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Letter

Sex chromosome turnover in hybridizing stickleback lineages

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Abstract

Recent discoveries of sex chromosome diversity across the tree of life have challenged the canonical model of conserved sex chromosome evolution and evoked new theories on labile sex chromosomes that maintain less differentiation and undergo frequent turnover. However, theories of labile sex chromosome evolution lack direct empirical support due to the paucity of case studies demonstrating ongoing sex chromosome turnover in nature. Two divergent lineages (viz. WL & EL) of nine-spined sticklebacks (*Pungitius pungitius*) with different sex chromosomes (linkage group [LG] 12 in the EL, unknown in the WL) hybridize in a natural secondary contact zone in the Baltic Sea, providing an opportunity to study ongoing turnover between coexisting sex chromosomes. In this study, we first identify an 80 kbp genomic region on LG3 as the sex-determining region (SDR) using whole-genome resequencing data of family crosses of a WL population. We then verify this region as the SDR in most other WL populations and demonstrate a potentially ongoing sex chromosome turnover in admixed marine populations where the evolutionarily younger and homomorphic LG3 sex chromosome replaces the older and heteromorphic LG12 sex chromosome. The results provide a rare glimpse of sex chromosome turnover in the wild and indicate the possible existence of additional yet undiscovered sex chromosome diversity in *Pungitius* sticklebacks.

Keywords: admixture, introgression, *Pungitius pungitius*, sex-determining region, SDR

Lay Summary

Sex chromosomes are highly conserved in mammals and birds but much more labile in other species, such as reptiles, amphibians, and fish. In the species having labile sex chromosomes, even different populations of the same species have the potential to evolve different sex chromosomes and sex-determining systems. In the nine-spined stickleback fish (*Pungitius pungitius*), the eastern European lineage has heteromorphic sex chromosomes on linkage group 12 (LG12), whereas our study identified the western European lineage to have homomorphic sex chromosomes on LG3, where an 80 kbp region determines sex. Interestingly, although the two lineages have different pairs of sex chromosomes, they mate and reproduce in the Baltic Sea. We show that in the hybrid marine populations, the western lineage's homomorphic sex chromosomes (LG3) are taking over the eastern lineage's heteromorphic sex chromosomes (LG12). This suggests that a transition of sex chromosomes (i.e., sex chromosome turnover) is happening in these populations. This study shows that sex chromosomes can be highly diverse in stickleback fish populations, even within the same species. Lastly, we also show a likely different and uncharacterized sex-determining system in UK populations of nine-spined sticklebacks, which indicates high sex chromosome diversity in *Pungitius* sticklebacks.

Introduction

In addition to genetic sex determination, sex chromosomes play an important role in reproductive isolation and speciation (Coyne & Orr, 2004; Presgraves, 2008). Sex chromosome evolution and diversification are thus crucial processes in biodiversification, and yet these processes remain understudied. The canonical model proposes that sex chromosomes evolve from autosomes by gaining the master sex-determining gene (e.g., *Sry* in mammals) in a sex-determining region (SDR) that ceases recombination. The nonrecombining SDR expands by accumulating repeats and deleterious mutations on the sex-limited chromosome (i.e., Y or W), which eventually becomes degenerated and structurally heteromorphic, as has been observed in highly conserved sex chromosomes of eutherian mammals, birds, and some insects (Bachtrog, 2013). On the other hand, recent studies of reptiles, amphibians, and fish have found highly labile sex chromosomes where different (sometimes unknown) genes may be recruited to trigger the sex-determining development (Myosho et al., 2012). In

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addition, the observation of diverse SDRs and homomorphic sex chromosomes indicates frequent turnovers where sex chromosomes can revert to autosomes before becoming highly diverged or degenerated (Dufresnes et al., 2015; Ieda et al., 2018; Jeffries et al., 2018). The diversity and evolutionary lability of sex chromosomes across the tree of life (Tree of Sex Consortium, 2014) have thus challenged the canonical model and inspired new theories of sex chromosome evolution (Furman et al., 2020; Kratochvíl et al., 2021; Perrin, 2021; Vicoso, 2019).

Sex chromosome turnover has been mostly inferred on the basis of sister lineages having different sex chromosomes (Ieda et al., 2018; Jeffries et al., 2018, 2022). In theory, the evolutionary process of sex chromosome turnover should involve a temporary stage when different SDRs coexist in the same population and are able to determine sex (Kitano et al., 2023; Myosho et al., 2012). Coexisting SDRs within species have been described in the polygenic sex determination (Moore & Roberts, 2013), but empirical cases of coexisting sex chromosomes in natural populations remain rare, partly because this stage is likely unstable (Kitano et al., 2023) and partly due to the difficulty of detecting young homomorphic sex chromosomes (Palmer et al., 2019). Recent studies of the Japanese soil frog found hybridization between populations having ZZ/ZW and XX/XY sex-determining systems (Ogata et al., 2018, 2021). However, these case studies focused on changes in SDRs in homologous sex chromosomes, which is different from the turnover between nonhomologous pairs of sex chromosomes where the replaced X or Z chromosome should revert to an autosome (Vicoso, 2019). Therefore, empirical case studies are needed to demonstrate coexisting nonhomologous sex chromosomes in natural populations and test theoretical mechanisms underlying their turnover.

The nine-spined stickleback (*Pungitius pungitius*) is a small cold-adapted fish with a circumpolar distribution in the northern hemisphere. The species colonized Europe from the North Pacific in multiple trans-Arctic waves, resulting in divergent western (WL) and eastern (EL) European lineages (Fang et al., 2021; Feng et al., 2022; Guo et al., 2019; Shikano et al., 2010; Teacher et al., 2011) with different sex chromosomes (Natri et al., 2019). The EL and non-European populations all have heteromorphic sex chromosomes (XX/XY) identified as linkage group 12 (LG12) with a large Y proposed to have evolved from a historically introgressed inversion from the closely related *P. sinensis* (Dixon et al., 2019; Natri et al., 2019). However, LG12 is not associated with phenotypic sex in the WL, which has unidentified homomorphic sex chromosomes that seem to have evolved more recently (Natri et al., 2019). In addition, WL and EL hybridize in the southeastern North Sea and southern Baltic Sea (Feng et al., 2022, 2024; Guo et al., 2019; Shikano et al., 2010; Teacher et al., 2011), indicating the coexistence of different sex chromosomes and a potential sex chromosome turnover.

