

1 Understanding the adaptive role of chromosomal  
2 inversions across large geographical scales: The  
3 potential of pool-seq data

4 Short title: Chromosomal inversions in adaptation

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## 20 *Abstract*

21 A growing body of research shows that chromosomal inversions, where each arrangement is associated  
22 with a certain environment and maintains a set of adaptive alleles, make an important contribution to local  
23 adaptation. However, inversions often remain unexplored across large geographical scales. It is unclear  
24 whether inversions contribute to adaptation across species ranges, which environmental factors affect  
25 arrangement frequencies, and whether the adaptive content of the same arrangement varies between  
26 locations. Here, we discuss ideas of how allele frequency data (e.g. pool-sequencing data) can be used to  
27 learn about inversions in a simple and cost-effective way. If populations connected by migration differ in  
28 arrangement frequency, plotting their SNP allele frequencies against each other will reveal a parallelogram  
29 whose corners reflect arrangement frequencies. We demonstrate the usefulness of this approach in locally-  
30 adapted populations of the intertidal snail *Littorina saxatilis* (Olivi). For twelve inversions, we estimate  
31 arrangement frequencies in 20 populations across the European species range. While roughly half of the  
32 inversions contribute to adaptation to the well-studied contrast between wave-exposed and crab-infested  
33 habitats, the other half likely contribute to adaptation to shore height. We also find evidence for  
34 geographical variation in arrangement content, suggesting variation in adaptive role.

## 35 *Introduction*

36 Local adaptation and speciation rely on the evolution of genetic differences between populations. How can  
37 such differences arise and be maintained when divergence happens in the face of gene flow, with  
38 recombination potentially breaking up favorable allele combinations and homogenizing allele frequencies  
39 (Felsenstein 1981)? Theory predicts that genetic architectures that group locally adaptive alleles in the  
40 genome or that reduce the negative effects of recombination in other ways will be favored (Smadja and  
41 Butlin 2011). Such architectures include chromosomal inversions (Kirkpatrick and Barton 2006;  
42 Charlesworth and Barton 2018). Kirkpatrick & Barton's (2006) model relies on the fact that recombination  
43 between the standard and the inverted arrangement in heterokaryotypic individuals (i.e., those with one  
44 standard and one inverted arrangement) is largely prevented, so that large "blocks" of alleles are protected  
45 from recombination. If the two arrangements contain multiple alleles adapted to alternative environments,  
46 inversions are therefore favored under divergent adaptation with gene flow.

47 Recent empirical genomics work supports these theoretical predictions. For example, cod populations in  
48 fjords are likely to be adapted to that particular environment via a chromosomal rearrangement containing  
49 alleles beneficial in low salinities (Barth et al. 2017). Adaptive differences between annual and perennial  
50 ecotypes of *Mimulus* monkeyflowers can be traced to a chromosomal inversion (Twyford and Friedman  
51 2015), and inversions are associated with marine-freshwater divergence in stickleback fish (Jones et al.  
52 2012).

53 While the role of inversions in local adaptation is becoming clearer, it is less well understood how this plays  
54 out on large geographical scales. Many taxa have wide geographical distributions, where similar  
55 environmental contrasts can occur repeatedly, but where selective factors can also be combined differently  
56 in different geographical locations. Then, the question is whether the same arrangements are repeatedly  
57 involved in adaptation and whether they are consistently associated with specific environmental factors.  
58 Inversions containing multiple co-adapted alleles might represent efficient "transport vehicles" between  
59 locations with similar environments (Westram et al. 2022). On the other hand, because of recombination

60 restriction, arrangement allelic content is somewhat inflexible, and difficulties in generating the “right”  
61 allelic combinations might prevent their repeated use when multiple environmental factors vary in complex  
62 environments (Roesti et al. 2022).

63 Some studies have found evidence for the re-use of inversions in adaptation across multiple locations: In  
64 *Drosophila subobscura*, the same inversions show repeated large-scale latitudinal clines on different  
65 continents and are associated with temperature adaptation (Kapun et al. 2014); in honeybees, the same  
66 inversions are repeatedly associated with altitude adaptation in different mountain regions (Christmas et  
67 al. 2019); and in seaweed flies, inversions show similar environmental associations in Europe and North  
68 America (Mérot et al. 2018). However, studies dissecting the adaptive role of inversions on large  
69 geographical scales are still relatively rare, especially in non-model species.

70 One advantage of gathering data on inversions across large geographical scales is that this might allow to  
71 disentangle different potential selective factors. Locally, multiple potential selective factors are often  
72 associated in space, e.g., different climate variables and biotic factors such as interacting species. In  
73 geographically distant sites, environmental factors might be associated in different ways, making it possible  
74 to identify those that are statistically associated with arrangement frequencies.

75 A directly related question is how arrangement content varies across space. Large inversions can contain  
76 large numbers of functional loci, and, within the same arrangement (e.g., the inverted arrangement), the  
77 genotypes at these loci may differ between geographical locations. The same arrangement could therefore  
78 also contribute to adaptation in different ways. For example, take the hypothetical example of a plant  
79 species adapted to different altitudes. If the same inversion contributes to altitude adaptation in two  
80 locations, the causal SNPs are not necessarily the same. The same inversion, if containing different adaptive  
81 alleles, could even contribute to altitude adaptation in one location and to herbivore adaptation in another.  
82 In other words, inversions are a useful evolutionary “toolbox” for local adaptation which, could be “filled”  
83 with different contents of adaptive alleles (i.e., different tools), depending on locally available genetic  
84 variation and local selection pressures.

86

87 Studying the adaptive role of inversions on large geographical scales therefore requires information about  
88 arrangement frequencies as well as information about SNP allele frequencies within arrangements for  
89 multiple locations. Recent studies have used individual next-generation sequencing data for inversion  
90 detection and genotyping via elevated linkage disequilibrium, caused by the reduction of recombination  
91 between arrangements (Kemppainen et al. 2015; Faria et al. 2019a; Huang et al. 2020; Mérot 2020).  
92 Individual sequencing data also provides information on inversion content. However, when including many  
93 locations this approach incurs high costs. In other population genetics contexts, pool-sequencing data,  
94 where multiple individuals from the same population are sequenced together without individual barcoding  
95 (Schlötterer et al. 2014), represents a cost-effective alternative to individual sequencing. This type of data  
96 provides population-level SNP allele frequency estimates but no individual genotypes and, therefore,  
97 cannot be used in the LD-based approaches mentioned above.  $F_{ST}$  scans with pool-seq data can provide  
98 indirect evidence for inversions under divergent selection via the presence of large blocks of elevated  $F_{ST}$   
99 between populations (e.g. Gould et al. 2017; Morales et al. 2019). However, to our knowledge, pool-seq  
100 data alone could so far not be used to determine arrangement frequencies, because the association  
101 between  $F_{ST}$  and arrangement frequencies is not straightforward. Determining arrangement frequencies in  
102 pool-seq data has therefore required prior knowledge about arrangement-diagnostic SNPs (i.e. SNPs that  
103 are fixed different between the two arrangements) (Kapun et al. 2014).

104 In this paper, we illustrate the use of pool-seq data for studying inversions across large geographical scales.  
105 We show that some LD information is retained in pool-seq data (or SNP allele frequency data from other  
106 sources), beyond the short-range LD within sequencing reads, and can be used to detect inversions *de-*  
107 *novo*, determine population arrangement frequencies, and study within-arrangement polymorphisms (i.e.,  
108 SNPs that vary between copies of the same arrangement). In the first part of the paper, we explain our  
109 approach conceptually. It relies on standard plots of allele frequency data; we aim at emphasizing the  
110 simplicity of applying this approach and therefore do not provide a specific data analysis pipeline or  
111 software. Our approach requires, at a minimum, SNP allele frequency data from two diverging populations

112 connected by gene flow and becomes especially useful when extended to multiple population pairs from  
113 the same taxon. We then use these ideas to test the role of inversions in local adaptation in the rough  
114 periwinkle *Littorina saxatilis* (Olivier), a marine snail, across large geographical scales, demonstrating the role  
115 of inversions in parallel evolution and showing within-arrangement differences between locations.

## 116 *Rationale*

### 117 *Determining arrangement frequencies from SNP allele frequency data*

118 When two populations diverge with gene flow, a plot of SNP allele frequencies in population 1 against  
119 frequencies in population 2 typically shows a correlation, with points scattered around the identity line  
120 (where  $x=y$ ) (Fig. 1A). When the two populations differ in arrangement frequency for an inversion, allele  
121 frequencies will still be correlated (because within each arrangement, gene flow and recombination  
122 between populations take place), but the correlation lines will be shifted from the identity line due to the  
123 different arrangement frequencies. This generates a “parallelogram” pattern that can be used to detect  
124 inversions and determine arrangement frequencies. We explain this pattern in the following.

