Hi-reComb: constructing recombination maps from bulk gamete Hi-C sequencing

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Summary:

Recombination is central to genetics and to evolution of sexually reproducing organisms. However, obtaining accurate estimates of recombination rates, and of how they vary along chromosomes, continues to be challenging. To advance our ability to estimate recombination rates, we present `Hi-reComb`, a new method and software for estimation of recombination maps from bulk gamete chromosome conformation capture sequencing (Hi-C). Simulations show that `Hi-reComb` produces robust, accurate recombination landscapes. With empirical data from sperm of five fish species we show the advantages of this approach, including joint assessment of recombination maps and large structural variants, map comparisons using bootstrap, and workflows with trio phasing vs. Hi-C phasing. With off-the-shelf library construction and a straightforward rapid workflow, our approach will facilitate routine recombination landscape estimation for a broad range of studies and model organisms in genetics and evolutionary biology. `Hi-reComb` is open-source and freely available at https://github.com/millanek/Hi-reComb.

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Introduction

Meiotic recombination is a hallmark of sexual reproduction. While mutations give rise to new genetic variants, recombination shuffles them to generate new haplotypes – that is, chromosomes with novel combinations of existing alleles which selection can act on. Therefore, recombination impacts key evolutionary processes such as adaptation and speciation, and it shapes the distribution of genetic variation along the genomes. Because organismal traits are usually influenced by many genetic variants (Shi et al. 2016; Boyle et al. 2017; Barton 2022), often with non-linear epistatic interactions among them (Phillips 2008; Domingo et al. 2019; Johnson et al. 2023), and because adaptation and speciation commonly require co-evolution of a whole suite of traits (Phillips and Arnold 1989; White and Butlin 2021), evolution is increasingly seen as multidimensional and combinatorial (Marques et al. 2019; Barton 2022), with recombination in a central role. Yet, routine and accurate genome-wide reconstruction of recombination maps remains a major challenge, and there is a need for novel approaches and methods for recombination inference.

Recombination rates vary by several orders of magnitude along the genome (Coop and Przeworski 2007; Stapley et al. 2017; Halldorsson et al. 2019). Accurate inference and representation of this variation requires ascertaining the chromosomal locations of many crossover events. Depending on how this is done, recombination rate inference methods can be divided into three catogories (Peñalba and Wolf 2020): (i) genetic linkage maps based on the transmission of polymorphic markers in crosses and pedigrees; (ii) population genetic approaches based on patterns of linkage disequilibrium (LD); and (iii) gamete sequencing, based on finding breakpoints in gamete haplotypes compared to the donor genome. The recombination maps inferred by the three approaches differ in several important respects, perhaps most notably in resolution, the measured time interval, and in how they are impacted by selection (Peñalba and Wolf 2020). For example, LD-based maps typically integrate over

26 thousands of generations of recombination, deliver high resolution, but are influenced by
27 selection occuring over that timeframe. In contrast, studies using crosses and pedigrees reflect
28 recombination over a small number of generations and rarely include the thousands of
29 samples needed to achieve resolution comparable to LD-based maps [but see (Morgan et al.
30 2017; Halldorsson et al. 2019) for high resolution maps in humans and mice]. Approaches
31 based on sequencing of sperm, egg, or pollen reveal all recombination during successful
32 gametogenesis, and thus deliver a direct snapshot, not affected by selection acting at other
33 life stages. The resolution of these gamete-based maps is largely determined by the number
34 of gametes that can be typed.

35 Single sperm whole-genome sequencing has been used for well over a decade (Lu et al. 2012;
36 Wang et al. 2012) and continues to deliver insights into the factors that influence meiotic
37 recombination (Hinch et al. 2019; Yang et al. 2022). However, practical limitations have
38 restricted studies to typing at most a few hundred sperm cells. One exception is the Sperm-
39 seq (Bell et al. 2020) protocol – a sperm specific variation of high-thoughput single cell
40 sequencing (Macosko et al. 2015) – which scaled to over 30,000 sperm cells. Nevertheless,
41 the adoption of this approach beyond the original study appears to be limited, likely due to the
42 custom and relatively complex laboratory procedure that is required. Bulk sequencing
43 approaches avoid the need for the isolation of individual cells. Three studies introduced linked-
44 read sequencing of bulk sperm and pollen (Dréau et al. 2019; Sun et al. 2019; Xu et al. 2019),
45 demostrating that single cell sequencing of individual gametes is not necessary to infer
46 crossover events. These studies use read-linkage information to find recombination
47 breakpoints by comparing individually barcoded gamete DNA fragments against the
48 haplotypes of the donor individual.

49 Here we present `Hi-reComb`, a new method and software for estimating individual
50 recombination maps from bulk gamete chromosome conformation capture (Hi-C) sequencing

data (Lieberman-Aiden et al. 2009; Rao et al. 2014; Oksuz et al. 2021), using a standard Hi-C library preparation protocol. By taking advantage of the long insert sizes delivered by Hi-C, this approach can achieve substantially greater effective coverage by crossover-informative read pairs than has been possible with comparable amount of linked-read sequencing. Moreover, Hi-C data provide information regarding large scale structural variation in the donor individual, allowing for simultaneous improvements of the reference genome and better interpretation of the genetic map constructed from this data. First, we show the accuracy of `Hi-reComb` by reconstructing genetic maps from simulated data. Next, to demonstrate the real-world utility of this approach, we constructed and sequenced several sperm Hi-C libraries, used the data to scaffold two reference genomes, and then inferred and evaluated several recombination maps for cichlid and stickleback fish. We show that the maps correspond well to LD-based maps and that the effects of donor haplotype phasing errors are limited. This approach is applicable to any species / individuals that produce at least hundreds of thousands of gametes. Many such species exist; therefore, `Hi-reComb` will be of utility to a broad range of researchers interested in genetics and recombination.

Materials and Methods

The Hi-reComb approach

Recombination map reconstruction using `Hi-reComb` starts with preparing a standard commercially available Hi-C library. We choose a library based on endonuclease digestion to provide a relatively uniform sequencing coverage (**Figure S1**). After sequencing and alignment to a reference genome, `Hi-reComb` detects meiotic crossovers by comparison of Hi-C fragments against donor haplotypes (**Figure 1**). Because the two reads from each pair of Hi-C fragments originate from the same haploid gamete, cases where the two fragments match different donor haplotypes indicate that a crossover took place between them. We denote this event as $X = C$. Conversely, if the two fragments match the same donor haplotype,

this suggests the absence of any crossover between them, denoted $X = N$. The match of Hi-C fragments to either of the two donor haplotypes is determined by sites at which the donor is heterozygous. Therefore, if the Hi-C fragments do not cover at least one heterozygous site each, such a read pair is not informative with regards to crossovers.

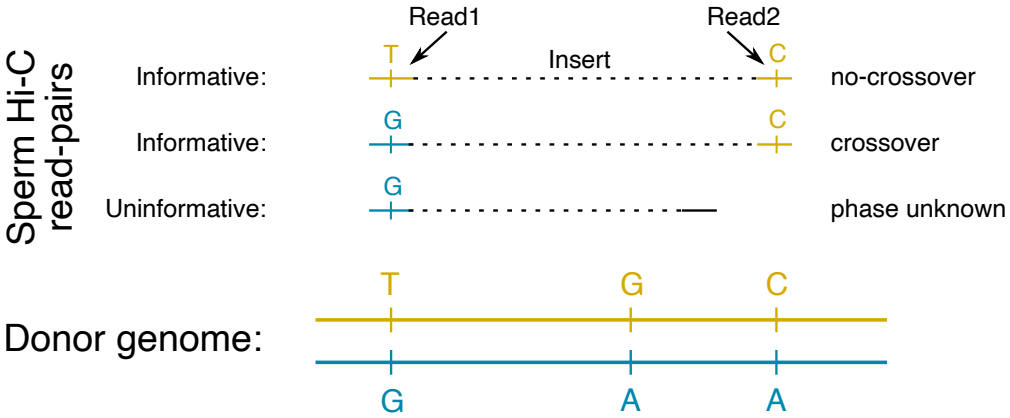

Figure 1: The principle of detecting crossovers with `Hi-reComb`. The donor genome is shown with heterozygous sites separating the two haplotypes indicated. For each sperm Hi-C read pair, if both fragments match the same donor haplotype, this indicates an absence of crossover (no-crossover). If the fragments match different donor haplotypes, this indicates a crossover between them. Finally, if both fragments do not cover haterozygous sites in the donor genome, the read pair is not informative with regards to recombination.

`Hi-reComb` estimates crossover likelihoods for each informative read pair i considering potential base-calling errors (base quality scores) and phasing errors (phase quality scores). Let c_i be a parameter indicating if a crossover did ($c_i = 1$) or did not ($c_i = 0$) take place between a pair of reads; thus $\theta = \{c_i | c_i \in \{0,1\}\}$. We then define a crossover likelihood $l_{ci} = P(X = C \mid c_i = 1)$ and non-crossover likelihood $l_{ni} = P(X = C \mid c_i = 0)$. While these likelihoods account for base calling and phasing, other sources of error, e.g. arising in variant calling/filtering or from non-crossover gene conversion events, are unaccounted for. We found that false positive crossover read pairs – i.e. overestimating l_{ci} and underestimating l_{ni} – can have substantial impact on the inferred recombination landscapes, creating false 'spikes' of recombination. This is true especially for read pairs with short inserts; that is, short genomic distances between the Hi-C fragments.

