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A Dense Linkage Map of Lake Victoria Cichlids Improved the *Pundamilia* Genome Assembly and Revealed a Major QTL for Sex-Determination

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ABSTRACT Genetic linkage maps are essential for comparative genomics, high quality genome sequence assembly and fine scale quantitative trait locus (QTL) mapping. In the present study we identified and genotyped markers via restriction-site associated DNA (RAD) sequencing and constructed a genetic linkage map based on 1,597 SNP markers of an interspecific F2 cross of two closely related Lake Victoria cichlids (*Pundamilia pundamilia* and *P.* sp. 'red head'). The SNP markers were distributed on 22 linkage groups and the total map size was 1,594 cM with an average marker distance of 1.01 cM. This high-resolution genetic linkage map was used to anchor the scaffolds of the *Pundamilia* genome and estimate recombination rates along the genome. Via QTL mapping we identified a major QTL for sex in a ~1.9 Mb region on Pun-LG10, which is homologous to *Oreochromis niloticus* LG 23 (Ore-LG23) and includes a well-known vertebrate sex-determination gene (*amh*).

The haplochromine cichlid lineage of the East African Great Lakes is famous for forming large adaptive radiations often in exceptionally short time, resulting in several hundred species each in Lakes Malawi and Victoria, and dozens of species each in several smaller East African Lakes (Kocher 2004; Seehausen 2015). The Lake Victoria

haplochromine cichlid radiation stands out in being the youngest (~15,000 years) showing a high degree of diversity in morphology, behavior and ecology (Greenwood 1974; Seehausen 1996). An abundance of studies have been published on the evolution of Lake Victoria cichlids, providing insight to colonization history (Nagl *et al.* 2000; Seehausen *et al.* 2003; Verheyen *et al.* 2003; Meier *et al.* 2017b), species formation (Seehausen *et al.* 1997; Seehausen and van Alphen 1999; Seehausen *et al.* 1999; Selz *et al.* 2014a), the interaction of sexual and natural selection (Seehausen 2000, Seehausen *et al.* 2008, Maan and Seehausen 2011), and the role of hybridization between distant relatives (Seehausen *et al.* 2003, Keller *et al.* 2013, Selz *et al.* 2014b, Meier *et al.* 2017a, Meier *et al.* 2017b). Recently, several cichlid genomes were published (Brawand *et al.* 2014), among them one from Lake Victoria. This genome is being used to investigate the genomic landscape of speciation (Meier *et al.* 2018). Detailed genetic linkage maps offer a powerful tool to improve the quality of genome assemblies (Fierst 2015) and set the framework for quantitative trait loci

(QTL) localization. In the past decade, a number of genetic linkage maps have been published for haplochromine cichlids using various molecular genetic markers (Streelman *et al.* 2003; Sanetra *et al.* 2009; O'Quin *et al.* 2013; Henning *et al.* 2014; 2017). For Lake Victoria cichlids three linkage maps based on two interspecific F2 hybrid crosses were published. The first was based on an F2 cross between *Paralabidochromis chilotes* and *Paralabidochromis sauvagei* and contained 184 microsatellites and two SNP markers with a mean marker spacing of 6.09 cM on 25 linkage groups (Kudo *et al.* 2015). The two others were based on F2 crosses between *Paralabidochromis sauvagei* and *Pundamilia cf. nyererei* (Henning *et al.* 2014) and *Paralabidochromis chilotes* and *Pundamilia cf. nyererei* (Henning *et al.* 2017). Linkage maps were constructed with 867 and 752 single-nucleotide polymorphism (SNP) markers resulting in a mean marker spacing of 1.30 and 1.09 cM, respectively on 22 linkage groups (Henning *et al.* 2014; 2017). These linkage maps were then used to identify QTL, such as for lateral stripes, lip size, and head morphology (Henning *et al.* 2014; 2017) as well as sex determination (Kudo *et al.* 2015). None of the linkage maps has been used to improve the Lake Victoria haplochromine genome assembly.

In haplochromine cichlids, some polymorphic color patterns are genetically linked to sex determination and are associated with segregating polymorphisms in sex determination (Holzberg 1978; Seehausen *et al.* 1999; Lande *et al.* 2001; Streelman *et al.* 2003; Kocher 2004). These observations supported the hypothesis that the rapid evolution of sex determination systems might play a role in the very rapid speciation of haplochromine cichlids (Seehausen *et al.* 1999; Lande *et al.* 2001; Kocher 2004; Ser *et al.* 2010). A high diversity of sex determination systems and high sex chromosome turnover rates are known in fish, including cichlids, with a variety of environmental and genomic factors resulting in male or female phenotypes (reviewed *e.g.*, in Heule *et al.* 2014a). In cichlids, very closely related species, populations within the same species, and even individuals within a population, can have different sex determination mechanisms or non-homologous sex chromosomes. This is evidenced by the presence of both XX-XY and ZZ-ZW sex determination systems within haplochromines of Lakes Victoria and Malawi and in oreochromine cichlids (Seehausen *et al.* 1999; Lande *et al.* 2001; Cnaani *et al.* 2008; Roberts *et al.* 2009; Ser *et al.* 2010). Some candidates for genetic sex determination in cichlids exist and could be associated with respective chromosomes, suggesting both XY and ZW systems. Across different species of *Oreochromis*, sex determination loci have been repeatedly mapped to three different linkage groups (LGs), namely LG 1 (XY), LG 3 (ZW), and LG 23 (XY) (Cnaani *et al.* 2008). In haplochromine cichlids, sex determination loci mainly mapped to LG 5 (ZW and XY) and LG 7 (XY) (Ser *et al.* 2010; Kudo *et al.* 2015; Roberts *et al.* 2016; Böhne *et al.* 2016; Peterson *et al.* 2017). Some genes that have repeatedly evolved as master sex determination genes in teleost fishes (Kikuchi and Hamaguchi 2013; Heule *et al.* 2014a) seem to play a role in sex determination in cichlids as well. Recent results published on *Astatotilapia calliptera*, a haplochromine cichlid from Lake Malawi, and *Oreochromis niloticus*, a distant relative of the East African adaptive radiations, indicate that two of these candidate genes, the gonadal soma-derived factor (*gsdf*) and the anti Müllerian hormone (*amh*) might have been re-used as sex determination loci (Eshel *et al.* 2014; Peterson *et al.* 2017). Those genes are often derived by duplication or allelic diversification from genes with a known function in sex differentiation or gonad development (Heule *et al.* 2014a).

