

1 **Cross-species Y chromosome function between malaria vectors of the *Anopheles***
2 ***gambiae* species complex**

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4 Federica Bernardini*, Roberto Galizi*, Mariana Wunderlich*, Chrysanthi Taxiarchi*, Nace
5 Kranjc*, Kyros Kyrou*, Andrew Hammond*, Tony Nolan*, Mara N. K. Lawniczak†,
6 Philippos Aris Papathanos‡, Andrea Crisanti* & Nikolai Windbichler*

7 Author information:

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9 * Department of Life Sciences, Imperial College London, South Kensington Campus,
10 London SW7 2AZ, UK.

11 † Malaria Programme, Wellcome Trust Sanger Institute, Hinxton, Cambridge CB10 1SA,
12 UK.

13 ‡Section of Genomics and Genetics, Department of Experimental Medicine, University of
14 Perugia, 06132 Perugia, Italy.

15 **Anopheles Y chromosome introgression**

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20 Corresponding authors:

21

22 **Nikolai Windbichler**

23 Address: Department of Life Sciences, Imperial College London, South Kensington Campus,

24 London SW7 2AZ, UK

25 Phone number: 02075941650

26 E-mail address: n.windbichler@imperial.ac.uk

27

28 **Federica Bernardini**

29 Address: Department of Life Sciences, Imperial College London, South Kensington Campus,

30 London SW7 2AZ, UK

31 Phone number: 02075945435

32 E-mail address: f.bernardini11@imperial.ac.uk

33 **Abstract**

34

35 Y chromosome function, structure and evolution is poorly understood in many species
36 including the *Anopheles* genus of mosquitoes, an emerging model system for studying
37 speciation that also represents the major vectors of malaria. While the Anopheline Y had
38 previously been implicated in male mating behavior, recent data from the *Anopheles gambiae*
39 complex suggests that, apart from the putative primary sex-determiner, no other genes are
40 conserved on the Y. Studying the functional basis of the evolutionary divergence of the Y
41 chromosome in the gambiae complex is complicated by complete F1 male hybrid sterility.
42 Here we used an F1xFO crossing scheme to overcome a severe bottleneck of male hybrid
43 incompatibilities and enabled us to experimentally purify a genetically labelled *A. gambiae* Y
44 chromosome in an *A. arabiensis* background. Whole genome sequencing confirmed that the
45 *A. gambiae* Y retained its original sequence content in the *A. arabiensis* genomic background.
46 In contrast to comparable experiments in *Drosophila*, we find that the presence of a
47 heterospecific Y chromosome has no significant effect on the expression of *A. arabiensis*
48 genes and transcriptional differences can be explained almost exclusively as a direct
49 consequence of transcripts arising from sequence elements present on the *A. gambiae* Y
50 chromosome itself. We find that Y hybrids show no obvious fertility defects and no
51 substantial reduction in male competitiveness. Our results demonstrate that, despite their
52 radically different structure, Y chromosomes of these two species of the gambiae complex
53 that diverged an estimated 1.85Myr ago function interchangeably, thus indicating that the Y
54 chromosome does not harbor loci contributing to hybrid incompatibility. Therefore, Y
55 chromosome gene flow between members of the gambiae complex is possible even at their
56 current level of divergence. Importantly, this also suggests that malaria control interventions
57 based on sex-distorting Y drive would be transferable, whether intentionally or contingent,

58 between the major malaria vector species.

59 **Introduction**

60

61 Sex chromosomes often play an important role in speciation, though the molecular factors
62 that influence this process remains an area of active investigation (Ellegren 2011). Y
63 chromosome sequence content in heterogametic animals is transmitted in a clonal manner due
64 to the lack of crossing over with the X across some or all of its length. This absence of
65 recombination promotes a progressive genetic degeneration including the accumulation and
66 rapid turnover of repetitive sequences (Charlesworth 1991; Rice 1996; Charlesworth &
67 Charlesworth 2000; Bachtrog 2013). It is generally thought that remaining Y-linked genes
68 represent the remnants of an inexorable process of inactivation, degradation, and gene loss,
69 and only genes with a selectable function, such as the male-determining factor are likely to
70 survive on the Y chromosome. However, while this view appears to hold true for mammals,
71 the birth of new genes on the Y has been described in *Drosophila* and may even dominate the
72 evolution of it's Y chromosome (Carvalho et al. 2015; Vibranovski et al. 2008). This
73 suggests an evolutionary dynamic that is particular to the Y of different phylogenetic groups.

74 Sex chromosomes play an important role in reproductive isolation, however it is mostly the
75 gene-rich X chromosome that has been implicated with three interrelated patterns of hybrid
76 incompatibility: Haldane's rule, the large X effect, and the asymmetry of hybrid viability and
77 fertility in reciprocal crosses (Wu et al. 1996; Masly & Presgraves 2007). In a few cases a
78 link between the Y and hybrid incompatibilities has been demonstrated. For example it has
79 been shown that the Y chromosome contributes to reproductive barriers between rabbit
80 subspecies (Geraldts et al. 2008). This can be explained by interactions, involved in
81 determining male dimorphism or fertility, between genes which diverged from allele pairs on
82 the Y and X and which break down in the heterospecific context. Such X-Y chromosome
83 incompatibilities have been demonstrated to contribute to hybrid male sterility in house mice

84 (Campbell & Nachman 2014). Additionally the introgression of heterospecific Y
85 chromosomes in *Drosophila* was found to affect male fertility and alters the expression of 2-
86 3% of all genes in hybrids (Sackton et al. 2011).

87 The varied picture of the Y's biological role emerging from work in mammals and
88 *Drosophila* suggests the need for additional studies using other model systems. The
89 *Anopheles* genus, which contains all human malaria-transmitting mosquito species has in
90 recent years received much attention, not only due to its stark medical importance but also as
91 a model system for studying speciation and chromosome evolution. In particular the
92 *Anopheles gambiae* species complex of eight sibling species including the most widespread
93 and potent vectors of malaria in Sub-Saharan Africa offers an excellent platform to further
94 our understanding of the biology of the Y and its possible role in reproductive isolation.

95 To this end, we decided to focus on the two species with prime medical importance, *A.*
96 *gambiae* and *A. arabiensis*, as they are the most anthropophilic members of the complex with
97 the widest distributions.

98 *A. gambiae* predominates in zones of forest and humid savannah whereas *A. arabiensis*
99 prevails in arid savannahs and steppes, including those of the South-Western part of the
100 Arabian Peninsula. In the sympatric areas, changes in seasonal prevalence are observed
101 showing an increase in the relative frequency of *A. arabiensis* during the dry season. In areas
102 where the distribution of *A. gambiae* and *A. arabiensis* overlaps, hybrids are detected at
103 extremely low frequency (0.02-0.76%) (Toure et al. 1998; Temu et al. 1997; Mawejje et al.
104 2013). However, a recent study conducted in Eastern Uganda to investigate hybridization
105 between these species showed that 5% of the samples analysed were hybrid generations
106 beyond F1 (Weetman et al. 2014). F1 male sterility and other post-zygotic isolation
107 mechanisms have been studied in *A. gambiae* and *A. arabiensis* hybrids. In addition to
108 mapping multiple loci contributing to male sterility, Slotman et al. demonstrated, by

109 observing the absence of particular genotypes in backcross experiments, that inviability is
110 caused by recessive factors on the X chromosome of *A. gambiae* incompatible with at least
111 one factor on each autosome in *A. arabiensis* (Slotman et al. 2004).

112 In the present study we wanted to establish whether the introgression of the *A. gambiae* Y
113 chromosome into an *A. arabiensis* genetic background is possible when selected for in a
114 controlled laboratory setting, whether the Y contributes to reproductive isolation and whether
115 a heterospecific Y, as it has been reported in *Drosophila*, would markedly modulate gene
116 expression patterns, fertility or behavior of Y hybrid males. In addition to basic biological
117 insights our attempt to better understand the biology of the mosquito Y is key for both the
118 development of male-specific traits for genetic control as well as predicting the behavior of
119 such traits in the field.

