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Phenotypic Divergence and Genomic Architecture Between Parallel Ecotypes at Two Different Points on the Speciation Continuum in a Marine Snail

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ABSTRACT

Speciation is rarely observable directly. A way forward is to compare pairs of ecotypes that evolved in parallel in similar contexts but have reached different degrees of reproductive isolation. Such comparisons are possible in the marine snail *Littorina saxatilis* by contrasting barriers to gene flow between parallel ecotypes in Spain and Sweden. In both countries, divergent ecotypes have evolved to withstand either crab predation or wave action. Here, we explore transects spanning contact zones between the Crab and the Wave ecotypes using low-coverage whole-genome sequencing, morphological and behavioural traits. Despite parallel phenotypic divergence, distinct patterns of differentiation between the ecotypes emerged: a continuous cline in Sweden indicating a weak barrier to gene flow, but two highly genetically and phenotypically divergent, and partly spatially overlapping clusters in Spain suggesting a much stronger barrier to gene flow. The absence of Spanish early-generation hybrids supported strong isolation, but a low level of gene flow is evident from molecular data. In both countries, highly differentiated loci were located in both shared and country-specific chromosomal inversions but were also present in collinear regions. Despite being considered the same species and showing similar levels of phenotypic divergence, the Spanish ecotypes are much closer to full reproductive isolation than the Swedish ones. Barriers to gene flow of very different strengths between ecotypes within the same species might be explained by dissimilarities in the spatial arrangement of habitats, the selection gradients or the ages of the systems.

Francesca Raffini and Aurélien De Jode contributed equally to this work.

1 | Introduction

The process of speciation involves the build-up of reproductive isolation. The precise meaning of 'reproductive isolation' has recently been debated (Westram et al. 2022 and associated commentaries); here, we use the term in a general sense to include both reduction in interbreeding and reduction in gene exchange. The timescale over which speciation occurs is highly variable (Coyne and Orr 2004), although it can be instantaneous (e.g., polyploidisation), it often occurs over timescales much longer than a human lifetime, which is challenging for direct observation. Therefore, inferences about the accumulation of reproductive isolation often depend on comparing contemporary pairs of populations at different points on a 'speciation continuum' (Seehausen and Wagner 2014; Stankowski and Ravinet 2021). Due to the complex set of processes involved in speciation, it can be helpful to think about this continuum in multiple dimensions (Bolnick et al. 2023; Johannesson et al. 2024). This approach can provide many insights, for example in considering whether there are early- and late-evolving components of reproductive isolation, or how patterns of gene exchange across the genome are modified at different stages of divergence (Feder et al. 2012). However, a monotonic progression from weak to strong isolation cannot be assumed (Bolnick et al. 2023; Stankowski and Ravinet 2021). More studies of population pairs across the speciation continuum are needed, particularly within clades so that comparisons among different levels of reproductive isolation are not confounded with differences among taxa that may not be relevant to the speciation process (Seehausen and Wagner 2014). Significant progress has been made in this direction, especially in terms of genome-wide patterns of genetic differentiation (Butlin and Faria 2024; Fang et al. 2020, 2021; Jiggins 2017; Reid et al. 2021; Riesch et al. 2017). The number of investigations in different study systems is, however, still limited and many questions about the mechanisms leading to completion of speciation remain open, particularly in the case of speciation with gene flow.

The appearance of the first components of reproductive isolation is often relatively well understood, but explaining the later accumulation of additional components of isolation and the final cessation of gene flow remains challenging (Butlin et al. 2008; Butlin and Smadja 2018; Kulmuni et al. 2020). Many factors have been suggested that might lead to stronger reproductive isolation. Time is needed for the accumulation of incompatibilities (Guerrero et al. 2017), and possibly also for response to divergent selection if this is mutation limited (Barrett and Schluter 2008), and so older population pairs might be more strongly isolated, as is often observed (Coyne and Orr 1989; Matute and Cooper 2021). Stronger divergent selection, for example between more distinct habitats, should also lead to stronger isolation (Funk et al. 2006). Some spatial arrangements of populations might be more conducive to the evolution of reproductive isolation than others, because gene flow opposes divergence (Coyne and Orr 2004) but contact also provides the opportunity for reinforcement (Servedio and Noor 2003; Yukilevich 2021). Furthermore, cycles of population expansion and contraction can act to bring components of reproductive isolation together (Butlin and Smadja 2018; Hewitt 1989). Opportunities for the evolution of assortative mating, either through mating behaviour or due to habitat association, can lead to strong reproductive isolation particularly when they are aligned to divergent selection (Kopp et al. 2018). Finally, one-allele barrier effects (Butlin et al. 2021; Felsenstein 1981), pleiotropy and multiple-effect traits (Servedio et al. 2011; Smadja and Butlin 2011) might promote the evolution of reproductive isolation. Likewise, genomic architecture, i.e., numbers and effect sizes of barrier loci, their genomic distribution and patterns of recombination, including the effects of structural variants, may be important because the coupling of individual barrier loci and of barrier effects can be opposed by recombination (Butlin and Smadja 2018; Dopman et al. 2024; Felsenstein 1981).

The intertidal gastropod genus, *Littorina*, is a model system in which several of these issues can be addressed (Johannesson et al. 2010, 2017, 2024; Johannesson 2016; Rolán-Alvarez et al. 2015). *Littorina saxatilis* is widespread and abundant on North Atlantic shores. Its ovoviviparous reproduction and resulting short lifetime dispersal have allowed it to adapt to many different habitat types (Reid 1996). Ecotypes adapted to habitats with a high risk of crab predation but less direct wave action (the 'Crab' ecotype), or habitats with strong wave action but low predation risk (the 'Wave' ecotype), occur in close proximity on many shores and form contact zones where habitats meet. These ecotypes differ in a suite of adaptive traits including size, shell shape, shell thickness and behaviour (Johannesson and Johannesson 1996). Demographic models suggest that they evolved in parallel in multiple countries (Butlin et al. 2014; Carvalho, Faria, et al. 2023) although this is likely to have involved repeated use of at least some adaptive genetic variants (Morales et al. 2019), a proportion of which are found in chromosomal inversions (Faria, Chaube, et al. 2019; Koch et al. 2021, 2022; Reeve et al. 2023; Westram et al. 2021). Background levels of genetic divergence between the Crab and Wave ecotypes and patterns of genomic differentiation determined using pooled sequencing data suggested that the strength of the barrier to gene flow between ecotypes varies widely among locations in Europe (Morales et al. 2019).

In this study, we compared and contrasted the patterns of divergence between Crab and Wave ecotypes in two geographic regions: the Swedish west coast, colonised since the last glaciation (divergence estimated ~15 kya), and the Galician coast in Spain (divergence estimated ~57 kya), where older populations survived the Pleistocene glaciations in situ (Bosso et al. 2022; Butlin et al. 2014; Carvalho, Faria, et al. 2023; Carvalho, Morales, et al. 2023; Doellman et al. 2011; Panova et al. 2011). In Sweden, the tidal range is small, the Crab–Wave axis is parallel to the shoreline (horizontal zonation) and the two ecotypes hybridise in narrow contact zones where sheltered boulder fields abut rocky headlands. There is a genome-wide barrier to gene exchange, but it is weak: background F_{ST} is about 0.04 (Johannesson et al. 2024), clinal changes in SNP frequency are widespread in the genome but fixed differences are rare, loci putatively contributing to barrier effects occur both within and outside polymorphic inversions (Koch et al. 2022; Westram et al. 2018, 2021). The barrier in Sweden appears to be due primarily to local adaptation, without noticeable intrinsic incompatibilities, but with some contribution of size-assortative mating (Hollander et al. 2005; Johannesson et al. 2008; Perini et al. 2020).

In Spain, the tidal range is much greater and the two ecotypes are distributed on a perpendicular up–down shore axis (vertical zonation) with the Crab ecotype primarily in the barnacle belt in the high shore, where predation is most intense, and the Wave ecotype among blue mussels in the low shore, where wave action is strongest. The Spanish ecotypes overlap in a contact zone in the mid shore, characterised by a mosaic distribution of barnacle and mussel patches (Johannesson et al. 1993, 1995; Rolán-Alvarez et al. 1997, 1999). There are some indications that the barrier to gene flow between ecotypes is stronger in Spain than in Sweden: background F_{ST} is higher, around 0.1 (Butlin et al. 2014; Morales et al. 2019; Westram et al. 2021), and only a few intermediate genotypes were observed in putatively hybrid samples using reduced representation (RADseq) data (Kess et al. 2018). Yet, an investigation of the pattern of differentiation in Spain with a combination of genome-wide data and detailed spatial coverage of the contact zone has been lacking.

Here, we take a step further into understanding the drivers of speciation, particularly at the molecular level. We describe the phenotypic and genomic patterns of differentiation between Crab and Wave snails in Spain using low-coverage whole-genome sequence data, shell features and behavioural traits for snails from dense transects across the contact zone. We make a direct comparison with a transect in Sweden as well as with published analyses based on pooled or capture sequencing. Despite studying populations of the same species and similar phenotypic divergence (Crab and Wave ecotypes), we report very different patterns of genomic differentiation in the contact zones of the two countries: in Spain, ecotypes partly overlap in space with evidence for only limited, almost unidirectional introgression, while in Sweden there is a gradual phenotypic as well as genetic transition from one ecotype to the other. Thus, despite being populations of the same species (*L. saxatilis*), pairs of ecotypes in different environmental and demographic contexts have very different positions on the speciation continuum and we discuss potential reasons for this.

2 | Materials and Methods

2.1 | Sampling

Snails were sampled to include the typical habitats of both the Crab and Wave ecotypes and the contact zone in between, in both Spain and Sweden. In Sweden, snails were sampled in a single transect along the shore from a boulder field ('Crab' habitat) to a rocky headland ('Wave' habitat) on the island of Ramsö (58°49′27.8″ N, 11°03′45.3″ E; a re-sampling of transect CZA_right from Westram et al. 2021). Note that the tidal amplitude is only 35 cm in Sweden, but high and low water level also vary with air pressure and wind direction up to a maximum amplitude of 1.5 m. In all parts of the transect, snails were collected from positions scattered throughout their vertical distribution (~1 m). Seven hundred snails were sampled in June 2015 along the transect, without reference to phenotype but aiming to avoid juveniles. The position of each snail was recorded in three dimensions using a total station (Trimble M3). For spatial analysis, we placed each snail on a 'least cost path' (as described in Westram et al. 2021) and distances were transformed to start from zero at the Crab ecotype end of the transect.

In Spain, snails were collected from Centinela on the west coast of Galicia (N 42°4′38.06″, W 8°53′47.47″) in spring (March) and autumn (September) of 2017. Each sample consisted of approximately 600 snails collected in the same way as in Sweden from two transects perpendicular to the shore, about 5 m wide and separated by 2–10 m, stretching from the upper limit of the *L. saxatilis* distribution in the splash zone to its lower limit close to low water of spring tides (tidal range ~4 m). Sampling was denser in the lower part of the shore where hybridisation between the two previously described ecotypes was expected (Galindo et al. 2013). For spatial analysis, we used the position of each snail on a single shore-position axis running through the middle of each transect. Distances were transformed such that each transect started from the top of the distribution (defined as zero) and ended at roughly low water level as indicated by the lowest collected snails. This corresponds to a vertical range of approximately 3 m and shore height was recorded relative to the position of the lowest individual sampled. To include habitat information, the presence or absence of barnacles (*Chthamalus* spp., typical of the mid to upper shore), mussels (*Mytilus galloprovincialis*, from mid to lower shore) and goose barnacles (*Pollicipes pollicipes*, lower shore) was recorded within 5 cm of each snail position and for approximately uniformly distributed positions across the area containing the two sampling transects. Snails were stored in individual tubes, moistened with seawater and kept at 4°C until phenotyping was complete. Then, the head and foot of each snail were dissected and preserved in 99% ethanol.

2.2 | Sequencing and Read Processing

Seventy-three adult snails from Spain in spring, 114 from Spain in autumn and 96 from Sweden were selected randomly relative to phenotype to cover the full transect range within each country. DNA was extracted from foot tissue using a modified CTAB protocol (Panova et al. 2016). In-house, high-throughput genomic DNA library preparation and whole-genome sequencing (Illumina HiSeq4000, 150 bp, eight lanes, paired-end) were performed by The Oxford Genomics Centre with a target coverage of 3× based on the estimated genome size of 1.35 Gb (Westram et al. 2018).

Raw reads were trimmed with Trimmomatic v. 0.38 (Bolger et al. 2014), retaining reads with a minimum length of 70 bp after filtering and mapped to the *L. saxatilis* draft reference genome (Westram et al. 2018) using bwa mem v. 0.7.17 (H. Li 2013) and default settings. Positions with base or map quality lower than 20 were discarded using Samtools v. 1.7 (Li 2011a; Li et al. 2009). PCR duplicates and overlap between paired-end reads were removed with Picard v. 2.0.1 (http://broadinstitute.github.io/picard/) and bamUtil (Jun et al. 2015). As specimens were sequenced in paired-ends in eight lanes, resulting in 16 outputs for each snail, the files belonging to the same individual were sorted and merged with Samtools.

To explore patterns of diversity within Spain and Sweden, variants were called separately in each country using samtools mpileup and bcftools call v. 1.11 (Li 2011a) including only the longest contigs covering 90% of the reference genome. Allelic read counts for each SNP rather than genotype calls were retained due to the low coverage. The resulting vcf files were filtered to retain only biallelic SNPs, positions where at least 50% of individuals had a read depth between one and 18, irrespective of the reference or alternative allele and a minor allele frequency higher than 0.05 using vcftools v. 0.1.14 (Danecek et al. 2011) and vcffilterjs (Lindenbaum and Redon 2018). Additionally, we retained only positions on the *L. saxatilis* linkage map (Westram et al. 2018), i.e., within 1000 bp of a SNP that could be positioned on this map.

Two types of datasets were generated for each country: unthinned and thinned. The first one included all the SNPs resulting from the processing described above without any further filtering. The second one was obtained by retaining one random SNP in each genomic window of 1000 bp to reduce the impact of linkage disequilibrium and avoid overweighting regions with high SNP density. To achieve this aim, the reference genome was sliced into bins of 1000 bp and one SNP was randomly picked in each window using the R v. 4.0.3 (R Core Team 2020) package GenomicAlignments v. 1.26.0 (Lawrence et al. 2013), vcftools and custom scripts. This random SNP subsampling was repeated three times in each country to create a total of six random SNP subsets from the unthinned datasets.