While false positives are equally likely across the insert length distribution, the proportion of true positives increases linearly with insert length: on average, there are 1,000x more true positives for read pairs with insert size of 1Mb compared with read pairs with insert size 1kb. This is because the further away two loci are from each other along the chromosome (the greater the insert length), the higher the probability that a crossover really occurred between these two loci (i.e., the greater the true positive rate). To estimate the overall false positive rate and to apply a correction to the likelihoods, we first use likelihoods of long-insert read pairs (default: > 1Mb), where the ratio of true to false positives is highly favorable, to estimate r_l^*, the average per-bp crossover rate for each chromosome. Then we calculate a correction factor by evaluating the crossover likelihoods of short-insert read pairs (default: < 1kb) against this baseline. Formally, the correction factor f is defined as $f = \frac{\sum_i l_{ci} - \sum_i d_i * r_l^*}{n_s}$ where i sums over all n_s short-insert informative read pairs with $X = C$ and where d_i is the insert size of the read pair i.

To calculate crossover probabilities, we incorporate the corrected likelihoods together with insert-length dependent priors in a Bayesian framework. We assign priors to read pairs under the assumption that the recombination rate is uniform along each chromosome. Therefore, the longer the insert length d_i the higher is the prior for each informative read pair i. The prior probability of crossover p_{ci} for each informative read pair i is then simply the product of r_l^* and of the insert length:

$$p_{ci} = r_l^* * d_i$$

Finally, the posterior crossover probability for each informative read pair i where $X = C$ is:

$$\mathbb{P}(c_i) = \frac{(l_{ci} - f) * p_{ci}}{(l_{ci} - f) * p_{ci} + (l_{ni} + f\ ) * (1 - p_{ci})}$$

For read pairs that do not indicate crossovers, we set $l_{ci} = P(X = N|c_i = 1) = 0$ and $l_{ni} = P(X = N|c_i = 0) = 1$. This is highly 'conservative' in terms of avoiding any risk of introducing

115 additional false positives. While it results in false negatives – i.e. underestimating l_{ci} and
116 overestimating l_{ni} – this approach does not affect the shape of the estimated recombination
117 landscapes because these false negatives are randomly distributed along the chromosomes.

118 To reconstruct the recombination map, we initially divide the crossover probability of each Hi-
119 C read pair uniformly along its insert length. That is, we assign the same probability to all
120 basepairs between the informative sites that determine the crossover. Then, we construct an
121 initial recombination map by dividing the sum of crossover and non-crossover probabilities at
122 each bp of the chromosome. While this initial map reflects crossover probabilities of individual
123 read pairs, our goal is to produce a recombination map that integrates probabilities over all
124 read pairs. To achieve this, `Hi-reComb` employs an Expectation-Maximization (EM)
125 procedure (Dempster et al. 1977), which is very similar to the EM procedure employed by
126 (Halldorsson et al. 2019). For a detailed description of the initial map construction and of our
127 EM procedure see **Supplementary Note 1**.

128 The core algorithm is supported by several practical heuristics that we found improve its
129 overall performance. The first heuristic concerns an 'edge effect' whereby the *effective*
130 *coverage* – i.e., the number of informative read pairs that span a given genomic interval –
131 drops towards chromosome ends (**Figure S1**). Reduced effective coverage leads to increased
132 sampling noise, which propagates through the EM algorithm and can affect genomic regions
133 far beyond the chromosome edges. To alleviate this problem, we have introduced a minimum
134 effective coverage cutoff around chromosome edges, whereby the genomic intervals beyond
135 the cutoff limits are excluded from the EM procedure and from the resulting recombination
136 maps. Second, if unexpectedly many read pairs are all bounded by the same SNP and/or if
137 unexpectedly many of these read pairs indicate crossovers (i.e., $X = C$), we eliminate these
138 read pairs and SNPs from consideration before starting the recombination map reconstruction.
139 This reduces errors arising from incorrect variant calling and/or phasing. Finally, the third

140 heuristic involves adjusting crossover likelihoods for long-insert read pairs (default >1Mb) to
141 consider the probability of double crossovers. In this calculation, the probability p_d of two
142 crossovers is given by a Poisson mass function: $p_d = \lambda^2 * e^{-\lambda}/2$, where $\lambda = r_l^*$.

143 **Implementation**

144 The `Hi-reComb` package is efficiently coded in C++, does not have any external
145 dependencies, and is straightforward to install, compile and use. It is open source and freely
146 available from https://github.com/millanek/Hi-reComb. `Hi-reComb` currently contains two
147 core modules and two additional utilities. The first core module, `FindInfoPairs` processes
148 aligned Hi-C read pairs to find the pairs that are informative with regards to crossovers (**Figure**
149 **1**). It takes as an input a set of phased heterozygous sites in the HapCUT2 format (Edge et
150 al. 2017) and Hi-C read pairs in SAM / BAM format (Danecek et al. 2021) and outputs only
151 pairs of Hi-C reads that cover at least one phased heterozygous site each. The second core
152 module, `RecombMap` performs recombination map inference as described above, with run
153 times of the order of minutes for each chromosome. In addition, `RecombMap` provides a
154 bootstrap option to evaluate uncertainty, whereby informative Hi-C read-pairs are resampled
155 with replacement to estimate additional genetic maps. This option also allows taking an
156 average across the bootstraps, which tends to result in smoother maps than a single run
157 estimate.

158 The `Hi-reComb` package also includes a tool to simulate informative Hi-C read pairs reflecting
159 a known recombination map. This `Simulate` utility enables users to evaluate the accuracy of
160 recombination map inference for a given effective coverage, error rate, insert-size distribution,
161 and map profile. The utility matches the insert size distribution of the simulated pairs to real
162 Hi-C read pair dataset provided as an input. Each simulated read pair is placed randomly onto
163 a chromosome, and its crossover status (i.e., $X = C$ or $X = N$) is determined by the
164 centimorgan (cM) distance from the input recombination map with an error rate (both false

165 positive and false negative) determined by the `--errorRate` parameter. Read pairs are
166 simulated until reaching a target effective coverage, which is specified by the `--
167 targetCoverage` parameter. The simulated reads are used to reconstruct a recombination
168 map as in the `RecombMap` module. The accuracy of map reconstruction for the given
169 parameters can then be evaluated by comparison with the input map. This procedure can be
170 repeated multiple times using the `--replicates` parameter.

171 Because the vast majority of read pairs do not have a crossover between them, it is possible
172 to use the sperm Hi-C reads to determine donor haplotype phasing, for example using
173 HapCUT2 (Edge et al. 2017). In this case, no other data is required. This approach works well
174 in practice, in part because of the robustness of `Hi-reComb` to errors, including phasing
175 errors, as we demonstrate in the Results section below. However, more accurate phasing can
176 be obtained, for example with Hi-C data from a somatic tissue or by using mother-father-
177 offspring trio data. For the latter purpose `Hi-reComb` provides the `TrioPhase` utility, which
178 takes as input a VCF file specifying heterozygous sites in the donor and another VCF file with
179 his parents who have been genotyped at the same loci. The `TrioPhase` utility outputs phased
180 haplotypes in the same format used by the core modules of `Hi-reComb`.

181 **Sperm Hi-C data**

182 We obtained sperm Hi-C data from one individual each of cichlid fish species *Aulonocara*
183 *stuartgranti* and *Astatotilapia calliptera* (from Lake Malawi), *Neolamprologus multifasciatus*
184 (from Lake Tanganyika), *Astatotilapia nubila* (from the swamps of Lake Victoria), and
185 threespine stickleback *Gasterosteus aculeatus* (from Walby Lake in Alaska), all obtained from
186 laboratory aquarium stocks. A single sexually mature male was sacrificed from each species
187 and his freshly harvested testes were flash-frozen in liquid nitrogen. Upon thawing at room
188 temperature, we cut open the testes and suspended the sperm in $\sim 100\mu l$ of TE buffer. Sperm
189 cells were counted with hemocytometer, aiming for between 100k and 1 million cells. This was

190 then used as input into the standard Dovetail Omni-C library preparation, following the
191 standard protocol. All libraries were sequenced on the Illumina Novaseq 6000 instrument,
192 obtaining paired end 2x150bp reads.

193 **Reference genomes and Hi-C scaffolding**

194 We first used to Hi-C reads from *A. calliptera* and from *N. multifasciatus* to produce accurate
195 reference genome assemblies with chromosome-scale scaffolds. For *A. calliptera*, we used
196 as a starting point the fAstCal1.2 genome (GenBank: GCA_900246225.3). This genome
197 assembly was already chromosome-scale, with scaffolding using 10x Genomics Chromium
198 linked reads, BioNano Irys optical maps and two low-resolution genetic maps (Quin et al. 2013;
199 Albertson et al. 2014). However, we found about a hundred disagreements between that
200 assembly and the Hi-C contact map. These discrepancies were corrected manually using the
201 PretextView software (https://github.com/sanger-tol/PretextView). The new manually curated
202 genome was used as a reference for recombination analyses and was deposited as fAstCal1.5
203 under GenBank accession GCA_900246225.6. For *N. multifasciatus*, we used as a starting
204 point the fNeoMul1.1 genome (GCA_963576455.1), which was not chromosome-scale but
205 was fragmented in 378 large contigs. To produce chromosome scale scaffolds for this species,
206 we used the YaHS Hi-C scaffolding tool with default parameters (Zhou et al. 2022). The new
207 scaffolded genome fNeoMul1.2 (GCA_963576455.2) was then used for recombination
208 analyses. Finally, for *G. aculeatus* we used the stickleback v5 reference under
209 GCA_016920845.1.