120 **Materials & Methods**

121

122 **Mosquito Strains and Rearing.** Wild-type *A. gambiae* and *A. arabiensis* mosquitoes of
123 strains G3 and Dongola were used respectively. Strain G3 was originally colonized from
124 West Africa (MacCarthy Island, The Gambia, in 1975) and obtained from the MR4 (MRA-
125 112). It is considered a hybrid stock with mixed features derived from both *Anopheles*
126 *gambiae s.s.* and *Anopheles coluzzii*. The Dongola strain of *A. arabiensis* was obtained from
127 the MR4 (MRA-856) and was originally isolated from Sudan. For the introgression
128 experiments we used two independently generated Y-linked *A. gambiae* transgenic strains
129 G^{Y1} (Referred to as YattP in Bernardini et al. 2014) carrying a Pax-RFP marker and G^{Y2}
130 (unpublished Roberto Galizi). Strain G^{Y2} contains a Y linked insertion of construct
131 pBac[3xP3-DsRed] β 2-eGFP::I-PpoI-124L (Galizi et al. 2014) also carrying a Pax-RFP
132 marker (no expression from the inactive β 2-eGFP::I-PpoI-124L locus is detectable in this
133 strain). Inverse PCR suggests position 17757 on the Y_unplaced collection as a likely
134 insertion site of this construct, however no assembly of the repetitive *A. gambiae* Y
135 chromosome exists. All mosquitoes were reared under standard condition at 28 °C and 80%
136 relative humidity with access to fish food as larvae and 5% (wt/vol) glucose solution as
137 adults. For eggs production, young adult mosquitoes (2–4 days after emergence) were
138 allowed to mate for at least 6 days and then fed on mice. Two days later, an egg bowl
139 containing rearing water (dH₂O supplemented with 0.1% pure salt) was placed in the cage.
140 One to two days after hatching, larvae were placed into rearing water containing trays. The
141 protocols and procedures used in this study were approved by the Animal Ethics Committee
142 of Imperial College in compliance with UK Home Office regulations.

143

144 **Genetic crosses and fertility assays.** Crosses were set up in BugDorm-1 cages with size

145 30x30x30 cm. Generally 100 female and 100 male mosquitoes were crossed during the
146 introgression experiment, although the number of males varied after the F3 bottleneck and
147 was dependent on the number of male progeny that could be recovered from the previous
148 generation. To assay fertility of the Y-introgressed males, after 11 generation of backcrossing
149 in cage, single crosses in cups were set up. Y-introgressed males were singularly introduced
150 into a cup together with one *A. arabiensis* female. Parallel the same number of cups were set
151 up for *A. arabiensis* males and females as a control. After 6 days of mating and blood feeding
152 of females, eggs were collected from every cup and the hatching rate (number of
153 larvae/number of eggs) relative to every cross was calculated.

154

155 **DNA sequencing.** Samples for DNA sequencing were 10 adult male A^{Y2} and 2 wild-type
156 control *A. arabiensis* males and were used individually for whole genome sequencing. A^{Y2}
157 males had been mated to *A. arabiensis* females prior to DNA extraction allowing us to
158 establish fertility for 7 of the 10 A^{Y2} males. The DNA libraries were prepared in accordance
159 with the Illumina Nextera DNA guide for Illumina Paired-End Indexed Sequencing. AMPure
160 XP beads were used to purify the library DNA and for size selection after which the resulting
161 libraries were validated using the Agilent 2100 bioanalyzer and quantified using a Qubit 2.0
162 Fluorometer. Sequencing runs were performed on 6 lanes (2 samples per lane) of an Illumina
163 flowcell (v3) on the HiSeq1500 Illumina platform, using a 2×100 bp PE HiSeq Reagent Kit
164 according to the manufacturer's recommendations. Raw reads were processed using FastQC
165 (Andrews 2010, available online at:
166 <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) and trimmomatic (Bolger et al.
167 2014).

168

169 **Variant calling and read coverage analysis.** Reads were aligned to the *A. gambiae* PEST

170 reference genome assembly (AgamP4) using BWA mem (Li 2013, bio-bwa v0.7.5a) and
171 sorted using Samtools (v1.2). We used the MarkDuplicates module from Picard tools (v1.9)
172 to remove PCR duplicates and the genome analysis tool kit (GATK, v3.3) to realign reads
173 around indels (McKenna et al. 2010). First we used the GATK modules HaplotypeCaller and
174 UnifiedGenotyper to call raw SNPs and merged them across the 10 A^{Y2} and 2 A male
175 samples using GenotypeGVCFs. Biallelic SNPs were selected using GATK VariantFiltration
176 and SelectVariants modules following the GATK best practices guideline. For the coverage
177 analysis we used bowtie (v1.1.1), exclusively reporting alignments for reads having only a
178 single reportable alignment and displaying no mismatches. We used the bedtools (v2.25)
179 makewindows tool to generate a sliding window bed file with 5kb windows overlapping by
180 2.5kb. We then used Samtools bedcov to generate per-window read counts and calculate the
181 group means for the 7 fertile A^{Y2} and 2 A male control samples.

182

183 **Analysis of Y signature elements.** Paired whole-genome sequencing (WGS) reads were
184 mapped using Bowtie2 (Langmead & Salzberg 2012) with standard parameters (bowtie2 -x
185 [consensus_build] -a -1 [x_R1] -2 [x_R2] -S [x.sam]) against a collection of consensus
186 sequences of all known Y chromosome loci of *A. gambiae* (Hall et al. 2016). Read counts at
187 every locus were generated with Samtools (Li et al. 2009) and normalized by library size and
188 locus length in FPKM. WGS data in the form of paired Illumina reads corresponding to the
189 control samples from *A. gambiae* males (NCBI SRA: SRR534285) and females (NCBI
190 SRA:SRR534286) and *A. arabiensis* females (NCBI SRA: SRR1504792) were taken from
191 the Hall *et al.* study. We also performed a separate analysis to evaluate Y chromosome
192 satellite DNA abundance in the introgressed male samples, in part because satellites Ag53A,
193 B and D due to their short length could not be appropriately assessed using Bowtie2. We used
194 jellyfish (Marçais & Kingsford 2011) to generate unique 25mers (kmers of 25bp long) from

195 each of the 6 known Y satellites consensus sequences (jellyfish count -C -m 25 [stDNA-
196 locus-x.fasta] -o [output] -c 5 -s 1000000000 -t [cores]). Using the same approach we then
197 generated and counted unique 25mers from each of the aforementioned WGS samples
198 (jellyfish count -C -L 5 -m 25 [x.fastq] -o [output] -c 5 -s 1000000000 -t [cores]) and assessed
199 the abundance of each of the stDNA specific kmers within the sample-specific kmers list,
200 resulting in a table providing abundance of each kmer in each sample (grouped by stDNA
201 locus). Raw kmer counts were normalized by library size (sequencing depth) and for the
202 WGS generated in this study (control and introgressed males) we calculated the median
203 abundance for each kmer in these two groups.

