

Braconidae revisited: *Bracon brevicornis* genome showcases the potential of linked-read sequencing in identifying a putative complementary sex determiner gene

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1 ABSTRACT

2 *Bracon brevicornis* is an ectoparasitoid of a wide range of larval-stage
3 Lepidoptera, including several pests of important crops, such as the corn borer,
4 *Ostrinia nubilalis*. It is also one of the earliest documented cases of complementary
5 sex determination in Hymenoptera. Here, we present the linked-read genome of *B.*
6 *brevicornis*, complete with an *ab initio*-derived annotation and protein comparisons
7 with fellow braconids, *Fopius arisanus* and *Diachasma alloem*. We demonstrate the

8 potential of linked-read assemblies in exploring regions of heterozygosity and search
9 for structural and homology-derived evidence of the *complementary sex*
10 *determiner* gene (*csd*).

11 **INTRODUCTION**

12 *Bracon brevicornis* (Wesmael) is a gregarious ectoparasitoid of various Lepidoptera
13 larvae, including many important pests, and is considered a cosmopolitan species
14 (Temerak 1983b; Venkatesan *et al.* 2009). In the past *B. brevicornis* has been
15 classified under the genus *Habrobracon* (Speicher and Speicher 1940),
16 *Microbracon* (Narayanan *et al.* 1954), or classified as one species with
17 *Habrobracon/Bracon hebetor* (Puttarudriah and Basavanna 1956), however recent
18 research shows that *B. brevicornis* and *B. hebetor* are genetically two distinct
19 species (Kittel and Maeto 2019). In the field, *B. brevicornis* has shown potential as a
20 biological control agent against important pest species in stored corn stalks, such as
21 *Ostrinia nubilalis* and *Sesamia cretica* (Kares *et al.* 2010), or against the coconut
22 moth, *Opisina arenosella* (Venkatesan *et al.* 2009). In the laboratory, *B. brevicornis*
23 attacks a wide range of larval host such as *Ephesthia kuehniella*, *Galleria* spp., and
24 *Spodoptera* spp. (Temerak 1983a).

25 Work on *B. brevicornis* has included both laboratory and semi-field set-ups to
26 determine both its efficacy as a biological control agent as well as its suitability as a
27 study system. There are several studies on the biology of *B. brevicornis*, e.g. on
28 population growth potential (Srinivasan and Chandrikamohan 2017), their host
29 range (Temerak 1983a), interspecific competition (Venkatesan *et al.* 2009), clutch
30 size and fitness (Villacañas de Castro and Thiel 2017), mate choice (Thiel *et al.* 2013;
31 Thiel and Weeda 2014), diet (Temerak 1983b), and efficacy (Kares *et al.* 2010).

32 Within a phylogenetic perspective, *B. brevicornis* falls within the subfamily
33 Braconinae, the largest of the cyclostome-forming braconid wasps (Chen and van
34 Achterberg 2019). The presence of a cyclostome (round mouthpart) is a defining
35 feature within braconid wasps, as it represents an unresolved evolutionary and
36 systematic question: is the cyclostome a derived trait within certain branches, or an
37 ancestral trait that has been lost in others (Chen and van Achterberg 2019)? Within
38 the Braconinae, there have been multiple switches from ectoparasitism to
39 endoparasitism and vice versa, and this combination of cyclostome and
40 endoparasitism has been described as a “controversial topic” by braconid
41 researchers and taxonomists (Chen and van Achterberg 2019). These systematic
42 issues are far from being resolved, and more genomic data would be useful for
43 future phylogenetic analyses (Chen and van Achterberg 2019). Yet, a
44 representative genome for the Braconinae is currently lacking. As previously stated,
45 *B. brevicornis* is an ectoparasitoid, and its position within a family that contains both
46 types of parasitism lifestyles holds promise for further phylogenetic comparisons.

47 In addition, as being part of the order Hymenoptera, *B. brevicornis* has a
48 haplodiploid sex determination system where males develop from unfertilized eggs
49 and females develop from fertilized eggs (Cook and Crozier 1995; Heimpel and de
50 Boer 2008). From a genetic perspective, *B. brevicornis* belongs to an interesting
51 genus where sex determination and diploid male production have been widely
52 studied (*B. hebetor*, Whiting and Whiting 1925, *B. brevicornis*, Speicher and Speicher
53 1940, *B. serinopae* Clark, Bertrand, and Smith 1963, reviewed in van Wilgenburg,
54 Driessen, and Beukeboom 2006; *B. spec. near hebetor*, Holloway *et al.* 1999; and *B.*
55 *variator*, A. Thiel, pers. comm.). Indeed, the first description of the complementary

56 sex determination (CSD) mechanism was provided for *B. hebetor* (= *B. juglandis* by
57 Whiting 1940, reviewed in Antolin *et al.* 2003), and recent work on *B. brevicornis* and
58 polyploidy studies include diploid male fitness as well as ploidy-dependent mate
59 choice behaviour (Thiel and Weeda 2014).

60 While straightforward to detect phenotypically through the formation of diploid
61 males following inbreeding (van Wilgenburg *et al.* 2006), the molecular mechanism
62 underlying CSD has thus far only been resolved to a small level of detail in the
63 honeybee *Apis mellifera* (L.) (Hymenoptera: Apidae), with the identification of the
64 *complementary sex determiner (csd)* gene (Beye *et al.* 2003). Heterozygosity at this
65 gene leads to female development, while hemi- and homozygous individuals
66 develop into haploid and diploid males respectively. Therefore, inbreeding often
67 leads to diploid male production in species with a CSD mechanism as it increases
68 homozygosity. *Csd* is a duplication of *feminizer (fem)*, a *transformer (tra)* ortholog
69 (Hasselmann *et al.* 2008) that is conserved across many insect orders as part of the
70 sex determination cascade (Geuverink and Beukeboom 2014). When heterozygous,
71 *csd* initiates the female-specific splicing of *fem*, which then autoregulates its own
72 female-specific splicing, ultimately resulting in female development. Within the
73 Hymenoptera, more duplications of *tra/fem* have been identified in species that are
74 presumed to have CSD (Geuverink and Beukeboom 2014), but these *tra/fem*
75 duplications have not been analysed for potential heterozygosity. Also, additional
76 hymenopteran genomes are necessary to understand the evolutionary history of
77 *tra/fem* duplications and identify the genes underlying CSD. However, an
78 assembled genome is usually haploid as areas of heterozygosity are collapsed in
79 the final stages of assembly. Yet recent advances in sequencing and analysis gave

80 us the ability to view heterozygous regions, known as “phases” in diploid assemblies,
81 within a genome which allow us to investigate potential *csd* regions.

