Divergent Gene Expression Following Duplication of Meiotic Genes in the Stick Insect *Clitarchus hookeri*

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Accepted: 17 March 2021

Abstract

Some animal groups, such as stick insects (Phasmatodea), have repeatedly evolved alternative reproductive strategies, including parthenogenesis. Genomic studies have found modification of the genes underlying meiosis exists in some of these animals. Here we examine the evolution of copy number, evolutionary rate, and gene expression in candidate meiotic genes of the New Zealand geographic parthenogenetic stick insect *Clitarchus hookeri*. We characterized 101 genes from a de novo transcriptome assembly from female and male gonads that have homology with meiotic genes from other arthropods. For each gene we determined copy number, the pattern of gene duplication relative to other arthropod orthologs, and the potential for meiosis-specific expression. There are five genes duplicated in *C. hookeri*, including one also duplicated in the stick insect *Timema cristinae*, that are not or are uncommonly duplicated in other arthropods. These included two sister chromatid cohesion associated genes (*SA2* and *SCC2*), a recombination gene (*HOP1*), an RNA-silencing gene (*AGO2*) and a cell-cycle regulation gene (*WEE1*). Interestingly, *WEE1* and *SA2* are also duplicated in the cyclical parthenogenetic aphid *Acyrthosiphon pisum* and *Daphnia duplex*, respectively, indicating possible roles in the evolution of reproductive mode. Three of these genes (*SA2*, *SCC2*, and *WEE1*) have one copy displaying gonad-specific expression. All genes, with the exception of *WEE1*, have significantly different nonsynonymous/synonymous ratios between the gene duplicates, indicative of a shift in evolutionary constraints following duplication. These results suggest that stick insects may have evolved genes with novel functions in gamete production by gene duplication.

Key words: meiotic gene, phylogenetic distribution, gene duplication, gene expression, parthenogenesis, Phasmatodea.

Significance

Many arthropod species have evolved nonsexual reproductive strategies such as parthenogenesis, yet knowledge of the genetic basis of their evolution is limited. We found several well-characterized meiotic genes, generally being single-copy within Arthropoda, that have more than one copy in the genome of the geographical parthenogenetic stick insect *Clitarchus hookeri*. These paralogs have relatively high level of sequence divergence and together with varied expression levels indicate potential functional divergence. These results establish a basis for further understanding the evolution of different reproductive strategies in these insects.

Introduction

Sexual reproduction is the predominant reproductive strategy in animals, yet many animal groups have repeatedly evolved alternative strategies such as parthenogenesis (obligate and facultative) and the related processes of androgenesis (Schwander and Oldroyd 2016) and gynogenesis (Schlupp 2005). This is particularly true of arthropods, which utilize all
the above strategies (Vershinina and Kuznetsova 2016). However, little is known about the genomic basis of meiosis in lineages that have repeatedly evolved alternative reproductive strategies. Gene family expansion and diversification have long been regarded as sources of evolutionary novelty (Ohno 1970; Lynch and Conery 2000), but their role in the evolution of alternative reproductive strategies is not well understood. Most gene duplicates likely lose function and deteriorate. However, some duplicates may be maintained and ultimately gain new functions, replace some of the functions of other genes, or contribute to dosage compensation (Zhang 2003).

Studies in cyclical parthenogenetic aphids, monogonont rotifers, and the crustacean *Daphnia* have provided evidence of gene duplications in conserved core meiotic genes (King and Serra 1998; Simon et al. 2002; DeCaestecker et al. 2009). Notably, most of these duplications are within the genes involved in cell-cycle regulation (Schurko et al. 2009, 2010; Hanson et al. 2013). The WEE1 G2 Checkpoint Kinase (WEE1) gene from the pea aphid (*Acyrthosiphon pisum*) and the Cell Division Cycle 20 (CDC20) gene from the rotifer (*Brachionus calyciflorus*) are downregulated in asexual compared with sexual forms. These findings suggest additional cell cycle controls during meiosis, and that suppression might alleviate meiotic arrests allowing parthenogenesis to develop (Schurko et al. 2010; Hanson et al. 2013). In addition, genomes of the rotifers (*B. calyciflorus* and *B. manjavacas*) and water flea (*Daphnia duplex*) also have duplicates of genes associated with sister chromatid cohesion (SCC) (*SMC1, SMC3, SMC6, REC8, SA*, and *TIM*) and recombination (*RECQ2*). Duplications of some genes involved in RNA silencing (*PIWI* and *AGO*) and DNA replication (*MCM7* and *SA*) also have duplicates of genes associated with sister chromatid cohesion (SCC) (*SMC1, SMC3, SMC6, REC8, SA, and TIM*) and recombination (*RECQ2*). These findings suggest additional cell cycle controls during meiosis, and that suppression might alleviate meiotic arrests allowing parthenogenesis to develop (Schurko et al. 2009; Hanson et al. 2013). However, whether such duplications are widespread in other animals with a high incidence of reproductive flexibility is yet to be determined.