204

205 **RNA and small RNA sequencing experiments.** Sample for RNA sequencing were
206 generated using the following experimental design. Four cages were set up with 40 *A.*
207 *arabiensis* wild-type males and 40 wild-type females and four cages with 40 A^{Y2} males and
208 their non-transgenic sibling females. After mating and blood feeding, progeny was collected
209 from the cages. 80 freshly hatched larvae were collected from each of both sets of cages and
210 combined in a single tray for rearing. At the pupal stage males were sexed and screened for
211 the fluorescent marker linked to the Y chromosome. From every tray 18 RFP positive and 18
212 RFP negative males were collected and placed in separate cages to allow emergence. Three
213 days after emergence males were dissected in order to separate three different tissues the
214 head, the abdominal segments harboring the reproductive tissues and the remainder of the
215 carcass. This experiment was performed in twice resulting in a total number of 8 replicates
216 for both controls and experimental samples. Libraries for total RNA sequencing were
217 prepared using the TruSeq RNA sample preparation kit by Illumina and sequenced on 3 lanes
218 of an Illumina HiSeq 2500 using 2x100 paired end reads. The samples described above were
219 also used for the construction of libraries for small RNA sequencing. Libraries were prepared

220 using the NEBNext Multiplex Small RNA kit for Illumina and sequenced on 3 lanes of a
221 MiSeq using the 1x42 single-read mode.

222

223 **Differential expression analysis.** Reads were aligned to the *Anopheles arabiensis* genome
224 supplemented with the 25 contigs corresponding to known *A. gambiae* Y loci (Hall et al.
225 2016) using HISAT2 (Pertea et al. 2016). We then used Stringtie (Pertea et al. 2016) in
226 conjunction with the *Anopheles arabiensis* reference transcriptome version AaraD1.3 to
227 predict novel genes and novel isoforms of known genes which were merged across samples
228 into a combined geneset using Cuffmerge (Ghosh & Chan 2016). Expression of all transcripts
229 was then quantified using Stringtie -B. We used the Ballgown (Pertea et al. 2016) suite to
230 determine transcripts showing significantly different expression levels using a cutoff of p-
231 value <0.05 adjusted for multiple testing, a mean expression greater than 1 FPKM across the
232 samples in the tissue analyzed and a log₂ fold-change greater than 1 between the introgressed
233 and control groups. To assign a repetitiveness score the sequence of each transcript was
234 blasted against the *Anopheles gambiae* PEST RepeatMasker library provided by
235 Vectorbase.org. For the small RNA dataset we generated a count matrix using Seqbuster and
236 SeqCluster suites (Pantano et al. 2011) and used DESeq2 (Love et al. 2014) for differential
237 expression analysis and log₂ transformation of the count data. Only putative small RNA loci
238 with a mean expression of more than 5 counts across samples were taken into account for this
239 analysis.

240

241 **Competition experiments.** Matings were performed in BugDorm-1 cages. Females and
242 competing males were allocated in cage with a 1:1:1 ratio. Every experiment was run in
243 triplicate. Crosses were set up as follows: 50 A females x 50 A males + 50 G^{Y2} males, 50 G
244 females x 50 G males + 50 A^{Y2} males, 50 A females x 50 A males + 50 A^{Y2} males, 50 G

245 females x 50 A males + 50 A^{Y2} males. After six days mating and blood feeding females were
246 collected from each experimental replicate and allowed to lay singularly in cups. The number
247 of eggs and hatched larvae was calculated for every cup in order to estimate the hatching rate
248 values. Progeny was screened for 3xP3 RFP to assess paternity and transgene ratio was
249 calculated in order to identify any occurring secondary mating.

250 **Results**

251

252 **Experimental introgression of the *A. gambiae* Y chromosome into *A. arabiensis***

253

254 We have previously established a number of transgenic strains in which different fluorescent
255 transgenes were inserted onto the *A. gambiae* Y chromosome (Bernardini et al. 2014)
256 (Roberto Galizi unpublished). Limited recombination between the *A. gambiae* sex
257 chromosomes has been suggested to occur in specific genetic backgrounds (Wilkins et al.
258 2007) but is generally believed to occur at very low frequencies (Mitchell & Seawright 1989;
259 Bernardini et al. 2014; Hall et al. 2016). We concluded, that for our purpose a Y-linked
260 fluorescent transgene would be a reliable tool to track the Y chromosome throughout a multi-
261 generational introgression experiment. It has previously been shown that F1 male hybrids
262 between *A. gambiae* and *A. arabiensis* suffer from complete male sterility, which precludes Y
263 chromosome introgression experiments in a straightforward manner. Our pilot experiments
264 confirmed this finding (Supplementary Table 1A). We employed a F1xFO crossing strategy
265 (we define this as a scheme where first generation female hybrids are crossed to pure species
266 males) in which transgenic Y *gambiae* males were backcrossed to F1 hybrid females that
267 were in-turn generated using either wild type *gambiae* or *arabiensis* mothers (Figure 1A). We
268 used two different transgenic Y strains herein referred to as G^{Y1} and G^{Y2} that both express an
269 RFP reporter gene driven by the neuronal 3xP3 promoter from different insertion sites on the
270 Y chromosome. In the F2 cross, the hybrid males containing the labeled Y are crossed again
271 to wild type *arabiensis* females, and then third generation hybrid males are crossed to both
272 hybrid females and wild type *arabiensis* females in the attempt to recover offspring. Using
273 this crossing scheme, we encountered a severe bottleneck at generation F3 when males are
274 predicted to have inherited a predominantly *A. gambiae* autosomal genome from their fathers

275 in conjunction with pure *A. arabiensis* genome (including the X chromosome) from their
276 mothers (Figure 1B). We recovered no larvae from >13,000 eggs when backcrossing of either
277 G^{Y1} or G^{Y2} males was carried on from F1 hybrid females originated from *A. gambiae*
278 mothers. In the reverse cross, using F1 hybrid females from *A. arabiensis* mothers, we
279 managed to recover 7 larvae (3 males, 4 females) with strain G^{Y2} in a direct cross to wild type
280 *A. arabiensis* females (Figure 1A). These 3 hybrid males obtained were used to progress the
281 introgression and we continuously maintained backcross purification by crossing hybrid
282 males recovered each generation to wild-type *arabiensis* females for a total of 11 generations
283 to establish the introgressed strains A^{Y2} used for all subsequent experiments in this study. The
284 occurrence of fertile phenotypes in these crosses is a rare event. In additional experiments
285 were either G^{Y1} or G^{Y2} males were crossed to F2 or F3 hybrid females (Figure 1A) the
286 majority of the resulting male progeny (15 larvae of which 7 were male and 34 larvae of
287 which 13 were male) failed to develop into fertile adults.

288

289 **Fertility of males carrying a heterospecific Y chromosome**

290

291 We first asked whether A^{Y2} males showed reduced levels of fertility when compared to wild-
292 type *A. arabiensis* males due to the presence of the heterospecific Y chromosome. In order to
293 assay individual males, rather than a population average, we performed single-copula mating
294 experiments of strain A^{Y2} or wild-type males mated to *A. arabiensis* females, measuring the
295 mating rates and oviposition and egg hatching rates of single females. Every generation the
296 males of the largest family were used for establishing the next round of single-copula
297 backcrosses and over the course of 7 generations a total of 344 wild type *A. arabiensis* and
298 350 A^{Y2} males were assayed. The rationale for this design was to exclude the possibility of *A.*
299 *gambiae* fertility loci having been retained by selection in introgressed males, because such

300 loci would be expected to segregate in this design. Figure 2 shows a summary of these
301 experiments. Single copula matings are inefficient, in fact less than 25% of females would
302 mate and oviposit under these condition (Figure 2A). No significant difference in the rate of
303 mating was observed between A^{Y2} (22%) and control *A. arabiensis* males (15.3%). Power
304 analysis suggested that the sample size of the successfully mated males would allow for the
305 reliable detection of an effect of medium size ($p=0.79$ for a two-sided t-test, $p\text{-value}=0.05$,
306 $d=0.5$). We observed that females mated to A^{Y2} males and females mated to *A. arabiensis*
307 wild-type males laid a comparable numbers of eggs (Figure 2B) that had comparable
308 hatching rates (Figure 2C). This analysis indicates that, under laboratory conditions and in the
309 absence of mate choice and male-male competition and taking into account above
310 considerations on power, A^{Y2} males show no significant difference in fertility when
311 compared to wild-type *A. arabiensis* males that retain their native Y chromosome.