To investigate the overall divergence between countries, variants were called and filtered jointly in Sweden and Spain, and a thinned dataset was generated using the procedures described above.

In all datasets (within and between countries, thinned and unthinned), the reference and alternative allele read depth was extracted from the vcf files and one random read per position and individual was subsampled using vcftools and custom scripts. As for the random SNP subsets, the random read subsampling was repeated three times to create replicates for each SNP dataset and subset. These 'single read' datasets (i.e., matrices with individuals and SNPs coded as 0 for the reference and 1 for the alternative allele) formed the basis of population genomic analysis, thereby avoiding the issues associated with calling genotypes from low-coverage data (Nielsen et al. 2011).

2.3 | Genomic Analyses

Overall, patterns of divergence within countries were explored in the thinned datasets through PCA and DAPC in the R packages adegenet (Jombart 2008; Jombart and Ahmed 2011) and factoextra v. 1.0.7 (https://github.com/kassambara/factoextra) treating individuals as haploid. If more than one genetic group was identified, the within-group PC1 scores among subsets were tested for normality using the Shapiro test (Shapiro and Wilk 1965). We compared group (if any) assignment of each individual between subsampled datasets and computed the correlation of the within-group PC1 scores using the Pearson or Spearman's coefficients, according to the data distribution, using the R package GGally v. 2.1.2 (https://ggobi.github.io/ggally). Loci with an allele frequency difference between groups (if any) > 0.5 were used to compute a Hybrid Index for each individual (mean over genotypes expressed as 1 for the allele more common in the 'Crab' environment and 0 for the allele more common in the 'Wave' environment). Individual ancestries were estimated through a maximum likelihood approach and cross-validation procedures using ADMIXTURE v. 1.3.0 (Alexander et al. 2009), PLINK v. 1.90b6.5 (Purcell et al. 2007) and custom scripts. We tested associations between genetic groups detected in Spain and environmental variables using Chi-squared or t-tests in R.

While the analyses described in the previous paragraph took advantage of the thinned datasets, the unthinned ones were used to compute global and per-locus F_{ST} (Weir and Cockerham 1984) between genetic groups (in Spain) or transect ends (in Sweden, defined as positions before 37 and after 92 m as in Koch et al. 2022), without imputation for missing values, using the R package hierfstat v. 0.5-7 (Goudet 2005). Global and per-locus F_{ST} were computed also between the Spanish Crab and Wave extremes, defined as transect positions before 20 and after 42 m, respectively, and excluding low-shore Crab individuals from the Wave group, in line with previous studies (Butlin et al. 2014; Morales et al. 2019; Westram et al. 2021). At a larger geographic scale, divergence between countries was explored using the joint thinned datasets through PCA and DAPC as described above.

Chromosomal inversions are known to contribute to ecotype differentiation in both Sweden and Spain (Faria, Chaube, et al. 2019; Koch et al. 2022; Morales et al. 2019; Westram et al. 2021, 2023). To investigate patterns of differentiation along the genome and identify additional chromosomal inversions in Spain that might or might not overlap with known ones, the unthinned datasets were analysed using two approaches. First, per locus F_{ST} values between genetic clusters were plotted along each linkage group (LG hereafter) to produce Manhattan plots using custom scripts in R. Genomic inversions differing in frequency between clusters are expected to generate blocks of higher differentiation compared to non-inverted regions (Le Moan et al. 2024; Mérot 2020). To test for differences in F_{ST} between inverted regions and collinear regions, we used a randomisation approach. For each LG, we computed the size of inversions in number of SNPs to control for recombination rate variation along the chromosome and a block of equal size was randomly positioned on the LG. Then, the difference between the average F_{ST} in that block and the average F_{ST} in the rest of the LG was computed. This procedure was repeated 1000 times and the observed F_{ST} difference between the inversion position and the rest of the LG was compared to the distribution of the permuted F_{ST} differences. If more than one inversion was present in a LG and the inversions overlapped, we considered them as one block in the permutation procedure. If the inversions did not overlap, two blocks were shuffled on the LG without being allowed to overlap and the F_{ST} difference was computed between the average of the two blocks and the rest of the LG. When the observed F_{ST} difference was higher than the 95th quantile of the distribution, we considered the F_{ST} difference between the inversions and the collinear regions to be significant. Second, we computed PCAs by map position using the same procedure illustrated above and custom scripts to extract the SNPs falling on each linkage map position. PC1 scores for each individual and map position were extracted and transformed by reversing the sign of the score when needed so that individuals belonging to the same genetic group always clustered on the same side of the PC1 axis. PC1 scores were then plotted along each LG and individuals were coloured according to their genetic group (Crab or Wave). The PCAs by map position were used to detect inversions without using prior knowledge regarding their positions in the genome.

However, in some cases, this approach failed to identify inversions in the positions of the original Swedish inversions, probably due to a lack of power. Therefore, additional PCAs were computed, in both Spain and Sweden independently, for each genomic region known to carry inversions in *L. saxatilis* and/or its sister species *L. arcana* (Reeve et al. 2023). We refer to these PCAs as 'PCAs by inversion'. Typically, a single chromosomal inversion would result in the presence of three distinct clusters on a PCA plot, with the two most distant groups comprising individuals that are homozygotes for one arrangement or the other and the cluster in between comprising individuals carrying both arrangements (Reeve et al. 2023). In the presence of overlapping inversions, the PCA plot displays a characteristic triangular pattern of six clusters with the three apical groups comprising homokaryotype individuals (Mérot 2020). An inversion was considered to be present when both the Manhattan plot and PCA by map position or by inversion displayed an inversion signal. Furthermore, to explore if the two ecotypes shared the same inversion arrangement in the two countries, loci that were present in both the Swedish and Spanish datasets were extracted and merged, values were centred and scaled together to preserve information about the geographical divergence within each inversion genotype, and individuals from Sweden were projected into the PCA space defined by the Spanish individuals using the suprow function in the *ade4* R package v. 1,7-18 (Dray and Dufour 2007), labelled projected PCA hereafter.

According to the patterns observed in both the independent and projected PCAs by inversion, these structural variants were classified into two categories: (i) regions showing a clear and shared inversion pattern in both Spain and Sweden and (ii) regions showing different patterns between countries or no obvious inversion signal. In the former cases, we computed arrangement frequencies in each country using the country-specific PCA for each single inversion. In both countries, the association between arrangements and genetic group (Spain) or end of the transect (Sweden) was tested using a Chi-squared test in the rstatix R package v. 0.7.2 (Kassambara 2023) and we used the projected PCAs to determine whether the same arrangement had a higher frequency in the same ecotype in both countries. To further explore the relationship between inversion genotype and shore position within the heterogeneous Spanish Crab ecotype, the correlation between the frequency of the arrangement most abundant in the Wave ecotype and shore position was tested in the Spanish Crab ecotype using a Kendall rank test in rstatix. For simple inversions, PC2 was associated with within-arrangement differentiation between the ecotypes. In some cases, homozygote individuals for the arrangement most abundant in the Wave ecotype were split into two distinct sub-clusters along PC2 corresponding to the two ecotypes. In most cases, those sub-clusters showed some overlap. We investigated the presence of gene flow between ecotypes within arrangement by plotting the PC2 scores against shore positions for individuals carrying the arrangement most abundant in the Wave ecotype, using the ggplot2 R package v. 3.4.4 (Wickham 2016).

To investigate the effect of chromosomal inversions on the observed patterns of divergence between ecotypes within countries, the genetic analyses described above were repeated excluding SNPs falling within known inversions (Faria, Chaube, et al. 2019; Koch et al. 2022; Reeve et al. 2023; Westram et al. 2021) from the original Swedish and Spanish thinned datasets using vcftools.

Consistency among the randomly sampled subsets of SNPs and reads was tested using Kendall's coefficient of concordance, *W*, computed using only the complete cases of the rankings with the R package DescTools v. 0.99.48 (Kendall 1948; Signorell 2023).

2.4 | Phenotypic Analyses

Parallel morphological differentiation of ecotypes in Sweden and Spain has been described previously (Butlin et al. 2014; Johannesson et al. 2010). Continuous variation in multiple phenotypic traits across the Swedish transect has also been described previously in genotyped individuals (Koch et al. 2021, 2022; Larsson et al. 2020; Westram et al. 2021). Here, we focused on quantitative phenotypic divergence in the Spanish transects, where the following traits were recorded in each snail: sex, wet weight, shell thickness (using NeoteckDTI Digital Dial Indicator Probe, 0.001 mm resolution at the widest point of aperture and average over three measures), shell ridging (presence/absence), shell striping (presence/absence) and boldness. To measure boldness, snails were disturbed to induce retraction and time until they emerged again (out time) and until they got back on the foot (crawl time) were recorded. This test was repeated three times for each snail and the average score for both out and crawl times between the three trials was used for subsequent analyses. Scores were attributed according to the out or crawl times in minutes, using the following categories for the two measures, respectively: $[0, <1]$, score $= 1$; $[0–1, 1–5] = 2$; $[1–5, 5–10] = 3$; $[5–10, 10–15] = 4$; $[10–15, >15] = 5$. Each trial was stopped after 15 min independently of the snail's response. Details of this method deviate slightly from previous studies (Koch et al. 2021) but result in a similar distinction between 'bold' (low score) and 'wary' (high score) behaviours.

Shell length and growth parameters were obtained from standardised pictures of each snail. These pictures were analysed using *ShellShaper* (Larsson et al. 2020) and aperture position parameters (r0_scaled = r0/shell_length, z0_scaled = z0/shell_length), aperture shape (extension factor c0/a0), aperture size (a0_scaled = a0/shell_length), the relative shell thickness (thickness/a0), height growth (log_gh), width growth (log_gw) and convexity (abs(log_gh-log_gw)) were computed as in previous studies (Koch et al. 2021; Larsson et al. 2020). For each phenotypic trait, we tested differences between the two genetic groups identified using the clustering analysis described above. Additionally, multivariate patterns were investigated through a PCA using the *prcomp* function from the R package *stats* v. 4.3.0 and the following scaled phenotypes: a0_scaled, r0_scaled, z0_scaled, log_gh, log_gw, relative thickness, weight, thickness and shell length. Finally, to investigate whether genetic groups and/or shore position had an effect on phenotypes, linear models were built using either phenotypic PCs or individual phenotypic variables as response variables.

To compare phenotypes between Sweden and Spain, we retrieved phenotypic data from Koch et al. (2022) for the 'CZA'sampling of the same transect in Ramsö (Sweden) a few years earlier (Westram et al. 2021). That dataset included the following variables: shell length, wet weight, aperture size (a0_scaled), aperture position (r0_scaled), aperture shape (c0/a0), height growth (gh), width growth (gw) and relative thickness. All these variables were scaled in each country and used

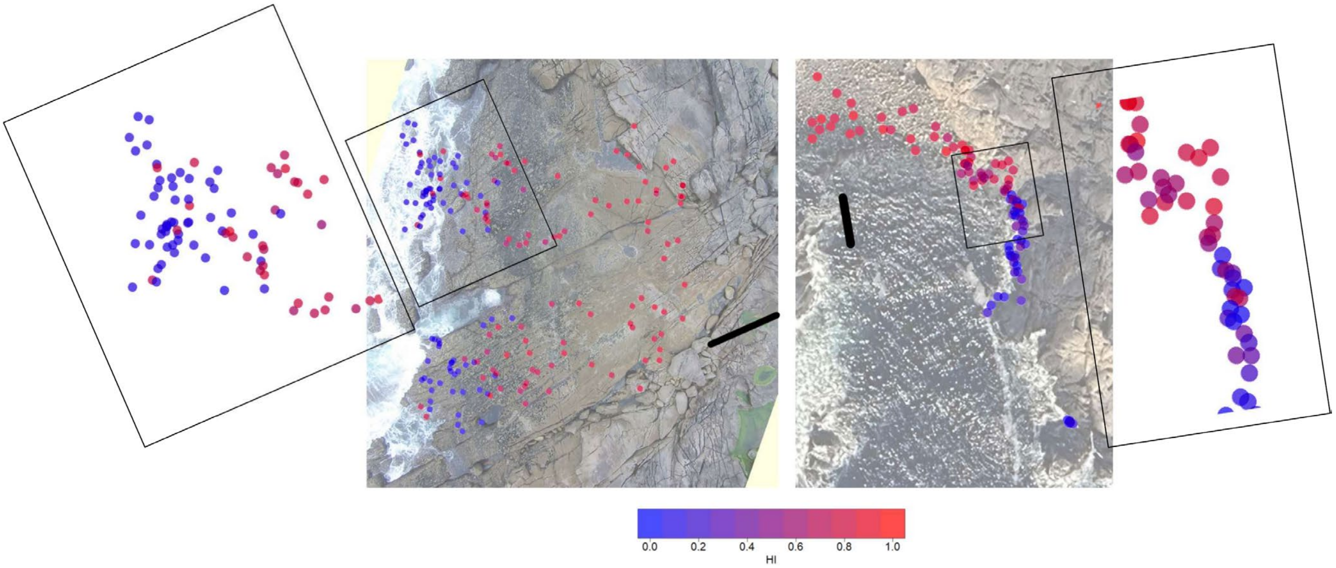


FIGURE 1 | Spatial distributions of ecotypes on the shore, along the sampling transects in Centinela (Spain, left panel) and Ramsö (Sweden, right panel). Each dot represents an individual and is coloured according to the Hybrid Index (HI), HI values closer to one describe the Crab ecotype (red) while the ones closer to zero denote the Wave (blue) ecotype. Scale (10 m) is represented by black bars. A zoom shows more detail for an area near the centre part of the transect in each location (black boxes). The centres of these plots are at N 42°4′38.06″, W 8°53′47.47″ (Centinela) and 58°49′27.8″ N, 11°03′45.3″ E (Ramsö) with North at the top of the figure.

in a PCA including all individuals from both CZA and Spain. In CZA, individuals found in the boulder habitat at least 15 m away from the boulder–rock transition were considered Crab ecotype, and individuals found in the rocky habitats at least 40 m away from the boulder–rock transition were considered Wave ecotype, following Koch et al. (2022). Average relative differences between ecotypes were computed as the difference between the Crab average and the Wave average divided by the Crab average for each variable both in Sweden and Spain, and bootstrap (10,000 iterations) confidence intervals were computed in R. Finally, to test for parallelism of phenotypic divergence, i.e., whether the vectors of relative differences between ecotypes in Spain and Sweden have a similar direction in a multi-dimensional phenotypic space, we used the TestOfAngle function from the GeometricMorphometricsMix v.0.08.7022 R package (Fruciano 2025). This approach uses the angleTest function from the Morpho v2.12 R package (Schlager 2017) that tests for the significance of the angle between two vectors taking dimensionality into account (Li 2011b; Klingenberg and Marugán-Lobón 2013). According to previous findings (Butlin et al. 2014), we predict that differences between ecotypes show similar trajectories (vectors) in Spain and Sweden, i.e., an angle close to zero is expected (De Lisle and Bolnick 2020).