The goal of this study was twofold. First, we aimed to identify the homomorphic sex chromosomes in the WL using whole-genome resequencing data of family crosses with known phenotypic sex. The homomorphic and evolutionarily young WL sex chromosomes are predicted to have low levels of degeneration and a narrow SDR with limited sequence differentiation (Palmer et al., 2019). Second, we aimed to characterize sex chromosome diversity using whole-genome resequencing data of 45 nine-spined stickleback populations across the species distribution range, including those in the natural secondary contact zone. We hypothesize that admixture between WL and EL results in a sex chromosome turnover, which predicts an unequal ratio of coexisting sex chromosomes in admixed populations where the more prevalent pair of sex chromosomes take over the other.

Materials and methods

WL family crosses

To identify sex chromosomes of the WL nine-spined sticklebacks, four family crosses were generated using individuals collected from Brugse polders, Maldegem, Belgium (51°10′ N, 03°28′ E) in 2011 under national and institutional ethical regulations and with permission from the Finnish Food Safety Authority (#1181/0527/2011 and #3701/0460/2011). The paired individuals were artificially mated in a zebrafish rack system (Aquaneering Inc., USA). Fish rearing and crossing followed the descriptions in Natri et al. (2019). Individuals were phenotypically sexed based on gonadal inspection. Genomic DNA was extracted from fin clip samples using the salting out method (Bruford et al., 1998). Whole-genome resequencing was done by BGI (Hong Kong) using the DNBseq PE 150 platform, targeting 10× for parents ($n = 8$) and 5× for offspring ($n = 15$ per sex per family, Supplementary Table S1). Demultiplexed raw sequencing reads were processed by AdapterRemoval version 2.3.1 (Schubert et al., 2016) to trim adapters, Ns (--trimns), and low-quality reads (--trimqualities). The retained pairs of reads and the collapsed (not truncated) reads were mapped to the ver.7 reference genome (Kivikoski et al., 2021) using bwa-mem in BWA v0.7.17 (Li, 2013) with the -M command and default parameters. The mapped reads were sorted and indexed and duplicates were marked using SAMtools version 1.16.1 (Danecek et al., 2021).

Identification of the WL SDR

First, sex-associated markers were inferred using the module Pileup2Likelihoods in LepMAP3 (Rastas, 2017). The duplicate-marked reads mapped to the 21 LGs were genotyped by mpileup in SAMtools (Li et al., 2009) using the minimum mapping quality 20 and the minimum base quality 30. Outputs were processed by ParentCall2 (Rastas, 2017) to call informative (removeNonInformative = 1) markers and identify sex-associated markers that are in non-Mendelian inheritance (diploid in the homogametic sex while haploid in the heterogametic sex) assuming XY male heterogamety (XLimit = 2) or ZW female heterogamety (ZLimit = 2).

Next, window-based analyses on SNP genotypes were used to narrow down the genomic region that has the largest intersex differentiation (hereafter, the SDR, which should include the sex-determining locus and its linked surrounding region that has ceased recombination). The duplicates-marked reads were genotyped by the Genome Analysis Toolkit (GATK) v4 following the best practice protocol (Depristo et al., 2011; Van der Auwera et al., 2013). Briefly, raw variants were called using HaplotypeCaller and merged by LG using CombineGVCFs, and all samples were jointly genotyped using GenotypeGVCFs. Biallelic SNPs on each LG were extracted using BCFtools (-m2 -M2 -v snps –min-ac = 1; Li, 2011) and filtered using VCFtools 0.1.16 (Danecek et al., 2011) by quality (--minGQ 20 --minQ 30), missingness (--max-missing 0.3), and minor allele frequency (--maf 0.01). Each LG was divided into 10 kbp nonoverlapping windows to calculate the percentage of heterozygous sites in each sex, the average ratio of female to male site depth in F_1 individuals, and the average per-site Weir and Cockerham's F_{st} between F_1 males and females in each family. These parameters were estimated using VCFtools (--site-mean-depth; --weir-fst-pop) and custom scripts available on Github. Results were visualized in R v4 (R Core Team, 2022). To verify candidate SDR windows, individual inbreeding coefficient (F-value) and missingness were estimated in VCFtools, and principal component analysis (PCA) was conducted in the R package adegenet

(Jombart & Ahmed, 2011) using the function glpca with all axes retained.

Genetic sexes and SDR in wild populations

Using the identified SDR, we characterized the genetic sexes and sex chromosomes of 887 wild-caught nine-spined sticklebacks from 45 globally distributed populations (Supplementary Table S2). The published raw whole-genome resequencing data (~10×; Feng et al., 2022) were remapped to the ver.7 reference genome and processed using GATK v3 following the best practice protocol. Biallelic SNPs were extracted from the identified LG3 SDR and the known sex-linked region on LG12 (1-16.9 Mb, hereafter the LG12 SDR for clarity, but this region probably lacks the most Y-specific SDR and might include some pseudoautosomal fragments; Kivikoski et al., 2021), respectively. SNPs were further filtered in VCFtools by quality (--minGQ 20 --minQ 30), depth (--min-meanDP 5 --max-meanDP 25), missingness (--max-missing 0.3), and minor allele frequency (--maf 0.01). Individual F-value and missingness were estimated in VCFtools. PCA was conducted using PLINK2 (--pca) with the default of keeping the first 10 PCs (Galinsky et al., 2016).

Because PCA indicated signals of genomic inversion (see results), we identified putative structural variants in the LG3 SDR using BreakDancer version 1.4.5 (Chen et al., 2009; Fan et al., 2014). The bwa-aligned and duplicates-marked bam files mapped to LG3 were processed by *bam2cfg.pl* to generate configuration files. Quality was checked following the protocol (Fan et al., 2014). Read groups were removed if they had the insert size coefficient of variation >0.4 or the percentage of inter-chromosomal read pairs >4%. The remaining individuals were processed by breakdancer-max to generate a list of putative structural variants, which were further filtered by confidence score (≥99) and the supporting number of reads (>the median of each dataset). BreakDancer analyses were performed separately on genetic males and females in the WL families (all parents and four randomly selected offspring per sex per family), the LG3-characterized WL populations (including POL-GDY, excluding UK), the LG12-characterized EL populations (excluding POL-GDY), and the non-European populations. The two UK populations were analyzed separately.