In the present study we construct a linkage map of an interspecific F2 cross between two very closely related Lake Victoria cichlid species (*Pundamilia pundamilia* and *P.* sp. 'red head'). The map was built using

1,597 SNPs identified and genotyped via restriction-site associated DNA (RAD) sequencing with an average marker distance of 1.01 cM. We then used the linkage map to anchor the scaffolds of the *P. nyererei* reference genome to the 22 linkage groups of the map and to perform a QTL analysis for putative sex determination loci in *Pundamilia*. We identify the LG determining sex in a Lake Victoria cichlid cross, as well as potential candidate genes for sex determination and put these findings into the context of sex determination evolution within a rapidly radiating clade of fish.

MATERIALS AND METHODS

Mapping family and RAD sequencing

The genetic cross was started with a lab bred *Pundamilia* sp. 'red head' (Seehausen 1996) male from Zue Island in Lake Victoria (lab strain established from wild caught fishes by OS in 1993, 4th or 5th lab generation) and a wild *P. pundamilia* female caught by OS at Makobe Island in Lake Victoria in 2003. Eggs were removed from the female's mouth five days after spawning and reared in isolation from the adults. After reaching maturity, four F1 individuals were crossed, resulting in two F2 families with together more than 300 individuals. When F2 individuals were adult and sexually mature sex was determined based on coloration, then sedated fish were killed with MS222 (50 mg/L for sedation; 300 mg/L for euthanization), and a fin clip was taken and stored in 98% ethanol for genetic analyses (Animal Permit numbers: BE18/15, BE47/11, LU04/07, LU02/13). Genomic DNA of 218 F2 progeny, the four F1 parents, and the two F0 grandparents was extracted using phenol-chloroform (Sambrook and Russell 2001). Restriction-site associated DNA (RAD) sequencing libraries were prepared following Marques *et al.* (2016) using a protocol slightly modified from Baird *et al.* (2008). In brief, genomic DNA was digested with *Sbf*I followed by shearing and size selection of 300 to 500 bp. Equimolar proportions of DNA from 11 to 48 individuals carrying different barcode sequences were pooled into one library. Each library was amplified in four reactions of 50 μl aliquots. A total of nine libraries were single-end sequenced (100 bp) each on a single lane of an Illumina HighSequation 2500 platform either at the Next Generation Sequencing Platform of the University of Bern or at the Genomic Technologies Facility of the University of Lausanne. Some individuals and all F0 grandparents were sequenced in up to three libraries to increase coverage. Together with each library, we sequenced about 10% reads of bacteriophage PhiX genomic DNA (Illumina Inc.) to increase complexity at the first 10 sequenced base pairs. During read processing, PhiX reads were further utilized to recalibrate libraries to equalize base quality scores across Illumina lanes utilizing GATK version 3.2 (McKenna *et al.* 2010).

Sequence processing and genotyping

Before recalibration, read qualities were inspected using fastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and filtered using FASTX Toolkit 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/index.html) requiring a minimum quality of 10 at all bases and of 30 in at least 95% of the read. After PhiX removal, reads were demultiplexed, cleaned, and trimmed to 92 bp with process_radtags implemented in Stacks v1.26 (Catchen *et al.* 2013). Reads were mapped against the *P. nyererei* reference genome (Brawand *et al.* 2014) using bowtie2 version 2.2.6 (using default parameters except for setting –N 1 allowing one mismatch in a seed alignment to increase sensitivity; Langmead and Salzberg 2012). Mapped reads of individuals run in multiple libraries were merged using Picard tools version 1.97 and filtered for a mapping quality of at least 30. After the filtering pipeline we were left
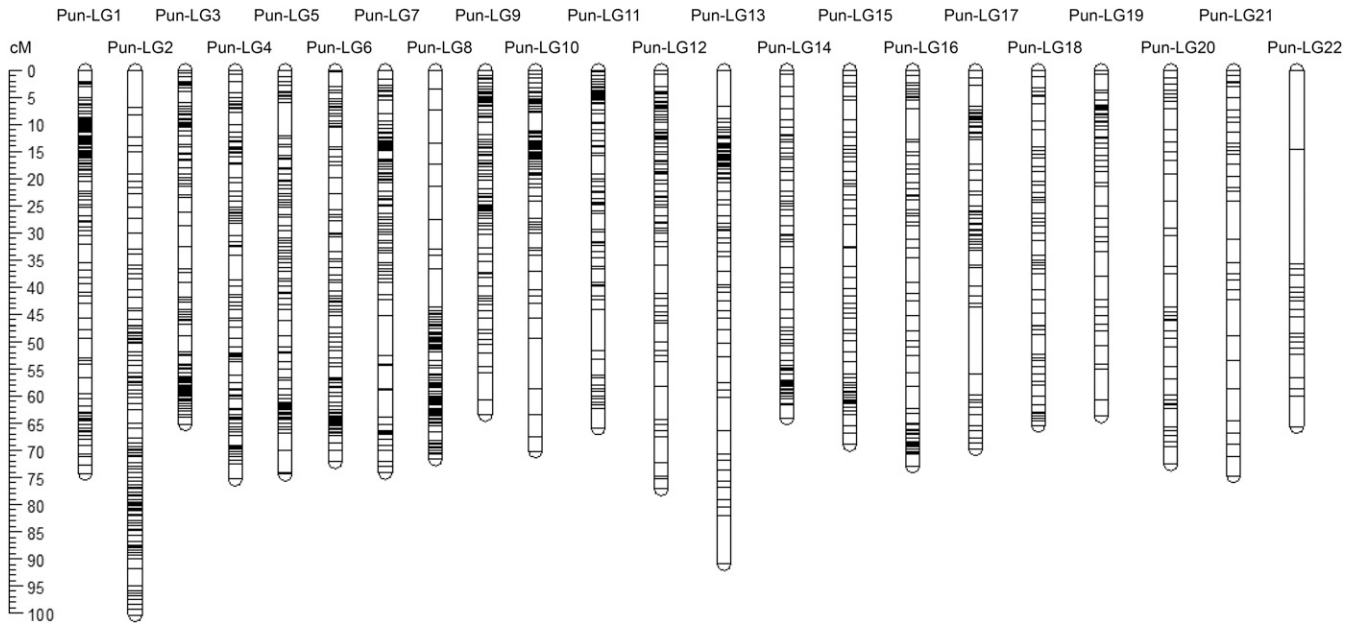
Figure 1 Linkage map indicating the positioning of 1,597 markers and Kosambi mapping length (cM) of 22 linkage groups.

