex-determining locus was mapped to LG12, with the peak value at 53.2 cM (95% C.I. = 52.0–55.0) in the male map ($F$ = 159.1; $LOD$ = 46.1; Figure 1). In this LG, the sex-specific maps of females and males were 233.7 cM and 56.8 cM, respectively, including six markers belonging to threespine stickleback LG7 (Figure 1).
In the male meiosis, no recombination was observed between the 15 markers belonging to threespine stickleback LG12 (Figure 1) or between phenotypic sex and male-linked alleles at these loci (Table S4).

**Mapping of pelvic reduction**

In the F2 progeny of F1 hybrids (all F1 fish had pelvic spines), pelvic spines were present in 218 individuals and absent in 65 individuals, in agreement with a 3:1 Mendelian ratio ($\chi^2 = 0.62$; $P = 0.43$). Mapping analyses detected a significant QTL for the lengths of left and right pelvic spines and girdles that exhibited extremely high $F$-values (196.1–309.6) and LOD scores (53.1–70.7) (Figure 2, Table 2). All of these traits were mapped to LG7 at 57 cM, the end of the LG (95% C.I. = 54–57 for left pelvic spine length and 56–57 for other traits) at marker Pun319, which is located within the *Pitx1* gene (Shapiro et al. 2009). This QTL region explained 58–69% of phenotypic variance in these traits (Table 2). In the analyses of different genotypes for three different alleles at Pun319, considerably lower mean values of these traits were observed for individuals with two alleles from Rytilampi than for those with other allelic combinations (ANOVA, $F_{3,267} = 118.3–227.0$; $P < 0.001$; Table 2). Individuals with alleles from both Helsinki and Rytilampi tended to show slightly lower mean values as compared to those with the homozygous alleles of Helsinki, exhibiting significant differences in four out of the eight comparisons (Tukey test, $P < 0.05$).

No significant or suggestive QTL were detected for these traits in other LGs, including LG4 (Figure 2), in which a strong QTL for these traits was identified in North American ninespine sticklebacks (Shapiro et al. 2009).
**DISCUSSION**

**Genetic basis of parallel pelvic reduction**

Our study uncovered a major genomic region determining pelvic reduction in Northern European ninespine sticklebacks. Based on the phenotypic segregation in the interpopulation cross, it is apparent that the reduction of pelvic spines is recessive and inherited in a simple Mendelian fashion, as observed in threespine sticklebacks (Cresko et al. 2004; Shapiro et al. 2004; Coyle et al. 2007). Both pelvic spine and girdle lengths were mapped to the same region where the Pitx1 gene is located, explaining large proportions of the variance in these traits. This gene is known to be responsible for pelvic reduction in threespine sticklebacks (Cole et al. 2003; Cresko et al. 2004; Shapiro et al. 2004; Coyle et al. 2007; Chan et al. 2010). Thus, the Pitx1 gene is a strong candidate for the determination of pelvic phenotypes in Northern European ninespine sticklebacks.

Shapiro et al. (2006) conducted interspecific crosses between threespine and ninespine sticklebacks to test whether the same gene or a different gene is responsible for pelvic reduction in these two species. Based on the phenotypes of parents and their F₁ hybrid progeny, they suggested that the same genetic mechanism is likely to be involved in pelvic reduction in the two species. However, in a mapping study of ninespine sticklebacks with the F₁ individuals of a cross between parents lacking pelvic structures from phenotypically monomorphic Canadian and polymorphic Alaskan populations, the major genomic region involved in pelvic reduction was identified on LG4, which was completely distinct from the Pitx1 locus on LG7 (Shapiro et al. 2009). Given that the Pitx1 gene appears to be involved in pelvic reduction in the Canadian population (Shapiro et al. 2006), it is likely that pelvic reduction is caused by different genes in these populations, implying that genetic variation resulting in similar phenotypes of pelvic structures exists in at least two different loci in North American ninespine sticklebacks. Based on our and previous studies (Shapiro et al. 2006, 2009), it appears that although the same genetic mechanism for pelvic reduction might have persisted in threespine and ninespine sticklebacks during 13 million years of divergence (Bell et al. 2009), an alternative genetic mechanism for pelvic reduction may have evolved in North American ninespine sticklebacks, possibly within the past 1.6 million years after the divergence from Northern European ninespine sticklebacks (Teacher et al. 2011) (Figure 3).