3 | Results

3.1 | Distinct Patterns of Genetic Divergence in Sweden and Spain

We explored genetic divergence along the transects spanning the Crab-hybrid-Wave axis in Sweden (boulder field to rocky headland) and Spain (high shore to low shore) using low-coverage whole-genome resequencing data in 283 snails (Figure 1). The Spanish transects did not show seasonal patterns (Figure S1); thus, the samples from spring and autumn were merged in the subsequent steps. A total of 311,549 (unthinned datasets) and 21,250 (thinned datasets) SNPs were obtained in Sweden, while the Spanish unthinned and thinned data included 339,614 and 21,212 SNPs, respectively. The joint thinned datasets, including SNPs polymorphic both within and among countries used to investigate the overall divergence between Sweden and Spain, included 7577 SNPs. As our filters were quite stringent, sites had to have reads in more than 50% of all 283 individuals to be included in this joint dataset. This resulted in a lower number of loci but was not biased against divergent sites between countries. We first describe genome-wide analyses, including both collinear loci and chromosomal inversions. Thereafter, we present results without inversions.

In both countries, the genome-wide PCA reveals separation along PC1 between Crab and Wave (Figure S2). The observed variance explained by PC1 (~3% and ~7% of variation in Sweden and Spain, respectively) exceeds random expectation despite sampling individuals from a single species across a geographically restricted area (within a few times the lifetime dispersal distance, Figure S2). However, Sweden and Spain showed distinct patterns of genetic differentiation that were also reflected in Hybrid Index distributions (Figures 1, 2, S2). In Sweden, snails at the Crab and Wave ends of the transect were distinguished by hybrid index and PC1, but with a continuous range of intermediates distributed clinally along the transect so that cluster analysis identified only a single genetic group (Figures 2, S2). This pattern was consistent among the random SNP and read subsets (Table S1) and in line with previous studies (Westram et al. 2018, 2021). In contrast to the Swedish population, the Spanish snails did not show a clinal pattern along the transect but instead formed two genetically distinct groups: one more genetically variable and spanning almost all of the transect (here referred to as the Crab ecotype

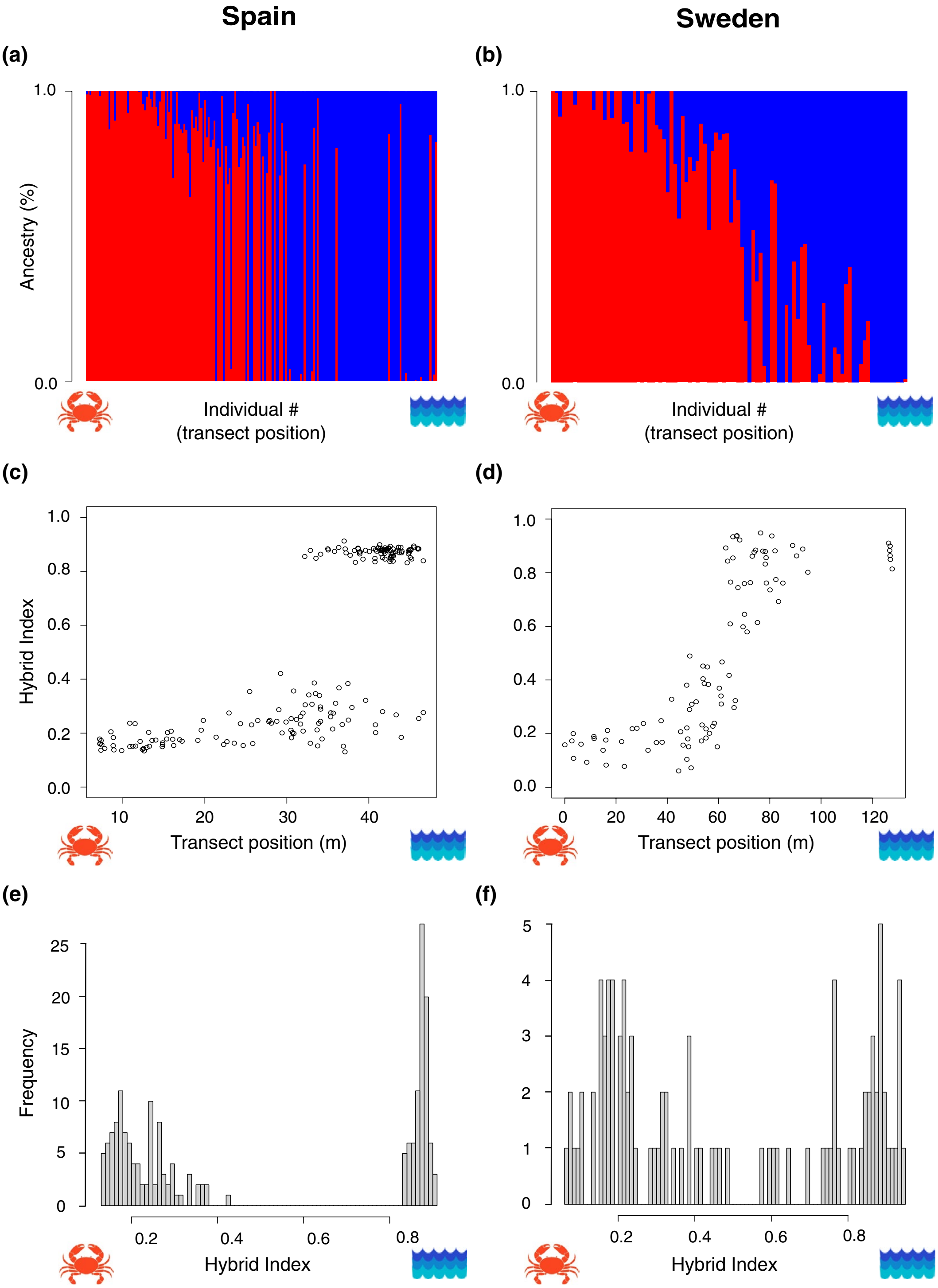


FIGURE 2 | Patterns of genomic divergence in Spain (a, c, e) and Sweden (b, d, f) as shown by admixture (a, b) and the Hybrid Index (c–f). Transect positions closer to zero correspond to the high shore (Spain) or boulder field (Sweden), where the Crab ecotype is typically found, while higher values represent the low shore (Spain) or rocky headland (Sweden), that typically hosts the Wave ecotype. Snails in the admixture plots (a, b) were ordered according to their position along transect. In Sweden, a sampling gap occurred at the transect positions 90–120 as seen in the plot of Hybrid Index along the shore (d, see also Figure 1) and a single genetic cluster was identified statistically but two groups were forced in the admixture plot (b) to facilitate comparisons with Spain. The Spanish Crab and Wave ecotype are coloured in red and blue, respectively. Please note that HI values of 0 and 1 are assigned to Crab and Wave randomly; in the shown subset, 0 denotes Crab while 1 represents Wave.

for consistency with previous studies, see Section 4), while the other was more homogeneous and mostly localised in the lower part of the shore (Wave ecotype). No intermediate genotype was observed between the two groups (Figures 2, S2–S4). However, some Spanish Crab individuals showed a hybrid index value close to 0.6, indicating admixture and significant contributions of alleles typical of the Wave ecotype in their genome, while individuals of the Wave ecotype had little or no evidence of Crab ancestry. None of the analysed Spanish snails had a Crab ancestry proportion between 0.1 and 0.6, or a hybrid index between 0.2 and 0.55, whereas such individuals were common in the Swedish transect (Figures 1, 2, Table S2). These results were consistent among the random SNP and read replicates (Figure S4, Table S1), including the assigned group membership, i.e., the same individuals were consistently classified as belonging to the Crab or Wave ecotype. Within-group PC1 scores were highly correlated among the random SNP and read subsets (range: 0.7999–0.9965, Figure S5). The two ecotypes in Spain exhibited different associations with habitat features (Figure S6). The Wave ecotype was more often associated with the goose barnacle *Pollicipes*. The *Mytilus* zone defined the overlap between the Crab and Wave ecotypes. Overall, the Wave ecotype was confined to the lower shore while the Crab ecotype was distributed over the whole vertical transect although at low densities in the lowest positions (Figures 1, 2, S6). At a larger spatial scale, the joint analyses consistently identified three genetic groups: Sweden and the two Spanish ecotypes. Most of the differentiation was explained by geographic separation between the two regions, followed by the Crab–Wave axis in Spain, and the divergence between the two Spanish ecotypes was larger than divergence in the whole Swedish transect (Figures S7 and S8).

3.2 | Differentiation Along the Genome and Chromosomal Inversions

In both Sweden and Spain, some genomic regions were far more differentiated than others (Table S3, Figures S9–S13). This is expected in young population pairs, where loci under selection tend to show greater divergence than the rest of the genome due to ongoing gene flow or a lack of time for differentiation to accumulate.

The genome-wide average differentiation between the two genetic groups was higher in Spain compared to the ends of the transect in Sweden (global F_{ST} values of 0.16 and 0.10, respectively, Table S3). The elevated genetic variability within the heterogeneous Spanish Crab group and the presence of individuals with a Wave genetic component likely account for the similar global F_{ST} values observed between ecotypes in the two countries, despite substantially higher divergence in Spain at the transect ends. In fact, F_{ST} values computed between the Spanish extremes (global $F_{ST}=0.19$) were higher than those between the two genetic groups (0.16) and higher than between extremes in Sweden (0.10, Table S3), in line with previous studies (Butlin et al. 2014; Morales et al. 2019; Westram et al. 2021). The average F_{ST} by LG was higher in Sweden than in Spain in LG8 and LG15; similar between the two countries for LG7, LG11, LG13 and LG16; and higher in Spain than in Sweden in all the other LGs (Table S3).

Some regions displayed higher F_{ST} in Sweden compared to Spain, while other regions presented high differentiation in both countries, both in the form of islands or narrow peaks of high differentiation relative to adjacent regions (Figures 3, S9, S10). A total of 53 contigs (4,711,312 bp) exhibited unusually high differentiation in both countries (average F_{ST} per contig >0.3 in both countries, Figure S11) accounting for approximately 43% (53/122) and 11% (53/476) of contigs with F_{ST} values exceeding 0.3 in Sweden and Spain, respectively. Highly differentiated genomic regions in both countries were located on LG1, LG2, LG3, LG5, LG6, LG8, LG9, LG12, LG14 and LG17, with LG6 and LG14 containing 26 and 10 of these contigs, respectively (Table S4). Forty-one (3,468,966 bp) of the 53 shared highly differentiated contigs were located in known inversions that were polymorphic between ecotypes in Sweden (Faria, Chaube, et al. 2019; Hearn et al. 2022; Westram et al. 2021) on LG6, LG9, LG12 and LG14.

To investigate the presence of inversions along the genome, we used Manhattan plots and PCA by map position or by inversion along each of the 17 LGs. Multiple chromosomal inversions were identified in both countries in the same positions across the genome. Inversion patterns were congruent among methods (F_{ST} and PCA by map positions) and generally more pronounced in Spain than in Sweden, with Manhattan plots showing blocks of high F_{ST} that were more differentiated from the background and PCA plots showing more distinct clusters (Figures 3, S9, S10, S12, S13). While the map-based approaches produced inconclusive patterns for LGC5.1, LGC6,1/2, LGC9.2, LGC10.1, LGC10.2, LGC11.1, LGC12.1/2/3/4 and LGC14.1/2, the PCAs by inversion confirmed the presence of these inversions in our dataset (Figures 4, S15).

Overall, a total of 19 inversions were polymorphic in our datasets, 14 shared between the two countries, 4 unique to Spain and 1 unique to Sweden (Table 1, Figures 4, S14). Compared to the set of previously known inversions (Reeve et al. 2023), our study did not detect any polymorphic rearrangements in LGC10, where two have been recorded elsewhere, and did not detect any new rearrangements. Inverted regions were generally significantly more differentiated than collinear regions, with similar patterns in Spain and Sweden (Figures S15, S16). However, in a few LGs, the differentiation gap between inverted and collinear regions was higher in Spain than in Sweden (Figures S9, S10 and S15, S16, Results S1: Differentiation between inverted and collinear regions).