125 We assume two populations connected by levels of gene flow high enough to generate correlations in allele  
126 frequencies in the background genome (e.g., Fig. 1A). We focus on a genomic region with a simple inversion  
127 polymorphism; i.e., two alternative arrangements, labelled A and B. It is irrelevant here which arrangement  
128 is the ancestral and which is the inverted arrangement. Arrangements A and B segregate at potentially  
129 different frequencies in the two populations. Thus, there are four arrangement frequencies,  $p_{A,1}$ ,  $p_{A,2}$ ,  
130  $p_{B,1} = 1 - p_{A,1}$ , and  $p_{B,2} = 1 - p_{A,2}$ .

131 Unless the inversion is very young, some SNPs will be (nearly) differentially fixed between the two  
132 arrangements (Fig. 1B, green and orange). These SNPs provide direct estimates of arrangement frequencies  
133 (Fig. 1C). If we assume allele frequencies are given in relation to a reference genome that sequencing data  
134 were mapped to, there are two sets of such diagnostic SNPs: SNPs where the A arrangement is fixed for the  
135 reference allele (“A-diagnostic SNPs”, green), and SNPs where the B arrangement is fixed for the reference

136 allele ("B-diagnostic SNPs", orange). The frequencies of A-diagnostic SNPs in population 1 and population 2  
137 provide estimates of the A arrangement frequencies in these two populations ( $p_{A,1}$  and  $p_{A,2}$ ), and thus also  
138 of  $p_{B,1} = 1 - p_{A,1}$  and  $p_{B,2} = 1 - p_{A,2}$ . Frequencies of B-diagnostic SNPs can be used in a similar way.  
139 These arrangement-diagnostic SNPs are also the strongest candidate loci for local adaptation – especially if  
140 they are diagnostic across multiple locations – as they achieve the highest differentiation between  
141 environments when the inversion is divergently selected.

142 SNPs polymorphic in both arrangements require recent exchange between arrangements, or repeated  
143 mutation, and are assumed to be rare. However, some SNPs will be fixed in arrangement A but  
144 polymorphic in arrangement B, or vice versa (Fig. 1B). These SNPs are not directly indicative of arrangement  
145 frequencies. However, they can only obtain a restricted range of allele frequencies. To give an example, we  
146 here focus on SNPs where arrangement A is fixed for the reference allele. We assume arrangement A has a  
147 frequency of  $p_{A,1} = 0.3$  in population 1 (Fig. 1B). In this case, all these SNPs must have a minimum allele  
148 frequency of 0.3 in population 1; each SNPs has an allele frequency between 0.3 and 1, depending on its  
149 frequency within arrangement B (one example shown in Fig. 1B, bold grey SNP). The same argument  
150 applies to population 2. Because there is gene flow between populations within arrangements, allele  
151 frequencies will be correlated between populations; but the correlation line will start at the minimum allele  
152 frequencies ( $p_{A,1}, p_{A,2}$ ) (Fig. 1C, dark grey circles).

153 Mathematically, the situation for a SNP fixed for the reference allele in arrangement A, but polymorphic in  
154 arrangement B, is as follows. The expected frequency for SNP  $i$  is

155  $p_{i,1} = p_{A,1} + p_{B,1}p_{i,B}$  in population 1 and

156  $p_{i,2} = p_{A,2} + p_{B,2}p_{i,B}$  in population 2,

157 where  $p_{i,B}$  is the frequency of the reference allele for this SNP within arrangement B. If gene flow is high,  
158  $p_{i,B}$  is expected to be roughly the same in both populations, and thus the two equations can be combined  
159 to express the correlation between the allele frequencies in the two populations:

160  $p_{i,2} = \frac{p_{B,2}}{p_{B,1}}p_{i,1} + p_{A,2} - \frac{p_{B,2}}{p_{B,1}}p_{A,1}$ .

161 This equation confirms that the line relating the allele frequencies in population 1 and 2 goes through  
162  $(p_{A,1}, p_{A,2})$  and through  $(1,1)$ , as explained in the example above, and shows that the slope is  $\frac{p_{B,2}}{p_{B,1}}$  (Fig. 1C,  
163 dark grey circles).

164 Analogous lines will form for the remaining three sets of SNPs (fixed for the alternative allele in A, variable  
165 in B; fixed for the reference allele in B, variable in A; fixed for the alternative allele in B, variable in A) (Fig.  
166 1B,C). Together, these four lines form a parallelogram, whose corners are at  $(0,0)$ ,  $(1,1)$ ,  $(p_{A,1}, p_{A,2})$  and  
167  $(p_{B,1}, p_{B,2})$  (Fig. 1C).

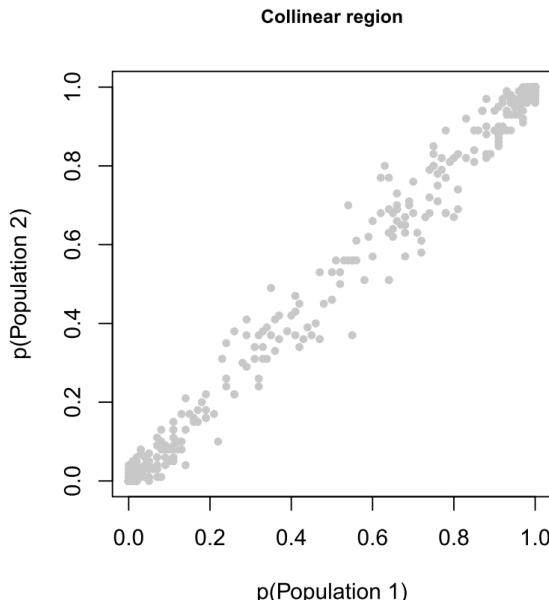
168 If arrangement frequencies do not differ between populations, no parallelogram will form as it “collapses”  
169 into a line (Fig. 1D). However, if arrangement-diagnostic SNPs are known, arrangement frequencies can still  
170 be determined (colored points in Fig. 1D).

171 The equations shown here assume no noise in the data and that within-arrangement frequencies are the  
172 same in population 1 and 2, leading to a perfect parallelogram. In empirical data, statistical sampling  
173 (included in Fig. 1) generates noise when sampling individuals from the population and pooling potentially  
174 slightly unequal amounts of DNA. In addition, due to evolutionary sampling (i.e. genetic drift), allele  
175 frequencies within arrangements will differ between populations, partly depending on the level of gene  
176 flow. Importantly, both sources of noise will generate scatter around the parallelogram but should not  
177 generate a bias or distortion of the shape.

178 Some rare SNPs may violate our assumptions: SNPs could be variable in both arrangement A and  
179 arrangement B, and therefore be located inside the parallelogram. SNPs could also show allele frequency  
180 differences within arrangements between populations (potentially due to selection) and could thus in  
181 principle be located anywhere in the plot. However, these issues are expected to affect a minority of the  
182 SNPs in an inversion region, and they will again not distort the overall parallelogram shape.

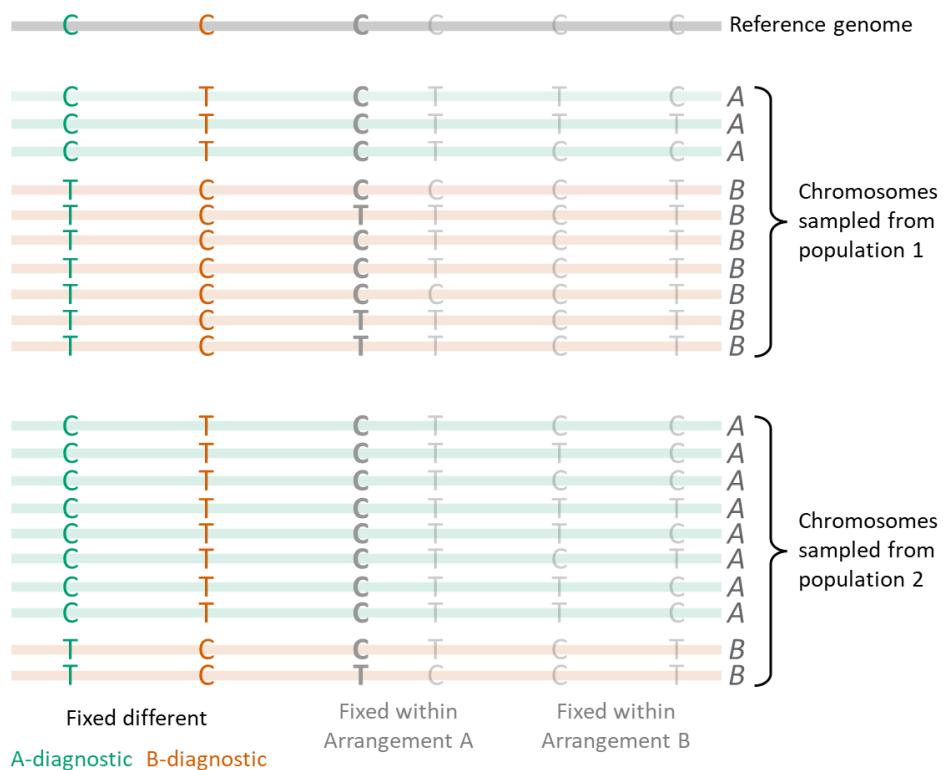
183 Thus, we expect that in many systems it should be possible to detect inversions by the presence of  
184 parallelograms in allele frequency plots, and to estimate arrangement frequencies by reading off the allele  
185 frequencies at the parallelogram corners.

