Genetic architectures of postmating isolation and morphology of two highly diverged rockfishes (genus *Sebastes*)

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Abstract
Speciation is a continuous process, and postmating isolation is thought to be an important driver of the late stages of speciation. However, relatively little is empirically known about the process compared to other isolating mechanisms that drive the early stages of speciation, especially in non-model organisms. In this study, we characterized the genetic architecture of postmating isolation between two rockfishes, *Sebastes schlegelii* and *S. trivittatus*, whose reproductive isolation is complete. We examined transmission ratio distortion (TRD) patterns of genetic markers in two reciprocal backcross populations. Markers showing either of the two types of TRD was widespread across the genome, with some of the distorted markers forming extensive clusters around the recombination coldspots. These suggest that the postmating

isolation effectively prevents gene flow across the genome and the recombination landscape contributes to the genetic architecture. Comparisons between two backcross families and two developmental stages showed little similarity in the distorted markers, suggesting asymmetry and stage-specificity of the isolation. This may be due to hybrid incompatibility involving maternal factors or extrinsic selection. The lack of sex-ratio distortion in the mapping families suggested that Haldane's rule in terms of hybrid inviability does not hold. Additionally, QTL mapping detected significant QTLs for sex and the morphological traits relevant to divergence and convergence of rockfishes, including the body colour patterns and the overall intensities of pigmentations. These findings, coupled with the first linkage map for rockfishes presented herein, constitute an essential step towards a comprehensive understanding of speciation and morphological diversification of rockfishes.

KEYWORDS

speciation, postzygotic isolation, hybrid inviability, extrinsic selection, recombination, convergence, body colouration, sex, linkage map, QTL

1. INTRODUCTION

Speciation is a continuous process whereby gene flow between diverging species diminishes with reproductive isolation. Existing species pairs can be ordered in a continuous sequence—a speciation continuum—according to the degree of divergence (Coyne & Orr, 1989a). Studying the species pairs at different points of the continuum enables the reconstruction of the order and the rate of evolution of different forms of reproductive barriers in a given system (Coyne & Orr, 1989a). A widely accepted view is that premating isolation due to local adaptation or sexual isolation tends to evolve early in speciation, whereas postmating isolation evolves later, thereby completing speciation or maintaining separate species (Coyne & Orr, 2004; Schemske, 2010). However, the latter process is relatively unclear, reflecting lesser attention being paid to highly diverged species pairs whose reproductive isolation is strong or complete (Matute & Cooper, 2021).

Postmating isolation may result from reduced fertilization success of heterospecific gametes (gametic isolation), intrinsic hybrid sterility and inviability (intrinsic postzygotic isolation), and environmentally-dependent hybrid sterility and inviability (extrinsic

postzygotic isolation) (Coyne & Orr, 2004). Among them, intrinsic postzygotic isolation is the most intensively studied in terms of both its genetic model and contribution to speciation; little is known about the other two mechanisms (Rometsch et al., 2020). Intrinsic postzygotic isolation is caused by negative epistatic interactions involving two or more genes in hybrids, i.e., Dobzhansky-Muller Incompatibility (DMI) (Maheshwari & Barbash, 2011). Previous empirical studies have shown that the strength of intrinsic postzygotic isolation, as well as the number of loci involved in DMI, often evolve exponentially with divergence ('snowball effect') (Matute et al., 2010; Stelkens et al., 2010), consistent with theoretical predictions (Orr & Turelli, 2001). Similarly, intrinsic postzygotic isolation characterises the isolation of most 'good species' examined (Coyne & Orr, 2004). However, it is still largely unknown what factors account for the variations in tempo and mode of evolution of intrinsic postzygotic isolation across different systems and how its genetic architecture relates to its efficacy (Coughlan & Matute, 2020). Therefore, understanding how postmating isolation contributes to the later stages of speciation requires more studies on various groups of organisms with diverse life histories and degrees of divergence, especially the understudied non-model organisms, and on gametic and extrinsic postzygotic isolation.

Postmating isolation between a pair of species can be detected by analysing the deviation of marker genotype frequencies from the Mendelian expectations (transmission ratio distortion, TRD) in hybrid mapping populations, such as backcross and F_2 generations (Fishman & McIntosh, 2019). Gametic isolation and hybrid inviability between parental species may cause variations in the fertilization success of gametes and mortality of progeny in hybrid crosses, resulting in TRD at genetic markers linked to the underlying loci of these barriers. Importantly, hybrid inviability due to DMI is largely masked in F_1, but manifests in later generation hybrids (Orr, 1995; Orr & Turelli, 2001). Extrinsic hybrid inviability can also be inferred from the patterns of TRD relative to the rearing conditions. Moreover, mapping the distorted markers on a reference genome or a linkage map can reveal the genetic architecture of the isolating barriers (e.g., Brennan et al., 2014). Using TRD as a proxy of postmating isolation is particularly useful when direct fitness measurement is difficult. For example, gametic isolation and prenatal hybrid inviability in internally fertilising animals could be inferred from TRD in the progeny immediately after parturition, which is otherwise, highly challenging.

Speciation and associated phenotypic diversification is sometimes accompanied by recurrent evolution of convergent traits (Schluter, 2000). While convergent traits between distant lineages are often interpreted as independent adaptations to similar ecological challenges, whether and to what extent they share similar genetic mechanisms varies across systems (Rosenblum et al., 2014). The similarity in genetic mechanisms of convergence between distant lineages ranges from independent fixations or introgression of identical mutations to shared molecular pathways with different nucleotides and genes (Chen et al., 1997; Evans et al., 2012; Stern, 2013). The first critical step toward understanding the convergence at the genetic level is to characterize the genetic architecture of the convergent trait in each lineage.

Rockfishes of the genus *Sebastes* are an ideal system to study speciation and the associated phenotypic diversification in marine environments. *Sebastes* is a genus comprising more than 100 species of viviparous marine fishes, most of which diverged in the last 8 million years (Hyde & Vetter, 2007; Kolora et al., 2021). These species have colonised diverse habitats from the near-shore rocky reefs of less than 1 m depth to the deep-sea continental slopes of up to 1,500 m depth (Love et al., 2002; Nakabo & Kai, 2013). Numerous incipient species pairs hybridising in nature are scattered across the phylogeny of the genus, enabling multiple independent studies on the earliest stage of speciation (Buonaccorsi et al., 2011; Muto et al., 2013; Saha et al., 2017; Schwenke et al., 2018). Convergence in some morphological traits among independent lineages has also been suggested either verbally or through phylogenetic comparative methods (Ingram & Kai, 2014; Kai et al., 2003), providing opportunities to study how those traits evolved along with the radiation in diverse environments. Furthermore, artificial crosses can produce fertile F_1 hybrids even between species pairs with no record of natural hybrids (T. Kawasaki, unpublished data). This feature enables genetic dissections of postmating isolation and quantitative trait loci (QTL) analysis of adaptive traits using later generation hybrids.

Nevertheless, little is known about the speciation and morphological diversification of rockfishes. Incipient species often occupy habitats of different depths and have distinct body colourations, implying the role of depth segregation or colour-related assortative mating in the early stages of speciation (Ingram, 2011; Longo et al., 2022; Muto et al., 2019; Shum et al., 2014; Venerus et al., 2013). Genes presumably related to these isolating barriers have also been identified (Behrens et al., 2021; Heras & Aguilar, 2019). However, the influence of other isolating barriers has not been tested,

and studies on isolating barriers between highly diverged species with strong reproductive isolation is lacking. Consequently, how reproductive barriers develop towards the completion of speciation in *Sebastes* remains elusive. As for morphological convergence, the genetic bases of the convergent traits or any other morphological traits have not been examined.

