Recombination in the eggs and sperm in a simultaneously hermaphroditic vertebrate

L. Theodosiou, W. O. McMillan, O. Puebla

Electronic Supplementary Material

Electronic Supplementary Methods

Experimental crosses

Crosses were carried out in Bocas del Toro (Panama) between April 7th and 13th 2013. Adult black (*H. nigricans*) and barred (*H. puella*) hamlets were collected in Punta Caracol (9° 21.967' N 082° 17.477' W) with hook-and-line while scuba diving. Individuals were collected no deeper than 30 feet, in the early afternoon to ensure that new eggs are hydrated on the day of capture while also leaving at least two hours to acclimate to the tanks before spawning. In order to limit decompression issues individuals were slowly brought up to 6 feet in a mesh bag and left at this depth in a mesh cage for at least 20 minutes. The fish were then transferred to the Smithsonian Tropical Research Institute's research station in a cooler filled with seawater and placed two by two (of the same species) in 29 x 45 x 30 cm tanks with continuous seawater flow and air bubbling. The top, back and sides of the tanks were covered with plywood boards to limit disturbances. The bottom was covered with sand, and a small structure (rock or dead coral) was placed in each tank to provide shelter.

Crosses were performed on the day of capture since wild-caught hamlets rarely hydrate new eggs in captivity (Fischer 1981). In order to limit disturbances during spawning and ensure that no eggs are discarded, seawater flow and air bubbling were turned off 90 minutes before sunset and the tank outflows were covered with 500 μm nylon mesh. Individuals were left undisturbed under natural light during the spawning period, i.e. during the hour preceding sunset. Thirty minutes after sunset, the tanks were checked for the presence of eggs with a flashlight. If present, eggs were collected using small custom sieves (10 cm diameter, 500 μm mesh) and rinsed with seawater into 500 ml cylindrical beakers. The mesh was then removed from the tank outflow, seawater flow and air bubbling were turned on again, and the eggs were brought to the lab where they transferred to petri dishes, with no more than about one hundred eggs per dish. From there on the eggs were checked every 8

hours under a stereomicroscope to remove unfertilized eggs and dead embryos or larvae and add fresh seawater. Larvae were collected at 70 hours post fertilization, at which point most of the yolk was resorbed but active feeding had not started yet. This strategy maximized the number of offspring, reduced the potential for DNA contamination from consumed prey items and maternal yolk cells and ensured that the larvae had enough DNA for RAD sequencing. Larvae from successful crosses (i.e. with at least 100 surviving larvae) were sieved, transferred to 95% ethanol and stored at 4°C. The two parents from successful crosses were photographed, fin clipped, and fin clips were stored in salt-saturated DMSO buffer. All adults were fed *ad libitum* after spawning and released within a few days of capture, on the same reef where they had been caught.

DNA extractions and sequencing

RAD libraries were prepared following a modified version of the RAD protocol by Etter *et al*. (2011) as described in Davey *et al*. (2013), using 115–250 ng of DNA per sample (mean = 170). Individual samples were identified with a combination of sixteen 8-bp indices on the P1 adapter and eight 8-bp indices on the reverse amplification primer that produce P2 adapters with 8 different Illumina indices (Table S1). In order to increase sequencing coverage for the two parents they were repeated four times each, providing a total of 128 samples (120 larvae plus 8 parents) that correspond to the number of possible index combinations (16 P1 x 8 P2). Genomic DNA was digested with the *SbfI* restriction enzyme (NEB) and the 128 samples were divided into 8 libraries of 16 samples each corresponding to the 16 P1 indices. For each library the 16 digested samples were ligated to one P1 adapter, pooled and sheared with a Covaris sonicator using a duty cycle of 10%, an intensity of 4 and 200 cycles per burst for a total of 48 s. The sheared libraries were run on individual agarose gels and the 300–600 bp range was manually excised and purified with MinElute columns (QIAGEN). P2 adapters were ligated after blunting and A-tailing, and each library was enriched with 16 amplification cycles in 10 individual PCR reactions containing 5 μl Phusion High-Fidelity PCR Master Mix with HF Buffer (NEB), 0.2 μl forward amplification primer (10 μM), 0.8 μl reverse amplification primer (2.5 μM, one indexed primer per library), 0.5 μl DMSO, 2.8 μl library (1-2 ng/μl) and 0.7 μl water (total 10 μl). Following purification, the eight libraries were pooled into two libraries of 64

individuals each and sequenced on two lanes (one per library) of a HiSeq 2000 Illumina sequencer (paired end, 2 x 100 bp) with a PhiX genomic library spiked in to increase diversity in the first base pairs of read 1 corresponding to the P1 adapter.

For mtDNA sequencing PCRs were carried out in 10 μl reactions containing 3 μl DNA, 0.5 μl primer FISH-BCL (5'-TCAACYAATCAYAAAGATATYGGCAC) and FISH-BCH (5'-TAAACTTCAGGGTGACCAAAAAATCA) at 10 μM concentration each (Baldwin *et al*. 2009), 0.1 μl Qiagen taq DNA polymerase at 5U/μl concentration, 1 μl 10x PCR buffer, 1 μl dNTPs at 2 mM concentration each, and 3.9 μl sigma water. The PCR thermal cycle consisted of one initial denaturation step of 6 min at 96°C, 30 cycles of 20 s at 94°C, 30 s at 55°C and 40 s at 72°C, and one final extension step of 20 min at 72°C. PCR products were purified with ExoSAP-IT (Affymetrix USB) using 2 μl of cleanup reagent, incubated for 15 min at 37°C and inactivated for 15 min at 80°C. Sequencing reactions were performed in 10 μl volume containing 2 μl of purified PCR product, 1 μl primer at 10 μM concentration, 1 μl BigDye Terminator v3.1 (Applied Biosystems), 3 μl BigDye buffer and 3 μl sigma water. The PCR thermal cycle consisted of one initial denaturation step of 1 min at 96°C followed by 30 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. Sequencing reactions were purified with Millipore Sephadex plates following the manufacturer instructions and sequenced on an ABI PRISM 3130xl automated genetic analyzer (Applied Biosystems). Sequence trace files were exported into Sequencer (Gene Codes Corporation) and the forward and reverse sequences were trimmed and assembled. Assembled contigs were examined and edited by hand and the consensus sequences of all contigs were aligned.

