Evolutionary transition to XY sex chromosomes associated with Y-linked duplication of a male hormone gene in a terrestrial isopod

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Abstract

Sex chromosomes are highly variable in some taxonomic groups, but the evolutionary mechanisms underlying this diversity are not well understood. In terrestrial isopod crustaceans, interactions with *Wolbachia*, a vertically transmitted endosymbiont causing male-to-female sex reversal, are thought to drive rapid evolutionary turnovers in sex chromosomes. Here, we use surgical manipulations and genetic crosses, plus genome sequencing, to examine sex chromosomes in the terrestrial isopod *Trachelipus rathkei*. Although an earlier cytogenetics study suggested a ZZ/ZW sex chromosome system in this species, we surprisingly find that in our study population, sex is determined by an XX/XY system. Consistent with a recent evolutionary origin for this XX/XY system, the putative male-specific region of the genome is small. The genome shows evidence of sequences horizontally acquired from past *Wolbachia* infections, as well as evidence of Y-linked duplications of the androgenic gland hormone gene, thought to be a possible target for sex reversal by *Wolbachia*. Overall, these results are consistent with the hypothesis that reproductive endosymbionts such as *Wolbachia* can promote quick turnover of sex determination mechanisms in their hosts.

Introduction

Although sexual reproduction is shared by most eukaryotes, a variety of different cues can trigger individuals to follow a male, female, or hermaphroditic developmental plan (Conover & Kynard 1981; Janzen & Phillips 2006; Ospina-Álvarez & Piferrer 2008; Tingley & Anderson 1986; Verhulst et al. 2010). In many eukaryotes, sex is primarily determined genotypically, and in most of those cases, sex chromosomes are the primary sex-determining factors, although some exceptions, including polygenic systems and haplodiploidy, are also known (Vandeputte et al. 2007; Heimpel & de Boer 2008). Sex chromosomes in animals are usually grouped into two main classes: XY systems, in which males are heterogametic (XY) and females are homogametic (XX); and ZW systems, in which females are heterogametic (ZW) and males are homogametic (ZZ). However, non-genetic cues can also play an important role in some species. For instance, environmental factors, such as temperature or population density, influence or determine phenotypic sex in reptiles, fishes, and invertebrates (Conover & Kynard 1981; Janzen & Phillips 2006; Tingley & Anderson 1986). In some cases, cytoplasmic factors, including sex-reversing endosymbionts, such as *Wolbachia*, microsporidia, and paramyxids can serve as a sex-determining signal (Bouchon et al. 1998; Pickup & Ironside 2018; Terry et al. 1998; Negri I et al. 2006; Kageyama et al. 2002).

Evolutionary theory holds that the formation of sex chromosomes begins when an autosome acquires a sex-determining locus (Rice 1996). Subsequently, recombination around the sex-determining locus is selected against because of sexually antagonistic selection (Bergero & Charlesworth 2009). For instance, selection should favor mutations that are beneficial in males but deleterious in females when those alleles are linked to a dominant male-determining allele; recombination, on the other hand, would break up this linkage and result in females that carry these male-beneficial alleles. The non-recombining region is then expected to spread in the

presence of continued sexually antagonistic selection, and may eventually span the whole sex chromosome, except for the usual presence of a small recombining pseudo-autosomal region (Charlesworth et al. 2005). Once recombination has ceased, the non-recombining sex chromosome, such as the Y chromosome in mammals or the W chromosome in birds, is expected to degenerate. Non-recombining genes frequently undergo pseudogenization, acquiring nonsense mutations or transposable element insertions (Charlesworth & Charlesworth 2000). At the same time, gene trafficking can occur when selection promotes the translocation of formerly autosomal genes to the sex chromosomes (Emerson et al. 2004).

Different species appear to be at different stages of sex chromosome evolution. For instance, the sex chromosomes of therian mammals are highly conserved, having originated ~160 million years ago (Potrzebowski et al. 2008; Veyrunes et al. 2008). The highly degenerated, heteromorphic Y chromosome represents an advanced stage of sex chromosome evolution. In other taxonomic groups, on the other hand, sex chromosomes appear to undergo more frequent evolutionary turnovers (Pennell et al. 2018; Cioffi et al. 2013; Myosho et al. 2015; Jeffries et al. 2018; Ross et al. 2009; Vicoso & Bachtrog 2015). Such young sex chromosomes may have little or no recombination suppression, differentiation in gene content, or sex chromosome dosage compensation, and may not be detectable by traditional cytogenetic methods because they are visually indistinguishable (homomorphic) (Vicoso & Bachtrog 2015; Gamble et al. 2014). Sex chromosomes may even be polymorphic within a species, with different sex-determining loci segregating within or among populations (Traut 1994; Meisel et al. 2016; Ogata et al. 2008; Orzack et al. 1980).

Unfortunately, we still have a limited understanding of why evolutionary turnovers of sex chromosomes are rare in some groups but frequent in others. A variety of models have been proposed to explain why these turnovers occur, including sexual antagonism, deleterious

92 mutations, and the 'hot potato' model (van Doorn & Kirkpatrick 2007; Blaser et al. 2013, 2014).
93 In some organisms, interactions with vertically transmitted reproductive endosymbionts are also
94 thought to influence the evolution of their hosts' sex determination mechanisms (Rigaud et al.
95 1997; Cordaux et al. 2011). However, many of these models have been difficult to test in nature.
96 This problem is exacerbated by the fact that, while sex chromosomes have been extensively
97 studied in model organisms like *Drosophila*, studies are more sparse in non-model organisms.
98
99 One group that has received relatively little attention is crustaceans. Different crustacean
100 species show a variety of distinct sex determining mechanisms, yet there are very few
101 crustacean species in which candidate master sex-determining genes have been identified
102 (Chandler et al. 2018, 2017). Within crustaceans, perhaps one of the best-studied groups in
103 terms of sex determination is the terrestrial isopods (Oniscidea). Terrestrial isopod species have
104 a mix of XY and ZW systems, along with reports of a few parthenogenic species and
105 populations (Rigaud et al. 1997; Johnson 1986; Fussey 1984). The bacterial endosymbiont
106 *Wolbachia* also influences sex determination by causing male-to-female sex reversal in some
107 isopod hosts (Bouchon et al. 1998; Cordaux et al. 2004). In fact, interactions with *Wolbachia* are
108 thought to drive rapid evolutionary turnover of the sex chromosomes in terrestrial isopods. This
109 hypothesis is supported by multiple lines of evidence. For instance, in the common pillbug
110 *Armadillidium vulgare*, a copy of the *Wolbachia* genome horizontally integrated into the host
111 genome (formerly known as the *f* element) led to the origin of a new W chromosome (Leclercq
112 et al. 2016). A recent phylogenetic analysis also identified, at minimum, several transitions in
113 heterogametic systems along the isopod phylogeny, including closely related species pairs with
114 different sex chromosome systems (Becking et al. 2017). Moreover, only a few species of
115 terrestrial isopods are known to have heteromorphic sex chromosomes, in which the X and Y, or
116 Z and W, chromosomes are visually distinguishable in cytogenetics experiments (Rigaud et al.
117 1997), and WW or YY individuals are often viable and fertile (Juchault & Rigaud 1995; Becking

et al. 2019), suggesting that the W and Y chromosomes have not lost any essential genes in these species.

In this study, we examined sex determination in the widespread species *Trachelipus rathkei.* This species was previously established by cytogenetic methods to have heteromorphic, albeit slightly, Z and W sex chromosomes (Mittal & Pahwa 1980). We sought to confirm female heterogamety by crossing females to sex-reversed neo-males (which have female genotypes but male phenotypes), and assessing the sex ratio of the resultant progenies. Surprisingly, we found that, at least in our focal population, sex is determined by an XX/XY system, suggesting a recent sex chromosome turnover. To test this hypothesis, we performed whole-genome sequencing. Consistent with a recent origin of an XX/XY sex determination system, we find evidence that the putative male-specific region of the genome is small, and we identified a male-specific partial duplication of the androgenic gland hormone (AGH) gene, a rare example of a candidate sex-determining gene in a crustacean. In addition, although our study population does not appear to harbor current *Wolbachia* infections, we find genomic evidence of past infections. Overall, our results are consistent with the hypothesis that sex-reversing endosymbionts like *Wolbachia* can drive rapid evolutionary turnover of sex chromosomes in their hosts.