To construct phylogenetic trees of the LG3 SDR, we selected two to three homozygous individuals (i.e., having high F-values in the LG3 SDR) from each of the 45 populations. Heterozygous individuals were excluded because bifurcating trees do not do well with heterozygotes. We also included 9 individuals of other stickleback species as outgroups (*Pungitius sinensis*, *P. platygaster*, *P. tymensis*, *P. kaibarae*, and *Gasterosteus aculeatus*). The outgroup samples were collected in previous projects (Guo et al., 2019), sequenced as described in Feng et al. (2022), and genotyped as described above. Biallelic SNPs in the LG3 SDR from these selected individuals were filtered together in VCFtools using the same parameters described above. The filtered data were transformed into phylip format using vcf2phylip (Ortiz, 2019), and the maximum likelihood (ML) tree was constructed in RAxML version 8 (Stamatakis, 2014) using the GTRGAMMA model, a rapid search (-f a), and 1000 bootstraps (-# 1000). The best ML tree was rooted by *Gasterosteus aculeatus* and visualized in FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Admixture between marine populations in the Baltic Sea

Next, we focused on the Baltic Sea hybrid zone, where different SDRs coexist. Because freshwater populations are strongly influenced by founder effects and genetic drift, we only analyzed marine populations exhibiting more gene flow and larger effective population sizes (Shikano et al., 2010; Feng, Löytynoja, et al., 2023). Individuals from the 12 marine populations were first filtered by a kinship cutoff of 0.177 (the convention for filtering first-degree relationships) in PLINK2 (Chang et al., 2015). Biallelic SNPs of the retained unrelated individuals were subset and filtered in VCFtools by quality (--minGQ 20 --minQ 30), mean sequencing depths (--min-meanDP 5 --max-meanDP 25), minor allele frequency (--maf 0.02), and missingness (--max-missing 0.7). Data mapped to the 19 autosomes (excluding LG12 and LG3) were further filtered in PLINK2 by linkage disequilibrium (--indep-pairwise 50 5 0.2), minor allele frequency (--maf 0.05), and missingness (--geno 0.1). The filtered autosomal data were analyzed by ADMIXTURE version 1.3.0 (Alexander et al., 2009) with 10-fold cross-validation (CV) and ten replicates per K (number of populations) from 1 to 12. The optimal K was indicated by the lowest CV errors. Outputs were compiled by CLUMPAK (Kopelman et al., 2015) and visualized using R. Identity-by-descent (IBD) tracks were estimated in IBDseq (Browning & Browning, 2013) to represent fragments of shared ancestry between individuals. IBDseq was performed with the minimum logarithm of the odds (LOD) score of 4 and all other parameters as default. The correlation between IBD-like track lengths and LOD scores was visualized as quality control. Individuals were further classified into early- or late-generation hybrids based on the joint estimation of ancestry (i.e., hybrid index) and interclass heterozygosity in HIest version 2.0 (Fitzpatrick, 2012). First, HIest was conducted on all populations using diagnostic autosomal SNPs that had no missing data and were fixed for different alleles in the parental populations DEN-NOR and RUS-LEV. The function HIest was run with default settings and the data type "allele count." Next, we focused on the most admixed Polish population and used its adjacent populations (GER-RUE and SWE-GOT) as parents. Because stringent filtering for diagnostic SNPs kept fewer than 50 SNPs, which would yield low statistical power (Fitzpatrick, 2012), we retained SNPs that had no missing data and delta allele frequencies ≥0.4 between parental populations.

Results

Identification of the WL SDR

Whole-genome resequencing of four family crosses generated a total of 2,894,080,759 QC-passed mapped reads. LepMAP3 identified 698 X-associated markers that were mostly (69.05%) located on the LG3 when assuming XY male heterogamety, but only 340 Z-associated markers that were relatively evenly distributed across chromosomes when assuming ZW female heterogamety (Supplementary Figure S1A). A total of 3,997,985 SNPs were retained from 21 LGs after filtering. All LGs showed equal sequencing depths in the two sexes and near-zero inter-sex F_{st}, except for LG3, which had elevated F_{st} toward the end of this linkage group in all family crosses (Supplementary Figure S1B). In addition, 20 windows on LG3 had absolute delta heterozygote percentages >30% between sexes with excessive male heterozygosity (Figure 1A). Individual-based inbreeding coefficient (F-value) and PCA showed that only one region on LG3 (17.26-17.34 Mb) fully differentiated males and females (Figure 1B), despite some minor effects of missing data on PCA (Supplementary Figure S2A; Yi & Latch, 2022). Accordingly, our results support WL nine-spined sticklebacks having the XX/XY sex-determining system and the sex chromosomes on LG3, where an 80 kbp region was identified to determine sex (hereafter the LG3 SDR).

