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A single QTL with large effect is associated with female functional virginity in an asexual parasitoid wasp

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Abstract

During the transition from sexual to asexual reproduction, a suite of reproduction-related sexual traits become superfluous, and may be selected against if costly. Female functional virginity refers to asexual females resisting to mate or not fertilizing eggs after mating. These traits appear to be among the first that evolve during transitions from sexual to asexual reproduction. The genetic basis of female functional virginity remains elusive. Previously, we reported that female functional virginity segregates as expected for a single recessive locus in the asexual parasitoid wasp *Asobara japonica*. Here, we investigate the genetic basis of this trait by quantitative trait loci (QTL) mapping and candidate gene analyses. Consistent with the segregation of phenotypes, we found a single QTL of large effect, spanning over 4.23 Mb and comprising at least 131 protein-coding genes, of which 15 featured sex-biased expression in the related sexual species *Asobara tabida*. Two of the 15 sex-biased genes were previously identified to differ between related sexual and asexual population/species: *CD151 antigen* and *nuclear pore complex protein Nup50*. A third gene, *hormone receptor 4*, is involved in steroid hormone mediated mating behaviour. Overall, our results are consistent with a single locus, or a cluster of closely linked loci, underlying rapid evolution of female functional virginity in the transition to asexuality. Once this variant, causing rejection to mate, has swept through a population, the flanking region does not get smaller owing to lack of recombination in asexuals.

KEYWORDS
asexuality, candidate genes, female functional virginity, introgression, linkage map, resistance to mating, single major QTL

1 | INTRODUCTION

A suite of reproduction-related sexual traits become superfluous during the transition from sexual to asexual reproduction (Carson et al., 1982; Jeong & Stouthamer, 2005; Kraaijeveld et al., 2009, 2016; Kremer et al., 2009; Ma et al., 2014a; Pannebakker et al., 2005; Parker et al., 2019; Russell & Stouthamer, 2011; Schwander et al., 2013). Asexual females do not require mating with males for offspring production, and produce only daughters. As a consequence, costly sexual traits are expected to be selected against in asexual lineages (Ma et al., 2014b; van der Kooi & Schwander, 2014). Consistent with this expectation, there is good evidence for rapid

reduction of sexual traits in asexual females, especially of traits linked to mate attraction and copulation, which are believed to be particularly costly in many species (Schwander et al., 2013; van der Kooi & Schwander, 2014).

Traits reducing mate attraction or copulation probability are of particular interest in hymenopteran insects, where they have been referred to as "female functional virginity" (Huigens & Stouthamer, 2003; Jeong & Stouthamer, 2005; Russell & Stouthamer, 2011; Stouthamer et al., 2010). In hymenopterans, virgin sexual females produce only sons, because sex determination relies on haplodiploidy (males develop from unfertilized, haploid eggs, females from diploid eggs) (Whiting, 1933). In many species, parthenogenesis is induced by endosymbionts, such as *Wolbachia*, *Cardinium* and *Rickettsia* (Ma et al., 2014b; Ma & Schwander, 2017). During the initial infection stage, an increasing number of asexual females cause sex ratios to become largely female biased. Infected females still can mate and fertilize their eggs in the initial infection stage, as found in *Trichogramma* wasps (Stouthamer & Kazmer, 1994). The female-biased sex ratio would then select for increased production of male offspring. Under haplodiploidy, an all-male progeny is produced when uninfected females remain virgin or mated females do not use stored sperm to fertilize their eggs. The mutations that increase production of male offspring were thus referred to as "female functional virginity" mutations (Huigens & Stouthamer, 2003; Stouthamer et al., 2010). These mutations will then spread into the infected population via matings between carrier males and infected females.

Reduced sexual traits associated with female functional virginity have been reported in at least six different asexual hymenopteran species (Gottlieb & Zchori-Fein, 2001; Jeong & Stouthamer, 2005; Ma et al., 2014a; Pannebakker et al., 2005; Pijls et al., 1996; Russell & Stouthamer, 2011; Stouthamer et al., 2010). The genetic and molecular basis underlying female functional virginity remains elusive, which is in part due to the inability to perform crosses in asexual organisms. However, crosses are possible in some hymenopteran asexual species, because asexuality is caused by infection with maternally transmitted endosymbionts, such as *Wolbachia*. When asexual females are cured from their endosymbionts via antibiotic treatment, they produce males and these males can be mated to females from related sexual strains (Jeong & Stouthamer, 2005; Ma et al., 2014a; Pannebakker et al., 2005; Russell & Stouthamer, 2011). The current evidence of the genetic mechanisms resulting in female functional virginity in asexual hymenopterans points to mutations in few genes with relatively strong phenotypic effects (reviewed in van der Kooi & Schwander, 2014), although these results may be biased by the Beavis effect, which postulates an overestimation of QTL effect sizes when sample size is small (Beavis, 1994).

Asobara japonica is a parasitic wasp with both sexual and all-female asexual populations that are geographically separated. Asexual populations are distributed across mainland Japan, whereas sexual populations largely occur on southern islands (Murata et al., 2009). *A. japonica*, like all hymenopterans, has haplodiploid reproduction (Giorgini et al., 2010; Kageyama et al., 2012; Leach et al., 2009; Ma et al., 2014a; Werren, 1997; Werren et al., 2008). Asexuality in *A. japonica* is induced by the bacterial endosymbiont *Wolbachia*,

and unfertilized haploid eggs laid by endosymbiont-infected females typically undergo diploidization and develop as females. Sexual populations are devoid of *Wolbachia* (Kremer et al., 2009; Reumer et al., 2012). A previous study has shown no variation between the *Wolbachia* strains in five asexual populations of *A. japonica* (Kraaijeveld et al., 2011), suggesting that the *Wolbachia* infection in this species is relatively young. Given the divergence between two large groups of mitochondial haplotypes in the parasitoid wasp, it was estimated that *Wolbachia* infection occurred between 8.2×10^5 and 2.2×10^5 generations (approximately 300,000–70,000 years) ago (Reumer et al., 2012). Removing the infection by antibiotic treatment results in the production of haploid males from unfertilized eggs. Asexual females also occasionally produce haploid males under natural conditions (Heath et al., 1999; Reumer et al., 2012). Although these males usually do not have any mating opportunities in nature, in the laboratory they can be mated with females of sexual strains.

A previous study in *A. japonica* revealed that asexual females are less attractive than sexual females, and completely refuse to mate (Ma et al., 2014a). Female functional virginity in asexual *A. japonica* is thus probably governed by genes mediating female mating propensity. Introgression of alleles from an asexual strain into a sexual one for four consecutive generations revealed that a single recessive locus with major effects caused introgressed females to produce only haploid sons, suggesting a relatively simple genetic architecture for female functional virginity (Ma et al., 2014a).

In the current study we identified the genomic regions and possible candidate genes associated with female functional virginity in *A. japonica*. We genotyped the sex-asex introgressed females that were previously analysed for resistance to mating and produce only haploid sons (Ma et al., 2014a). We constructed a genetic map, a genome assembly of *A. japonica* from PacBio long reads sequencing, and subsequently performed a quantitative trait loci (QTL) analysis. In addition, Illumina short-reads of sexual and asexual strains of *A. japonica*, and a transcriptome data set of the sexual species *Asobara tabida*, were used to infer candidate genes.