186 A)



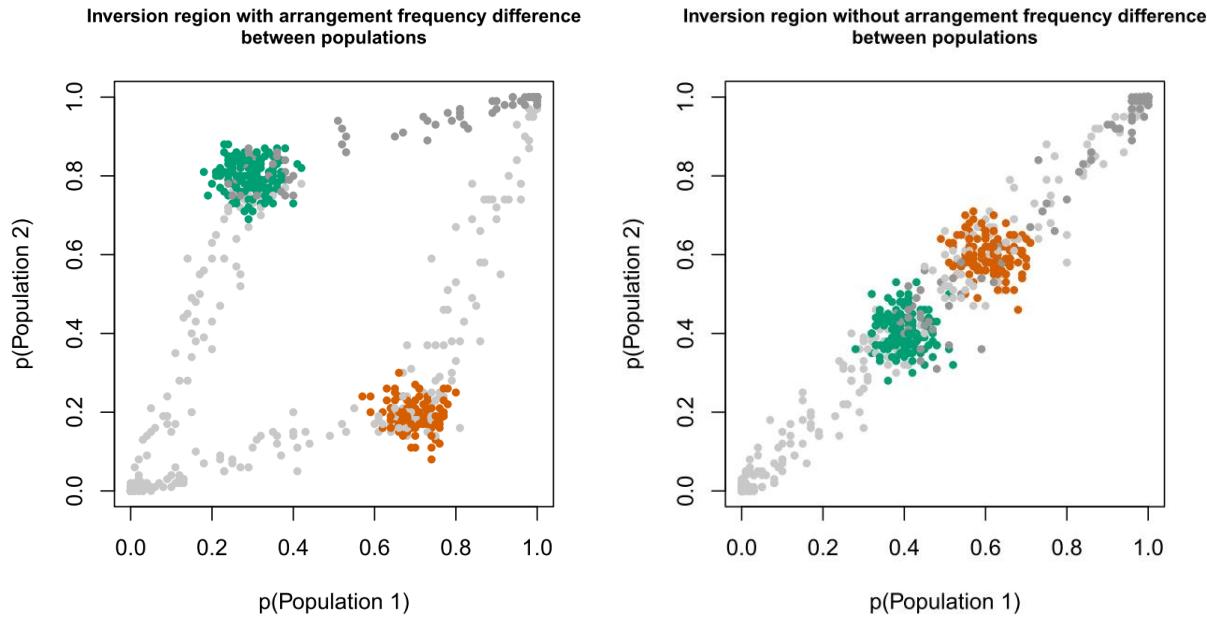
187

188 B)



189

190 C) D)



191

192 Fig. 1: The emergence of parallelograms in allele frequency plots. A) Simulation of a collinear region. B)  
193 Illustration of a chromosomal region with inversion polymorphism (light green horizontal line: arrangement  
194 A; light orange line: arrangement B; shown in the same orientation for illustrative purposes). The reference  
195 genome (top) is assumed to have a C allele at each SNP position for simplicity. SNPs fixed different between  
196 arrangements might have the reference allele either in arrangement A (A-diagnostic SNPs, green) or  
197 arrangement B (B-diagnostic SNPs, orange). Alternatively, SNPs can be fixed in only one arrangement  
198 (grey). C) Simulation with  $p_{A,1} = 0.3$  and  $p_{A,2} = 0.8$  (as in panel B). D) Simulation with  $p_{A,1} = p_{A,2} = 0.4$ . In  
199 C) and D), SNPs considered arrangement-diagnostic are indicated in green (A-diagnostic) and orange (B-  
200 diagnostic). The SNP type variable in one arrangement discussed in the main text is shown in bold in B) and  
201 by darker grey points in C) and D). Details about simulations in Supplementary Text.

## 202 *Study system*

203 To use the above ideas for studying inversion evolution on large geographical scales, we focused on a  
204 system with known inversion polymorphism, the marine snail *Littorina saxatilis*, that shows parallel  
205 divergent adaptation in many geographical locations. *L. saxatilis* occurs on rocky shores across Europe and  
206 North America. The two most well-studied ecotypes of *L. saxatilis* are the “Crab” ecotype - large, thick-

207 shelled, wary snails adapted to crab predation - and the “Wave” ecotype - small, thin-shelled, bold snails  
208 adapted to wave exposure. Both ecotypes occur across Europe and have probably evolved repeatedly at  
209 least in Spain, Sweden, and the UK (Butlin et al. 2014). The ecotypes are locally connected by gene flow and  
210 form hybrid zones where their habitats adjoin (Westram et al. 2021).

211 In addition, *L. saxatilis* also shows local adaptation to different shore levels (Johannesson et al. 1995). High  
212 shore levels are associated with increased air and sun exposure compared to low shores, and thus high-  
213 shore snails experience more extreme temperature and higher desiccation risk. Importantly, Crab-Wave  
214 and Low-High adaptation are not independent (explained in detail in Supplementary Text; Morales et al.  
215 2019), as high shore levels correspond to Crab habitat and low shore levels correspond to Wave habitat in  
216 Spain, while this pattern is reversed in the UK and France (Fig. 2). In Sweden, both Crab and Wave occur  
217 across both shore levels (Fig. 2).

218 Previous work on the Swedish west coast has shown that the Crab and Wave ecotype differ in arrangement  
219 frequency for multiple chromosomal inversions (Faria et al. 2019, Westram et al. 2021) and that divergent  
220 traits map to these inversions (Koch et al. 2021). A study based on pool-seq from Spain, France, the UK, and  
221 Sweden found that multiple genomic regions corresponding to polymorphic inversions in Sweden show  
222 high levels of differentiation (e.g., elevated  $F_{ST}$ ) between Crab and Wave ecotypes in other European  
223 locations as well (Morales et al. 2019). Some of the inversion regions also showed elevated differentiation  
224 between the low and high shore (Morales et al. 2019).

225 While these results suggest a role of inversions in parallel evolution in *L. saxatilis*, three important aspects  
226 are not well understood. 1) The arrangement frequencies are only known for four Swedish locations and  
227 could thus far not be determined for other European locations due to the use of pool-seq data. In  
228 particular, for locations where  $F_{ST}$  is low between Crab and Wave or Low and High pool-sequencing  
229 samples, it is unclear whether this is due to the absence of inversion polymorphism or due to similar  
230 arrangement frequencies in both populations. 2) As a consequence, the association of different  
231 arrangements with the different selective axes could be addressed only indirectly (Morales et al. 2019) and

232 needs confirmation with actual arrangement frequency data. 3) It is unclear whether the adaptive alleles  
233 inside arrangements differ between geographical locations.

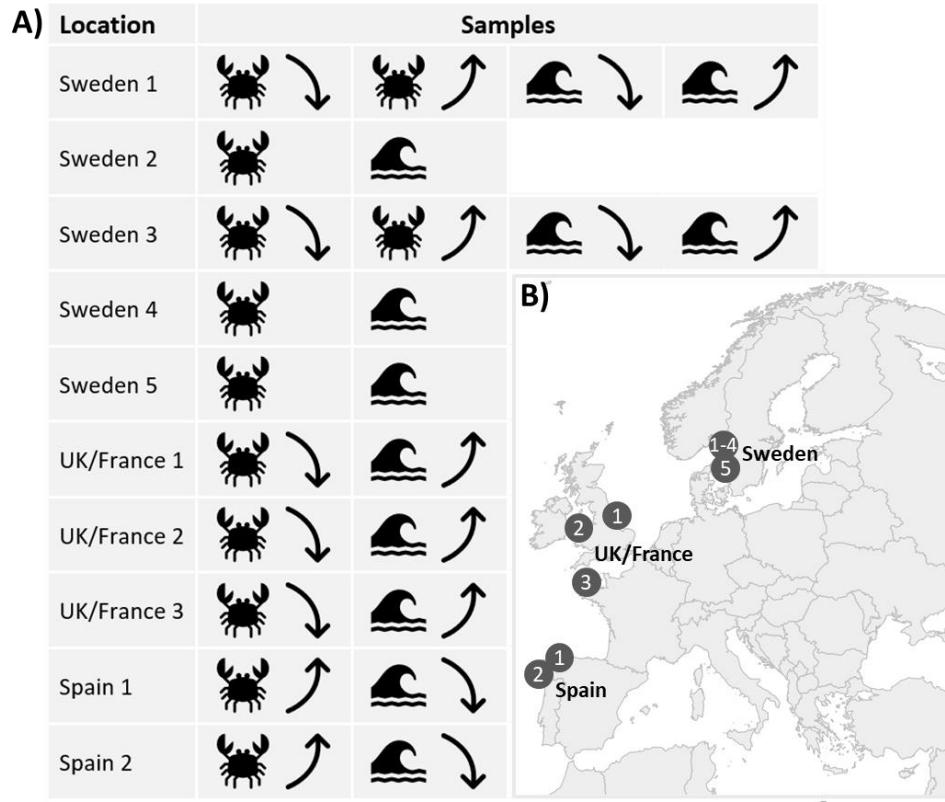
234 In this paper, we address these three points, using pool-seq data according to the ideas explained above,  
235 with three main aims: 1) Determining arrangement frequencies in multiple geographical locations across  
236 Europe; 2) Testing whether inversions are consistently associated with Crab-Wave or Low-High selection; 3)  
237 Analyzing the allelic content of different arrangements and comparing it between locations. We confirm  
238 our inferences of arrangement frequencies with individual sequencing data.