82 Here we report on the whole-genome sequencing of a pool of females from an
83 isolated *B. brevicornis* strain using 10X Genomics technology that relies on linked-
84 read sequencing (10x Genomics Inc., Pleasanton, CA, USA). Due to their long history
85 of genetic isolation during laboratory rearing, the females in this strain are assumed
86 to have a high level of homozygosity, whereas a *csd* locus would retain its
87 heterozygosity. The 10X Genomics technology allows for generating phased data in
88 which allelic variants can be identified after assembly. High-molecular weight DNA
89 is partitioned into small droplets containing a unique barcode and adapter in such
90 a way that only a few DNA molecules are present within each droplet. Within each
91 droplet the DNA is broken into pieces and the barcode (Gel Bead-in-Emulsion,
92 “GEM”) is ligated to each of the DNA fragments. This resulting library can then be
93 sequenced on an Illumina sequence platform. In the assembly step the reads
94 originating from the same fragment are organized by barcode and put together
95 into synthetic long-read fragments. Importantly, it is nearly impossible that two
96 fragments with opposing allelic-variances are together in the same droplet
97 (Weisenfeld *et al.* 2017). This technique therefore allowed us to identify potential *csd*
98 candidates in the female-derived *B. brevicornis* genome after sequencing by
99 studying the phased data containing the different haplotypes. Moreover, as *B.*
100 *brevicornis* is a potential biological control agent of several pests, the availability of
101 a full genome may provide effective ways to study and improve this species to grow
102 it into an established biological control agent for Lepidopteran pests.

103 **METHODS**

104 **Species description and general rearing:**

105 Individuals of *B. brevicornis* were taken from the laboratory colony L06. The colony
106 was initiated in 2006 from naturally parasitized *O. nubilalis* larvae collected in maize
107 fields near Leipzig, Germany. Species identification was first carried out by Matthias
108 Schöller and Cornelis van Achterberg based on morphological characteristics
109 (Bernd Wührer, AMW Nützlinge, pers. comm.) Since collection, parasitoids have
110 been reared on late instar larvae of the Mediterranean flour moth, *E. kuehniella*
111 (Thiel and Weeda 2014). The species identity of strain L06 was recently revalidated
112 based on molecular data and is entirely separate from its congeneric *B. hebetor*
113 (Kittel and Maeto 2019).

114 **DNA extraction:**

115 Immediately following emergence, 100 to 120 female wasps were flash frozen in
116 liquid nitrogen and ground with a mortar and pestle. Genomic DNA was extracted
117 using a protocol modified from Chang, Puryear, and Cairney (Chang *et al.* 1993).
118 Modifications include adding 300 µL BME to extraction buffer just before use. Instead
119 of 10M LiCl, 0.7 volume isopropanol (100%) was added to the initial supernatant,
120 after which it was divided into 1.5 mL Eppendorf tubes as 1 mL aliquots for
121 subsequent extractions. The initial centrifugation step occurred at a slower rate and
122 for a longer period of time to adjust for machine availability. Final pellets were
123 dissolved in 50 µL autoclaved MQ and recombined at the end of the extraction
124 process (1.0 mL). DNA concentration was measured with an Invitrogen Qubit 2.0
125 fluorometer using the dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, USA)

126 with final assessments for DNA quality, amount, and fragment size confirmed via
127 BioAnalyzer 2100 (Agilent, Santa Clara, California, USA).

128 **10X Genomics library preparation and sequencing:**

129 As the genome of *B. brevicornis* is relatively small for the scale of the 10X platform,
130 there is a higher risk of overlapping fragments within single GEMs. In order to reduce
131 this risk, genomic DNA of a larger and previously analysed genome (Tomato,
132 *Solanum lycopersicon* (L.) (Solanaceae), commercial variety Heinz 1607) (Hosmani
133 *et al.* 2019) was used as 'carrier DNA'. DNA extraction of *S. lycopersicon* followed
134 the protocol of Hosmani *et al.* (Hosmani *et al.* 2019). The DNA of both *B. brevicornis*
135 and *S. lycopersicon* was pooled in a 1:4 molar ratio.

136 One nanogram of this pooled DNA was used for 10X Genomics linked-read library
137 preparation following the Chromium Genome Reagent Kits Version 1 User Guide
138 (CG-00022) (10x Genomics, Pleasanton, USA). Barcoded linked read DNA fragments
139 were recovered for final Illumina library construction (Illumina, San Diego, USA). The
140 library was used for 2 x 150 bp paired-end sequencing on one lane of an Illumina
141 HiSeq 2500 at the business unit Bioscience of Wageningen University and Research
142 (Wageningen, The Netherlands). Sequencing data was then used for basecalling
143 and subsequent demultiplexing using Longranger (v2.2.2) (10X Genomics)
144 (command `-mkfastq`), yielding 212,910,509 paired-end reads with a read length of
145 150 bp.

146 **Assembly:**

147 To filter sequence data from Heinz tomato (*S. lycopersicon*) carrier DNA sequences,
148 23bp (16bp GEM + 7 bp spacer) were removed from forward reads and all reads
149 were subsequently mapped to an in-house high quality reference assembly of the

150 Heinz genome using BWA-MEM v0.7.17 (Li 2013). Using samtools v1.9 (Li *et al.* 2009),
151 all unaligned read pairs (`-F=12`) were extracted and labelled non-Heinz. The
152 assembly of the non-Heinz labelled read set was performed with 10X Supernova
153 assembler v2.1.0 (10X Genomics), using default settings including commands for
154 both pseudohap (`--style=pseudohap`) and pseudohap2 (`--style=pseudohap2`)
155 outputs (Weisenfeld *et al.* 2017). These commands determine the output from
156 Supernova, the first being the final scaffold output (pseudohap), while the second
157 is the so-called 'parallel pseudohaplotype' (pseudohap2) scaffolds that represent
158 areas of divergence or phases (Weisenfeld *et al.* 2017). Phasing is flattened in the
159 pseudohap output by selecting the region with higher mapping coverage, whereas
160 in the pseudohap2 output is differentiated by ".1" and ".2" at the end of each
161 scaffold name to denote phasing, though not all scaffolds are phased at this point
162 due to lack of divergence during assembly.

163 To verify whether there were no Heinz leftovers in the assembly, minimap2 v2.17-r941
164 (Li 2018) was used to align the assembly against the same Heinz assembly. Further
165 examination on presence of possible non-*B. brevicornis* scaffolds, i.e. bacterial
166 scaffolds from sample microbiome, was performed with BlobTools (v1.0) (Laetsch
167 and Blaxter 2017), relying on megaBLAST against the NCBI NT-NR database (Acland
168 *et al.* 2014)(2018-11-19) (`max_target_seqs=1`, `max_hsps=1`, `value=1e-25`) for
169 taxonomical classification and BWA-MEM mapping of reads against scaffolds for
170 coverage statistics. Reads mapping only against "Arthropoda" classified scaffolds
171 were then extracted and used for a final k-mer analysis using jellyfish v2.1.1 (`-C m=21`
172 `-s=2000000000`) (Marçais and Kingsford 2011) and GenomeScope (Vurture *et al.*
173 2017) to infer heterozygosity.