Few animal groups display such a wide array of reproductive strategies as the insect order Phasmatodea, more commonly known as stick insects. Of the approximately 3,000 species, around 10% are parthenogenetic and scattered in different families (*Scali 2009*). The females may reproduce by obligate or facultative parthenogenesis (*Pijnacker 1966, 1968, 1969; Koch et al. 1972; Pijnacker and Ferwerda 1978; Marescalchi et al. 1991, 1993; Mantovani et al. 1999; Scalì et al. 2003; Schwander and Crespi 2009; Buckley and Bradler 2010; Morgan-Richards et al. 2010, 2019; Myers et al. 2013; Alavi et al. 2018; Parker et al. 2019). In addition, other reproductive strategies are known including androgenesis and hybridogenesis (*Mantovani and Scalì 1992; Scavariello et al. 2017*). In Phasmatodea, unfertilized oocytes can organize the first division of embryos without the donation of a centriole from sperm and this is thought to be at least one factor responsible for the repeated evolution of parthenogenesis (*Marescalchi et al. 2002*). Variations to meiosis include apomixis, automixis, and the modification of meiotic products, which allow the parthenogenetic offspring to achieve a somatic chromosome number (*Pijnacker 1969; Marescalchi et al. 1991, 1993; Alavi et al. 2018*). However, the molecular basis of these modifications to meiosis in stick insects is largely unknown.

In the New Zealand stick insect fauna, obligate and geographical parthenogenesis is also frequent (*Salmon 1991; Jewell and Brock 2002; Buckley et al. 2009, 2010; O’Neill et al. 2009; Morgan-Richards et al. 2010, 2019*). *Citarchus hookeri*, one of the most widespread New Zealand stick insect species, is a geographical parthenogen with sexual populations predominant in the northern and western areas of the North Island and parthenogenetic populations being distributed mostly in the South Island and eastern North Island (*Buckley et al. 2010, 2014; Buckley and Bradler 2010; Morgan-Richards et al. 2010, 2019*). This species is diploid (2n = 36–39), with males lacking one sex chromosome (XO) compared with females (XX) (*Parfitt 1980; Morgan-Richards and Trewick 2005*). Recent evidence has shown isolated parthenogens can produce sons after mating with males for two generations, which together with an observed deficiency in heterozygotes, suggests that parthenogenesis in *C. hookeri* is automatic where haploid gametes are produced followed by the restoration of the chromosomal number within each gamete (*Morgan-Richards et al. 2019*).

In this study, we sequenced gonadal transcriptomes from female and male *C. hookeri* using RNA-Seq and identified 97 candidate meiotic genes that have been well-characterized for their roles in meiosis in other systems. We used this gene set to identify gene duplications in candidate *C. hookeri* meiotic genes that are rare or absent in other arthropods, with an emphasis on comparison with species exhibiting flexible reproduction, like stick insects. To determine whether these duplications occurred prior to or following the diversification of Phasmatodea, we also examined these meiotic candidate genes in the genome assembly of the stick insect *Timema cristinae*, sister taxon to all other stick insects (*Euphasmatodea*) (*Whiting et al. 2003; Simon 2019*). We then estimated the expression of *C. hookeri* candidate meiotic genes in non-germline and germline tissues and identified parthenogens can produce sons after mating with males for two generations, which together with an observed deficiency in heterozygotes, suggests that parthenogenesis in *C. hookeri* is automatic where haploid gametes are produced followed by the restoration of the chromosomal number within each gamete (*Morgan-Richards et al. 2019*).

**Materials and Methods**

**Sample Collection, Preparation, and RNA Sequencing**

Biological replicates of three males and three females were collected from the same population at Totara Park, Auckland,
New Zealand (37° 0.111 S, 174° 55.039 E) for gonadal transcriptome sequencing (supplementary table S1, Supplementary Material online). Female nymphs (third to fifth instars) were collected and then reared until maturation to allow developmental consistency of ovaries for comparison. Specifically, they were reared in separate culture boxes, exposed to room temperature with natural photoperiod in Auckland (35° 52.300 S, 173° 49.290 E). They were fed Manuka (Leptospernum scoparium) leaves, which were replaced every 2 days. After maturing, they were checked for egg laying once every day. Each insect was then snap frozen and stored at −80°C following the first instance of egg laying. Female reproductive tract including approximately 18–20 ovarioles, early developing eggs and oviducts were dissected in ethanol (100%) for RNA extraction. Males were adults when collected, snap frozen and stored at −80°C directly. Male testicle pairs, appearing to be mature but not degraded, were dissected in ethanol (100%) for RNA extraction. Total RNA extraction and library preparation were performed as described in Wu et al. (2016). The extractions were barcoded individually and then pooled for sequencing on the HiSeq2000 platform with three lanes to generate 100 bp PE reads at New Zealand Genomics Limited (NZGL). The pooled data were separated by individual barcode to achieve three individual replicates for each sex.