with a total of 719,720,265 sequences across the nine RAD libraries (on average 79,970,000 reads per library). For the female and male parental samples, 1,364,225 and 6,459,242 reads respectively, were mapped and remained after filtering. For the 222 progeny individuals (including the F1) we obtained on average 2,008,826 reads per individual. All 224 individuals (218 F2, the two grandparents and four F1) were genotyped using freebayes version 1.0.0 (Garrison and Marth 2012). As a first filter, sites were kept if bi-allelic, had less than 50% missing data, a quality of more than 2, a minor allele frequency of more than 5%, and a minimal depth of 3. Utilizing a script established to filter freebayes genotype calls based on RAD sequencing (https://github.com/jpuritz/dDocent/blob/master/scripts/dDocent_filters), genotypes were further excluded (thresholds given in brackets) on criteria related to allelic balance at heterozygote sites (< 0.28 allele balance between reads), quality *vs.* depth (ratio <0.5), strand presentation (overlapping forward and reverse reads), and site depth (one standard deviation from mean and a quality score lower than twice the depth first, followed by an additional maximum mean depth cutoff of 67). Multi-allelic variants and indels were removed, resulting in 7,401 SNPs. Of the 7,401 filtered SNPs 2,052 were alternative homozygous in the grandparents, and were used to build the genetic linkage map.

Linkage map

A linkage map was constructed with JoinMap 4.0 (Van Ooijen 2006) using 212 F2 progeny derived from two F1 families. Out of the 224 genotyped individuals (including the 2 F0 and 4 F1), 2 F1 and 6 F2 were removed due to missing data (>25%). Out of the 2,052 loci homozygous for alternative alleles in the grandparents, we placed 1,597 in the final linkage map. Loci were excluded if positioned identically with another locus. Markers showing segregation distortion ($\chi 2$ test, $P < 0.001$) were excluded for linkage map reconstruction. Linkage groups were identified based on an independent logarithm of odds (LOD) threshold of 12. Unlinked markers were excluded. The strongest cross-link (SCL) in the final map is 5.4. The linkage map was built using the regression mapping algorithm, a recombination frequency smaller than 0.40, and an LOD larger than 3. Up to three rounds of marker positioning were conducted with a jump threshold of 5. A ripple was performed after the addition of each new marker. Map distances were calculated using the Kosambi mapping function. All markers resolved onto 22 linkage groups were matched to positions in the *Oreochromis niloticus* genome using a chain file (Brawand *et al.* 2014) with liftover (UCSC Genome Browser LiftOver tool; Hinrichs *et al.* 2006), which converts genome coordinates between

■ **Table 1 Summary of length and number of markers for each linkage group. Synteny between this study (Pun-LG) and the *Oreochromis niloticus* reference (Ore-LG) is indicated**

Pundamilia LG	*Oreochromis* LG	length [cM]	# SNPs
Pun-LG1	Ore-LG3	74.182	120
Pun-LG2	Ore-LG7	100.271	103
Pun-LG3	Ore-LG22	65.086	100
Pun-LG4	Ore-LG16-21	75.325	94
Pun-LG5	Ore-LG1	74.372	90
Pun-LG6	Ore-LG11	72.082	90
Pun-LG7	Ore-LG15	74.093	88
Pun-LG8	Ore-LG19	71.579	88
Pun-LG9	Ore-LG18	63.352	82
Pun-LG10	Ore-LG23	70.089	77
Pun-LG11	Ore-LG10	65.94	76
Pun-LG12	Ore-LG17	77.124	75
Pun-LG13	Ore-LG5	90.878	74
Pun-LG14	Ore-LG9	63.956	71
Pun-LG15	Ore-LG8-24	70.914	61
Pun-LG16	Ore-LG14	72.914	60
Pun-LG17	Ore-LG20	69.652	58
Pun-LG18	Ore-LG6	65.41	54
Pun-LG19	Ore-LG2	63.732	44
Pun-LG20	Ore-LG4	72.481	39
Pun-LG21	Ore-LG13	74.665	33
Pun-LG22	Ore-LG12	65.627	20
		1593.724	1597

different assemblies. This matching allows examining the synteny between *Pundamilia* and *Oreochromis* chromosomes and comparisons with other published studies.