174 Assembly completeness was determined using *BUSCO* (v3.0.2) with the
175 *insect_odb9* ortholog set and the fly training parameter (Simão *et al.* 2015) while
176 assembly statistics were determined using *QUAST* (Gurevich *et al.* 2013). The
177 aforementioned pseudohap2 scaffolds were used in *csd* analysis, while the
178 pseudohap scaffolds are now the assembly used for annotation.

179 ***Ab initio* gene finding and protein comparison:**

180 The coding sequences of two additional braconids (members of the subfamily
181 Opiinae, and similar to the Braconinae belonging to the cyclostome subgroup (Li *et*
182 *al.* 2013; Chen and van Achterberg 2019)) were used for gene prediction and
183 protein comparisons: *Fopius arisanus* (Sonan) (Hymenoptera: Braconidae) and
184 *Diachasma alloem* (Muesebeck) (Hymenoptera: Braconidae). Both sets of coding
185 sequences were retrieved from the NCBI Assembly Database, version ASM8063v1
186 for *F. arisanus* and version Dall2.0 for *D. alloem* (Acland *et al.* 2014; Geib *et al.* 2017;
187 Tvedte *et al.* 2019).

188 For gene prediction, Augustus (v2.5.5) was first used to predict genes from the *B.*
189 *brevicornis* assembly (Stanke and Morgenstern 2005). Using BLAST, coding
190 sequences of *F. arisanus* were set as a query to the genome of *B. brevicornis* using
191 default parameters (except `minIdentity=50`) (Camacho *et al.* 2009). The result
192 was converted into a hints file that was used to predict the genes of *B. brevicornis*
193 using *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) as the species
194 parameter in Augustus (`--species=nasonia -`
195 `extrinsicCfgFile=extrinsic.E.cfg`).

196 After prediction, the protein sequences were retrieved and compared to both *F.*
197 *arisanus* and *D. alloem* (version Dall2.0) using Proteinortho (v6.0, `-p=blastp, -`

198 $e=0.001$) (Lechner *et al.* 2011). From the orthology grouping generated by
199 Proteinortho, gene names could be allocated to the predicted genes. Lengths of
200 both these *B. brevicornis* genes and the orthologs of *F. arisanus* and *D. alloem* were
201 retrieved using samtools for comparison (Li *et al.* 2009). Errors within the annotation
202 related to genome submission and validation were corrected with manual
203 annotation of exons (three cases) and removal of two predicted genes that were
204 more than 50% ambiguous nucleotides.

205 ***In silico* identification of feminizer as a putative csd locus:**

206 The pseudohap2 files were deduplicated using the dedupe tool within BBTools
207 (sourceforge.net/projects/bbmap/) (`ac=f`) to remove all parallel
208 pseudohaplotypes that were complete duplicates as these scaffolds were not
209 heterozygous. The remainder of the set contained both scaffolds that previously had
210 a duplicate, as well as solitary scaffolds that did not have a partner scaffold. These
211 unique scaffolds were removed using the “filter by name” tool in BBTools, leaving
212 258 scaffolds, or 129 pairs of pseudohap2 scaffolds. Pairs were pairwise aligned in
213 CLC Genomics Workbench 12 (Qiagen, Hilden, Germany) using default settings
214 (`gap open cost=10, gap extension cost=1, end gap cost=free,`
215 `alignment=very accurate`).

216 A local tBLASTn search against the entire *B. brevicornis* assembly was performed
217 using the *Apis mellifera* Feminizer protein (NP_001128300) as query in Geneious Prime
218 2019.1.3 (<http://www.geneious.com>, (Kearse *et al.* 2012)). The protein of gene
219 “g7607” (locus tag = BBRV_LOCUS33129) was used in an NCBI BLASTp against the nr
220 database with default settings (Camacho *et al.* 2009; Acland *et al.* 2014). Next a
221 region stretching from ~10Kbp upstream and downstream of the first and last

222 tBLASTn hit in scaffold 12, respectively, was annotated using HMM plus similar protein-
223 based gene prediction (FGENESH+, Softberry, <http://www.softberry.com/>) with
224 *Nasonia vitripennis tra* (NP_001128299) and *N. vitripennis* for the specific gene-
225 finding parameters (Solovyev 2007). Only this combination of settings resulted in a
226 full-length annotation from TSS to poly-A with seven exons. The resulting protein
227 prediction was used in a BLASTp search with default settings against the nr
228 database. To annotate the potential *fem* duplication, a stretch of ~10Kbp directly
229 upstream of the annotated putative *fem* was again annotated using FGENESH+
230 (Softberry) with *Nasonia vitripennis tra* (NP_001128299) and *N. vitripennis* for the
231 specific gene-finding parameters (Solovyv et al. 2007). The predicted annotation
232 contained five exons but lacked the last coding segment with stop codon. A protein
233 alignment was made in Geneious Prime 2019.1.3 with *A. mellifera csd* (ABU68670)
234 and *fem* (NP_001128300); *N. vitripennis tra* (XP_001604794) and *B. brevicornis*
235 putative *fem* and *B. brevicornis* putative *fem* duplicate (*fem1*), using MAFFT v7.450
236 with the following settings: **Algorithm=auto, Scoring matrix=BLOSUM62, Gap**
237 **open penalty=1.53, Offset value=0.123** (Kato 2002; Kato and Standley
238 2013).

239 **Microsynteny analysis:**

240 A microsynteny analysis was achieved by comparing the arrangement of a set of
241 homologous genes directly upstream and downstream of *tra* or *fem* in *A. mellifera*
242 and *N. vitripennis* using a combination of the online tool SimpleSynteny (Veltri et al.
243 2016) and tBLASTn searches using default settings in Geneious Prime. The scaffolds
244 containing *fem* (*A. mellifera*, scaffold CM000059.5, 13.2Mbp in length), *tra* (*N.*
245 *vitripennis*, scaffold NW_001820638.3, 3.7Mbp in length) or the putative *fem* (*B.*

246 *brevicornis*, scaffold 12, 4.5 Mbp in length) were extracted from their respective
247 genomes (*Apis*: GCA_000002195.1_Amel_4.5_genomic, *Nasonia*: nvi_ref_Nvit_2.1,
248 *Bracon*: *B. brevicornis* assembly from this study) and searched with protein sequence
249 from the following genes: *tra* (GeneID: 00121203), LOC100121225, LOC100678616,
250 LOC100680007 originating from *N. vitripennis*; and *fem* (GeneID:724970), *csd*
251 (GeneID:406074), LOC408733, LOC551408, LOC724886 originating from *A. mellifera*.
252 The advanced settings for SimpleSynteny were as follows: **BLAST E-value**
253 **Threshold=0.01**, **BLAST Alignment type=Gapped**, **Minimum Query Coverage**
254 **Cutoff=1%**, **Circular Genome Mode=Off**. If the gene was not found within the
255 extracted scaffold, it was searched for in the full genome assembly. For the image
256 settings, **Gene Display Mode=Project Full-Length Gene**. This generated image
257 was used together with results from the tBLASTn searches as template to draw the
258 final figure. The final figure that we present in the Results and Discussion section
259 depicts ~0.9Mbp of genomic region for all three species.