In this study, we characterise genetic architectures of postmating isolation and the morphological difference between the two rockfishes *S. trivittatus* and *S. schlegelii*. They are estimated to have diverged 6–7 million years ago (mya) (Hyde & Vetter, 2007; Kolora et al., 2021), with no documented record of natural hybridisation (Figure S1). They have partially overlapping geographical distributions in the western Pacific: *S. trivittatus* is distributed in higher latitudes, from the coasts of Sakhalin Island and the southern Kamchatka Peninsula to the Yellow Sea; whereas *S. schlegelii* is found from the coasts of Sakhalin and the southern Kuril Islands to the Bohai Sea (Nakabo & Kai, 2013; Parin et al., 2014). Their morphological difference involves several traits important in the contexts of speciation and convergence of rockfishes. Using two reciprocal backcross families (single F_1 male × females of *S. trivittatus* and *S. schlegelii*), we first constructed a hybrid linkage map, which is the first linkage map for rockfishes. We then explored the TRD of genetic markers as an approximation of genetic architectures of postmating isolation. Comparisons of the patterns of TRD between two developmental stages and between the families revealed stage-specificity and asymmetry of the isolation. We also performed a QTL analysis for the morphological traits and sex. This study signifies the first critical step toward a better understanding of speciation and morphological diversification of *Sebastes*, which in turn will provide a valuable contrast to those in well-studied model organisms.

2 MATERIALS AND METHODS

2.1 Experimental crosses and sampling

Rockfishes are viviparous and have prolonged storage periods of sperm and zygotes in a female ovary (each from one to several months) (Love et al., 2002). A female releases hundreds of thousands to millions of hatched larvae per spawning. Nutrients are supplied to the progeny both maternally (matrotrophy) and via yolk (Macfarlane & Bowers, 1995; Tengfei et al., 2021). We artificially inseminated a wild female *S.*

trivittatus in November 2012 using sperm obtained from a wild male *S. schlegelii*, both collected off Hokkaido, Japan, according to Kawasaki et al. (2017). Briefly, a sperm suspension was prepared by mincing testis and sperm reservoir in urine obtained from the male fish euthanized with tricaine methanesulfonate (MS222), then deposited into the external genital pore of the female fish. Parturition of the F_1 yolk-sac larvae occurred ca. seven months from the insemination. Subsequently, a single male of the F_1 progeny was crossed with a wild female *S. trivittatus* (different individual from the founder female) and a wild female *S. schlegelii* in December 2015 according to Kawasaki et al. (2017). The parturitions occurred ca. six months later. The resulting backcross families are hereafter referred to as BC_Str and BC_Ssc, respectively. Rearing conditions for the fishes and a schematic diagram of the experimental design are provided in Appendix S1 and Figure S2. We sampled approximately 100 progeny of each family at each of the two stages: yolk-sac larvae (0 d after parturition) for genotyping and immature subadult (ca. 270 d: see Figure S2) for both phenotyping and genotyping. All the females used for the crosses had been separated from males at least for one year to prevent unintended mating. We could not use F_1 females for the cross because female rockfishes naturally require a few years more than males for maturation (Love et al., 2002).

2.2 Phenotyping

Independent lineages of *Sebastes* sometimes have strikingly similar colour patterns, including the presence of vertical bands on the dorsum, a horizontal stripe along the lateral line, and a horizontal stripe along the dorsal-fin base, but not necessarily similar colours. Besides, both colour and colour patterns are often the most important trait to distinguish sister species of the genus (Ingram, 2011; Longo et al., 2022; Muto et al., 2019; Shum et al., 2014; Venerus et al., 2013). Convergence in meristic and morphometric traits involves gill-raker number and length, eye size, body depth and width, pectoral-fin length and width, upper- and lower-jaw lengths, and head spination (Ingram & Kai, 2014; Kai et al., 2003). We phenotyped these traits and other taxonomically important traits in subadults of the mapping families (Tables S1 and S2). As for sex determination, the XY system controlled by the male master sex-determining gene *amhy* was demonstrated for *S. schlegelii*, *S. koreanus*, and *S. pachycephalus*. The latter two species belong to the same subclade of *Sebastes* with *S. schlegelii* and

S. trivittatus (Song et al., 2021) (Figure S1). The gene *amhy* is present in a ca. 5 kb male-specific region of the chromosome (CHR) 12 in the chromosome-level genome assembly of *S. schlegelii* (He et al., 2019), which we used as a reference genome in this study.

We measured standard length (SL) and body weight of each subadult fish and took a digital image of the left side of the body along with a KODAK Colour Separation Guide using Pentax K200D immediately after euthanasia. We kept the distance between the camera and the fish constant across individuals. The sex of subadults was determined through visual inspections of the overall appearance of the fresh gonads: males have a pair of elongated, almost thread-like white gonads, whereas females have partly rounded orange gonads. The visual inspection was validated for a subset of individuals via histological observation. The fish was then fixed in 10% formalin and preserved in 70% ethanol.

The body colour and the colour patterns of each fish were characterised in three ways using the digital image (Figure S3). First, we coded the presence/absence of the vertical bands on the dorsum and the horizontal stripes along the lateral line and along the dorsal-fin base in each fish as binary traits based on visual inspection. The coding was done twice by the same person for consistency. Second, we quantified the overall intensities of black pigmentation (supposedly melanophore-based) and yellow pigmentation (xanthophore-based) on the body by counting the number of corresponding pixels. Each image was colour-corrected by setting the grey patch of the colour separation guide as the neutral grey using the eyedropper tool in the Curves adjustment implemented in Adobe Photoshop CS6. We then cropped the image by a polygon defined by 11 landmarks on the body to restrict the analysis to an unambiguously definable area, excluding the fins, which were sometimes damaged. We counted the number of pixels on the cropped image that fell within the ranges of the hue/saturation/brightness (HSB) colour space using the Color Threshold function of ImageJ 1.53c (Schneider et al., 2012); black: H = 0–255, S = 0–255, B = 0–100; yellow: H = 10–50, S = 0–255, B = 100–200. These ranges were determined by preliminary visual inspections of the colour of the extracted pixels, of a subset of individuals, under variable combinations of HSB values. Third, we quantified the colour patterns of the fish using the ‘patternize’ v0.0.2 package (Van Belleghem et al., 2018) on R v3.6.3 (R Core Team, 2020). We cropped the colour-corrected digital image by a polygon defined by six landmarks on the body to restrict the analysis to the upper half

of the flank, where the focal colour patterns are expressed. We used the *patRegK* function to automatically align all images, assign pixel colours to a predefined number of clusters (K), and extract the colour patterns from the image based on the distribution of each cluster. We analysed with K = 4–6 (Figure S4). The variation in the extracted pattern was summarised by a Principal Component Analysis (PCA), performed for each colour cluster of each K using the *patPCA* function. For QTL mapping, we used the PC scores derived from three colour clusters from K = 5, which captured the colour pattern variation most successfully (see Appendix S1 for more detail).

We quantified the overall body shape from the lateral view using geometric morphometrics. We used tpsDig2 (Rohlf, 2017) to digitize 16 landmarks on the digital image of each fish (Figure S3). Generalized Procrustes Analysis was performed on these landmarks using the *gpagen* function implemented in the R package 'geomorph' v4.0.0 (Adams et al., 2021). The variation in the obtained Procrustes shape coordinates among individuals was summarized by a PCA using the *gm.prcomp* function. Linear measurements and counts were made on 34 and 13 traits, respectively, on the left side of the body of the fixed samples. Methods for measuring and counting followed Muto et al. (2011, 2019) with the following additions: head spines, the number of spines on the dorsal aspect of the cranium; lacrimal spines, the number of spines on the lacrimal.