210 **Alignment and Hi-C contact maps**

211 Hi-C reads were mapped to the reference genomes using `bwa mem` v 0.7.17 (Li 2013) with
212 the `-5SP` and `-T0` options. To generate Hi-C contact maps, we used the `pairtools`
213 (Open2C et al. 2024) software (v. 1.1.0), using the `--min-mapq 30`, `--walks-policy`
214 `5unique`, `--max-inter-align-gap 30`, and `--chroms-path` options for the `parse`

215 command, and using the `dedup` command with default parameters. The 'pairs' files were then
216 used as input into the `juicer_tools` (v 1.22.01) `pre` command from the Juicebox package
217 (Durand et al. 2016) which was also used for contact map visualization.

218 **Variant calling, filtering, phasing**

219 To remove duplicates for all purposes other than the Hi-C contact maps above, we used the
220 `MarkDuplicates` command from the `picard` package (v 2.26.6) with the option
221 `REMOVE_DUPLICATES=true`. Variant calling was done separately for each individual with
222 bcftools v.1.16 (Danecek et al. 2021) using `mpileup --count-orphans -Ou` output piped
223 into the `call` program with `-mv -Oz` options. For variant filtering we used a mappability mask,
224 whereby we broke down the genome into overlapping k-mers of 150bp (matching the read
225 length), mapped these k-mers back to the genome, and masked all sites where fewer than
226 90% of k-mers mapped back to their original location perfectly and uniquely. Next, we filtered
227 variants based on sequencing depth, with limits based on examining the coverage histogram
228 for each sample: removing variants with depth ≤ 12 and ≥ 75 for *A. calliptera*, ≤ 35 and $\geq$
229 140 for *A. stuartgranti*, ≤ 20 and ≥ 120 for *N. multifasciatus*, ≤ 35 and ≥ 120 for *A. nubila*, and
230 ≤ 8 and ≥ 100 for *G. aculeatus*. Finally, we applied the following hard filters, removing variants
231 where any of these applied: `%QUAL<20`, `MQ<40`, `MQ0F>0.4`, `RPBZ<-5.0`, or `RPBZ > 5.0`.
232 After variant filtering, we kept only biallelic SNPs with heterozygous genotypes. To estimate
233 the allelic phase of these SNPs, we used `hapcut2` (v 1.3.4). After dividing the VCF files and
234 the alignment bam files per chromosome, for each chromosome we ran first the
235 `extractHAIRS` and then the `hapcut2` commands, both with the `--hic 1` option.

236 **Stickleback trio-based phasing – data and processing**

237 The *G. aculeatus* sperm donor individual originated from an aquarium F1 cross from wild-
238 caught parents. We sequenced the DNA of his parents to trio-phase the heterozygous SNPs
239 in this individual using Mendelian inheritance logic (trio phasing). The sequencing was done

240 on the Illumina Novaseq 6000 instrument with 2x150bp reads as a part of a larger DNA
241 sequencing of 24 individuals, each at approximately 25x coverage. For processing this dataset
242 we used alignment with `bwa mem` v 0.7.17 (Li 2013) with default options, followed by the
243 `MarkDuplicates` command from the `picard` package (v 2.26.6) with default options. Then
244 we used GATK v 4.2.3 (DePristo et al. 2011) to call variants, using `HaplotypeCaller` in
245 GVCF mode for each individual separately followed by joint genotyping using
246 `GenotypeGVCFs` with the `--include-non-variant-sites` option. For variant filtering,
247 we generated a callability mask to identify and filter out regions where we were unable to
248 confidently call variants. This included: (i) sites determined by overall read depth cutoffs based
249 on examining a depth histogram (≤ 300 and ≥ 700 on autosomes; ≤ 200 and ≥ 600 on the X
250 chromosome), (ii) sites where > 6 individuals had missing genotypes, (iii) sites identified by
251 GATK as low quality (with the `LowQual` tag) and (iv) sites with poor mappability. The
252 mappability mask was determined in the same way as for the Hi-C analyses above: we broke
253 down the genome into overlapping k-mers of 150bp (matching the read length), mapped these
254 k-mers back to the genome, and masked all sites where fewer than 90% of k-mers mapped
255 back to their original location perfectly and uniquely. Finally, we used several hard filters based
256 on GATK best practices, specifically focusing on overall genotype quality (`QUAL<20`), mapping
257 quality (`MQ<40`), mapping strand bias (`FS>40`), variant quality normalized by depth (`QD<2`) and
258 excess heterozygosity when compared with Hardy-Weinberg equilibrium (`ExcessHet>40`).

259 **Stickleback trio-based phasing – Hi-reComb trio phasing module**

260 We used the utility `TrioPhase` from `Hi-reComb` to estimate the haplotype phase of the *G.*
261 *aculeatus* sperm donor individual using mother-father-offspring trio genotype calls and simple
262 Mendelian logic. For each heterozygous SNP in the offspring, the utility first checks that both
263 alleles are present in the parents at the same site. If both parents are heterozygous then the
264 SNP cannot be phased because the parental genotypes are not informative. In all other cases,

265 the allele inherited from the first parent is added to haplotype 1 and the allele inherited from
266 the second parent is added to haplotype 2. The phased haplotypes are output as a single
267 block in the `hapcut2` format.

268 **Stickleback LD-based map**

269 We used a dataset of 334 Alaskan stickleback individuals sequenced to mean 19.6x coverage
270 (min: 13.4x; max: 25.8x) on the Illumina Novaseq 6000 instrument with 2x150bp reads.
271 Alignment, variant calling, and variant filtering was done in the same way as for the trio-based
272 phasing dataset (see above), except that the overall depth filter was set at ≤ 4000 and ≥ 8000
273 on autosomes; ≤ 3000 and ≥ 7000 on the X chromosome and the maximum number of
274 missing genotypes to 66 (i.e. $\leq 20\%$ missingness).

275 This dataset included 23 individuals from Walby Lake, the same population as the sperm
276 donor. To estimate changes in effective population size (N_e) through time, we used smc++
277 v.1.15.4 (Terhorst et al. 2017), with the commands: vcf2smc -> estimate. Then we used the
278 pyrho (Spence and Song 2019) software to infer recombination rates along the genome based
279 on patterns of LD. To build likelihood tables for pairs of biallelic sites, we used the
280 `make_table` command with demographic history as inferred by smc++, and the Moran
281 approximation specified by the `--approx` and `--moran_pop_size N` flags where N equals
282 1.5× the number of haplotypes. This was followed by the pyrho `optimize` command to infer
283 the recombination maps with a window size of 50 SNPs and block penalty of 15.

284 # Results

285 **Sperm Hi-C datasets, contact maps, and genome scaffolding**

286 Key characteristics of the sperm Hi-C datasets presented in this manuscript are summarized
287 in **Table 1**. A statistic that has a crucial effect on recombination inference is the *effective*
288 *coverage*. Effective coverage reflects the total length of DNA segments that can be assessed

289 for presence of crossovers and is determined not only by the depth and quality of the Hi-C
290 library but is also substantially influenced by the heterozygosity of the donor individual. The
291 greater the heterozygosity, the greater is the chance that each of the two fragments of a Hi-C
292 read pair covers a heterozygous site and thus is informative, as illustrated in **Figure 1**. This
293 effect is clearly seen in the *G. aculeatus* dataset, where the Hi-C library was of relatively low
294 quality and contained only ~2.6 million read pairs mapping to the same chromosome with >1kb
295 insert, an order of magnitude lower than all the other samples, likely due to the much lower
296 amount of sperm cells used as a starting material. Despite this, the effective coverage for *G.*
297 *aculeatus* is comparable to the other datasets.

298 **Table 1: Sperm Hi-C data overview.**

Species	Raw coverage	Cis >1kb reads pairs (millions)	Heterozygous sites per kb	Effective coverage (chr 1)
A. calliptera	92x	18.7	1.16	292x
A. nubila	140x	48.2	1.55	3,587x
A. stuartgranti	185x	60.1	0.94	1,253x
G. aculeatus	270x	2.6	2.61	655x
N. multifasciatus	165x	40.4	1.55	1,369x

299 The use of the Hi-C data for scaffolding of the *N. multifasciatus* genome resulted in 863.6Mb
300 (98.2%) of sequence being assigned to 22 chromosomes, while the remaining 67 unplaced
301 scaffolds comprise 16Mb of sequence. The *N. multifasciatus* Hi-C contact map mapped to this
302 new fNeoMul1.2 assembly (GCA_963576455.2) is shown in **Figure S2**. The use of the Hi-C
303 data for scaffolding of the *A. calliptera* genome resulted in 863.0Mb (98.3%) of sequence being
304 assigned to 22 chromosomes, while the remaining 122 unplaced scaffolds comprise 14.7Mb
305 of sequence. The *A. calliptera* Hi-C contact map mapped to this new fAstCal1.5 assembly
306 (GCA_900246225.6) is shown in **Figure S3A** and examples of how disagreements between
307 the Hi-C contact map and the previous version of the assembly were resolved are shown in
308 **Figure S3B**. For *A. calliptera*, the chromosome count is as expected, based on known
309 karyotypes of Lake Malawi cichlids (Poletto et al. 2010; Conte et al. 2019), and matches the
310 previous assembly. On the other hand, karyotypes of other species of the cichlid tribe

311 Lamprologini, to which *N. multifasciatus* belongs, showed only 21 chromosomes (Ozouf-
312 Costaz et al. 2017); therefore, our results reveal previously unknown chromosome number
313 polymorphism in this tribe.