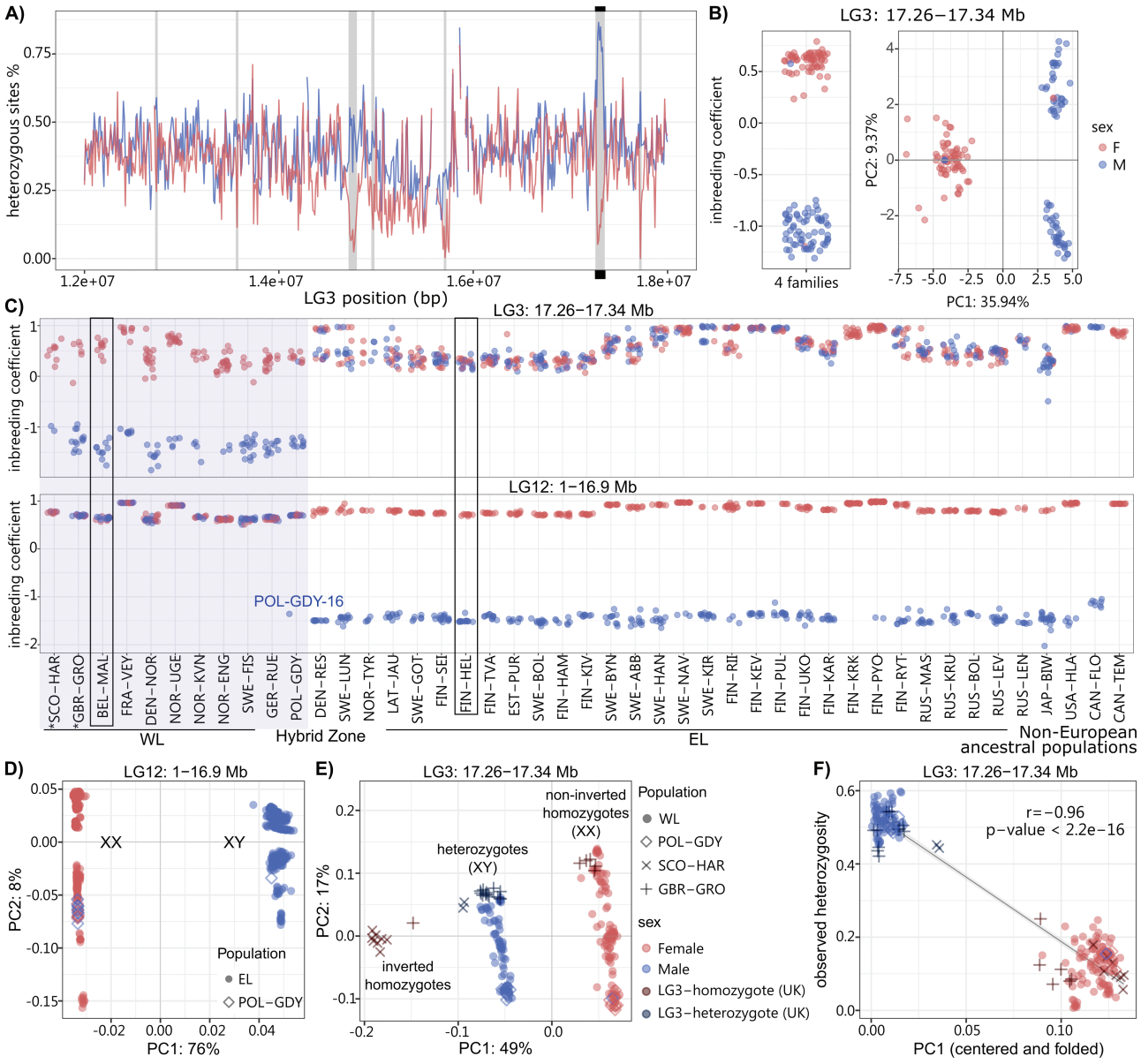

Figure 1. Identification of the LG3 SDR and individual genetic sex. (A) Window-based heterozygosity using family crosses. The x-axis represents the end positions of 10 kbp windows. Colours depict the phenotypic sex (same in B). Gray vertical lines highlight the windows having absolute delta heterozygote percentages >30% between sexes. Black bars label the position of the identified SDR. (B) *F*-values and PCA of family crosses in the LG3 SDR. Each dot represents one individual (same in the following panels). Two F_1 individuals (M15 and F15) from one family cross (Bel_Mal_14) had conflicting genotypic and phenotypic sexes, indicating swapped labels or misidentified phenotypic sex. (C) *F*-values of wild-caught individuals in the LG3 (top) and LG12 (bottom) SDR. Colors represent the identified genetic sex (same in D). Populations (Supplementary Table S2; Figure 2) are ordered by their indicated sex chromosomes and the previously described nuclear ancestry (Feng et al., 2022). The populations in black boxes had known phenotypic sexes and were consistent with the identified genetic sexes. (D) PCA of the LG12-characterized EL populations and POL-GDY (diamond shapes) in the LG12 SDR. (E) PCA of the LG3-characterized WL populations (highlighted in C) in the LG3 SDR. Shapes represent population, and colors represent genetic sex or LG3 heterozygosity (in UK populations). (F) Observed heterozygosity (percentages of heterozygous sites per individual in the LG3 SDR) against the centered and folded PC1 scores in (E). Legends are the same as in (E).

The genetic sex of wild-caught individuals and the SDR diversity in European populations

Filtering of the 887 wild-caught nine-spined sticklebacks retained 1,447 SNPs in the LG3 SDR (17.26–17.34 Mb) and 318,850 SNPs in the LG12 SDR (1–16.9 Mb). Genetic sex was identified based on individual *F*-values: low *F*-values indicate excessive heterozygosity in the SDR of XY males (similar to the F_{IS} in (Shikano et al., 2011)), whereas high *F*-values indicate excessive homozygosity in the SDR of XX females. In the homologous autosomal regions of each SDR (i.e., LG12 in WL or LG3 in EL individuals), both sexes tend to show high *F*-values due to the Wahlund effect caused by the joint analysis of all populations. The identified genetic sexes (Figure 1C) were consistent with known phenotypic sexes available in one WL (BEL-MAL) and one EL population (FIN-HEL, except for one phenotypic female, 16-f, which was also identified as a genetic male in Feng et al., 2022). Three populations were identified with only genetic females (FIN-KRK, CAN-TEM) or genetic males (CAN-FLO), all of which are expected to have LG12 sex chromosomes (Feng et al., 2022; Natri et al., 2019). This observation indicates potentially biased sampling or different sex