De Novo Transcriptome Assembly and Quality Assessment

Raw reads were quality assessed with FastQC (Andrews 2010) and then preprocessed as follows: 1) ribosomal RNAs were filtered using SortMeRNA (v2.1) (Kopylova et al. 2012); 2) Illumina universal adapters were screened and trimmed from the 3′ end using CUTADAPT (v1.15) (Martin 2011); 3) 12 bases from the 5′ end, low quality 3′ end (< Q30) and poly-A tail (10 continuous A/T from 3′ end) were trimmed using PRINSEQ (v0.20.4) (Schmieder and Edwards 2011); 4) PRINSEQ was also used to filter reads containing more than one ambiguous base (N) and then maintain pairs with both reads longer than 50 bases; 5) all the maintained read pairs were kept first 68 bases. The resulting reads were then subjected to de novo assembly using TRINITYRNASEQ (v2.6.5) (Grabherr et al. 2011) with applied parameters “–min_contig_length 200; –CPU 8.” The assembled transcript set was then cleaned of duplicates, fragments, and alternate transcripts using EvidentialGene (v18-01-2018) (Gilbert 2013), which screens transcripts by converting large, redundant mRNA assembly set to best protein coding sequences. The raw and filtered assemblies were then quality evaluated with Benchmarking Universal Single-Copy Orthologs (BUSCO v3.0.2) (Simao et al. 2015) software (e-value: 1e-3), to detect the presence of a core set of 1,066 highly conserved arthropod proteins encoded as single copies.

Gene Identification

Clitarchus hookeri candidate meiotic genes were initially identified by searching protein homologs from other insects against the protein set generated from EvidentialGene. The search set included sequences that were collected from the publications of meiotic amino acid sequences according to the reported sequence IDs from species including Daphnia pulex (Schurko et al. 2009), Acyrthosiphon pisum (Srivisan et al. 2010), Nasonia vitripennis (Schurko et al. 2010), and Drosophila melanogaster (a wings apart-like protein sequence; GenBank ID: NP_001284804.1), as well as gene coding sequences from Brachionus calyciflorus and B. manjavacas (Hanson et al. 2013). The homologous search was carried out using BLAST (v2.2.30) (McGinnis and Madden 2004) and the BLASTP and TBLASTN (e-value < 10−5) were used with amino acid sequences and gene coding sequences as queries, respectively. The ten best hits of every query sequence were kept and then combined to remove duplicated results. To further determine gene identities, all the resulting C. hookeri protein matches were used as query sequences to search against the GenBank RefSeq protein database (BLASTP: e-value < 10−5; Database was updated on September 28, 2018) and those without the matches to our targeted meiotic proteins were discarded. The resulting C. hookeri meiotic proteins were then used to self-search (BLASTP: e-value < 10−5) against the same EvidentialGene generated protein set in order to capture genes that were not present as hits from BLAST searches using genes from distantly related species as queries. Similarly, the resulting ten best hits of every query sequence were searched against the GenBank RefSeq protein database to determine their gene identities. Finally, to determine whether the putative gene copies are paralogs and not just different alleles, we identified their genomic location by aligning transcripts to the C. hookeri genome scaffolds (Wu et al. 2017) using GMAP (v2018-05-30; sequence identities >95%) (Wu and Watanabe 2005).

We searched for orthologs and paralogs of the C. hookeri gene duplicates in the stick insect T. cristinae genome assembly (v3.0) downloaded from http://nosil-lab.group.shef.ac.uk/?page_id=25 (last accessed March 30, 2021) (Riesch et al. 2017). The identified T. cristinae meiotic genes were used as a search set to BLAST (TBLASTN, e-value < 10) against the T. cristinae genome assembly, followed by a prediction of coding regions and amino acids from the scaffolds containing the best blast matches using the online FGENESH+ protein-based gene predictor (Solovyev 2004) (C. hookeri protein homologs as comparison and Tribolium castaneum as the specific gene-finding parameters). The predicted T. cristinae protein sequences were then used as queries to search against the NCBI RefSeq database to confirm gene identities. The C. hookeri gene copies were predicted as single genes in T. cristinae if the two copies had the best hit to one T. cristinae homolog.
Phylogenetic Reconstruction and dN/dS Ratio

Phylogenies were built from gene coding sequences identified in this study and the arthropod species; *Nasonia vitripennis*, *Pediculus humanus*, *Zootermopsis nevadensis*, *Apis mellifera*, *Acythosiphon pisum*, *Tribolium castaneum*, *Drosophila melanogaster*, *Aedes aegypti* and *Bombyx mori*, retrieved from InsectBase (Yin et al. 2016) or JGI (*Daphnia pulex* v1.0 Filtered gene models v1.0 and v1.0 Frozen Gene Catalog 7/3/07) (Colbourne et al. 2011). All sequence IDs are provided in supplementary table S2, Supplementary Material online. The alignments were generated using “translation align” with default parameters (MUSCLE alignment) implemented within Geneious (v10.0.9) (Kearey et al. 2012) and then curated on Gblocks online Server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html; last accessed March 30, 2021) (Castresana 2000) using “Codons” and allowing “smaller final blocks” and “gap positions within the final blocks.” The degree of nucleotide substitution saturation was assessed using DAMBE (v7.2.152) (Xia 2018). The inferred transition or transversion distances were plotted against the GTR distance. Substitution saturation was considered where the transition distances begin to exceed the transversion distance when plotted against the GTR distance. Maximum-likelihood trees were constructed using PhyML (v2.0) (Guindon et al. 2009) with the best-fit model selected by Jmodeltest (v2.1.10) (Posada 2008; Darriga et al. 2012) under the Akaike Information Criteria (AIC) (Brooks 1989; Posada and Buckley 2004). All the selected best-fit models, calculated gamma values, and proportion of variable sites for the construction of maximum-likelihood phylogeny are listed in supplementary table S2, Supplementary Material online. Bootstrap support values were calculated by nonparametric bootstrap analysis (n = 1,000 bootstrap pseudoreplicates). The resulting phylogenies were rendered using FigTREE (v1.4.3) (Rambaut 2009) and were drawn with *Daphnia pulex* as the outgroup if possible.