314 Hi-C contact maps provide information regarding large-scale structural variation present in the
315 donor individual. Perhaps the most prominent of these is an inversion in *A. stuartgranti* with
316 respect to the *A. calliptera* reference, located in the middle of chromosome 2 (~ 12.7Mb –
317 16.7Mb), corresponding to the 'small' inversion previously reported by (Blumer et al. 2024).
318 Notably, we found that this inversion is surrounded by an extended region of very low
319 recombination in *A. stuartgranti* (**Figure S4**), illustrating how sperm-based Hi-C contact maps
320 and recombination maps can be used together to better understand the interaction between
321 structural variation and recombination.

322 **Hi-reComb recombination inference from simulations**

323 To evaluate the accuracy of recombination map inference with `Hi-reComb`, we used the
324 `Simulate` utility and explored how the performance is influenced by key parameters: the error
325 rate and the effective coverage. For each run, we supplied the `Hi-reComb Simulate` utility
326 with a reference map, simulated ten replicate Hi-C datasets from this map, and then ran
327 recombination map inference for each replicate.

328 **Figure 2A** shows an example of ten replicate maps reconstructed from simulations with 1%
329 error rate and 3,000x effective coverage, with chr 2 of *A. stuartgranti* as a reference map. With
330 these parameters, the reconstructed maps showed correlation with the truth of between 0.75
331 and 0.97 (orange line in **Figure 2B**), depending on the resolution at which we measured the
332 correlations. In subsequent runs, we varied the effective coverage between 500x and 3,000x,
333 matching approximately the range of coverage found in our empirical datasets (**Table 1**), while
334 keeping the error rate at 1%. The results, shown in **Figure 2B**, revealed that decreasing the

335 effective coverage reduces the accuracy of inferred maps, as expected. However, even at the
336 relatively low effective coverage of 500x, the reconstructed maps show substantial positive
337 correlation with the truth – on average 0.53 at 2kb scale and 0.90 at 5Mb scale.

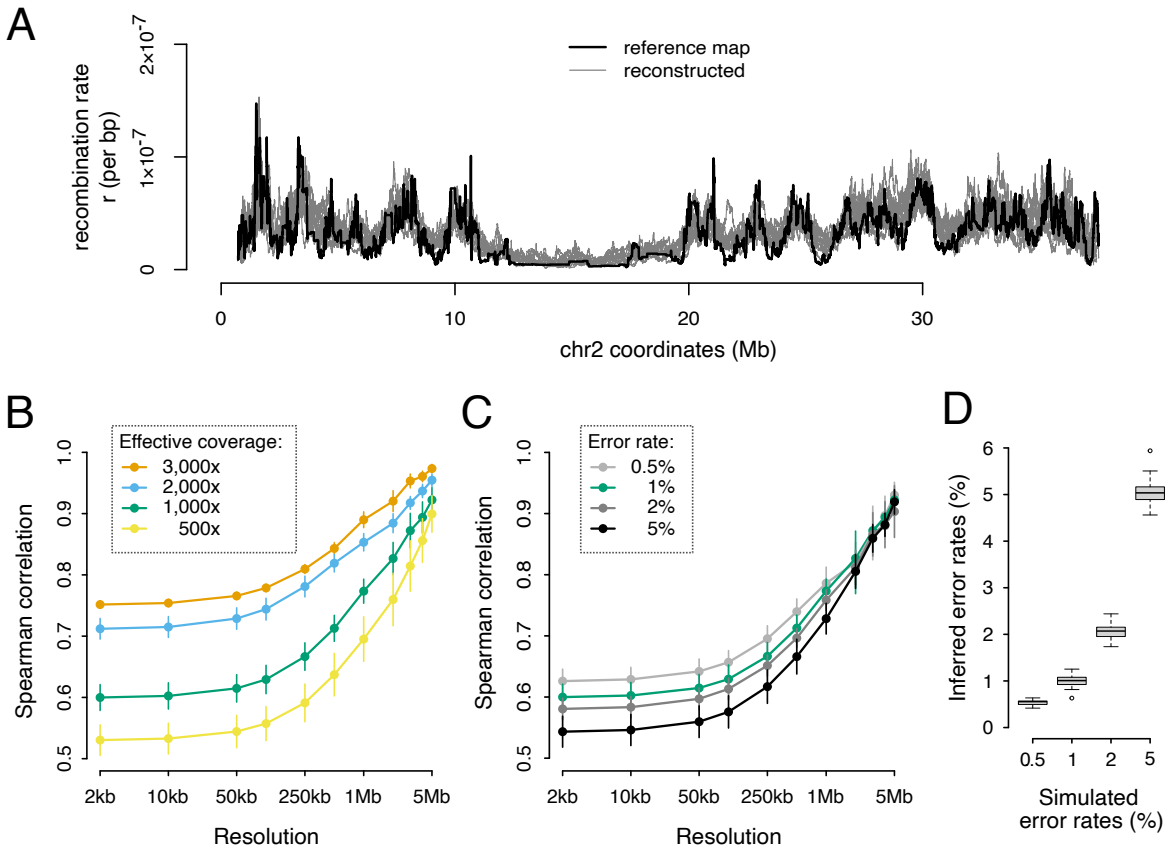

Figure 2: Hi-reComb recombination map inference from simulated data. (**A**) A comparison of a reference map against ten maps reconstructed by `Hi-reComb` from simulated data at 3,000x effective coverage and 1% error rate. (**B**) The dependence of accuracy of recombination map reconstruction on the effective coverage. (**C**) The dependence of accuracy of recombination map reconstruction on the error rate. (**D**) The accuracy of error rate estimation by `Hi-reComb`.

338 Next, we explored the impact of the error rate on recombination map inference. We fixed the
339 coverage at 1,000x and varied the error rate from 0.5% to 5%. We found that at the high error
340 rate of 5%, `Hi-reComb` still infers maps with highly positive correlation with the truth (0.54 at
341 2kb; 0.92 at 5Mb), which is only a fraction lower than at the 0.5% error rate where the
342 correlations are 0.63 at 2kb and 0.93 at 5Mb scale (**Figure 2C**). The resilience to a relatively
343 high degree of error is partly due to the ability of `Hi-reComb` to accurately infer the error rate

344 from the data: at 1,000x effective coverage, all inference runs reported error rate estimates
345 within a very narrow range of the truth (**Figure 2D**).

346 **Empirical recombination maps**

347 We used `Hi-reComb` to infer genetic maps for the five datasets described in **Table 1**. Error
348 rates estimated from these empirical datasets were within the range where simulations
349 demonstrated reliable performance, with means between 0.8 and 1.9% in cichlids and 0.6%
350 in stickleback (**Figure 3A**). The highest error rates of over 4% were found in cichlids on
351 chromosome 3, which contains by far the most highly repetitive sequence where variant calling
352 is difficult (**Figure S5**). This, along with the lower error rate found in sticklebacks, which have
353 a genome with a much lower proportion of repetitive elements than cichlids (**Figure S5**), shows
354 the extent to which error rates are affected by miscalled SNPs in repetitive regions of the
355 genome. Among the cichlids, we see a link between error rates and effective coverage, with
356 the low-coverage *A. calliptera* having the highest error rate, followed by the medium coverage
357 *A. stuartgranti* and *N. multifasciatus*, and the lowest error rate among cichlids is in the high-
358 coverage *A. nubila*.

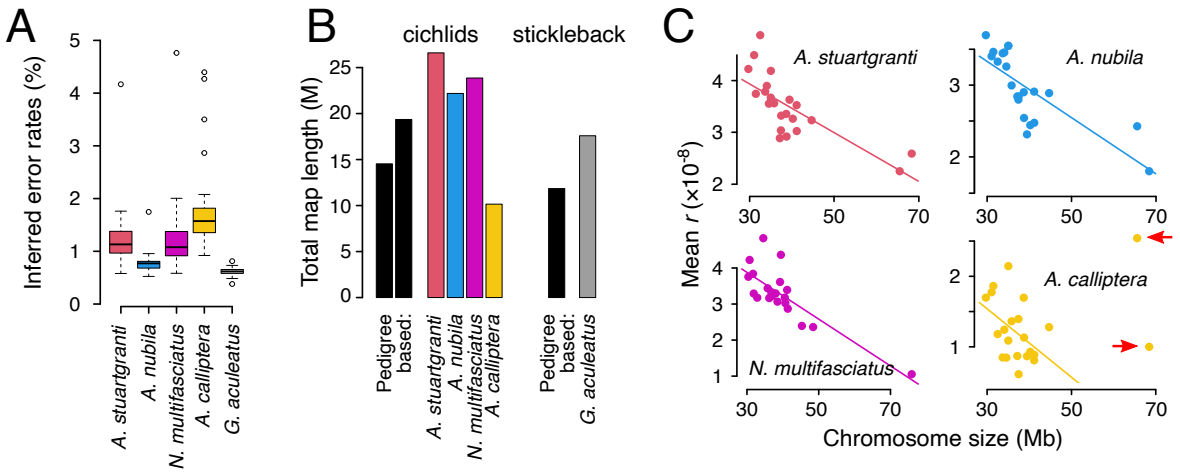

Figure 3: An overview of Hi-C inferred maps. (**A**) The error rates estimated by `Hi-reComb` from empirical data. (**B**) Recombination map lengths in Morgans (M) compared against three previously published pedigree-based maps (for cichlids, left: Albertson *et al. (Albertson et al. 2014)* ; right: O'Quin *et al. (Quin et al. 2013)*; for stickleback: Roesti *et al. (Roesti et al. 2013)*. (**C**) The negative relationship between the chromosome size and the mean recombination rate in cichlids. The two large chromosomes that were excluded in *A. calliptera* as outliers are highlighted with arrows.