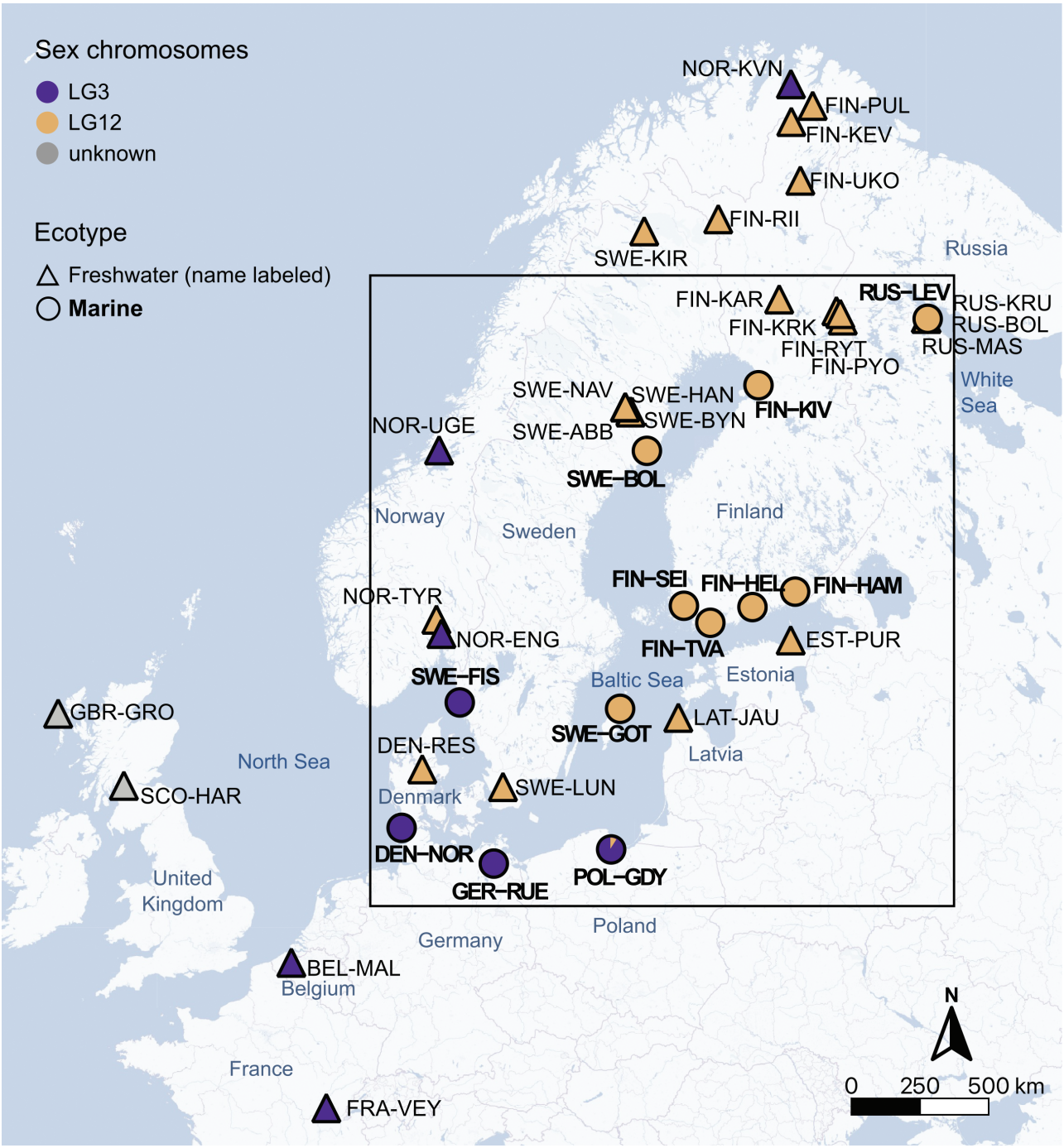

Figure 2. Sex chromosome diversity in the 40 European nine-spined stickleback populations. Each dot represents one population, and shapes depict ecotypes. Names of the 12 marine populations are bolded. Colors represent the identified sex chromosomes in each population (both LG3 and LG12 SDRs coexist in a skewed ratio in POL-GDY). The black rectangle highlights the Baltic Sea area.

chromosomes or SDRs in these populations. Overall, most EL populations had the LG12 SDR while WL populations had the LG3 SDR, and both SDRs were found in the admixed Polish population (POL-GDY) which had ten LG3-characterized males and one LG12-characterized male (Figures 1C and 2).

To further support the genetic sex identification based on F-values, we conducted PCA on the LG3 SDR using the 11 LG3-characterized WL populations (highlighted in Figure 1C; 221 individuals) and PCA on the LG12 SDR using the 30 LG12-characterized EL populations (618 individuals). The non-European populations were excluded for clarity, and no effect of missing data bias was detected (Supplementary Figure S2B and C). The Polish population was included in both PCAs, which supported our genetic sex identification (Figure 1D and E). Surprisingly, while PCA of the LG12 SDR in EL showed the expected two clusters of genetic males and females (Figure 1D), PCA of the LG3 SDR in WL showed three clusters (Figure 1E) in a three-stripe pattern that has been used as a signature of genomic inversion (Ma & Amos, 2012). Consistently, SV analyses detected a putative inversion around 17.275–17.328 Mb on LG3 using WL males (Supplementary Figure S3A). Not all males were detected with this putative inversion (Supplementary Table S3), probably due to limitations of

next-generation short read sequencing, but no large inversion (>10 kbp) was detected using WL females, EL, or non-European individuals (Supplementary Figure S3A). Therefore, these results indicate a putative inversion in the SDR unique to the LG3-Y chromosome. Accordingly, the three PCA clusters represent non-inverted homozygotes (XX females), heterozygotes (XY males) in the middle, and the putatively inverted homozygotes, the last of which were only found in UK populations (Figure 1E). The inverted haplotype in the UK may not be strictly sex-linked because YY genotype is biologically unlikely in such a high frequency, even considering rare cases reported in other species (e.g., mating of sex-reversed individuals, Dufresnes et al., 2015; viable YY individuals, Ieda et al., 2018). In addition, several UK individuals were intermediate between the heterozygous and homozygous clusters on PCA (Figure 1E) and the plot of heterozygosity against PC1 (Figure 1F), which indicates rare recombination events of this region in these individuals and thus weaker recombination suppression of the LG3 region in UK populations compared to the other WL populations. SV detection using UK populations showed two putative inversions in this LG3 region (Supplementary Table S3; Supplementary Figure S3B), which is intriguing but hard to interpret due to the limitations of the next-generation sequencing data. However, at minimum, these results indicate a different evolutionary history of this LG3 region in UK populations. The ML phylogenetic tree of the LG3 SDR showed that homozygotes of the putative LG3 inversion from UK diverged after the divergence of a basal Western Atlantic lineage (Supplementary Figure S4), indicating that the WL-specific LG3 SDR is evolutionarily younger than the LG12 SDR shared between EL and ancestral non-European populations.

Hybridization between populations having different SDRs

A total of 237 unrelated individuals were retained from 12 marine populations (Supplementary Table S2), including three LG3-characterized WL populations, eight LG12-characterized EL populations, and the Polish population where both SDRs coexist (Figure 2). The Danish (DEN-NOR) and Russian (RUS-LEV) populations were included as representatives of WL- and EL-like ancestors, respectively (Feng et al., 2022). ADMIXTURE using 152,546 filtered autosomal SNPs indicated optimal four genetic clusters ($K = 4$) based on CV errors (Supplementary Figure S5A) and results at $K = 5$ further distinguished the German population (GER-RUE; Figure 3A). All Polish individuals were highly admixed, including the one LG12-characterized male which is thus not a migrant from an EL population (Figure 3A). Similar genetic admixture patterns were found when males ($n = 119$) and females ($n = 118$) were analyzed separately (Supplementary Figure S5B–E), indicating that the prevalence of LG3-characterized males in the highly admixed Polish population could not be explained by higher male-specific gene flow from WL populations.