To infer differences in selective patterns between *C. hookeri* duplicates, dN/dS ratios were calculated using branch models within the CODEML package of PAML (v4.5) (Yang 2007). Each analysis had both *C. hookeri* duplicates assigned an independent dN/dS ratio with respect to the rest of the phylogeny. Likelihood ratio tests were conducted between the model allowing for independent ratios, and the associated null model that does not allow for independent ratios. This enables the detection of shifts in the pattern of selection between the two *C. hookeri* duplicates and the background pattern over the rest of the tree (Yang 2007). *HOP1* was not included in the phylogenetic analysis due to a lack of homologous sequences from other sampled arthropod genome assemblies.

Read Quantification and Differential Expression Analysis

Transcript quantification was carried out using “salmon quant” from Salmon (v0.9.1) (Patro et al. 2017) with parameters “I A -p 8 –gcBias.” The cleaned short reads were mapped to the *C. hookeri* transcripts using the Salmon-implemented mapping procedure. Differential expression (DE) analyses were performed in R (v3.5.2) (Ihaka and Gentleman 1996) using the DESeq2 Bioconductor package (Love et al. 2014). This program takes read counts to estimate sample size factors, followed by estimating dispersions by expected mean values from the maximum likelihood estimate of log2 fold changes, and finally fits a negative binomial distribution. The transcripts with an adjusted P value less than 0.05 and a minimum fold change (FC) of 2 were reported as significantly differentially expressed. The volcano plot was generated using an R package EnhancedVolcano (Bligh 2018).

Estimation of Gonad-Specific Expression

To infer meiosis-specific expression, four RNA-Seq data sets derived from non-gonadal tissues (antennae, head and prothorax and leg, midgut, and male terminalia) obtained from our previous study (Wu et al. 2016) were aligned to the transcriptome assembly. These were then used to generate transcript per million (TPM) values using Salmon. All the raw data sets were quality re-evaluated and trimmed due to different use of these data sets in this study. The antennae, midgut, and male terminalia reads had duplicated read pairs removed and then had 15 bases trimmed from the 5’ end using FastUniq (Xu et al. 2012) and PRINSEQ, respectively. The reads derived from the head, prothorax, and legs had duplicated pairs removed using FastUniq. Reads were then trimmed using PRINSEQ at the low-quality 3’ ends and filtered for low quality sequences, resulting in lengths of at least 50 bases of cleaned read pairs. A transcript was considered a candidate for gonad-specific expression if it had a TPM value of less than one in all the non-gonadal samples (supplementary table S3, Supplementary Material online).

Results

De Novo Assembly and Assessment

A total of 271,860,387 read pairs (supplementary table S1, Supplementary Material online) were used to generate a de novo assembly of the *C. hookeri* gonadal transcriptome. The initial TRINITY assembly was then filtered by EvidentialGene with a comparison shown in table 1. Approximately one-third of the TRINITY transcripts were maintained after filtering with EvidentialGene. BUSCO results show the EvidentialGene processed transcript set had a significant reduction in duplicated sequences, from 66.9% to 2.6%, whereas the estimation of complete proteins remained similar. Therefore, we used the EvidentialGene filtered assembly as the gene pool to search for candidate meiotic genes.

Meiotic Gene Inventory and Expression

We compiled a meiotic core gene list from previous studies on several arthropod species that can switch between sexual and
table S3, Supplementary Materialonline). Most of these genes were located on the same genomic scaffold. For example, HOP2 and RAD21 genes that were homologous to one of the core meiotic genes from previous studies were inferred to be candidate meiosis genes if they were expressed in gonadal tissue (testis and ovarian), but not in the non-gonadal tissues of antennae, head, prothorax, leg, and midgut. We also found that some genes were simply mis-assemblies. We also found that some genes were indicating they may represent alternatively spliced variants or paralogues in other insects (AGO2 with these two genes also having rare double gene duplications, identified from both C. hookeri and T. cristinae phylogeny due to its absence in most of other taxa (presence in T. castaneum and Z. nevadensis). However, the two identified copies in C. hookeri are much more similar to each other in terms of amino acid identity (45.1%) compared with sequences from other arthropod genomes (between 22.2% and 43.3%), suggestive of a duplication within Phasmatodea.