Raw sequences filtering, assembly and SNP selection

Raw sequences were filtered using the *process_radtags* pipeline in Stacks version 1.21 (Catchen *et al*. 2011, 2013). This included removal of low-quality reads (with an average raw phred score <10 within a 15-bp sliding window or with one or more uncalled base), of reads with an ambiguous index at position 1–8 of read 1 (where one of the 16 P1 indices is expected), of reads with an ambiguous *SbfI* restriction site at position 9–15 of read 1 (where TGCAGG is expected) and of reads including adapter sequences. This also included the clipping of the 8-bp P1 indices at the beginning of read 1, and of the last 10 bp of all reads

since sequence quality dropped after read position 90. Since all pairs of indices differed by more than two bp, sequences that differed by a single bp from any expected index (or the restriction site sequence) were corrected and retained. The pipeline *clone_filter* in Stacks was used to filter out pairs of paired-end reads that match exactly, since these are expected to represent PCR clones in the vast majority of cases. Finally, the raw reads were mapped to the PhiX genome with bbmap (Bushnell B, sourceforge.net/projects/bbmap/) to double-check that the PhiX reads had been effectively removed by the sequencing facility.

In the absence of a reference genome for *Hypoplectrus*, reads were assembled *de novo* using the *denovo_map.pl* pipeline in Stacks. At least five identical raw reads were required to create a stack (m=5) and three to create a stack in progeny individuals (P=3). Two mismatches between loci were allowed when processing a single individual (M=2) and none when building the catalog (n=0). Four mismatches were allowed when aligning secondary reads to primary stacks (N=4) and highly repetitive stacks were removed or broken up. The pipeline *genotypes* in Stack was used to filter and export genotypes. We followed the recommendations by Henning *et al*. (2014) for linkage maps and applied stringent filtering, considering only data from stacks that were present in at least 90% of the larvae with at least 15x coverage. When more than one single nucleotide polymorphism (SNP) per stack was identified we used only the first one for genotyping.

Electronic Supplementary Results

Experimental crosses and COI sequencing

Our methodology resulted in a spawning rate of 50%, with nine successful spawns out of 18 assays. The number of eggs produced by each pair that spawned varied but was always in the hundreds. Fertilization success was high, with the vast majority of eggs (>90%) fertilized. Survival rates of the eggs and larvae were variable, yet six of the nine crosses produced more than one hundred 70-hour larvae. The cross with the highest number of larvae was produced by two black hamlets (sample ID 27680 and 27681, thereafter parent 1 and 2, respectively) and was selected for genotyping. Both fertilization success and survival rate were high for this cross (>90%), which is expected to limit the effects of selection on the linkage maps. The COI sequences of the two parents differed by 4 SNPs at positions 307 (G in parent 1 and A in

parent 2), 364 (T/A), 403 (A/C) and 556 (C/T), providing the opportunity to distinguish them unambiguously. One hundred and nineteen of the 120 larvae were sequenced successfully and all had a COI sequence that matched unambiguously one of the two parents, with 65 larvae (55%) matching parent 1 and 54 larvae (45%) matching parent 2.

Filtering, assembly and SNP selection

We generated a total of 335 217 503 paired-end reads (2x100 bp) for the two parents and all offspring. Of these 26.6% were discarded because pairs of paired-end reads matched exactly (putative PCR clones), 16.9% due to ambiguous indices, 0.4% due to low quality, 0.3% due to the presence of adapter sequence in the read and 0.2% due to ambiguous restriction site. Thus, we retained 54.9% of the raw reads and the final dataset consisted of 184 182 283 paired-end reads. Mapping of the raw reads to the PhiX genome returned <0.0002% hits after filtering.

As expected, the number of stacks recovered per sample was highest in the two parents, with 66 284 and 67 678 stacks for parent 1 and 2, respectively, and plateaued at about 65 000 for the larvae (Figure S1). Yet a number of larvae had a substantially lower sequencing coverage and number of stacks, which could result from poor DNA extraction or poor library preparation for these individuals. Preliminary analyses indicated that the 25 larvae with lowest number of stacks (<32 669) provided a large proportion of missing data for linkage analysis and map construction. These were therefore excluded from downstream analyses, leaving a total of 95 offspring. The mean number of stacks for these 95 offspring was 59 714. Mean sequencing coverage for parent 1 and 2 was 100x and 143x, respectively, and mean coverage for the 95 larvae was 19x (min=12x, max=28x). Our final dataset consisted of 3515 stacks that were present in at least 86 of the 95 larvae (90%) with at least 15x coverage.

Linkage analysis and map construction

A total of 1216 and 1324 recombination events were observed for parent 1 and parent 2, respectively. The eggs and sperm maps for each parent were based on 42 F_1 larvae for the parent 1/sperm and parent 2/egg maps, and on 53 larvae for the parent 1/egg and parent 2/sperm maps.

Electronic Supplementary Discussion

Comparison with previous RAD dataset

Considering all samples, we identified an average of 49 807 RAD stacks (putative loci) per individual, which is 7% lower than the mean value of 53 811 obtained in our previous RAD hamlet dataset, generated with the same restriction enzyme (Puebla *et al*. 2014). The difference may be due to lower sequencing coverage in this study (20x versus 31x in Puebla *et al*. 2014) and to the different assembly parameters used for linkage mapping versus population genetic analysis.

The proportion of putative PCR clones was substantially higher in this study (26.6% versus 5.9% in Puebla *et al*. 2014). This may be due to the fact that the 70-hour larvae that we used were tiny (<3 mm) and provided lower amounts of genomic DNA for library preparation (mean = 170 ng per sample) than the gill tissue used in our previous study (mean = 936 ng per sample), requiring therefore more PCR cycles for library amplification. This underscores the importance of filtering out PCR clones (Andrews *et al*. 2014), particularly when starting with low amounts of DNA.

The proportion of reads discarded due to ambiguous barcodes was also much higher in this study (16.9% versus 0.1% in Puebla *et al*. 2014). This may have been driven by low sequence quality in the first base pairs of read 1 associated with low sequence diversity in the P1 adapters, since we used only 16 P1 indices in this study versus 64 in Puebla *et al*. (2014). Quality control of the raw data confirmed that sequence quality was noticeably lower in the first three to four base pairs of reads 1, and visual inspection of the 20 most commonly discarded reads due to ambiguous barcodes revealed that all of them differed from expected barcodes in the first two to four positions of the P1 barcode, often with four Ts (e.g TTTTCAGT). This suggests that the high proportion of reads discarded due to ambiguous barcodes is indeed due to low sequence quality in the first base pairs of read 1, even though a PhiX control library had been mixed with our libraries to increase diversity in the first base pairs of read 1. All in all, these observations illustrate how the quality of RAD data is highly dependent on the details of the library preparation and sequencing protocols. These should therefore be considered with care, particularly when library preparation and/or sequencing are outsourced to external laboratories, genome centers or companies.

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Table S1. Adapters and amplification primers used. Indices highlighted in red.