Further mapping analyses with more populations from Northern Europe and North America should provide a more refined picture of the evolutionary process of different genetic architectures for pelvic reduction in sticklebacks.

It has been suggested that the likelihood of genetic parallelism underlying similar phenotypic changes decreases with increasing evolutionary distance between taxa (Elmer and Meyer 2011; Conte et al. 2012). There are several known cases in which the same genes
are responsible for phenotypic parallelism within the same or closely related species (Wood et al. 2005; Arendt and Reznick 2008; Nadeau and Jiggins 2010). However, the same gene can underlie similar phenotypes even across disparate taxonomic groups (Arendt and Reznick 2008; Nadeau and Jiggins 2010; Conte et al. 2012). The Melanocortin 1 receptor (Mc1r) gene is a case in point; it is known to control for pigmentation variation in diverse vertebrate taxa, including mammals, birds, and reptiles (Manseau et al. 2010). The most probable form of genetic parallelism among distantly related taxa is provided by repeated independent mutations in the same gene rather than shared ancestral genetic variation (Elmer and Meyer 2011). Thus far, empirical studies to infer parallel genetic evolution have been mostly based on candidate gene approaches, which rely on a priori hypothesis with respect to coding sequence or expression differences in a particular gene of interest (Wood et al. 2005; Arendt and Reznick 2008; Nadeau and Jiggins 2010). Consequently, inferences about different genetic mechanisms can be made solely on the basis of the lack of evidence for genetic parallelism in candidate genes. In contrast, genetic mapping provides powerful means to identify the locations and magnitudes of genomic regions controlling various phenotypic traits on a genome-wide scale. Despite the fact that intraspecific studies have been rarely performed to identify different genes or genomic regions involved in similar phenomena, within the same species (Gross et al. 2009; Thurber et al. 2013) as well as between closely related species (Ng et al. 2008; Lee et al. 2011). Although generalizations about the genetic mechanisms and evolutionary processes that lead to similar phenotypes may as yet be too early (Kopp 2009; Elmer and Meyer 2011), the case of pelvic reduction in ninespine sticklebacks highlights the fact that alternative QTL resulting in similar phenotypes can evolve over a short evolutionary time scale. This emphasizes the importance of both interspecific and intraspecific comparisons in understanding the genetic architectures of parallel phenotypic evolution.

In addition to a major genomic region explaining 81–87% of the variance in pelvic structures, a modifier locus of smaller effect was mapped to LG1 in North American ninespine sticklebacks (Shapiro et al. 2009). Similarly, four modifier loci were identified in threespine sticklebacks in which the Pitx1 locus explained 65% and 47% of the variance in pelvic spine and girdle lengths, respectively (Shapiro et al. 2004). Although the Pitx1 locus explained similar proportions of the variance in these traits in Northern European ninespine sticklebacks as observed in threespine sticklebacks (Shapiro et al. 2004), no significant modifier loci were detected in our study. Based on these results, it is likely that the number and location of modifier loci with small effects can differ between populations or species in sticklebacks, possibly because of their different genetic backgrounds. It should be also noted that the possible existence of modifier loci with small effects in