359 The inferred cichlid recombination maps varied in length from 1,015 centimorgan (cM) for *A.*
360 *calliptera* to 2,660 cM for *A. stuartgranti*, which falls both below and above two previously
361 published pedigree-based maps for Lake Malawi cichlids that had lengths of 1,453 cM
362 (Albertson et al. 2014) and 1,935 cM (Quin et al. 2013). For stickleback, the inferred map had
363 a length of 1,758 cM which is somewhat above the 1,184 cM previously reported from a
364 pedigree-based map (Roesti et al. 2013) (**Figure 3B**). However, we note that the absolute
365 map lengths should be interpreted with caution for at least two reasons. First, differing levels
366 of somatic cell 'contamination' across samples would affect the overall rate comparisons.
367 While we tried to separate sperm from the surrounding tissues, examination under the
368 microscope showed that our biological samples contained a small number of somatic cells,
369 and we did not conduct further cell purification or sorting. Second, the edge effect where
370 effective coverage decreases towards the ends of each chromosome (see **Methods**) can have
371 different impact across individuals with different mean effective coverage.

372 It is known that in many species, there is a negative association between the chromosome
373 length (in Mb) and the mean per-bp recombination rate (mean r)(Haenel et al. 2018; Brazier
374 and Glémin 2022) . Consistent with these previous studies, our results also indicate a strong
375 negative correlation in both cichlids and in stickleback. In cichlids (**Figure 3C**), the negative
376 link was very clear across all chromosomes for *A. stuartgranti* ($r^2 = 0.55$; $p = 7.9 \times 10^{-5}$), *A.*
377 *nubila* ($r^2 = 0.59$; $p = 3.1 \times 10^{-5}$), and *N. multifasciatus* ($r^2 = 0.64$; $p = 7.1 \times 10^{-6}$). In *A.*
378 *calliptera*, the two large chromosomes (chr 3 and chr 7) were outliers and the negative
379 association between chromosome length and the mean recombination rate was present only
380 if these two chromosomes were excluded (complete dataset: $r^2 = 0.02$; $p = 0.53$; outliers
381 excluded: $r^2 = 0.20$; $p = 0.049$). In stickleback, the negative association between the
382 chromosome length and mean r was very strong in maps based on the trio phasing, which
383 will be described below ($r^2 = 0.71$; $p = 3.0 \times 10^{-6}$; **Figure S6**).

384 To estimate uncertainty in reconstructed recombination landscapes, `Hi-reComb RecombMap`
385 includes a bootstrap procedure whereby informative read pairs are resampled with
386 replacement. As an example, **Figure 4A** shows a recombination landscape for chr 4 of *A.*
387 *stuartgranti* with 95% confidence intervals (95% CIs) estimated based on 50 bootstrap
388 replicate runs. Importantly, the bootstrap estimates facilitate comparisons among
389 recombination landscapes. We define the areas where 95% CIs of two maps do not intersect
390 as areas of significant recombination rate differences, or $\Delta(r)$ regions. To illustrate this
391 functionality, **Figure 4B** shows comparisons between the maps of *A. stuartgranti* and *A.*
392 *nubila*. Overall, we found that $\Delta(r)$ regions between these two species comprised 62.3 Mb of
393 sequence, or 7.23% of the genome, with variation across chromosomes between 3.1% and
394 14.7%. To account for the fact that mean rates differ between the two maps, we also
395 normalized the means before calculating the $\Delta(r)$ regions. On these mean-normalized maps,
396 $\Delta(r)$ regions comprised 45.7 Mb of sequence, or 5.30% of the genome, with variation across
397 chromosomes between 2.8% and 11.0%.

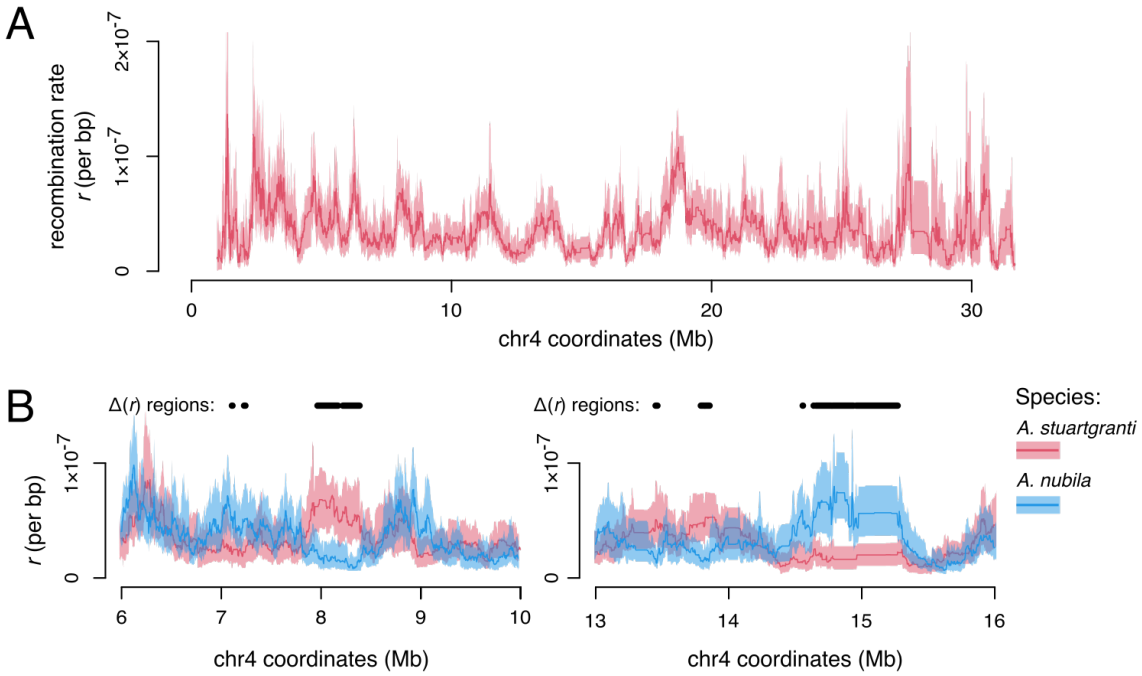

Figure 4: Bootstrap facilitates comparisons between recombination landscapes. (**A**) Chromosome 4 of *A. stuartgranti* with recombination landscapes based on the mean of 50 bootstrap replicates (thick line) and 95% confidence intervals (shaded areas). (**B**) Examples of comparisons between the maps of *A. stuartgranti* and *A. nubila*, highlighting areas of significant recombination rate differences between the maps (the $\Delta(r)$ regions).

398 **Comparisons with trio phasing and with LD-based maps**

399 Our standard workflow uses the same Hi-C dataset for both haplotype phasing and for
400 recombination map inference (see **Methods**). To compare this approach with independent
401 trio-based phasing, we took advantage of the fact that the *G. aculeatus* donor individual was
402 bred in an aquarium from known parents. We obtained short read whole genome data from
403 the parents and, after variant calling and filtering, we used `Hi-reComb TrioPhase` to obtain
404 haplotype phase information for ~840 thousand SNPs across the 20 stickleback autosomes.
405 We found that this approach reduced the crossover (false positive/negative) error rate
406 estimated by `Hi-reComb` by more than a third, down to below 0.4% for the *G. aculeatus* data
407 (**Figure 5A**). This result suggests that about a third of the errors in the hapcut2-phased
408 datasets arose due to incorrect phasing.

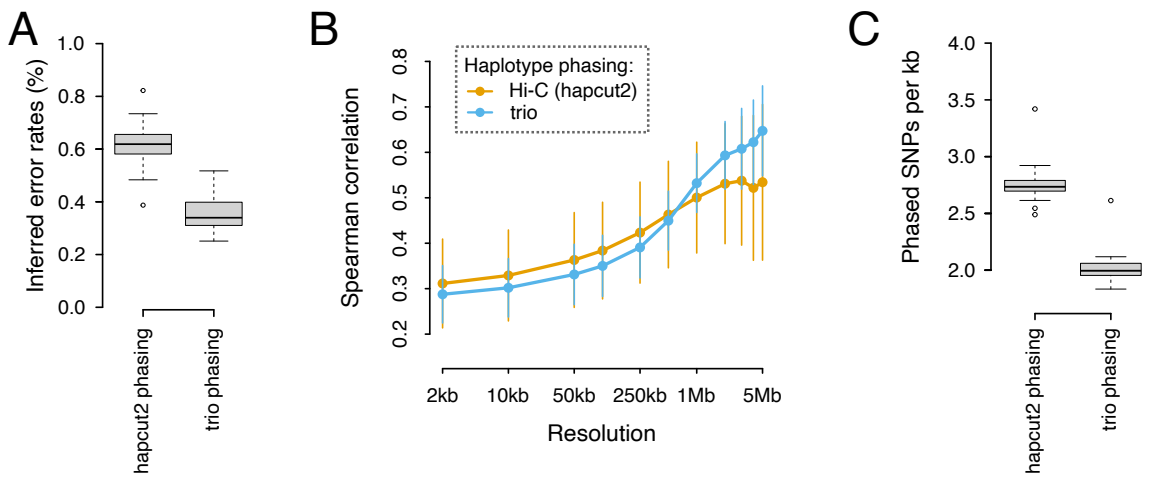

Figure 5: Hapcut2 vs. trio phasing of stickleback data. (**A**) The error rates estimated by `Hi-reComb` for the hapcut2 and trio-phased *G. aculeatus* datasets. (**B**) Correlations between gamete based `Hi-reComb` maps and an LD-based map from the same stickleback population. (**C**) The average density of SNPs phased with the hapcut2 approach and the trio approach. Each datapoint corresponds to one chromosome.