Adapter/primer	Sequence
P1 adapters	
P1-TOP-G1-102	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACACTGACTGC*A
P1-TOP-G1-103	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACGTAGCATGC*A
P1-TOP-G1-127	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCACACAGTTGC*A
P1-TOP-G1-128	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAGTCTCATGC*A
P1-TOP-G1-167	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTACTCGTTGC*A
P1-TOP-G1-169	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTCATGTGTGC*A
P1-TOP-G1-193	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGCATCGTTGC*A
P1-TOP-G1-195	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTGACTGTGC*A
P1-TOP-G2-101	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACACGACATGC*A
P1-TOP-G2-104	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACGTCTACTGC*A
P1-TOP-G2-129	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAGTGTGTTGC*A
P1-TOP-G2-130	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCATGATCATGC*A
P1-TOP-G2-166	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTACGCTGTGC*A
P1-TOP-G2-170	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTCAGTGTTGC*A
P1-TOP-G2-194	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTGTGACTGC*A
P1-TOP-G2-196	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTGCAGTTGC*A
P1-BOTTOM-G1-102	/5Phos/GTCAGTGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P1-BOTTOM-G1-103	/5Phos/TGCTACGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P1-BOTTOM-G1-127	/5Phos/ACTGTGTGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P1-BOTTOM-G1-128	/5Phos/TGAGACTGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P1-BOTTOM-G1-167	/5Phos/ACGAGTACAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P1-BOTTOM-G1-169	/5Phos/CACATGACAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P1-BOTTOM-G1-193	/5Phos/ACGATGCAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P1-BOTTOM-G1-195	/5Phos/CAGTCACAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P1-BOTTOM-G2-101	/5Phos/TGTCGTGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P1-BOTTOM-G2-104	/5Phos/GTAGACGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P1-BOTTOM-G2-129	/5Phos/ACACACTGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P1-BOTTOM-G2-130	/5Phos/TGATCATGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P1-BOTTOM-G2-166	/5Phos/CAGCGTACAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P1-BOTTOM-G2-170	/5Phos/ACACTGACAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P1-BOTTOM-G2-194	/5Phos/GTCACACAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P1-BOTTOM-G2-196	/5Phos/ACTGCACAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P2 adapter	
Short P2 Adapter Top	/5Phos/GATCGGAAGAGCGGTTCAGCAGGTCCATTC
Short P2 Adapter Bottom	GAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T
PCR amplification primers	
Forward amplification primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC*T
Reverse iPCRtagT1	CAAGCAGAAGACGGCATACGAGATAACGTGATGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T
Reverse iPCRtagT2	CAAGCAGAAGACGGCATACGAGATAAACATCGGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T
Reverse iPCRtagT9	CAAGCAGAAGACGGCATACGAGATCGCTGATCGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T
Reverse iPCRtagT11	CAAGCAGAAGACGGCATACGAGATCTGTAGCCGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T
Reverse iPCRtagT52	CAAGCAGAAGACGGCATACGAGATGCTAACGAGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T
Reverse iPCRtagT53	CAAGCAGAAGACGGCATACGAGATGCTCGGTAGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T
Reverse iPCRtagT67	CAAGCAGAAGACGGCATACGAGATTGGTGGTAGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T
Reverse iPCRtagT68	CAAGCAGAAGACGGCATACGAGATTTCACGCAGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T
P2 index sequencing primer	AAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTC

*= Phosphorothioate bond

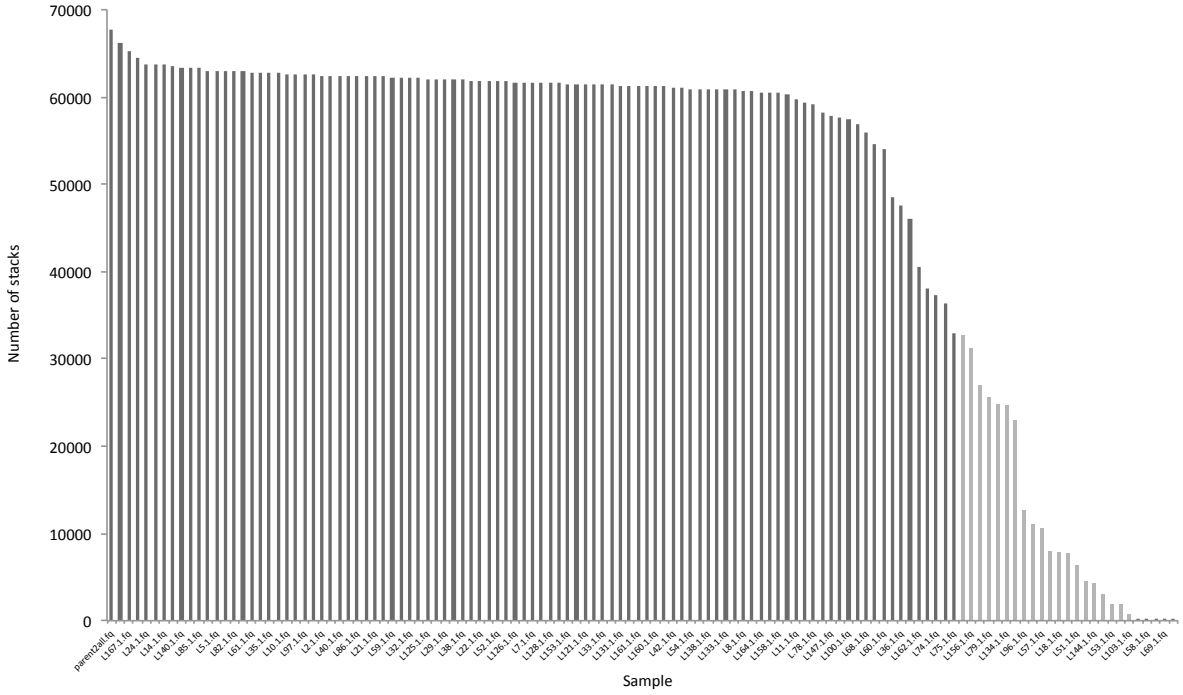

Figure S1. Number of stacks recovered per sample, in decreasing order. The number of stacks was highest in the two parents, with 66 284 and 67 678 stacks for parent 1 and 2, respectively. The number of stacks in the larvae plateaued at about 65 000 (left side of the plot), but was substantially lower in a number of them (right side). The larvae highlighted in light grey were discarded for linkage analysis and map construction.