2 | MATERIALS AND METHODS

2.1 | Wasp strains and culturing

A sexual and a *Wolbachia*-infected, asexual strain of *Asobara japonica* were used. The sexual strain originated from the Amami-Oshima island (AO) and the asexual strain from Kagoshima (KG), both in Japan, and have been cultured in the laboratory since 2009. These two strains are closely related (Murata et al., 2009; Reumer et al., 2012), which minimizes the probability of genetic incompatibility in crosses. *A. japonica* was cultured on second-instar *Drosophila melanogaster* larvae as hosts at 25°C, with a 16L: 8D light-dark cycle and 60% relative humidity (for details see Ma et al., 2013, 2014a).

Sons from asexual females (so called "asexual" males) were obtained by antibiotic treatment of *Wolbachia*-infected females (see below) or directly collected from mass cultures in which males occur

at low frequency (i.e. ~0.7%, N = 6,000 individuals). As spontaneously occurring males are sometimes diploid (Ma et al., 2015), flow cytometry was used to ensure that all males used in experiments were haploid (for details see Ma et al., 2013).

Antibiotic treatment (to obtain "asexual" males) was performed by providing *Wolbachia*-infected females with *Drosophila* hosts cultured on food containing 10 mg of rifampicin for 1 g yeast powder (see Ma et al., 2014a). Rifampicin reduces the *Wolbachia* titre, but has little impact on development in *Asobara* (Dedeine et al., 2001). Complete removal of *Wolbachia* was confirmed by the production of only male offspring by the treated females.

2.2 | Introgression cross design

To investigate the genetic basis of female functional virginity, measured as females producing haploid sons only, asexual KG males were mated to virgin sexual AO females in the first generation, and then to introgressed F1 females for three consecutive generations (details in Ma et al., 2014a; Figure 1b). Briefly, virgin females were collected by individually isolating wasp pupae in plastic vials (diameter 2.4 cm, height 7.5 cm) containing a layer of agar to control humidity (Ma et al., 2013). These virgin females were individually paired with a male from the asexual strain for 24 h, and subsequently offered

approximately 100 s-instar *D. melanogaster* larvae for oviposition for 36 h. The resulting wasp pupae were isolated from parasitized hosts to prevent mating upon emergence. Females emerging from these pupae (the F1 generation) were collected and backcrossed to males of the asexual KG strain, by individual pairing, to produce the offspring of the next generation (Ma et al., 2013, 2014a).

Under this cross design, the proportion of asexual alleles in females of each generation is expected to increase from 50% in F1, to 75% in BC2, 87.5% in BC3 and a 93.8% in BC4 (Ma et al., 2014a; Figure 1b). For each backcross, the emerging wasps were anaesthetized with CO_2, counted and sexed (Ma et al., 2013, 2014a). For each backcross generation, a subset of introgressed females (N = 49–225 depending on the generation) was sampled from the introgression experiment to determine their offspring sex ratio, by pairing a single female with a sexual male for 24 h and allowing 36 h for oviposition. Each female was scored for production of exclusively male or male and female offspring. The thus obtained binomial trait was used as the phenotype for QTL mapping.

2.3 | Genome-wide SNP markers development

To enable genetic analysis, SNP markers were generated from Illumina short reads of 30-pooled individuals of both the sexual
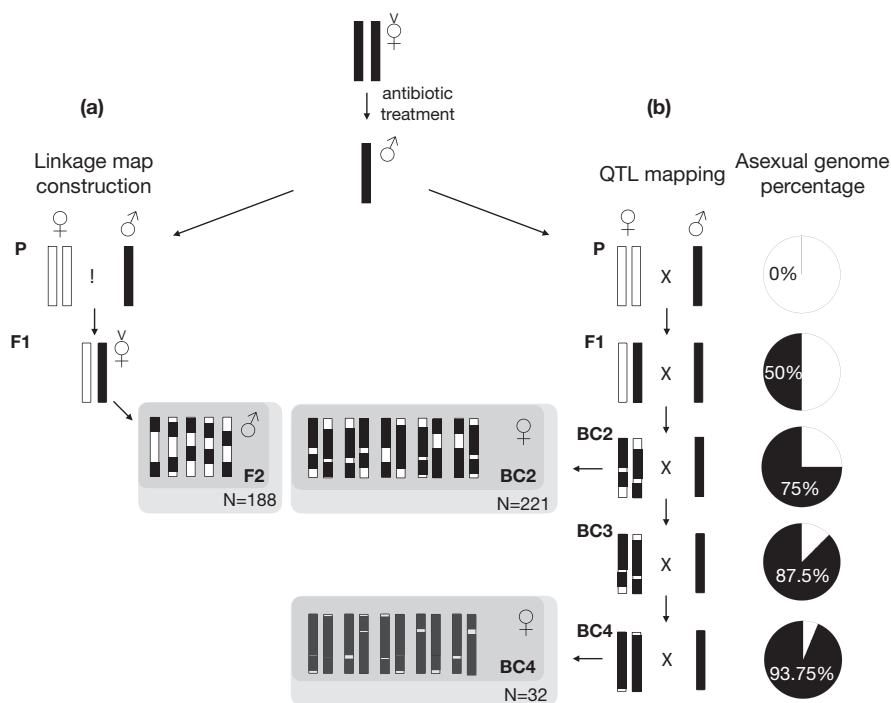

FIGURE 1 Cross schemes used to generate F2 recombinant males for linkage map construction (a), and four generations of backcrosses for QTL mapping of the female functional virginity trait (b). The AO sexual female (white) is crossed to the KG asexual male (black) to generate F1 females that were set up as virgins to produce recombinant F2 males. The F2 males (a) in grey shade were used for genotyping and linkage map construction, and females from backcross generation 2 (BC2) and 4 (BC4) (b) in grey shade were used for determining female mating success and QTL mapping. Black and white mosaic bars denote relative component of sexual and asexual genomes in consecutive generations. The pie chart shows the percentage of asexual genome component (in black) in diploid females in consecutive introgressed generations (modified from Ma et al., 2014a)

AO and asexual KG strains (BioProject: PRJNA661661, accession number: SRR12618696-SRR12618699). Genomic DNA was extracted with a modified high-salt protocol (adjusted from Aljanabi & Martinez, 1997, see details in the next section). DNA libraries were prepared at the University Medical Center Groningen Sequencing Facility and sequenced on an Illumina HiSeq2000. SNP markers were developed from the Illumina short reads using the de novo genome-wide DIAL (De novo Identification of ALleles) computational pipeline, which identifies SNPs between two closely related genomes without needing a reference genome (Ratan et al., 2010). To prevent long CPU times, only a subsample of the reads (1,172.5 Mb or 3.9–4.2x coverage of the estimated wasp genome of 282–304 Mb, see K-mer analysis below) were analysed. The DIAL pipeline first removes duplicate reads and builds a short consensus sequence (ranging from 95 to 300 bp), which it then aligns to reads from previous runs to form clusters. Clusters with few nucleotide differences are selected and putative SNPs are detected and filtered based on the read quality at the variant position (minimum Phred-quality score used was 20).