A mix of simple and complex inversions was detected, with patterns not always consistent between Spain and Sweden (Figure 4, Table 1, Results S1: Inversion results). In Spain, arrangement frequencies were associated with ecotypes for all inversions, whereas this was true for only a subset of inversions in Sweden. In all except three cases (LGC9.1, LGC10.2 and LGC11.1, where arrangement frequency differences between ecotypes were not significant in Sweden) the arrangement with higher frequency in Wave was the same in both countries. In Spain, for many inversions, one arrangement was fixed in the Wave ecotype while arrangement frequencies varied with shore position in the Crab ecotype, with Crab individuals in the low shore more likely to carry the Wave arrangement (Figure S17). In some cases, the Crab and Wave ecotype homozygote individuals carrying the Wave arrangement formed distinct clusters in the PCA-by-inversion analyses along PC2. This suggests that, while both Crab and Wave individuals can carry the Wave arrangement, the content of this arrangement differs between ecotypes, indicating low or absent gene flow. However, for other inversions, Crab and Wave individuals were spread along PC2 and for LGC1.2, the arrangement found in Crab individuals was more Wave-like in the lower part of the shore (Figure S18), suggesting

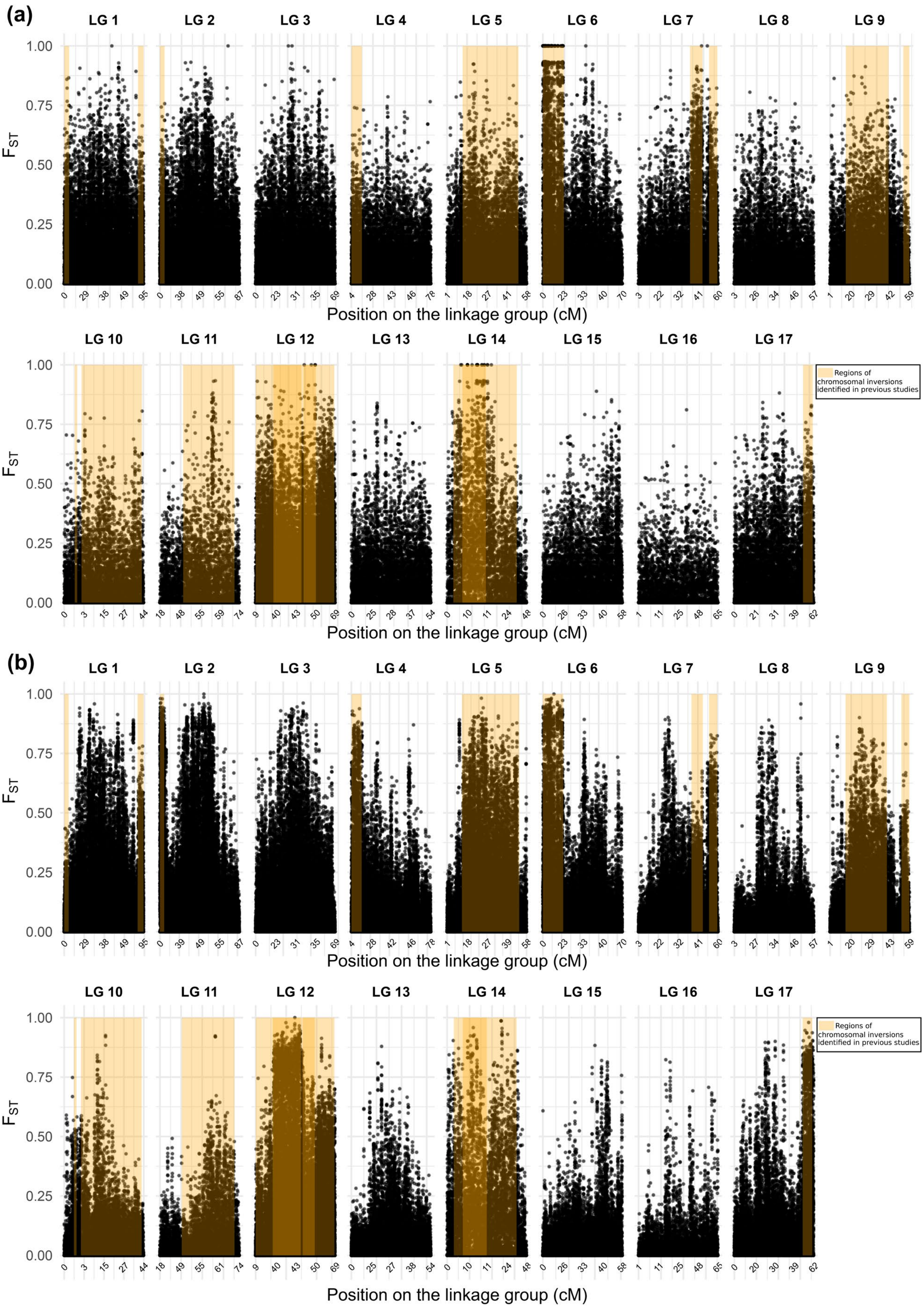


FIGURE 3 | Legend on next page.

FIGURE 3 | Manhattan plots of per locus F_{ST} values along the genome in Sweden (a) and Spain (b). The linkage groups and positions in centimorgans are indicated at the top and the bottom, respectively. Region of chromosomal inversions identified in previous studies are highlighted in orange. Overlapping rectangles indicate the presence of overlapping inversions.

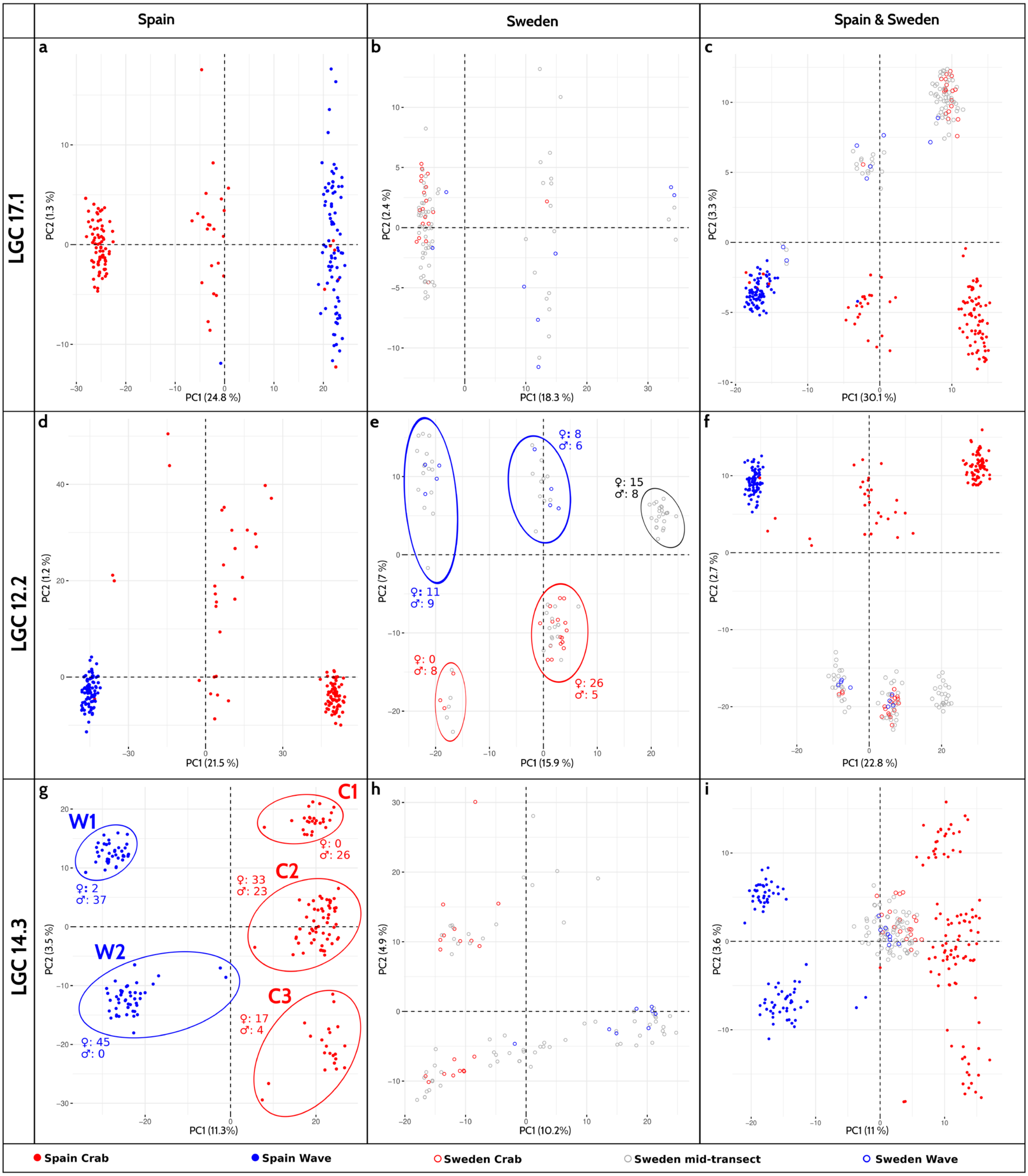
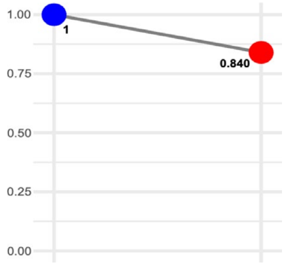
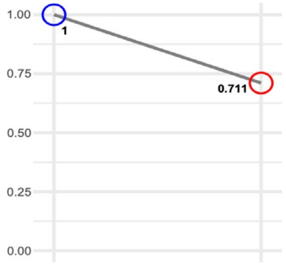
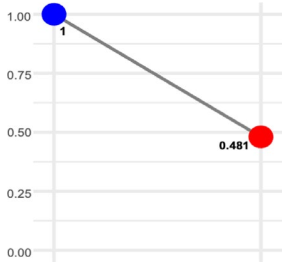
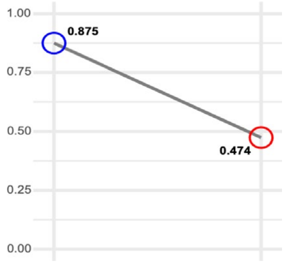
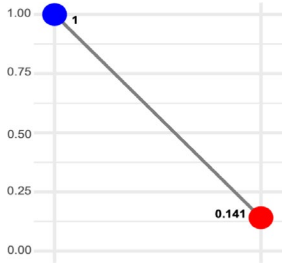
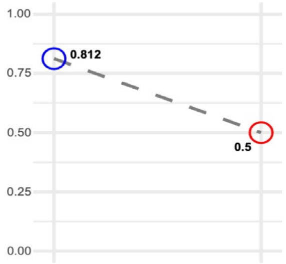


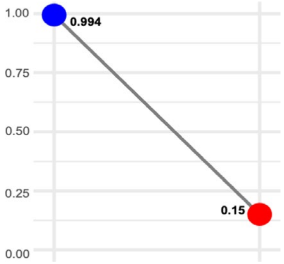
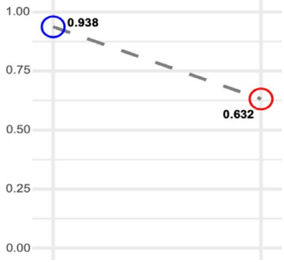
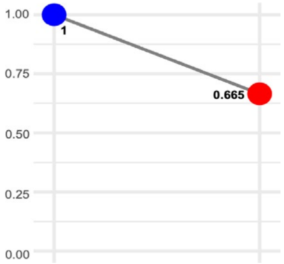
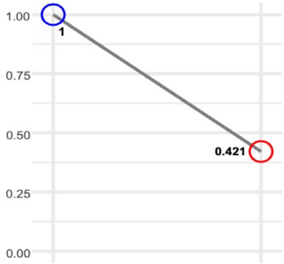
FIGURE 4 | Genetic PCAs by inverted region in Spain (a, d, g), Sweden (b, e, h), and both countries (suprow approach; c, f, i) showing examples of inversions with similar (simple: LGC17.1, a, b, c; or complex: LGC12.2; d, e, f) or contrasting (LGC14.3; g, h, i) patterns between countries. Filled dots represent the Spanish individuals, empty circles the Swedish ones. The Crab and Wave ecotype (Spain) or ends of the transect (Sweden) are denoted in red and blue, respectively, while grey circles represent individuals in or close to the Swedish contact zone (middle portion of the transect).

TABLE 1 | Inversions identified in Spain and Sweden through the PCA by inversion approach in this study.

	Spain					Sweden			
			Arrangement frequency					Arrangement frequency	
Inversion	**PCA pattern**	**Association between arrangement & ecotype (Chi-square)**	**Wave ecotype**	**Crab ecotype**	**Association between arrangement & shore position in Crab ecotype (Kendall test)**	**PCA pattern**	**Association between arrangement & ecotype (Chi-square)**	**Wave ecotype**	**Crab ecotype**
LGC1.1	Simple	***			*	Simple	*		
LGC1.2	Simple	***			NS	Simple	*		
LGC2.1	Simple	***			NS	Simple	NS		

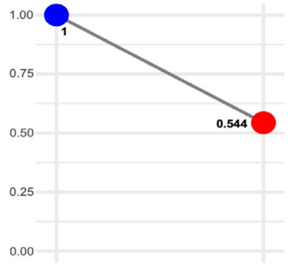
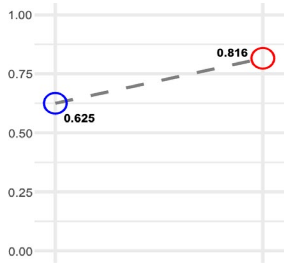
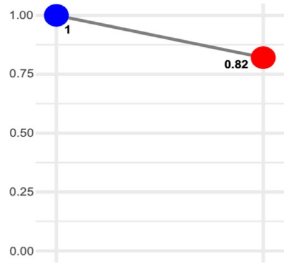
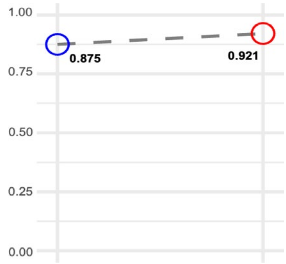
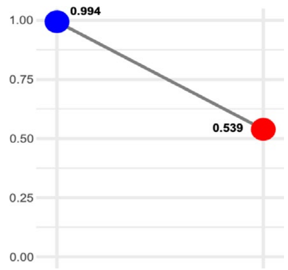
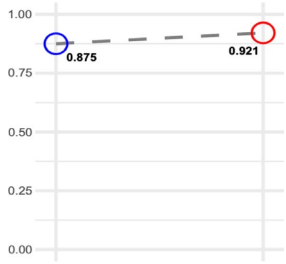
(Continues)

TABLE 1 | (Continued)

	Spain					Sweden			
			Arrangement frequency					Arrangement frequency	
Inversion	PCA pattern	Association between arrangement & ecotype (Chi-square)	Wave ecotype	Crab ecotype	Association between arrangement & shore position in Crab ecotype (Kendall test)	PCA pattern	Association between arrangement & ecotype (Chi-square)	Wave ecotype	Crab ecotype
LGC4.1	Simple	***			NS	Simple	NS		
LGC5.1	Complex	***	0.00595 (0.827)	0.903 (0.0971)	*	Two distinct clusters	—	—	—
LGC6.1/2	Simple	***	0	0.917	**	Complex	***	0 (0.688)	0.974 (0)
LGC7.1	Simple	***			*	Simple	***		
LGC7.2	Complex	***	1 (0)	0.277 (0.646)	**	Simple	**	0.938	0.5