Anchoring of reference scaffolds

In order to reconstruct a chromosomal reference genome for *Pundamilia*, we used the linkage map to anchor the scaffolds of the *Pundamilia* genome from Brawand *et al.* (2014) onto the 22 *Pundamilia* linkage groups (Pun-LGs) identified during mapping (see paragraph above). We ordered and oriented the scaffolds with ALLMAPS (Tang *et al.* 2015). Gaps between the scaffolds were then estimated using interpolated recombination rate estimates based on the conversion between map distances (cM) and physical distances (bp) as implemented in the ALLMAPS function "estimategaps" (Tang *et al.* 2015). We caution, that gap sizes estimated from interpolated recombination rates are associated with high uncertainty. In addition to an improved reference version, resolving linkage groups, we compiled a chain file for converting positions on the original *Pundamilia nyererei* reference (Brawand *et al.* 2014) to our new reference (*Pundamilia* reference version 2.0). Chain files were produced with ALLMAPS and in the opposite direction using chainSwap from kentUtils (https://github.com/ENCODE-DCC/kentUtils). We could then use the chain file to liftover the position of all 7,401 genotyped loci, using Picard liftoverVcf (http://broadinstitute.github.io/picard/index.html). In addition, we generated a new version of the NCBI *Pundamilia nyererei* RefSeq annotation file with the positions for reference version 2.0 by lifting over the positions from the NCBI PunNye1.0 annotation release 101 (https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Pundamilia_nyererei/101/#BuildInfo) using the UCSC liftOver tool (Hinrichs *et al.* 2006) and custom-made chain files (see Table 2). By comparing physical (bp) and recombination distances (cM), we estimated recombination rates along the different linkage groups. First, we pruned the linkage map for markers generating negative recombination rates and markers that were less than 20 kb apart. Then we fitted a cubic smoothing spline to the physical (bp) and recombination (cM) distances using the R function "smooth.spline" setting the smoothing parameter (spar) to 0.7 and inferred the recombination positions in cM for the genomic positions as the first derivative of the "predict.smooth.spline" function.

QTL mapping of sex

QTL mapping of the sex-determining region was performed with Rqtl (Broman *et al.* 2003) based on 209 individuals (3 F2 were discarded prior to analysis as they were juveniles) and 1,597 SNP markers. 137 males and 72 females were included. Sex was mapped by standard interval mapping as a binary trait and significance was determined by permutation (n = 1000). Bayesian confidence intervals were estimated as implemented in Rqtl and the highest LOD score was used to calculate the percent variance explained following $1 - 10^{-2 \, LOD / n}$ (Broman and

Sen 2009). Plotting phenotypic sex against the genotypes for the marker most strongly associated with sex, revealed two individuals labeled as females, but carrying a male genotype. Those individuals were dissected and the post-hoc inspection revealed undeveloped gonads. The same plot also revealed both males (n = 74) and females (n = 32) that were heterozygous at the locus strongly associated with sex. To investigate if sex in those individuals, was explained by another locus, we extracted the genotypes of these individuals, sub-setting the data set and repeated the interval mapping. Further, we made use of 366 markers positioned on the linkage group containing the sex QTL and investigated segregation patterns at those loci in more detail in the larger of our mapping families (n = 122 F2 offspring). Based on the improved, annotated reference (v.2.0) we determined the number of annotated genes in the QTL interval and screened for candidate genes for sex determination.

Data availability

All genomic resources (see Table 2), the genotype (vcf format), and phenotype file are available at the dyrad repository https://doi.org/10.5061/dryad.59q56g6. Raw read sequencing files (fastq files for all 224 individuals) are deposited on short read archive SRA accession SRP136207. Supplemental material available at Figshare: https://doi.org/10.25387/g3.6221921.

RESULTS AND DISCUSSION

Linkage map

The linkage map comprises 22 linkage groups containing 1,597 markers with an average marker distance of 1.01 cM adding up to a total map length of 1593.72 cM (Figure 1, Table 1). It is slightly longer than other maps published on Lake Victoria cichlids (1130.63 cM in Henning *et al.* 2014, 1133.2 cM in Kudo *et al.* 2015 and 1225.68 cM in Henning *et al.* 2017), but contains more markers with a lower average marker distance (1.30 cM (Henning *et al.* 2014), 1.09 cM (Henning *et al.* 2017) and 6.09 cM (Kudo *et al.* 2015)). The detection of 22 linkage groups is consistent with the expected number of chromosomes in haplo-tilapiine cichlids (Guyon *et al.* 2012). Out of 1,597 markers used to build the *Pundamilia* linkage map, 1,182 markers could be positioned onto *Oreochromis niloticus* linkage groups (Ore-LG). Figure 2 reveals extensive synteny between the chromosomes of these distantly related cichlid species. The linkage map presented here will facilitate comparative genomics and will enable comparisons of previous QTL results with newly established results (for an example see paragraph below on QTL for sex-determination) using Ore-LGs as a reference point.

Improvement of the genomic resources for Lake Victoria cichlids (Pundamilia)

The *Pundamilia* linkage map provides a new chromosome framework for whole genome sequence assembly and map integration with more anchoring points then previous published maps. The anchored genome encompasses 78.7% of the total bases (653,642,680 bp) of the original *P.*

■ **Table 2 List of genomic resources provided with this manuscript and available at the dyrad repository** https://doi.org/10.5061/dryad.59q56g6