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**Table 2 QTL and phenotypic variation of pelvic spine and girdle lengths**

| Trait                  | LG  | Position in cM (C.I.) | F  | LOD  | PVE (%) | Closest Marker | Phenotypic Mean (± SE) for Each Genotype at Pun319 (mm) |
|------------------------|-----|-----------------------|----|------|---------|----------------|--------------------------------------------------------|
| Left pelvic spine      | LG7 | 57 (54–57)            | 288.34 | 67.80 | 67     | Pun319        | HEL/HEL (N = 70) 3.62 ± 0.09 HEL/RYT1 (N = 60) 3.22 ± 0.11 HEL/RYT2 (N = 73) 3.41 ± 0.07 RYT1/RYT2 (N = 68) 0.47 ± 0.13 |
| Right pelvic spine     | LG7 | 57 (56–57)            | 309.61 | 70.73 | 69     | Pun319        | HEL/HEL (N = 70) 3.49 ± 0.09 HEL/RYT1 (N = 60) 3.23 ± 0.11 HEL/RYT2 (N = 73) 3.32 ± 0.07 RYT1/RYT2 (N = 68) 0.35 ± 0.12 |
| Left pelvic girdle     | LG7 | 57 (56–57)            | 275.72 | 65.99 | 66     | Pun319        | HEL/HEL (N = 70) 7.98 ± 0.13 HEL/RYT1 (N = 60) 7.44 ± 0.16 HEL/RYT2 (N = 73) 7.41 ± 0.09 RYT1/RYT2 (N = 68) 3.90 ± 0.18 |
| Right pelvic girdle    | LG7 | 57 (56–57)            | 196.05 | 53.11 | 58     | Pun319        | HEL/HEL (N = 70) 7.61 ± 0.13 HEL/RYT1 (N = 60) 7.14 ± 0.16 HEL/RYT2 (N = 73) 7.11 ± 0.08 RYT1/RYT2 (N = 68) 4.37 ± 0.17 |

C.I., 95% confidence interval; PVE, proportion of phenotypic variation explained; HEL, allele from Helsinki; RYT, allele from Rytilampi.

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**Figure 3** Phylogenetic relationships and candidate genes for pelvic reduction in threespine and ninespine sticklebacks. The phylogeny is based on molecular (Haglund et al. 1992b; Orti et al. 1994; Aldenhoven et al. 2010; Teacher et al. 2011) and fossil data (Bell et al. 2009). The candidate genes were determined by the previous (Cole et al. 2003; Cresko et al. 2004; Shapiro et al. 2004, 2006, 2009; Coyle et al. 2007; Chan et al. 2010) and current studies.
Chromosomal rearrangements

The comparative mapping revealed that a segment of one autosome corresponding to LG7 in threespine sticklebacks is linked to LG12 in Northern European ninespine sticklebacks. Our results also indicated that none of the loci located on this segmental part shows linkage to the markers mapped to LG7. Hence, the rearrangement of genetic linkage patterns is likely attributable to a chromosomal rearrangement that has occurred after the divergence between threespine and ninespine sticklebacks. Because no linkage was detected between the segmental part and either the remaining region of LG7 or LG12 in the genetic map of North American ninespine sticklebacks (Shapiro et al. 2009), it is not certain if the arrangement of linkage patterns has occurred before or after the split between Northern European and North American populations. Our study also identified possible chromosomal inversions in several LGs as compared to the threespine stickleback genome, although potential errors in the genome sequences cannot be ruled out (Ross and Peichel 2008; Natri et al. 2013). Further cytogenetic analyses would clarify whether the linkage between LG12 and the segment of LG7 has formed via physical or pseudo linkage, as well as verify the occurrence of intrachromosomal rearrangements in Northern European ninespine sticklebacks.