312 As an additional control, we back-crossed A^{Y2} males to *A. gambiae* females. Despite the
313 presence of the *A. gambiae* Y chromosome in these males we expected this experiment to re-
314 create hybrid incompatibility in the form of male infertility in the resulting progeny. Indeed
315 we found hybrid males to be fully sterile (Supplementary Table 1B) thus confirming that X-A
316 incompatibilities are sufficient to explain this phenotype (Slotman et al. 2004).

317

318 **Genomic analysis of males with a heterospecific Y chromosome**

319

320 After $n=11$ generations of backcrossing, assuming no selection for sections of the *A. gambiae*
321 genome, the expected autosomal genome proportion of the *A. gambiae* donor would be $1/2^n$
322 or less than 0.05%. Given that *gambiae* genomic regions contributing to hybrid
323 incompatibilities would be selected against in males this is likely an underestimation due to
324 such detrimental haplotypes being selectively removed. To confirm that our backcrossing

325 scheme had eliminated the *A. gambiae* autosomal genome but retained the *A. gambiae* Y
326 chromosome we performed whole genome DNA sequencing from 10 A^{Y2} males and 2 wild-
327 type control males of our *A. arabiensis* lab colony. To determine whether introgressed males
328 were fertile, they were singularly mated to wild-type *arabiensis* females before their genomic
329 DNA was extracted. WGS reads were mapped to the *A. gambiae* genome to identify genomic
330 regions with fixed allele differences between introgressed and control groups by confining
331 our analysis to biallelic SNPs. No assembly of the *A. gambiae* Y exists, however the PEST
332 assembly includes the Y_unplaced sequence collection that includes ~230kB of unscaffolded
333 contigs that have been assigned to the Y. Our analysis (Table 1) showed that the autosomes
334 of introgressed-Y and pure species contained a small number of differentially represented
335 SNPs comparable in number to the X chromosome which, since it is replaced in every
336 backcross generation, serves as a background control. In contrast, the majority of
337 differentially represented SNPs (74.7% of the total number of differential fixed SNP and
338 35.6% of the total number of SNPs on the Y) arose from reads mapping to the Y_unplaced
339 portion of the *A. gambiae* genome that represents less than 0.1% of the total genome
340 assembly. Within the Y_unplaced collection we found that most SNPs mapped to the largest
341 contig, which also contains the male-determine gene (Supplementary Figure 1). In addition
342 12.7% of of the total number of SNPs arose from the UNKN collection (unassigned contigs)
343 that is also expected to contain a number of unassigned Y sequences and repetitive elements.
344 We performed an additional sliding-window analysis where we considered only reads
345 mapping uniquely to the *A. gambiae* genome and allowed for no mismatches. The rationale
346 was that, given the observed levels of divergence between these genomes, perfectly matching
347 reads are expected to predominantly map to the genome of origin. When comparing the mean
348 number of reads of A^{Y2} fertile introgressed males and the samples of the *A. arabiensis* control
349 group we find that the Y_unplaced collection experiences significant coverage only in A^{Y2}

350 males as do parts of the UNKN collection. For the autosomes and the X, few windows accrue
351 a significant number of reads and we find no substantial differences between the groups in
352 the direction of A^{Y2}, with the possible exception of an intergenic region on chromosome 2L
353 (Supplementary Figure 2). This suggests that, apart from the Y, both groups have a similar *A.*
354 *arabiensis* background and we concluded that the transgenic *A. gambiae* Y had been
355 successfully purified in an *A. arabiensis* genomic background, although our data can't rule
356 out that some fraction of *A. gambiae* genomic DNA other than the Y chromosome persists in
357 the A^{Y2} strain.
358

359 **Analysis of Y chromosome sequence content in introgressed males**

360

361 Y chromosomes of *A. gambiae* and *A. arabiensis* have been shown to differ dramatically in
362 their structure and sequence content (Hall et al. 2016). To assess whether introgression of the
363 *A. gambiae* Y chromosome into the *A. arabiensis* genome coincided with any detectable
364 structural re-arrangements of the Y, for example the selective elimination of sequences that
365 would be detrimental in hybrids, we mapped DNA reads from all A^{Y2} individuals as well as
366 pooled control datasets from Hall et al. against a collection of known Y chromosome genes
367 and repeats of *A. gambiae*. This collection includes consensus sequences of all known and
368 putative genes, repetitive elements as well as satellite DNA (Hall et al. 2016). We assessed
369 normalized read depth at each locus (Fragments Per Kilobase of transcript per Million
370 mapped reads) as a proxy for copy number of each sequence element in a given background.
371 This analysis is complicated by the occurrence of autosomal copies of many of these
372 elements as well as possible variation between Y chromosome isolates. Figure 3 shows
373 normalized read counts for these elements in strain A^{Y2} plotted versus males and females of
374 both *A. gambiae* and *A. arabiensis*. We observe an excellent correlation in the representation
375 of these Y signature sequence elements between of *A. gambiae* males and A^{Y2} males.
376 Because these signature elements are derived from and to some degree specific to the *A.*
377 *gambiae* Y chromosome, they were under-represented in *A. arabiensis* control males and in
378 females of both species. Interestingly, the AgY280 satellite shows an approximately 15x
379 higher coverage in *A. gambiae* males compared to the A^{Y2} strain. However this satellite is
380 known to be highly variable between Y isolates (Hall et al. 2016). In order to better assess the
381 content of DNA satellites, in particular satellites Ag53A, B and C that are too short for
382 standard read mapping, we performed an additional analysis based on kmer counts in the read
383 libraries. This analysis showed that A^{Y2} males resemble *A. gambiae* males with regards to the

384 content of DNA satellites attributed to the Y (Supplementary Figure 3). Together these
385 analyses therefore confirm the presence of the *A. gambiae* Y chromosome in the introgressed
386 strain and suggest that the introgression experiment did not impact in any evident way the
387 original content of this Y chromosome.

388

389 **Transcriptomic analysis of males carrying a heterospecific Y chromosome**

390

391 We next asked to what extent the presence of a heterospecific Y chromosome would alter the
392 expression of autosomal or X-linked genes of the *A. arabiensis* background. This could occur
393 by (i) the action of *gambiae* Y trans-acting factors or absent *A. arabiensis* Y trans-acting
394 factors, (ii) by Y sequences that recruit cellular trans-acting factors and/or modulate the
395 chromatin structure and epigenetic state of other chromosomes or (iii) by the activation of
396 mobile genetic elements present on the *A. gambiae* Y that trigger a cellular response in X or
397 autosomal sequences. We focused our analysis on 3 tissues, the head, the terminal abdominal
398 segments containing the reproductive tissues and the remainder of the carcass. We performed
399 RNA sequencing of a total of 8 control and 8 experimental samples for each tissue from A^{Y2}
400 and wild-type males that had been reared in the same larval tray and that were sexed and
401 separated at the pupal stage. Paired-end reads were mapped against the 1214 *A. arabiensis*
402 genomic scaffolds supplemented by 25 consensus sequences corresponding to known *A.*
403 *gambiae* Y loci previously mentioned, as well as the reporter gene construct. Due to the
404 incomplete annotation of the *A. arabiensis* genome we performed an isoform level analysis
405 where we first predicted novel genes and novel isoforms of known genes across all samples.
406 Gene-level expression of the experimental groups as well as sample relationships are
407 summarized in Supplementary Figure 4 and Supplementary File 1. Finally, in order to
408 indicate whether differentially expressed transcripts potentially represented known mobile