260 **Data availability:**

261 Raw sequence data for *B. brevicornis* after removal of carrier DNA and
262 contamination, as well as the assembly, can be found in the EMBL-EBI European
263 Nucleotide Archive (ENA) under BioProject PRJEB35412, however, are currently
264 being updated due to error in initial upload. In the meantime, both the assembly file
265 (.fasta) (<https://doi.org/10.6084/m9.figshare.12674189.v2>) and the complete
266 annotation file (.gff) (<https://doi.org/10.6084/m9.figshare.12073911.v2>) are available
267 in a separate repository. Contaminated pseudohap scaffolds are available for
268 download alongside the two pseudohap2 FASTA files, more details are provided in
269 the supplementary materials at <https://doi.org/10.17026/dans-xn6-pjm8>.

270 **RESULTS AND DISCUSSION**

271 A total of 172 ng of *B. brevicornis* DNA was extracted, which was then reduced to 1
272 ng/ μ L for library preparation. Sequencing of the Heinz diluted library resulted in a
273 total yield of 54 Gbp of data (corrected for 10X 23bp segment of forward reads).
274 Mapping against the Heinz genome assembly showed a mapping percentage of
275 84.8%. There was a total of 30,278,915 unmapped pairs, comprising ~8.39 Gbp of
276 data. This corresponds to the 4:1 ratio between Heinz and *B. brevicornis* DNA in the
277 library. Further scaffold decontamination with BlobTools resulted in a separation of
278 the assembly into *B. brevicornis* scaffolds and microbiome scaffolds. The final
279 genome is 123,126,787 bp (123 Mbp) in size, comprised of 353 scaffolds (5.5%
280 ambiguous nucleotides). This is similar to the projected physical genome size of 133
281 Mbp (J. G. de Boer, unpublished data, flow cytometry). BUSCO analysis indicates a
282 completeness of 98.7% (single orthologs 97.0%, duplicate orthologs 1.7%).

283 K-mer analysis of the *B. brevicornis*-only read set showed an expected haploid
284 genome length of ~115 mbp (105 Mbp unique, 10 Mbp repeat) and a heterozygosity
285 of ~0.54%. Peak coverage was 27x.

286 ***Ab initio* gene finding and protein comparison:**

287 In total, 12,686 genes were predicted, with an average coding sequence length of
288 529.86 amino acids. The number of genes correspond well to those found in *F.*
289 *arisanus* (11,775) and *D. alloem* (13,273), the two closest relatives of *B. brevicornis* for
290 which public data is available. Proteinortho analysis resulted in 7660 three-way
291 orthology groups (7,830 *B. brevicornis* genes), while 362 orthology groups contained
292 proteins of *B. brevicornis* and *F. arisanus* (382 *B. brevicornis* genes), and 451 groups
293 contained *B. brevicornis* and *D. alloem* genes (479 *B. brevicornis* genes). A large

294 number of orthology groups (2,492) had no *B. brevicornis* genes, while 3,995
295 predicted genes remain ungrouped.

296 Compared to *F. arisanus*, the mean relative length of predicted *B. brevicornis* genes
297 was 1.016, while the mean relative length for the two- and three-way orthology
298 groups was 0.996. Similar results were obtained for comparisons to *D. alloem*, where
299 mean relative length for *B. brevicornis* genes was 1.011 and 0.988 for the two- and
300 three-way orthology groups. Furthermore, the pairwise lengths of all these proteins
301 resemble each other very well (Figure 1).

302 **Identification of a putative feminizer ortholog and duplication event:**

303 After deduplicating the similar parallel pseudohaplotype files, 6,706 scaffolds in
304 total, the remainder of the set contained 3,420 scaffolds, of which 3,286 scaffolds
305 were solitary and did not have a counterpart pseudohap2 for comparison. Some
306 had had a previous duplicate removed in the deduplication, while others never had
307 a partner scaffold in the first place. These unique scaffolds were removed, leaving
308 258 scaffolds, or 129 pairs of pseudohap2 scaffolds. These putatively heterozygous
309 scaffolds were good candidates to search for potential *csd* loci as these are
310 presumed to be heterozygous in females.

311 So far, a *csd* gene has been sequenced only in species of bees of the genus *Apis*,
312 and it is highly polymorphic, even within subspecies (Wang *et al.* 2012). It is located
313 adjacent to the more conserved *feminizer* (*fem*) (Hasselmann *et al.* 2008), and we
314 therefore started with localizing *feminizer* in the genome. As *feminizer* (or its ortholog
315 *transformer*, *tra*) was not identified in the *ab-initio* annotation, we used a local
316 tBLASTn search to find *fem* in the assembly. Four hits with E-value from 5.86e-04 to
317 8.59e-08 were found in scaffold 12. Searching the annotation using part of the

318 tBLASTn result shows that it is annotated as “g7607” (locus tag = BBRV_LOCUS33129)
319 which gave a first hit with protein O-glucosyltransferase 2 (*Diachasma alloeum*) after
320 a BLASTp search, and no *fem* or *tra* hits were found. A closer inspection showed that
321 “g7607” is annotated as fusion protein with the N-terminal part resembling *fem* and
322 the C-terminal part putatively encoding *O-glucosyltransferase 2*. Next, we used
323 FGENESH+ to re-annotate the genomic region, resulting in a full-length putative *B.*
324 *brevicornis feminizer* (*Bbfem*) ortholog containing seven exons (Figure 3). We found
325 that the two *fem/tra* signature domains in Hymenoptera, the Hymenoptera domain
326 (Verhulst *et al.* 2010) and CAM domain (putative autoregulatory domain) (Hediger
327 *et al.* 2010), are present in the putative *fem* ortholog, but are also duplicated
328 upstream of putative *Bbfem*. A second manual re-annotation step showed that a
329 partial *fem*-duplicate is encoded directly upstream of putative *Bbfem* containing
330 five exons (Figure 3), which we denote here as *Bbfem1* as suggested by Koch *et al.*
331 (Koch *et al.* 2014). The level of potential heterozygosity in the area encoding *Bbfem*
332 and *Bbfem1* is the highest when compared across all 129 pairs of pseudohap2
333 scaffolds (Figure 3).

334 A protein alignment showed that the full-length putative *Bbfem* as well as *Bbfem1*
335 are highly similar to each other and both contain all known *fem/tra* domains (Figure
336 S1). *Bbfem1* lacks a notably long first Arginine/Serine (RS)-rich region which is present
337 only in *Bbfem* (124-153aa), but it otherwise appears to encode for a full-length
338 protein. The *csd*-specific hypervariable domain (Figure S1, purple text; (Beye *et al.*
339 2003)) is not present in *Bbfem* nor in *Bbfem1*. Therefore, the gene name has been
340 updated as “g7607 putative *Bbfem*-*Bbfem1 csd*” in the official annotation.