239 **Methods**

240 *Sampling and pool-sequencing*

241 We reused the whole-genome pool-seq data published by Morales et al. (2019). Briefly, snails were  
242 collected from 10 locations in three geographical areas - Sweden, UK/France, and Spain (Fig. 2, Table S1).  
243 UK and France are grouped together here as they are geographically relatively close and share the same  
244 shore distribution of ecotypes (Fig. 2). Snails were collected from Crab and Wave habitat as well as from the  
245 Low and High shore (i.e., the lowest and highest part of the local distribution).

246 In Spain and UK/France, the Crab-Wave and the Low-High axes are associated, but with different  
247 directionalities (Fig. 2). In these cases, there are only two possible types of samples (Crab-Low and Wave-  
248 High in UK/France; Crab-High and Wave-Low in Spain), each of which is represented by a pool of 100  
249 females. In Sweden, both the Crab and the Wave ecotype can occur on the low and high shore (in separate  
250 parts of the shore), so that four different types of samples are possible. For two locations (Sweden 1 and 3),  
251 we generated pools representing all four possible combinations, each containing 50 females. When doing  
252 Crab-Wave comparisons for these locations (see below), we combined allele counts for the Low and High  
253 sample for each of Crab and Wave. For the remaining three Swedish locations (Sweden 2, 4 and 5), only  
254 Crab and Wave ecotypes were collected randomly across shore levels. Each of these pools contained DNA  
255 from 24 individuals (including both sexes).



257 Fig. 2: Overview of the samples (pools) used in this study (see also Morales et al. 2019). A) Up to four  
258 samples were obtained in each of ten geographical locations. Crab and Wave ecotype are indicated by  
259 symbols; Low and High are indicated by an upward and a downward arrow, respectively. Note that the  
260 association between the Crab-Wave and the Low-High axis varies between geographical areas. For Sweden  
261 2, 4 and 5, pools contained individuals sampled randomly across both shore levels. B) Partial map of Europe  
262 showing the ten sampling sites.

263 For each pool ("sample" in the following), tissue of the different individuals was combined (i.e., pooled),  
264 DNA was extracted, genomic libraries were generated and sequenced on an Illumina HiSeq2500 machine.  
265 Reads for each sample were trimmed and mapped to the *L. saxatilis* reference genome. After further  
266 bioinformatic processing, we obtained allele count data for each sample and used these to calculate allele  
267 frequencies. For details of the pooling procedure and bioinformatic processing, see Morales et al. (2019).  
268 We only retained biallelic SNPs with a coverage depth of at least 25x in each pool and a minor allele  
269 frequency of 0.02 across all pools together.

270 *De-novo detection of inversion polymorphism*

271 In this study, we focus mainly on inversion regions described previously by Westram et al. (2018) and Faria  
272 et al. (2019) for the Swedish west coast, determining arrangement frequencies in other European locations  
273 (next section). However, as we mention above that pool-seq data might be used for inversion de-novo  
274 detection as well, we also ask whether de-novo detection would have been possible with our data.

275 We generated allele frequency plots analogous to Fig. 1C / Fig. 1D for each Crab-Wave pair in 4cM windows  
276 along the genome. 4cM were used to ensure enough SNPs in most windows. One of us, unaware of  
277 chromosome and location IDs, evaluated each plot, noting in which window a parallelogram was visible  
278 ("Yes", "No", or "Uncertain"). For this, each set of windows belonging to the same chromosome was kept  
279 intact, as seeing differentiation in the collinear background is necessary to evaluate the presence of a  
280 parallelogram; but the chromosome and location IDs were removed, and the order of the sets was  
281 randomized. We then compared the results of the scoring with the locations of known inversions.

282 *Estimation of arrangement frequencies*

283 Our main goal was to estimate arrangement frequencies. We included 12 inversion regions on 9 different  
284 chromosomes from Faria et al. (2019), excluding arrangements on linkage group 14, which showed  
285 inconsistent patterns between Spain and the other countries and might contain complex or overlapping  
286 rearrangements, and regions on linkage group 12, which is probably a sex chromosome (Hearn et al. 2022).  
287 The inversions are labelled LGC1.1, LGC1.2, LGC2.1, LGC4.1, LGC6.1/2, LGC7.1, LGC7.2, LGC9.1, LGC10.1,  
288 LGC10.2, LGC11.1, and LGC17.1, where the first number in each label refers to the linkage group the  
289 inversion region is located in. We used the chromosomal coordinates from Westram et al. (2021; innermost  
290 coordinates).

291 As shown in Fig. 1C, arrangement frequencies could be estimated for a single Crab-Wave or Low-High  
292 sample pair from a single location. Here, as we sampled multiple locations, we first identified "globally  
293 arrangement-diagnostic SNPs" (SNPs that were arrangement-diagnostic across many locations) and used  
294 only those to estimate arrangement frequencies. This approach has three advantages: 1. It ensures that the

295 “A arrangement” and the “B arrangement” are labelled consistently across locations, as the same  
296 diagnostic SNPs are used; 2. It improves the detection of the parallelogram corners for locations where the  
297 allele frequency data are noisy; 3. It allows for the estimation of arrangement frequencies in locations  
298 where the frequency does not differ between populations (i.e. where the parallelogram collapses into a  
299 line) (Fig. 1D).

300 To determine A- and B-diagnostic SNPs for each known inversion region, we performed three steps,  
301 explained in detail in the Supplementary Text. Briefly, we first plotted allele frequency data for all SNPs  
302 from the inversion region analogous to Fig. 1C / Fig. 1D for each Crab-Wave population pair and ensured  
303 visually that multiple pairs showed a parallelogram pattern (this was the case for all studied inversions). We  
304 then identified the SNPs with the highest  $F_{ST}$  (>80% quantile of the  $F_{ST}$  distribution) for each pair. These  
305 SNPs roughly correspond to the arrangement-diagnostic SNPs (i.e., the SNPs in the  $(p_{A,1}, p_{A,2})$  and  
306  $(p_{B,1}, p_{B,2})$  corners of the parallelogram). The reason is that differentiation between populations with  
307 different arrangement frequencies is highest for those SNPs that are fixed different between arrangements  
308 (again assuming no large allele frequency differences within arrangements between populations, which is  
309 appropriate for the majority of SNPs). As we were looking for globally arrangement-diagnostic SNPs, we  
310 then retained only those SNPs as candidates that were in the high- $F_{ST}$  class in at least 50% of the included  
311 locations. Finally, we ensured to retain only SNPs whose allele frequencies are strongly associated with  
312 arrangement frequencies across locations. For that, we ran a PCA for all populations, including all candidate  
313 SNPs. The first axis of this PCA sorts populations roughly by arrangement frequency. The SNPs that have the  
314 greatest impact on this axis are the ones most strongly associated with arrangements across locations. We  
315 therefore retained only those candidate SNPs above the 90% quantile of the distribution of absolute  
316 loadings on PC1.

317 Each inversion arrangement might be associated with the reference genome allele at some SNPs and with  
318 the alternative allele at others (Fig. 1). For that reason, some SNPs have positive loadings on PC1 while  
319 others have negative loadings. We therefore obtained two sets of arrangement-diagnostic SNPs, “A-

320 diagnostic SNPs" (positive loadings; reference allele associated with the arrangement arbitrarily labelled  
321 "A") and "B-diagnostic SNPs" (negative loadings; reference allele associated with arrangement B).

322 Having obtained two sets of globally diagnostic SNPs for each inversion, we calculated an estimate of the  
323 frequency of arrangements A and B for each sample shown in Fig. 2 by averaging across the frequencies of  
324 the diagnostic SNPs in that sample.

325 The inversion on linkage group 6 (labelled LGC6.1/2) contains a part where most likely a second inversion  
326 has occurred, overlapping the original, larger inversion. Therefore, three mostly non-recombinant  
327 haplotypes exist (Faria et al. 2019a). In that case we did not expect to observe a simple parallelogram when  
328 including SNPs from the whole inversion region. We therefore restricted all analyses of this inversion to the  
329 region affected by only a single inversion, which contains only two haplotypes, applying a 1cM buffer (0-  
330 7.73cM).

331 We validated our arrangement frequency results using individual sequencing data. A recent study obtained  
332 capture sequencing data for hundreds of individuals using probes randomly distributed throughout the  
333 genome for two of the Swedish locations included here (Sweden 3 and Sweden 4). As multiple probes  
334 covered each inversion, reliable arrangement frequency estimates could be obtained using an LD-based  
335 method (Westram et al. 2021, Faria et al. 2019). Here, we compared the arrangement frequency estimates  
336 obtained in the two studies.