The DIAL pipeline initially generated a database of 21,976 SNPs with their flanking region sequences and information about coverage depth and phred-quality scores for each of the two strains. The SNP coverage ratio R is defined as the DIAL-generated coverage depth (d_SNP) of the SNP nucleotide, divided by the average coverage depth (d_reads) of the selected reads (against the estimated genome size): $R = d\_SNP/d\_reads$. Following author recommendations (Ratan et al., 2010), only markers with $R > 1.5$ were selected for further verification (n = 10,001). Next, minimum coverage three and minimum base pair quality score 30 were used for further filtering which resulted in 951 remaining SNPs. Lastly, mitochondrial, bacterial and human homologous fragments were removed from the candidate SNP cluster list based on the BLAST results from NCBI sequence database, yielding 420 usable SNPs of which 130 were randomly selected for linkage map construction.

A subset of these 130 SNPs (57) was tested for amplification in both strains with primer pairs designed with Perlprimer (version 1.1.21, Marshall, 2004, Table S1) and Sanger sequenced (see below). This yielded 47 confirmed SNPs, to which another 66 randomly selected SNPs from the filtered SNP pool were added, for a total of 113 SNPs to be used for in the linkage map and QTL analyses.

The Illumina short reads (KG and AO strains) were also used for estimating the genome size for each strain of *A. japonica* (KG: 281–304 Mb; AO: 280–284 Mb). The genome size was estimated by fitting the k-mer distribution of the Illumina short reads, obtained from JELLYFISH v2.1.0 (Marçais & Kingsford, 2011), using GENOMESCOPE v2 (http://qb.cshl.edu/genomescope/genomescope2.0/) (Vurture et al., 2017), with recommended k-mer size of 21 (jellyfish count -m 21 -o fastq.counts -C -s 500000000 -t 5 Genome_R1.fastq GenomeR2.fastq).

2.4 | Genotyping and linkage map construction

A cross between the sexual and asexual strains was set up to construct a linkage map for QTL mapping (Figure 1a). Virgin sexual AO females were individually crossed to asexual KG males (n = 20) and provided with 100 hosts for oviposition (Figure 1a). After approximately two weeks, wasp pupae were isolated to collect virgin F1 females (n = 23) that were again offered 100 hosts for oviposition. Recombinant F2 (haploid) males (n = 188) were collected and stored at –80°C until DNA extraction and genotyping. DNA was extracted using a modified high salt protocol (adjusted from Aljanabi & Martinez, 1997). In short, each wasp was homogenized in a 1.5 ml Eppendorf tube with homogenization buffer (0.4 M NaCl, 10 mM Tris:HCl pH 8.0, 2 mM EDTA) using a plastic pestle. After adding proteinase K, the tissue was incubated overnight at 55°C. After isopropanol precipitation, the DNA was dissolved in 20 μl MQ water.

The 188 F2 males were genotyped for the 113 SNPs using the high-throughput Kompetitive Allele-Specific PCR robot genotyping system (KASP, Semagn et al., 2014) at the Institute of Biology Leiden (The Netherlands). Two allele-specific forward primers with a single difference at the SNP nucleotide position, and one common reverse primer (Table S2), were designed for each SNP fragment sequence. Each forward primer is attached to a unique unlabelled tail sequence at the 5′ end (15 bp) which can be detected by fluorescence (Semagn et al., 2014).

The initial genotyping data set was first cleaned up by removing SNPs with >50% missing genotypes, which resulted in 96 suitable SNPs for linkage mapping. MAPDISTO software (version 1.7.7; Lorieux, 2012) was used to construct the genetic linkage map using the "BC" (backcross) population type setting to accommodate the haplodiploid sex determination system and cross design (Figure 1a). The "autoripple" function was used to order the loci per linkage group. Kosambi's mapping function (Kosambi, 1943) was used to translate recombination fractions into map distances. The linkage map was visualized with MAPCHART (version 2.2, Voorrips, 2002).

2.5 | QTL mapping

In total, 221 BC2 and 32 BC4 introgressed females were genotyped for the 96 SNP markers using the KASP platform. DNA was extracted from homogenized whole bodies with a Qiagen DNeasy kit after overnight treatment with 10% proteinase K (Qiagen) at 56°C. The samples were processed on a BioSprint 96 workstation (Qiagen) with standard BS 96DNA program, resulting in 200 ul Buffer AE (Qiagen) DNA elution.

QTL analyses were conducted in the R package R/qtl v 1.46–2 (Broman & Sen, 2009; Broman et al., 2003). The genotype data were converted to the backcross format, with homozygotes encoded as "AA" and heterozygotes as "AB" (Table S3) (Broman & Sen, 2009). Duplicated markers were identified and removed with the "findDupMarkers" function. After removing markers with no genotype data, 92 markers remained. For each G2 or G4 recombinant female (n = 253 in total), the probability of the allelic state at every map position, conditional to the observed genotype for the segregating

SNP markers, was estimated with a hidden Markov model, allowing for 0.001 genotyping error rate and missing genotype data. The genetic map was also re-estimated from the backcross by the Lander-Green algorithm (hidden Markov model) for further QTL analysis using R/QTL (Broman & Sen, 2009, Figure S1). QTLs for the female functional virginity were identified by Haley–Knott regression (HK) or the expectation maximization (EM) algorithm (Haley & Knott, 1992). The G2 and G4 generations were analysed jointly. Function "scanone" was run first for a binary model using 1,000 permutations, and generation as a covariate. A 5% threshold of logarithm of the odds (LOD) was applied in both methods. The "FIND.MARKER" function was then used to identify the closest markers to the QTL and calculate the phenotypic effects of the markers on either side of the QTL using the function "EFFECTPLOT". The 95% confidence interval of the QTL region was calculated with function "BAYESINT". Finally, a linear regression, generalized linear model (GLM, y ~ Generation +QTL + Generation:QTL, generation and the interaction with QTL were not significant), was applied to investigate the effect of generation and an interaction between generation and the QTL (see details in Results section), and to estimate the percent of phenotypic variance explained by the QTL.