None of these stick insect gene copy pairs are arranged tandemly, as assessed by inspection of genomic scaffolds. The C. hookeri gene copy pairs exhibited varying numbers of exons and dVdS ratios compared with paralogous sequences (table 2). The saturation plots (supplementary fig. S2, Supplementary Material online) did show a degree of saturation in all genes and more so at higher levels of overall sequence divergence. However, we are focusing on the dN and dS rates estimated on the C. hookeri tip branches, which are less effected by saturation. To test for differences in selective pressures between the duplicated copies, branch tests were carried out by labelling the two C. hookeri gene copies as independent foreground branches. This allows both copies to have independent dN and dS rates from each other, and the rest of the phylogeny. All the genes tested except for WEE1 had significant likelihood ratios indicating different selective expression, whereas only 13 non-DE genes (22.4%) were detected with gonad-specific expression.

### Meiotic Gene Duplications

We focused on five candidate meiotic genes that showed lineage-specific expansion in either C. hookeri or in both C. hookeri and T. cristinae. Two of these genes are involved in SCC. SCC2 was the only gene found with two copies in both stick insect species. SA2 has two copies in D. melanogaster, five copies in Daphnia pulex and two copies in C. hookeri. The chromosomal recombination gene HOP1, which can be absent in other assembled insect genomes (Ramesh et al. 2005), has two copies in C. hookeri. AGO2 from the RNA silencing and WEE1 from cell-cycle regulatory categories were both found with two copies in C. hookeri, with these two genes also having rare double gene paralogues in other insects (AGO2 in N. vitripennis and T. castaneum; WEE1 in N. vitripennis and A. pisum). Phylogenetic reconstructions of SA2, SCC2, WEE1, and AGO2 are given in figure 2. The SA2, AGO2, and WEE1 trees indicate that the two C. hookeri duplicates are sister lineages of each other; whereas the SCC2 duplications, identified from both C. hookeri and T. cristinae, do not group by species. We were unable to reconstruct the HOP1 phylogeny due to its absence in most of other taxa (presence in T. castaneum and Z. nevadensis). However, the two identified copies in C. hookeri are much more similar to each other in terms of amino acid sequence identity (45.1%) compared with sequences from other arthropod genomes (between 22.2% and 43.3%), suggestive of a duplication within Phasmatodea.

### Differential Gene Expression

We compared the transcriptome-wide patterns of DE between females and males. Principle component analysis (PCA) shows that the gene expression patterns from the two tissues are clearly separated with 98% of the variance explained by PC1 (as fig. 1A). Compared with male samples, the three females have expression patterns that are much more dispersed even though they were reared in the laboratory and sampled at the same developmental stage (after the first egg was laid) for organ harvest. There were 49 meiotic candidates showing DE, all of which had much higher abundance of transcripts in testis (as fig. 1B). Among all the meiotic genes, nearly half of the DE genes (23) also show gonad-specific expression, whereas only 13 non-DE genes (22.4%) were detected with gonad-specific expression.
codon substitution dynamics between the duplicated gene pairs and the rest of the phylogeny. However, in all cases, the omega values were all less than zero, indicative of overall purifying selection.

Differential Expression between Gene Copies
Aligning short reads from non-gonadal tissues to all of the gene duplicates indicated that the a copies of the SA2, SCC2, and WEE1 genes are all specifically expressed in the gonads. On the other hand, both copies of HOP1 and neither of the AGO2 copies display gonad-specific expression. For each gene, the expression patterns of the two copies vary between females and males (as fig. 3). The gonad-specific a copies from SA2, SCC2, and WEE1 had much higher expression levels in male than female gonads, whereas the b copies had lower expression differences between the two sexes. The two gonad-specific copies of HOP1 had higher expression in males. The HOP1a copy was expressed at a higher level than b in females, whereas it showed the opposite pattern in males. The non-gonad-specific copies of AGO2 showed higher levels of expression of a compared with b in both sexes and the expression of b showed a higher variance among the sample replicates. In addition, AGO2a was expressed at a much higher level in males compared with females.

Discussion
The wide array of alternative reproductive strategies in stick insects suggests that meiosis has been modified many times,
yet the molecular basis underpinning modifications to meiosis are not well understood. In this study, we used *C. hookeri*, a geographical parthenogenetic stick insect, and *T. cristinae*, the sister taxon to all the other stick insects, as model species to investigate meiotic gene duplication, shifts in gene expression and patterns of selection at the codon level. In *C. hookeri*,