Although rapid turnover of sex chromosome systems is often observed in fish, including sticklebacks (Devlin and Nagahama 2002; Mank and Avise 2009), the sex-determining gene was mapped to LG12 in Northern European ninespine sticklebacks, as in North American fish (Shapiro et al. 2009). In the male meiosis of both North American and Northern European ninespine sticklebacks, no recombination was observed in the chromosomal region corresponding to threespine stickleback LG12. Nevertheless, it is noteworthy that the interchromosomal rearrangement of linkage patterns was found for the sex chromosomes in our study. Cytogenetic analyses have shown that the Y chromosome of Northern European and North American ninespine sticklebacks is much larger than the X chromosome because of a Y chromosome rearrangement, possibly as a result of a duplication of the ancestral Y chromosome or an insertion of a duplicated autosomal segment into the Y chromosome (Ocalewicz et al. 2008; Ross et al. 2009). However, because the linkage between LG12 and the segment of LG7 was identified both in female and male maps in our study, it is unlikely that the Y chromosome rearrangement is a proximate cause of the rearrangement of linkage patterns. Although it is not certain whether the linkage between LG12 and the segment of LG7 is caused by physical or pseudo linkage, it appears that the segmental region of LG7 co-segregates with the sex-determining locus in Northern European ninespine sticklebacks. Theoretical studies have shown that the formation of linkage between the sex-determining locus and autosomal genes under sexually antagonistic selection has significant consequences on both population fitness and sex chromosome evolution (Charlesworth and Charlesworth 1980; van Doorn and Kirkpatrick 2007). As such, it would be of particular interest to assess if genes underlying sexually dimorphic traits and mating behavior reside in the chromosomal region newly linked to the sex-determining locus.

CONCLUSIONS

Our study demonstrated that the Pitx1 gene is a strong candidate for the determination of pelvic reduction in Northern European ninespine sticklebacks. The interspecific and intraspecific comparative analyses indicated that although the same genetic mechanism for pelvic reduction might have persisted in threespine and ninespine sticklebacks, alternative QTL for pelvic reduction might have evolved in ninespine sticklebacks, possibly within the past 1.6 million years after the split between Northern European and North American populations. Hence, our study gives empirical support for the view that alternative genetic mechanisms leading to similar phenotypes can evolve over a relatively short evolutionary time scale.

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LITERATURE CITED

Aldenhoven, J. T., M. A. Miller, P. S. Corneli, and M. D. Shapiro, 2010 Phylogeography of ninespine sticklebacks (Pungitius pungitius) in North America: glacial refugia and the origins of adaptive traits. Mol. Ecol. 19: 4061–4076.
Arendt, J., and D. Reznick, 2008 Convergence and parallelism reconsidered: what have we learned about the genetics of adaptation? Trends Ecol. Evol. 23: 26–32.

Barrett, R. D., and D. Schluter, 2008 Adaptation from standing genetic variation. Trends Ecol. Evol. 23: 38–44.

Bell, M. A., 1976 Evolution of phenotypic diversity in Gasterosteus aculeatus superspecies on the Pacific coast of North America. Syst. Zool. 25: 211–227.

Bell, M. A., and S. A. Foster, 1994 Introduction to the evolutionary biology of the threespine stickleback, pp. 1–27 in The Evolutionary Biology of the Threespine Stickleback, edited by M. A. Bell, and S. A. Foster. Oxford University Press, Oxford.

Bell, M. A., J. D. Stewart, and P. J. Park, 2009 The world’s oldest fossil threespine stickleback fish. Copeia 2009: 256–265.

Bookstein, F. L., 1991 Morphometric Tools for Landmark Data: Geometry and Biology, Cambridge University Press, Cambridge.

Chan, Y. F., M. E. Marks, F. C. Jones, G. Villarreal, Jr., M. D. Shapiro, Ciof, C. Wilson, J. Murphy, M. Currey, Cresko, W. A., A. Amores, P. F. Colosimo, P. F., K. E. Hosemann, S. Balabhadra, G. Villarreal, Jr., M. Dickson, 2010 Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a Pitx5 enhancer. Science 327: 302–305.

Charlesworth, D., and B. Charlesworth, 1980 Sex differences in fitness and selection for centric fusions between sex chromosomes and autosomes. Genet. Res. 35: 205–214.