Methods

Animal collection and husbandry

We sampled wild isopods from Rice Creek Field Station (RCFS) at SUNY Oswego in Oswego, NY. We captured animals using a combination of methods. First, we haphazardly searched through leaf litter, logs, and rocks. We also used “potato traps”, made by carving out a 1-2 cm

144 diameter core from a potato and placing it in the litter for 1-2 weeks. Finally, we constructed
145 pitfall traps from plastic cups buried in the ground with the rim of the cup flush with the ground.
146 The primary species captured were *Oniscus asellus* and *Trachelipus rathkei*, but we also
147 captured *Philoscia muscorum*, *Hyloniscus riparius*, *Trichoniscus pusillus*, and occasionally
148 *Cylisticus convexus*. Species identification was performed in the field and confirmed in the lab,
149 where we also determined the phenotypic sex of specimens.
150
151 Isopods were housed in plastic food storage containers with holes in the lids for air exchange,
152 on a substrate of moistened soil. Containers were checked twice weekly. Animals were fed
153 carrots and dried leaves *ad libitum*. The photoperiod was kept on a schedule of 18:6 light
154 hours:dark hours in the summer and and 14:10 in the winter. We isolated ovigerous females in
155 individual containers, and separated offspring from their mothers upon emergence from the
156 marsupium. We initially sexed offspring at six to eight weeks old, and separated males from
157 females to prevent sibling mating. We then double-checked offspring sex at roughly two week
158 intervals thereafter until four months of age to watch for individuals that might have shown late
159 signs of sexual differentiation. Terrestrial isopods are known to store sperm from a single mating
160 to fertilize future broods. Therefore, for experimental crosses we only used *T. rathkei* females
161 that were born in the lab, separated from brothers as soon as they could be sexed, and which
162 had not produced any offspring by 12 months of age.
163
164 *Wolbachia* testing
165
166 We used PCR assays to detect *Wolbachia* in *T. rathkei* individuals. DNA was extracted from
167 one or two legs, depending on the size of the animal. We ruptured the leg tissue in 400 µL
168 deionized water along with a few 0.5 mm zirconia/silica beads (enough to cover the bottom of
169 the tube) using a bead beater machine. Samples were lysed following a protocol of 2500 RPM

170 for 10 seconds, followed by 4200 RPM for 10 seconds, and finally 4800 RPM for 10 seconds.
171 The tube was then visually inspected to confirm the leg was sufficiently pulverized. We then
172 transferred the lysate to a new tube, added 60 µL of a 5% Chelex® 100 molecular biology grade
173 resin suspension, and incubated for 15 minutes at 100° C. After incubation, we centrifuged the
174 extract at 16,000g for 3 minutes, and reserved 80 µL of supernatant for PCR testing. We
175 confirmed successful DNA extraction using the mitochondrial primers HCO2198/LCO1490
176 (Folmer et al. 1994). We performed PCRs in 10 µL reactions, using 4.95 µL of molecular biology
177 grade water, 2 µL NEB OneTaq Buffer, 1 µL of mixed dNTPS at a final concentration of 2mM for
178 each dNTP, 1 µL of a 5 µM solution of each primer, and 0.05 µL of NEB OneTaq. For the
179 mitochondrial primer set, PCR conditions included an initial denaturation of 94° C for 1 minute; 5
180 cycles of 94° C denaturation for 30s, 45° C annealing for 90s, and 68° C extension for 60s. The
181 samples then underwent 35 cycles of 94° C for 30s, 51° C for 90s, and 68° C for 60s. This was
182 followed by a final extension step of 68° C for 5 minutes. To test for *Wolbachia*, we performed
183 PCR using *Wolbachia*-specific primers targeting the *wsp* (81f/691r) and *ftsZ* (ftsZf1/ftsZr1)
184 genes (Braig et al. 1998; Werren John H. et al. 1995). We performed PCRs in 10 µL reactions,
185 using 4.95 µL of molecular biology grade water, 2 µL NEB OneTaq Buffer, 1 µL of mixed dNTPs
186 at a final concentration of 2mM for each dNTP, 1 µL of either *wsp* or *ftsZ* primers, and 0.05 µL
187 of NEB OneTaq. PCR conditions contained an initial denaturation of 95°C for 5 minutes,
188 followed by 36 cycles of 95° C for 60s, 54° C for 60s, and 68°C for 3 minutes. This was followed
189 by a final extension step at 68° C for 10 minutes.
190
191 <u>Androgenic gland implantation and crosses</u>
192
193 To test whether sex is determined by a ZZ/ZW or XX/XY system of sex determination in our
194 population of *T. rathkei*, we performed crosses between females and experimentally sex-
195 reversed neo-males. Juvenile female *T. rathkei* were implanted with live androgenic glands,

196 according to (Becking et al. 2017). Male donors and female recipients were selected from large
197 lab-reared broods with even (~1:1) sex ratios. An adult male was sacrificed by decapitation, and
198 live androgenic glands were dissected into Ringer solution (393 mM NaCl, 2 mM KCl, 2 mM
199 $CaCl_2 \cdot 2H_2O$, 2 mM $NaHCO_3$). Female recipients were between 5 and 8 weeks old, an age at
200 which males and females begin to become distinguishable, but at which sexual development is
201 not complete. The gland was injected using a pulled glass pipette into a hole pierced with a
202 dissecting needle in the 6th or 7th segment of the juvenile female's pereon. Recipients were
203 isolated in a small plastic container with a moist paper towel for recovery and observation.
204 Experimental animals were monitored for signs of male development. Any animal that failed to
205 develop male genitalia by 4 months post-implantation was considered to be a failed injection.
206 After maturation, adult neo-males were placed in individual containers with 1-3 previously
207 unmated females. Crosses were monitored twice weekly to check for signs of reproduction in
208 females. Gravid females were then isolated into their own containers until parturition.
209
210 Genome sequencing
211
212 All raw sequencing data and the draft genome assembly are available under NCBI BioProject
213 PRJNA633105. Data analysis scripts are available at
214 https://github.com/chandlerlab/trachelipus_genome.
215
216 We performed whole-genome sequencing using a combination of Illumina, PacBio, and Oxford
217 Nanopore sequencing, with multiple sequencing samples of each sex (Table 1). Because we
218 expected the *T. rathkei* genome to be large, repetitive, and highly polymorphic, and because we
219 expected to need to isolate DNA from multiple individuals, we established a partially inbred
220 laboratory line using offspring from a single female collected from RCFS. We mated brothers
221 and sisters from this female for two generations in the lab prior to collecting genetic samples

222 from the third generation for sequencing. DNA was collected for sequencing using the Qiagen
223 DNEasy Blood and Tissue Kit following the manufacturer's instructions. DNA was quantified
224 using the Qubit DNA Broad Range assay kit, and the A260/280 value was checked with a
225 Nanodrop spectrophotometer. Samples were stored at -80°C prior to being shipped to the
226 sequencing center. Illumina sequencing was performed at the State University of New York at
227 Buffalo Genomics and Bioinformatics Core Facility.
228
229 For PacBio sequencing, we had to pool DNA from multiple individuals to obtain sufficient
230 quantities of DNA for library preparation. We performed separate DNA extractions from three
231 individuals of each sex as above. Then, we pooled the DNA from the three individuals of each
232 sex and concentrated it using Ampure XP beads (Beckman-Coulter). Briefly, we washed the
233 beads three times in molecular biology grade water, once in Qiagen buffer EB, and finally re-
234 suspended the beads in their original buffer. We then added equal volumes of Ampure XP
235 suspension to the DNA samples, mixed them on a shaker for 15 minutes, placed the tubes on a
236 magnetic bead separator, and removed the supernatant. We washed the beads twice with 1.5
237 mL of 70% ethanol, and finally eluted the DNA samples in 30 µL of Qiagen buffer EB.
238 Sequencing libraries were prepared and sequenced at the University of Delaware Sequencing &
239 Genotyping Center on a PacBio RSII.
240
241 We also supplemented our PacBio dataset with Oxford Nanopore sequencing data. We isolated
242 DNA from a single *T. rathkei* female and two separate males using a Qiagen DNEasy Kit as
243 described above. We then performed sequencing on Oxford Nanopore Minion flowcells (R9.4)
244 with the Rapid Sequencing Kit (SQK-RAD004) following the manufacturer's instructions.
245
246 <u>Genome assembly</u>
247