| Type	Name
Text file giving the position of 1,597 loci on the *Pundamilia* linkage map and the respective positions on *Pundamilia* and *Oreochromis* references	P_cross.MarkerPositions.txt
Fasta file of the improved *Pundamilia* reference genome (v2.0)	P_nyererei_v2.fasta.gz
Chain files to convert position between original (v1.0) and new reference (v2.0)	P_nyererei_v1.To.P_nyererei_v2.chain, P_nyererei_v2.To.P_nyererei_v1.chain
Annotation file matching reference v2.0 position with the NCBI annotation release 101	P_nyererei_v2.gff.gz
Text file giving the extrapolated recombination rates along *Pundamilia* reference genome	P_nyererei_v2.RecRates.txt
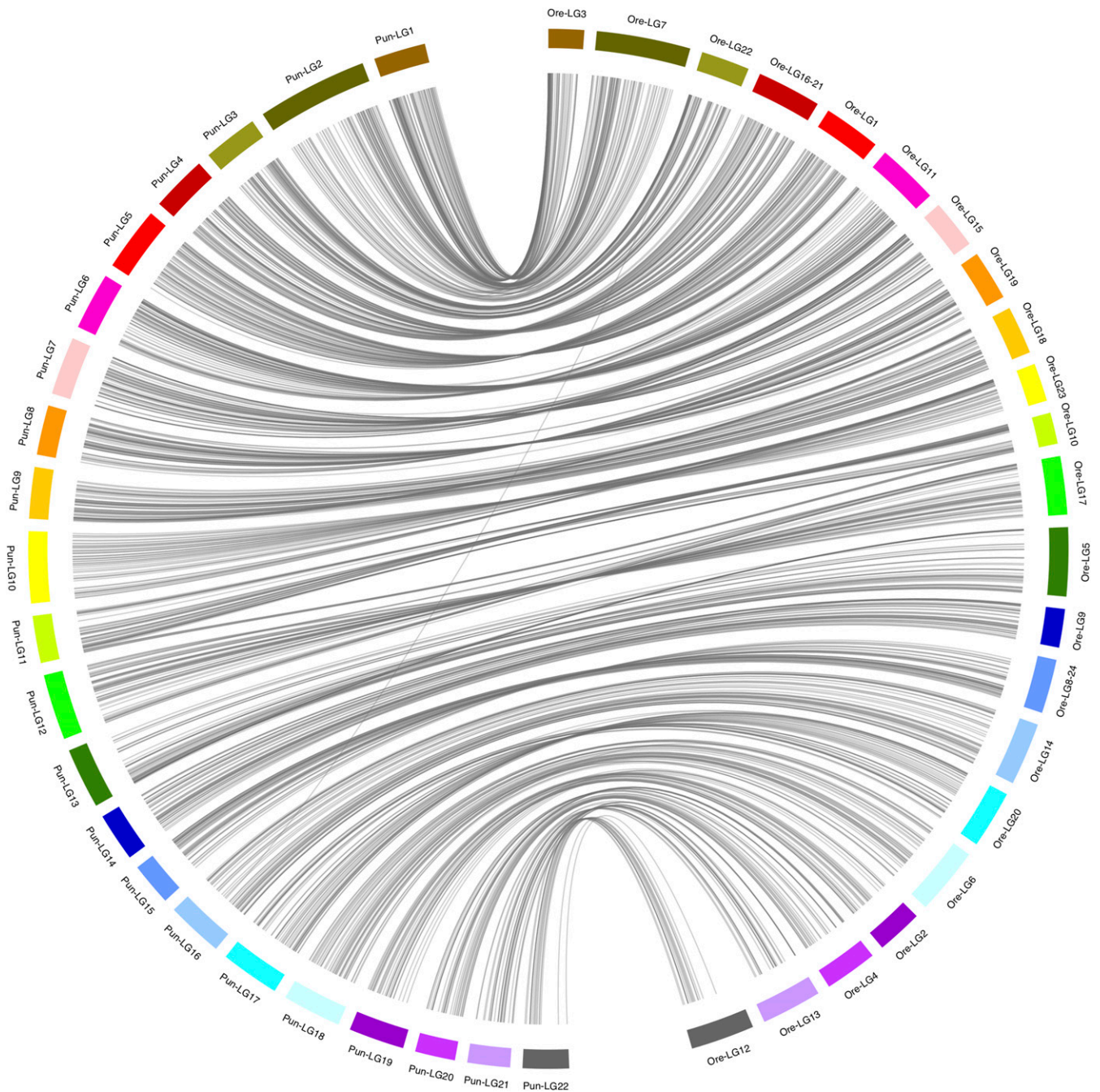
Figure 2 Synteny plot showing the correspondence of *Pundamilia* linkage groups (Pun-LG) with *Oreochromis niloticus* linkage groups (Ore-LG). Lines indicate markers used in linkage map construction, which could be positioned in the *Pundamilia* reference (v2.0) and lifted over to the *Oreochromis* reference.

nyererei reference genome based on 383 anchored scaffolds, of which 233 are now oriented. This is a slightly higher fraction than in the Lake Malawi cichlid *Metriaclima zebra*, where 564,259,264 bp (66.5%) of the genome sequence could be anchored to linkage groups (O'Quin *et al.* 2013). The mean marker density is 2.4 per megabase (Mb). The 6,853 remaining scaffolds could not be anchored due to lack of informative markers. This improved resolution of the new reference assembly (v2.0) will greatly facilitate genome scan approaches in Lake Victoria cichlids. Such approaches rely on the information from neighboring genomic positions to identify signatures of selection due to genetic

hitchhiking. Any approaches evaluating or making use of linkage information, like linkage disequilibrium (LD) based genome scans, association studies or evaluations of the genomic landscape of divergence will now become more powerful. Together with the improved reference, we provide chain files to liftover positions from the previous version (v1.0) to the new chromosome level resolved reference version (v2.0). We further provide a matching annotation file based on the NCBI annotation (see Table 2 for a complete list of all genomic resources). Finally, we estimated recombination rates and show that those are highly variable across the genome ranging from
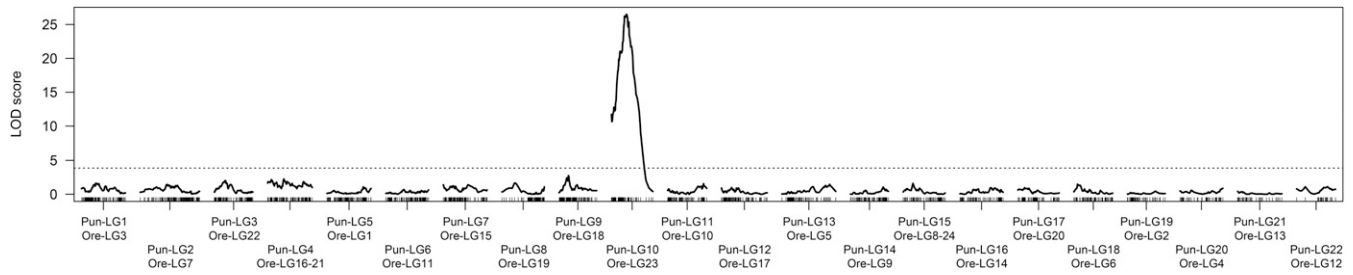
Figure 3 QTL mapping of sex. LOD scores across the 22 linkage groups are shown. Genome-wide significance levels are indicated by horizontal lines (alpha = 0.05 dotted line). Marker loci are indicated along the x-axis.