Cioffi, M. B., O. Moreira Filho, L. F. Almeida-Toledo, and L. A. C. Bertollo, 2012 The contrasting role of heterochromatin in the differentiation of sex chromosomes: an overview from Neotropical fish. J. Fish Biol. 80: 2125–2139.

Cole, N. J., M. Tanaka, A. Prescott, and C. A. Tickle, 2003 Expression of limb initiation genes and clues to the basis of morphological diversification in threespine sticklebacks. Curr. Biol. 13: R951–R952.

Colosimo, P. F., C. L. Peichel, K. Nergen, B. K. Blackman, S. D. Shapiro et al., 2004 The genetic architecture of parallel armor plate reduction in threespine sticklebacks. PLoS Biol. 2: 635–641.

Colosimo, P. F., K. E. Hosemann, S. Balabhadra, G. Villarreal, Jr., M. Dickson et al., 2005 Widespread parallel evolution in sticklebacks by repeated fixation of Ectodysplasin alleles. Science 307: 1928–1933.

Conte, G. L., M. E. Arnegard, C. L. Peichel, and D. Schluter, 2012 The probability of genetic parallelism and convergence in natural populations. Proc. Biol. Sci. 279: 5039–5047.

Coyle, S. M., F. A. Huntingford, and C. L. Peichel, 2007 Parallel evolution of Pitx5 underlies pelvic reduction in Scottish threespine stickleback (Gasterosteus aculeatus). J. Hered. 98: 581–586.

Cresko, W. A., A. Amores, C. Wilson, J. Murphy, M. Currey et al., 2004 Parallel genetic basis for repeated evolution of armor loss in Alaskan threespine stickleback populations. Proc. Natl. Acad. Sci. USA 101: 6050–6055.

Devlin, R. H., and Y. Nagahama, 2002 Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208: 191–364.

Elmer, K. R., and A. Meyer, 2011 Adaptation in the age of ecological genomics: insights from parallelism and convergence. Trends Ecol. Evol. 26: 298–306.

Elphinstone, M. S., G. N. Hinten, M. J. Anderson, and C. J. Nock, 2003 An inexpensive and high-throughput protocol to extract and purify total genomic DNA for population studies. Mol. Ecol. Notes 3: 317–320.

Endler, J. A., 1986 Natural Selection in the Wild, Princeton University Press, Princeton.

Ferguson-Smith, M. A., and V. Trifonov, 2007 Mammalian karyotype evolution. Nat. Rev. Genet. 8: 950–962.

Green, P., K. Falls, and S. Crooks, 1990 Documentation for CRI-MAP (version 2.4), Washington University School of Medicine, St. Louis.

Gross, J. B., R. Borowsky, and C. J. Tabin, 2009 A novel role for McIr in the parallel evolution of depigmentation in independent populations of the cavefish Astyanax mexicanus. PLoS Genet. 5: e1000326.

Haglund, T. R., D. G. Buth, and R. Lawson, 1992a Allozyme variation and phylogenetic relationships of Asian, North American, and European populations of the threespine stickleback, Pungitius pungitius, pp. 438–452 in Systematics, Historical Ecology, and North American Freshwater Fishes, edited by R. L. Mayden Stanford University Press, Stanford.

Haglund, T. R., D. G. Buth, and R. Lawson, 1992b Allozyme variation and phylogenetic relationships of Asian, North American, and European populations of the threespine stickleback. Copeia 1992: 432–443.

Heckel, G., M. Zbinden, D. Mazzi, A. Kohler, G. Reckeweg et al., 2002 Microsatellite markers for the three-spined stickleback (Gasterosteus aculeatus L.) and their applicability in a freshwater and an anadromous population. Conserv. Genet. 3: 79–81.

Herczeg, G., M. Turtiani, and J. Merilä, 2010 Morphological divergence of North-European nine-spined sticklebacks (Pungitius pungitius): sign-natures of parallel evolution. Biol. J. Linn. Soc. Lond. 101: 403–416.