2.6 | Genome assembly and annotation

We assembled the genome of the asexual KG strain of *A. japonica* using PacBio long-read Single-Molecule-Real-Time (SMRT) sequencing (BioProject: PRJNA661661, accession number: JADHZF000000000). The mechanism of asexuality in this species is probably gamete duplication, which causes genome-wide homozygosity (Ma, 2014). As expected, homozygosity estimates obtained with JELLYFISH v2.1.0 (Marçais & Kingsford, 2011) were extremely high (98.4%–100%). Samples of 50 milligrams of asexual KG females (approximately 120 individuals) were used for DNA extractions with Genomic-tip 100/G (Qiagen) according to the manufacturer's protocol. A single library was constructed with SMRTBELL, and BLUEPIPPIN was used to select the >10 kb long fragments. The library was run on 30 PacBio RSII cells. In total 2,189,795 (post-filter) reads were obtained, representing an approximate coverage of 94x. PacBio reads were then de novo assembled with FALCON (v0.2.2) with default parameters for insect data sets, but with modifications to fit our study species (Chin et al., 2016), i.e. length_cutoff_pr =12,000, pa_HPCdaligner_option = -v -dal128 -t16 -e0.75 -M24 -l4800 -k18 -h480 -w8 -s100, pa_Dbsplit_option = -x500 -s400, falcon_sense_option = --output_multi –min_idt 0.70 –min_cov 5 –max_n_read 200 –n_core 8. The draft genome was then polished with QUIVER (2.2.1) (Chin et al., 2013). The quality and summary statistics of the assembled genome were analysed with QUAST v4.6.3 (Gurevich et al., 2013). BUSCO3 was used to estimate the completeness of the assembled PACBIO KG genome (Simão et al., 2015).

RNAseq data sets originally generated for a different study were used to annotate the KG genome. These data sets were obtained from ovary tissues of both sexual and asexual *A. japonica* strains. Long-read SMRT sequencing of RNA was conducted on 100 pooled ovaries of asexual KG females, and ovaries from sexual females of the population IR (Iriomote-jima island Japan, Murata et al., 2009). Dissected ovaries were disrupted in Trizol (Invitrogen) and transferred to phase lock gel tubes. 200 µl chloroform was added to the 1 ml Trizol solution, shaken for 15 s and centrifuged for 10 min at 12,000 *g* at 4°C. The resulting upper layer was transferred to the gDNA Eliminator spin columns of the RNeasy Plus Micro Kit (Qiagen) and further RNA extraction proceedings followed the kit protocol. The Teloprime (Lexogen) method was used to synthesize and amplify full-length cDNA. The library was constructed with SMRTBELL and sequenced on two cells of the PacBio Sequel. High-quality circular consensus sequences were used to identify full-length mRNA sequences, based on the presence of the primers and polyA sequences. ISOSEQ PIPELINE (v3) was then used to perform the analysis and polish isoform sequences (Gordon et al., 2015). The high-quality isoform sequences were subsequently aligned with GMAP against the PACBIO genome assembly of the KG strain (Wu & Watanabe, 2005). Finally, the generated transcriptome was functionally annotated with BLAST2GO v4.1 (Götz et al., 2008).

2.7 | Candidate gene analysis

Our QTL mapping approach uncovered a large QTL peak spanning over at least 4.23 Mb of the *A. japonica* genome assembly, in two nonoverlapping contigs (see results). To obtain a list of candidate genes in this region, we used the original annotations from the PacBio genome (based on *A. japonica* transcriptomes from ovary tissue), and improved these using publicly available transcriptomes of three parasitoid wasp species (*Diachasma alloeum*, *Fopius arisanus*, and *Microplitis demolitor*; NCBI BioProject accession numbers: PRJNA284396, PRJNA259570, PRJNA214515 respectively) to ensure genes not expressed in ovaries were included. We were able to annotate 131 candidate genes in the QTL region (see results).

We hypothesized that female resistance to mating may be a female-specific trait and be regulated by genes featuring sex-biased expression. We screened all (131) candidate genes for sex-biased expression in a data set of the sexual sister species *A. tabida*, which was originally generated for a different purpose. The *A. tabida* data set was obtained by extracting RNA from whole-bodies of male and female *A. tabida*, with TriZol (Invitrogen) according to manufacturer's protocol. Individual RNA extractions of 11 females were pooled for one female library, and of 25 males for one male library. RNA libraries were prepared and sequenced on the Illumina HiSeq2000 at the UMCG Sequencing Facility. In total, 20.2 million and 26.6 million reads were retained in the female and male libraries respectively (BioProject: PRJNA661661, SAMN16067724, SAMN16067725), after the trimming step to remove adaptors and low quality reads using TRIMMOMATIC v0.33 with default parameters (Bolger et al., 2014).

Generation	Percentage of asexual genome	Sample size	No. of successful matings	No. of failed matings	Percentage of successful matings
G0 (sexual strain)	0%	56	50	6	89.29%
F1	50%	49	46	3	93.88%
G2	75%	225	121	104	53.78%
G3	87.50%	NA	NA	NA	NA
G4	93.75%	51	21	30	41.18%
G0 (asexual strain)	100%	20	0	20	0.00%

Sample sizes, number and proportion of successful and failed matings for four consecutive generations (Ma et al., 2014a, 2014c).

We performed de novo transcriptome assembly with Trinity v 2.4.0 using default parameters (Haas et al., 2013), and performed differential gene expression analysis between sexes with the R package EDGER v3.4 (Robinson et al., 2010; McCarthy et al., 2012; Chen et al.,2016). The details of differential expression analysis was described previously (Ma, Veltsos, Toups, et al., 2018; Ma, Veltsos, Sermier, et al., 2018). Briefly, the trimmed reads of the female and male sample were mapped to the assembled transcriptome with Kallisto v.0.43.0 (Bray et al., 2016). Read counts of the output from Kallisto mapping were imported for gene expression analysis in EDGER v3.4 (McCarthy et al., 2012; Robinson et al., 2010) and filtered with average Log_2(CPM) > 0 per sample, followed by normalizing the expression by trimmed mean M values (TMM). Normalized expression counts for each sample were used to calculate sex bias using standard measures and Benjamini-Hochberg correction for multiple-testing with false discovery rate (FDR) of 5%. As we did not have biological replicates, the recommended dispersion value 0.4 was used in EDGER (Chen et al., 2016), and stringent criteria were applied when calling sex bias ($|log_2FC| \geq 2$, or fold change ≥ 4).