409 elements or repetitive DNA arising from the Y (but matching paralogous sequences present
410 on the *A. arabiensis* scaffolds which could thus be misreported as differentially expressed)
411 we blasted each predicted transcript to the *A. gambiae* repeat library and assigned a
412 repetitiveness score. We then predicted differential expression of transcripts between A^{Y2} and
413 wild-type males (Figure 4, Supplementary File 2). Few transcripts were expressed
414 significantly lower in A^{Y2} males. This is partially expected because *A. arabiensis* genomic
415 scaffolds are derived from the DNA of females (Neafsey et al. 2015) and it is thus not
416 possible to identify any *A. arabiensis* Y-linked genes that would have fallen into this class.
417 However, it also indicates that few if any endogenous genes are down-regulated as a result of
418 the presence of the heterospecific Y chromosome. In contrast a number of transcripts
419 displayed significantly higher levels of expression in A^{Y2} males. However, the majority of
420 these correspond to the known *A. gambiae* Y loci (purple triangles in Figure 4) and their
421 expression is also summarized in Supplementary figure 5. Of the remaining upregulated *A.*
422 *arabiensis* transcripts (colored circles in Figure 4) the majority had a high repetitiveness
423 score i.e. significant homology to *A. gambiae* repeats. A manual homology search using all *A.*
424 *arabiensis* transcript sequences passing the significance threshold and cut-off for expression
425 confirmed that virtually all differentially expressed transcripts are related to repetitive DNA.
426 In addition to the above analysis we also measured small RNA expression in these tissues
427 finding no evidence for the differential expression of small non-coding RNAs between A^{Y2}
428 and wild-type males (Supplementary Figure 6). While it is possible that such effects could
429 occur in other developmental stages or under specific environmental conditions we find little
430 evidence that the heterospecific Y-chromosome markedly affects expression of the non-
431 repetitive, autosomal or X-linked gene repertoire of the *A. arabiensis* genome.

432

433 **Female mate-choice and male competition experiments**

434

435 Although we find no strong effect of the heterospecific Y chromosome on the transcriptome
436 and fertility of individual A^{Y2} males it is possible that Y-linked sequences do play a role in
437 male fitness or female choice which would also limit the practical use of introgressed Y-
438 linked traits e.g. for vector control. In order to test this hypothesis we set up a panel of
439 competitive mating experiments where two strains of males were allowed to compete for
440 mating with females in population cages (Figure 5). Both wild-type *A. arabiensis* and A^{Y2}
441 males performed substantially worse (winning only 13.7% and 11.1% of matings
442 respectively) than *A. gambiae* males in competition experiments for either *A. gambiae* or *A.*
443 *arabiensis* females. These findings confirms previous observations (Schneider et al. 2000)
444 and demonstrates that, irrespective of the female type *gambiae* males are superior to *A.*
445 *arabiensis* males in the lab setting. In contrast A^{Y2} males were only slightly less competitive
446 compared to wild type *arabiensis* males winning ~40% of matings with *arabiensis* females
447 (p=0.0146) and no significant difference was observed when they competed against
448 *arabiensis* males for *gambiae* females (p=0.156). The particular set up of the experiments also
449 allowed to score for secondary mating measured by the percentage of transgenic male
450 progeny. With the possible exception of one case, re-mating (showing a significant deviation
451 from a 50% transgene ratio in the progeny) was not observed in these experiments. Overall a
452 slight reduction in male competitiveness was observed which could relate, in addition to the
453 effect of the heterospecific Y, to inbreeding or fitness costs associated with expression of the
454 fluorescent marker gene. Importantly, in all cases no significant difference to the wild type *A.*
455 *arabiensis* males observed in terms of the number of laid and hatching eggs from matings
456 with A^{Y2} males confirming our previous analysis.

457 **Discussion**

458

459 In a classic study Slotman et al. mapped quantitative trait loci related to male sterility in
460 hybrids between *A. gambiae* and *A. arabiensis* and at least five or six sterility factors were
461 detected in each of the two species. The X chromosome was found to have a
462 disproportionately large effect on male hybrid sterility (Slotman et al. 2004), which is likely
463 related to divergent alleles present within multiple fixed chromosomal rearrangements on the
464 X. A possible role of the Y chromosome in hybrid incompatibilities was suggested by the
465 authors but not followed up on experimentally. Using an F1xF0 crossing scheme we
466 generated F3 males with an *arabiensis* X chromosome and a set of *arabiensis* autosomes as
467 well as an *A. gambiae* Y chromosome. The second set of autosomes is expected to contain on
468 average 75% *A. gambiae* sequences. The majority of such F3 males were expected to be
469 sterile; however we hypothesized that it should be possible to select a small fraction of fertile
470 males that lacked the *A. gambiae* incompatibility loci that cause sterility when interacting
471 with the *A. arabiensis* background. Indeed, we recovered 56 larvae out of a total 29,776 eggs
472 laid (0.18%) from pooled backcrosses of ~600 F3 males sampled.

473 Surprisingly, after multiple generations of backcross purification we found that the *A.*
474 *gambiae* Y chromosome does not markedly influence male fertility, fitness or gene
475 expression in Y hybrids. This rules out the Y chromosome as a major factor contributing to
476 hybrid incompatibilities and, more importantly, it is in stark contrast to findings in the
477 *Drosophila* model (Sackton et al. 2011). Despite the Y's relative paucity of genes in the fly
478 even intraspecific Y chromosome variants profoundly affect the expression a substantial
479 number of genes located on the X or the autosomes. The introgression of heterospecific Y
480 chromosomes in *Drosophila*, to the extent that it is even possible (Johnson et al. 1993), has
481 consequently been found to markedly affect male fertility and gene expression in interspecific

482 hybrids. In a *D. simulans* background the *D. sechellia* Y has little effect on viability, but it
483 reduced male fecundity by 63% as well as sperm competitiveness (*D. simulans/sechellia*
484 divergence time has been estimated at only 0.25 Myr). Y introgression differentially affected
485 genes involved in immune function and spermatogenesis suggesting a trade-off in investment
486 between these processes. However, it has also been suggested that a significant part of the
487 observed effect in *Drosophila* may be attributed to the ribosomal DNA (rDNA) clusters that
488 are abundant on both the X and the Y in the fly. Importantly, in the *A. gambiae* strain G3 we
489 used for this study (unlike some strains such as (Wilkins et al. 2007)) and in *A. arabiensis* the
490 rDNA is located exclusively on the X-chromosome and thus does not confound introgression
491 experiments the same way. Our results may thus be more indicative than similar experiments
492 in *Drosophila* of the true effect a gene-poor heterospecific Y exerts on the genome of a
493 related species.

494 It is possible that more subtle effects of the introgressed Y, not detectable in our experimental
495 setup, exist. Future work could involve hybrid performance testing in mating swarms or
496 under semi-field conditions. The fact however that, despite its radically different structure
497 and an estimated divergence time of ~1.85 Myr or more than 7 times that between *D.*
498 *simulans* and *D. sechellia*, the *A. gambiae* Y seems to be able to fully replace the *A.*
499 *arabiensis* Y, suggests that in *Anopheles* the Y either carries no important factors that
500 diverged between these two species or that no such factors are present at all. Although, early
501 work had implicated the Anopheline Y chromosome in mating behavior in a study using *A.*
502 *labranchiae* and *A. atroparvus* species (Fraccaro et al. 1977) our work is more in line with a
503 recent study that leveraged long single-molecule sequencing to determine the content and
504 structure of the Y chromosome of the primary African malaria mosquito, *A. gambiae* in
505 comparison to its sibling species in the *gambiae* complex (Hall et al. 2016). This study
506 revealed rapid sequence turnover within the species complex with only a single gene, recently

507 shown to act as a male-determining factor (Krzywinska et al. 2016), being conserved and
508 exclusive to the Y in all species. This gene called YG2 (alternatively referred to as Yob) was
509 not detectable in the genome of the more distant mosquito relative *Anopheles stephensi*,
510 overall suggesting rapid evolution of Y chromosome in this highly dynamic genus of malaria
511 vectors.