### 337 *Association of arrangements with selective axes*

338 We first tested, for each inversion and geographical location, whether an inversion showed elevated  
339 differentiation between two samples (i.e., the Crab and Wave sample or the Low and High sample). We  
340 used the empirical distribution of SNP  $F_{ST}$  values as the null distribution, considering only genomic regions  
341 outside inversions. We considered an inversion region as highly differentiated if the  $F_{ST}$  calculated from the  
342 estimated arrangement frequencies was above the 95% quantile of this distribution. However, because  
343 arrangement frequencies and SNP frequencies are not directly comparable and were obtained using

344 different methods, the results should be interpreted as a general indication of high differentiation rather  
345 than a thorough test for divergence under selection.

346 Frequency differences between the two samples from the same location in Spain or UK/France could  
347 reflect a response to either the Crab-Wave or the Low-High selective axis. The distinction can only be made  
348 when including samples from multiple geographical areas where the two selective axes are associated in  
349 different ways (Fig. 2). For each inversion, we therefore ran a Wilcoxon signed rank test including all Crab-  
350 Wave pairs to test whether one arrangement is significantly more common in one ecotype. We performed  
351 the analogous test for the Low-High pairs.

352 *Analysis of arrangement content*

353 Our aim here was to test whether the same arrangement might contain different adaptive alleles in  
354 different geographical areas. If that was the case, one would expect that SNPs that are arrangement-  
355 diagnostic in some locations may show different patterns in other locations. First, within each geographical  
356 area, we identified SNPs that show a (nearly) fixed difference between the two arrangements (“locally  
357 arrangement-diagnostic SNPs”). For that, for each SNP, we calculated the Euclidean distance between a  
358 vector of the allele frequencies for the focal SNP and a vector of the frequencies of arrangement A,  
359 including all populations in the focal geographical area. We did the same for a vector of the frequencies of  
360 arrangement B and retained the smaller Euclidean distance (as the SNP could be either A- or B-diagnostic).  
361 We then selected the SNPs with the Euclidean distance below the 0.01 quantile of the distribution of  
362 Euclidean distances. We visualized the associations of these SNPs with the inversion in all geographical  
363 areas by plotting the regression line (lm() function in R) of SNP frequency against arrangement frequency  
364 for each area separately. If a SNP contributes to adaptation only locally, we expect that it will not be  
365 strongly associated with the inversion in other geographical areas. If a SNP contributes to adaptation in  
366 multiple locations, but does not contribute to adaptation to the same selective axis as the inversion, its  
367 association with the inversion might be reversed between locations. For example, if arrangement A  
368 contributes to Crab adaptation and arrangement B to Wave adaptation, alleles contributing to high shore  
369 levels in this inversion would be found in arrangement A in Spain, but in arrangement B in the UK. We note

370 that, of course, the existence of these expected patterns does not necessarily indicate that the respective  
371 SNPs directly contribute to adaptation – they might be neutral SNPs in LD with adaptive SNPs, or they might  
372 show the pattern purely by chance. Our analysis is simply aimed at testing whether location-specific  
373 arrangement contents exist in general.

374 The regression between SNP and inversion frequency is most informative when inversion frequency varies  
375 strongly between populations. We therefore performed this analysis only on two arrangements showing  
376 clear associations with one environmental axis each (LGC6.1/2: Crab-Wave axis; LGC9.1: Low-High axis).

377 All analyses were performed in R version 4.2.1 (R Core Team 2021).

## 378 *Results*

### 379 *Allele frequencies and de-novo detection of inversion polymorphism*

380 Allele frequencies were generally correlated between samples within locations (Fig. S1), but some genomic  
381 regions showed visually identifiable parallelograms. We visually detected parallelograms in at least two  
382 locations for each of the inversion regions known from Faria et al. (2019) (Fig. S1, Fig. S2). Allele frequencies  
383 for one inversion region are shown as an example in Fig. 3. The corners and edges of the parallelograms  
384 were often clearly visible by eye; however, some parallelograms were quite noisy, and there were always  
385 some SNPs "inside" the parallelograms, not clearly associated with the parallelogram corners or edges (Fig.  
386 3, Fig. S1, Fig. S3).

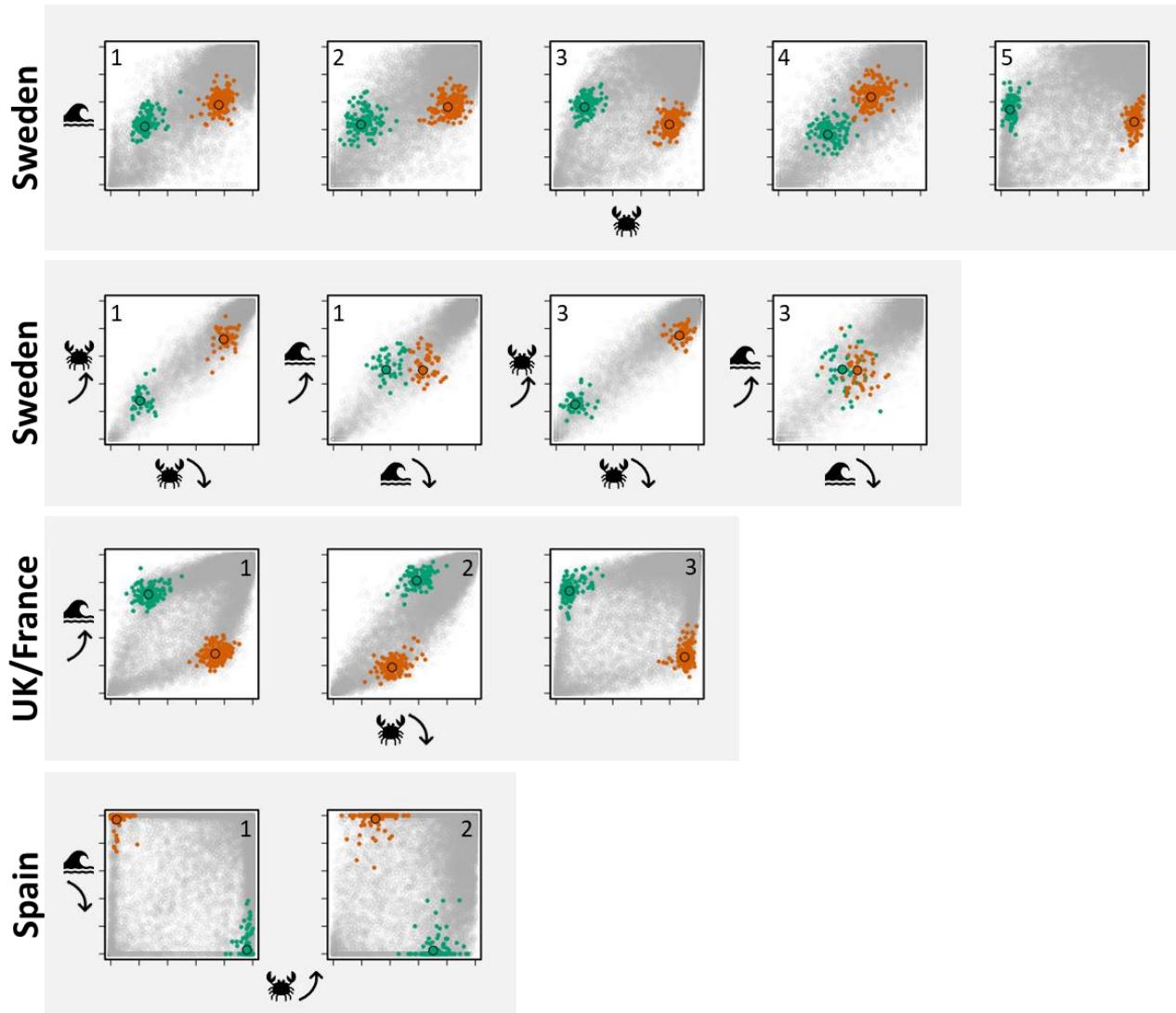
387 After randomization, one of us scored 4cM windows along chromosomes by eye for the presence of a  
388 parallelogram. We then asked whether the regions with visible parallelograms correspond to regions  
389 containing known inversions (Westram et al. 2021). We found that many windows could not be scored  
390 visually (56% of windows were labelled “uncertain”), due to noisy data and / or small numbers of SNPs.  
391 However, where scoring was possible, inversions could be detected relatively reliably: Of 243 windows  
392 overlapping with an inversion that could be scored, 197 contained a detectable parallelogram (false  
393 negative rate 19%). Where no parallelogram was visible, this was often because arrangement frequency did

394 not differ much between the two populations (as inferred below), and thus no parallelogram was expected  
395 despite the presence of an inversion polymorphism (Fig. 1D, Fig. S2). The false positive rate (parallelogram  
396 inferred without the presence of a known inversion) was 11% (42 out of 384 windows). For details, see Fig.  
397 S2.