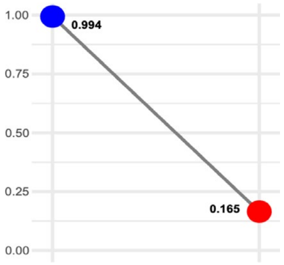
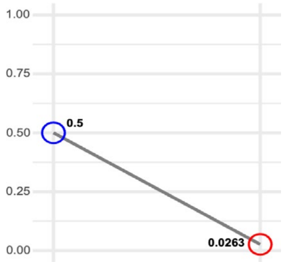
(Continues)

TABLE 1 | (Continued)

	Spain					Sweden			
			Arrangement frequency					Arrangement frequency	
Inversion	PCA pattern	Association between arrangement & ecotype (Chi-square)	Wave ecotype	Crab ecotype	Association between arrangement & shore position in Crab ecotype (Kendall test)	PCA pattern	Association between arrangement & ecotype (Chi-square)	Wave ecotype	Crab ecotype
LGC9.1	Simple	***			***	Simple	NS		
LGC9.2	Simple	***	1	0.495	***	No clear pattern	—	—	—
LGC10.2	Simple	***			***	Simple	NS		
LGC11.1	Simple	***			***	Simple	NS		
LGC12.1	Two clusters	—	—	—	—	Simple	**	0.75	1

(Continues)

TABLE 1 | (Continued)

	Spain					Sweden			
			Arrangement frequency					Arrangement frequency	
Inversion	**PCA pattern**	**Association between arrangement & ecotype (Chi-square)**	**Wave ecotype**	**Crab ecotype**	**Association between arrangement & shore position in Crab ecotype (Kendall test)**	**PCA pattern**	**Association between arrangement & ecotype (Chi-square)**	**Wave ecotype**	**Crab ecotype**
LGC12.2	Complex	***	1 (0)	0.0534 (0.835)	***	Complex	***	0.25 (0.75)	0.421 (0)
LGC12.3	Complex	***	1 (0)	0.0728 (0.835)	***	Complex	***	0.25 (0.75)	0.421 (0)
LGC12.4	Complex	***	1 (0)	0.348 (0.5891)	NS	Simple	***	0.938	0.342
LGC14.1/2	Complex	—	—	Top cluster: 17♀/ 4 ♂ Middle cluster: 33♀/ 23 ♂ Bottom cluster: 0♀/ 26 ♂	—	No clear pattern	—	—	—
LGC14.3	Complex	—	Top cluster: 2♀/ 37 ♂ Bottom cluster: 45♀/ 0 ♂	Top cluster: 0♀/26 ♂ Middle cluster: 33♀/ 23 ♂ Bottom cluster: 17♀/ 4 ♂	—	No clear pattern	—	—	—
LGC17.1	Simple	***			***	Simple	***		

Note: Frequencies of the same arrangement in clear inversion patterns identified in both countries (see main text and Figure S14 for further information on the interpretation of inversions in Spain and Sweden). In the miniplots, solid lines indicate statistically significant arrangement frequency differences between ecotypes, while dashed lines represent non-significant differences. When the projected PCAs did not allow identifying the same arrangement across countries, we reported the frequencies of the most abundant arrangement in each country. For complex inversions, with three arrangements present, the frequency of the next most abundant arrangement is given in parentheses. In the Spanish LG14, the number of male (♂) and female (♀) individuals in each ecotype is reported. Dashes denote untested cases due to unclear inversion signals. The statistical significance level is indicated using NS (p-value > 0.05), * (p-value < 0.05), ** (p-value < 0.01), *** (p-value < 0.001).

that gene flow might occur in this particular arrangement between the Crab and Wave ecotypes. In Sweden, arrangements for LGC12.2 were associated with sex in the Crab ecotype, as previously reported (Hearn et al. 2022), but this was not true in Spain. Instead, arrangements in LG14 showed associations with sex (Figure 4, Figure S14) suggesting that the sex-determining locus is on different chromosomes in the two countries. More detailed descriptions of these inversion patterns are provided in Supporting Information: Inversion results.

To investigate the contribution of chromosomal inversions to genetic divergence along transects, the genetic analyses illustrated in Figures 2 and S2–S5 described above were repeated using the thinned datasets and removing the SNPs in known inversions. In Sweden, clinal patterns remained consistent after removing the positions within known inversions (Figures S19, S20, Table S2). A similar trend was observed in Spain, where no substantial differences were detected between the patterns with and without inversions: both showed two distinct genetic groups without intermediate genotypes, and the down-shore variation was still present within the Crab ecotype (Figures 2, S2–S4, S19, S20). The Wave ecotype was less homogeneous, and the percentage of variance attributed to the divergence between the two genetic groups (Wave and Crab) in PC1 was slightly lower (5.5%) compared to the analyses with inversions (6.93%, Figures S2, S19). As in the analyses with inversions, results were consistent among the random subsets, which showed the same individual assignment to the Crab or Wave ecotype and a high correlation of the within-ecotype PC1 scores (Figure S21, Table S1). After removing inversions, genome-wide average F_{ST} decreased both in Spain and Sweden (Table S3). The genome-wide variability of F_{ST} (standard deviation of F_{ST} in the genome) was higher in Spain than in Sweden and decreased in both countries with the removal of the inversions (from 0.162 to 0.138 in Spain and from 0.137 to 0.121 in Sweden). It remained high in Spain and Sweden, indicating heterogeneity in the barrier to gene flow over and above the direct contribution of inversions. Given the widespread differentiation also within the analysed chromosomal rearrangements and strong effect of inversions, suggesting a major contribution of indirect selection due to linkage disequilibrium, we did not attempt functional annotation of outlier regions.

3.3 | Phenotypic Divergence

Analyses of individual traits in 185 Spanish snails revealed significant phenotypic differences between the two genetic groups. Some phenotypes could not be measured in all snails (see sample sizes in Figure S22). Overall, the Crab snails were bigger, heavier and possessed thicker shells with lower height and width growth, a higher aperture height, a smaller aperture size and higher size-independent relative thickness compared to Wave (Figure S22). Most of the Crab shells (93%) displayed ridges, while they were present only in a single Wave snail. In Sweden, ridging is observed in Crab, but it is much less pronounced compared to Spain and infrequently present in Wave (Castillo et al. 2023). Striped shells, a phenotype never present in Sweden, were observed in most snails except 11 individuals belonging to the Wave ecotype. Stripes were mostly black in the Crab snails, while they were both black and brown in Wave. Behavioural tests indicated that the Crab snails were more wary than the Wave ecotype (Figure S22). In fact, more than half of the Crab snails took more than ten minutes to come out of their shell (median out boldness score of 4.33) whereas less than five minutes (median out boldness score of 1.33) were needed for most of the Wave individuals (Figure S22). Using data from Koch et al. (2022) for the CZA transect, relative differences between average individual phenotypic traits in Crab and Wave varied between Spain and Sweden. Except for aperture shape and aperture position in Spain, relative differences between ecotypes were all different from zero. Wherever differences between ecotypes were found in both countries, they were in the same direction. While relative differences between average wet weight and shell length were similar between ecotypes in Sweden and Spain, there were greater and significant differences in average aperture size, aperture position, aperture shape, relative thickness, width growth and height growth between ecotypes in Sweden compared to Spain (Figure S23).

The multivariate analysis indicated a more continuous variation in phenotypic than genomic data in the Spanish samples, and partial overlap between the Crab and Wave ecotypes (Figure 5). The first axis of the PCA, accounting for around 51% of the observed variation, was highly correlated with shell length parameters, while the second axis, describing around 19% of variance, was highly correlated with the aperture position (r0 scaled and z0 scaled, Figure 5a). Overall, the two ecotypes were phenotypically different. However, one individual of Crab genotype showed phenotypic features typical of the Wave ecotype. In general, the Crab snails showed a down-shore phenotypic cline, as in the genomic analyses, individuals located closer to the lower shore exhibiting more Wave-like phenotypic characteristics (Figure 5b).

Linear models revealed that both the genetic group and transect position had a significant effect on the PC1 phenotype. However, a closer look at the patterns within groups indicated that shore position had a significant effect on the PC1 phenotype only within Crab (Figure S24, Tables S5 and S6). The PC2 phenotype was only influenced by the genetic group. The genetic group had a significant effect on every individual phenotypic trait except aperture shape, convexity and z0 scaled (influencing aperture position). The transect position had a significant effect on most individual phenotypic traits (Table S5).

The joint phenotypic PCA, including the common subset of traits from both the Spanish snails and the Swedish CZA dataset from Koch et al. (2022), also showed continuous phenotypic variation between individuals (Figure S25). Divergence between Crab and Wave ecotypes was very similar in direction in both countries but higher in Sweden than in Spain. Moreover, vectors of relative differences between ecotypes were significantly similar (angle = 15.75°, p-value $< 1.63\mathrm{e}{-05}$) indicating parallelism in phenotypic divergence between ecotypes among countries (Figure S25). Ecotype difference was mostly carried by the first axis, which accounted for around 66% of the total variation and was highly correlated with aperture size, aperture shape and height growth. Wave ecotype snails appeared more similar between countries than Crab ecotype snails, and the Crab ecotype in Spain was, on average, more ‘Wave-like’ than the Crab ecotype in Sweden.

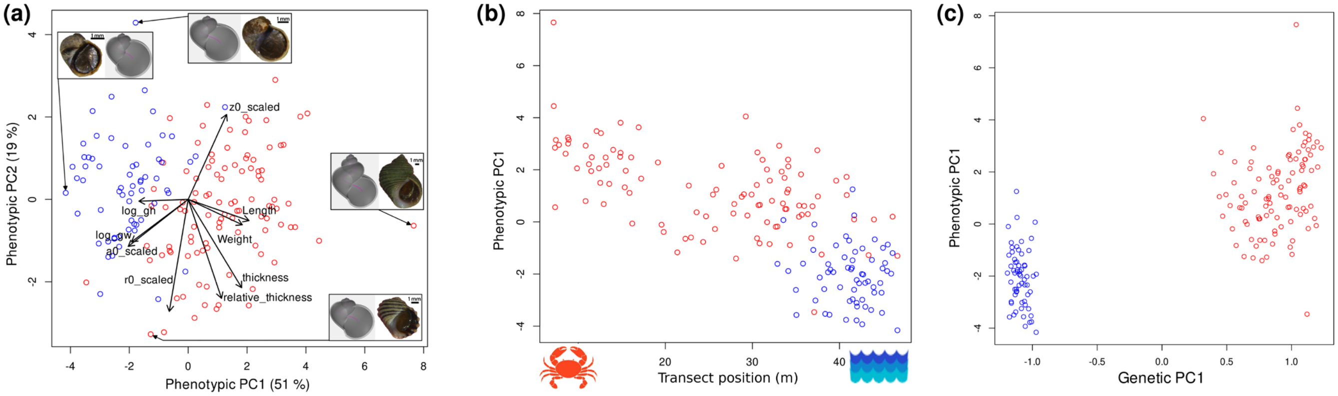


FIGURE 5 | Multivariate phenotypic divergence in Spain as shown by the first two PCA axes (a), PC1 along the transect (b), and its relation to genome-wide differentiation (c). Transect positions closer to zero correspond to the high shore while higher values represent the low shore. The Crab and Wave ecotype are coloured in red and blue, respectively.

4 | Discussion

Littorina saxatilis in Sweden and Spain illustrate the evolution of replicated reproductive isolation in two independent lineages of the same species, i.e., parallel speciation (Schluter and Nagel 1995). The two systems analysed here have a relatively recent common ancestry (50-278ky, Carvalho, Faria, et al. 2023; Panova et al. 2011) and share some genomic patterns of differentiation (Morales et al. 2019; Reeve et al. 2023). Hence, the speciation processes of both should be promoted or limited by roughly the same genomic potentials and constraints. Moreover, there is evidence that divergence into Crab and Wave ecotypes has evolved without extended periods of isolation, that is, mostly in the presence of gene flow in both countries (Butlin et al. 2014; Carvalho, Faria, et al. 2023). These natural replicates offer an outstanding opportunity to investigate the impact of different factors contributing to reproductive barriers in the two countries.

While much of the genetic and phenotypic structure of this Swedish contact zone was known from earlier studies (Westram et al. 2018, 2021), our understanding of the Spanish ecotypes was based on reduced representations of the genome and analysis of parental and hybrid groups identified from phenotypic traits, rather than genetically assigned individuals (Johannesson et al. 1993, 1995; Kess et al. 2018; Rolán-Alvarez et al. 1997). This could not reveal the full pattern of differentiation, either spatially or genetically, and so restricted understanding of gene flow. Our dense and, with respect to phenotype, random sampling in a Spanish contact zone in Centinela revealed two distinct genetic clusters and no F1 hybrid individuals or potential backcrosses to the Wave ecotype. A lack of admixture, consistent with our observations, was previously described in a SNP-based study with targeted sampling of phenotypically intermediate individuals (Kess et al. 2018). The data we provide here show a homogeneous Wave ecotype, very similar in phenotype to the Swedish Wave, and in Spain found exclusively in the lowest part of the shore. Conversely, the Spanish Crab ecotype, phenotypically resembling the Swedish Crab one, is mostly present in the high shore but is also distributed throughout the shore and overlaps spatially (but not genetically) with the Wave ecotype in the lowest zone. Previous work shows that the pattern observed in the Swedish site studied here is repeated across multiple sites (Westram et al. 2021). Although no other sites have been studied in a similar way in Spain, the results of Kess et al. (2018) for three other sites in North Western Spain strongly suggest the presence of distinct genetic groups with few intermediates and measurements of assortative mating between ecotypes at multiple locations have demonstrated isolation indices between 0.64 and 1.0, including Centinela that was one of the sites with the lowest value (Rolán-Alvarez et al. 1999). Our insights suggest that, while the two regions share many similarities, they also show significant differences and represent different points on the speciation continuum.