512

513 The role of gene flow between *A. gambiae* and *A. arabiensis* in leading to adaptive
514 introgression and the implications for vector control has been highlighted by Weetman and
515 colleagues (Weetman et al. 2014). Post-F1 gene flow occurs between *A. gambiae* and *A.*
516 *arabiensis*, and, especially for traits under strong selection, could readily lead to adaptive
517 introgression of genetic variants relevant for vector control. Introgression of the Y
518 chromosome between species is generally viewed as unlikely and markers found on the Y
519 generally show restricted gene flow relative to other loci. However, this contrasts with the
520 recent hypothesis (Neafsey et al. 2015; Hall et al. 2016) of Y gene flow between *A. gambiae*
521 and *A. arabiensis*. Contrary to the established species branching order, the YG2 gene tree
522 suggested that Y chromosome sequences may have crossed species boundaries between *A.*
523 *gambiae* and *A. arabiensis* (Hall et al. 2016) and the authors suggested that Y chromosome
524 introgression could have predated the development of male F1 hybrid sterility barriers that
525 exist between this pair of species (Hall et al. 2016). Our study lends support to this hypothesis
526 as it experimentally demonstrates the possibility of a cross-species Y chromosome transfer
527 and shows that the Y is functional in this context.

528 Our findings thus also suggest that Y-linked genetic traits generated in *A. gambiae* could be
529 transferred to its sister species. For example strain A^{Y1} carries a site-specific docking site that
530 now also allows the generation of male-exclusive genetic traits in *A. arabiensis*. Recent
531 progress towards the elusive goal of efficient sex-ratio distortion by a driving Y chromosome

532 (Galizi et al. 2014; Bernardini et al. 2014) could lead to the development of invasive distorter
533 traits in *A. gambiae* that may then be transferred to sibling species. This could be done
534 deliberately in the lab as we have demonstrated here, but it could also occur contingently in
535 the wild after a large-scale release of transgenic males. While one should always be wary
536 extrapolating lab studies to conditions in the field, the notion that the Y chromosome does not
537 represent a genetic barrier for gene flow between two members of the *A. gambiae* species
538 complex (*A. gambiae* and *A. arabiensis*) should inform the design and implementation of
539 genetic control interventions based on transgenic mosquitoes.

540 **Figure Legends**

541

542 **Figure 1.** F0xF1 hybrid crossing scheme and resulting progeny. **(A)** Observed number of
543 eggs and progeny arising for each indicated cross where the F1 hybrid females had either an
544 *A. gambiae* mother (top) or an *A. arabiensis* mother (bottom). The asterisk indicates strain
545 G^{Y1} or G^{Y2} respectively. In the bottleneck generation (F3) males were crossed to either pure
546 species *arabiensis* females ($\text{♀}A$) or crossed to female hybrids with decreasing levels of *A.*
547 *gambiae* genome content ($\text{♀}H_{(F2)}$, $\text{♀}H_{(F3)}$). **(B)** Crossing scheme indicating the *A. gambiae*
548 (red) or *A. arabiensis* (white) genomic contributions in generations F1 to F3 with autosomes
549 represented as a single pair labeled A. In the F1 cross *A. gambiae* males of strains G^{Y1} or G^{Y2}
550 are crossed to hybrid F1 females. Each generation the resulting males are backcrossed to *A.*
551 *arabiensis* wild-type females. In the bottleneck generation F3 hybrid males harbour an *A.*
552 *arabiensis* X with a fraction (~25% on average) of autosomal regions expected to be
553 homozygous for the *arabiensis* background (red arrow).

554

555 **Figure 2.** Single copula mating experiments. **(A)** Number of females ovipositing following
556 single matings with A or A^{Y2} males. Counts of eggs **(B)** and hatching larvae **(C)** for each
557 individual family.

558

559 **Figure 3.** Analysis of the content of the introgressed Y chromosome. The plots show the
560 number of normalized reads mapping to the *A. gambiae* Y chromosome reference loci
561 calculated as \log_{10} transformed FPKM values for A^{Y2} males on the x-axis compared to either
562 wild type *A. arabiensis* males (top left panel), wild-type *A. gambiae* males (top right panel),
563 wild-type *A. arabiensis* females (bottom left panel) and *A. gambiae* females (bottom right
564 panel) on the y-axis. The dashed linear regression line and associated r^2 coefficient indicate

565 the best correlation in read counts of signature Y elements between A^{Y2} and *A. gambiae*
566 *males*.

567

568 **Figure 4.** Differential expression analysis by RNA-seq. Volcano plots showing log₂ fold-
569 change values (x-axis) by -log₁₀ corrected p-values (y-axis) for all transcripts between
570 introgressed A^{Y2} males and *A. arabiensis* control males. The analysis was performed
571 separately for the head (left panel), the carcass (middle panel) and the abdominal segments
572 containing the reproductive tract (right panel). Transcripts derived from *A. arabiensis*
573 scaffolds are represented as circles and colored based on the percentage of their sequence
574 masked by sequences in the *A. gambiae* repeats library. Transcripts from the reference set of
575 *A. gambiae* Y loci are indicated by purple triangles and the name of the signature locus.
576 Dashed lines represent the thresholds used for adjusted p-value ($p < 0.05$) and log₂ fold
577 change (>1.0).

578

579 **Figure 5.** Mating competition experiments. The genotypes of the females and the two types
580 of competing males is indicated at the top for each experiment. The second, third and fourth
581 row of panels show the number of eggs laid by individual females, the hatching rate for each
582 family as well as the ratio of transgenic to wild-type larvae respectively. P-values were
583 calculated using Welch's two-tailed t-test.

584

585 **Table 1.** Analysis of single nucleotide polymorphisms between A and A^{Y2} males

586

587 **Supplementary Table 1.** F1 hybrid male crosses and observed progeny. G: *A. gambiae*; A:
588 *A. arabiensis*; H: hybrid; A^{Y2}: introgressed strain.

589

590 **Supplementary Figure 1.** Allele frequency and position of biallelic SNPs predicted between
591 2 wild-type *A. arabiensis* and 10 A^{Y2} males within the Y_unplaced sequence collection.
592 Dashed lines indicate breaks between Y_unplaced sequence contigs whose order is not
593 continuous. The black triangle indicates the presumed location of the transgene construct.

594

595 **Supplementary Figure 2.** Genome-wide read density maps showing the mean number of
596 reads of the introgressed (7 fertile A^{Y2} males) and *A. arabiensis* control groups (2 A males).
597 The x-axis represents the chromosomal position and the y-axis the normalized number of
598 reads per 5kb sliding window with 2.5kb overlaps for a maximum of 10⁶ reads per window.
599 Only uniquely mapping reads and those lacking mismatches were considered for this
600 analysis. The panels show the five major chromosome arms in the *A. gambiae* genome
601 assembly plus the UNKN and Y_unplaced sequence collections. The black arrow indicates
602 the presumed location of the transgene construct within the Y_unplaced collection.

603

604 **Supplementary Figure 3.** Violin plot showing the kmer abundance analysis for 6 *Anopheles*
605 *gambiae* Y-linked DNA satellites represented as normalized log₁₀ transformed count values
606 in males (blue) and females (red) of control and introgressed strains.

607

608 **Supplementary Figure 4.** (A) Heatmaps of all RNAseq sample libraries based on the
609 Euclidean distance of each library summed across all transcripts. (B) Distribution of gene-
610 level FPKM expression values across the experimental groups and the 3 tissues analyzed.

611

612 **Supplementary Figure 5.** RNA expression levels from *Anopheles gambiae* Y signature
613 elements as fragments per kilobase of element per million fragments mapped (FPKM) values
614 represented as log₁₀ transformed FPKM values for males of the introgressed (A^{Y2}) and

615 control groups (A) calculated for each of the analyzed tissues.