We $\log_{10}$ transformed SL, weight, all linear measurements, and intensities of pigmentations. We tested the dependence of each trait on size (SL) and sex in each family, and the difference between the two families, using either linear regression, the Wilcoxon's rank-sum test, or the Fisher's exact test, depending on the distribution of the trait (Tables S1, S2). The traits used for QTL mapping were tested for pairwise correlations using the *corr.test* function implemented in the R package 'psych' v2.1.6 (Revelle, 2021), with the false discover rate (FDR) correction. We used the residuals of linear regression of each trait on size or sex for the correlation test, if the trait was dependent on these variables.

2.3 Genotyping

All parental individuals, 137 BC_Str individuals (48 larvae and 89 subadults) and 136 BC_Ssc individuals (48 larvae and 88 subadults) were subjected to genotyping by double-digest restriction-site-associated DNA sequencing (ddRAD-seq), according to

Peterson et al. (2012) with minor modifications (see Appendix S1 for details). For subadults of BC_Str and their parents, a single library was prepared and sequenced with 50-bp single-end reads on an Illumina HiSeq 2500 system. A single library was prepared and sequenced with 150-bp paired-end reads on an illumina HiSeq X system for the remaining individuals. In either case, the sequencing was performed by Macrogen Japan Corporation (Kyoto, Japan). We used two different sequencing platforms because the availability of related facilities changed during the study. Only the forward sequences from the paired-end reads were used for consistency across the platforms. Parental individuals were sequenced twice or thrice for stringent genotyping.

We processed the ddRAD-seq reads using fastp v0.23.1 (Chen et al., 2018) to remove adapter sequences and trim the lengths to 50 bp. Reads with one or more bases with q-values < 20 were discarded. Two individuals of BC_Str subadults and an individual of BC_Ssc subadults were removed because of the few reads. The trimmed sequences were mapped to the chromosome-level reference genome assembly of a male *S. schlegelii* (He et al., 2019) (China National GeneBank Sequence Archive accession No. CNA0000824), using BWA-mem v0.7.17 (Li, 2013) with a mismatch penalty of 5. Unmapped reads and reads with a mapping quality < 5 were removed by Samtools v1.12 (Danecek et al., 2021). The resulting BAM files were used for identifying SNPs using 'gstacks' implemented in Stacks v2.5.4 (Catchen et al., 2013) with the default settings. The subsequent filtering steps were performed separately for each family to maximize the number of usable SNPs in the downstream analyses. We created a vcf file using 'populations' implemented in Stacks, retaining only the first SNP of a locus if two or more were detected. Genotypes with read depths < 10 or > 300 were removed, and SNPs were discarded if the resulting genotyping rate per SNP was < 0.6, using VCFtools v0.1.16 (Danecek et al., 2011). We then used SnpSift v4.3 (Cingolani et al., 2012) to retain only the SNPs for which the alleles observed in backcross progeny could be unambiguously determined to be inherited from either *S. trivittatus* or *S. schlegelii*. This was done by keeping only SNPs for which genotypes of grandparents and parents conformed to all the following criteria: 1) either homozygous in both grandparents or heterozygous in only one of the grandparents, 2) heterozygous in the F_1 male parent, and 3) homozygous in the pure female parent. We then removed the SNPs genotyped in < 60 BC individuals.

2.4 Linkage map and QTL

We first constructed a hybrid linkage map for each family using the R package ‘R/qtl’ (Arends et al., 2010) and then a consensus map of the two families using the R package ‘LPmerge’ v1.7 (Endelman & Plomion, 2014). The map for each family was constructed as follows: We removed markers showing deviation of genotype frequency from the expected even ratio of homozygotes and heterozygotes (hereafter referred to as single-locus TRD; chi-square test, $p < 0.001$ after FDR correction). Linkage groups (LGs) were initially formed with a minimum LOD score of 6 and a maximum recombination fraction (RF) of 0.35. The initial linkage grouping was highly consistent with the reference genome assembly of *S. schlegelii*, most markers on each LG being mapped to one of the 24 CHRs of the reference genome. However, deviations from this pattern were observed; a few markers on each LG mapped to an alternative (non-syntenic) CHR, and some markers formed an extra 25th LG. Such markers were either discarded, manually reassigned to another LG, or left unmodified based on examinations of individual LOD scores and RFs (Appendix S1; Figure S5; Table S3). Next, we ordered markers in each LG using the *orderMarkers* function. We checked the quality of the initial order by inspecting the plots of LOD score and RF between pairs of markers on each LG and corrected putative errors using either the *switch.order* or the *orderMarkers* function. We also sought better orders for LOD score and chromosome length using the *ripple* function. To identify problematic markers, we investigated the change in the length of each LG by eliminating one marker at a time using the *droponemarker* function. We removed the interior marker whose omission decreased the length by > 10 centimorgans (cM). We inferred genotyping errors using the *calc.errorlod* function and removed the genotypes with error LOD scores > 6. We also removed one outlier individual of each of BC_Str larvae and subadults concerning the numbers of crossovers. We then used the *ripple* function to search for marker orders with higher LOD scores. Finally, we compared the LOD score of the resulting marker order of each LG with that of the alternative order based on the physical position of markers on the corresponding CHR of the reference genome, and retained the order with a higher LOD score. If the order based on the physical position was chosen, we applied the *ripple* function again to search for orders with further higher LOD scores.

We conducted QTL mapping separately for each family based on the consensus map using the R package ‘R/qtl2’ v0.24 (Broman et al., 2019). Among the phenotyped

traits, colour-related traits, head spination, numbers of gill-rakers, lateral-line pores, and fin rays have been shown to differ between *S. trivittatus* and *S. schlegelii*; however, the difference in the other traits is unknown (Nakabo & Kai, 2013). Therefore, for QTL mapping, we used only the traits for which significant differences were observed between the two backcross families, assuming that such differences represent the difference between the parental species. We used family-specific markers as well as markers shared by the two families. We first inserted pseudomarkers in the linkage map at intervals of 1 cM. We conducted a genome scan for each trait using the *scanone* function based on the Haley-Knott regression (Haley & Knott, 1992). We accounted for the effects of size (SL) or sex by using these variables as additive covariates if the focal trait was significantly associated with them. Genome scans for body weight were performed with or without size as a covariate to differentiate potential QTLs of growth and condition. We conducted 10,000 permutations to estimate the genome-wide significance threshold of LOD score at alpha = 0.05 using the *scan1perm* function. We estimated a 95% confidence interval (CI) for each QTL using the *bayes_int* function. We estimated the effect of a QTL by calculating the proportion of phenotypic variance explained (PVE): PVE = (sum of squares between genotypes) / (sum of squares total) (e.g., Takahashi et al., 2021). For the calculation of PVE and the visualisation of phenotypic variation by genotype, we used the residuals of the regression of each trait on size or sex, if the trait was dependent on these variables; otherwise, we used raw phenotype values.

2.5 TRD

We tested two classes of TRD. First, we tested the single-locus TRD of each marker and assessed the distribution of the distorted markers on the reference genome. We used both the markers that were or were not filtered out during the construction of the linkage map for this analysis. Second, we tested non-random associations of genotypes between pairs of markers. In the present mapping families, the following two-marker genotypes are possible for a given pair of markers: StSt/StSt, StSt/StSc, StSc/StSt, and StSc/StSc for BC_Str; ScSc/ScSc, ScSc/StSc, StSc/ScSc, and StSc/StSc for BC_Ssc, with St and Sc representing alleles from *S. trivittatus* and *S. schlegelii*, respectively, and genotypes of two markers separated by a slash. Negative epistatic interaction (DMI) between alleles at loci near the markers results in

underrepresenting one or more of the two-marker genotypes, which is detectable as non-random associations of genotypes (Berdan et al., 2021; Pritchard et al., 2011). We calculated the expected two-marker genotype frequency from the observed genotype frequency of each marker. We then compared the observed and the expected two-marker genotype frequencies to detect non-random associations of genotypes using the chi-square test. For this analysis, we reduced the markers so that the minimum distance between adjacent markers became minimum 1 cM using the *pickMarkerSubset* function in R/qtl. Consequently, a total of 47,694 tests among 316 markers and 59,580 tests among 353 markers were performed for BC_Str and BC_Ssc, respectively. Distributions of the pairs of markers showing significant non-random associations of genotypes were assessed on the linkage map rather than the reference genome since the analysed markers had been filtered based on their positions on the linkage map. Both types of TRD were analysed separately for larvae and subadults of each family and corrected for FDR using the *p.adjust* function in R.