The systems have evolved parallel phenotypic divergence resulting in similar Crab and Wave ecotypes driven by strong divergent natural selection established by high predation from crabs in one environment and strong physical stress from wave action in the other micro-habitat (Janson 1983; Johannesson et al. 2010; Koch et al. 2022; Rolán-Alvarez et al. 1997). Snails from these two sites located in distant countries share a similar genomic architecture that shows high levels of differentiation between genetic groups or ends of transects (this study, Koch et al. 2022), including genomic loci located within chromosomal inversions (this study, Morales et al. 2019; Reeve et al. 2023). Moreover, we here show that most of these inversions present the same arrangement at higher frequency in the same ecotype (Crab/Wave) in both countries, indicating common sets of adaptive alleles within inversions that might have a shared origin. Inversions are key components in divergence and local adaptation, especially under gene flow (Barth et al. 2017; Faria, Johannesson, et al. 2019; Kirkpatrick and Barton 2006; Wellenreuther and Bernatchez 2018), and they host genes contributing to traits under selection in *L. saxatilis*, at least in Sweden (Koch et al. 2021, 2022). Shared genetic loci may have allowed for allele reuse in response to similar environmental pressures and kick-started the adaptive and diversifying processes in parallel, while reduced recombination has contributed to overcoming the homogenising effects of gene flow and aided the establishment of separate evolutionary paths in the face of permeable reproductive barriers. Furthermore, size-assortative mating seems to be present in both locations (Johannesson et al. 1995; Perini et al. 2020) and provides a potential additional barrier to gene flow, although, as discussed below, this might not be more than marginally important. Interestingly, inversions alone did not fully explain the two distinct genetic clusters observed in Spain, as is also true in Sweden (this study, Koch et al. 2022; Westram et al. 2021). In fact, the two groups in Spain remained clearly discrete after removing inversions, suggesting that gene flow between the Spanish Crab and Wave ecotypes is highly restricted throughout the genome. Yet, genome-wide divergence between ecotypes is low in both countries, potentially due to their recent origin or ongoing gene flow, albeit weak.

However, some country-specific patterns emerged in the two target sites. Our results support the presence of two inversions in these Spanish transects (LGC5.1 and LGC9.2), previously identified as 'new putative inversions' (Reeve et al. 2023) that were not reported in the Swedish site, hinting that the contribution of structural variants to evolution might differ between populations. Sex determination is strongly though not perfectly linked to inversions in both lineages, as previously reported in several organisms (Bachtrog 2013; Blumer et al. 2024; Peichel et al. 2020). Remarkably, our findings suggest that sex determination in Spain and Sweden did not involve the same loci, a rare example of intra-specific variation in the genetic determination of sex. In Sweden, LGC12.2 and LGC12.3 showed a strong association with sex in Crab (this study, Hearn et al. 2022; Koch et al. 2021). Conversely, LGC14.1/2 and LGC14.3 are related to sex in Centinela (Spain), with a particularly strong relationship displayed by Wave individuals in the latter inversion. Additionally, Wave in Sweden presents the highest abundance of the LGC9.1 inversion arrangement found in the Spanish Crab snails (this study, Morales et al. 2019). This locus is most likely implicated in local shore height-related adaptation to temperature and/or desiccation stress as Crab in Spain and Wave in Sweden are found higher on the shore than Wave in Spain and Crab in Sweden.

A key distinction between the two lineages is that reproductive isolation between Crab and Wave ecotypes is substantially stronger in Spain than in Sweden (this study, Kess and Boulding 2019; Morales et al. 2019). The overall level of genome-wide divergence between ends of transects or genetic groups is lower in the site in Sweden than in Spain. The Swedish contact zones show a continuous, unimodal pattern with frequent hybrids at the contact (this study, Westram et al. 2021); in contrast, the Spanish contact zone analysed here is genetically discrete, bimodal with no early hybrids and evidence of only weak, unidirectional gene flow. Chromosomal inversions showed more distinct and ecotype-associated differences in frequencies in Spain, with several arrangements fixed in the Wave ecotype, while most of them remain polymorphic at transect ends in Sweden (this study, Westram et al. 2021). In some Spanish inversions, distinct haplotypes in the Crab and Wave ecotypes were observed within the same arrangement ('sub-clusters' in PCAs), indicating very low levels of gene exchange between these ecotypes for a substantial period and/or additional divergence due to some form of selection or genetic drift. Secondary contact between the Spanish ecotypes could also contribute to their divergence, although the available evidence does not support extended periods of previous isolation (Carvalho, Faria, et al. 2023) and short vicariance events are unlikely to contribute to strong reproductive barriers. Taken together, these differences indicate that the Swedish barriers to gene flow are considerably weaker than those in Spain; the Spanish ecotypes are closer to completion of speciation than the Swedish ones.

The Spanish snails belonging to the genetic Crab group become phenotypically more Wave-like in a down-shore gradient while still retaining their genetic group identity (this study, Rolán-Alvarez et al. 1997). The two Spanish genetic groups analysed here are mostly distinguished by shell ridging and colour, especially in the overlap zone, as earlier described (Johannesson et al. 1993, 1995; Rolán-Alvarez et al. 1997, 1999). The Wave-like phenotypic appearance of the Crab individuals in the low shore is also accompanied by an increase in genetic similarity in collinear loci and in the frequency of inversion arrangements typically found in the Wave snails. This genetic and phenotypic convergence of the Crab and Wave ecotypes in the low shore is likely driven by microhabitat-related selection, supported by rare unidirectional weak gene flow from Wave to Crab, favouring some introgressed variants but working against others. Introgression would provide an important source of standing variation that can facilitate adaptation of Crab ecotype individuals to the Wave environment in the low shore. Alternatively, and possibly complementarily, the more Wave-like background in Crab in the low shore could have emerged from ancestral polymorphisms facilitating local adaptation. The absence of fixed arrangements in any inversion in the Spanish Crab (contrasting with high rates of fixation in the Spanish Wave, Figure S17) further lends support to the role of chromosomal inversions in the adaptation of this group over a much more heterogeneous environment than Wave in Spain (but not in Sweden where environmental heterogeneity is greater in the Wave habitat), which raises a concern with the conventional naming of the Spanish genetic group distributed from high to low shore as a 'Crab ecotype'.

Why do these two systems exhibit such different extents of reproductive isolation? We discuss five non-mutually exclusive potential explanations:

1. The Swedish ecotypes are much younger than the Spanish ones (~15 and ~57 ky, respectively, Carvalho, Faria, et al. 2023). The Spanish populations survived the Pleistocene glaciations in situ, whereas the Swedish populations are the result of post-glacial colonisation (Bosso et al. 2022; Doellman et al. 2011; Panova et al. 2011; Stankowski et al. 2023). If the divergence is limited by available genetic variation, a longer time of divergence would have increased the chances for the addition of mutations that increased local adaptation and hence, by involving more loci, the barrier strength.
2. The two contact zones are arranged differently in the shore space: a narrow and gradual environmental transition from boulder field to rocky headland in Sweden versus a large mosaic of barnacle and mussel patches in Spain. Individual life-time local cruising range is rather limited in this species (a few meters, Cruz et al. 2004; Erlandsson et al. 1998; Janson 1983; Westram et al. 2018). In the Swedish contacts, most snails will never come close to the other ecotype, while in Spain, the majority of snails live close to or in the contact zone. Selection for reinforcement of reproductive barriers would not take place except in the contact zone (Fernández-Meirama

et al. 2022), and thus in Sweden, only a small proportion of the population would be under selection for reinforcement of barriers. By contrast, in Spain, many snails would be involved, and reinforcement alleles would be more likely to be established.

3. Selection reinforcing prezygotic barriers to gene flow might be weak in Sweden. Preliminary data suggest that hybrid snails in Sweden are as fit in the intermediate habitat of the contact zone as pure ecotypes (Janson 1983; Sadedin et al. 2009). The survival of the Spanish hybrids has not been measured in this study as they are rare. However, fertility data of hybrids from laboratory cross-breeding of Spanish Crab and Wave individuals show that F1 hybrids have very high rates of embryo abortion (60%, Figure S26) compared to what is found in Swedish hybrids present in contact zones (12%, Johannesson et al. 2020; Sá-Pinto et al. 2013). Low fertility of Spanish hybrids would represent an additional component of reproductive isolation in itself and would favour selection reinforcing prezygotic barriers in Spain.
4. Size is a multiple-effect trait that may be conducive to coupling of reproductive barriers (Butlin and Smadja 2018; Smadja and Butlin 2011), and size-assortative mating is present in both Sweden and Spain (Johannesson et al. 1995; Perini et al. 2020). However, in Sweden, where snail size changes gradually across the contact zone, the barrier effect of the size-assortment becomes minor due to the extensive number of hybrids (Perini et al. 2020). This might also apply to Spain, though less strongly, due to the convergence in size in the low shore. However, in Spain, there is assortative mating caused by Crab and Wave being non-randomly distributed in the patchy environment and snails of divergent size overlapping in the mid-shore (Boulding et al. 2017; Rolán-Alvarez et al. 1999). Earlier experimental work based on phenotypic identification of ecotypes suggested microhabitat choice causing assortative mating in the Spanish contact zone (Cruz et al. 2004; Johannesson et al. 1995), but these experiments need to be repeated with individuals in which the mating pattern is confirmed by genotyping. In Sweden, such *habitat* choice would be difficult to achieve, as the contact zone is more a continuous environmental transition than patchy, such that most snails experience only one habitat type (Janson 1983; Westram et al. 2018).
5. The Swedish genetic and phenotypic transitions are formed by mainly one selection gradient running from crab selection in boulder fields to wave selection on rocky cliffs. A second axis of selection runs from high to low shore with strong gradients in temperature and desiccation (Sokolova et al. 2000), but in Sweden, this axis is perpendicular to the Crab–Wave selection axis. In Spain, the two selection gradients are parallel and act in synergy as both the temperature/desiccation axis and the Crab–Wave axis run from high shore to low shore. The distribution of inversion arrangements somewhat reflects the effects of the different selection axes in Sweden and Spain. In six of the inversions that showed differences between ecotypes in both Spain and Sweden, the same arrangement was more frequent in Wave than Crab in both countries (e.g., inversions on linkage groups LG6, LG12 and LG14). This is consistent with previous findings (Morales et al. 2019), and genes involved in shell traits that discriminate between Crab and Wave ecotypes are present in these inversions (Koch et al. 2021, 2022). However, one inversion (LGC9.1) showed contrasting arrangement frequencies between Crab and Wave ecotypes in the two countries, supporting earlier findings that some loci are instead under divergent selection for adaptation over the high–low shore environmental axis (Morales et al. 2019). Interestingly, we also found a clear association between arrangement frequency and position on the shore within the Spanish Crab ecotype in our study, further supporting the role of this inversion in adaptation to high–low shore selection gradients. Hence, in Spain, where the two selection gradients coincide, the total divergent selection between ecotypes and the proportion of the genome under divergent selection are likely to be inflated compared to in Sweden. This may result in a stronger barrier to gene flow in the Spanish system.

5 | Conclusions

Our findings underscore the value of high resolution and multidimensional data in natural replicated experiments within the same species in characterising the intricate nature of speciation. Thanks to a remarkable study system provided by a marine snail in which two parallel ecotypes have advanced to different points on the speciation continuum, we show that reproductive isolation can arise despite a history of gene flow but its progress towards completion revolves around complex ecological and/or evolutionary dynamics. Future studies will clarify the interplay and the relative contributions of extrinsic environmental factors (e.g., spatial configuration, divergent selection) and intrinsic components (e.g., time of divergence, chromosomal rearrangements, genetic incompatibilities, opportunities for reinforcement) in the different levels of reproductive isolation observed between the Swedish and Spanish systems. Differences and similarities between the *L. saxatilis*' Swedish and Spanish pairs together offer an outstanding opportunity to compare barriers to gene flow of diverse strengths without being confounded with differences that might have accumulated in comparisons among distinct taxa, and so contributing to unravelling the mystery of speciation.

Author Contributions

R.K.B., K.J., A.M.W., F.R. and A.D.J. designed the study. R.K.B., K.J., A.M.W., Z.B.Z., R.F., J.G. and E.R.-A. collected data. R.K.B., F.R., A.D.J. and R.F. analysed data. F.R., A.D.J., R.K.B. and K.J. drafted the manuscript. All the authors revised and agreed to the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Raw sequence reads are deposited in NCBI SRA (BioProject accession ID PRJNA781449). Phenotypic data (maturity, sex, wet weight, shell thickness, shell ridging, shell striping, shell length, shell growth parameters and boldness scores), scripts and all Supporting Information have been archived in Zenodo repositories (https://doi.org/10.5281/zenodo.15368131, https://doi.org/10.5281/zenodo.15450273, https://doi.org/10.5281/zenodo.15482727, respectively).

Benefits Generated: A research collaboration was developed with scientists from the countries where fieldwork was conducted; all collaborators are included as co-authors. Data and results of research have been shared with the provider communities and the broader scientific community through appropriate biological databases, as described above. The research addresses a priority concern regarding how biodiversity is generated and maintained. More broadly, our group is committed to international scientific partnerships, as well as institutional capacity building.

References

Alexander, D. H., J. Novembre, and K. Lange. 2009. "Fast Model-Based Estimation of Ancestry in Unrelated Individuals." *Genome Research* 19, no. 9: 1655–1664. https://doi.org/10.1101/gr.094052.109.

Bachtrog, D. 2013. "Y-Chromosome Evolution: Emerging Insights Into Processes of Y-Chromosome Degeneration." *Nature Reviews Genetics* 14, no. 2: 113–124. https://doi.org/10.1038/nrg3366.

Barrett, R. D. H., and D. Schluter. 2008. "Adaptation From Standing Genetic Variation." *Trends in Ecology & Evolution* 23, no. 1: 38–44. https://doi.org/10.1016/j.tree.2007.09.008.

Barth, J. M. I., P. R. Berg, P. R. Jonsson, et al. 2017. "Genome Architecture Enables Local Adaptation of Atlantic Cod Despite High Connectivity." *Molecular Ecology* 26, no. 17: 4452–4466. https://doi.org/10.1111/mec.14207.

Blumer, L. M., V. Burskaia, I. Artiushin, et al. 2024. "Introgression Dynamics of Sex-Linked Chromosomal Inversions Shape the Malawi Cichlid Adaptive Radiation." *bioRxiv*, 2024.07.28.605452. https://doi.org/10.1101/2024.07.28.605452.