248 We performed a hybrid assembly combining the short- and long-read sequence data. We first
249 removed adapters and trimmed the Illumina sequencing data using Trimmomatic v. 0.36 (Bolger
250 et al. 2014); we removed leading and trailing bases, as well as internal windows of at least 4bp,
251 with a quality score of 5 or lower, and discarded any reads shorter than 36bp after trimming. We
252 then used SparseAssembler (Ye et al. 2012) to assemble the cleaned Illumina data from sample
253 Mpool (to minimize the number of sequence polymorphisms that would be present in the data
254 with additional samples), using two different kmer sizes (k=51 and k=61). After performing
255 preliminary quality checks using Quast (Gurevich et al. 2013), we decided to proceed with the
256 k=61 assembly, which had the longer total length and N50 (Supplementary Table 1). However,
257 because we suspected the genome might still contain high levels of heterozygosity despite two
258 generations of inbreeding, we used Redudans (Pryszcz & Gabaldón 2016) to remove putative
259 allelic contigs from the Illumina-only assembly; we set identity and overlap thresholds of 95%.
260
261 Prior to performing hybrid assembly, we used the short reads to correct sequencing errors in the
262 long reads using FMLRC (Wang et al. 2018) with the default settings, except requiring a
263 minimum count of 3 to consider a path (-m 3). PacBio and Oxford Nanopore reads derived from
264 female isopods were corrected using Illumina sample Fpool, while long reads from male
265 samples were corrected using sample Mpool.
266
267 We next performed hybrid assembly using DBG2OLC (Ye et al. 2016), which accepts a short-
268 read assembly (rather than raw short-read sequence data) and long-read sequence data (in this
269 case, our PacBio and Oxford Nanopore reads) as input. We tested out a range of different
270 parameter values: from the Redundans-filtered assembly, we first removed contigs less than
271 100bp or 200bp; we tested kmer sizes of 17 and 19; for the kmer coverage threshold, we tried
272 values of 2 and 5; and for the minimum overlap, we tried values of 10 and 30. We used an

273 adaptive threshold of 0.01. These assemblies ranged in size from ~5.2 Gb to 8.5 Gb; we
274 selected three assemblies across the range of total sizes for further processing.
275
276 We next corrected errors in these assemblies, caused by the relatively high error rates in long-
277 read sequence data. In the standard DBG2OLC pipeline, the resulting contigs are corrected
278 using the contigs from the short-read assembly and from the long reads using Sparc (Ye & Ma
279 2016); however, in our initial attempts, large portions of the assemblies went uncorrected,
280 perhaps because we had relatively low-coverage long-read data. Therefore, instead we
281 performed three rounds of error correction using Pilon (Walker et al. 2014), by mapping the raw
282 Illumina sequence reads to each assembly using bbmap (first two rounds; (Bushnell et al.
283 2017)) and bwa mem (third round; with the parameters -A 1 -B 1 -O 1 -E 1 -k 11 -W 20 -d 0.5 -L
284 6 for mapping to an error-prone assembly; (Li 2013)).
285
286 Finally, we assessed the quality of each assembly using BUSCO v.3.0.2 (Simão et al. 2015),
287 with the arthropod reference gene set, and selected the assembly with the greatest number of
288 BUSCO reference genes present for further analysis.
289
290 To remove contaminants from the final assembly, we generated blob plots using Blobtools v.1.0
291 (Laetsch & Blaxter 2017). To accomplish this, we BLASTed all contigs against the NCBI
292 nucleotide (nt) database using megablast (Morgulis et al. 2008) and against Uniprot reference
293 proteomes using diamond blastx (Buchfink et al. 2015). We then removed any contigs that were
294 identified as coming from plants, fungi, viruses, or bacteria, except for those matching
295 *Wolbachia*.
296
297 <u>Genome annotation</u>
298

299 We used RepeatModeler v.1.0.10, which uses RECON (Bao & Eddy 2002), RepeatScout (Price
300 et al. 2005), and Tandem Repeat Finder (Benson 1999), to construct a custom repeat library for
301 *T. rathkei*. Because we were unable to run RepeatModeler successfully using the full assembly,
302 we ran RepeatModeler on a random subset 40% of the contigs; this should still successfully
303 identify most repetitive elements in the genome as long as all repeat families are still well
304 represented in the subset. We then masked the assembly using RepeatMasker 4.0.7 (Tarailo-
305 Graovac & Chen 2009).
306
307 We annotated coding sequences using the MAKER pipeline (Cantarel et al. 2008). We initially
308 ran MAKER using assembled transcript sequences (est2genome=1) from previously available
309 data from one wild-caught male and one wild-caught female *T. rathkei* from the same population
310 (Becking et al. 2017) (SRR5198727, SRR5198726), along with protein alignments against
311 Uniprot-Swissprot (version March 2020) and used the resulting output to train SNAP (Korf
312 2004). To train AUGUSTUS (Stanke et al. 2006) we used the output from the BUSCO quality
313 assessment described earlier. We then completed a final round of MAKER using the trained
314 gene models.
315
316 <u>Development of sex-linked PCR markers</u>
317
318 We used multiple approaches to develop male-specific, putatively Y-linked PCR markers. Initial
319 attempts to perform a genomic assembly with male samples and then identify contigs with zero
320 coverage in females were unsuccessful. We therefore developed a complementary approach by
321 looking for male-specific k-mers in the raw sequencing reads. We used kmc v.3.1.0 (Kokot et al.
322 2017) to count all the canonical 21-mers in each of the Illumina sequencing datasets (in other
323 words, each 21-mer and its reverse complement were considered to be the same k-mer during
324 counting). We then searched for k-mers that occurred at least 8 times in the Mpool Illumina

325 sequencing dataset and a total of at least 3 times combined across the lower coverage M2, M5,
326 M6, and wildM samples, but which were completely absent from all female samples. We then
327 extracted all sequence reads containing these candidate male-specific k-mers using mirabait
328 v.4.0.2 (Chevreux et al. 1999), and assembled them using Spades v.3.11.1 (Bankevich et al.
329 2012).
330
331 To test male-specificity of these contigs, we used polymerase chain reaction (PCR). We
332 developed PCR primers for a subset of candidate male-specific contigs. To identify the best
333 candidates, we first mapped raw sequencing reads from all male and female samples to the full
334 genome sequence plus the candidate male-specific contigs, and identified contigs that had
335 coverage in male samples but not female samples; we also avoided contigs that showed
336 evidence of containing repeat elements, after BLAST searches against the whole genome
337 assembly. We designed primers using PRIMER3 (Untergasser et al. 2012, 3). In these PCRs,
338 primers were initially screened using template DNA from two male samples and two female
339 samples; primers that showed evidence of sex specificity after this first PCR were re-tested
340 using a larger number of samples. PCR primers were initially tested using a cycle of 98°C for 3
341 minutes, followed by 40 cycles of 98°C for 15s, 50°C for 35s, and 68°C for 60s; this was
342 followed by a final extension step of 68°C for 10 minutes. For samples that did not amplify under
343 this program, a gradient PCR was run to determine optimal annealing temperature. All PCRs
344 were performed using the same recipe and reaction conditions as the *Wolbachia* PCRs
345 described above.
346
347 We also identified open reading frames (ORFs) in these candidate male-specific contigs using
348 Transdecoder v.4.0.0 (Haas & Papanicolaou 2016), and annotated the ORFs using Trinotate
349 v.3.1.1 (Bryant et al. 2017). Subsequently, we designed additional primers targeting these
350 ORFs.

351

352 **Results**

353

354 No *Wolbachia* and balanced sex ratios in *T. rathkei*

355

356 Among the 100+ individuals captured and tested between 2015 and 2017, no *T. rathkei* from
357 RCFS conclusively tested positive for *Wolbachia*. This was not due to inadequate testing
358 protocols; for instance, a captive population of *Porcellio laevis* housed in our lab shows nearly a
359 100% infection rate using the same methods (not shown). Approximately 150 *T. rathkei* broods
360 were raised in the lab from either mated, wild-caught females or first-generation crosses. The
361 mean and median brood sizes of this species in our lab were 27.1 and 22.5 offspring,
362 respectively, and the vast majority of these broods had a balanced sex ratio (Supplementary
363 Table 2). Thus, the prevalence of *Wolbachia* and other sex ratio distorters is at most very low in
364 this population of *T. rathkei*. In addition, some wild-caught females produced broods even after
365 several months to a year in isolation in the lab (Supplementary Table 2), confirming that this
366 species is capable of long-term sperm storage.