0 to 9.4 cM/Mb (Table 2), with a mean recombination rate of 2.3 cM/Mb. Knowledge of fine-scale patterns of recombination rate variation (see Figure 4C) will be useful for future studies of adaption and speciation (Stapley *et al.* 2017) in the exceptional species radiation of Lake Victoria cichlids (Meier *et al.* 2018).

Characterization of sex-determination in Pundamilia

Our knowledge of sex determination in *Pundamilia*, a prime model system of sympatric speciation in Lake Victoria, had been limited. Here, we mapped sex to Pun-LG10, which is homologous with Ore-LG23 (Figure 3; $P << 0.001$, LOD = 26.5). We did not find any further associations on any of the other LGs. Ore-LG23 has been previously identified as one potential XY sex-determining LG in *Oreochromis*

(Cnaani *et al.* 2008; Palaiokostas *et al.* 2013) and in four cichlid tribes from Lake Tanganyika overexpression of male specific genes accumulates on Ore-LG23 (Böhne *et al.* 2014). Early work on sex determination in Lake Victoria cichlids had suggested polymorphisms in several unlinked genomic regions to be associated with sex, and invoked a major effect locus and some modifiers (Seehausen *et al.* 1999). Recent QTL mapping identified genomic regions involved in sex determination in Lake Victoria cichlids on Ore-LG5 and Ore-LG2 (Kudo *et al.* 2015) or on derived, female specific B chromosomes (Yoshida *et al.* 2011). Ore-LG5 was repeatedly found to be involved in sex-determination (both ZW or XY) in other cichlids, *e.g.*, in the riverine haplochromine cichlids *Astatotilapia burtoni* and *Astatotilapia calliptera* from Lakes Tanganyika and Malawi
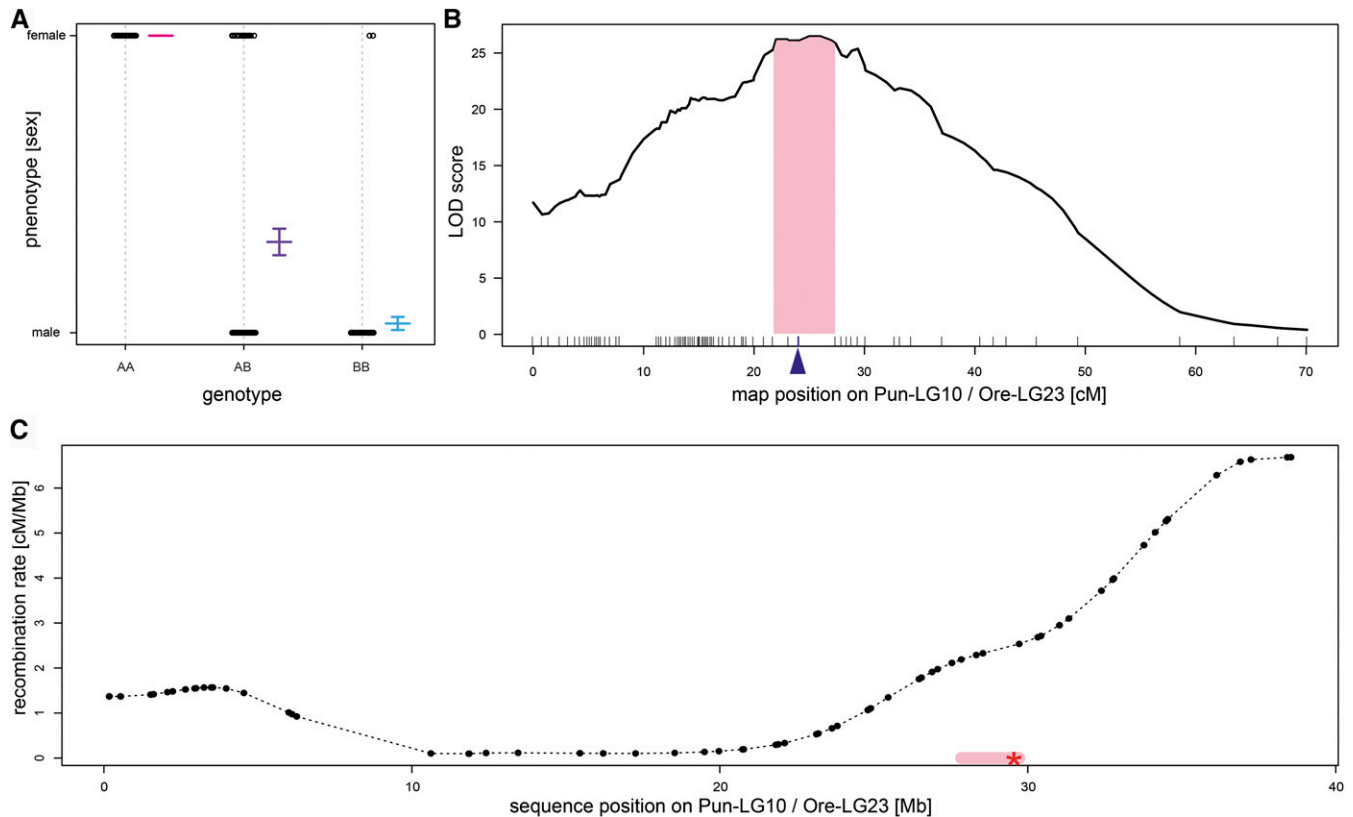