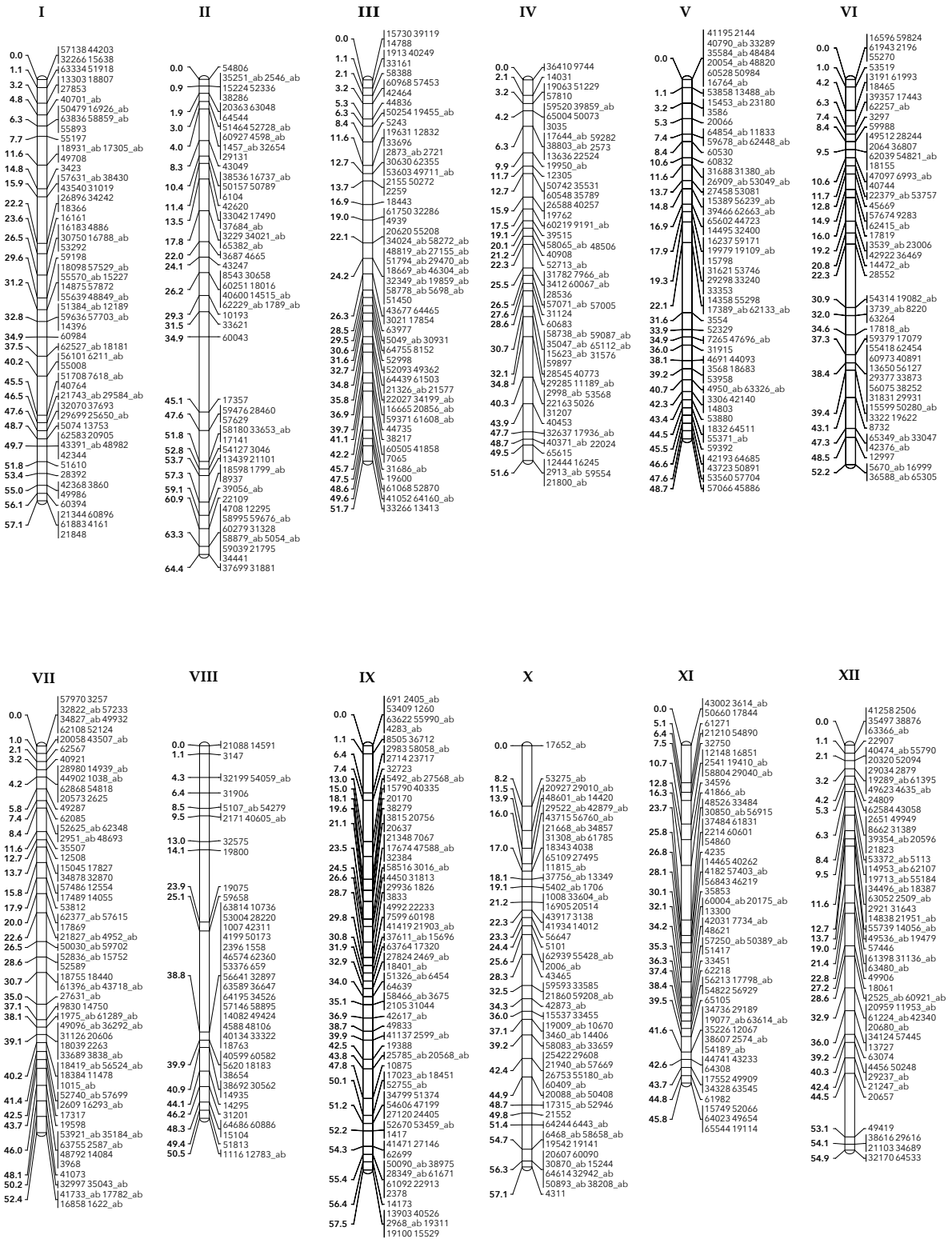

Figure S2 (part 1/1). Linkage map for parent 1 (considering both sexes). Double heterozygote markers are identified with _ab following the marker names.

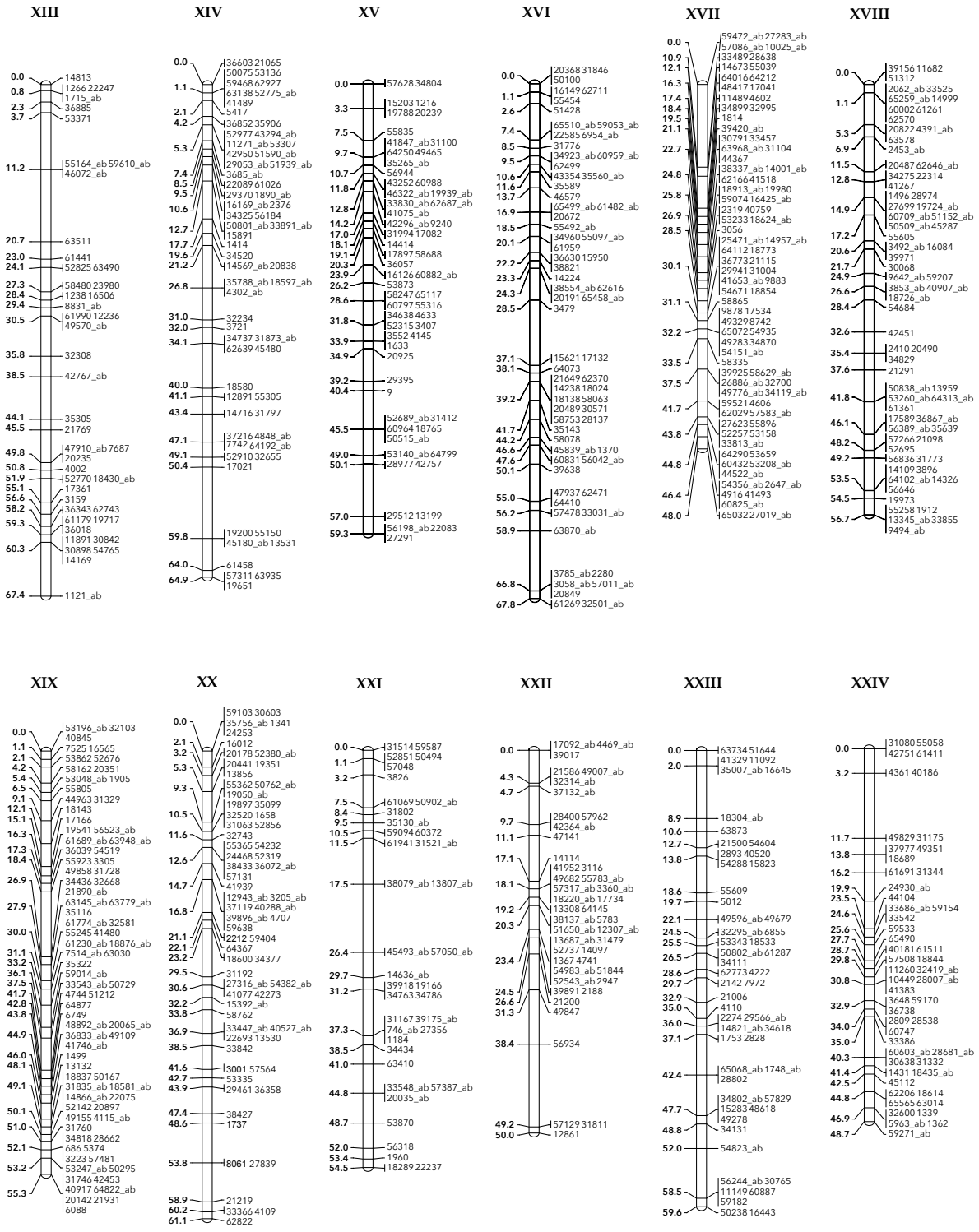

Figure S2 (part 2/2). Linkage map for parent 1 (considering both sexes). Double heterozygote markers are identified with _ab following the marker names.