398 *Estimation of arrangement frequencies*

399 We determined a set of diagnostic SNPs for each arrangement. The numbers of such SNPs varied between  
400 twelve and 265 per inversion. By averaging the frequencies of diagnostic alleles, we estimated the  
401 frequencies of the A and B arrangement in each of the samples (Fig. 3 and Fig. S3). The frequencies  
402 obtained in this way generally corresponded very well to the visible parallelogram corners (Fig. 3 and Fig.  
403 S3) and were also very similar to frequencies obtained from individual sequencing data (Westram et al.  
404 2021) (Fig. S4, Fig. S5), validating our pool-seq approach.

405 Note that the data for the Swedish Low-High pairs had been processed separately from the rest of the  
406 dataset and does not contain all diagnostic SNPs. Arrangement frequency estimates are therefore based on  
407 a reduced number of SNPs (Fig. S3). However, there were always at least 10 diagnostic SNPs, except for the  
408 frequencies of LGC6.1/2 and LGC10.1 (5 SNPs and 1 SNP, respectively), which therefore need to be treated  
409 with caution.

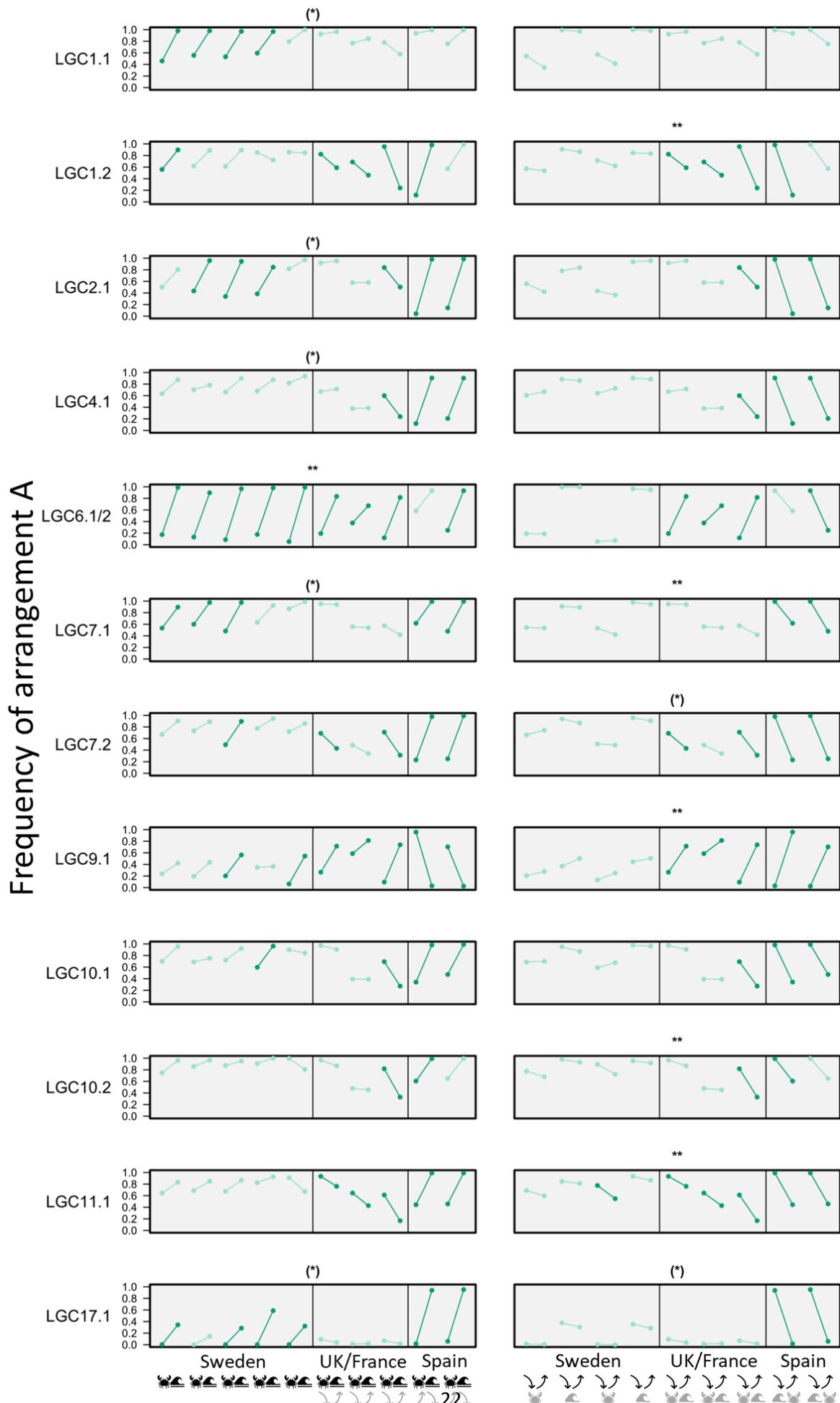


411 Fig. 3: Allele frequency plots for the inversion region LGC9.1 for 14 different pairs of samples (axes ranging  
412 from 0 to 1; axis symbols as in Fig. 2). Each small point represents the frequencies of the reference genome  
413 allele for a SNP in the two local samples. Colored points represent arrangement-diagnostic SNPs (green:  
414 arrangement A; orange: arrangement B). Larger points with black borders indicate the frequencies of  
415 arrangement A and B obtained by averaging frequencies across arrangement-diagnostic SNPs. The  
416 geographical area of each sample pair is indicated on the left; the location ID is shown within each plot.  
417 Analogous plots for all other inversion regions can be found in Fig. S3.

418 *Association of arrangements with selective axes*

419 Our results show that all inversions known from studies in Sweden are also segregating in other European  
420 locations (Fig. 4). Inversions were almost always segregating at the location level, i.e., usually both

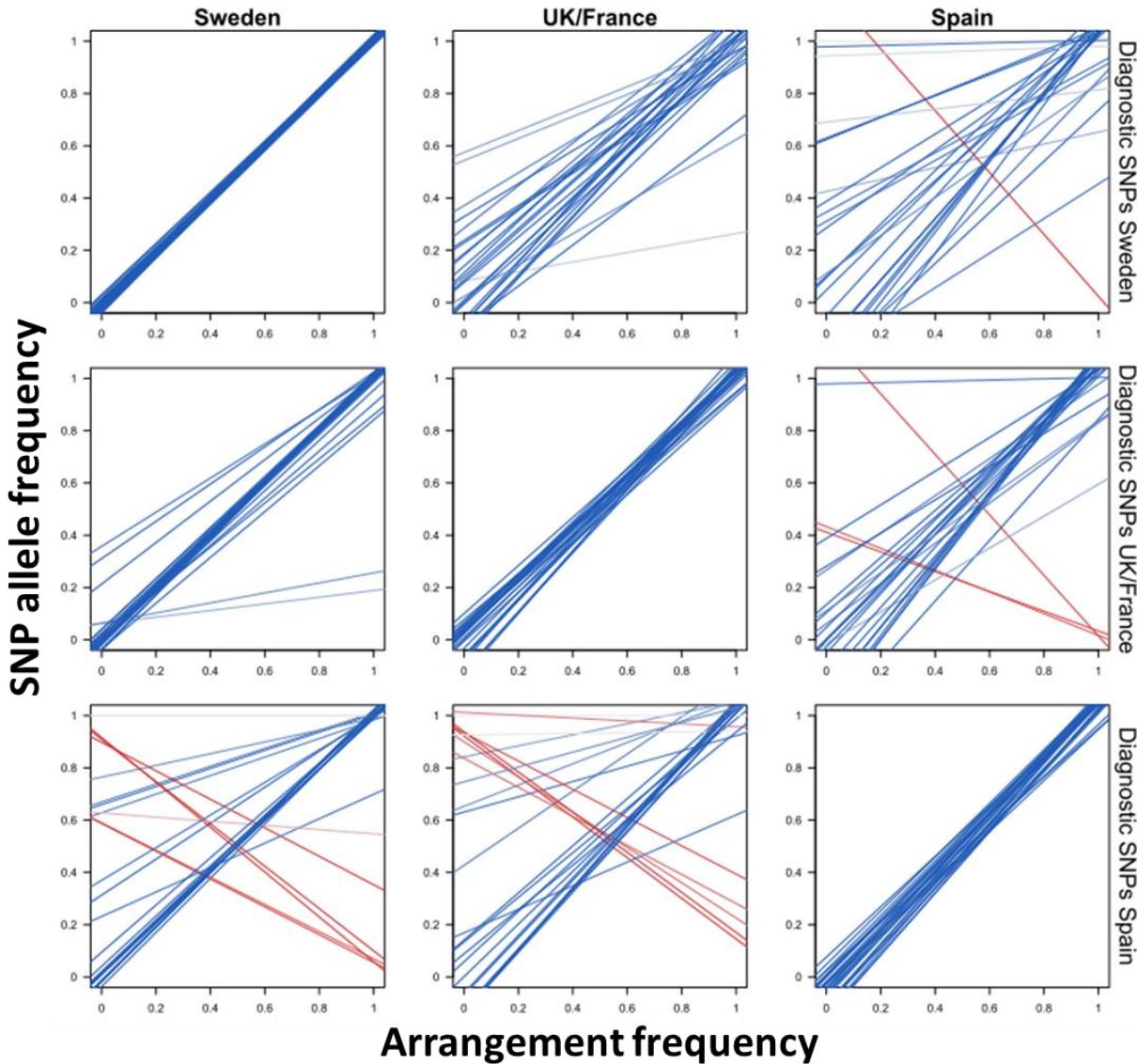
421 arrangements were present in each location, with the rarer arrangement having a frequency higher than  
422 0.05. A notable exception is LGC17.1, which was near fixation overall in UK/France (Fig. 4). In contrast,  
423 within the Crab or Wave ecotype within a given location, near-fixation was more common. In particular, the  
424 two Spanish Wave ecotype samples were close to fixation for one arrangement for most inversions (Fig. 4).  
  