367

368 Crossing sex-reversed individuals indicates an XY sex determination system

369

370 Five juveniles implanted with androgenic glands survived to mature into males; they were
371 crossed with virgin females from families with normal sex ratios. Each putative neo-male was
372 paired with 2 to 3 females, and each female produced 1-3 broods of offspring. Two of these
373 males sired broods with balanced sex ratios (not significantly different from a 1:1 ratio of males
374 to females; Table 2); these were likely individuals that would have developed into males even
375 without the AG implantation, and thus are uninformative with respect to the sex determination
376 system (Becking et al. 2017). However, three other males produced only female offspring,

377 consistent with an XX/XY system (XX neo-male × XX female yields all XX and therefore 100%
378 female offspring) but not a ZZ/ZW system (ZW neo-male × ZW female expected to produce 1/4
379 ZZ, 1/2 ZW, and 1/4 WW offspring, thus 75% female or 66.7% female depending on whether
380 WW genotypes are viable).
381
382 Genome assembly
383
384 The draft genome assembly of *T. rathkei* is approximately 5.2 Gb in total length. The genome is
385 highly repetitive, consisting of approximately 70% repetitive elements. Transposable elements
386 constitute the largest repeat category, with LINEs, followed by DNA elements and LTRs, being
387 the most represented (Figure 1). All repeat families seem to have a single divergence peak of
388 around 7-10% (Figure 1).
389
390 Despite its large size, the draft assembly is likely only partially complete, with ~25% of arthropod
391 BUSCO genes missing (Table 3). There were 15,805 transcripts assembled from the previously
392 available transcriptome dataset whose best hits in blastn searches against the NCBI nt
393 database and diamond blastx searches against Uniprot-Swissprot were from other arthropods;
394 of those, only 53% had nearly full-length matches in the genome (≥ 90% of the transcript length
395 at ≥ 90% sequence identity), suggesting some missing data and/or remaining uncorrected
396 sequencing errors in the draft assembly as well.
397
398 We screened the *T. rathkei* genome for *Wolbachia* nuclear insertions by BLASTing the
399 assembled contigs against a collection of *Wolbachia* genome sequences, and then BLASTing
400 the matching regions against all representative bacterial genomes from RefSeq to rule out false
401 positives. After this filtering step, we were left with 1010 high confidence matches (best BLAST
402 hit in a *Wolbachia* genome, e-value < 1×10^{-6}) spread across 719 contigs, with a total length of

403 ~350 kb for the matching sequences (Supplementary Table 3), much smaller than a typical full
404 *Wolbachia* genome of about 1 - 1.6 Mb on average (Sun et al. 2001). These likely horizontally
405 acquired sequences clustered closely with *Wolbachia* strain *wCon* from the isopod *Cylisticus*
406 *convexus* (Badawi et al. 2018) with 100% bootstrap support, in a group sister to *wVulC* and the *f*
407 element of *Armadillidium vulgare* (Leclercq et al. 2016) (Figure 2). Moreover, there were
408 multiple insertions carrying sequences similar to the cytoplasmic incompatibility genes *cifA* and
409 *cifB* genes (LePage et al. 2017; Beckmann et al. 2017; Lindsey et al. 2018). These *cifA*- and
410 *cifB*-like sequences probably represent nonfunctional pseudogenes, however, as they show
411 evidence of being broken up by insertions (Supplementary Figure 1).
412
413 Searching for candidate sex-determining genes
414
415 We identified ~6.04 x 10^6 21-mers as potentially male-specific, suggesting there is
416 approximately 6 Mb of male-specific sequence content in the genome. However, when we
417 isolated the raw sequencing reads containing those 21-mers and assembled them, we obtained
418 89.4 Mb of assembled sequences, suggesting the male-specific region may be as large as ~90
419 Mb, but still shares significant similarity with the X chromosome. Even if up to 90 Mb of
420 sequence is partially sex-linked, this represents just 1.7% of the genome, consistent with an
421 evolutionarily young Y chromosome in this species.
422
423 Of the initial 16 candidate Y-linked PCR markers designed from anonymous sequences, none
424 showed the expected pattern of male-specific amplification in our early tests (Supplementary
425 Table 4). This may be due to the highly repetitive nature of the *T. rathkei* genome, despite our
426 best efforts to target primers to non-repetitive sequences.
427

428 Because the candidate male-specific contigs were assembled from Illumina data only and thus
429 short and fragmented, we were unable to screen them for annotated candidate sex-determining
430 genes using the typical MAKER pipeline. However, we were able to identify open reading
431 frames (ORFs) and annotate them like transcripts using Trinotate (Bryant et al. 2017). Two
432 contigs in the male-specific assembly showed homology to the androgenic gland hormone
433 (AGH) gene upon annotation, suggesting there may be a Y-linked duplication of the AGH gene.
434 Therefore, we designed PCR primers specifically targeting one of the Y-linked AGH-like
435 sequences (AGHY1 on NODE_44048_length_535; F: 5'-ATTCTTGACTCTCCCCACGA-3'; R:
436 5'-TCTCCAACTACGATTTCGTTAATT-3'). These primers resulted in a PCR product of the
437 expected size (195 bp) in all male samples screened (7/7), but not in any of the female samples
438 (0/7), all of which were unrelated wild-caught individuals, confirming the male-specificity of this
439 AGH allele.
440
441 This AGH sequence could be either a male-specific duplication of the AGH gene, or a Y-linked
442 allele that has diverged from an X-linked copy (in other words, gametologs). To distinguish
443 between these possibilities, we examined the sequencing depth of these genes and of other
444 putatively single-copy genes (identified in the BUSCO analysis) in male and female sequencing
445 samples. If the male-specific AGH sequence is a gametolog of an X-linked sequence, we would
446 expect the total sequencing depth of all AGH sequences (putative autosomal and putative Y-
447 linked) to be the same in both the pooled male and pooled female samples, with the female
448 sample having a higher average sequencing depth for the putative X-linked AGH sequences
449 (since they would be homozygous for the X-linked gametologs, while males would be
450 hemizygous for the X-linked gametologs). If, on the other hand, the male-specific AGH
451 sequences are Y-linked duplicates, and not allelic to the other AGH sequences in our assembly,
452 we would expect the shared autosomal AGH sequences to have similar sequencing depth in
453 both male and female samples, and the combined sequencing depth of all AGH sequences

454 (putative autosomal and putative Y-linked) would be higher in the male sample. Our results
455 were consistent with the latter scenario, suggesting these are Y-linked duplicates rather than
456 gametologs (Figure 3). Note that sequencing depth of AGHY1 and AGHY2, though much lower
457 in the female sample than in the male sample, is still non-zero in the female sample, probably
458 because of ambiguously mapped reads due to high similarity between the Y-linked and
459 autosomal copies.
460
461 Because the male-specific AGH sequences were found only in our Illumina data, we were
462 unable to assemble them into long contigs, even after repeated attempts to assemble them
463 individually with different assemblers and parameter values (not shown); all these contigs were
464 ~600bp or less in length. Thus we are unable to determine whether these are complete
465 duplicates of the whole gene, or fragments. Nevertheless, a phylogenetic analysis suggests that
466 one of the Y-linked duplicates is a copy of the other, rather than an independent duplication of
467 an autosomal copy, and based on branch lengths they are as divergent from one another as
468 AGH orthologs in different species (Figure 3). In addition, these Y-linked copies seem to lack an
469 intron that is present in the autosomal copies (Figure 4), suggesting they may have originated
470 via retrotransposition.
471
472 We also find some evidence of additional autosomal duplicates of the androgenic gland
473 hormone (AGH) gene. Two contigs in the full assembly contained annotated transcripts with at
474 least partial homology to the expressed transcript identified as the AGH sequence, and a third
475 contained no annotated genes but still showed high sequence similarity to AGH in BLAST
476 searches. However, not all of the annotated exons in the first two copies matched the expressed
477 transcript, and there were unannotated portions of the same contigs that did show sequence
478 similarity to the transcript (Figure 4). Moreover, some of the matching portions of the assembled
479 contigs had less than 90% sequence identity to the expressed transcript, and analysis of the