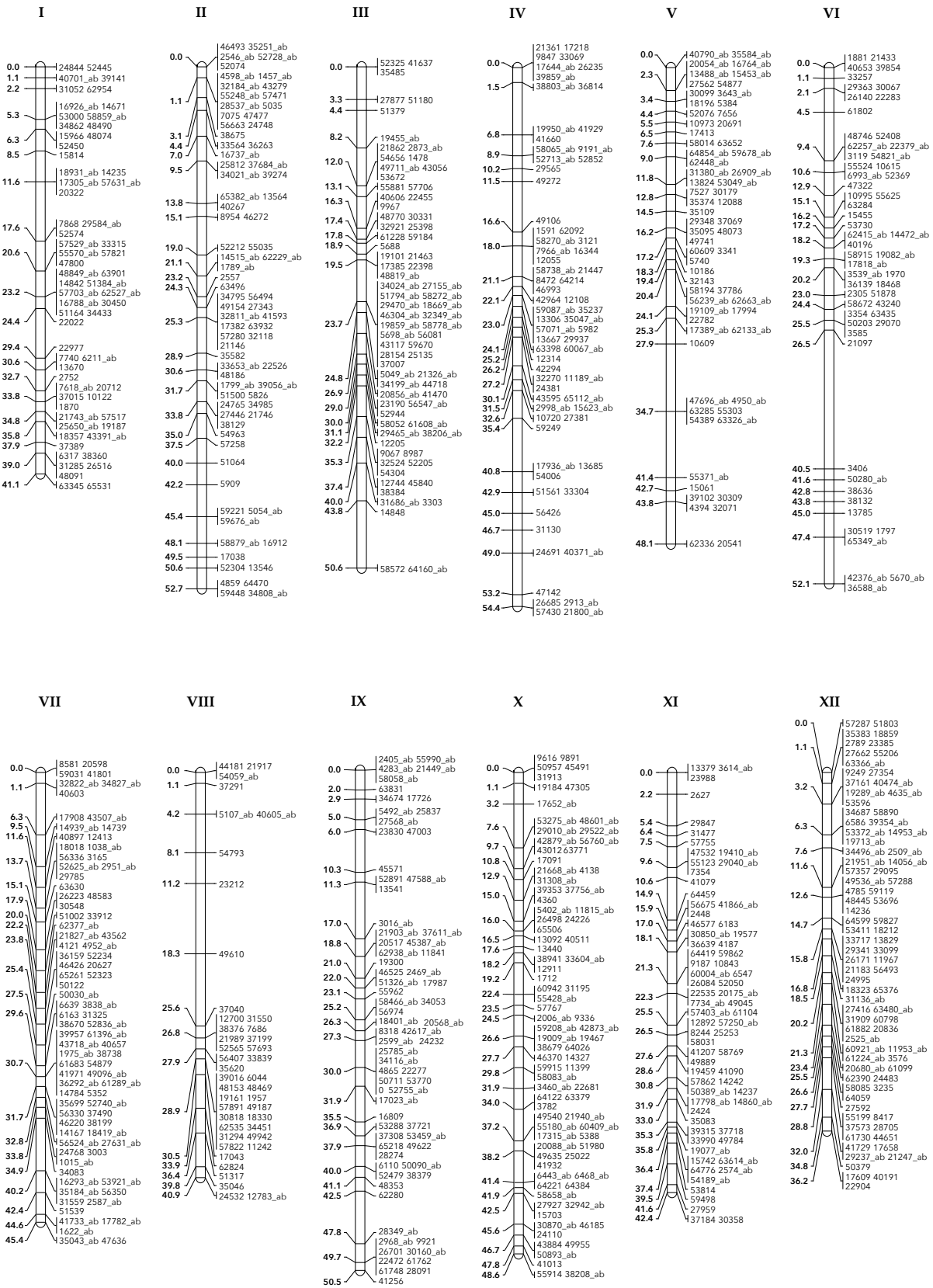

Figure S3 (part 1/2). Linkage map for parent 2 (considering both sexes). Double heterozygote markers are identified with _ab following the marker names.