Higuchi, M., and A. Goto, 1996 Genetic evidence supporting the existence of two distinct species in the genus Gasterosteus around Japan. Environ. Biol. Fishes 47: 1–16.

Hu, Z., and S. Xu, 2008 A simple method for detecting the statistical power for detecting a QTL located in a marker interval. Heredity 101: 48–52.

Huey, R. B., G. W. Gilchrist, M. L. Carlson, D. Berrigan, and L. Serra, 2000 Rapid evolution of a geographic cline in size in an introduced fly. Science 287: 308–309.

Hufton, A. L., D. Groth, M. Vingron, H. Lehrach, A. J. Pousta et al., 2008 Early vertebrate whole genome duplications were predated by a period of intense genome rearrangement. Genome Res. 18: 1582–1591.

Huttley, G. A., M. J. Wakefield, and S. Eastal, 2007 Rates of genome evolution and branching order from whole genome analysis. Mol. Biol. Evol. 24: 1722–1730.

Jallon, O., J. M. Aury, F. Brunet, J. L. Petit, N. Stange-Thomann et al., 2004 Genome duplication in the teleost fish Tetraodon nigroviridis reveals the early vertebrate proto-karyotype. Nature 431: 946–957.

Jeffery, W. R., 2009 Regressive evolution in Astyanax cavefish. Annu. Rev. Genet. 43: 25–47.

Jones, R. C. D., and H. W. Thomas, 1992 Are parallel morphologies of cave organisms the result of similar selection pressures? Evolution 46: 353–365.

Kopp, A., 2009 Metamodels and phylogenetic replication: a systematic approach to the evolution of developmental pathways. Evolution 63: 2771–2789.

Laine, V. N., C. R. Primmer, G. Herczeg, J. Merilä, and T. Shikano, 2012 Isolation and characterization of 13 new nine-spined stickleback, Pungitius pungitius, microsatellites located nearby candidate genes for behavioural variation. Ann. Zool. Fenn. 49: 123–128.

Landry, L., W. F. Vincent, and L. Bernatchez, 2007 Parallel evolution of lake whitefish dwarf cytoplasts in association with limnological features of their adaptive landscape. J. Evol. Biol. 20: 971–984.

Largiárd, C. R., V. Fries, B. Kobler, and T. C. M. Bakker, 1999 Isolation and characterization of microsatellite loci from the threespined stickleback (Gasterosteus aculeatus L.). Mol. Ecol. 8: 342–344.

Lee, S. F., L. Rako, and A. A. Hoffmann, 2011 Genetic mapping of adaptive wing variation size in Drosophila simulans. Heredity 107: 22–29.

Losos, J. B., T. R. Jackman, A. Larson, K. de Queiroz, and L. Rodríguez-Schettino, 1998 Historical contingency and determinism in replicated adaptive radiations of island lizards. Science 279: 2113–2118.

Mäkinen, H. S., J. M. Cano, and J. Merilä, 2008 Identifying footprints of directional and balancing selection in marine and freshwater three-spined stickleback (Gasterosteus aculeatus) populations. Mol. Ecol. 17: 3565–3582.
Manceau, M., V. S. Domingues, C. R. Linnen, E. B. Rosenblum, and H. E. Hoekstra, 2010 Convergence in pigmentation at multiple levels: mutations, genes and function. Phil. Trans. R. Soc. B. 365: 2439–2450.

Mank, J. E., and J. C. Avise, 2009 Evolutionary diversity and turn-over of sex determination in teleost fishes. Sex. Dev. 3: 60–67.

Miller, C. T., S. Beleza, A. A. Pollen, D. Schluter, R. A. Kittles et al., 2007 cis-Regulatory changes in Kit ligand expression and parallel evolution of pigmentation in sticklebacks and humans. Cell 131: 1179–1189.