341 **Synteny analysis of putative fem encoding region:**

342 We compared the orthologous gene arrangement of a number of genes up- and
343 downstream of *N. vitripennis tra* and *A. mellifera fem* and *csd*, with the genomic
344 organization of the *Bbfem* region (Figure 2). *N. vitripennis* LOC100680007 is present
345 in the *tra/fem* containing scaffolds of all three genomes, while *A. mellifera*
346 LOC408733 has both translocated closer to *Nasonia tra* and to a different scaffold
347 in *B. brevicornis*. *N. vitripennis* LOC100121225 and LOC100678616 are encoded in
348 opposing directions in both *A. mellifera* and *N. vitripennis* but are both downstream
349 of *tra* in *N. vitripennis* and upstream of *fem* and *csd* in *A. mellifera*. There is no match
350 for both genes in *B. brevicornis*. *A. mellifera* LOC724886 and LOC551408 are
351 encoded in opposing directions with the same orientation in both *N. vitripennis* and
352 *A. mellifera* but are reversed in *B. brevicornis* and downstream of *Bbfem* and *Bbfem 1*
353 while they are upstream of *csd* and *fem* in *A. mellifera*. In *N. vitripennis* both genes
354 are not located in the *tra* containing scaffold but in another scaffold indicating that
355 this region has undergone chromosomal rearrangements.

356 **CONCLUSIONS AND PERSPECTIVES**

357 Here, we present the genome of the braconid wasp *Bracon brevicornis*, a parasitoid
358 wasp that not only has biological control applications, but also offers potential as a
359 study system for future analyses into braconid phylogenetics and gene evolution.
360 With no previous genomes available for the subfamily Braconinae, the most
361 speciose of the braconid wasps, the resources and investigations presented here fill
362 this gap. Our linked-read library, assisted by carrier DNA of *S. lycopersicon*, has
363 resulted in a highly contiguous, very complete assembly, comprised of just 353
364 scaffolds and 12,686 genes. This gene count is similar to related species, and in

365 further protein length comparisons, the proteins are highly similar. This indicates that
366 the predicted genes are highly complete, a necessary feature for any future
367 phylogenetic comparisons between species or families.

368 We utilized the 10X Genomics linked-read approach to obtain pseudohaploid
369 information that would allow us to search for potential *csd* loci *in silico*. As a
370 substantial number of scaffolds were putatively heterozygous, we used the notion
371 that in *A. mellifera*, *csd* is located adjacent to *fem* (Hasselmann *et al.* 2008) to limit
372 our search for *csd* candidates. We manually annotated a putative *B. brevicornis*
373 *fem* and a partial *Bbfem* duplicate that is highly similar, and both genes encode all
374 known *tra/fem* protein domains (Figure S1) (Verhulst *et al.* 2010). Both genes are in
375 a small region that is highly heterozygous, especially when compared to the
376 remainder of the scaffold, which would suggest true heterozygosity and not
377 assembly error, but also when compared to the level of heterozygosity in the other
378 128 aligned pseudohap2 scaffolds.

379 Our synteny analysis showed only little structural conservation between *B.*
380 *brevicornis*, *A. mellifera*, and *N. vitripennis* with the translocation of LOC408733 (*A.*
381 *mellifera*) and the absence of LOC100121225 and LOC100678616 (*N. vitripennis*) in
382 the *B. brevicornis* genome region. It is known that genomic regions encoding sex
383 determination genes are dynamic in nature, showing both duplications and
384 translocations (Dechaud *et al.* 2019). Also, *tra/fem* duplications have been shown
385 in CSD systems before, most notably in *A. mellifera* where a *fem* gene duplication
386 event resulted in it becoming a *csd* locus (Hasselmann *et al.* 2008; Gempe *et al.*
387 2009). However, also in non-CSD systems *tra* duplications have been observed
388 (Geuverink and Beukeboom 2014; Jia *et al.* 2016; Geuverink *et al.* 2018). Although

389 there is some debate on whether *fem* paralogs originated due to a single
390 duplication event and functions as *csd* (Schmieder *et al.* 2012), or evolved multiple
391 times independently and may have other functions (Koch *et al.* 2014), we suggest
392 that the *Bbfem* paralog, *Bbfem1*, is a good *csd* gene candidate in *B. brevicornis*.
393 However, in-depth analyses are required to verify this. Ultimately, our presented
394 genome with its pseudohaploid information provides multiple opportunities for
395 future studies, such as to improve the biological control opportunities with this
396 species, but also to shed light on the evolutionary history of complementary sex
397 determination systems.

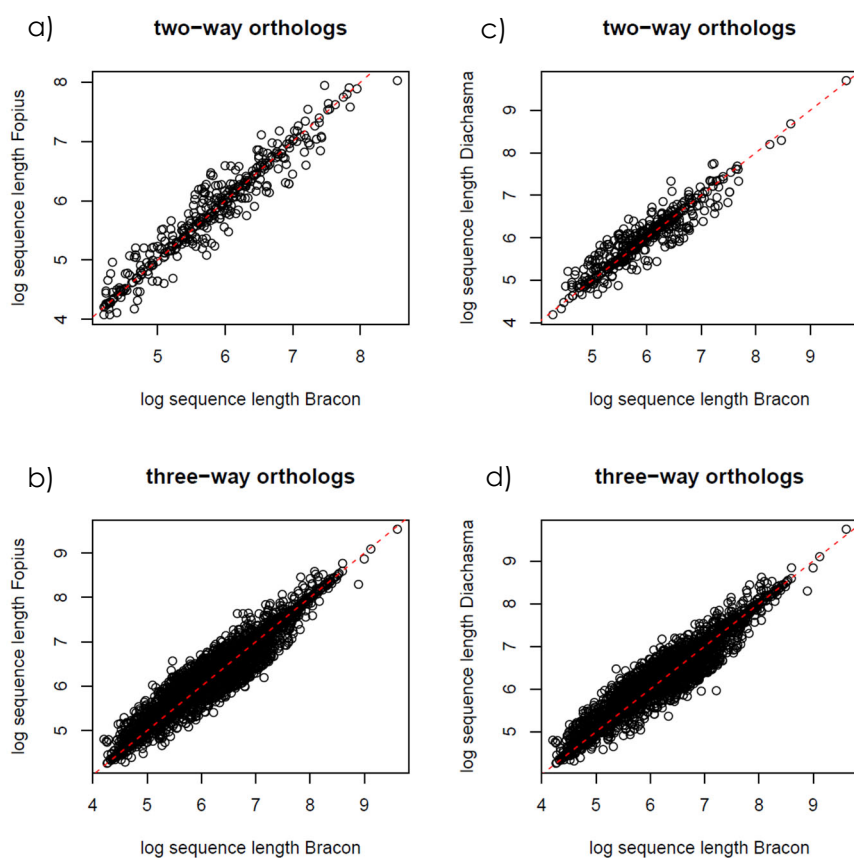
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401 Geuverink for discussions on *tra/fem* duplicates in Hymenoptera. This project was
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404 **SUPPLEMENTARY MATERIALS**