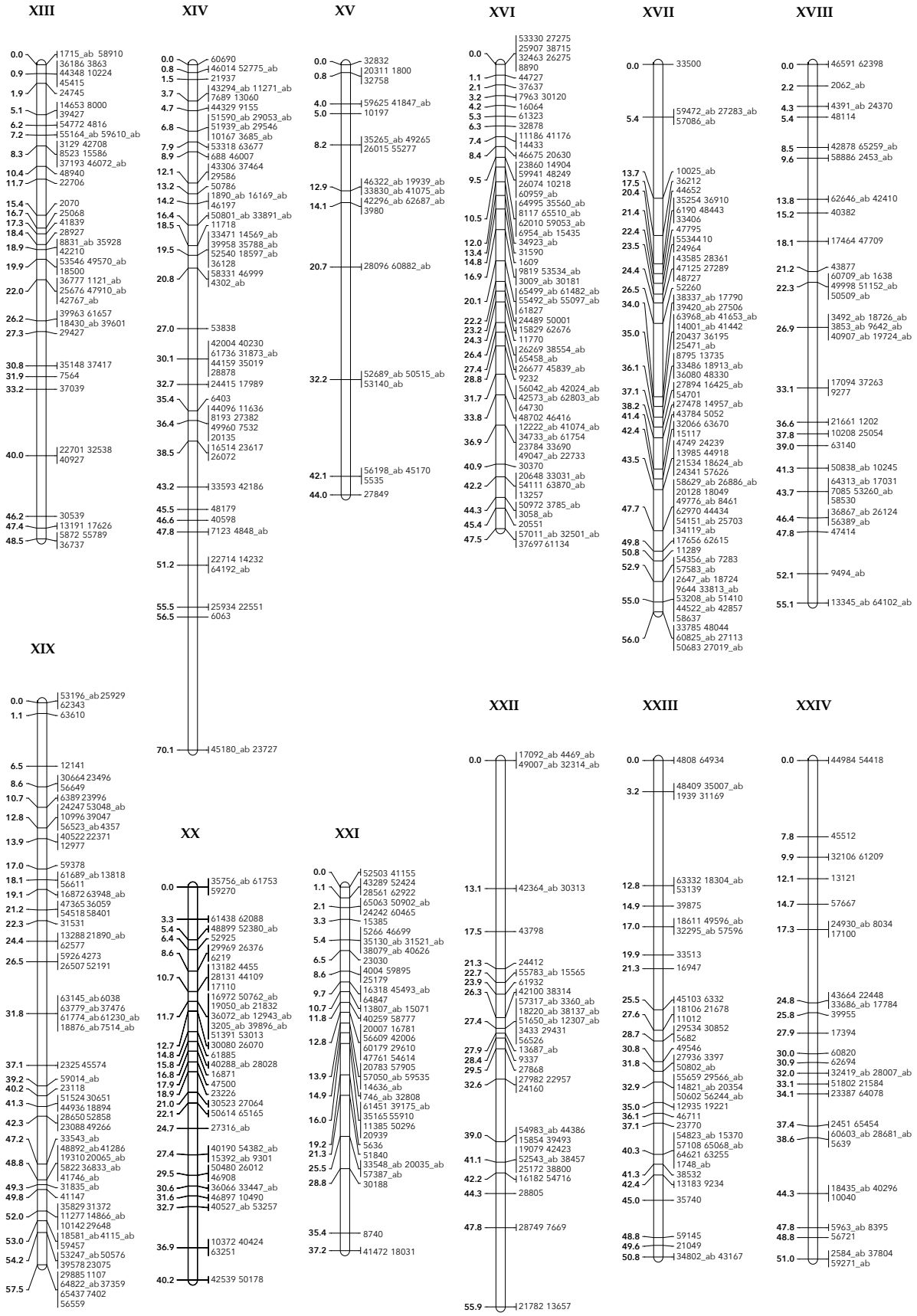

Figure S3 (part 2/2). Linkage map for parent 2 (considering both sexes). Double heterozygote markers are identified with _ab following the marker names.

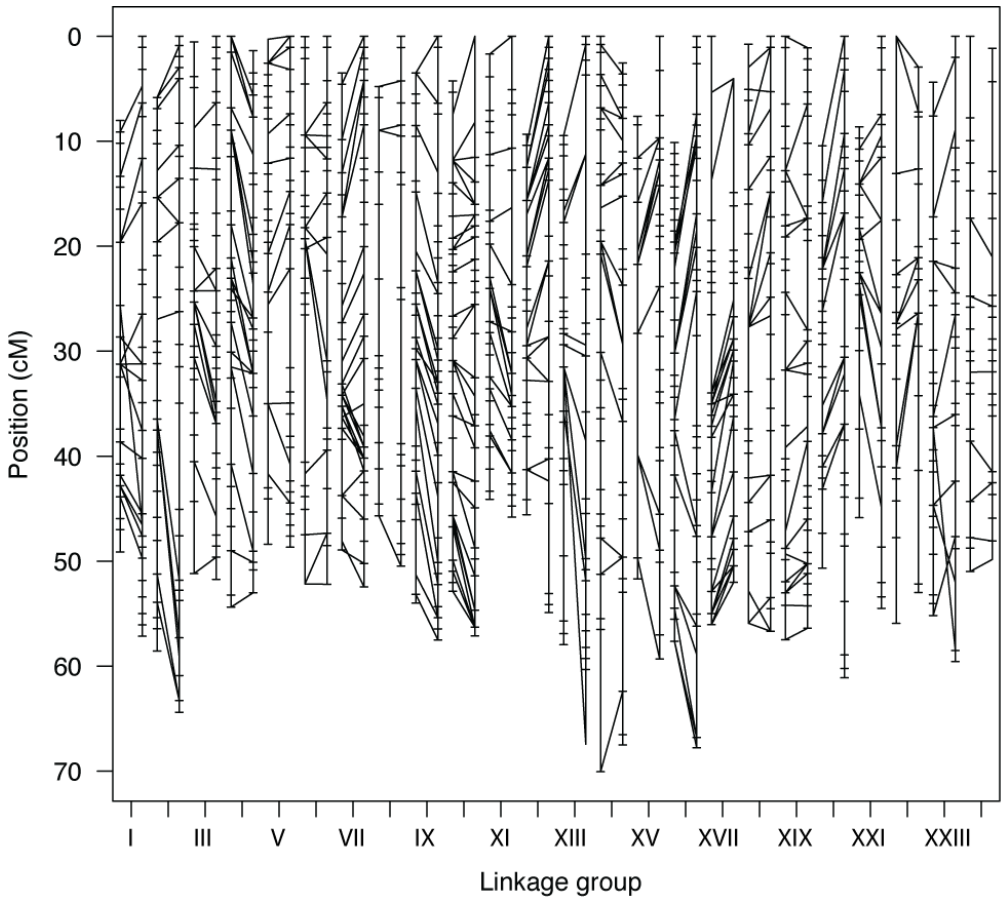

Figure S4. Comparison between parent 1 (left) and parent 2 (right) maps, considering both sexes and using double heterozygote markers as anchors. The two maps are in perfect agreement regarding LG homology, i.e. all double heterozygote markers assigned to one LG in one map are also assigned to a unique LG in the other map. The two maps are also in good agreement regarding the order of markers on each LG, although a few conflicts (criss-crossing lines) are apparent.