Merilä, J., 2013 Nine-spined stickleback (Pungitius pungitius): an emerging model for evolutionary biology research. Ann. N. Y. Acad. Sci. 1289: 18–35.

Nadeau, N. J., and C. D. Jiggins, 2010 A golden age for evolutionary genomics. Genomic studies of adaptation in natural populations. Trends Genet. 26: 484–492.

Naruse, K., M. Tanaka, K. Mita, A. Shima, J. Postlethwait et al., 2004 A medaka gene map: the trace of ancestral vertebrate proto-chromosomes revealed by comparative gene mapping. Genome Res. 14: 820–828.

Nattr, H. M., T. Shikano, and J. Merilä, 2013 Progressive recombination suppression and differentiation in recently evolved neo-sex chromosomes. Mol. Biol. Evol. 30: 1131–1144.

Ng, C. S., A. M. Hamilton, A. Frank, O. Barmina, and A. Kopp, 2008 Genetic basis of sex-specific color pattern variation in Drosophila malerkotliana. Genetics 180: 421–429.

Oculewicz, K., D. Fopp-Bayat, P. Woznicki, and M. Jankun, 2008 Heteromorphic sex chromosomes in the ninespine stickleback Pungitius pungitius. J. Fish Biol. 73: 456–462.

Ortí, G., M. A. Bell, T. E. Reimchen, and A. Meyer, 1994 Global survey of mitochondrial-DNA sequences in the threespine stickleback: evidence for recent migrations. Evolution 48: 608–622.

Östlund-Nilsson, S., and I. Mayer, 2007 The biology of other sticklebacks, pp. 353–372 in Biology of the Three-Spined Stickleback, edited by Östlund-Nilsson S., I. Mayer, and F. A. Huntingford. CRC Press, Boca Raton.

Peichel, C. L., K. Nereng, K. A. Olbi, B. L. E. Cole, P. F. Colosimo et al., 2001 The genetic architecture of divergence between threespine stickleback species. Nature 414: 901–905.

Pritchard, J. R., and D. Schluter, 2001 Declining interspecific competition during character displacement: summoning the ghost of competition past. Evol. Ecol. Res. 3: 209–220.

Pritchard, J. K., J. K. Pickrell, and G. Coop, 2010 The genetics of human adaptation: hard sweeps, soft sweeps, and polygenic adaptation. Curr. Biol. 20: R208–R215.

Ravi, V., and B. Venkatesh, 2008 Rapidly evolving fish genomes and teleost diversity. Curr. Opin. Genet. Dev. 18: 544–550.

Reznick, D. N., F. H. Rodd, and M. Cardenas, 1996 Life-history evolution in guppies (Poecilia reticulata: Poeciliidae). 4. Parallelism in life-history phenotypes. Am. Nat. 147: 319–338.

Rogers, S. M., and L. Bernatchez, 2007 The genetic architecture of ecological speciation and the association with signatures of selection in natural lake whitefish (Coregonus sp. Salomoniidae) species pairs. Mol. Biol. Evol. 24: 1423–1438.

Rohlf, F. J., 2006 TpsDig version 2.10, Department of Ecology and Evolution, State University of New York, Stony Brook, New York.

Ross, J. A., and C. L. Peichel, 2008 Molecular cytogenetic evidence of rearrangements on the Y chromosome of threespine stickleback fish. Genetics 179: 2173–2182.

Ross, J. A., J. R. Urton, J. Boland, M. D. Shapiro, and C. L. Peichel, 2009 Turnover of sex chromosomes in the stickleback fishes (Gasterosteidae). PLoS Genet. 5: e1000391.

Schlüter, D., 2000 The ecology of adaptive radiation, Oxford University Press, Oxford.

Seaton, G., J. Hernandez, J. Grunche, I. White, J. Allen et al., 2006 GridQTL: a grid portal for QTL mapping of compute intensive datasets. Proceedings of the 8th World Congress on Genetics Applied to Livestock. Belo Horizonte, Brazil.