Results

3.1 Linkage map

Genotype data after filtering consisted of 642 and 823 markers for BC_Str and BC_Ssc, respectively, with 545 markers common between them, and both families consisted of 135 individuals (87 subadults; 47 larvae). No marker was mapped to the unanchored sequences of the reference genome assembly. The hybrid linkage maps constructed separately for each family consisted of 24 LGs, consistent with karyotypes of both parental species as well as the reference genome (Figure 1) (Arai, 2011). The consensus map consisted of 898 markers, including 636 markers for BC_Str, 799 markers for BC_Ssc, and 537 shared markers, with a total length of 1413.5 cM and an average marker interval of 1.6 cM (Table S4). Each LG was syntenic to one of the 24 CHRs of the reference genome, showing high collinearity (Figure S6). Thus, the LGs were numbered according to the corresponding CHRs. Exceptions to the syntenic relationships were one or a few markers on LGs 5, 12, 15, 19, 20, and 22, unequivocally assigned to these LGs in both BC_Str and BC_Ssc maps while being mapped to CHRs 7, 7, 11, 21, 22, 18, and 16, respectively (Table S3). Each LG had a ‘recombination coldspot’, where little or no recombination was observed across a wide

408 range of the corresponding CHR of the reference genome (Figure S6). These regions
409 were positioned distally on each LG except for that observed in the middle of LG 4,
410 consistent with 23 acrocentric and one metacentric chromosome in both parental
411 species (Ida et al., 1982).
412
413 **3.2 TRD**
414
415 Distributions of genotype frequencies and single-locus TRD across the reference
416 genome are shown in Figures 2, S7, and S8. Significant TRD at $p < 0.05$ after FDR
417 correction was observed for a total of 131 markers, most of which showing TRD in only
418 the subadults of either family (Figure 3; Table S5). In BC_Str, none of the distorted
419 markers was common in both stages. In BC_Ssc, six distorted markers were shared
420 between the stages, and one of them ('scaffold6_37022538') additionally showed TRD
421 in the subadults of BC_Str. This marker, positioned terminally on CHR 6, showed an
422 interesting pattern of distortion in which heterozygotes and homozygotes were
423 overrepresented in BC_Str and BC_Ssc, respectively. Only 34.1% of the distorted
424 markers of BC_Str subadults showed excesses of homozygotes. This largely reflects
425 the presence of a few CHRs harbouring extended regions with excesses of
426 heterozygotes (see below). In contrast, 100%, 92.6%, and 83.3% of the distorted
427 markers showed excesses of homozygotes in BC_Str larvae, BC_Ssc larvae, and
428 BC_Ssc subadults, respectively.
429 Several extended genomic regions, harbouring more than ca. 20 markers with
430 single-locus TRD in the same direction, were recorded (Figure 2). In BC_Str subadults,
431 CHR 2 exhibited an excess of heterozygotes across its entire length. Approximately 27
432 Mb of CHR 6 showed an excess of heterozygotes in BC_Str and, in turn, an excess of
433 homozygotes in BC_Ssc. CHRs 10 and 14 in BC_Ssc and BC_Str subadults,
434 respectively, showed excesses of homozygotes across more than ca. 20 Mb. These
435 regions on CHRs 6, 10, and 14 roughly corresponded to the recombination coldspots
436 (Figure S6). Smaller clusters of 2–5 distorted markers spanning ca. 1.2–3.8 Mb were
437 also observed in CHRs 7, 21, and 23 in BC_Str subadults and CHRs 4 and 19 in
438 BC_Ssc subadults (Figures S7, S8). The remaining markers showing single-locus TRD
439 were interspersed across the genome without forming a distinct cluster.
440 Tests for non-random associations of genotypes between pairs of markers on
441 separate LGs yielded no significant result after FDR correction (all $p > 0.42$), partly

because of numerous tests, making the tests highly conservative. We, therefore, focused on the marker pairs showing associations at nominal $p < 0.01$ and additional supports for the associations. The additional supports were the clustering of multiple marker pairs whereby two or more adjacent markers on a LG (hereafter referred to as a marker block) showed associations with another marker block on a different LG. Fifty five such associations (pairs of marker blocks) were found (Figure 4; Table S6); however, none of these was common across developmental stages or families, i.e., none of the associations observed in different stages/families involved marker blocks with overlapping ranges on both LGs. The numbers of the observed associations were comparable between the developmental stages in each family (Figure 3).

Sex ratio was slightly male-biased in subadults of BC_Str but did not differ significantly from parity (female:male = 0.40:0.60, n = 80; $\chi^2 = 0.89$, df = 1, $p = 0.35$), and was more even in BC_Ssc (female:male = 0.48:0.52, n = 95; $\chi^2 = 0.09$, df = 1, $p = 0.75$).

3.3 QTL mapping

Thirty-nine morphological traits were subjected to QTL mapping, showing significant differences between the two families (Figure S9; Tables S1, S2). Significant QTLs with LOD scores greater than the genome-wide thresholds were detected for 11 and 9 traits in BC_Str and BC_Ssc, respectively (Figure 1; Table 1; Figures S10, S11). Single significant QTL for each trait was detected, except for yellow pigmentations of BC_Str, where two QTLs were detected on different LGs. As for colour-related traits, QTLs for the following traits were detected: yellow pigmentations for both families (on different LGs); black pigmentations and PC1 scores based on the 1st and the 3rd colour clusters of the patternize analysis for BC_Str; vertical bands on dorsum and horizontal stripe along the lateral line for BC_Ssc. The 1st and the 3rd colour clusters of the patternize analysis represented the variations in a stripe along the lateral line and vertical bands on the dorsum, respectively. As for body shape and counts, QTLs for the following traits were detected: lower caudal-peduncle length in both families (on different LGs); PC2 scores based on the geometric morphometrics, predorsal length, anal-fin base length, 2nd anal-fin spine length, and number of head spines in BC_Str; orbit length, interorbital width, and numbers of pectoral-fin rays and gill-rakers on lower gill arch in BC_Ssc. PC2 scores based on the geometric morphometrics represented

variations in the relative positions of the head and body. Directions of the QTLs were consistent with the differences between the parental species except for the following traits: predorsal length and the 3rd colour cluster of the patternize analysis in BC_Str; lower caudal-peduncle length and yellow pigmentations in BC_Ssc. The QTL for a horizontal stripe along the lateral line exhibited the highest PVE among all morphological traits. In addition, QTL for sex with a high LOD score was detected on LG 12 in each family, with the position roughly corresponding to that of the *amhy* gene: 26.4 Mb on the reference genome (Song et al., 2021), ca. 25 cM on the consensus map (Figure S6).

The QTLs for the following traits had overlapping CIs: PC2 scores of geometric morphometrics and 2nd anal fin length on LG 1, yellow and black pigmentations on LG 5, sex, yellow pigmentations, and the 1st colour cluster of the patternize analysis on LG 12 of BC_Str; orbit and lower caudal-peduncle lengths on LG 15 of BC_Ssc.