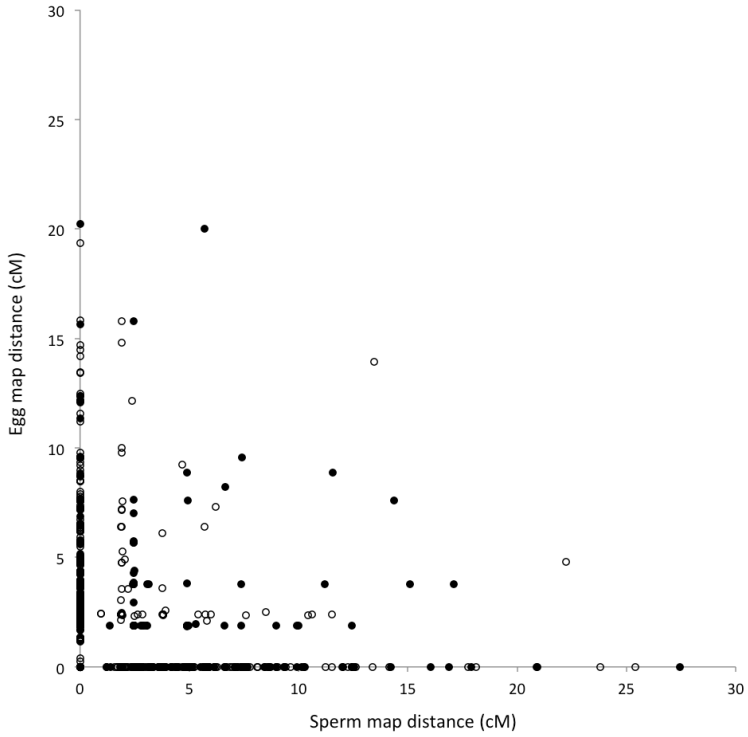

Figure S5. Eggs versus sperm inter-marker distances (cM) for all pairs of adjacent markers in parent 1 (black dots) and parent 2 (open dots). Pairs of adjacent markers with equal recombination in the two sexes would fall on the 1:1 diagonal, but we observe instead a large proportion of points close to either the eggs or sperm axis, indicating eggs- or sperm-biased recombination for the majority of adjacent pairs of markers.

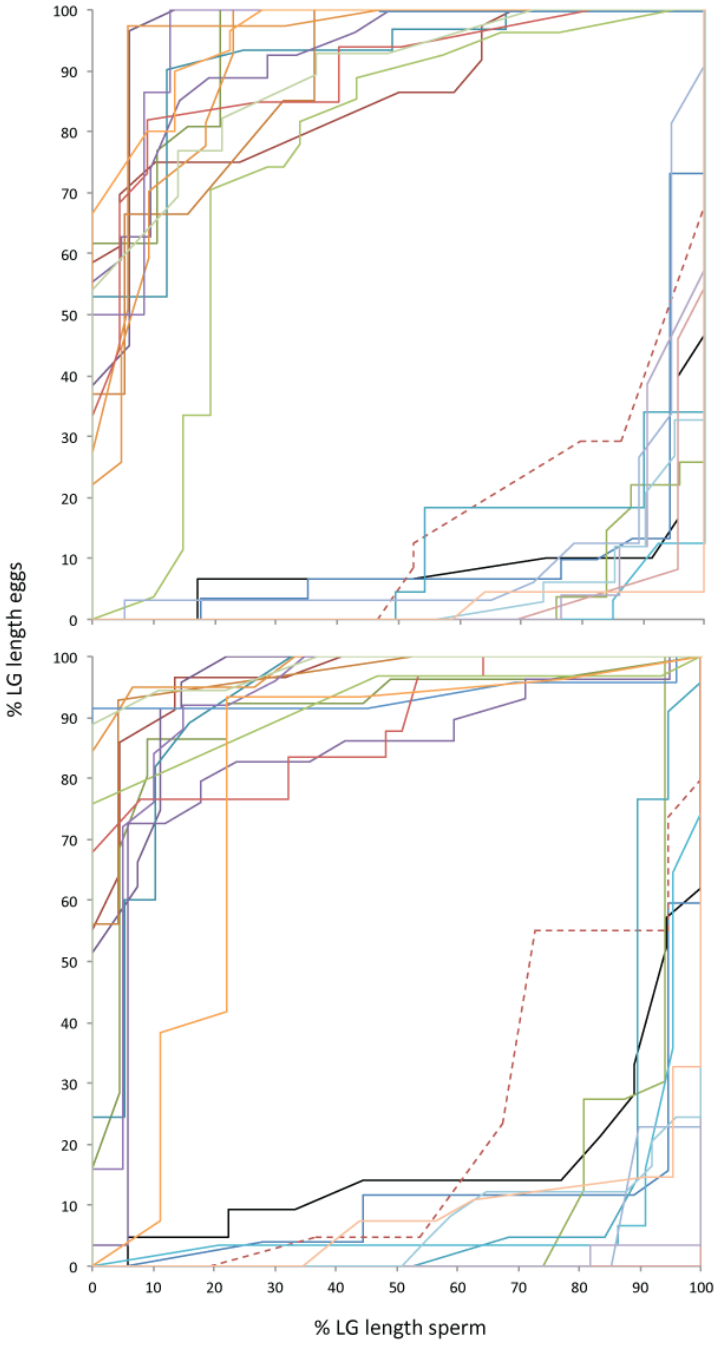

Figure S6. Comparison between standardized eggs and sperm linkage maps in parent 1 (above) and parent 2 (below). Each line corresponds to one LG, and each point to the position of markers in the sperm and eggs maps in terms of percentage of total LG length. A slope of 1 indicates a homogenous difference in recombination rate between the sperm and eggs along LGs, a slope >1 relatively higher recombination in the eggs, and a slope <1 relatively lower recombination in the sperm. All LGs present a marked difference between recombination patterns in the sperm and egg, with relatively higher recombination in the eggs and relatively lower recombination in the sperm at one LG extremity and the opposite at the other extremity. LG VIII is represented with a dashed line.