425 All inversions showed elevated differentiation (when compared to collinear SNPs) between samples in at  
426 least three locations (Fig. 4, dark green lines). Within geographical areas (Sweden, France/UK, Spain), the  
427 direction of differentiation was always consistent (when considering only locations showing elevated  
428 differentiation according to our test), e.g., the Crab sample always had a higher frequency of arrangement  
429 A than the Wave sample of the same location.  
  
430 We compared patterns between geographical areas to reveal whether arrangements were consistently  
431 associated with the Crab-Wave axis or the Low-High axis. Wilcoxon signed rank tests indicated that out of  
432 12 inversions analyzed, 11 showed significant consistent associations with at least one of the two  
433 environmental axes (six remained significant after sequential Bonferroni correction; Fig. 4). Four inversions  
434 were significantly associated with the Crab-Wave axis, 5 with the Low-High axis, and two with both axes.  
435 However, it is clear that in some of these cases, while directionalities are similar across countries, local  
436 arrangement frequency differences between environments are often small and not indicative of selection  
437 (light green lines in Fig. 4; e.g. LGC7.1 Low-High). Clear environment-inversion associations consistent  
438 across countries with at least 5 instances of elevated differentiation within locations (dark green lines in Fig.  
439 4) were found in five cases: LGC6.1/2 (Crab-Wave), LGC7.1 (Crab-Wave), LGC17.1 (Crab-Wave), LGC9.1  
440 (Low-High), and LGC11.1 (Low-High).



442 Fig. 4: Frequency of arrangement A for each inversion, location, and sample. Lines connect samples from  
443 the same location. Samples within locations are shown in the order Crab-Wave (left) or Low-High (right).  
444 Inversions associated with Crab-Wave (Low-High) are expected to show consistent directionalities of the  
445 lines in the left (right) plot. Dark green color indicates significantly elevated arrangement frequency  
446 difference between environments within locations. Asterisks indicate the significance of a Wilcoxon signed  
447 rank test for general frequency differences between Crab and Wave / Low and High (\* p<0.05; \*\* p<0.01;  
448 \*\*\* p<0.001; brackets: not significant after sequential Bonferroni correction).

449 *Analysis of arrangement content*

450 We analyzed the arrangement content for two inversions that showed particularly strong associations with  
451 an environmental axis, LGC6.1/2 (associated with Crab-Wave) and LGC9.1 (associated with Low-High). We  
452 found that most SNPs associated with the inversion in one geographical area also showed a similar  
453 association in the other two geographical areas (Fig. 5, Fig. S6). This is consistent with the fact that for all  
454 inversions we could find “globally” diagnostic SNPs that work across all studied countries. However, we also  
455 found some SNPs that were associated with the inversion in one area but invariant in other areas (lines in  
456 grayish colors in Fig. 5, Fig. S6). In addition, some SNPs showed reverse associations with the inversion in  
457 different countries, particularly when Spain was compared to UK/France and Sweden (red lines in Fig. 5,  
458 Fig. S6).



459

460 Fig. 5: Patterns of locally arrangement-diagnostic SNPs in other geographical areas for the LGC6.1/2  
461 inversion. Each line represents the regression of SNP allele frequency against the frequency of arrangement  
462 A for one SNP. The geographical area where the SNPs are arrangement-diagnostic is indicated on the right  
463 (SNPs can be arrangement-diagnostic in multiple countries, so they can occur in multiple rows); regression  
464 lines always show the allele that is positively associated with arrangement A in this geographical area. Line  
465 color indicates Pearson's correlation coefficient ( $r$ ), with a color gradient from negative (red) via 0 (grey) to  
466 positive (blue).

467 **Discussion**

468 *Using SNP allele frequency data to analyze inversion regions*

469 The importance of chromosomal rearrangements in adaptive divergence has become increasingly clear,  
470 and testing for their presence and frequency in natural populations is important for understanding adaptive  
471 processes. In particular, it is a largely open question whether inversions contribute to adaptation  
472 repeatedly across large geographical scales, and whether they differ in adaptive content between  
473 geographical areas, representing versatile “toolboxes”. While multiple methods exist to determine the  
474 presence and population frequency of inversions, they usually require individual sequencing data (which  
475 can generate high costs if many locations are studied) and sophisticated analyses. Here, we present ideas of  
476 how simple allele frequency data (here, cost-effective pool-seq data) could be used to study inversions.

477 Our approach relies on a parallelogram shape in plots of allele frequencies in population 2 against  
478 population 1, which appears when populations are connected by gene flow but differ in arrangement  
479 frequency for an inversion. These parallelograms are essentially an outcome of the extensive LD in the  
480 inversion region, generating associated frequency shifts across many SNPs. Our simulations and empirical  
481 results thus demonstrate that LD information is not entirely lost in allele frequency data, as sometimes  
482 assumed, and extends beyond the read- or paired read-level previously targeted by LD analyses using pool-  
483 seq data (Feder et al. 2012). The parallelograms are useful both for de-novo detection (presence of a  
484 parallelogram indicates presence of a polymorphic inversion) and frequency determination (parallelogram  
485 corners indicate arrangement frequencies) and thus provide an alternative to often more costly and labor-  
486 intensive methods based on cytogenetics, LD analysis or long-read sequencing data.

487 Inversion de-novo detection by eye worked best for sample pairs with limited general differentiation, many  
488 individuals per pool, and larger arrangement frequency differences between populations. LD-based  
489 approaches in individual data are certainly generally more reliable and would often be required to confirm  
490 findings from pool-seq data. However, we note that for high-quality samples parallelogram patterns are

491 very clear and many rearrangements can be discovered with little doubt. For example, our French pools  
492 showed very little noise and all inversions were reflected by extremely clear parallelograms that not only  
493 allowed for accurate inversion detection but also for reliable frequency estimation (Fig. S3). We conclude  
494 that for researchers studying local adaptation using pool-seq data, it is certainly worth screening the  
495 genome for inversions using allele frequency plots. Regions with clear parallelograms will usually also show  
496 elevated  $F_{ST}$ ; however, the advantage of the parallelogram method is that a parallelogram clearly hints at a  
497 genomic rearrangement (while high  $F_{ST}$  can also occur in collinear regions), and in contrast to  $F_{ST}$  it allows  
498 for arrangement frequency estimation. Allele frequency plots could also be a quick way to scan a genome  
499 for candidate inversion regions even if individual sequencing data is available, being faster than a full LD  
500 analysis. One important thing to note here is that other chromosomal rearrangements, e.g., large  
501 duplications, might potentially also lead to parallelogram shapes. Therefore, studies relying on de-novo  
502 detection (in contrast to our study, where inversions were previously known), should check the type of  
503 rearrangement in follow-up analyses, potentially using individual sequencing data.

504 Determining the location of parallelogram corners visually, or using allele frequencies of diagnostic SNPs, it  
505 is possible to estimate arrangement frequencies in each sample. Applying the latter approach, we obtained  
506 arrangement frequency estimates that were highly reliable. To find "arrangement-diagnostic SNPs", we  
507 made use of the fact that we had sampled multiple population pairs, identifying the SNPs most strongly  
508 associated with parallelogram corners across different locations. Using these diagnostic SNPs, it was  
509 possible to detect inversion polymorphism even in locations with very noisy parallelograms (e.g., Fig. 3,  
510 Sweden 1 Crab-Wave) and in locations where arrangement frequencies were very similar in the two  
511 samples and thus no parallelogram appeared (e.g., Fig. 3, Sweden 4). Finding arrangement-diagnostic SNPs  
512 directly from pool-seq data thus provides an alternative to first determining them from individual  
513 sequencing data (Kapun et al. 2014). Of course, even SNPs detected in this way might not be fully reliable –  
514 for example, SNPs that are arrangement-diagnostic in 9 locations might not be so in the 10<sup>th</sup> location, and  
515 while our approach finds statistically associated SNPs, unless very stringent thresholds are used this

516 association may not be perfect (see SNPs deviating from the parallelogram corners in Fig. S3). Nevertheless,  
517 if multiple putatively arrangement-diagnostic SNPs are used, reliable estimates should be possible.