480 sequencing depth of these regions reveals that one has very low coverage, suggesting it may
481 be an assembly artifact (see below). Thus, we cannot rule out the possibility that some of these
482 possible autosomal duplicates represent assembly and/or annotation artifacts. If they are real,
483 these autosomal duplicates appear to be specific to *Trachelipus*, occurring after its divergence
484 from *Porcellio* (Figure 3), but they may still be nonfunctional.
485
486 **Discussion**
487
488 Possible sex chromosome polymorphism and recent transition to XY sex chromosomes in
489 *Trachelipus rathkei*
490
491 We have shown that, at least in our upstate New York population, sex determination in the
492 terrestrial isopod *Trachelipus rathkei* is based on an XX/XY sex chromosome system. Two
493 independent lines of evidence support this finding: first, crosses between females and sex-
494 reversed neo-males yielded all female offspring (Table 2), consistent with an XX/XY system but
495 not a ZZ/ZW system (Becking et al. 2017); second, we have identified PCR primers that only
496 amplify a product in male samples, indicating the presence of a male-specific genomic region,
497 i.e., a Y chromosome.
498
499 Our findings run counter to a previously published study showing evidence of female
500 heterogamety in this species based on cytogenetics; in that study, female germ cells contained
501 one set of unpaired chromosomes (presumably, the Z and W sex chromosomes), while male
502 germ cells did not (Mittal & Pahwa 1980). There are multiple possible explanations for this
503 contradiction. First, it is possible that the previous study incorrectly identified the species of
504 study specimens, as no information on identification is given in the paper; however, *T. rathkei* is
505 relatively easy to distinguish from other cosmopolitan terrestrial isopod species by its five pairs

506 of pleopodal “lungs” (most superficially similar species such as *Porcellio scaber* have only two
507 pairs; (Hatchett 1947; Shultz 2018)). In addition, that study was published before feminizing
508 *Wolbachia* was widely recognized in terrestrial isopods. It is therefore theoretically possible that
509 the females used in that study carried an XY genotype but were feminized by *Wolbachia*, while
510 the males in that study might have carried a YY genotype, perhaps resulting from a cross
511 between an XY father and a sex-reversed XY or YY mother which failed to transmit *Wolbachia*
512 (Becking et al. 2019). However, we found no evidence of sex-reversing *Wolbachia* in our *T.*
513 *rathkei* population.
514
515 Perhaps the most likely explanation is sex chromosome polymorphism. Indeed, this would not
516 be unprecedented, as sex determination in terrestrial isopods is thought to evolve rapidly
517 (Rigaud et al. 1997; Cordaux et al. 2011; Becking et al. 2017), and within-species sex
518 chromosome polymorphisms are documented in a few other species. For instance, two
519 subspecies of *Porcellio dilatatus*, *P. dilatatus dilatatus* and *P. dilatatus petiti* have XX/XY and
520 ZZ/ZW systems, respectively (Juchault & Legrand 1964; Legrand et al. 1974; Becking et al.
521 2017). In addition, multiple sex determining elements segregate in populations of the common
522 pillbug *Armadillidium vulgare* (Juchault et al. 1992), including a novel W chromosome that
523 resulted from the integration of an almost entire *Wolbachia* genome into the host genome
524 (Leclercq et al. 2016). Outside terrestrial isopods, sex chromosome polymorphisms are also
525 documented in a range of other arthropods and vertebrates (Rodrigues et al. 2013; Orzack et al.
526 1980; Franco et al. 1982; Ogata et al. 2008). *T. rathkei* is probably non-native in North America
527 where this study was conducted (Jass & Klausmeier 2000), as well as perhaps in India where
528 the prior study on cytogenetics was done (Mittal & Pahwa 1980). Given its cosmopolitan
529 distribution, and the fact that other terrestrial isopods have moderate to high levels of genetic
530 diversity (Romiguier et al. 2014), it might not be especially surprising for *T. rathkei* to harbor

multiple polymorphic sex-determining loci. Hopefully future follow-up work can further characterize geographic variation in sex determination in this species.

Regardless of whether or not sex determination is polymorphic in *T. rathkei*, the sex chromosomes in this species are likely evolutionarily young because it is nested within a clade that mostly consists of ZZ/ZW species (Becking et al. 2019). In addition, the putative male-specific region of its genome is relatively small, displaying only moderate divergence from candidate gametologous sequences, similar to other terrestrial isopods examined so far (Chebbi et al. 2019; Becking et al. 2019). Given that we found genomic evidence of a past association with *Wolbachia* in this species and that infection by *Wolbachia* has been found in other *T. rathkei* populations (Cordaux et al. 2012), this observation is consistent with the hypothesis that transitions in sex determination mechanisms may be triggered by *Wolbachia* and other endosymbionts that manipulate host reproduction (Rigaud et al. 1997; Cordaux et al. 2011). If other populations of *T. rathkei* with different sex determination mechanisms can be identified, it may be possible to leverage this system to further study the mechanisms and selective forces influencing transitions in sex determination mechanisms. In addition, studies of sex determination in a phylogenetic context involving other members of the family Trachelipodidae would shed further light on the origins of the X and Y chromosomes in *T. rathkei*.

<u>Genome size, structure, and repetitive elements</u>

The draft genome assembly of *T. rathkei* is especially large, at around 5.2 Gb, with approximately 29% GC content. The actual genome is likely to be even larger, given that ~25% of the BUSCO arthropod orthologs were missing in our assembly. By comparison, genomes of pillbugs in the genus *Armadillidium* tend to be smaller at around 1.2 - 2 Gb in size (Chebbi et al. 2019; Becking et al. 2019), but other terrestrial isopods have genomes ranging to over 8 Gb

557 (Gregory 2020), and other crustacean relatives such as amphipods also have large genomes
558 (Rees et al. 2007; Rivarola-Duarte et al. 2014; Kao et al. 2016), so *T. rathkei* is not out of the
559 ordinary for this group.
560
561 The *T. rathkei* genome contains a large proportion of repetitive elements, in particular
562 transposable elements (Figure 1). The most common transposable element families are LINEs,
563 DNA elements, and LTRs, similar to *Armadillidium vulgare* and *A. nasatum* (Chebbi et al. 2019;
564 Becking et al. 2019). The distribution of divergence values, with a single mode around 7-10%
565 divergence, suggests that most repeat families expanded around the same time as in *A. vulgare*
566 and *A. nasatum*; however, unlike in *A. vulgare*, *T. rathkei* shows no evidence of a second more
567 recent burst in DNA element activity. Simple repeats also comprise a substantial portion of the
568 genome; even manually looking through the assembled contigs reveals a high abundance of
569 (TA)x repeats.
570
571 *Wolbachia* insertions
572
573 We found many contigs with high similarity to the *Wolbachia* genome (Supplementary Table 3),
574 even though we were unable to detect current *Wolbachia* infections in our population using
575 PCR. This is not surprising given that *Wolbachia* is relatively common in terrestrial isopods and
576 arthropods in general (Cordaux et al. 2012; Pascar & Chandler 2018; Medina et al. 2019), has
577 been found in other populations of *T. rathkei* (Cordaux et al. 2012), and that horizontal transfers
578 of *Wolbachia* DNA into host genomes is also common (Dunning Hotopp 2011). These
579 *Wolbachia* insertions seem to be most closely related to strain *wCon* from *C. convexus*, which
580 does not induce male-to-female sex reversal, but rather causes cytoplasmic incompatibility
581 (Moret et al. 2001; Badawi et al. 2018). Consistent with this, we find that several copies of the
582 cytoplasmic incompatibility genes *cifA* and *cifB* among these insertions in the *T. rathkei* genome