**Table 2**

Duplications of Candidate Meiotic Genes from the *Clitarchus hookeri* Genome

| Gene | Copies | Transcript ID | Protein Length (aa) | Protein Amino Acid Identities* (%) | Genomic Scaffold ID | Exon Number | eS (dN/dS) | Ratio |
|------|--------|---------------|---------------------|-----------------------------------|---------------------|-------------|-----------|-------|
| SA2  | a      | TRINITY_DN59275_c4_g1_i2 | 1,107               | 63                                | scaffold2017_size403015 | 20          | 0.1726    |       |
|      | b      | TRINITY_DN59744_c3_g2_i6 | 1,190               |                                    | scaffold96_size1435301 | 6           | 0.00877   |       |
| SCC2 | a      | TRINITY_DN54282_c2_g1_i2 | 1,451               | 23                                | scaffold2590_size338625 | 1           | 0.23236   |       |
|      | b      | TRINITY_DN59006_c0_g1_i1 | 2,181               |                                    | scaffold8460_size69818 | 16          | 0.00616   |       |
| WEE1 | a      | TRINITY_DN56459_c4_g1_i2 | 528                 | 52                                | scaffold1083_size585250 | 1           | 0.00115   |       |
|      | b*     | TRINITY_DN52807_c2_g1_i1 | 520                 |                                    | scaffold204_size1131059, scaffold28031_size7424 | 1, 1         | 0.00814   |       |
| HOP1 | a      | TRINITY_DN58075_c4_g1_i3 | 573                 | 38                                | scaffold2018_size403633 | 8           | —         |       |
|      | b      | TRINITY_DN58899_c1_g1_i1 | 661                 |                                    | scaffold2609_size333860 | 11          | —         |       |
| AGO2 | a*     | TRINITY_DN59887_c5_g2_i1 | 786                 | 44                                | scaffold5587_size154872, scaffold4972_size179711 | 1, 1         | 0.00113   |       |
|      | b      | TRINITY_DN53431_c1_g2_i1 | 608                 |                                    | scaffold548_size808714 | 3           | 0.04382   |       |

*Indicates genes are found to be split across two scaffolds.

*Amino acid identities were measured from aligning amino acid sequences of the gene a and b using MUSCLE alignment implemented within Geneious.*
we identified multiple lineage-specific gene duplicates together with varied expression and evolutionary rates between each pair of duplicate genes. This finding contrasts with *T. cristinae* where only a single duplication event was observed (Bradler et al. 2015).

**Meiotic Gene Inventory and Expression**

All the meiosis-associated genes found with expressed homologs in the *C. hookeri* transcriptome also have *T. cristinae* gene orthologs, suggesting a high degree of conservation in these genes between the two stick insect genomes. Given that *Timema* is the sister taxon to all other stick insects (Whiting et al. 2003), it is also possible that this meiotic gene repertoire is conserved across the Phasmatodea. The expression of a complete set of the core meiotic recombination gene homologs in the *C. hookeri* reproductive organs is consistent with the same meiotic recombination machinery. Although some genes from this inventory are absent in other sexual and cyclical parthenogenetic arthropods (Ramesh et al. 2005; Schurko et al. 2009, 2010; Hanson et al. 2013; Tvedte et al. 2017), they seem to be maintained in at least these two stick insect species.

The comparison between gonadal and a wide range of non-gonadal tissues identified 36 predicted meiotic gene candidates with gonad-specific expression, consistent with their previously hypothesized role in meiosis. As expected, many of these genes (15) are involved in recombination-related functions. Similar to many obligate sexual organisms, candidate meiosis-specific genes (genes with gonad-specific expression) from *C. hookeri* also include the genes encoding the cohesion subunit *REC8* (Stoop-Myer and Amon 1999), the synaptonemal complex components *HOP1* and *HOP2* (Leu et al. 1998; Anuradha and Muniyappa 2004), the double-strand break (DSB) initiator *SPO11* (Keeney et al. 1997; Keeney 2008), the mismatch repair protein *MSH4* (Paquis-Flucklinger et al. 1997) and *MSH5* (Snowden et al. 2004), as well as the DSB end resect protein *MRE11* (Johzuka and Ogawa 1995). Although *DMC1* encoding proteins that play a role in DSB strand innovation and displacement have been reported as meiosis specific (Sehorn et al. 2004), in our data they were found to be expressed in both gonad and non-gonad tissues (leg, head, antennae, and midgut), indicating possible functions in mitosis (Maciver et al. 2019). The seven genes involved in cell-cycle regulation that displayed gonad-specific expression may contribute to determining whether the cell cycle is

![Fig. 3.—Boxplots showing expression difference of each duplicate pair between sexes.](image-url)

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meiotic rather than mitotic; for example, additional time controls for allowing recombination and the meiosis I to meiosis II transition (Marston and Amon 2004). Comparative analysis of gene expression between female and male gonads revealed that all differentially expressed meiotic genes were male biased and mostly gonad specific. This pattern of higher expression levels of meiotic genes in males matches our expectations. Female gonads only contain a limited number of cells from their ovarian tips that are undergoing meiosis, compared with testis, that include a far greater percentage of cells in the process of spermatogenesis.