One candidate gene out of 15 with differential expression between the sexes in *A. tabida*, *hormone receptor 4*, was analysed in further detail because of its suggested role in mating behaviour. To compare the gene sequences between sexual and asexual strains of *A. japonica*, we mapped the Illumina short reads of the sexual AO strain against the PacBio genome using bwa mem with default settings (Li & Durbin, 2009). Before and after read filtering, read mapping quality was assessed with Samtools (v1.3) FLAGSTAT function (Li et al., 2009), and filtered by quality threshold >20. SNP calling was conducted on the mapped SAM files with BCFtools (v1.7, Narasimhan et al., 2016), and mapped reads with mapping depth coverage below three and above 80 were filtered out. Final SNPs were then called with vcfutils.pl scripts from BCFtools (v1.7) and plotted with a 500 bp sliding window for the two QTL contigs using GGPLOT2 in R (v3.6.3, R Core Team, 2020).
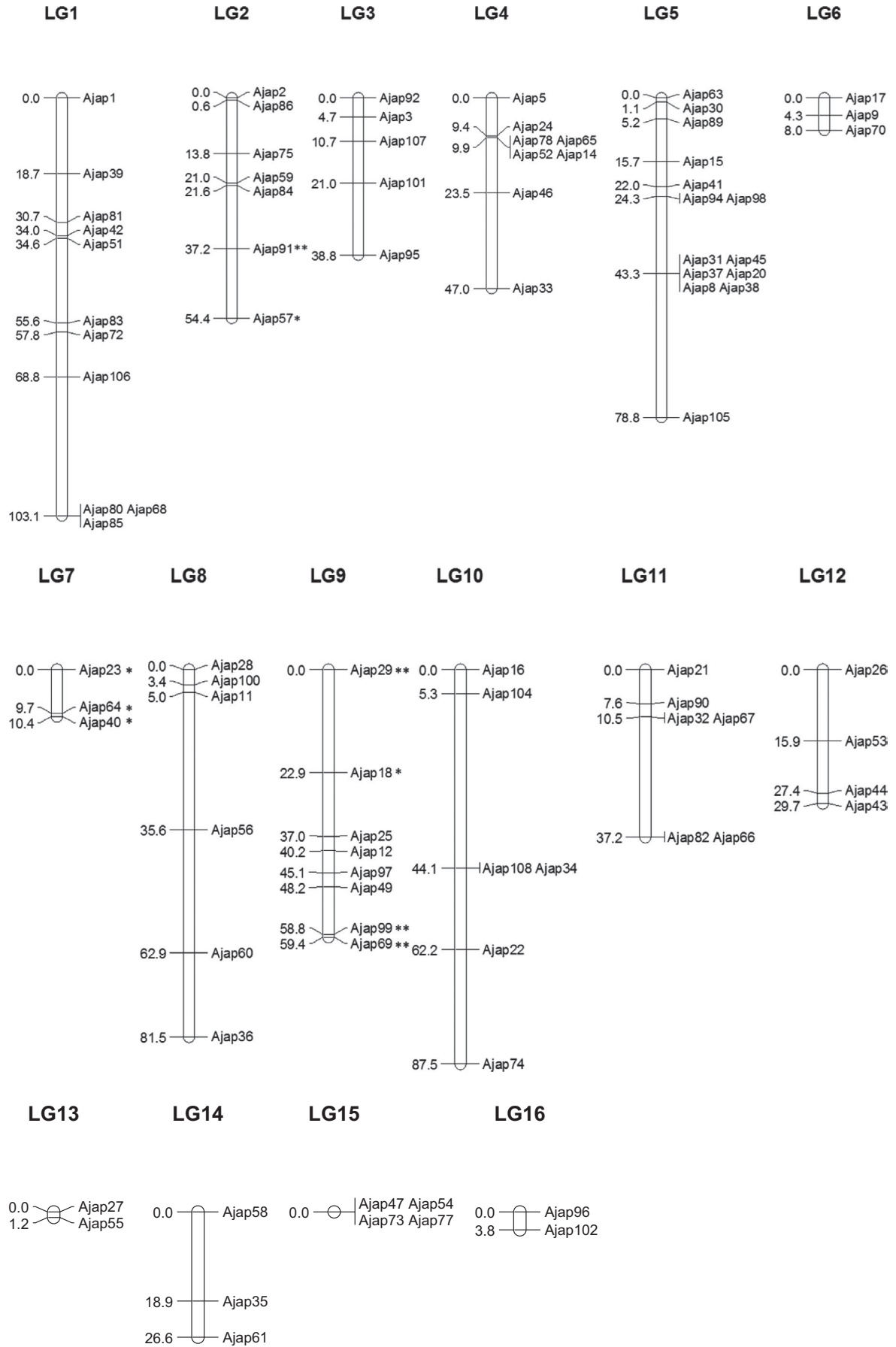
3 | RESULTS

3.1 | Female functional virginity

We previously documented that asexual females of *A. japonica* rejected all mating attempts when confronted with males (Ma et al., 2014a). We classified this female resistance to mating as representing female functional virginity (sensu Huigens & Stouthamer, 2003). To determine the genetic basis of female functional virginity, we previously introgressed alleles from the asexual KG strain into the sexual AO strain for four consecutive generations (summarized in Table 1, see also Ma et al., 2014a). In the present study, females from these same introgression generations were used for QTL analysis.

3.2 | Linkage map construction

The DIAL pipeline initially identified 21,976 SNPs, and a series of filtering steps led to 420 SNPs for further testing and selection (see Methods for details). Eventually, a set of 96 SNPs was used to construct a linkage map from the genotypes of 188 F2 haploid males (Figure 2). Linkage groups were determined with a minimum LOD score of 3 and a maximum recombination frequency of 0.35 between the linked pairs of SNP markers. After this first mapping, the double recombinants were used to identify potential genotyping errors with an error detection $p < .05$. Using this threshold, 25 erroneous candidates were removed which were coded as missing data. Next, Chi-square tests were used to check for segregation distortion from Mendelian expectations (1:1 for haploid F2 males). Segregation distortion was observed for 13.5% of SNP markers (13 out of 96, Chi-square test, $p < .05$), nine SNPs were skewed to the asexual genome and four to the sexual genome. Interestingly, one linkage group (LG7) consisted of seven markers that all significantly deviated from Mendelian expectations, with the asexual genome variant

FIGURE 2 Genetic linkage map of *Asobara japonica*. For each linkage group, the relative position (centimorgans) is indicated on the left side and the SNP markers are shown on the right side. **$p < .01$, *$p < .05$ in chi-square tests for segregation distortion. LG, linkage group

LG1

0.0 — Ajap1
18.7 — Ajap39
30.7 — Ajap81
34.0 — Ajap42
34.6 — Ajap51
55.6 — Ajap83
57.8 — Ajap72
68.8 — Ajap106
103.1 — Ajap80 Ajap68
         Ajap85

LG2

0.0 — Ajap2
0.6 — Ajap86
13.8 — Ajap75
21.0 — Ajap59
21.6 — Ajap84
37.2 — Ajap91**
54.4 — Ajap57*

LG3

0.0 — Ajap92
4.7 — Ajap3
10.7 — Ajap107
21.0 — Ajap101
38.8 — Ajap95

LG4

0.0 — Ajap5
9.4 — Ajap24
9.9 — Ajap78 Ajap65
      Ajap52 Ajap14
23.5 — Ajap46
47.0 — Ajap33

LG5

0.0 — Ajap63
1.1 — Ajap30
5.2 — Ajap89
15.7 — Ajap15
22.0 — Ajap41
24.3 — Ajap94 Ajap98
43.3 — Ajap31 Ajap45
       Ajap37 Ajap20
       Ajap8 Ajap38
78.8 — Ajap105

LG6

0.0 — Ajap17
4.3 — Ajap9
8.0 — Ajap70

LG7

0.0 — Ajap23 *
9.7 — Ajap64 *
10.4 — Ajap40 *

LG8

0.0 — Ajap28
3.4 — Ajap100
5.0 — Ajap11
35.6 — Ajap56
62.9 — Ajap60
81.5 — Ajap36

LG9

0.0 — Ajap29 **
22.9 — Ajap18 *
37.0 — Ajap25
40.2 — Ajap12
45.1 — Ajap97
48.2 — Ajap49
58.8 — Ajap99 **
59.4 — Ajap69 **