A total of 147,503 IBD-like tracks were detected from the 19 autosomes (Supplementary Figures S6A and S7). Track lengths decayed with increasing geographic distances between genetic clusters (Supplementary Figure S8), consistent with isolation by distance. Longer tracks were shared within populations, and similar patterns were observed when using all individuals combined (Figure 3B) or separated by sex (Supplementary Figure S6B), consistent with ADMIXTURE plots and indicating a lack of sex-specific patterns. HIest classified marine individuals as late-generation hybrids (e.g., not F_1 or F_2; Fitzpatrick, 2012; Lipshutz et al., 2019), both when using the Danish and Russian populations as parents (59 autosomal SNPs, all 237 individuals; Figure 3C) and when using the German and Swedish populations as parents (154 autosomal SNPs, 66 individuals; Figure 3D). Therefore, the observed skewed ratio of LG3- and LG12-characterized males in the Polish population cannot reflect putative sex-specific migration during early hybridization, but is the result of evolutionary forces working on several generations of admixture.

Discussion

Recent studies have demonstrated an incredible diversity of sex chromosomes across the tree of life (Tree of Sex Consortium, 2014) and inspired new theories of labile sex chromosome evolution (Furman et al., 2020; Kratochvíl et al., 2021; Vicoso, 2019). However, empirical case studies of coexisting sex chromosomes and their turnover are rare, but they are needed to test these theories. Using the nine-spined stickleback as the study system, here we identify LG3 as the previously unknown homomorphic sex chromosomes of WL. Our results indicate the possible existence of an additional, yet uncharacterized, sex-determining system in UK populations, which have also been suggested to have a unique historical ancestry (Feng et al., 2022) and experienced strong genetic drift in isolated freshwater habitats. Additional sampling of individuals with known sexes is required to identify the SDR in UK populations. We hypothesize that the LG3 SDR evolved in an ancestral lineage that colonized western Europe during an early wave of migration (Feng et al., 2022) and was subsequently spread to other WL and admixed populations by gene flow (Figure 4A). Moreover, the results suggest a potentially ongoing sex chromosome turnover in the natural WL-EL hybrid zone, where the evolutionarily younger homomorphic LG3 appears to be taking over the older heteromorphic LG12 as the sex chromosome (Figure 4B and C). Below, we discuss how these findings further our understanding of labile sex chromosome evolution.

The early evolution of sex chromosomes requires gaining a master sex-determining locus and recombination suppression around this locus in the new SDR (Figure 4B). Several master sex-determining genes have been identified in teleost fishes (reviewed in Vicoso, 2019), including a duplicated and translocated copy of the anti-Müllerian hormone gene (*AmhY*; Hattori et al., 2012; Li et al., 2015; Peichel et al., 2020; Sardell et al., 2021) which was also found in the sister genus of *Pungitius* (Jeffries et al., 2022). However, no master sex-determining gene has been identified so far in any *Pungitius* species, which might have recruited different genes to trigger sex development. Two genes annotated in the LG3 SDR have been associated with sex in other vertebrates (*RNF31*, *DNAJB6A*; Supplementary Table S4), but it should be noted that these annotations were based on the EL reference genome which represents the autosomal LG3 and does not contain the most Y-specific regions in the WL. Therefore, we were not able to identify the candidate sex-determining gene or sexually antagonistic genes that might contribute to recombination suppression of the LG3 SDR (Ponnikas et al., 2018). However, we hypothesize that the putative inversion in the LG3 SDR might have facilitated the initial recombination suppression (Figure 4B), similar to previous findings that the LG12 sex chromosomes evolved from an introgressed inversion and that recombination suppression might have predated the sex-determining role of the LG12 SDR (Dixon et al., 2019; Natri et al., 2019). Future studies require high-quality reference assemblies of WL populations to verify the putative inversion and identify the gene content of the LG3 SDR.

We demonstrated coexisting SDRs in the natural WL-EL hybrid zone where either LG12-Y or LG3-Y is proposed as sufficient to determine male development. While nine-spined sticklebacks