Bolger, A. M., M. Lohse, and B. Usadel. 2014. "Trimmomatic: A Flexible Trimmer for Illumina Sequence Data." *Bioinformatics* 30, no. 15: 2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

Bolnick, D. I., A. K. Hund, P. Nosil, et al. 2023. "A Multivariate View of the Speciation Continuum." *Evolution* 77, no. 1: 318–328. https://doi.org/10.1093/evolut/qpac004.

Bosso, L., S. Smeraldo, D. Russo, et al. 2022. "The Rise and Fall of an Alien: Why the Successful Colonizer *Littorina saxatilis* Failed to Invade the Mediterranean Sea." *Biological Invasions* 24, no. 10: 3169–3187. https://doi.org/10.1007/s10530-022-02838-y.

Boulding, E. G., M. J. Rivas, N. González-Lavín, E. Rolán-Alvarez, and J. Galindo. 2017. "Size Selection by a Gape-Limited Predator of a Marine Snail: Insights Into Magic Traits for Speciation." *Ecology and Evolution* 7, no. 2: 674–688. https://doi.org/10.1002/ece3.2659.

Butlin, R. K., and R. Faria. 2024. "Local Adaptation and Reproductive Isolation: When Does Speciation Start?" *Evolutionary Journal of the Linnean Society* 3, no. 1: kzae003. https://doi.org/10.1093/evolinnean/kzae003.

Butlin, R. K., J. Galindo, and J. W. Grahame. 2008. "Sympatric, Parapatric or Allopatric: The Most Important Way to Classify Speciation?" *Philosophical Transactions of the Royal Society, B: Biological Sciences* 363, no. 1506: 2997–3007. https://doi.org/10.1098/rstb.2008.0076.

Butlin, R. K., M. Saura, G. Charrier, et al. 2014. "Parallel Evolution of Local Adaptation and Reproductive Isolation in the Face of Gene Flow." *Evolution* 68, no. 4: 935–949. https://doi.org/10.1111/evo.12329.

Butlin, R. K., M. R. Servedio, C. M. Smadja, et al. 2021. "Homage to Felsenstein 1981, or Why Are There So Few/Many Species?" *Evolution* 75, no. 5: 978–988. https://doi.org/10.1111/evo.14235.

Butlin, R. K., and C. M. Smadja. 2018. "Coupling, Reinforcement, and Speciation." *American Naturalist* 191, no. 2: 155–172. https://doi.org/10.1086/695136.

Carvalho, J., R. Faria, R. K. Butlin, and V. C. Sousa. 2023. "Parallel Evolution of Local Adaptation in *Littorina saxatilis* Inferred With Whole-Genome Pool-Seq Data." *bioRxiv*, 2023.09.18.558243. https://doi.org/10.1101/2023.09.18.558243.

Carvalho, J., H. E. Morales, R. Faria, R. K. Butlin, and V. C. Sousa. 2023. "Integrating Pool-Seq Uncertainties Into Demographic Inference." *Molecular Ecology Resources* 23, no. 7: 1737–1755. https://doi.org/10.1111/1755-0998.13834.

Castillo, D. G., N. Barton, R. Faria, et al. 2023. "Predicting Rapid Adaptation in Time From Adaptation in Space: A 30-Year Field Experiment in Marine Snails." *bioRxiv*, 2023.09.27.559715. https://doi.org/10.1101/2023.09.27.559715.

Coyne, J. A., and H. A. Orr. 1989. "Patterns of Speciation in Drosophila." *Evolution* 43, no. 2: 362–381.

Coyne, J. A., and H. A. Orr. 2004. *Speciation*. Sinauer Associates.

Cruz, R., C. Vilas, J. Mosquera, and C. García. 2004. "Relative Contribution of Dispersal and Natural Selection to the Maintenenace of a Hybrid Zone in *Littorina*." *Evolution* 58, no. 12: 2734–2746. https://doi.org/10.1554/03-644.

Danecek, P., A. Auton, G. Abecasis, et al. 2011. "The Variant Call Format and VCFtools." *Bioinformatics* 27, no. 15: 2156–2158. https://doi.org/10.1093/bioinformatics/btr330.

De Lisle, S. P., and D. I. Bolnick. 2020. "A Multivariate View of Parallel Evolution." *Evolution* 74, no. 7: 1466–1481. https://doi.org/10.1111/evo.14035.

Doellman, M. M., G. C. Trussell, J. W. Grahame, and S. V. Vollmer. 2011. "Phylogeographic Analysis Reveals a Deep Lineage Split Within North Atlantic *Littorina saxatilis*." *Proceedings of the Royal Society B: Biological Sciences* 278, no. 1722: 3175–3183. https://doi.org/10.1098/rspb.2011.0346.

Dopman, E. B., K. L. Shaw, M. R. Servedio, R. K. Butlin, and C. M. Smadja. 2024. "Coupling of Barriers to Gene Exchange: Causes and Consequences." *Cold Spring Harbor Perspectives in Biology* 16, no. 8: a041432. https://doi.org/10.1101/cshperspect.a041432.

Dray, S., and A. B. Dufour. 2007. "The ade4 Package: Implementing the Duality Diagram for Ecologists." *Journal of Statistical Software* 22, no. 4: 1–20. https://doi.org/10.18637/jss.v022.i04.

Erlandsson, J., E. Rolán-Alvarez, and K. Johannesson. 1998. "Migratory Differences Between Ecotypes of the Snail *Littorina saxatilis* on Galician Rocky Shores." *Evolutionary Ecology* 12, no. 8: 913–924. https://doi.org/10.1023/A:1006559904596.

Fang, B., P. Kemppainen, P. Momigliano, X. Feng, and J. Merilä. 2020. "On the Causes of Geographically Heterogeneous Parallel Evolution in Sticklebacks." *Nature Ecology & Evolution* 4, no. 8: 1105–1115. https://doi.org/10.1038/s41559-020-1222-6.

Fang, B., P. Kemppainen, P. Momigliano, and J. Merilä. 2021. "Population Structure Limits Parallel Evolution in Sticklebacks." *Molecular Biology and Evolution* 38, no. 10: 4205–4221. https://doi.org/10.1093/molbev/msab144.

Faria, R., P. Chaube, H. E. Morales, et al. 2019. "Multiple Chromosomal Rearrangements in a Hybrid Zone Between *Littorina saxatilis* Ecotypes." *Molecular Ecology* 28, no. 6: 1375–1393. https://doi.org/10.1111/mec.14972.

Faria, R., K. Johannesson, R. K. Butlin, and A. M. Westram. 2019. "Evolving Inversions." *Trends in Ecology & Evolution* 34, no. 3: 239–248. https://doi.org/10.1016/j.tree.2018.12.005.

Feder, J. L., S. P. Egan, and P. Nosil. 2012. "The Genomics of Speciation-With-Gene-Flow." *Trends in Genetics* 28, no. 7: 342–350. https://doi.org/10.1016/j.tig.2012.03.009.

Felsenstein, J. 1981. "Skepticism Towards Santa Rosalia, or Why Are There So Few Kinds of Animals?" *Evolution* 35, no. 1: 124–138. https://doi.org/10.2307/2407946.

Fernández-Meirama, M., E. Rolán-Alvarez, and A. Carvajal-Rodríguez. 2022. "A Simulation Study of the Ecological Speciation Conditions in the Galician Marine Snail *Littorina saxatilis*." *Frontiers in Genetics* 13: 680792. https://doi.org/10.3389/fgene.2022.680792.

Fruciano, C. 2025. "GeometricMorphometricsMix: Miscellaneous Functions Useful for Geometric Morphometrics_." R Package Version 0.0.8.7022, commit 778e586da7108cb82f7a2991114f3d2880c3293e. https://github.com/fruciano/GeometricMorphometricsMix.

Funk, D. J., P. Nosil, and W. J. Etges. 2006. "Ecological Divergence Exhibits Consistently Positive Associations With Reproductive Isolation Across Disparate Taxa." *Proceedings of the National Academy of Sciences* 103, no. 9: 3209–3213. https://doi.org/10.1073/pnas.0508653103.

Galindo, J., M. Martínez-Fernández, S. T. Rodríguez-Ramilo, and E. Rolán-Alvarez. 2013. "The Role of Local Ecology During Hybridization at the Initial Stages of Ecological Speciation in a Marine Snail." *Journal of Evolutionary Biology* 26, no. 7: 1472–1487. https://doi.org/10.1111/jeb.12152.

Goudet, J. 2005. "Hierfstat, a Package for r to Compute and Test Hierarchical F-Statistics." *Molecular Ecology Notes* 5, no. 1: 184–186. https://doi.org/10.1111/j.1471-8286.2004.00828.x.

Guerrero, R. F., C. D. Muir, S. Josway, and L. C. Moyle. 2017. "Pervasive Antagonistic Interactions Among Hybrid Incompatibility Loci." *PLoS Genetics* 13, no. 6: e1006817. https://doi.org/10.1371/journal.pgen.1006817.

Hearn, K. E., E. L. Koch, S. Stankowski, et al. 2022. "Differing Associations Between Sex Determination and Sex-Linked Inversions in Two Ecotypes of *Littorina saxatilis*." *Evolution Letters* 6, no. 5: 358–374. https://doi.org/10.1002/evl3.295.

Hewitt, G. M. 1989. "The Subdivision of Species by Hybrid Zones." In *Speciation and Its Consequences*, 85–110. Sinauer Associates.

Hollander, J., M. Lindegarth, and K. Johannesson. 2005. "Local Adaptation but Not Geographical Separation Promotes Assortative Mating in a Snail." *Animal Behaviour* 70, no. 5: 1209–1219. https://doi.org/10.1016/j.anbehav.2005.03.014.

Janson, K. 1983. "Selection and Migration in Two Distinct Phenotypes of *Littorina saxatilis* in Sweden." *Oecologia* 59, no. 1: 58–61. https://doi.org/10.1007/BF00388072.

Jiggins, C. D. 2017. *The Ecology and Evolution of Heliconius Butterflies*. Oxford University Press.

Johannesson, B., and K. Johannesson. 1996. "Population Differences in Behaviour and Morphology in the Snail *Littorina saxatilis*: Phenotypic Plasticity or Genetic Differentiation?" *Journal of Zoology* 240: 475–493.

Johannesson, K. 2016. "What Can Be Learnt From a Snail?" *Evolutionary Applications* 9, no. 1: 153–165. https://doi.org/10.1111/eva.12277.

Johannesson, K., R. K. Butlin, M. Panova, and A. M. Westram. 2017. "Mechanisms of Adaptive Divergence and Speciation in *Littorina saxatilis*: Integrating Knowledge From Ecology and Genetics With New Data Emerging From Genomic Studies." In *Population Genomics: Marine Organisms*, edited by M. F. Oleksiak and O. P. Rajora, 277–301. Springer International Publishing. https://doi.org/10.1007/13836_2017_6.

Johannesson, K., R. Faria, A. Le Moan, et al. 2024. "Diverse Pathways to Speciation Revealed by Marine Snails." *Trends in Genetics* 40, no. 4: 337–351. https://doi.org/10.1016/j.tig.2024.01.002.

Johannesson, K., J. N. Havenhand, P. R. Jonsson, M. Lindegarth, A. Sundin, and J. Hollander. 2008. "Male Discrimination of Female Mucous Trails Permits Assortative Mating in a Marine Snail Species." *Evolution* 62, no. 12: 3178–3184. https://doi.org/10.1111/j.1558-5646.2008.00510.x.

Johannesson, K., B. Johannesson, and E. Rolan-Alvarez. 1993. "Morphological Differentiation and Genetic Cohesiveness Over a Microenvironmental Gradient in the Marine Snail *Littorina saxatilis*." *Evolution* 47, no. 6: 1770–1787. https://doi.org/10.2307/2410220.

Johannesson, K., M. Panova, P. Kemppainen, C. André, E. Rolán-Alvarez, and R. K. Butlin. 2010. "Repeated Evolution of Reproductive Isolation in a Marine Snail: Unveiling Mechanisms of Speciation." *Philosophical Transactions of the Royal Society, B: Biological Sciences* 365, no. 1547: 1735–1747. https://doi.org/10.1098/rstb.2009.0256.

Johannesson, K., E. Rolán-Alvarez, and A. Ekendahl. 1995. "Incipient Reproductive Isolation Between Two Sympatric Morphs of the Intertidal Snail *Littorina saxatilis*." *Evolution* 49, no. 6: 1180–1190. https://doi.org/10.1111/j.1558-5646.1995.tb04445.x.

Johannesson, K., Z. Zagrodzka, R. Faria, A. Marie Westram, and R. K. Butlin. 2020. "Is Embryo Abortion a Post-Zygotic Barrier to Gene Flow Between *Littorina* Ecotypes?" *Journal of Evolutionary Biology* 33, no. 3: 342–351. https://doi.org/10.1111/jeb.13570.

Jombart, T. 2008. "Adegenet: A R Package for the Multivariate Analysis of Genetic Markers." *Bioinformatics* 24, no. 11: 1403–1405. https://doi.org/10.1093/bioinformatics/btn129.

Jombart, T., and I. Ahmed. 2011. "Adegenet 1.3-1: New Tools for the Analysis of Genome-Wide SNP Data." *Bioinformatics* 27, no. 21: 3070–3071. https://doi.org/10.1093/bioinformatics/btr521.

Jun, G., M. K. Wing, G. R. Abecasis, and H. M. Kang. 2015. "An Efficient and Scalable Analysis Framework for Variant Extraction and Refinement From Population Scale DNA Sequence Data." *Genome Research* 25: 918–925. https://doi.org/10.1101/gr.176552.114.

Kassambara, A. 2023. "*rstatix: Pipe-Friendly Framework for Basic Statistical Tests* (Version 0.7.2) [Computer Software]." https://rpkgs.datanovia.com/rstatix/.

Kendall, M. 1948. *Rank Correlation Methods*. Griffin.

Kess, T., and E. G. Boulding. 2019. "Genome-Wide Association Analyses Reveal Polygenic Genomic Architecture Underlying Divergent Shell Morphology in Spanish *Littorina saxatilis* Ecotypes." *Ecology and Evolution* 9, no. 17: 9427–9441. https://doi.org/10.1002/ece3.5378.

Kess, T., J. Galindo, and E. G. Boulding. 2018. "Genomic Divergence Between Spanish *Littorina saxatilis* Ecotypes Unravels Limited

Admixture and Extensive Parallelism Associated With Population History." *Ecology and Evolution* 8, no. 16: 8311–8327. https://doi.org/10.1002/ece3.4304.