4. Discussion

4.1 TRD and postmating isolation

4.1.1 General pattern

We observed tens of markers showing two classes of TRD: single-locus TRD and non-random associations of genotypes between pairs of markers. These markers, presumably linked to the loci that conferred survival variation among hybrid progeny and hence the postmating isolation between *S. trivittatus* and *S. schlegelii* (barrier loci), were widely distributed across the genome and some of them encompassed large proportions of particular chromosomes. Such a genetic architecture suggests genetic redundancy and the multifarious nature of the postmating isolation. These features would strongly reduce gene flow across the genome if the two species did mate, thereby enabling the postmating isolation to play a critical role in completing speciation or maintaining distinct gene pools (Coughlan & Matute, 2020; Nosil et al., 2009). Three of the four extensive genomic regions with single-locus TRD in subadults of one or both families coincided with the recombination coldspots. This suggests that each of these three regions represents the isolating effects of one or a few barrier loci affecting broad surrounding regions due to the tight linkage, rather than the effects of more numerous and widespread barrier loci. Overall, the combined effects of the recombination

landscape and the effects of individual barrier loci created the observed complex genetic architecture.

TRD was observed in each of the four samples differing in the developmental stages and the directions of the crosses, with only a few distorted loci shared between them (Figure 3). TRD observed in larvae can be ascribed to postmating isolating barriers acting at the prenatal stage, from the artificial insemination until the parturition, including gametic isolation and hybrid inviability. TRD in subadults can be based on hybrid inviability at the postnatal stage from the parturition until sacrifice. In fact, the distinct genetic architectures observed for larvae and subadults suggest that largely different isolating mechanisms have been acting at the pre and postnatal stages. On the other hand, asymmetric isolation has been frequently reported in previous studies employing reciprocal interspecific crosses, and its primary cause is thought to be hybrid incompatibility involving uniparentally inherited genetic factors or maternal effects (Turelli & Moyle, 2007). More discussion on specific mechanisms of TRD in different samples is provided in section 4.1.2.

We found no significant bias in the sex ratio in either family, suggesting little or no difference in the fitness between sexes. We also observed no tendency for markers on the sex chromosome CHR 12 to be more frequently involved in either type of TRD. These results suggest that, regarding hybrid inviability, the present system does not obey the two ‘rules’ concerning reproductive isolation: disproportionate contribution of X or Z chromosomes to postmating isolation (large X effect) and more severe fitness reduction of heterogametic sex in hybrid crosses (Haldane’s rule) (Coyne & Orr, 1989b). This observation is consistent with homomorphic, recombining sex chromosomes in *Sebastes* (Arai, 2011; Song et al., 2021; this study) since most explanations for the two rules require heteromorphic sex chromosomes (Coyne, 2018). Teleosts generally do not obey Haldane’s rule regarding hybrid inviability (reviewed in Schilthuizen et al., 2011); the present system is considered one of such cases.

We found discordance between the current linkage map and the reference genome of *S. schlegelii* regarding the assignments of some markers to LGs/CHRs (Figure S6). Some of the markers also showed non-random associations of genotypes with markers on another LG (Figure 4). These results are reminiscent of reciprocal translocations between the parental species. However, reciprocal translocation in parental species generally has more extensive effects on a hybrid linkage map whereby LGs involved in the translocation are connected into a single LG via markers

on the translocated region (Farré et al., 2011; Fishman et al., 2013), which was not observed herein (Figure S5). We suspect that this discordance resulted from slight errors in our linkage map or the genome assembly. Further studies will be necessary to clarify whether the discordance represents some form of chromosomal rearrangements, an important mechanism of reproductive isolation and adaptive divergence between taxa (e.g., Matschiner et al., 2022).

A previous genomic study by Behrens et al. (2021) has identified several genomic regions showing elevated values of both F_{ST} and D_{XY} between each of the two pairs of incipient species of *Sebastes* (*S. carnatus*–*S. chrysomelas* and *S. miniatus*–"*S. crocotulus*"), both pairs belonging to different subclades from the present parental species. These regions are supposedly involved in adaptive divergence and reproductive isolation in the presence of gene flow in each pair. The regions were located at 38.5–39.0 Mb on CHR 3, 18.5–20.0 Mb on CHR 4, 0.5–1.5 Mb on CHR 7, 10.5–11.0 Mb on CHR 9, and 30.0–32.5 Mb on CHR 13 on the same reference genome as that used in the present study. None of these regions overlaps with the extended TRD possibly associated with extrinsic selection in the present system (see below). Instead, these regions except that on CHR 3 coincided with the recombination coldspots (Figure S6), implicating conserved recombination landscapes across the phylogeny of rockfishes and their role in determining the genetic architecture of reproductive isolation (Burri et al., 2015; Nachman & Payseur, 2012).

4.1.2 Possible mechanisms of isolation

Owing to the present cross design and the distinct (asymmetric) genetic architectures of the two families, we suggest that he most likely isolating mechanisms causing TRD at the prenatal stage are gametic isolation and hybrid inviability involving maternal factors. Under the present cross design, both F_1 sperm and embryos of the two families have been stored in ovaries of reciprocal maternal species during sperm storage and gestation, with the embryos possessing maternally inherited genetic factors from their respective maternal species (Figure S2). DMI involving maternally inherited factors such as mito-nuclear incompatibility is a predominant mechanism of asymmetric reproductive isolation in reciprocal hybrid crosses (Turelli & Moyle, 2007). Empirical evidence that mitochondrial genomes from reciprocal parental populations cause TRD in distinct sets of chromosomes in reciprocal F_2 crosses has been reported for marine

copepod (Lima et al., 2019). Besides, interactions between maternally expressed factors and sperm or zygotes within the mother's body can result in asymmetric reproductive isolation (e.g., Devigili et al., 2018). Notably, such interactions are predicted to be driven by mother-offspring conflict in viviparous, matrotrophic animals, including rockfishes (viviparity-driven conflict hypothesis; Zeh & Zeh, 2000). In any case, the mechanisms of the TRD at the prenatal stage might be genetically complex, involving epistatic interactions among multiple nuclear loci, as well as the interactions with maternally inherited or expressed factors.

The TRD at the postnatal stage may also be explained by multiple factors, including intrinsic hybrid inviability due to DMI involving maternally inherited factors and environmentally-dependent (extrinsic) inviability. The former can have a common mechanistic and genetic basis with that acting at the prenatal stage. For instance, mito-nuclear incompatibility may disrupt the metabolic function of mitochondria essential to all developmental stages (Burton & Barreto, 2012). In fact, five loci showing significant excess of homozygotes on CHRs 1, 4, 7, and 13 were shared between the two stages in BC_Ssc. These may represent loci that confer fitness effects across the developmental stages via intrinsic asymmetric hybrid inviability. The remaining distorted loci that were not shared with the prenatal stage are considered to have been caused by stage-specific intrinsic hybrid inviability or extrinsic inviability.