Lineage-Specific Gene Duplication and Expression

A previous study assembled the genome of *C. hookeri* to a size of approximately 4.2 Gb, roughly four times that of *T. cristinae* (Wu et al. 2017). The difference in genome size can largely be attributed to a dramatically higher repetitive element content and longer introns in *C. hookeri* (Wu et al. 2017) relative to *T. cristinae*. *Citarchus hookeri* also has numerous duplicated genes relative to *T. cristinae* including those encoding phosphoglucose isomerase (Dunning et al. 2013) and cellulase (Shelomi et al. 2016; Wu et al. 2016). In this study, we found four meiotic genes with lineage-specific gene duplications in *C. hookeri*, but not in *T. cristinae*, evidence of further gene family expansion in *C. hookeri* relative to *T. cristinae*. It is also possible that there are more meiotic gene duplications in the *C. hookeri* genome, that were not detected from our current transcriptome due to, for example, low levels of gene expression. However, we did not perform a search against the available *C. hookeri* genome assembly because: 1) genes identified from the genome assembly can be pseudogenes or expressed in other tissues that are not associated with reproduction, 2) the genomic scaffolds are still highly fragmented with over 2% of bases being gaps (21 meiotic transcripts were split across multiple genomic scaffolds), and 3) more complete genes are likely to be absent in the genome assembly than the transcriptome (91.6% of BUSCO proteins are present in the genome assembly whereas 96.7% are present in the transcriptome assembled in this study). However, it is possible that apparent gene duplicates identified from our short read assembled transcriptomes are alleles or isoforms rather than true duplicates. We suggest this is not likely to be the case for our proposed duplicate pairs because each gene copy was present on separate genomic scaffolds, varied in exon number and were highly diverged from each other on the basis of amino acid identities (less than 64%, table 2) and phylogenetic analysis (as fig. 2). The duplicates from *SA2* and *WEE1* also have expression bias measured between gonadal and non-gonadal tissues. The genes *SA2a* and *WEE1a* were found to be expressed specifically in gonadal tissues, whereas the copy “b” of these two genes also had expression in non-gonadal tissues (supplementary table S3, Supplementary Material online). For each gene, the expression patterns of the two copies varied between females and males (as fig. 3).

Sister Chromatid Cohesion

SCC is a process required for connecting the newly formed sister chromatids from DNA duplication and lasts from S phase to anaphase onset during mitosis and meiosis (Peters and Nishiyama 2012). *SA2* is a subunit of the SCC complex (cohesin) that forms a ring structure together with *SMC3* and *RAD21* (mitosis)/REC8 (meiosis) to bind sister chromosomes (Peters and Nishiyama 2012). In *Drosophila*, *Stromalin* (*SNM*) is the paralogous duplicate of *SA2*, and encodes proteins present along the lengths of homologous chromosomes that are essential for homologous pairing and chromosome segregation in male meiosis (Thomas et al. 2005). *SNM* has a faster rate of evolution than *SA* (Thomas et al. 2005; Beekman 2013). Similarly, the genome of *C. hookeri* also harbors two copies of *SA2* and the a copy has gonad-specific expression along with a longer branch length compared with the more widely expressed b copy. Unlike *Drosophila*, the a copy also shows expression in the female gonads. Interestingly, this gene has five copies in the genome of the cyclical parthenogenetic *Daphnia pulex* (Schurko et al. 2009, 2010).

*SCC2* is one of the components of another protein complex, which is responsible for loading cohesin onto the chromosomes (Ciosk et al. 2000). This complex also interacts with cohesin and other proteins, such as *PDSS*, *ESPL1* (Separase) and *WAPL*, to stabilize the ring structure and enable cohesion success and effective release of the cohesin from chromosomes (Panizza et al. 2000; Peters and Nishiyama 2012). *SCC2* is a highly conserved gene, with homologs found across a wide range of eukaryote species (Seitz et al. 1996; Furuya et al. 1998; Rollins et al. 1999). In meiosis, proteins encoded by this gene, in addition to being involved in recruiting cohesins, are also involved in synaptonemal assembly and maintenance (Gause et al. 2008). The *SCC2* gene was duplicated in both stick insect genomes before the two species diverged. Similar to *SA2*, the *C. hookeri* a copy is expressed only in gonads and has a longer branch length than the universally expressed b copy. These results indicate the meiotic functions of this gene might have shifted a long time ago and whether the b copy still maintains a role in meiosis requires further investigation. The gonad-specific copies *SA2a* and *SCC2a* may have been under less stringent selective constraints due to tissue-limited expression and subsequently accumulated more substitutions compared with the widely expressed b copy.

Synaptonemal Complex Component

*HOP1* was found to have an extra copy in the *C. hookeri* genome relative to *T. cristinae*. In yeast, this gene is specific to meiosis, acting as a component of the synaptonemal complex, and plays a significant role in chromosomal pairing as
well as participating in inter-homolog recombination and crossing over (Hollingsworth and Byers 1989; Hollingsworth et al. 1990; Anuradha and Muniyappa 2004; Anuradha et al. 2005). Interestingly, this gene, although generally conserved in most other eukaryotes is missing in some insect lineages, such as the sexually reproducing Drosophila and Anopheles (Ramesh et al. 2005). In our study, the survey of HOP1 in Daphnia pulex and 11 insect species demonstrates that it is only present in the three Polynoeoptera species (Z. nevadensis: XM_022072157.1, T. cristinae, and C. hookeri) and the beetle Tribolium castaneum (TCOGS2: TC000115). The expression of the two C. hookeri copies appears to be gonad specific, but the expression of α is higher than β in the females, whereas the opposite pattern is apparent in males, indicating their involvement in sex-specific processes, possibly during meiotic recombination. It is interesting that in arthropods, HOP1 can be missing in both sexual (Ramesh et al. 2005; Srinivasan et al. 2010; Hanson et al. 2013) and cyclical parthenogenetic species (A. pisum and D. duplex from this study), indicating its presence does not correlate well with reproductive mode. However, our discovery of a HOP1 duplication in arthropods raises interesting questions about its function and evolution.