Sémon, M., and K. H. Wolfe, 2007 Rearrangement rate following the whole-genome duplication in teleosts. Mol. Biol. Evol. 24: 860–867.

Shapiro, M. D., M. E. Marks, C. L. Peichel, B. K. Blackman, K. S. Nereng et al., 2004 Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. Nature 428: 717–723.

Shapiro, M. D., M. A. Bell, and D. M. Kingsley, 2006 Parallel genetic origins of pelvic reduction in vertebrates. Proc. Natl. Acad. Sci. USA 103: 13753–13758.

Shapiro, M. D., B. R. Summers, S. Balabhadra, A. L. Miller, C. B. Cunningham et al., 2009 The genetic architecture of skeletal convergence and sex determination in ninespine sticklebacks. Curr. Biol. 19: 1140–1145.

Shikano, T., K. Shinada, G. Herguez, and J. Merilä, 2010a History vs. habitat type: explaining the genetic structure of European nine-spined stickleback (Pungitius pungitius) populations. Mol. Ecol. 19: 1147–1161.

Shikano, T., J. Ramadevi, Y. Shimada, and J. Merilä, 2010b Utility of sequenced genomes for microsatellite marker development in non-model organisms: a case study of functionally important genes in nine-spined sticklebacks (Pungitius pungitius). BMC Genomics 11: 334.

Shikano, T., H. M. Natri, Y. Shimada, and J. Merilä, 2011 High degree of sex chromosome differentiation in stickleback fishes. BMC Genomics 12: 474.

Shimada, Y., T. Shikano, and J. Merilä, 2011 A high incidence of selection on physiologically important genes in the three-spined stickleback, Gasterosteus aculeatus. Mol. Biol. Evol. 28: 181–193.

Slade, J. A. W. Santure, P. G. D. Feulner, E. A. Brown, A. D. Ball et al., 2010 Genome mapping in intensively studied wild vertebrate populations. Trends Genet. 26: 275–284.

Takahashi, H., and A. Goto, 2001 Evolution of East Asian ninespine sticklebacks as shown by mitochondrial DNA control region sequences. Mol. Phylogenet. Evol. 21: 135–155.

Teacher, A. G. F., T. Shikano, M. E. Karjalainen, and J. Merilä, 2011 Phylogeography and genetic structuring of European nine-spined sticklebacks (Pungitius pungitius) - mitochondrial DNA evidence. PLoS ONE 6: e19476.

Thurber, C. S., M. H. Jia, Y. Jia, and A. L. Caicedo, 2013 Similar traits, different genes? Examining convergent evolution in related weedy rice populations. Mol. Ecol. 22: 685–698.

van Doorn, G., and M. Kirkpatrick, 2007 Turnover of sex chromosomes induced by sexual conflict. Nature 449: 909–912.

Voorrips, R. E., 2002 MapChart: Software for the graphical presentation of linkage maps and QTLs. J. Hered. 93: 77–78.

Walker, J. A., and M. A. Bell, 2000 Net evolutionary trajectories of body shape evolution within a microgeographic radiation of threespine sticklebacks (Gasterosteus aculeatus). J. Zool. (Lond.) 252: 293–302.

Watanabe, K., S. Mori, and M. Nishida, 2003 Genetic relationships and origin of two geographic groups of the freshwater threespine stickleback, Hariyoi. Zoolog. Sci. 20: 265–274.

Wood, T. E., J. K. Burke, and I. H. Rieseberg, 2005 Parallel genotypic adaptation: when evolution repeats itself. Genetics 123: 157–170.

Woods, I. G., C. Wilson, B. Friedlander, P. Chang, D. K. Reyes et al., 2013 Similar traits, different genes? Examining convergent evolution in related weedy rice populations. Mol. Ecol. 22: 685–698.

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