Extrinsic hybrid inviability occurs when ecologically divergent species produce hybrids with intermediate phenotypes, and the hybrid suffers reduced fitness in the parental environments (Nosil, 2012). The present study was not specifically designed to examine this process. Nonetheless, ca. 20 Mb region of CHR 6 showing heterozygote excess in BC_Str and homozygote excess in BC_Ssc suggest additive selection against *S. trivittatus* alleles, implying extrinsic hybrid inviability under an environment where *S. trivittatus* is less fit than *S. schlegelii* (Rundle & Whitlock, 2001). The pattern also implicates immigrant inviability, a class of premating isolation whereby immigrants of parental species suffer reduced fitness in their non-native habitats (Nosil, 2012). Additionally, regions with homozygote excess in BC_Ssc subadults (e.g., CHR 10) or heterozygote excess in BC_Str subadults (e.g., CHR 2) with no concurrent TRD in the other family could also be associated with extrinsic selection against *S. trivittatus* alleles with dominance effects. A possible environmental factor underlying the extrinsic selection is water temperature. Species distribution models for *S. schlegelii* have suggested that water temperature is the most important environmental variable

determining the natural distribution of the species, and it prefers water temperatures of 3–20 ºC (Chen et al., 2021). Although the thermal tolerance of *S. trivittatus* is unknown, it is reasonable to assume that this species is adapted to colder waters than *S. schlegelii*, given the more northern distributional of the former. On the other hand, the water temperatures of the tanks were 3.1–21.3 ºC in the present study (see Appendix S1). These temperatures are within the range tolerated by *S. schlegelii* but may have deviated from that by *S. trivittatus*, thus, selecting against *S. trivittatus* alleles at relevant loci.

The terminal locus ('scaffold6_37022538') of the distorted region of CHR 6 additionally showed TRD in larvae, although the distortion in BC_Str larvae was not statistically significant (Figure 2; Table S5). The directions of the distortion were the same between stages in each family (heterozygote excess in BC_Str; homozygote excess in BC_Ssc), suggesting that the extrinsic selection may have acted at the prenatal stage as well. Effects of ambient thermal conditions on prenatal viability of offspring via mother's body temperature of viviparous snake have been previously documented (Lourdais et al., 2004).

4.2 QTLs for morphological traits and sex determination

Among the morphological traits with significant QTLs detected, the following traits have been previously suggested to be related to convergence or speciation of rockfishes: orbit diameter, number of gill-rakers on the lower arch, head spination, intensities of pigmentations, stripe along the lateral line, and dark bands on the dorsum. The colour clusters based on the patternize analysis are also considered among such traits since these are the quantitative representations of the colour patterns. We do not nominate candidate genes underlying these traits, considering the broad CIs for each QTL, presumably due to the limited sample sizes. Nonetheless, the present study constitutes the first step toward understanding the genetic bases of the traits at the individual gene level, which will, in turn, provide insights into the traits' adaptive significance and the evolutionary trajectories within and across lineages (e.g., Kokita et al., 2021; Kratochwil et al., 2018).

The variations in the body colour (overall intensities of pigmentations) and the colour patterns in the present system were suggested to have partially independent genetic bases. More specifically, genomic regions containing the QTLs for colour

related traits were divided into three classes: containing the QTLs for the intensities of pigmentations, the colour patterns, and both (Figure 1). We suspect that these classes reflect the functions of genes that confer the association of each genomic region with the variations in the traits. For example, in the melanocortin system of teleosts, tyrosinase-related protein 1 is involved in pigment formation and therefore can affect the overall body colour, leaving the patterns unaffected, whereas Melanocortin 1 receptor is involved in the proliferation of chromatophores and therefore can affect both the colour and the colour patterns (Cal et al., 2017; Singh & Nüsslein-Volhard, 2015). Mutations in genes such as *leopard* in zebrafish are known to affect interactions among chromatophores, affecting only the colour patterns (Singh & Nüsslein-Volhard, 2015). The supposed partially independent genetic bases for the colour and the colour-patterns in the present system is consistent with the colour pattern convergence in rockfishes, where independent lineages have similar colour patterns but not necessarily similar colours.

The present result implicated a common sex-determination system for *S. schlegelii* and *S. trivittatus*, i.e., male-determination by *amhy*. The role of *amhy* has been originally demonstrated for *S. schlegelii* and additionally confirmed in *S. pachycephalus*, and *S. koreanus* (Song et al., 2021). Under the present cross design, *amhy* inherited from the male F_0 *S. schlegelii* segregated in each backcross family. Besides, a highly significant QTL for sex was detected on LG 12 in each family, with CIs encompassing the presumed position of *amhy*. Taken together, *amhy* from F_0 *S. schlegelii* successfully determined sex in both families despite the contrasting degrees of genetic contributions from *S. schlegelii* and *S. trivittatus*, implying that the sex of *S. trivittatus* is also controlled by this gene. The sex-determination role of *amhy* might be conserved across the members of the northwest Pacific subclade of the genus, to which the aforementioned four species belong (Figure S1).

4.3 Concluding remarks

We provided a snapshot of postmating isolation in the speciation continuum of rockfishes at the point where reproductive isolation is complete in nature. It is most likely characterised by 1) the involvement of tens of loci across the genome, 2) asymmetry of the underlying loci concerning the direction of the crosses, and 3) multifarious mechanisms including asymmetric gametic isolation and hybrid inviability

due to interaction with maternal factors, and extrinsic selection. Future studies employing direct observations of the isolating mechanisms at the phenotypic level, and comparisons with other pairs of species at various points in the speciation continuum, will contribute to a more comprehensive understanding of the speciation in rockfishes. Besides, several limitations of the present study should also be noted. First, some of the observed TRD may reflect isolating mechanisms that have evolved after the completion of reproductive isolation. Second, the slight difference in rearing conditions for the two families could have affected the results. Third, some of the TRD that were unique to larvae of each family might be false positives. This is because markers showing TRD in larvae should also do so in subadults unless the TRD in larvae is counterbalanced by selective mortality at the postnatal stage. Finally, TRD may occur due to mechanisms other than reproductive isolation between parental species, such as inbreeding depression and DMI involving alleles segregating within species (Corbett-Detig et al., 2013; Plough & Hedgecock, 2011). These issues can be partly addressed by careful control of rearing conditions and appropriate cross designs (e.g., reciprocal F_2 crosses; Lima et al., 2019).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

NM, TK, YoS, and HT conceived the study. TK and YoS performed the crosses and oversaw the fish husbandry. NM, TK, YuS, SI, YoS, and HT performed the phenotyping.

NM, RK, and AJN performed the genotyping. NM and RK performed the analyses. NM wrote the manuscript. All authors read and approved the manuscript.

DATA AVAILABILITY STATEMENT

Sequence data have been deposited in Sequence Read Archive in DDBJ/EMBL-EBI/NCBI under accession number DRA014198. Genotype and phenotype data and linkage maps are available from Dryad.

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1016 **TABLES**

1017

1018 **TABLE 1** Significant QTLs for body colouration, shape, counts, and sex. The 'Direction' column indicates whether *S.*
1019 *trivittatus* (Str) or *S. schlegelii* (Ssc) alleles cause higher trait values (for continuous traits) or the presence of the focal trait
1020 (for binary traits). Asterisks indicate allelic effects in the direction expected from the divergence between the parental species.