405 Additional supplementary material from this study (contaminated scaffolds,
406 pseudohap2 scaffolds) are available on the DANS EASY Repository,
407 <https://doi.org/10.17026/dans-xn6-pjm8>

408 **FIGURES**

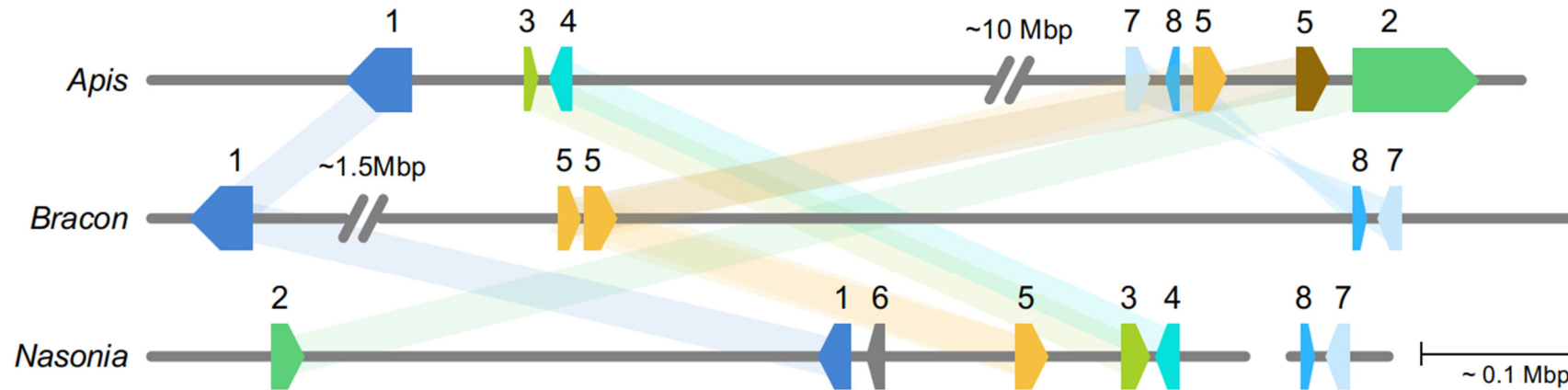


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410 Figure 1. Protein length comparison between *Bracon brevicornis* and *Fopius*
411 *arisanus*, a) two- and b) three-way orthologs, and *B. brevicornis* and *Diachasma*
412 *alloem*, c) two- and d) three-way orthologs. Sequence lengths have been log-
413 transformed; red dashed line indicates synteny.

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417 Figure 2. Microsynteny of genomic regions containing *tra/fem* paralogues. Shown is ~0.9 Mbp of genomic region of *Apis*
 418 *mellifera*, *Bracon brevicornis* and *Nasonia vitripennis*, containing the approximate coding region for 1. LOC100680007 (dark
 419 blue), 2. LOC408733 (green), 3. LOC100121225 (lime), 4. LOC100678616 (cyan), 5. *tra/fem/fem1* (yellow) and *csd* (brown), 6.
 420 LOC107980471 (gray), 7. LOC724886 (blue), 8. LOC551408 (light blue). Locus 2 is located on a different scaffold in *B.*
 421 *brevicornis*, locus 3 and 4 are not present in *B. brevicornis*. Locus 6 is unique to *N. vitripennis*, and locus 7 and 8 are located on
 422 a different scaffold in *N. vitripennis*, which is depicted on the right. Both 7 and 8 are in the same order and orientation as in *B.*
 423 *brevicornis*, but reversed in *A. mellifera*.

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428 Figure 3. *Bracon brevicornis* annotation of *Bbfem* and *Bbfem1* on the alignment of pseudohaplotype track 1 and 2 in
 429 Geneious Prime 2019.1.3 (<http://www.geneious.com>, (Kearse *et al.* 2012)). Within the assembled genome, this section
 430 corresponds to a region on scaffold 12. The *Bbfem1* annotation lacks the last coding segment with stop codon. The identity
 431 track shows the amount of sequence identity across an arbitrary window (depending on zoom setting) and can be used as a
 432 proxy for heterozygosity. Green is identical, yellow is mismatch, and red is no match due to introduced gaps during
 433 alignment. The coding regions of *Bbfem1* and *Bbfem* are in a high putatively heterozygous region.

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438 Figure S1. Protein alignment of *A. mellifera csd* (ABU68670) and *fem*
439 (NP_001128300), *N. vitripennis tra* (XP_001604794), *B. brevicornis fem* and *fem1*.
440 Purple shading indicates Hymenoptera domain (Verhulst *et al.* 2010), yellow
441 shading indicates CAM domain (Hediger *et al.* 2010), blue shading indicates
442 Proline (P)-rich region, red text colour indicates Arginine/Serine (RS)-rich regions,
443 and purple text colour indicates hypervariable region in *csd* (Beye *et al.* 2003).