RNA Silencing and Cell-Cycle Regulation

Both stick insect genomes express all the genes included in the categories of RNA silencing and cell-cycle regulation from our a priori gene list. Clitarchus hookeri has two duplication events of the genes AGOB (n = 2) and WEE1 (n = 2), whereas only one copy of each was found in the T. cristinae genome. Argonaute proteins form the functional core of the RNA-induced silencing complexes, which mediate RNA silencing in eukaryotes. In mammals, AGO1-4 forms the complex that mediates RNA cleavage targeted by micro-RNAs (miRNAs) and small-interfering RNAs (siRNAs) (Liu et al. 2004; Meister et al. 2004), some of which are also involved in the biogenesis of miRNAs and siRNAs (Yang and Lai 2010; Meister 2013). Inactive AGO2 in mouse results in aberrant meiotic maturation and defects in spindle formation and chromosome alignment during oogenesis (Stein et al. 2015). MEL1, an AGO homolog in rice (Oryza sativa) regulates cell division of premeiotic germ cells, and is involved in the proper modification of meiotic chromosomes and meiotic progression (Nonomura et al. 2007). It is interesting that there are multiple gene duplication events in the argonaute family (piwi: n = 8; AGO3: n = 2) from the cyclical parthenogenetic A. pisum and that some of the gene members (piwi3, piwi8, and AGO3a) are differentially expressed in ovaries between sexual and asexual forms (Lu et al. 2011; Srinivasan et al. 2014). Multiple copies of AGOB have been also reported in N. vitripennis (n = 3) and the cyclical parthenogenetic B. calyciorus (n = 2) (Schurko et al. 2010; Hanson et al. 2013). In addition, we found that the Tribolium castaneum genome has two copies of AGOB. The six members of the Argonaute family that we found in the C. hookeri transcriptome have provided candidates to study the evolution of geographic parthenogenesis in this species.

WEE1 is a subunit of the G2/M checkpoint protein complex, which inhibits cyclin-dependent kinase CDK1 activities through phosphorylation, thereby ensuring mature entry into mitosis and meiosis (Nurse and Thuriaux 1980; Den Haese et al. 1995). It also plays a role in the cell size checkpoint by coordinating cell size and cell cycle progression (Kellogg 2003). In Drosophila, proper regulation of the early syncytial cycles of embryogenesis requires inhibitory phosphorylation of CDK1 by WEE1 (Price et al. 2000; Stumpff et al. 2004). Interestingly, the expansion of this gene family seems to frequently occur in species that display alternative reproductive modes. The cyclical parthenogenetic pea aphid has three WEE1 duplicates, and most interestingly, they are all down-regulated in asexual pea aphids compared with sexual forms, suggesting an evolved additional cell cycle checkpoint control that may be associated with reproductive flexibility (Srinivasan et al. 2010). WEE1 duplicates (n = 2) have also been found in the cyclical parthenogenetic rotifer Brachionus manjavacas (Hanson et al. 2013). In our study, we found an additional WEE1 duplication event (n = 2) in the C. hookeri transcriptome, and so far, duplication of this gene in arthropods has only been seen in these three species displaying reproductive flexibility.

Conclusion

We have identified 101 candidate meiotic genes and their expression patterns in the stick insect C. hookeri. Five of these genes were duplicated in C. hookeri and in 1 in Timema that are rarely or never duplicated in the other arthropod genome assemblies we surveyed. Three of the five genes showed gonad-specific expression in one of the duplicates. These duplicates also showed evidence of shifts in substitution pattern between the duplicate pairs but with overall purifying selection. These inferences have shed light on the genes underlying meiosis in stick insects. Future work will be needed to test the function of the candidate genes examined here. Also, an examination of differential gene expression in these candidate genes between sexual and parthenogenetic populations of C. hookeri, may reveal the molecular switches underlying parthenogenesis.

Supplementary Material

Supplementary data are available at Genome Biology and Evolution online.

Acknowledgments

This work was supported by the Allan Wilson Centre, Core funding for Crown Research Institutes from the New Zealand
Ministry of Business, Innovation and Employment’s Science and Innovation Group, and the University of Auckland. We thank New Zealand eScience Infrastructure (NeSI) for high-performance computing facilities; Robyn Howitt, Julia Allwood, Ana Ramon-Laca, and Duckchul park for laboratory support; and Ross Crowhurst for analysis advice.

**Author Contributions**

T.R.B. and R.D.N. conceived and designed the project and advised on analyses. C.W. collected samples and performed de novo assembly, gene identification, and comparative transcriptomic analysis. V.G.T. and C.W. performed RNA extraction. V.G.T. performed PAML analysis. C.W. prepared the manuscript draft. T.R.B. and R.D.N. edited and commented extensively on the draft manuscript. All authors reviewed and approved the final manuscript.

**Data Availability**

RNA sequencing (RNA-Seq) reads and the de novo transcriptome assembly are deposited in the National Centre for Biotechnology Information (NCBI) under the project PRJNA395945.

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