Trait	Family	LG	Pos (cM)	Pos (Mb)[a]	LOD	CI lower	CI upper	PVE	Direction
Colouration									
Colour cluster 1: PC1	BC_Str	12	0.0	0.7	3.00	0.0	26.2	14.6	Ssc*
Colour cluster 3: PC1	BC_Str	16	60.8	25.8	3.36	51.6	60.8	16.6	Str
Black pigmentation	BC_Str	5	11.0	1.3	2.77	0.0	45.9	6.2	Ssc*
Yellow pigmentation	BC_Str	5	34.1	11.4	3.21	0.0	47.4	12.2	Str*
Yellow pigmentation	BC_Str	12	15.0	16.7	3.31	0.0	18.5	11.8	Str*
Yellow pigmentation	BC_Ssc	22	58.0	23.4	5.86	43.9	60.5	22.9	Ssc
Stripe along lateral line	BC_Ssc	1	51.0	29.6	9.27	40.8	51.5	46.9	Str*
Dark bands on dorsum	BC_Ssc	2	35.3	23.0	3.41	17.5	48.7	19.0	Str*
Shape									
GM: PC2	BC_Str	1	40.8	27.2	3.05	37.8	60.2	15.8	Str*
Orbit diameter	BC_Ssc	15	6.0	12.9	3.25	0.0	54.0	6.4	Ssc*
Interorbital width	BC_Ssc	13	2.0	2.7	3.75	0.0	51.6	4.2	Ssc*
Lower peduncle length	BC_Str	12	35.6	32.8	3.19	29.2	42.5	5.7	Ssc*
Lower peduncle length	BC_Ssc	15	24.9	23.9	3.04	0.0	54.0	5.0	Str
Predorsal length	BC_Str	20	37.0	6.8	2.70	11.5	51.3	2.2	Ssc
Anal-fin base length	BC_Str	24	24.0	12.1	2.51	0.8	39.6	17.4	Str*

Anal-fin 2nd spine length	BC_Str	1	51.0	29.6	4.84	43.9	60.2	10.5	Str*
Counts									
Gill rakers on lower arch	BC_Ssc	3	23.0	32.3	2.78	0.0	60.5	15.5	Str*
Pectoral-fin rays	BC_Ssc	11	37.0	17.5	2.79	0.0	58.3	14.7	Ssc*
Spines on head	BC_Str	11	54.0	21.5	3.42	0.0	60.2	16.7	Str*
Sex	BC_Str	12	25.2	26.1	7.71	15.4	29.2	49.6	NA
Sex	BC_Ssc	12	21.0	27.6	20.80	18.5	23.2	94.9	NA

[a] Based on the marker position on the reference genome assembly of *S. schlegelii*. For the QTL on pseudomarker, the physical position of the nearest marker is provided.

Abbreviations: CI, confidence interval; cM: centimorgan; GM, geometric morphometrics; LG, linkage group; Mb, megabase; PVE, per cent variance explained.

Figures

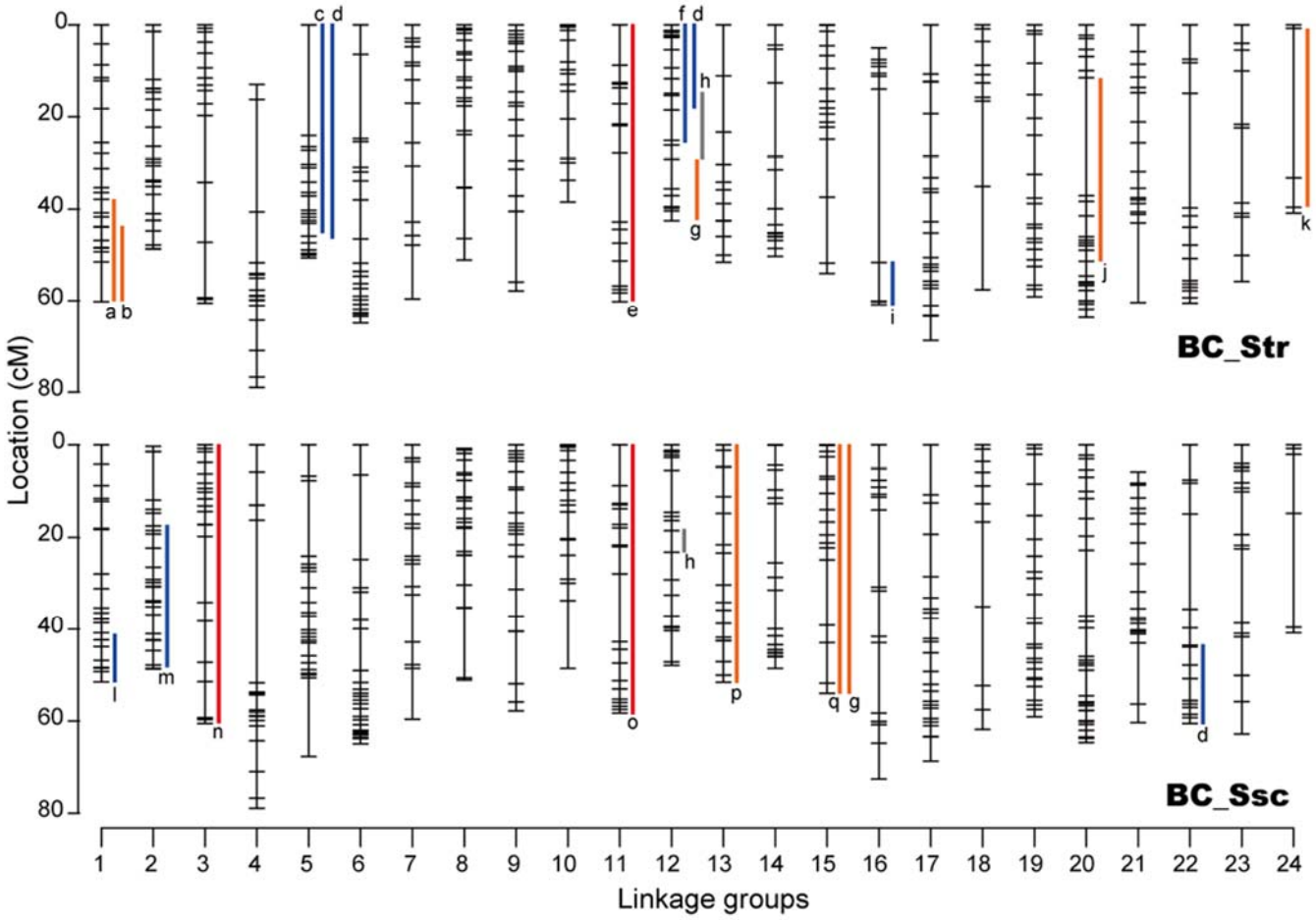
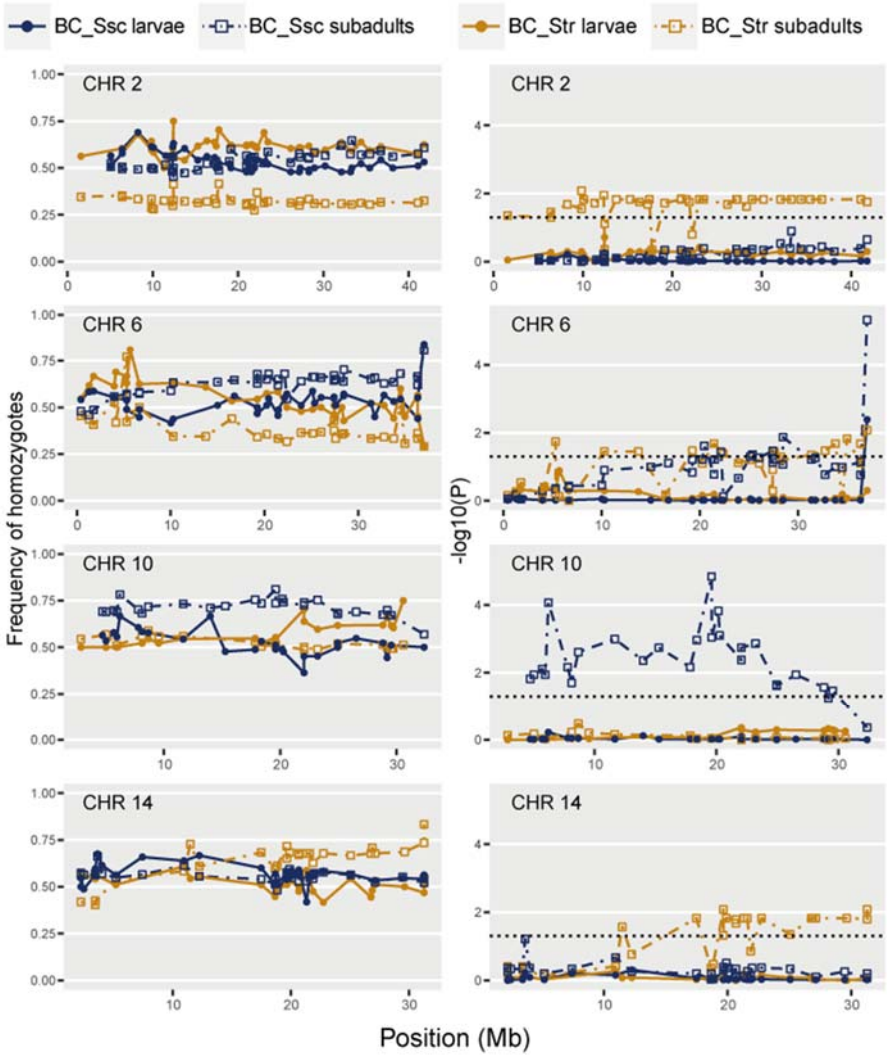