LITERATURE CITED

- Acland, A., R. Agarwala, T. Barrett, J. Beck, D. A. Benson *et al.*, 2014 Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res.* 42: 8–13. <https://doi.org/10.1093/nar/gkt1146>.
- Antolin, M. F., P. J. Ode, G. E. Heimpel, R. B. O'Hara, and M. R. Strand, 2003 Population structure, mating system, and sex-determining allele diversity of the parasitoid wasp *Habrobracon hebetor*. *Heredity (Edinb.)*. 91: 373–381. <https://doi.org/10.1038/sj.hdy.6800337>.
- Beye, M., M. Hasselmann, M. K. Fondrk, R. E. Page, and S. W. Omholt, 2003 The gene *csd* is the primary signal for sexual development in the honeybee and encodes an SR-type protein. *Cell* 114: 419–429. [https://doi.org/10.1016/S0092-8674\(03\)00606-8](https://doi.org/10.1016/S0092-8674(03)00606-8).
- Camacho, C., G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos *et al.*, 2009 BLAST+: Architecture and applications. *BMC Bioinformatics* 10: 421. <https://doi.org/10.1186/1471-2105-10-421>.
- Chang, S., J. Puryear, and J. Cairney, 1993 A simple and efficient method for isolating RNA from pine trees. *Plant Mol. Biol. Report.* 11: 113–116. <https://doi.org/10.1007/BF02670468>.
- Chen, X., and C. van Achterberg, 2019 Systematics, Phylogeny, and Evolution of Braconid Wasps: 30 Years of Progress. *Annu. Rev. Entomol.* 64: 335–358. <https://doi.org/10.1146/annurev-ento-011118-111856>.
- Clark, A. M., H. A. Bertrand, and R. E. Smith, 1963 Life Span Differences between Haploid and Diploid Males of *Habrobracon serinopae* after Exposure as Adults to X Rays. *Am. Nat.* 97: 203–208. <https://doi.org/10.1086/282271>.
- Cook, J. M., and R. H. Crozier, 1995 Sex determination and population biology in the hymenoptera. *Trends Ecol. Evol.* 10: 281–286. [https://doi.org/10.1016/0169-5347\(95\)90011-X](https://doi.org/10.1016/0169-5347(95)90011-X).
- Dechaud, C., J. N. Volff, M. Scharf, and M. Naville, 2019 Sex and the TEs: Transposable elements in sexual development and function in animals. *Mob. DNA* 10: 42. <https://doi.org/10.1186/s13100-019-0185-0>.
- Geib, S. M., G. H. Liang, T. D. Murphy, and S. B. Sim, 2017 Whole Genome Sequencing of the Braconid Parasitoid Wasp *Fopius arisanus*, an Important Biocontrol Agent of Pest Tephritid Fruit Flies. *G3 Genes, Genomes, Genet.* 7: 2407–2411. <https://doi.org/10.1534/g3.117.040741>.
- Gempe, T., M. Hasselmann, M. Schiøtt, G. Hause, M. Otte *et al.*, 2009 Sex determination in honeybees: Two separate mechanisms induce and maintain the female pathway (B. S. Baker, Ed.). *PLoS Biol.* 7: e1000222.

<https://doi.org/10.1371/journal.pbio.1000222>.

- Geuverink, E., and L. W. Beukeboom, 2014 Phylogenetic Distribution and Evolutionary Dynamics of the Sex Determination Genes *doublesex* and *transformer* in Insects. *Sex. Dev.* 8: 38–49. <https://doi.org/10.1159/000357056>.
- Geuverink, E., K. Kraaijeveld, M. van Leussen, F. Chen, J. Pijpe *et al.*, 2018 Evidence for involvement of a transformer paralogue in sex determination of the wasp *Leptopilina clavipes*. *Insect Mol. Biol.* 27: 780–795. <https://doi.org/10.1111/imb.12522>.
- Gurevich, A., V. Saveliev, N. Vyahhi, and G. Tesler, 2013 QUAST: Quality assessment tool for genome assemblies. *Bioinformatics* 29: 1072–1075. <https://doi.org/10.1093/bioinformatics/btt086>.
- Hasselmann, M., T. Gempe, M. Schjøtt, C. G. Nunes-Silva, M. Otte *et al.*, 2008 Evidence for the evolutionary nascence of a novel sex determination pathway in honeybees. *Nature* 454: 519–522. <https://doi.org/10.1038/nature07052>.
- Hediger, M., C. Henggeler, N. Meier, R. Perez, G. Saccone *et al.*, 2010 Molecular Characterization of the Key Switch F Provides a Basis for Understanding the Rapid Divergence of the Sex-Determining Pathway in the Housefly. *Genetics* 184: 155–170. <https://doi.org/10.1534/genetics.109.109249>.
- Heimpel, G. E., and J. G. de Boer, 2008 Sex Determination in the Hymenoptera. *Annu. Rev. Entomol.* 53: 209–230. <https://doi.org/10.1146/annurev.ento.53.103106.093441>.
- Holloway, A. K., G. E. Heimpel, M. R. Strand, and M. F. Antolin, 1999 Survival of diploid males in *Bracon* sp. near *hebetor* (Hymenoptera: Braconidae). *Ann. Entomol. Soc. Am.* 92: 110–116. <https://doi.org/10.1093/aesa/92.1.110>.
- Hosmani, P. S., M. Flores-Gonzalez, H. van de Geest, F. Maumus, L. V Bakker *et al.*, 2019 An improved de novo assembly and annotation of the tomato reference genome using single-molecule sequencing, Hi-C proximity ligation and optical maps. *bioRxiv* 767764. <https://doi.org/https://doi.org/10.1101/767764>.
- Jia, L.-Y., J.-H. Xiao, T.-L. Xiong, L.-M. Niu, and D.-W. Huang, 2016 The transformer genes in the fig wasp *C. eratosolen solmsi* provide new evidence for duplications independent of complementary sex determination. *Insect Mol. Biol.* 25: 191–201. <https://doi.org/10.1111/imb.12210>.
- Kares, E. A., I. A. El-Sappagh, G. H. Ebaid, and I. M. Sabra, 2010 Efficacy of releasing *Bracon brevicornis* wesm. (Hymenoptera: Braconidae) for controlling hibernated *Ostrinia nubilalis* (Hübner) and *Sesamia cretica* led. larvae in stored corn stalks. *Egypt. J. Biol. Pest Control* 20: 155–159.

- Katoh, K., 2002 MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* 30: 3059–3066. <https://doi.org/10.1093/nar/gkf436>.
- Katoh, K., and D. M. Standley, 2013 MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol. Biol. Evol.* 30: 772–780. <https://doi.org/10.1093/molbev/mst010>.
- Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung *et al.*, 2012 Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28: 1647–1649. <https://doi.org/10.1093/bioinformatics/bts199>.
- Kittel, R. N., and K. Maeto, 2019 Revalidation of *Habrobracon brevicornis* stat. rest. (Hymenoptera: Braconidae) Based on the CO1, 16S, and 28S Gene Fragments. *J. Econ. Entomol.* 112: 906–911. <https://doi.org/10.1093/jee/toy368>.
- Koch, V., I. Nissen, B. D. Schmitt, and M. Beye, 2014 Independent evolutionary origin of femparalogous genes and complementary sex determination in hymenopteran insects (W. Hughes, Ed.). *PLoS One* 9: e91883. <https://doi.org/10.1371/journal.pone.0091883>.
- Laetsch, D. R., and M. L. Blaxter, 2017 BlobTools: Interrogation of genome assemblies [version 1; peer review: 2 approved with reservations]. *F1000Research* 6: 1287. <https://doi.org/10.12688/f1000research.12232.1>.
- Lechner, M., S. Findeiß, L. Steiner, M. Marz, P. F. Stadler *et al.*, 2011 Proteinortho: Detection of (Co-)orthologs in large-scale analysis. *BMC Bioinformatics* 12: 124. <https://doi.org/10.1186/1471-2105-12-124>.
- Li, H., 2013 Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv*.
- Li, H., 2018 Minimap2: Pairwise alignment for nucleotide sequences. *Bioinformatics* 34: 3094–3100. <https://doi.org/10.1093/bioinformatics/bty191>.
- Li, X. Y., C. van Achterberg, and J. C. Tan, 2013 Revision of the subfamily Opiinae (Hymenoptera, Braconidae) from Hunan (China), including thirty-six new species and two new genera. *Zookeys* 268: 1–186. <https://doi.org/10.3897/zookeys.268.4071>.
- Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan *et al.*, 2009 The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25: 2078–2079. <https://doi.org/10.1093/bioinformatics/btp352>.
- Marçais, G., and C. Kingsford, 2011 A fast, lock-free approach for efficient parallel counting of occurrences of k-mers. *Bioinformatics* 27: 764–770.