616

617 **Supplementary Figure 6.** Analysis of small RNAs. The left panels show MA plot
618 representations of the expression of small non-coding RNAs. The analysis was performed
619 separately for the head (A), the carcass (B) and the segments containing the male
620 reproductive tract (C). No small RNA locus passed the significance (adjusted p-value <0.05)
621 and log₂ fold change (>1.0) threshold that would indicate differential expression between
622 introgressed and control samples. The right panels indicate the size distribution of small
623 RNAs analyzed on the x-axis and the number of unique RNA species identified of each
624 length is shown on the y-axis. The color of the bars indicates the total number of reads for all
625 the RNA species in each bin as defined in the legends.

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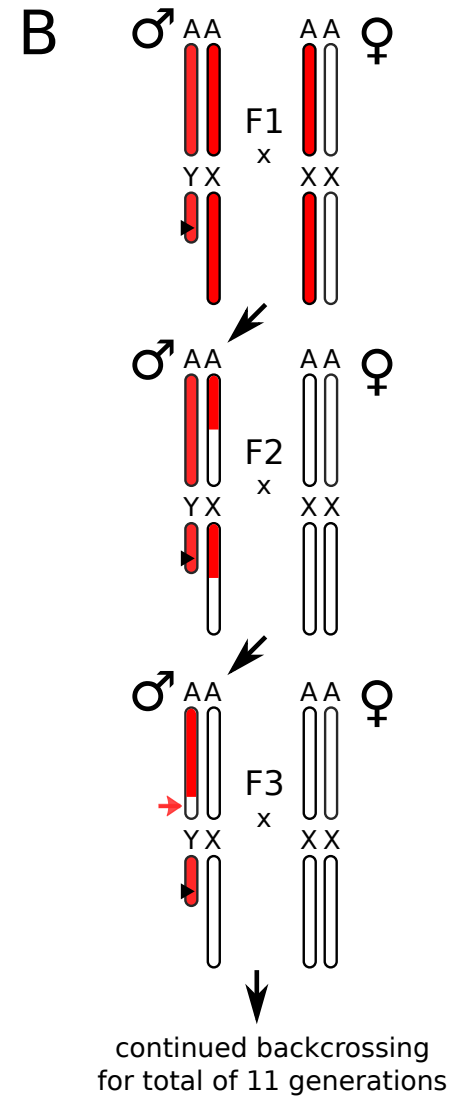
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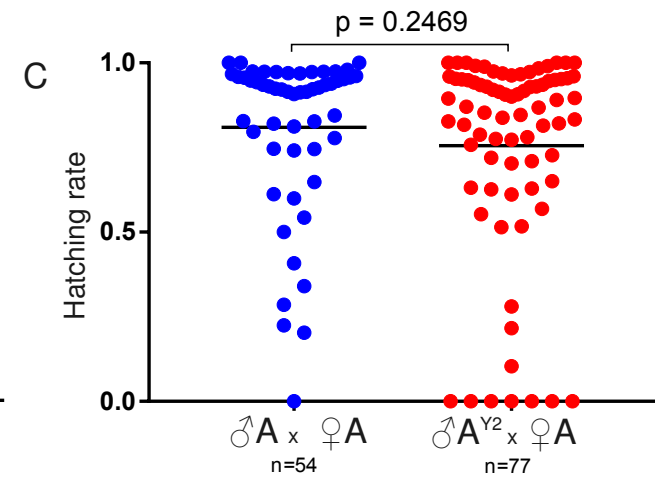
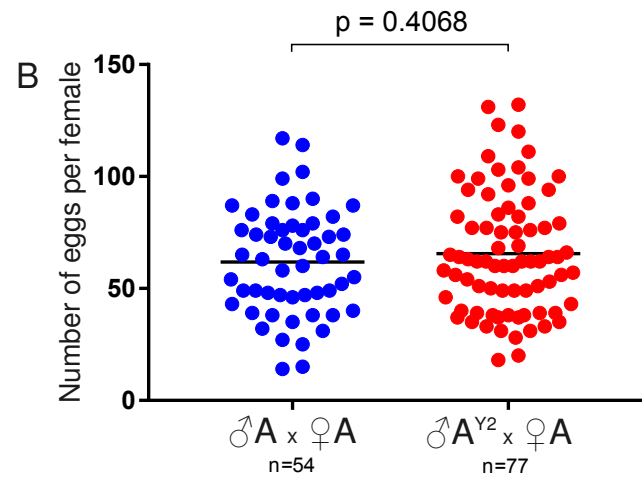
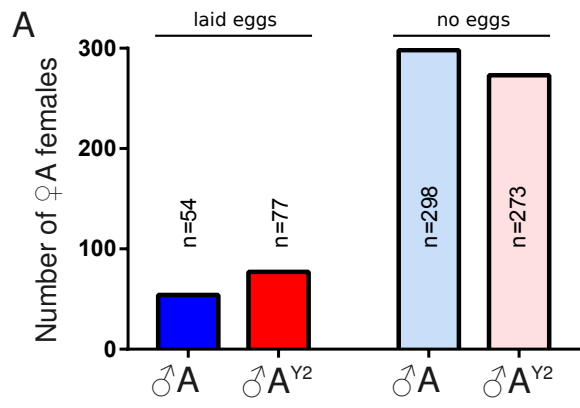
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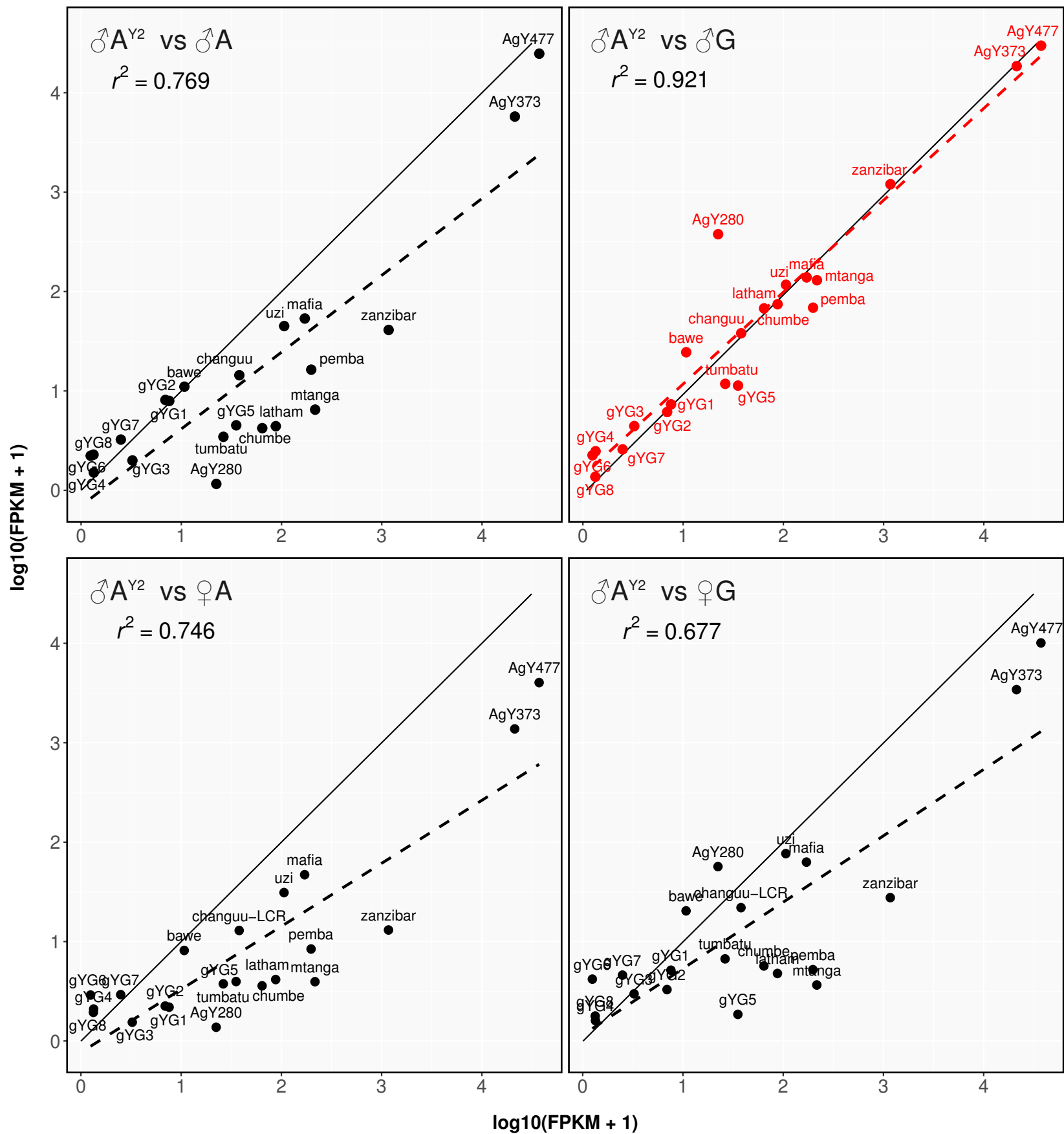
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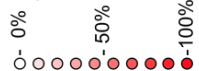
				strain G ^{Y1}		strain G ^{Y2}	
				eggs	progeny	eggs	progeny
	♂A	X	♀G		>2000		>2000
F1	♂G*	X	♀H _(A/G)		>2000		>2000
F2	♂H*	X	♀A		>2000		>2000
F3	♂H*	X	♀A	408	0	827	0
		X	♀H _(F2)	300	0	3347	0
		X	♀H _(F3)	3344	0	5210	0
	♂G	X	♀A		>2000		>2000
F1	♂G*	X	♀H _(G/A)		>2000		>2000
F2	♂H*	X	♀A		>2000		>2000
F3	♂H*	X	♀A	400	0	2095	3♂,4♀
		X	♀H _(F2)	6992	13♂,21♀	2795	0
		X	♀H _(F3)	2638	0	1420	7♂,8♀