Kirkpatrick, M., and N. Barton. 2006. "Chromosome Inversions, Local Adaptation and Speciation." *Genetics* 173, no. 1: 419–434. https://doi.org/10.1534/genetics.105.047985.

Klingenberg, C. P., and J. Marugán-Lobón. 2013. "Evolutionary Covariation in Geometric Morphometric Data: Analyzing Integration, Modularity, and Allometry in a Phylogenetic Context." *Systematic Biology* 62, no. 4: 591–610. https://doi.org/10.1093/sysbio/syt025.

Koch, E. L., H. E. Morales, J. Larsson, et al. 2021. "Genetic Variation for Adaptive Traits Is Associated With Polymorphic Inversions in *Littorina saxatilis*." *Evolution Letters* 5, no. 3: 196–213. https://doi.org/10.1002/evl3.227.

Koch, E. L., M. Ravinet, A. M. Westram, K. Johannesson, and R. K. Butlin. 2022. "Genetic Architecture of Repeated Phenotypic Divergence in *Littorina saxatilis* Ecotype Evolution." *Evolution* 76, no. 10: 2332–2346. https://doi.org/10.1111/evo.14602.

Kopp, M., M. R. Servedio, T. C. Mendelson, et al. 2018. "Mechanisms of Assortative Mating in Speciation With Gene Flow: Connecting Theory and Empirical Research." *American Naturalist* 191, no. 1: 1–20. https://doi.org/10.1086/694889.

Kulmuni, J., R. K. Butlin, K. Lucek, V. Savolainen, and A. M. Westram. 2020. "Towards the Completion of Speciation: The Evolution of Reproductive Isolation Beyond the First Barriers." *Philosophical Transactions of the Royal Society, B: Biological Sciences* 375, no. 1806: 20190528. https://doi.org/10.1098/rstb.2019.0528.

Larsson, J., A. M. Westram, S. Bengmark, T. Lundh, and R. K. Butlin. 2020. "A Developmentally Descriptive Method for Quantifying Shape in Gastropod Shells." *Journal of the Royal Society Interface* 17, no. 163: 20190721. https://doi.org/10.1098/rsif.2019.0721.

Lawrence, M., W. Huber, H. Pagès, et al. 2013. "Software for Computing and Annotating Genomic Ranges." *PLoS Computational Biology* 9, no. 8: e1003118. https://doi.org/10.1371/journal.pcbi.1003118.

Le Moan, A., S. Stankowski, M. Rafajlović, et al. 2024. "Coupling of Twelve Putative Chromosomal Inversions Maintains a Strong Barrier to Gene Flow Between Snail Ecotypes." *Evolution Letters* 8, no. 4: 575–586. https://doi.org/10.1093/evlett/qrae014.

Li, H. 2011a. "A Statistical Framework for SNP Calling, Mutation Discovery, Association Mapping and Population Genetical Parameter Estimation From Sequencing Data." *Bioinformatics* 27, no. 21: 2987–2993. https://doi.org/10.1093/bioinformatics/btr509.

Li, H. 2013. "Aligning Sequence Reads, Clone Sequences and Assembly Contigs With BWA-MEM." *arXiv: Genomics*. https://doi.org/10.48550/arXiv.1303.3997.

Li, H., B. Handsaker, A. Wysoker, et al. 2009. "The Sequence Alignment/Map Format and SAMtools." *Bioinformatics* 25, no. 16: 2078–2079. https://doi.org/10.1093/bioinformatics/btp352.

Li, S. 2011b. "Concise Formulas for the Area and Volume of a Hyperspherical Cap." *Asian Journal of Mathematic and Statistics* 4: 66–70. https://doi.org/10.3923/ajms.2011.66.70.

Lindenbaum, P., and R. Redon. 2018. "Bioalcidae, Samjs and Vcffilterjs: Object-Oriented Formatters and Filters for Bioinformatics Files." *Bioinformatics* 34, no. 7: 1224–1225. https://doi.org/10.1093/bioinformatics/btx734.

Matute, D. R., and B. S. Cooper. 2021. "Comparative Studies on Speciation: 30 Years Since Coyne and Orr." *Evolution* 75, no. 4: 764–778. https://doi.org/10.1111/evo.14181.

Mérot, C. 2020. "Making the Most of Population Genomic Data to Understand the Importance of Chromosomal Inversions for Adaptation and Speciation." *Molecular Ecology* 29, no. 14: 2513–2516. https://doi.org/10.1111/mec.15500.

Morales, H. E., R. Faria, K. Johannesson, et al. 2019. "Genomic Architecture of Parallel Ecological Divergence: Beyond a Single Environmental Contrast." *Science Advances* 5, no. 12: eaav9963. https://doi.org/10.1126/sciadv.aav9963.

Nielsen, R., J. S. Paul, A. Albrechtsen, and Y. S. Song. 2011. "Genotype and SNP Calling From Next-Generation Sequencing Data." *Nature Reviews Genetics* 12, no. 6: 443–451. https://doi.org/10.1038/nrg2986.

Panova, M., H. Aronsson, A. R. Cameron, et al. 2016. "DNA Extraction Protocols for Whole-Genome Sequencing in Marine Organisms." In *Marine Genomics, Methods in Molecular Biology*, edited by A. Blomberg, vol. 1452, 13–44. Springer.

Panova, M., A. M. H. Blakeslee, A. W. Miller, et al. 2011. "Glacial History of the North Atlantic Marine Snail, *Littorina saxatilis*, Inferred From Distribution of Mitochondrial DNA Lineages." *PLoS One* 6, no. 3: e17511. https://doi.org/10.1371/journal.pone.0017511.

Peichel, C. L., S. R. McCann, J. A. Ross, et al. 2020. "Assembly of the Threespine Stickleback Y Chromosome Reveals Convergent Signatures of Sex Chromosome Evolution." *Genome Biology* 21, no. 1: 177. https://doi.org/10.1186/s13059-020-02097-x.

Perini, S., M. Rafajlović, A. M. Westram, K. Johannesson, and R. K. Butlin. 2020. "Assortative Mating, Sexual Selection, and Their Consequences for Gene Flow in *Littorina*." *Evolution* 74, no. 7: 1482–1497. https://doi.org/10.1111/evo.14027.

Purcell, S., B. Neale, K. Todd-Brown, et al. 2007. "PLINK: A Tool Set for Whole-Genome Association and Population-Based Linkage Analyses." *American Journal of Human Genetics* 81, no. 3: 559–575. https://doi.org/10.1086/519795.

R Core Team. 2020. "*R: A Language and Environment for Statistical Computing* [Computer Software]." R Foundation for Statistical Computing. http://www.R-project.org/.

Reeve, J., R. K. Butlin, E. L. Koch, S. Stankowski, and R. Faria. 2023. "Chromosomal Inversion Polymorphisms Are Widespread Across the Species Ranges of Rough Periwinkles (*Littorina saxatilis* and *L. arcana*)." *Molecular Ecology* 33: e17160. https://doi.org/10.1111/mec.17160.

Reid, D. G. 1996. "*Systematics and Evolution of Littorina* (2009/05/11 ed., Vol. 76)." The Ray Society; Cambridge Core. https://www.cambridge.org/core/article/dg-reid-systematics-and-evolution-of-Littorina-x-463p-london-the-ray-society-1996-volume-164-of-the-series/C42A72C8BA749D9BEDA00F2D76BAE7CB.

Reid, K., M. A. Bell, and K. R. Veeramah. 2021. "Threespine Stickleback: A Model System for Evolutionary Genomics." *Annual Review of Genomics and Human Genetics* 22, no. 1: 357–383. https://doi.org/10.1146/annurev-genom-111720-081402.

Riesch, R., M. Muschick, D. Lindtke, et al. 2017. "Transitions Between Phases of Genomic Differentiation During Stick-Insect Speciation." *Nature Ecology & Evolution* 1, no. 4: 0082. https://doi.org/10.1038/s41559-017-0082.

Rolán-Alvarez, E., C. J. Austin, and E. G. Boulding. 2015. "The Contribution of the Genus Littorina to the Field of Evolutionary Ecology." In *Oceanography and Marine Biology*, 166–223. CRC Press.

Rolán-Alvarez, E., J. Erlandsson, K. Johannesson, and R. Cruz. 1999. "Mechanisms of Incomplete Prezygotic Reproductive Isolation in an Intertidal Snail: Testing Behavioural Models in Wild Populations." *Journal of Evolutionary Biology* 12, no. 5: 879–890. https://doi.org/10.1046/j.1420-9101.1999.00086.x.

Rolán-Alvarez, E., K. Johannesson, and J. Erlandsson. 1997. "The Maintenance of a Cline in the Marine Snail *Littorina saxatilis*: The Role of Home Site Advantage and Hybrid Fitness." *Evolution* 51, no. 6: 1838–1847. https://doi.org/10.2307/2411006.

Sadedin, S., J. Hollander, M. Panova, K. Johannesson, and S. Gavrilets. 2009. "Case Studies and Mathematical Models of Ecological Speciation.

3: Ecotype Formation in a Swedish Snail." *Molecular Ecology* 18, no. 19: 4006–4023. https://doi.org/10.1111/j.1365-294X.2009.04320.x.

Sá-Pinto, A., M. Martínez-Fernández, C. López-Fernández, et al. 2013. "Incipient Post-Zygotic Barrier in a Model System of Ecological Speciation With Gene Flow." *Journal of Evolutionary Biology* 26, no. 12: 2750–2756. https://doi.org/10.1111/jeb.12269.

Schlager, S. 2017. "Morpho and Rvcg – Shape Analysis in R." In *Statistical Shape and Deformation Analysis*, edited by G. Zheng, S. Li, and G. Szekely, 217–256. Academic Press.

Schluter, D., and L. M. Nagel. 1995. "Parallel Speciation by Natural Selection." *American Naturalist* 146, no. 2: 292–301. https://doi.org/10.1086/285799.

Seehausen, O., and C. E. Wagner. 2014. "Speciation in Freshwater Fishes." *Annual Review of Ecology, Evolution, and Systematics* 45, no. 1: 621–651. https://doi.org/10.1146/annurev-ecolsys-120213-091818.

Servedio, M. R., G. S. V. Doorn, M. Kopp, A. M. Frame, and P. Nosil. 2011. "Magic Traits in Speciation: 'Magic' but Not Rare?" *Trends in Ecology & Evolution* 26, no. 8: 389–397. https://doi.org/10.1016/j.tree.2011.04.005.

Servedio, M. R., and M. A. F. Noor. 2003. "The Role of Reinforcement in Speciation: Theory and Data." *Annual Review of Ecology, Evolution, and Systematics* 34, no. 1: 339–364. https://doi.org/10.1146/annurev.ecolsys.34.011802.132412.

Shapiro, S. S., and M. B. Wilk. 1965. "An Analysis of Variance Test for Normality (Complete Samples)†." *Biometrika* 52, no. 3–4: 591–611. https://doi.org/10.1093/biomet/52.3-4.591.

Signorell, A. 2023. "*DescTools: Tools for Descriptive Statistics* (Version 0.99.48) [Computer Software]." https://cran.r-project.org/package=DescTools.

Smadja, C. M., and R. K. Butlin. 2011. "A Framework for Comparing Processes of Speciation in the Presence of Gene Flow." *Molecular Ecology* 20, no. 24: 5123–5140. https://doi.org/10.1111/j.1365-294X.2011.05350.x.

Sokolova, I. M., A. I. Granovitch, V. J. Berger, and K. Johannesson. 2000. "Intraspecific Physiological Variability of the Gastropod *Littorina saxatilis* Related to the Vertical Shore Gradient in the White and North Seas." *Marine Biology* 137, no. 2: 297–308. https://doi.org/10.1007/s002270000343.

Stankowski, S., and M. Ravinet. 2021. "Defining the Speciation Continuum." *Evolution* 75, no. 6: 1256–1273. https://doi.org/10.1111/evo.14215.

Stankowski, S., Z. B. Zagrodzka, J. Galindo, et al. 2023. "Whole-Genome Phylogeography of the Intertidal Snail *Littorina saxatilis*." *Evolutionary Journal of the Linnean Society* 2, no. 1: kzad002. https://doi.org/10.1093/evolinnean/kzad002.

Weir, B. S., and C. C. Cockerham. 1984. "Estimating F-Statistics for the Analysis of Population Structure." *Evolution* 38, no. 6: 1358–1370. https://doi.org/10.2307/2408641.

Wellenreuther, M., and L. Bernatchez. 2018. "Eco-Evolutionary Genomics of Chromosomal Inversions." *Trends in Ecology & Evolution* 33, no. 6: 427–440. https://doi.org/10.1016/j.tree.2018.04.002.

Westram, A. M., R. Faria, K. Johannesson, and R. Butlin. 2021. "Using Replicate Hybrid Zones to Understand the Genomic Basis of Adaptive Divergence." *Molecular Ecology* 30, no. 15: 3797–3814. https://doi.org/10.1111/mec.15861.

Westram, A. M., H. E. Morales, K. Johannesson, R. Butlin, and R. Faria. 2023. "Understanding the Adaptive Role of Chromosomal Inversions Across Large Geographical Scales: The Potential of Pool-Seq Data." *bioRxiv*, 2023.08.20.553987. https://doi.org/10.1101/2023.08.20.553987.

Westram, A. M., M. Rafajlović, P. Chaube, et al. 2018. "Clines on the Seashore: The Genomic Architecture Underlying Rapid Divergence in the Face of Gene Flow." *Evolution Letters* 2, no. 4: 297–309. https://doi.org/10.1002/evl3.74.

Westram, A. M., S. Stankowski, P. Surendranadh, and N. Barton. 2022. "What Is Reproductive Isolation?" *Journal of Evolutionary Biology* 35, no. 9: 1143–1164. https://doi.org/10.1111/jeb.14005.

Wickham, H. 2016. *Ggplot2*. Springer. https://link.springer.com/book/10.1007/978-0-387-98141-3.

Yukilevich, R. 2021. "Reproductive Character Displacement Drives Diversification of Male Courtship Songs in Drosophila." *American Naturalist* 197, no. 6: 690–707. https://doi.org/10.1086/714046.

Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Appendix S1.**