583 (Supplementary Figure 1). Thus, these past *T. rathkei* infections may have caused cytoplasmic
584 incompatibility rather than host sex reversal, but it is possible that the same *Wolbachia* strain
585 may have multiple effects on host phenotypes.
586
587 Candidate sex-determining genes and repeated duplication of the AGH gene
588
589 Male differentiation in terrestrial isopods is controlled by the androgenic gland hormone, AGH.
590 AGH is a peptide hormone similar in structure to insulin, and is secreted by the androgenic
591 gland (Martin et al. 1999). AGH expression is sufficient to transform juvenile female isopods into
592 fertile males (Martin et al. 1999). Presumably, in wild-type males, the primary sex-determining
593 signal triggers the differentiation of the androgenic glands during development, which then
594 secretes AGH. Interestingly, the draft genome of *T. rathkei* contains multiple AGH-like
595 sequences. While some of these may be assembly artifacts, there is evidence three at least
596 partial Y-linked sequences, of which one was confirmed by PCR to be male-specific. These
597 duplications seem to be specific to *T. rathkei* (Figure 5), though other members of the genus
598 *Trachelipus* or the family Trachelipodidae have yet to be examined. Consistent with this, a past
599 study found no evidence of any expressed AGH duplications in other terrestrial isopod species
600 except *Porcellio gallicus* (Cerveau et al. 2014).
601
602 In many other species, novel sex chromosomes have arisen via duplication of a sex-determining
603 gene. For instance, duplicates of the vertebrate gene *Dmrt1* have evolved into master sex-
604 determining signals on the W and Y chromosomes, respectively, in the frog *Xenopus laevis*
605 (Yoshimoto et al. 2008) and the medaka *Oryzias latipes* (Nanda et al. 2002; Matsuda et al.
606 2002, 2007), while a Y-linked duplicate of the anti-Müllerian hormone gene is a candidate
607 master sex-determining gene in the teleost fish *Odontesthes hatcheri* (Hattori et al. 2012). The
608 presence of Y-linked AGH copies in *T. rathkei*, and no other obvious open reading frames

homologous to known sex determination or sex differentiation genes, makes these genes obvious candidates for the master male-determining signal in *T. rathkei*. Unfortunately, we were unable to assemble full copies of these Y-linked AGH homologs because they only showed up in our Illumina data, not in our low-coverage long read data. Future deep sequencing using long reads should further clarify the molecular evolution of these genes. In addition, expression studies should determine which of these genes are expressed, in what tissues, and at what stages.

If one of these AGH duplicates is indeed the master sex-determining signal in *T. rathkei*, this could support the idea of a transition in sex determination mechanisms triggered by *Wolbachia* and other reproductive endosymbionts (Rigaud et al. 1997; Cordaux et al. 2011). AGH may be a primary molecular target by which *Wolbachia* causes male-to-female sex reversal in isopod hosts, as injection with AGH does not cause female-to-male sex reversal in individuals infected by *Wolbachia* (Juchault & Legrand 1985; Cordaux & Gilbert 2017). Thus, *Wolbachia* may impose strong selection (via female-biased sex ratios) favoring duplication and divergence of the AGH gene to escape this sex reversal. The finding that *T. rathkei* may have historically been infected by a *Wolbachia* strain causing cytoplasmic incompatibility does not necessarily negate this finding, as the same *Wolbachia* strain can have multiple effects on host phenotypes.

Conclusions

We have shown that the terrestrial isopod *Trachelipus rathkei* uses an XX/XY sex chromosome system, at least in upstate New York, in contrast to a past cytogenetic study suggesting a ZZ/ZW mechanism (Mittal & Pahwa 1980). In line with this, whole-genome sequencing and follow-up PCRs demonstrate the existence of male-specific, Y-linked copies of the androgenic gland hormone gene in this species. These findings highlight the role of gene duplication in the

635 evolution of sex chromosomes and support the possibility that reproductive endosymbionts like

636 *Wolbachia* may favor evolutionary transitions in host sex determination mechanisms.

637

638

639

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644 **Table 1.** *Trachelipus rathkei* DNA samples used for genomic sequencing.

Sample name	Sex	Notes	Platform/ read length	Total data (Gb)	Ref.	Accession Number
M-pool	M	Pool of three brothers, lab-reared	Illumina, 2x100	191.6	This study	SRR11797365
F-pool	F	Pool of three sisters, lab-reared	Illumina, 2x100	191.9	This study	SRR11797364
M2	M	One male, lab-reared	Illumina, 2x250	22.3	This study	SRR11797360
M5	M	One male, lab-reared	Illumina, 2x250	20.6	This study	SRR11797359
M6	M	One male, lab-reared	Illumina, 2x250	23.6	This study	SRR11797358
F3	F	One female, lab-reared	Illumina, 2x250	131.5	This study	SRR11797357
F4	F	One female, lab-reared	Illumina, 2x250	26.8	This study	SRR11797356
Wild-M	M	One male, wild-caught	Illumina, 2x100	14.1	(Chandler et al. 2015)	SRR4000567
Wild-F	F	One female, wild-caught	Illumina, 2x100	14.4	(Chandler et al. 2015)	SRR4000573
PB-F1	F	Pool of three sisters, lab-reared	PacBio	22.2	This study; (Peccoud et al. 2017)	SRR11797355
PB-F2	F	Pool of three sisters, lab-reared	PacBio	1.3	This study; (Peccoud et al. 2017)	SRR11797354
PB-M	M	Pool of three brothers, lab-reared	PacBio	3.9	This study; (Peccoud et al. 2017)	SRR11797353
ONT-F	F	One female	ONT	0.4	This study	SRR11797363
ONT-M1	M	One male	ONT	3.9	This study	SRR11797362
ONT-M2	M	One male	ONT	3.3	This study	SRR11797361

645

646

647 **Table 2.** Sex ratios from crosses between putative neo-males (juvenile females implanted with
648 an androgenic gland) and females. *p* gives the probability of the observed or a more extreme
649 result (pooling the data for each neo-male) under a balanced sex ratio (i.e., assuming each
650 individual offspring has an equal probability of being male or female).
651

Neo-male	Female	Number of female offspring	Number of male offspring	*p*
D-4-7	1	23	0	5.7×10^{-14}
	2	14	0	
	3	7	0	
G-4-22	1	16	8	0.13
	2	17	14	
	3	13	13	
F-4-9	1	36	0	1.7×10^{-18}
	2	23	0	
10-8	1	24	35	0.19
	2	40	40	
AGS169-2	1	45	0	2.1×10^{-25}
	2	37	0	

652
653

654 **Table 3.** Assembly statistics for the *T. rathkei* draft genome.

Total length	5,181,251,014 bp
Number of contigs	421,784
N50	39,761 bp
GC content	29.0%
Complete BUSCO genes	533 single copy (51.9%); 39 duplicated (3.7%)
Fragmented BUSCO genes	203 (19.0%)
Missing BUSCO genes	271 (25.4%)

655

656

657 **Figure Legends**

658

659 **Figure 1.** Distribution of divergence levels for repetitive elements in the *Trachelipus rathkei*
660 genome.

661

662 **Figure 2.** Phylogenetic tree showing the position of candidate horizontally transferred
663 *Wolbachia* segments in the *Trachelipus rathkei* genome. Numbers by nodes indicate bootstrap
664 support. The tree was generated by concatenating all candidate *Wolbachia* insertions in *T.*
665 *rathkei* longer than 1000 bp, along with the best-matching regions in the reference *Wolbachia*
666 genomes, aligning with MUSCLE v. 3.8.31 (Edgar 2004), filtering alignments with trimal v.
667 1.2rev59 (Capella-Gutiérrez et al. 2009), selecting a model using ModelTest-NG v. 0.1.6
668 (Darriba et al. 2020), and running the analysis in RAxML-NG v. 0.9.0 (Stamatakis 2014) with
669 100 bootstrap replicates.

670

671 **Figure 3.** Distribution of sequencing depth for single-copy BUSCO genes in male and female
672 Illumina sequencing datasets (M-pool and F-pool). Labeled dots indicate the sequencing depth
673 for the different AGH copies in each sample.

674

675 **Figure 4.** Possible duplicates of the androgenic gland hormone gene in the *Trachelipus rathkei*
676 genome. The green bars represent the sequence of the expressed AGH sequence, assembled
677 from previously available transcriptome data. Gray bars represent contigs in the draft genome
678 assembly, and the pink bars on contigs represent annotated exons. Purple segments
679 connecting portions of the transcript to portions of contigs represent BLAST hits. The
680 incongruence between annotated exons and BLAST matches between the transcript and
681 contigs suggests the annotation still contains some errors.