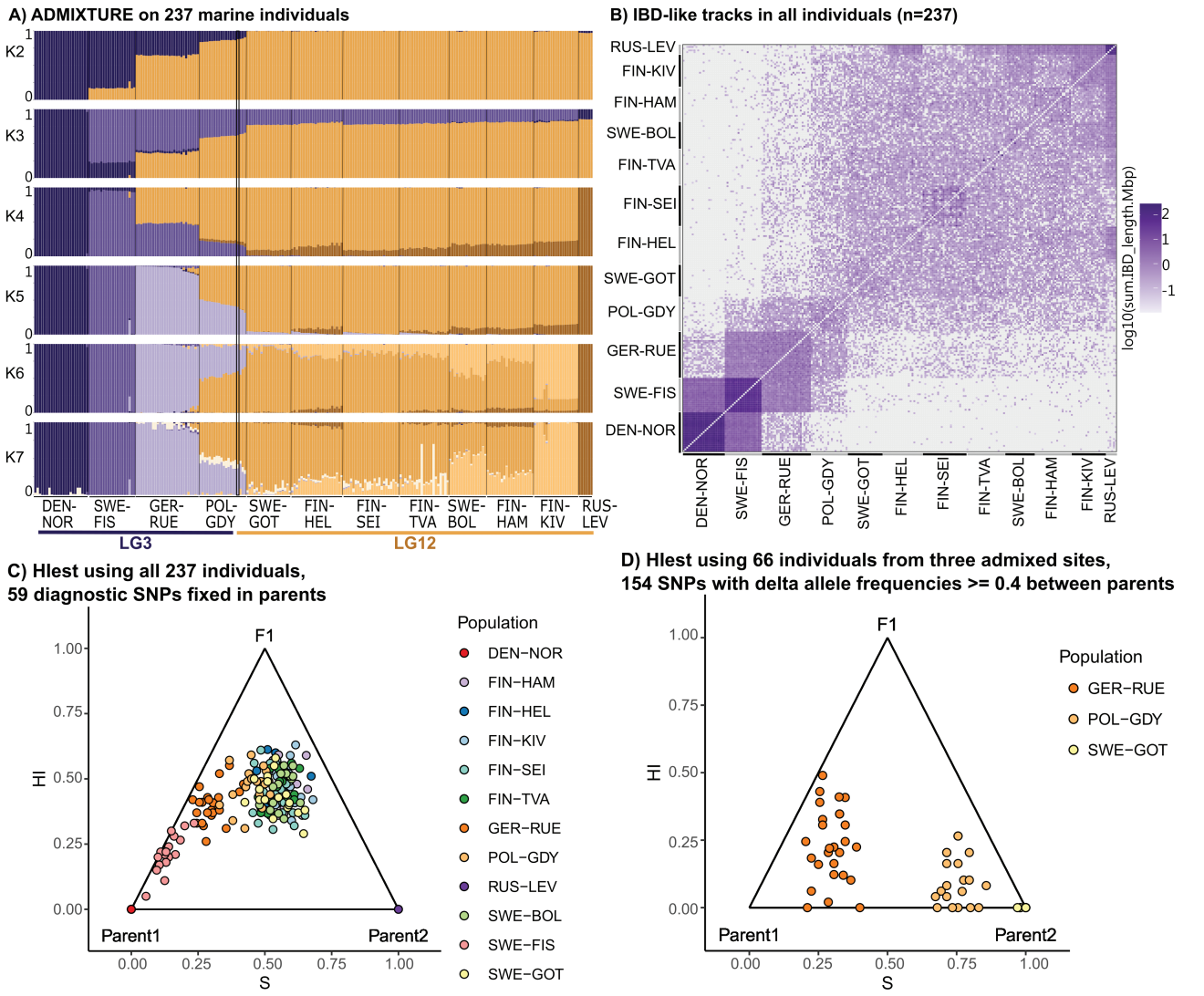

Figure 3. Autosomal analyses of marine populations. (A) ADMIXTURE plot of all 237 individuals. The y-axis shows the percentage of inferred ancestry classes indicated by color. Bars represent individuals ordered by sampling sites and the identified sex chromosomes (labeled at the bottom). The only LG12-characterized male in the Polish population is highlighted. (B) The total length of identity-by-descent-like tracks shared between individual pairs. Darker colors indicate longer shared tracks (in Mbp on the log10 scale). (C) and (D) Hybrid classification based on the joint distribution of ancestry (S) and interclass heterozygosity (HI). The expected positions of parental populations and their F_1-generation individuals are labeled. Each dot represents one individual, and colors depict populations.

in the hybrid Polish population had both SDRs present, it should be noted that the LG3-Y and LG12-Y should not co-occur within the same individual as males should not mate with each other (unless there is mating of sex-reversed XY females, which has not been reported in this species). The slightly higher EL-like ancestry of the Polish population predicts relatively even proportions of LG3- and LG12-characterized males (or even more of the latter). However, we observed one LG12-characterized male but ten LG3-characterized males, a highly skewed ratio that is unlikely to be caused by sampling effects alone and cannot be explained by migration or sex-specific patterns of gene flow based on our results. Therefore, we propose that the Polish population has evolved from initially LG12-characterized males into the observed state of mostly LG3-characterized males after multigenerational admixture. This observation might result from the higher fitness of males carrying the LG3-Y compared to males carrying the LG12-Y in the hybrid zone, indicating a potentially ongoing turnover where LG3 gradually replaces LG12 as sex chromosomes in the Polish population (Figure 4C). The admixed German population might have completed the turnover under this scenario.

Our observations are consistent with the mechanism of sex chromosome turnovers driven by selection against a higher deleterious mutation load (Blaser et al., 2013, 2014). This mechanism proposes that when the heterogametic sex is conserved (e.g., XY males in both EL and WL), the evolutionarily younger Y chromosome will take over the older Y chromosome to purge the higher deleterious mutation load accumulated on the non-recombining region of the older Y, while the older X will revert to an autosome to complete the sex chromosome turnover (Blaser et al., 2013, 2014). As a result, frequent sex chromosome turnovers can maintain evolutionarily young sex chromosomes that do not go through major degeneration (Palmer et al., 2019; Vicoso, 2019). In nine-spined sticklebacks, both the longer evolutionary time and the larger physical genomic region of the LG12 SDR indicate more accumulated deleterious mutations compared to the evolutionarily younger and shorter LG3 SDR. The