Figure 4 A) Phenotypic effect of genotypes at the locus most strongly associated with sex. The plot identifies two females as likely phenotyping errors and 106 individuals heterozygote at that locus. B) Plot of LOD scores indicating the region of strongest association with sex on Pun-LG10 (Ore-LG23). The 95% Bayesian confidence interval is highlighted in light pink. Marker loci are indicated along the x-axis. The locus shown in panel A is indicated by a blue arrow. C) Variation in recombination rates (sex-averaged) along Pun-LG10 (Ore-LG23). The Bayesian confidence interval (pink highlight) is situated next to a region of low recombination. The red star indicates the position of *amh* (candidate gene for sex determination).

and associated rivers (Roberts *et al.* 2016; Böhne *et al.* 2016; Peterson *et al.* 2017), in *Cyprichromis leptosoma* from Lake Tanganyika (Gammerdinger *et al.* 2018) and in *Labeotropheus trewavasae* and across some *Metriaclima* species from Lake Malawi (Ser *et al.* 2010; Parnell and Streelman 2013). We find no indications of sex determining factors on Ore-LG5 in *Pundamilia*.

The mapping interval (Bayesian confidence interval of 5.7 cM, 21.7 to 27.4 cM; Figure 4B) in total covers four markers and spans a region of ~1.9 Mb (Figure 4C). The marker showing the strongest association with sex in our study (Figure 4A) explains 44% of the phenotypic variance in sex. We have misidentified two likely subadults (gonads appear not to be developed on post-hoc inspection. However, sex is not entirely explained by this marker as we also identified 106 individuals, both males and females, which are heterozygous at this position. Repeating the mapping procedure for those heterozygous individuals again identified a region on Pun-LG10 (Ore-LG23) as weakly associated with sex ($P = 0.177$, LOD = 3.33, position right to previous interval at 28.8 cM). This suggests that none of the markers used to build the linkage map are determining sex directly, but that the causal locus can be found close by and indicates that there are no further major genetic determiners of sex segregating in this cross. Investigating the segregation patterns in the larger of the F2 mapping-families (n = 122) more in detail revealed that the loci selected to build the map (reciprocally homozygous in F0 female (AA) and male (BB) and heterozygous in both F1 (AB)) segregate as expected in a 50:50 ratio of AA:AB in F2 females and AB:BB in F2 males (Figure 5A). However, evaluating segregation patterns of the additional markers genotyped but not used for the construction

of the linkage map, indicate that the sex determination system on Pun-LG10 is male heterogametic (XY, Figure 5B). We identified 57 loci between 0 and 35 Mb that were homozygous in the F0 and F1 females and heterozygous in the F0 and F1 males; these markers are similarly homozygous in all F2 females and heterozygous in all F2 males, consistent with females being XX and males being XY (Figure 5B, the plot also shows 13 loci > 35 Mb). Additional evidence comes from markers heterozygous in the F0 female (AB) and homozygous in the F0 male (BB), for which we find all 35 loci for positions < 33 Mb heterozygous (AB) for both, the male and the female F1 individual of the mapping family. The heterozygous loci in both, male and female, F1 are a segregation pattern only consistent with male heterogametic (XY) segregation. If females would be heterogametic (ZW) those loci would need to be homozygous (BB) in one of the F1 sexes and not heterozygous (AB) in both as observed in the first 33 Mb of Pun-LG10 including the mapping interval of our sex QTL. Sex-averaged recombination rates around the QTL are similar to genome wide average rates. However, recombination rates to one side of the QTL are low and even close to zero within 20 Mb proximity to the mapping interval (Figure 4C). Such a pattern, potentially due to suppressed recombination in the heterogametic sex (males), might indicate initial steps toward the evolution of a heteromorphic (degenerated) sex (Y) chromosome (Charlesworth 1991).