<https://doi.org/10.1093/bioinformatics/btr011>.

Narayanan, E. S., G. W. Angalet, B. R. Subba Rao, and G. I. D'Souza, 1954 Effect of refrigeration of the pupæ of *Microbracon brevicornis* Wesm. on the pigmentation of the adult. *Nature* 173: 503–504.
<https://doi.org/10.1038/173503b0>.

Puttarudriah, M., and G. P. C. Basavanna, 1956 A study on the identity of *Bracon hebetor* Say and *Bracon brevicornis* Wesm. (Hymenoptera: Braconidae). *Bull. Entomol. Res.* 47: 183–191. <https://doi.org/10.1017/S0007485300046617>.

Schmieder, S., D. Colinet, and M. Poirié, 2012 Tracing back the nascence of a new sex-determination pathway to the ancestor of bees and ants. *Nat. Commun.* 3: 895. <https://doi.org/10.1038/ncomms1898>.

Simão, F. A., R. M. Waterhouse, P. Ioannidis, E. V. Kriventseva, and E. M. Zdobnov, 2015 BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* 31: 3210–3212.
<https://doi.org/10.1093/bioinformatics/btv351>.

Solovyev, V., 2007 Statistical Approaches in Eukaryotic Gene Prediction, pp. 97–159 in *Handbook of Statistical Genetics*, edited by D. Balding, C. Cannings, and M. Bishop. Wiley-Interscience,.

Speicher, B. R., and K. G. Speicher, 1940 The Occurrence of Diploid Males in *Habrobracon brevicornis*. *Am. Nat.* 74: 379–382.
<https://doi.org/10.1086/280904>.

Srinivasan, T., and Chandrikamohan, 2017 Population growth potential of *Bracon brevicornis* Wesm. (Braconidae: Hymenoptera): A life table analysis. *Acta Phytopathol. Entomol. Hungarica* 52: 123–130.
<https://doi.org/10.1556/038.52.2017.010>.

Stanke, M., and B. Morgenstern, 2005 AUGUSTUS: a web server for gene prediction in eukaryotes that allows user-defined constraints. *Nucleic Acids Res.* 33: W465–W467. <https://doi.org/10.1093/nar/gki458>.

Temerak, S. A., 1983a Host preferences of the parasitoid *Bracon brevicornis* Wesm. (Hym., Braconidae) and host sensitivity to its venom. *Zeitschrift für Angew. Entomol.* 96: 37–41. <https://doi.org/10.1111/j.1439-0418.1983.tb03638.x>.

Temerak, S. A., 1983b Longevity of *Bracon brevicornis* [Hym: Braconidae] adults as influenced by nourishment on artificial and natural foods. *Entomophaga* 28: 145–150. <https://doi.org/10.1007/BF02372138>.

Thiel, A., and A. C. Weeda, 2014 Haploid, diploid, and triploid - Discrimination ability against polyploid mating partner in the Parasitic Wasp, *Bracon*

brevicornis (Hymenoptera: Braconidae). *J. Insect Sci.* 14: 1–7.
<https://doi.org/10.1093/jisesa/ieu153>.

Thiel, A., A. C. Weeda, J. G. de Boer, and T. S. Hoffmeister, 2013 Genetic incompatibility drives mate choice in a parasitic wasp. *Front. Zool.* 10: 43.
<https://doi.org/10.1186/1742-9994-10-43>.

Tvedte, E. S., K. K. O. Walden, K. E. McElroy, J. H. Werren, A. A. Forbes *et al.*, 2019 Genome of the parasitoid wasp *diachasma alloeum*, an emerging model for ecological speciation and transitions to asexual reproduction (J. Gonzalez, Ed.). *Genome Biol. Evol.* 11: 2767–2773. <https://doi.org/10.1093/gbe/evz205>.

Veltri, D., M. M. Wight, and J. A. Crouch, 2016 SimpleSynteny: a web-based tool for visualization of microsynteny across multiple species. *Nucleic Acids Res.* 44: W41–W45. <https://doi.org/10.1093/nar/gkw330>.

Venkatesan, T., S. K. Jalali, and K. Srinivasamurthy, 2009 Competitive interactions between *Goniozus nephantidis* and *Bracon brevicornis*, parasitoids of the coconut pest *Opisina arenosella*. *Int. J. Pest Manag.* 55: 257–263.
<https://doi.org/10.1080/09670870902914155>.

Verhulst, E. C., L. van de Zande, and L. W. Beukeboom, 2010 Insect sex determination: it all evolves around transformer. *Curr. Opin. Genet. Dev.* 20: 376–383. <https://doi.org/10.1016/j.gde.2010.05.001>.

Villacañas de Castro, C., and A. Thiel, 2017 Resource-Dependent Clutch Size Decisions and Size-Fitness Relationships in a Gregarious Ectoparasitoid Wasp, *Bracon brevicornis*. *J. Insect Behav.* 30: 454–469.
<https://doi.org/10.1007/s10905-017-9632-2>.

Vurture, G. W., F. J. Sedlazeck, M. Nattestad, M. C. Schatz, J. Gurtowski *et al.*, 2017 GenomeScope: fast reference-free genome profiling from short reads. *Bioinformatics* 33: 2202–2204. <https://doi.org/10.1093/bioinformatics/btx153>.

Wang, Z., Z. Liu, X. Wu, W. Yan, and Z. Zeng, 2012 Polymorphism analysis of *csd* gene in six *Apis mellifera* subspecies. *Mol. Biol. Rep.* 39: 3067–3071.
<https://doi.org/10.1007/s11033-011-1069-7>.

Weisenfeld, N. I., V. Kumar, P. Shah, D. M. Church, and D. B. Jaffe, 2017 Direct determination of diploid genome sequences. *Genome Res.* 27: 757–767.
<https://doi.org/10.1101/gr.214874.116>.Freely.

Whiting, P. W., 1940 Multiple alleles in sex determination of *Habrobracon*. *J. Morphol.* 66: 323–355. <https://doi.org/10.1002/jmor.1050660208>.

Whiting, P. W., and A. E. Whiting, 1925 Diploid males from fertilized eggs in hymenoptera. *Science* (80-.). 62: 437.

<https://doi.org/10.1126/science.62.1611.437>.

van Wilgenburg, E., G. Driessen, and L. W. Beukeboom, 2006 Single locus complementary sex determination in Hymenoptera: an “unintelligent” design? *Front. Zool.* 3: 1. <https://doi.org/https://doi.org/10.1186/1742-9994-3-1>.