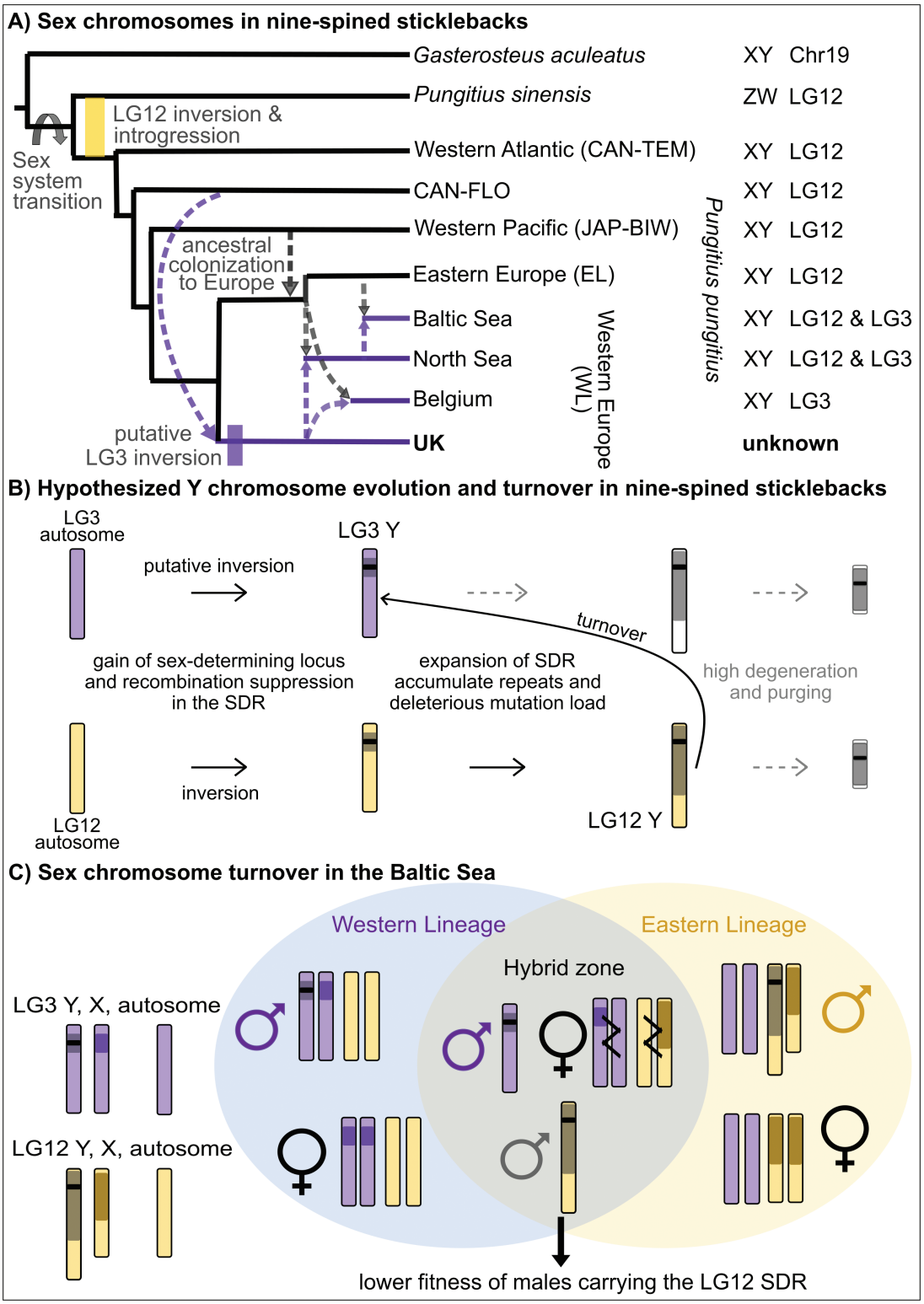

Figure 4. The hypothesized sex chromosome evolution and turnover in nine-spined sticklebacks. (A) Sex chromosome diversity in nine-spined stickleback lineages. The network is adapted from Feng et al., (2022). Dashed arrows represent ancestral waves of colonization to Europe and admixture events. Vertical bars represent the previously described LG12 inversion (Dixon et al., 2019; Natri et al., 2019) and the putative LG3 inversion (indicated in this study) that might have occurred after an ancestral lineage colonized Western Europe. Coloured arrows and lines highlight the hypothesized spread of the putative LG3 inversion and possible sex chromosome turnovers. (B) Hypothesized evolution of the LG3- and LG12-Y chromosomes. Horizontal bars represent the master sex-determining locus in the nonrecombining SDR (gray areas). High Y-degeneration is proposed by the canonical theory but is not found in these labile sex chromosomes. (C) A possible sex chromosome turnover in the WL-EL hybrid zone where the LG3 SDR appears to be taking over the LG12 SDR. Dark lines indicate recombination between X chromosomes and their homologous autosomes in hybrid individuals.

deleterious mutation load on LG12 might not have been purged by degeneration, considering the cytogenetically larger Y (Natri et al., 2019). Therefore, selection against deleterious mutation load would result in the observed sex chromosome turnover from LG12 to LG3.

Alternatively, sex chromosome turnover in the WL-EL hybrid zone might be driven by natural selection favoring the LG3-Y, which might be linked with genes adapted to high-salinity environments in the western Baltic Sea (Feng et al., 2024). These fitness-related genes might also make the LG3-Y less adapted to the environments of the less saline eastern Baltic Sea compared to the LG12-Y, which might explain why the proposed sex chromosome turnover was not observed in the other EL marine populations. However, it is also possible that the LG3-characterized males have not reached these EL populations in high frequency or quantity, in which case this sex chromosome turnover might slowly expand to the other EL populations given enough time and continuous gene flow. In addition, sex chromosome turnover may be driven by other mechanisms, including Dobzhansky-Muller incompatibility (e.g., higher incompatibility between the LG12-Y and the WL genome), sex ratio selection (Eshel, 1975; Kozielska et al., 2006), sexually antagonistic selection (van Doorn & Kirkpatrick, 2007), and meiotic drive (Úbeda et al., 2015). It is likely that multiple selection forces contribute to the WL-EL sex chromosome turnover. The available data cannot distinguish between these hypotheses, and these hypotheses need to be tested in future studies.

In conclusion, our empirical study of nine-spined sticklebacks furthers the understanding of labile sex chromosome evolution in several ways. First, we identified the homomorphic sex chromosomes of WL and indicated a putative inversion in the SDR, which might have facilitated the initial recombination suppression. Second, we demonstrated a rare evolutionary snapshot of a potentially ongoing sex chromosome turnover in the natural hybrid zone where two sex chromosome systems can coexist in a single population. The observed sex chromosome turnover is consistent with selection against higher deleterious mutation load on the LG12-Y, but other mechanisms may also contribute to this turnover, and future studies are needed to test alternative hypotheses. Lastly, our results showed a possibly still unrecognized sex-determining system in the UK populations of nine-spined sticklebacks, indicating potentially high intraspecific sex chromosome diversity, which might be maintained by frequent turnovers. Stickleback fishes thus provide a model system for future studies to further test hypotheses of labile sex chromosome evolution and turnover.

Supplementary material

Supplementary material is available online at *Evolution Letters*.

Data and code availability

Raw sequencing data of the four family crosses are available on NCBI (BioProject PRJNA1023372). The alignment for the phylogenetic analyses is available in Supplementary Materials. Bioinformatic codes and scripts used in this study are available on Github (https://github.com/xuelingyi/SexChromosome_nine-spined_stickleback). The raw sequencing data of wild-caught individuals have been published in Feng et al. (2022) and can be accessed on the European Nucleotide Archive (ENA) using access code PRJEB39599.

Author contributions

X.Y., K.R., and J.M. conceived the study. X.F., A.L., and J.M. collected the raw data. X.F. and A.L. processed and remapped the raw sequencing data of wild-caught individuals. X.Y. processed the raw sequencing data of family crosses. X.Y. analyzed the processed data with help from D.W. and K.R. X.Y. visualized the results and wrote the first draft of the manuscript. All authors edited the subsequent versions of the manuscript.

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