Within our mapping interval of ~1.9 Mb, 65 genes, based on the NCBI annotation for the new *Pundamilia* reference assembly, can be found (Table S1). Among them is the anti-müllerian hormone (*amh*), a master gene for sex determination in other fish. *Amh* is part of the transforming growth factor beta pathway, responsible for the
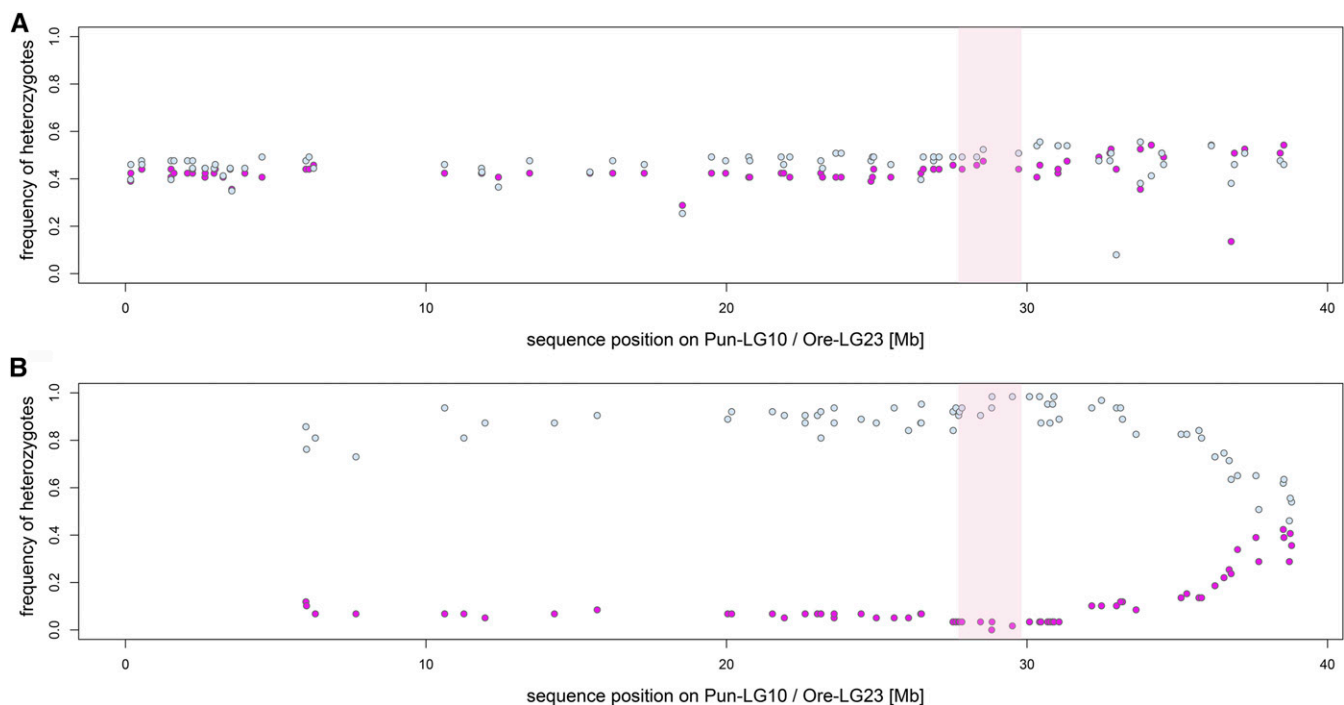
Figure 5 Frequency of heterozygote individuals (n = 122), separated by sex (63 males: light blue, 59 females: pink) for markers selected by their segregation pattern in the larger mapping family and by their position on Pun-LG10 (Ore-LG23). A) 78 markers, selected as reciprocally homozygous (AA/BB) in the F0 and heterozygote in both, the male and the female F1 (AB/AB), segregate as expected in a 50:50 ratio of AA:AB in F2 females and AB:BB in F2 males, resulting in frequency of heterozygous F2 individuals around 0.5 for both sexes. B) 70 markers, selected as homozygote in the F0 and F1 females and heterozygote in the F0 and F1 males, segregate similarly in the F2; *i.e.*, the frequency of heterozygous individuals is approaching 0 in females and 1 in males for positions < 35 Mb. The QTL region (Bayesian confidence interval) for sex determination in *Pundamilia* is located between 27.8 and 29.7 Mb (shaded region).

regression of Müllerian ducts in tetrapods (Josso *et al.* 2001). Even though teleost fish do not have Müllerian ducts, the *amh* pathway has a prominent role in sex determination for several distantly related fish species. In the Japanese pufferfish (*Takifugu rubripes*), a mutation in the receptor of the *amh* (amhrII) determines sex (Kamiya *et al.* 2012). The *amhy* (Y chromosome-specific anti-müllerian hormone) gene has been inserted upstream of *amh* in the cascade of male development in the neotropical silverside *Odonthestes hatcheri* (Hattori *et al.* 2012). Similarly, in *Oreochromis niloticus*, a Y-linked duplicate of *amh* acts as a major sex determination locus (Eshel *et al.* 2012; Li *et al.* 2015). In *Oryzias luzonensis*, a mutation of an *amh* related ligand gsdf^y is responsible for sex determination (Myosho *et al.* 2012). The same ligand is suggested to be involved in sex determination in the haplochromine cichlid *Astatotilapia calliptera* (Peterson *et al.* 2017). Beside the two master sex determination genes in *Oreochromis niloticus* on LG23 (*amh*) and in *Astatotilapia calliptera* on LG7 (*gsdf*) (Peterson *et al.* 2017), no other candidate for sex determination have been shown to be directly involved in sex determination in any other cichlid species (Heule *et al.* 2014b, Böhne *et al.* 2016, Gammerdinger *et al.* 2018, but see Böhne *et al.* 2014). They might act as so-called "newcomers" (Herpin and Schartl 2015). Our results indicate that in the Lake Victoria cichlid *Pundamilia* Pun-LG10 (Ore-LG 23) acts as an (evolving) sex chromosome, even though it might not be the only region controlling sex in *Pundamilia*. The anti-müllerian hormone *amh* (or a derived copy) appears to be a strong candidate influencing sexual development in *Pundamilia*, but further work is warranted to characterize the genomic candidate region and the impact of this candidate gene on sex determination.

A recent meta-analysis showed that transitions between sex determination systems are frequent across fish species, including transitions to and between heteromorphic sex chromosomes (Pennell *et al.* 2018). In cichlids a high turnover of sex determination systems was described in Lake Malawi (Ser *et al.* 2010), Lake Tanganyika (Böhne *et al.* 2014; Gammerdinger *et al.* 2018), and in oreochromine cichlids (Cnaani *et al.* 2008). Neither *amh*, nor Pun-LG10 or a homologous region was invoked in sex determination in other Lake Victoria cichlids that have previously been used for mapping sex (Kudo *et al.* 2015; Yoshida *et al.* 2011). This circumstance implies that multiple sex determining systems segregate among Lake Victoria cichlids as well. This is consistent with early work on sex determination in this group (Seehausen *et al.* 1999). Given the extreme youth of the Lake Victoria species radiation (~15,000 years; Seehausen 2006), this may be surprising at first. Recent work, however, has shown that much of the genetic variation in the radiation is much older than the species radiation and originated from a hybridization event between two anciently divergent cichlid lineages from which all 500+ species of the radiation evolved (Meier *et al.* 2017a). It is tempting to speculate that the variation in sex determination systems between and within species of this radiation traces its roots to these ancient lineages too, something that should be tested in the future.

ACKNOWLEDGMENTS

AUTHOR'S CONTRIBUTIONS

PGDF, JS, MPH, and JIM performed the experiment and the analysis. OS conceived the original idea, made the F1 cross and supervised the project. PGDF and JS took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Communicating editor: R. Doerge