LG10

0.0 — Ajap16
5.3 — Ajap104
44.1 — Ajap108 Ajap34
62.2 — Ajap22
87.5 — Ajap74

LG11

0.0 — Ajap21
7.6 — Ajap90
10.5 — Ajap32 Ajap67
37.2 — Ajap82 Ajap66

LG12

0.0 — Ajap26
15.9 — Ajap53
27.4 — Ajap44
29.7 — Ajap43

LG13

0.0 — Ajap27
1.2 — Ajap55

LG14

0.0 — Ajap58
18.9 — Ajap35
26.6 — Ajap61

LG15

0.0 — Ajap47 Ajap54
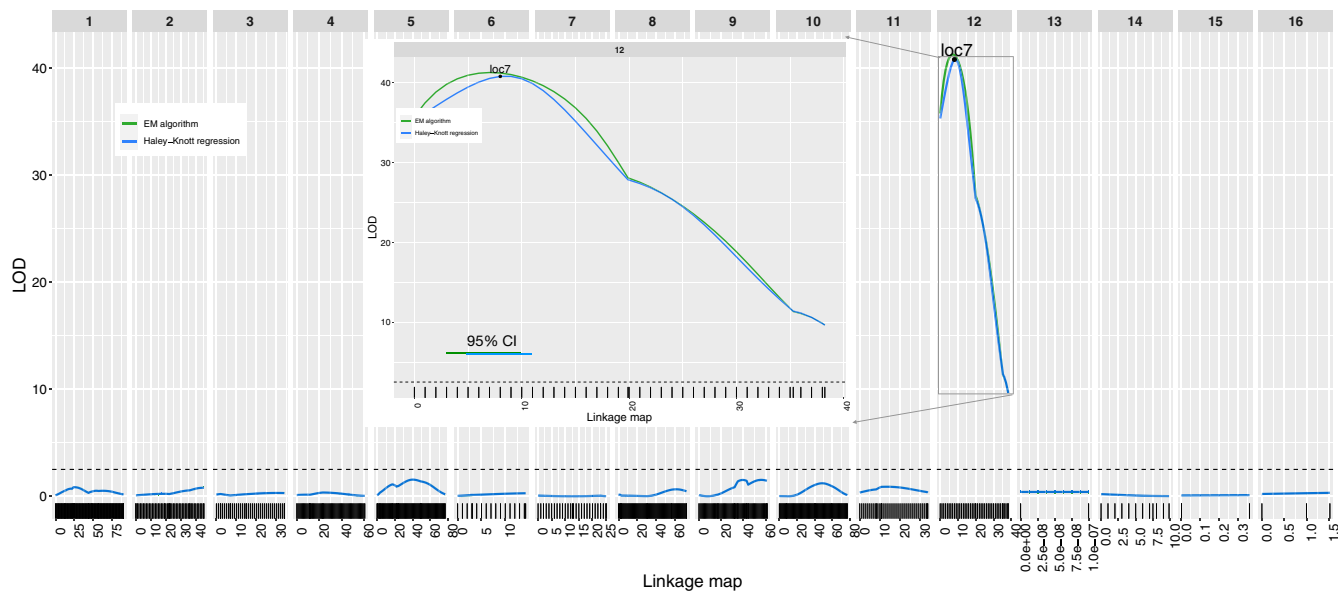   Ajap73 Ajap77

LG16

0.0 — Ajap96
3.8 — Ajap102

FIGURE 3 A single QTL for female functional virginity in *A. japonica*. The horizontal line corresponds to the 5% genome-wide significant threshold from permutation tests. The inserted figure shows 95% confidence interval for the detected QTL at LG12, with a range of 4–10 cM for expectation maximization (EM) and 6–11 cM for Haley-Knott regression (HK) method. LG, linkage group

being more common than expected. Four of the seven markers with the most extreme segregation distortion (p < .000001) were removed from LG7 to enable proper QTL analysis (Table S4), although including these four distorted SNPs did not change the QTL mapping results. The final map consisted of 16 linkage groups, and the total length was 667.32 cM with an average distance between markers of 7.3 cM (Figure 2).

3.3 | QTL of asexual female functional virginity

In total, 253 sex-asex introgressed females (221 G2 and 32 G4) were genotyped (Figure 1, Table 1). QTL analysis identified a single QTL explaining 48.8% of the variation among females for the presence or absence of functional virginity (Figure 3), It was located on LG12 at 7 cM (based on the EM algorithm) or 8 cM (on the HK regression), with very high LOD values (41.3 and 40.8 respectively) (GLM model, p < e-16). No other significant QTL was detected across the genome above the 5% LOD threshold of >2.51, estimated from 1,000 permutations. Furthermore, no significant interactions of paired markers between linkage groups were detected, neither by the scantwo function nor by the multiple QTL function in R/QTL. The 95% confidence interval of the QTL location ranged between 4 and 10 cM with the EM method and 6–11 cM with the HK method (Figure 3). The generation (BC2 or BC4) was not a significant variable in the QTL model.

Two markers, Ajap26 (7–8 cM away from the QTL peak) and Ajap53 (7.9–8.9 cM away, Figure 3), delineate the QTL 95% confidence intervals (4–11 cM from EM and HK model estimate). Females produced daughters (>80%) when the markers were heterozygous (C:G for Ajap26 and A:T for Ajap53), and only sons when they were

homozygous (G:G and A:A respectively, which are the genotypes in the asexual strain; Figure 4). Note that, given the cross scheme (sex-asex introgressed females always mated to asexual males), the alternative homozygote C:C or T:T (fixed for the sexual strain) did not occur in the sex-asex introgressed females. These results corroborate the prediction of a single recessive locus to be the determinant of female functional virginity (Ma et al., 2014a).

3.4 | Candidate gene analysis

The assembled genome length is 271.51 Mb, suggesting that our PacBio genome assembly is 89.3%–96.4% complete (see Table 2 for additional statistics). Furthermore, BUSCO v3.0.1 identified 97% complete (C), 0.5% fragmented (F), and 96.4% single-copy (S) genes. BUSCOs using the Insecta data set (n = 1,658, C: 97.0% [S:96.4%, D (duplicated):0.6%], F:0.5%, M (missing): 2.5%, n:1,658). The assembled transcriptome of *A. tabida* was composed of 34,140 transcripts longer than 300 bp, and 2,810 (8.2%) transcripts were differentially expressed between the sexes with stringent criteria of $|log_2FC| \geq 2$ (Table S5).

The two SNP markers that identified the QTL mapped to two nonoverlapping contigs of the KG PacBio genome, contig0058 (1.78 Mb) and contig0035 (2.45 Mb). Based on the total genetic map length of 667.43 cM, assuming even marker density and using an estimated genome size 281.6–304.1 Mb, the two contigs comprise 58%–63% of the genomic region under the QTL peak. As female functional virginity trait (i.e., female resistance to mating) is probably a female-specific trait, we speculated that genes underlying its regulation may show sex-biased expression in sexual species.