409 To evaluate the accuracy of the recombination maps inferred by `Hi-reComb` from the hapcut2
410 and trio-phased datasets, we compared both against a recombination map obtained for the
411 same stickleback population (Walby Lake, Alaska) using linkage disequilibrium (LD) patterns
412 in an independent population genetic dataset (see **Methods**). While the LD-based map does
413 not represent the truth, we can nevertheless draw some conclusions from the map

414 correlations. As expected, given its lower error rate, the trio-phased dataset delivers more
415 accurate maps reflected in better correlations with the LD-based dataset at resolutions ≥1Mb
416 (**Figure 5B**). However, surprisingly, the pattern changes at finer resolutions (2kb to 500kb),
417 where the hapcut2-phased dataset seems to deliver a more accurate recombination map. The
418 apparent greater accuracy at high-resolution is likely explained by the fact that hapcut2-based
419 phasing, although less accurate, delivers a greater density of phased heterozygous sites along
420 the genome and thus more fine-scale information (**Figure 5C**).

421 Discussion

422 Mapping the distribution of meiotic recombination along chromosomes is a crucial step in
423 many genomic analyses. The `Hi-reComb` software provides a new, straightforward, and cost-
424 effective approach for inferring recombination maps, based on sequencing of a Hi-C library
425 from gametes from a single individual. In this manuscript we show results based on fish sperm,
426 but the method will be applicable to a broad range sexually reproducing species. Nevertheless,
427 at least two factors can limit the applicability of `Hi-reComb`. First, it is necessary to obtain a
428 relatively large number of gametes from a single individual, in the range between 100
429 thousand and 1 million for the Hi-C protocol we used. While this is easily achievable for males
430 of many larger species (e.g., most vertebrates), the cell count requirement will be challenging
431 to fulfil for males of smaller species and almost always for females who rarely produce such
432 large numbers of gametes. Second, it is necessary that the donor individual has sufficient
433 heterozygosity. We have demonstrated that heterozygosity of ~1 SNP per thousand basepairs
434 is sufficient (**Table 1**), a value that is towards the lower end of nucleotide diversity range across
435 sexually reproducing species(Leffler et al. 2012; Romiguier et al. 2014). Therefore, individuals
436 from most natural populations will be sufficiently heterozygous. However, the heterozygosity
437 requirement will pose a limitation for obtaining recombination maps from individuals who are
438 inbred in nature or due to human manipulation.

439 `Hi-reComb` is well suited for comparisons of recombination among individuals of the same or
440 closely related species, facilitated by the bootstrapping option as illustrated in **Figure 4**. It will
441 be interesting to learn more about how much variation there is across individuals, where
442 current knowledge of recombination landscape variation is limited(Johnston et al. 2016;
443 Peñalba and Wolf 2020), with studies mostly focusing on genome-wide crossover
444 counts(Payseur 2024). An important aspect of inter-individual variation in recombination is the
445 difference between sexes (known as heterochiasmy), which is known to be considerable, at
446 least in some species(Sardell and Kirkpatrick 2019). In this context, it should be noted that the
447 `Hi-reComb` approach is limited to diploid sequences and, therefore, we were not able to
448 obtain the X or Y chromosome maps for *G. aculeatus* in which there are large regions of
449 hemizygosity due to degeneration on the Y chromosome(Peichel et al. 2020) .

450 We used a straightforward protocol to illustrate the potential of `Hi-reComb` for routine
451 recombination map inference. At the same time, it is possible to envisage several
452 improvements to the protocol. For example, cell sorting or purification prior to library
453 preparation that ensures that only gametes are used would deliver recombination rate
454 quantification that is more accurate and comparable across individuals. It could also be
455 beneficial to take into account that chromatin in gametes can be distinct from somatic cells,
456 especially in sperm where chromatin is highly condensed by protamines(Okada 2022) or by
457 specific histone proteins in flowering plants(Buttress et al. 2022). Chromatin decompaction
458 treatment of sperm cells could deliver an even more uniform coverage and a higher quality of
459 the Hi-C library (e.g., fewer duplicates, larger insert sizes).

460 We envisage that `Hi-reComb` will contribute to our understanding of patterns and of the
461 ultimate causes of recombination rate variation by substantially easing the production of
462 gamete-based recombination maps. Given the strengths and weaknesses of this approach,
463 we also see great potential in combining `Hi-reComb` maps with pedigree- and LD-based

464 maps. Measuring recombination in gametes while assessing which haplotypes are transmitted
465 across generations will shed light on the multifaceted interaction between recombination and
466 selection.

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471 **Data Availability Statement**

472 All raw sequence data are available on NCBI under the following accessions: BioProject
473 PRJNA1133007 (sperm Hi-C of cichlids), BioProject PRJNA1192732 (stickleback sperm Hi-C
474 and whole genome sequences of parents for trio based phasing), BioProject PRJEB49185
475 (stickleback population genetic data). The Hi-C scaffolded cichlid genomes are available
476 under GCA_900246225.6 for *A. calliptera* and GCA_963576455.2 for *N. multifasciatus*.

477 **Author contributions**

478 M.M., R.D., B.S., and R.E.G. conceived and designed the study; M.M. developed the Hi-
479 reComb software and conducted the analyses; M.T., M.M., N.M., and S.S. prepared the Hi-C
480 libraries; C.Z. performed scaffolding of the fNeoMul1.2 genome; M.T. produced the stickleback
481 LD-based maps; J.N.W. bred stickleback for Hi-C sequencing and trio phasing; J.N.W., C.L.P.
482 and D.B. led the fieldwork obtaining stickleback population genetic data; W.S. and O.S. bred
483 cichlid fish for Hi-C sequencing; M.M. wrote the manuscript with comments and input from
484 other co-authors.

485 **Acknowlegements**

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487 discussions, Matthew Chotlos and Adrian Indenmaur for fish husbandry, Carolin Sommer-

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489 sequencing core for DNA sequencing.

490 **Competing interests**

491 R.E.G. is a co-founder of Dovetail Genomics.

492

Supplementary Figures

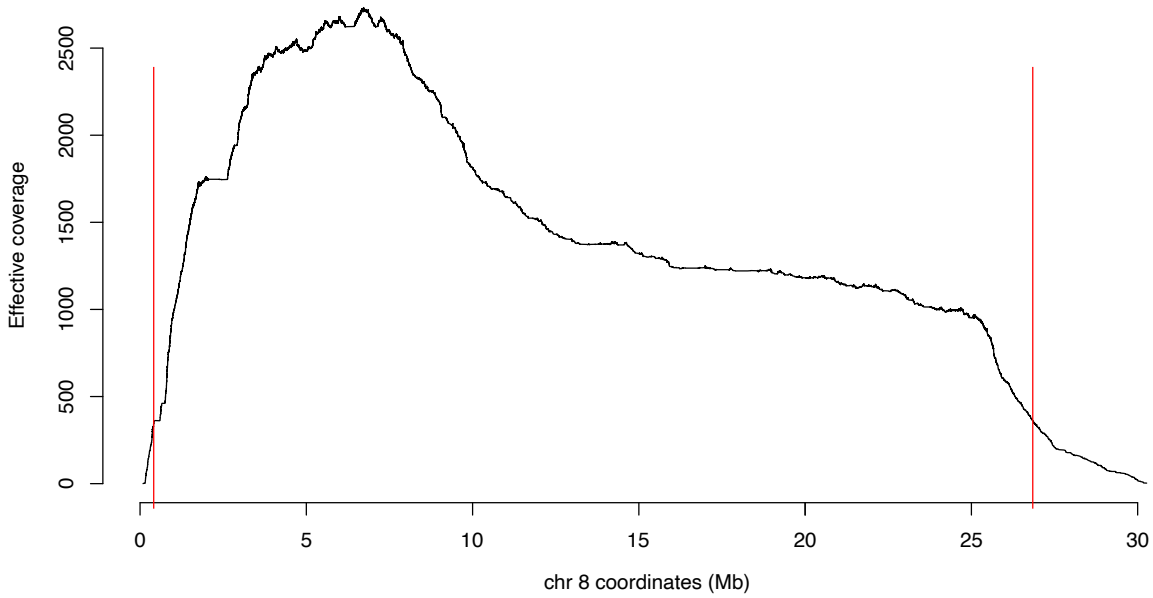

Figure S1: Effective coverage along the chromosome and the 'edge effect'.