682

683 **Figure 5.** Phylogenetic tree showing relationships among AGH sequences from terrestrial
684 isopods. AGH2 and AGHY3 are missing from this phylogeny because those sequences were
685 omitted because of their short length. The tree was generated using all AGH-like sequences
686 from *T. rathkei* of at least 100 bp, along with reference AGH nucleotide sequences from other
687 species, aligning them with MUSCLE v. 3.8.31 (Edgar 2004), selecting a model using
688 ModelTest-NG v. 0.1.6 (Darriba et al. 2020), and running the analysis in RAxML-NG v. 0.9.0
689 (Stamatakis 2014) with 100 bootstrap replicates.
690
691
692
693

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Figure 1

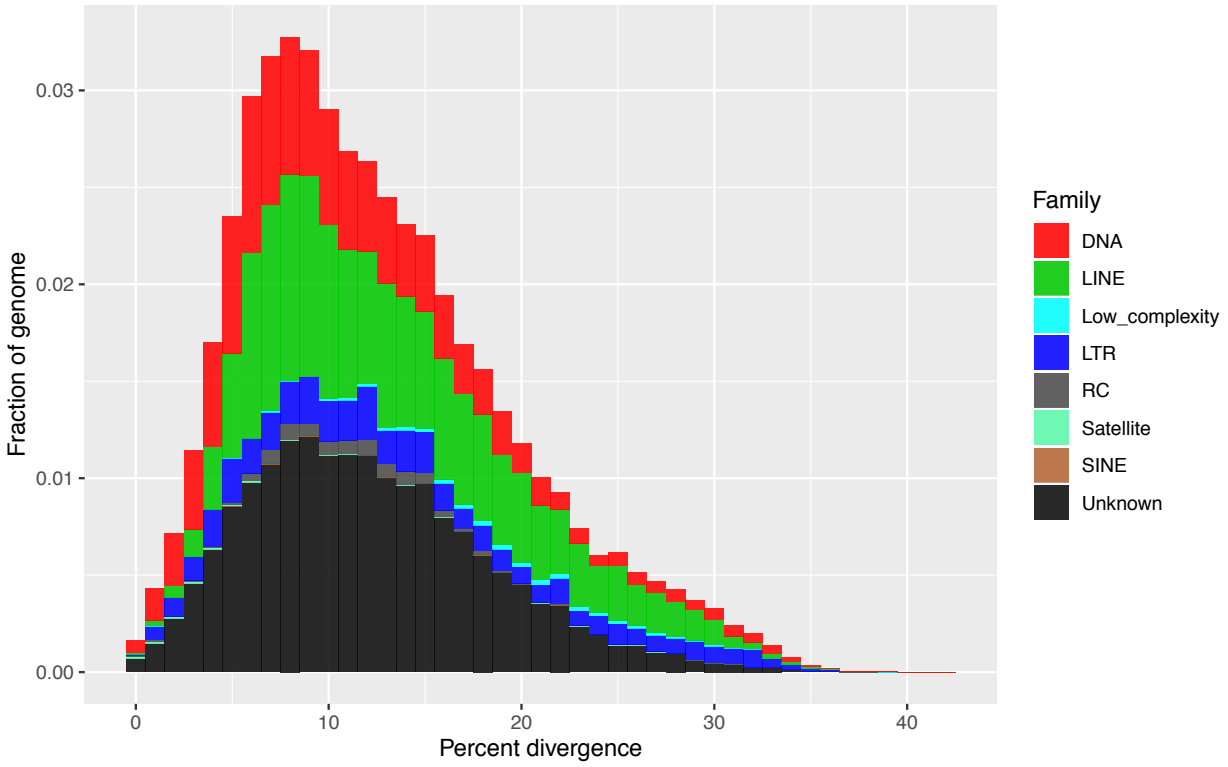
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Figure 2

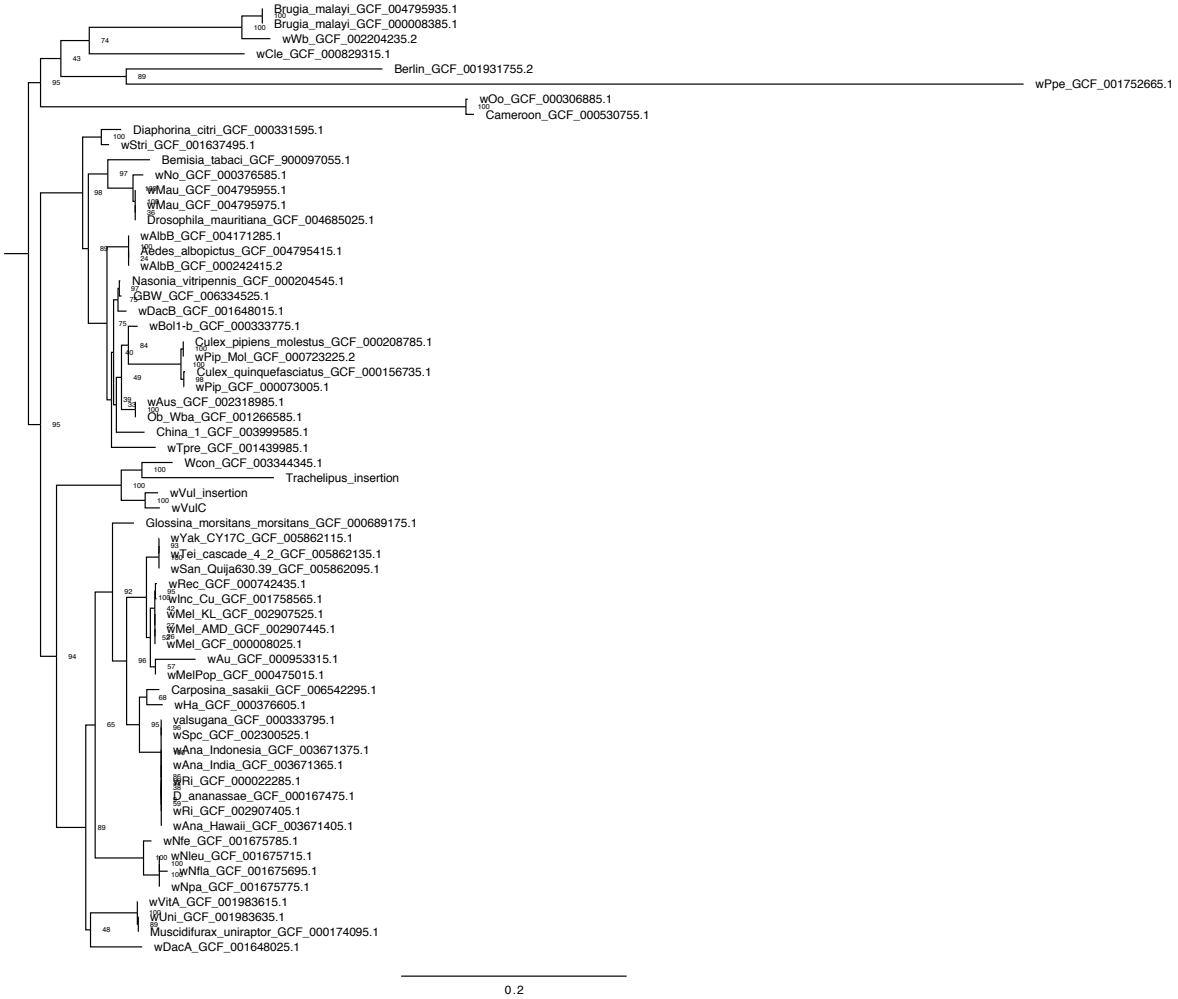
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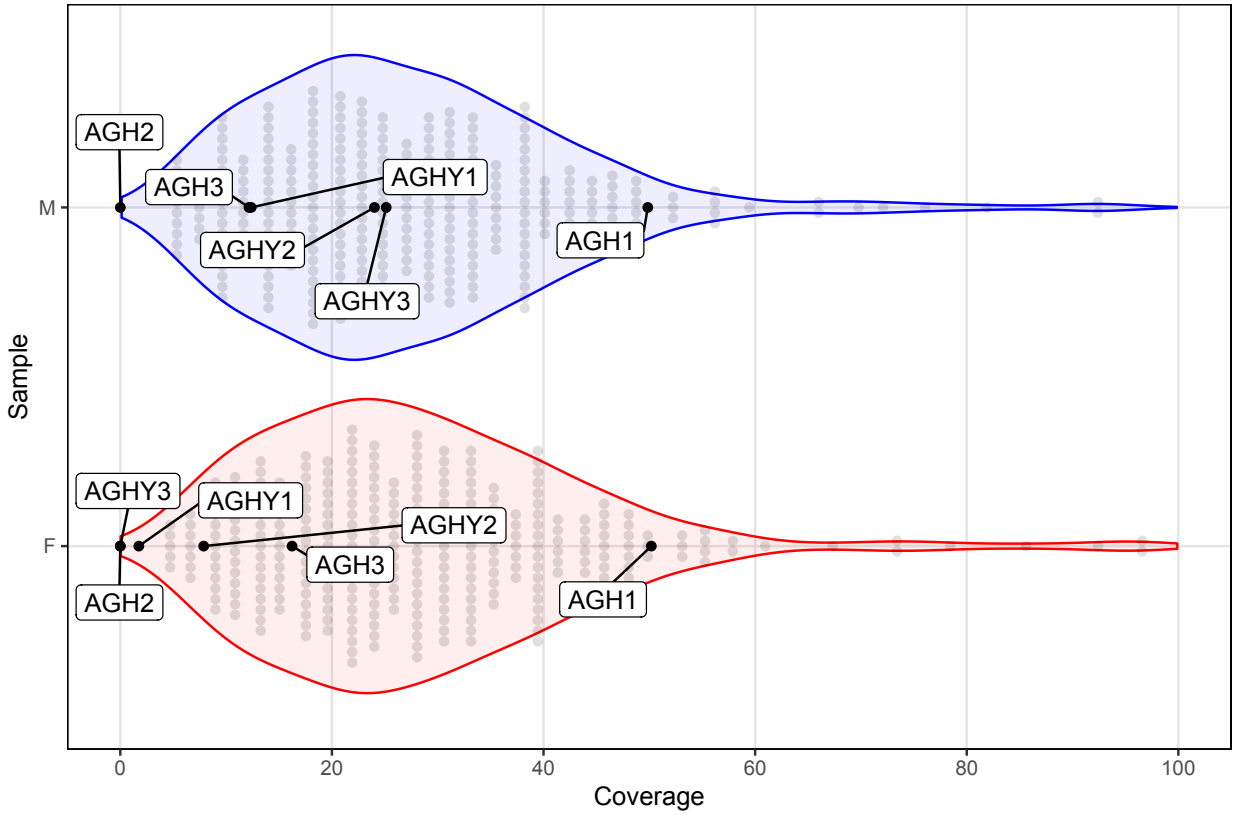
Figure 3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Coverage