FIGURE 4 Genotype and associated proportion of successful mating for the introgressed sex-asex females at each of the two most closely linked markers flanking the detected QTL. Marker Ajap26 (a) and marker Ajap53 (b)
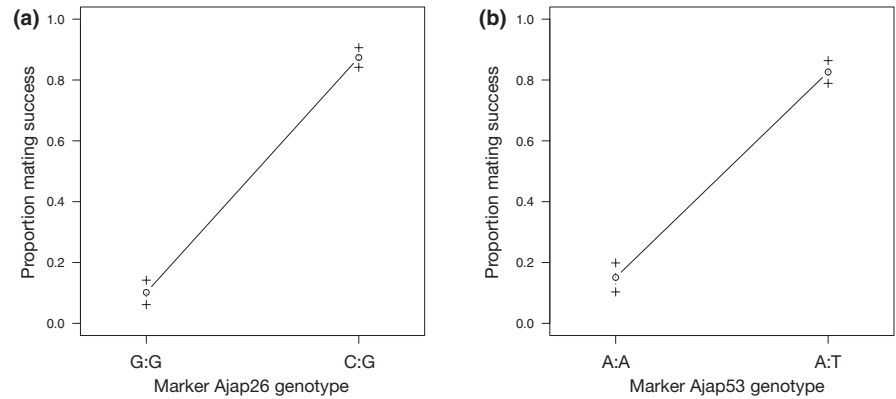

TABLE 2 Summary of the assembled PacBio genome of the asexual KG female of *A. japonica*

Size (bp)/category	Contig number	Total length (bp)
≥5,000	739	271,381,159
≥10,000	656	270,751,034
≥25,000	471	267,578,667
≥50,000	375	264,211,158
Longest	1	5,810,954
N75	NA	610,177
N50	NA	1,651,859
GC%	NA	38.61%
Total	784	271,509,217

TABLE 3 Among the total 131 candidate genes under the QTL (Table S6), 15 genes showed sex-biased expression in the sexual species *Asobara tabida*. All but one show female-biased expression

Species	Gene/transcript	Gene function	Sex bias	Log2(male/female)
A. japonica	transcript/15564	**CD151 antigen**[a]	Male	3.98
A. japonica	transcript/543	DNA replication ATP-dependent helicase/nuclease DNA2	Female	−3.62
A. japonica	transcript/11757	PREDICTED: uncharacterized protein LOC105264533	Female	−4.13
A. japonica	transcript/349	Cordon-bleu protein-like 1	Female	−4.2
A. japonica	transcript/4407	**Nuclear pore complex protein Nup50**[b]	Female	−11.04
A. japonica	transcript/7189	Protein lin−9 homologue	Female	−3.02
A. japonica	transcript/1434	Putative ankyrin repeat domain-containing protein 31	Female	−3.88
A. japonica	transcript/15419	Ubiquitin-conjugating enzyme E2 L3	Female	−3.62
A. japonica	transcript/16014	Vesicle-trafficking protein SEC22b-B	Female	−7.23
D. alloeum	XM_015253230.1	Homeobox protein Nkx−6.2	Female	−3.6
D. alloeum	XM_015260178.2	Unconventional myosin-XIX	Female	−4.07
D. alloeum	XM_015262299.1	Phospholipase D1	Female	−10.43
D. alloeum	XM_029125918.1	Uncharacterized LOC107035734	Female	−4.2
F. arisanus	XM_011307786.1	Hormone receptor 4	Female	−3.56
F. arisanus	XM_011317030.1	Open rectifier potassium channel protein 1	Female	−4.85

[a]Bold indicates shared putative candidate gene with differential expression between sexual and asexual stick insects in *Timema* (Parker et al., 2019).
[b]Bold indicates shared putative candidate gene with mutations in asexual genome of *Leptopilina clavipes* (Kraaijeveld et al., 2016).

The two contigs associated with the QTL comprised 131 genes annotated with transcriptomes from different wasp species (Table S6). Among the 131 genes, several are encoding zinc finger proteins, ubiquitin-like proteins, protein kinase C-binding proteins, in addition to 23 (18%) uncharacterized genes (Table S6). A total of 15 genes showed sex-biased expression in whole-body RNA libraries of the sexual species *A. tabida* (Table 3), of which 14 had higher expression in females. Two (*CD151 antigen*, *nuclear pore complex protein Nup50*)

of these 15 sex-biased genes were also reported in previous studies (Table 3), in which genes were found to accumulate deleterious mutations following transitions from sexual reproduction to asexuality (Kraaijeveld et al., 2016), or showed differential expression between asexual and sexual sister species (Parker et al., 2019). Furthermore, *hormone receptor 4* also showed significantly differential expression with female bias (Log$_2$(male/female) = −3.56). Null alleles, harboring a premature stop codon were detected in both the asexual KG and sexual AM strain. A relatively higher density of SNPs between sexual and asexual *A. japonica* strains was found in the putative promoter region (up to 5 kb upstream of the start codon) compared to the intron and exon regions (Figure S2).

4 | DISCUSSION

Our aim was to investigate the genetic basis of female functional virginity in *A. japonica*. This required, as a first step, to generate a linkage map and genomic data sets that will be useful for comparative genomic studies across hymenopteran species. With an average intermarker distance of 7.3 cM, the *A. japonica* map has a comparable resolution to maps for other hymenopterans, including 4.0 cM for the bumblebee *Bombus terrestris* (Stolle et al., 2011), 7.5 cM for the honeybee *Apis mellifera* (Solignac et al., 2004), 11.1 cM for *Nasonia vitripennis* (Pannebakker et al., 2011), 17.0 cM for *Bracon hebetor* (Antolin et al., 1996) and 17.7 cM for *Trichogramma brassicae* (Laurent et al., 1998). Moreover, based on the estimated genome size of 281–304 Mb, the recombination rate of *A. japonica* is 2.20–2.37 cM/Mb, compared to ~1.5 cM/Mb for *Nasonia* (Pannebakker et al., 2011), ~4.76 cM/Mb for *Bombus terrestris* (Stolle et al., 2011) and ~22.8 cM/Mb for *Apis mellifera* (Solignac et al., 2004). This, and the fact that the 16 linkage groups defined by the SNP map agree with earlier estimates of the number of chromosomes (12–17 chromosomes based on cytological data; Gokhman, 2009; G. Massimo, personal communication), indicate the accuracy of the constructed linkage map.

Our QTL analysis revealed that female functional virginity is associated with a single QTL with major effect. This is consistent with the previous prediction of a single recessive locus based on the segregation of phenotypes in our introgression lines (Ma et al., 2014a). In this previous study, *Wolbachia*-cured asexual females of *A. japonica* were found to have reduced attractiveness to males (only 55% asexual females attracted sexual males vs. 100% sexual females), and a complete loss of mating acceptance resulting in a total absence of matings. As a result, these females produced only haploid sons (Ma et al., 2014a). The reduced attractiveness is probably not due to within-population mating preferences when this asexual population was sexual in the past, different from the situation reported from asexual *Leptopilina* wasps (Kraaijeveld et al., 2009). Indeed, in *A. japonica*, sexual females are equally attractive to sexual males and to males produced by asexual females, suggesting the reduced attractiveness in the reciprocal pairing is not due to lineage divergence between asexual and sexual strains (Ma et al., 2014a). Similar to our findings of a single QTL underlying female functional virginity, a

simple genetic architecture for this trait was also found in two other studies of wasps with endosymbiont-induced asexuality. Inability to fertilize eggs segregated as expected for one or few loci with recessive effects in *Telenomus nawai*, and as expected for few loci with dominant effects in *Trichogramma pretiosum* with additional minor-effect modifiers (Jeong & Stouthamer, 2005; Russell & Stouthamer, 2011).