518 It is likely that some SNPs violate the assumptions of being variable in one arrangement only and not  
519 strongly differing in within-arrangement frequency between local populations (see Rationale). However,  
520 these violations only introduce noise; as arrangement frequency estimation relies on SNPs found  
521 consistently near the parallelogram corners, these SNPs do not strongly affect our approach. Similarly,  
522 some collinear SNPs accidentally included when inversion boundaries are not exactly known, or because of  
523 assembly errors, may generate points inside the parallelogram but should not generate major problems in  
524 frequency estimation. However, inversions affected by very large amounts of gene conversion or double  
525 cross-over might be harder to analyze as this will generate a larger number of SNPs "inside" the  
526 parallelogram.

527 In this paper, we have aimed at introducing the general rationale of using allele frequency data for  
528 inversion frequency estimation and demonstrating its usefulness in an empirical system. We have not  
529 developed a fully automated approach to detect and quantify inversion polymorphism, but rather use a  
530 heuristic approach. Automation could be an interesting possibility for the future. A challenge for such a tool  
531 is the incorporation of SNPs violating assumptions (see above) as well as sampling noise; in our pilot  
532 attempts, automation (results not shown) always performed much worse than the heuristic approach  
533 presented here and even just visual examination.

534 Extensive simulations exploring the limits of parallelogram detection (visually or via a heuristic approach)  
535 and optimizing parameter settings for the detection of diagnostic SNPs are well beyond the scope of this  
536 paper. However, they could be an interesting avenue for future work. Relevant factors to vary include the  
537 extent of gene flow between populations, the inversion histories (e.g., time to accumulate differences), the  
538 level of gene flux between arrangements, and the extent of noise in the allele frequency data (e.g.,  
539 numbers of individuals in the pool; relative DNA contribution of each individual to the pool).

540 *Inversions across large geographical scales in Littorina*

541 In our study system, we used the approach discussed above to confirm the presence of polymorphic  
542 inversions across Europe, determine arrangement frequencies (which was impossible based on earlier FST  
543 approaches; Morales et al. 2019) and analyze arrangement content.

544 A first main finding is that the known inversions are polymorphic across most of the studied European  
545 locations. This extends the work on inversion polymorphism in Sweden (Faria et al. 2019a; Westram et al.  
546 2021) and earlier pool-seq work, which could often show high divergence in inversion regions, but could  
547 not detect inversion polymorphism in locations where the different ecotypes did not differ in arrangement  
548 frequency (e.g. LGC7.1 in the UK and France). This widespread polymorphism might indicate balancing  
549 selection on at least some of the inversions (Wellenreuther and Bernatchez 2018; Faria et al. 2019a,  
550 2019b).

551 One obvious question is whether parallel evolution of Crab-Wave and Low-High ecotypes in *Littorina* is  
552 driven by the same inversions across Europe. Using the arrangement frequency data in this study we could  
553 address this question. We found that 5 arrangements are clearly associated with either the Crab-Wave  
554 (LGC6.1/2, LGC7.1, LGC17.1) or the Low-High (LGC9.1, LGC11.1) selection axis, while other inversions show  
555 less clear associations. These results are consistent with work on other systems showing the same  
556 arrangement repeatedly associated with the same environment in different geographical locations (e.g.  
557 Mérot et al. 2018; Christmas et al. 2019), and also overall consistent with earlier analyses of the same  
558 dataset (Morales et al. 2019). However, this previous work did not infer arrangement frequencies and  
559 relied on elevated  $F_{ST}$  in inversion regions.  $F_{ST}$  in inversion regions is affected by various factors, including  
560 not only arrangement frequency but also inversion age, selection, and the extent of gene flux. Here, we  
561 obtain arrangement frequency estimates that are independent of these factors and therefore more reliable  
562 (Fig. 4). However, the associations with environmental axes still need to be interpreted with some caution:  
563 It is not possible to fully disentangle the Crab-Wave and the Low-High selective axes, partly because  
564 locations within geographical areas all have the same association between environmental axes (Fig. 2) but  
565 are treated as independent here. It is only possible to resolve this with a modified sampling design, where

566 Crab and Wave individuals are sampled at exactly the same shore level, and Low and High individuals are  
567 sampled within Wave or Crab, for each of the geographical area.

568 Arrangement-environment associations that are consistent between geographical areas might partly be  
569 driven by a set of selected SNPs strongly differentiating the arrangements across different locations.  
570 Finding causal SNPs under selection in high-LD regions like inversions is notoriously difficult as many SNPs  
571 show correlated patterns (Ayala et al. 2017). However, as part of our approach to determine arrangement  
572 frequencies, we determined " arrangement-diagnostic" SNPs associated with arrangements across multiple  
573 locations; these are the strongest candidates for SNPs driving consistent arrangement frequency  
574 differences and warrant further study. Thus, comparisons across multiple geographical locations might be  
575 one way of narrowing down candidate SNPs within inversion regions and our approach makes this feasible  
576 without the high cost of individual sequencing.

577 Interestingly, most inversions studied here, even if significantly associated with a single selective axis, did  
578 not show fully consistent associations across all locations. For example, LGC7.2 was significantly associated  
579 with the Low-High axis, but visual inspection also suggests a Crab-Wave association pattern in some  
580 Swedish locations. Associations with multiple selective axes were already indicated in Morales et al. (2019).  
581 For LGC17.1, the clear association with the Crab-Wave axis is limited to Sweden and Spain but absent in the  
582 UK/France. These examples of differences between geographical areas might indicate location-specific  
583 patterns or strengths of selection. We use a crude categorization of samples into "Crab" and "Wave" or  
584 "Low" and "High" habitats, but the details and strengths of selective pressures likely vary between  
585 countries. In stickleback lake-stream divergence, Stuart et al. (2017) discovered that non-parallel  
586 divergence patterns can be attributed to slight variations in local selection pressures. These nuances are  
587 often overlooked when habitats are simply categorized as "lakes" and "streams". Such effects could explain  
588 some of the complex patterns we see.

589 Additionally, or alternatively, the differences between geographical areas could indicate that inversions  
590 have different functional contents in different locations – i.e., contain different “tools” adjusted to local  
591 requirements. For example, LGC9.1 might contain only Low-High adaptive variants in UK/France and Spain

592 but might additionally contain Crab-Wave adaptive variants in Sweden. In line with this, we find that some  
593 of the SNPs differentiating arrangements in Sweden do not show such associations in the other  
594 geographical areas (Fig. S6).

595 Such differences in arrangement content were generally found when focusing on locally arrangement-  
596 diagnostic SNPs in LGC6.1/2 and LGC9.1. Each of the geographical areas has a small number of SNPs that do  
597 not differentiate arrangements elsewhere. These could contribute to location-specific adaptive differences  
598 (e.g., differences in shell sculpture that exist in Spain but not elsewhere), contribute to adaptation to  
599 environmental contrasts that do not exist in the other areas, or fulfil adaptive functions that are fulfilled by  
600 other parts of the genome elsewhere. While we cannot currently say whether any of these SNPs are  
601 actually functional, these results nevertheless demonstrate the potential for geographical variation in  
602 arrangement content. In particular, we find some SNPs with reversed associations with the inversion in the  
603 UK/France vs. Spain for both of the studied inversions. In LGC6.1/2 (which is generally associated with the  
604 Crab-Wave axis), the genomic regions around these SNPs could contribute to adaptation to the Low-High  
605 axis, which is reversed between the UK/France and Spain. Similarly, in LGC9.1 (which is generally associated  
606 with the Low-High axis), these regions could contribute to Crab-Wave adaptation.

607 These results and hypotheses emphasize the need to analyze patterns of selection within arrangements.  
608 While some studies have focused on selection within arrangements – for example, an experimental study  
609 on *Drosophila subobscura* showed how markers within arrangements changed more than expected by  
610 chance in the course of a selection experiment (Santos et al. 2016) – arrangement content remains largely  
611 unexplored for many systems with inversion-facilitated adaptation.

612 In summary, we show that allele frequency data, e.g., from pool-seq, has the potential to increase our  
613 understanding of the role of inversions in adaptive evolution. Allele frequency plots are straightforward to  
614 generate, by-eye analysis is sufficient in some cases, and heuristic approaches are easy to implement.  
615 These plots can be used to detect undiscovered inversions, determine arrangement frequencies, and  
616 analyze inversion content. Applying these ideas to the *L. saxatilis* system, we obtain results strongly  
617 consistent with inversions being associated with divergent adaptation, as predicted by theory and

618 suggested by previous work on this system (Morales et al. 2019). We find that while arrangements clearly  
619 show repeated associations with the same environmental axes, there are also differences between  
620 geographical areas and variation in arrangement content. This highlights the need to go beyond focusing on  
621 an inversion as a single unit: While inversions might be useful toolboxes for adaptation, what matters are  
622 the tools inside.

## 623 *Data and Code Accessibility Statement*

624 Raw sequencing reads can be found in the Sequence Read Archive (BioProject PRJNA494650). The allele  
625 count data from Morales et al. (2019), used as input in this study, can be found on Dryad (doi...). R scripts  
626 for all customs steps in this manuscript are deposited in GitHub (link...).

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