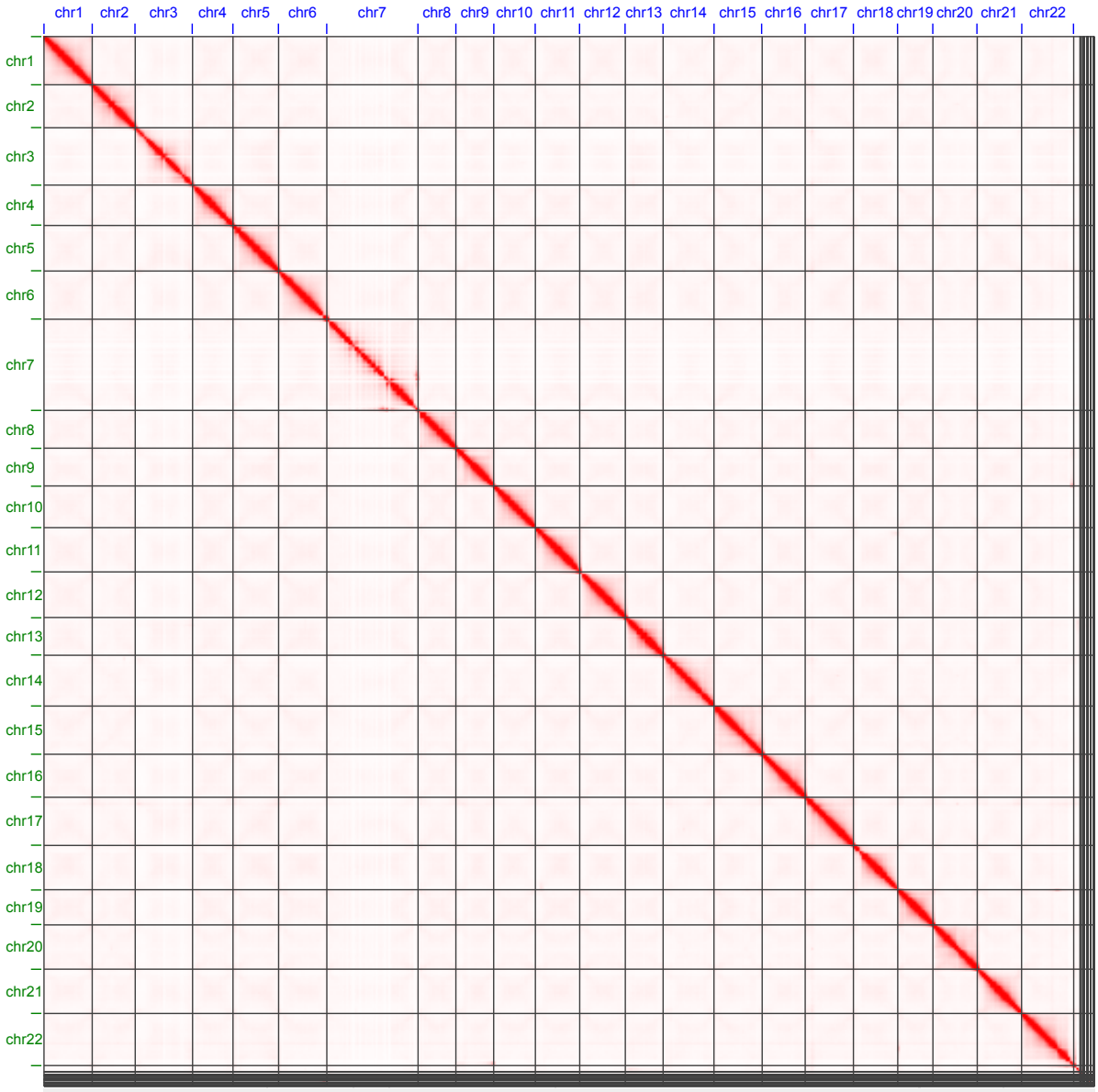

Figure S2: Hi-C contact map for read pairs mapped to the newly scaffolded *Neolamprologus multifasciatus* fNeoMul1.2 reference genome.

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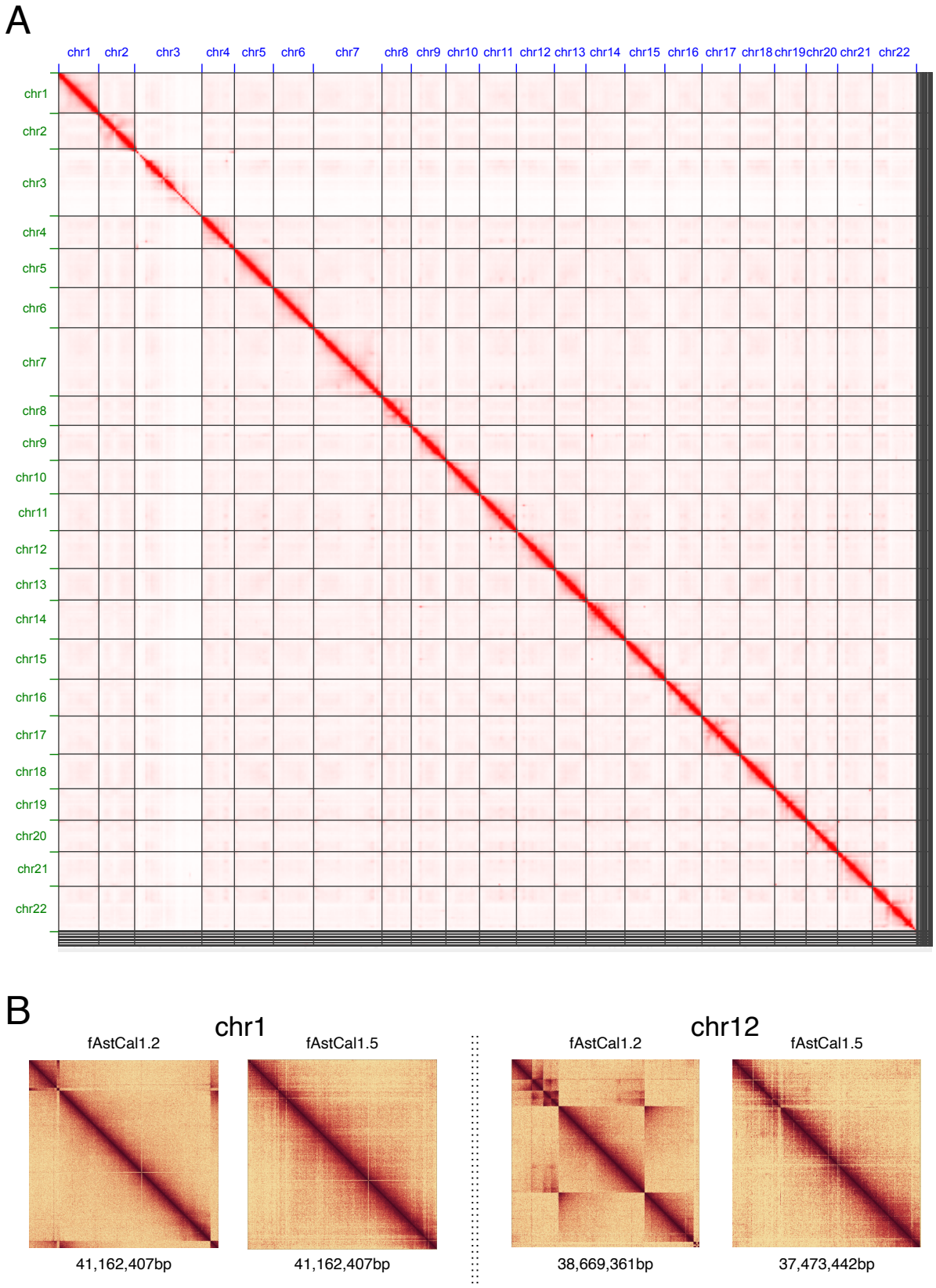

Figure S3: (A) Hi-C contact map for read pairs mapped to the newly scaffolded *Astatotilapia calliptera* fAstCal1.5 reference genome. (B) A comparison of the original fAstCal1.2 vs. the new fAstCal1.5 assemblies for two example chromosomes, showing the resolution of disagreements between the Hi-C contact map and the original assembly.