FIGURE 1 Consensus linkage maps and 95% confidence intervals of positions of QTLs for colouration (blue bars), shape (orange), counts (red), and sex (grey): a, PC2 scores based on geometric morphometrics; b, Anal-fin 2nd spine length; c, black pigmentation; d, yellow pigmentation; e, head spination; f, PC1 scores based on colour cluster 1 derived from patternize analysis; g, lower peduncle length; h, sex; i, PC1 scores based on colour cluster 3; j, predorsal length; k, anal-fin base length; l, stripe along lateral line; m, dark bands on dorsum; n, gill-rakers on lower arch; o, pectoral-fin rays; p, interorbital width; q, orbit diameter. The difference in the length of each linkage group between families is due to the presence of markers unique to either family (see also Table S4).



1038 **FIGURE 2** Genotype frequencies and transmission ratio distortion of markers on
1039 selected chromosomes, shown on the reference genome assembly of *S. schlegelii*. For
1040 each marker, frequencies of homozygotes (left column) and *p* values as $-\log_{10}p$ from the
1041 chi-square tests for deviations of genotype frequencies from Mendelian expectations
1042 corrected for false discovery rates (FDR) (right column) are given. Dotted line indicates
1043 FDR-corrected $p = 0.05$.
1044

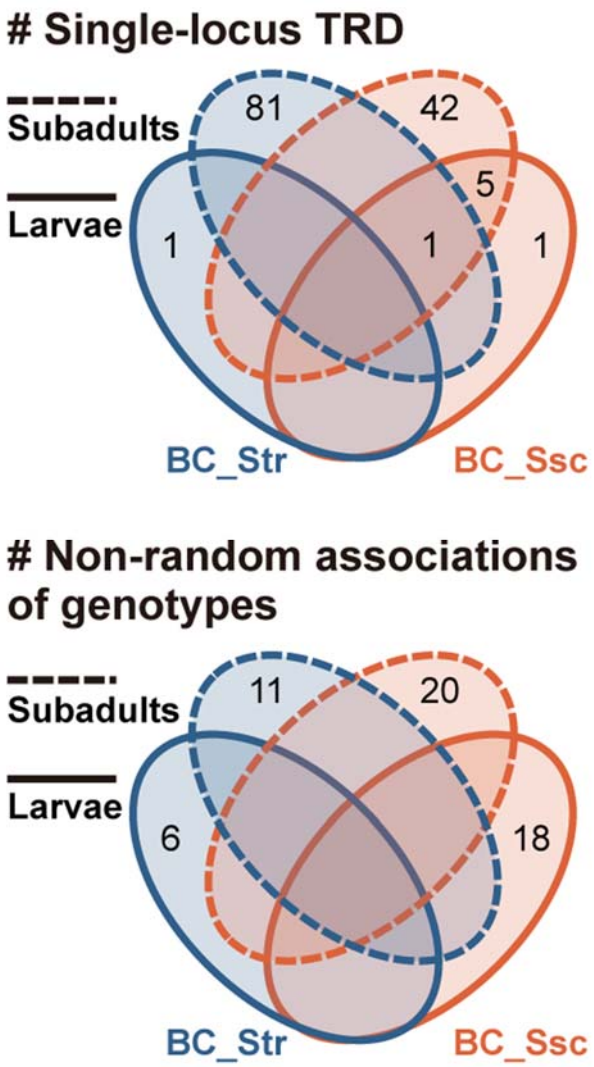


FIGURE 3 Venn diagrams for the number of markers showing significant single-locus transmission ratio distortion (TRD) and the number of pairs of marker blocks (groups of two or more adjacent markers) on separate linkage groups (LGs) showing non-random associations of genotypes. The non-random associations of genotypes between pairs of marker blocks were regarded as common across samples of different developmental stages/families if the associations in different samples involved marker blocks with overlapping ranges on both LGs (none observed in the present study).

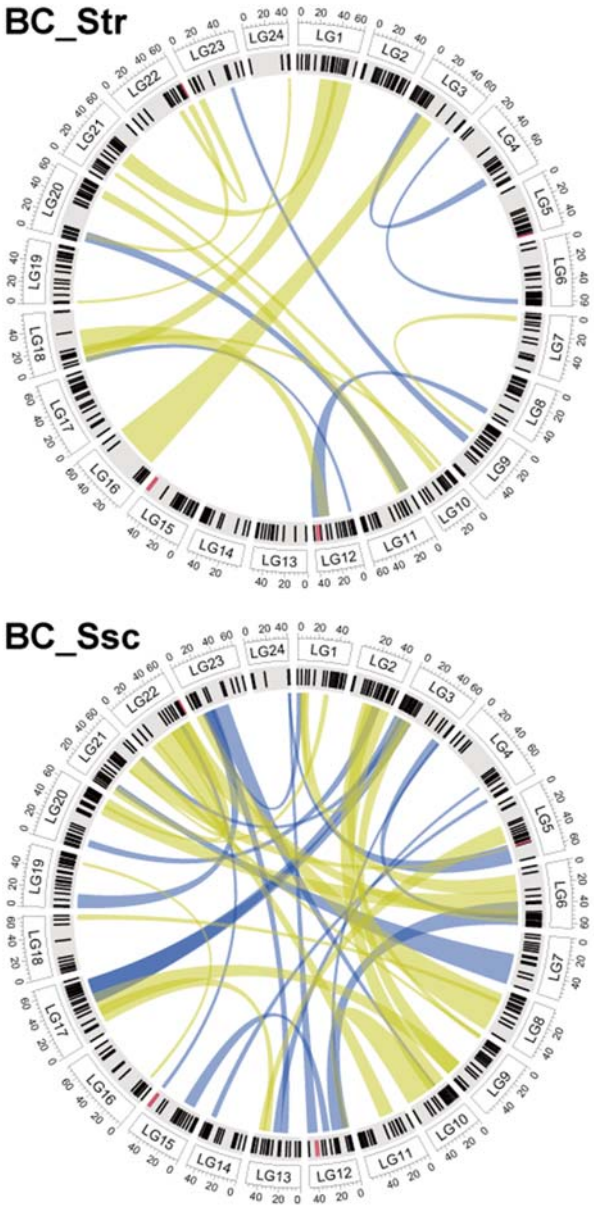


FIGURE 4 Possible epistatic interactions between pairs of markers on separate linkage groups (LGs). Each link represents a pair of marker blocks showing a non-random association of genotypes (chi-square test; nominal $p < 0.01$). Blue and yellow links correspond to the associations observed in larvae and subadults, respectively. The outer and inner circles represent each LG of the consensus linkage map and marker positions used for this analysis, respectively. Red ticks indicate the markers assigned to different (non-syntenic) LGs/chromosomes on the consensus linkage map and on the reference genome assembly of *S. schlegelii*.