991 **Figure 4**

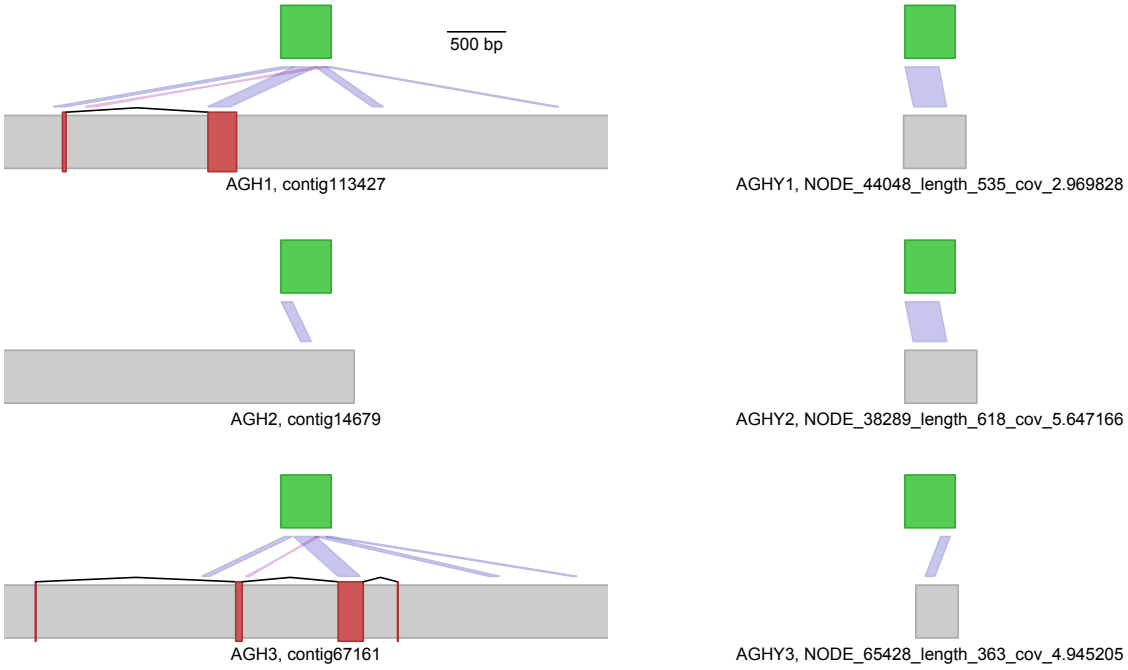

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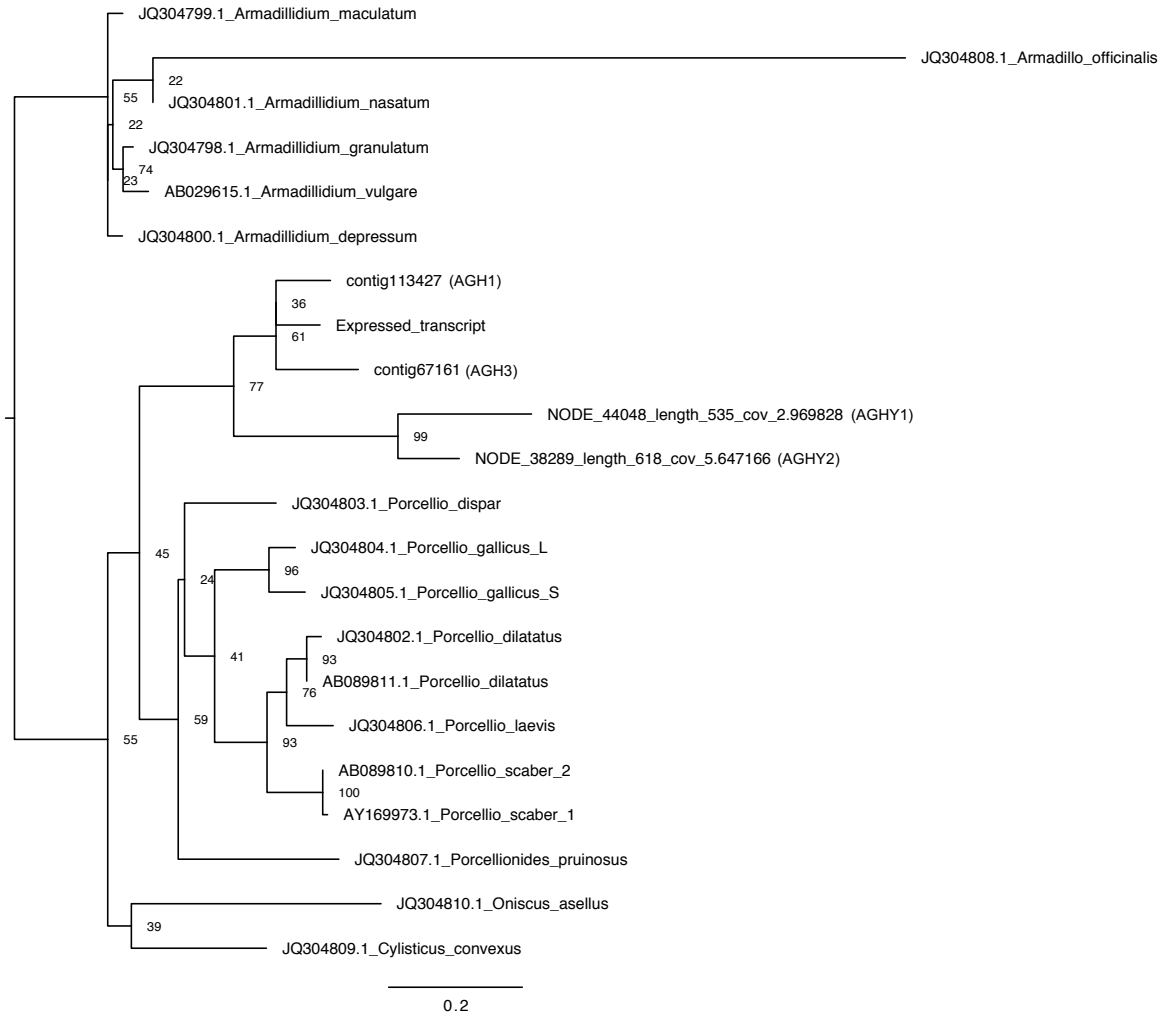
Figure 5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