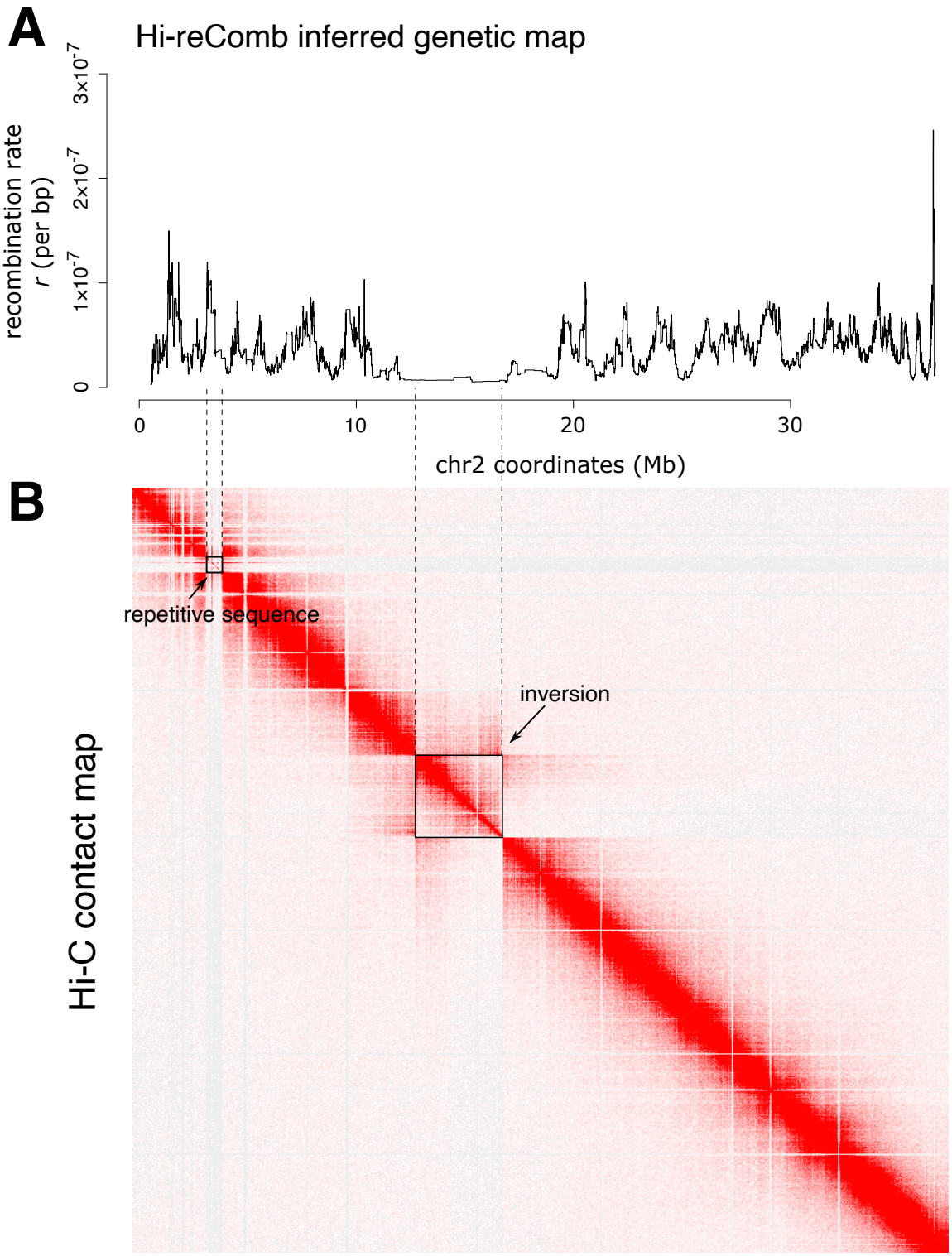

Figure S4: An inversion and suppressed recombination on *A. stuartgranti* chromosome 2. (**A**) The `Hi-reComb` inferred genetic map. (**B**) Hi-C contact map. Both panels are aligned along chr2 coordinates of the *A. calliptera* fAstCal1.5 reference. The figure illustrates a useful advantage of the Hi-C approach: at the same time as being used for recombination inference, the Hi-C contact map provides information about structural variation in the donor individual. Thus, we can see that the region of low recombination in the centre of the chromosome corresponds to and extends beyond the inversion.

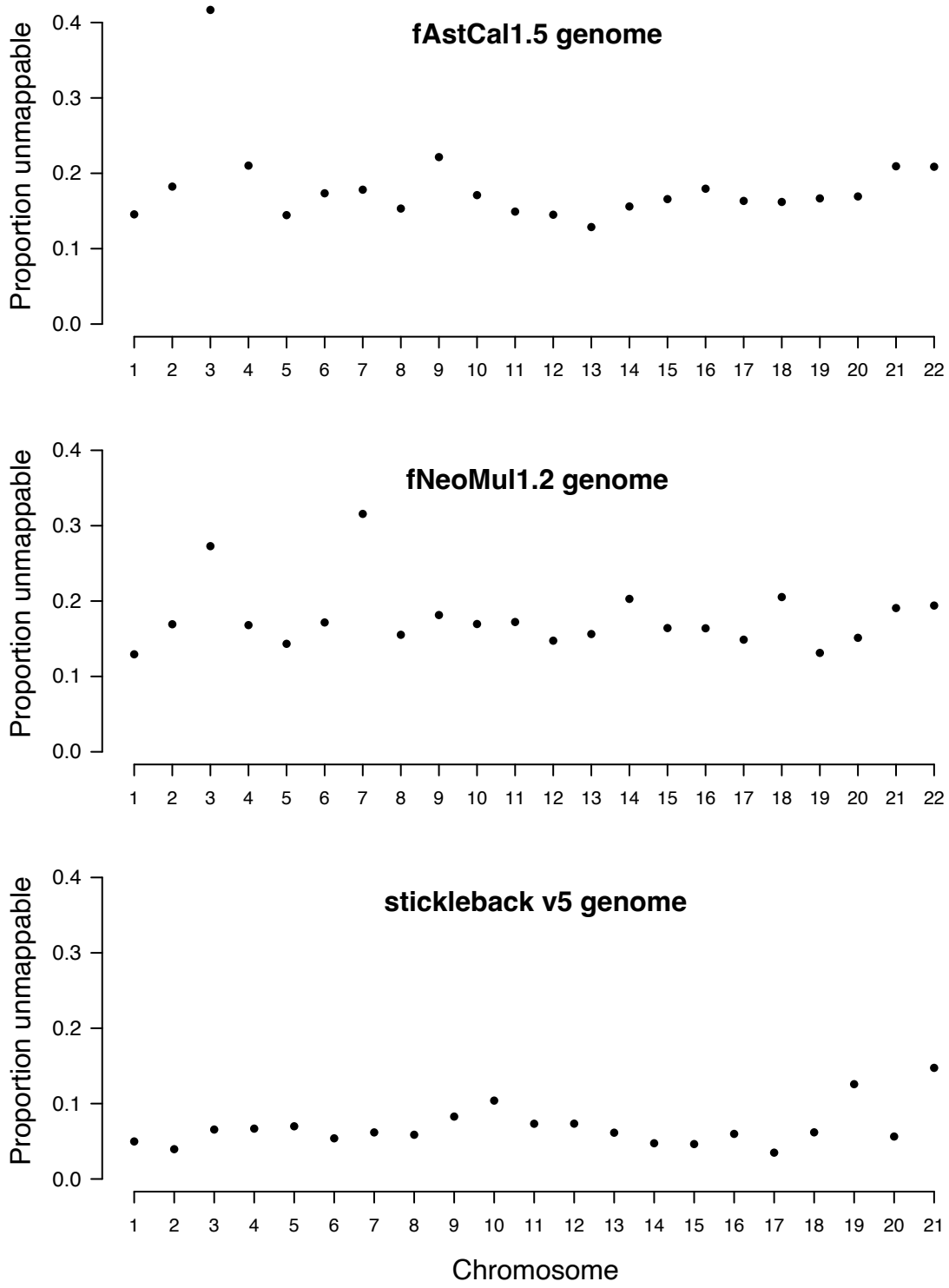

Figure S5: The proportion of unmappable sequence on different chromosomes in the three genome asseblies used in this study. Unmappable sequences are defined as regions covered by the mappability mask (see **Methods**). In the fAstCal1.5, chromosome 3 has by far the most unmmapable sequence (42%), while the next highest number is 22% on chromosome 9. It is also notable that the stickleback assembly contains much less unmappable sequence than the cichlid genomes (stickleback mean: 6.9%; cichlid 17.7% in fNeoMul1.2 and 18.2% in fAstCal1.5).

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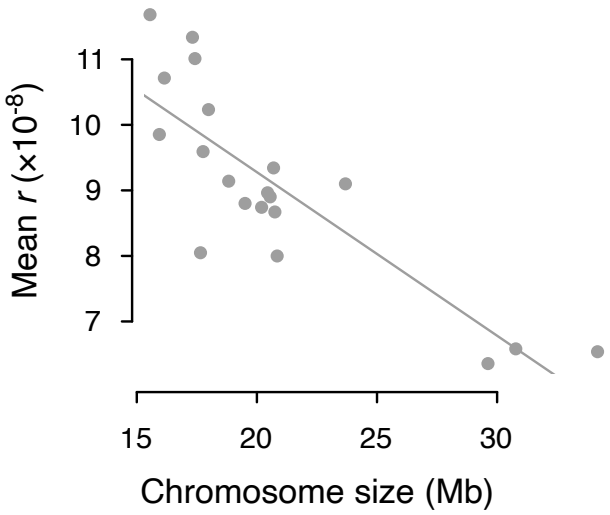

Figure S6: The negative relationship between the chromosome size and the mean recombination rate in stickleback. These results reflect recombination maps based on trio-phased data.

500

Supplementary Note 1: Map inference and the EM procedure

For each chromosome, `Hi-reComb` produces a recombination map that integrates crossover probabilities from all informative read pairs mapping onto that chromosome. The procedure is similar to the algorithm used by Halldorsson et al. (2019)(Halldorsson et al. 2019). Let $\mathbb{P}(c_i)$ be the crossover probability for a read pair i between a pair of defining phase-informative SNPs at physical positions along the chromosome g_{li} and g_{ui}. We use d_i to denote the physical distance between g_{li} and g_{ui}. The per-bp crossover probability p_i^{bp} for the read pair i is then $p_i^{bp} = \mathbb{P}(c_i)/d_i$. Next, let $(g_j)_0^N$ be the ordered sequence of defining SNPs for all informative read pairs and, within this sequence, let l_i and u_i be the indices of SNPs that define the informative read pair i.

We estimate the per-bp recombination rate r_j^{bp} for each genomic interval $I_j = [g_j, g_{j+1})$. We initialize the genetic map by finding all the K read-pairs that span each I_j and setting $r_j^{bp} = \sum_{k=1}^{K} p_k^{bp} / \sum_{k=1}^{K}(1 - \mathbb{P}(c_k))$. The proportion of recombination expected to occur within the interval, the recombination fraction r_j, is estimated as $r_j = r_j^{bp} * (g_{j+1} - g_j + 1)$. The sequence of recombination rate values $(r_j^{bp})_0^{N-1}$ specifies the entire genetic map m on the interval $[g_0, g_N)$.

We use the initial genetic map as an input into the EM procedure. First, we calculate for each read pair with positive crossover probability (i.e., $p_i^{bp} > 0$), the probability that the crossover occurred within a genomic interval I_j. This probablity p_i^j depends on the recombination rates in all the intervals that are covered by the read pair; specifically:

$$p_i^j = \frac{r_j}{\sum_{k=l_i}^{u_i} r_k}$$

An updated genetic map is calculated from these probabilities, with recombination fraction values given by $r_j = \sum_{k=1}^{K} p_k^j / \sum_{k=1}^{K}(1 - \mathbb{P}(c_k))$, where the sums are again over all the K read-

pairs that span the interval I_j. With the updated map, we then calculate new values for p_i^j then update genetic map again, and continue for `--maxEM` iterations or until convergence is reached, as defined by $\sum_{j=0}^{N-1}\left|r_j^m - r_j^{m-1}\right| < \varepsilon$, where the superscript m denotes iteration number m and ε is provided by the user via the `--epsilon` parameter.

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