Our QTL analysis identified a single large-effect QTL on LG12 at 7 or 8 cM (95% CI: 4–11 cM), strongly associated with female functional virginity. Annotation of the QTL region identified 131 physically-linked candidate genes. To our knowledge, this is the first study to identify candidate genes for female functional virginity, a trait that is predicted to evolve in the transition from sexuality to asexuality (Huigens & Stouthamer, 2003). Because female functional virginity is female-specific, we hypothesized that the underlying gene(s) would show differential expression between sexes in sexual species. In total, 15 of the 131 genes were sex-biased in *A. tabida*, the sexual sister of *A. japonica*, and all but one had female-biased expression. The molecular changes in genes associated with the decay of sexual traits are still poorly known. A review (Arbuthnott, 2009) on the genetic architecture of sexual traits influencing premating isolation, such as behavioural signaling and courtship traits, found 69% (25 of 36) to be encoded by few loci with relatively large effects, and suggested changes in courtship behaviour may often evolve or decay quickly. Little is still known about the genetic basis of female receptivity, but auditory and/or olfactory receptors are likely candidates (Laturney & Moehring, 2012). Among the 15 differentially expressed genes, gene functions are, among others, associated with hormone receptor, homeobox protein, ubiquitin-conjugating enzyme, nuclear pore complex protein, DNA replication ATP-dependent helicase. We further analysed *hormone receptor 4*, because this gene encodes a steroid hormone receptor, and may therefore be relevant to mating resistance and female functional virginity. *Hormone receptor 4* was shown to mediate the specific timing and expression of steroid hormone 20-hydroxyecdysone (20E) during the onset of metamorphosis in *Drosophila* and *Lepidoptera* (Kang et al., 2019; Ou et al., 2011). Steroid hormone 20E can be transferred from males to females during mating in the malaria mosquito *Anopheles gambiae*, leading to the loss of female receptivity to mating with other males (Gabrieli et al., 2014). It is thus possible that *hormone receptor 4*, among other steroid hormone receptors, interacts with steroid hormone 20E to induce the loss of mating receptivity in the malaria mosquito. Similar explanation could be applied to the asexual females of *A. japonica* with loss of mating propensity. We detected a premature stop codon in *hormone receptor 4* in both the sexual and asexual strains. Moreover, we found multiple SNPs in the upstream (putative promoter) region, and in the gene itself, differentiating the sexual and asexual *A. japonica* strains. Further functional analyses are required to confirm this speculative role of *hormone receptor 4* in female functional virginity of *A. japonica*.

Few studies have identified genes that are subject to change following transitions to asexuality. Kraaijeveld et al., (2016) compared genomes of sexual and asexual linages of the parasitoid wasp

Leptopilina clavipes, and identified 16 genes with deleterious mutations (including frame shifts and early stop codons) associated with asexuality. One of our candidate genes, *nuclear pore complex protein Nup50*, was also among these 16 genes of *L. clavipes*. *Nup50* is involved in mRNA transport, or positive regulation of transcription by RNA polymerase II and predominantly interacts with transcriptionally active genes inside the nucleoplasm, in particular those involved in developmental regulation and the cell cycle (Buchwalter et al., 2014; Kalverda et al., 2010). However, although this gene may be involved in the fertilization process, it probably does not directly cause female reluctance to mate, because asexual females mate readily but are unable to fertilize eggs in *L. clavipes* (Kraaijeveld et al., 2009, 2016). Another of our candidate genes, *CD151 antigen*, was also reported in a study identifying differential gene expression between females of sexual and asexual stick insects (Parker et al., 2019). *CD151 antigen* is a member of the transmembrane 4 superfamily, involved in cellular processes such as cell signalling, and may regulate integrin trafficking and function (Shigeta et al., 2003; Termini & Gillette, 2017). In future studies, a near chromosomal-level genome assembly to completely cover the QTL region would be helpful to evaluate whether there are more candidate genes for female functional virginity in *A. japonica*. We were unable to improve our available genome assembly (e.g., bridge the large gap between the two contigs underlying the QTL) with the current data sets, and further scaffolding would require Hi-C and/or optical mapping sequencing approaches (Belton et al., 2012; Howe & Wood, 2015; Li et al., 2019). Meanwhile, functional analyses of the identified 131 candidate genes, especially the 15 ones with sex-biased expression, can be undertaken to further elucidate the molecular basis of female functional virginity in this species. Whether certain genes are particularly affected by a shift to asexuality requires more comparative studies on a broader array of species.

Sexual traits, including female mating behaviours, are costly, which may lead to such traits being selected against under asexuality (King & Hurst, 2010; Russell & Stouthamer, 2011; Stouthamer et al., 2010). Theoretical modeling of the spread of asexuality-inducing endosymbionts in mixed populations with uninfected and infected females, has confirmed that female functional virginity is favoured under sex-ratio selection (Stouthamer et al., 2010). Such selection might be stronger in haplodiploid species because unmated females will produce only haploid male progeny that balances the sex ratio. Once a mutation for rejection to mate has swept through an asexual population, the region comprising the genes associated with mating resistance does not get smaller, due to lack of recombination in asexuals. Moreover, this linked region may be maintained by selection if the trait has a polygenic basis. This may explain the large size of the single QTL detected in our study. Overall, our results are consistent with mutation(s) in the genomic region consisting of a single gene or a cluster of linked genes underlying rapid evolution of female functional virginity in the transition to asexuality.

AUTHOR CONTRIBUTIONS

Conceptualization: W.-J.M., B.P., T.S., L.W.B., L.v.d.Z.. Data curation: W.-J.M., X.L., E.G., S.Y.A. Genome, transcriptome assembly and annotation: X.L., E.G., S.Y.A. Data analyses: W.-J.M., P.V. Funding acquisition: L.W.B., L.v.d.Z., T.S. Supervision: B.P., T.S., L.v.d.Z., L.W.B. Visualization: W.-J.M., P.V. Writing – original draft: W.-J.M. Writing – review & editing: W.-J.M., P.V., L.W.B., T.S., L.v.d.Z., B.P., E.G., X.L., S.Y.A.

DATA AVAILABILITY STATEMENT

The data presented in this study can be accessed on NCBI Bioproject (PRJNA661661) with Accession nos SRR12618696-SRR12618699, JADHZF000000000, SAMN16067724, SAMN16067725). All relevant scripts and data files to perform these analyses have been deposited in Zenodo at https://doi.org/10.5281/zenodo.4557574, and Github at https://github.com/Wen-Juan/Decaytrait_qtl.

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SUPPORTING INFORMATION

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