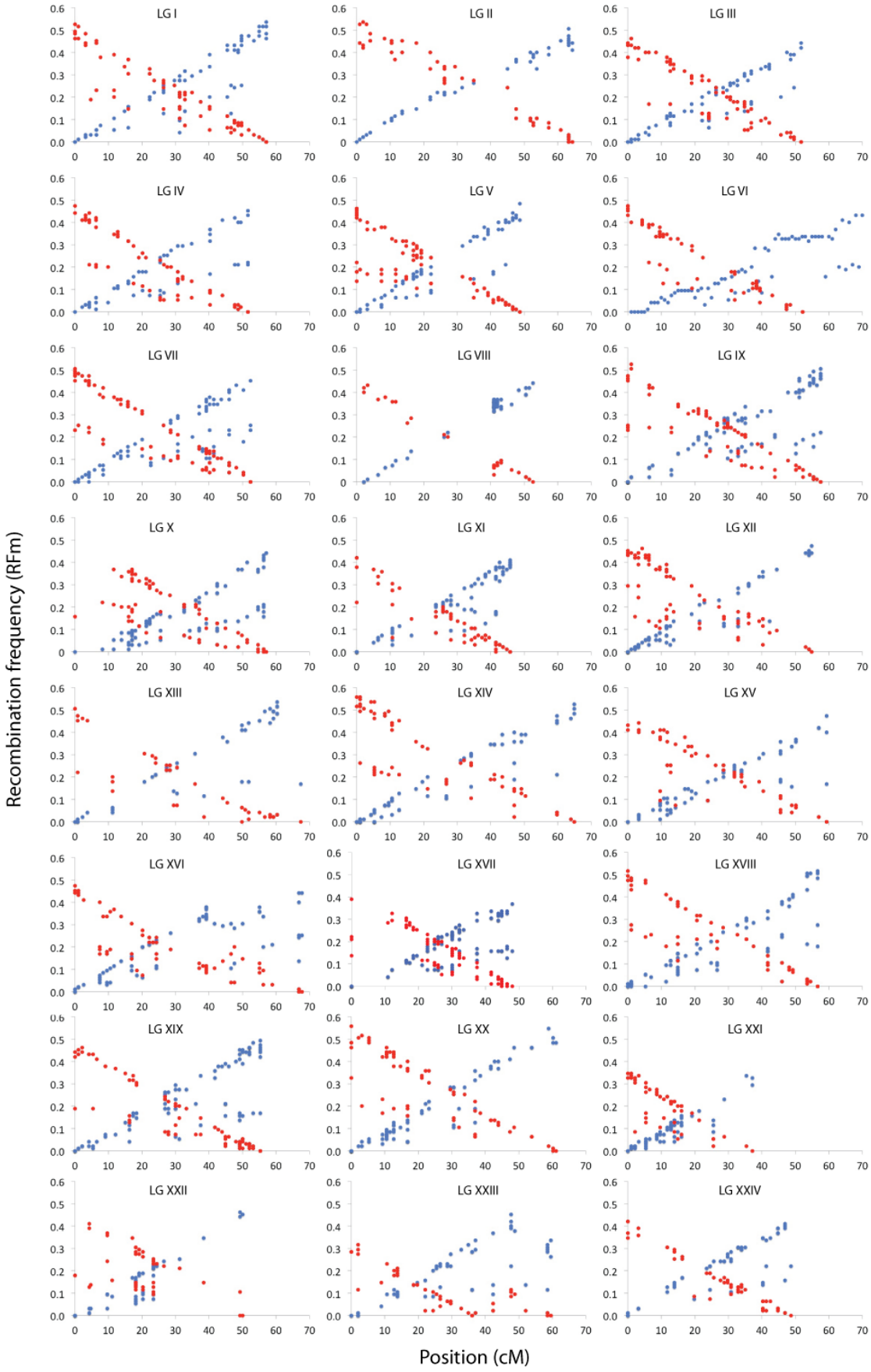

Figure S7. Cumulative recombination frequencies (RF_m) along LGs, starting from each end (read and blue, respectively) of each LG. RF_m tends to increase from 0 at the terminal reference marker towards a value of 0.5 at the opposite end for all LGs, which is expected in the case of acrocentric or telocentric chromosomes.

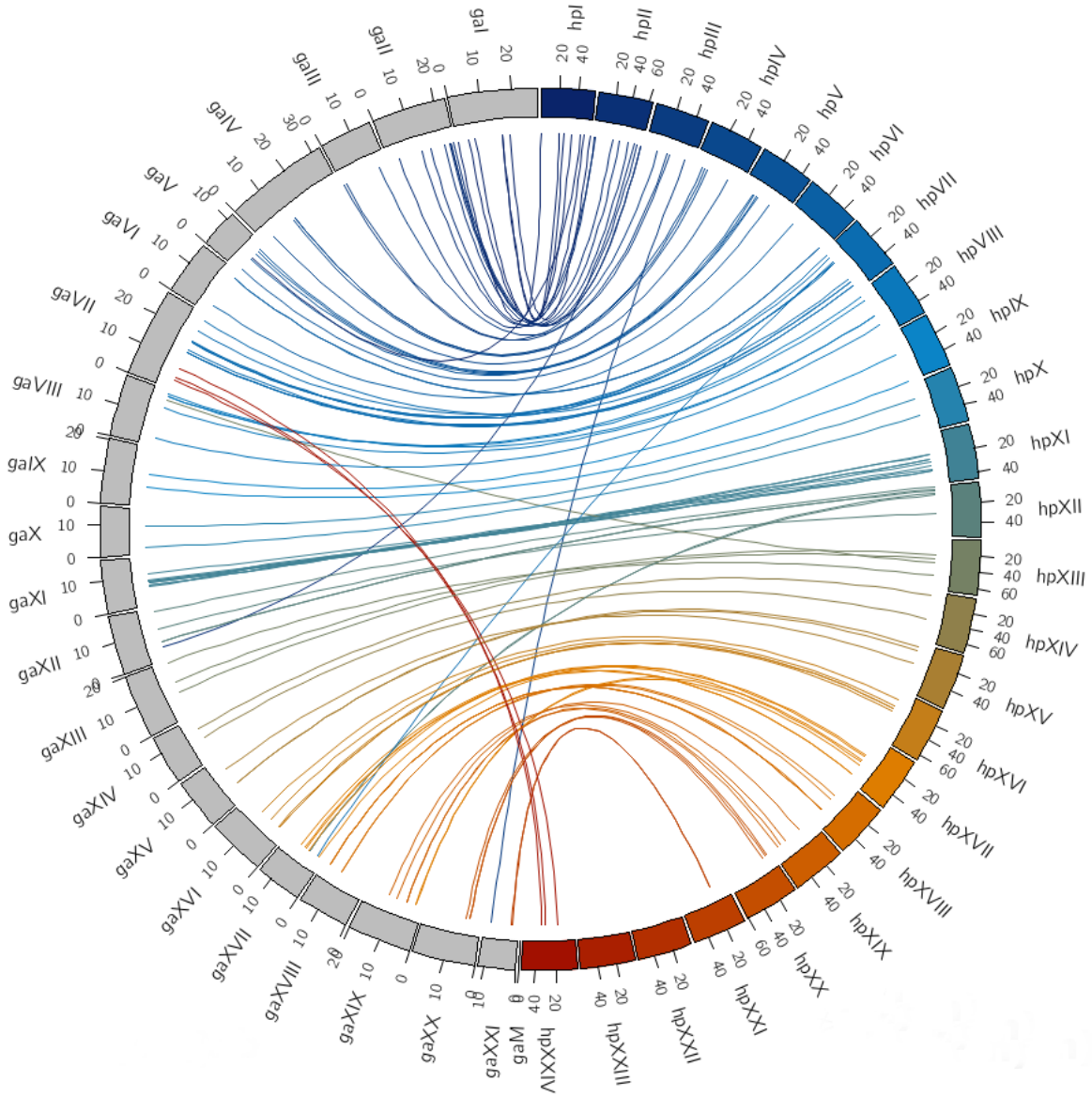

Figure S8. Synteny between stickleback (left, grey) and hamlet (right, colors) LGs. Numbers within each LG correspond to position, in cM for the hamlet linkage map (hp) and in Mb for the stickleback genome (ga). gaM: stickleback mitochondria.