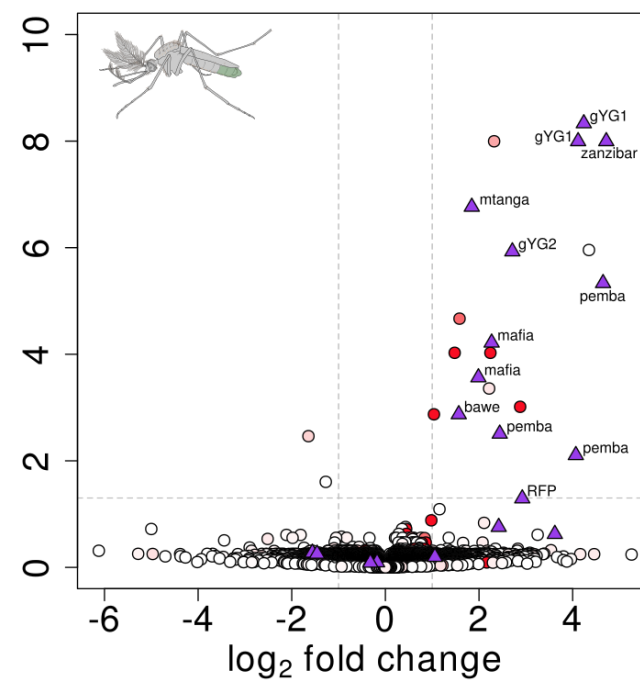
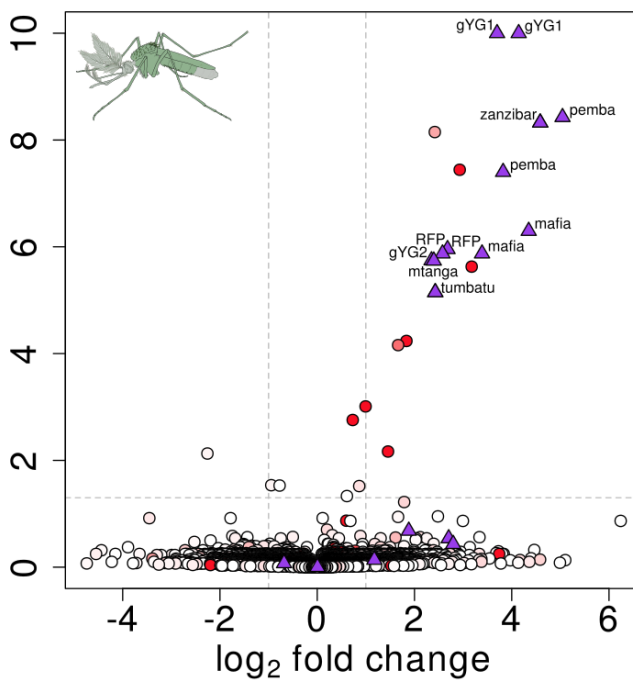
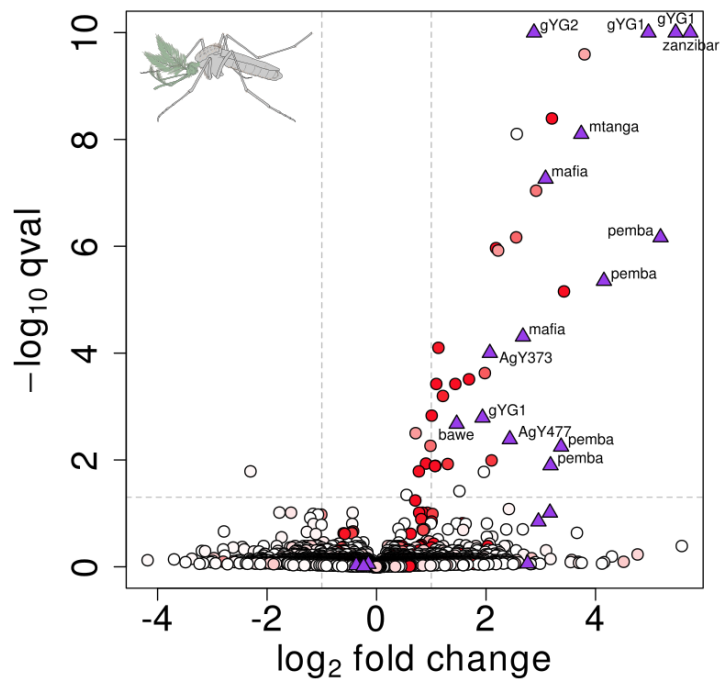


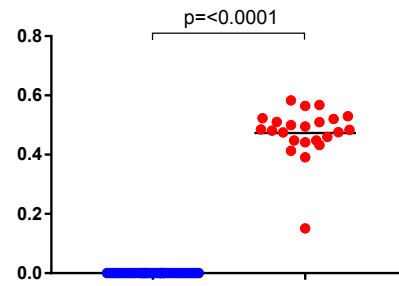
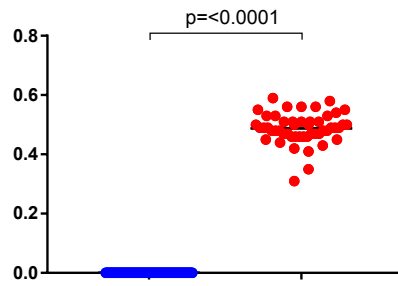
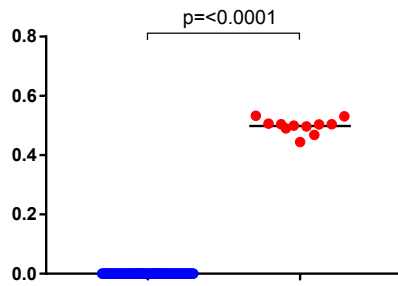
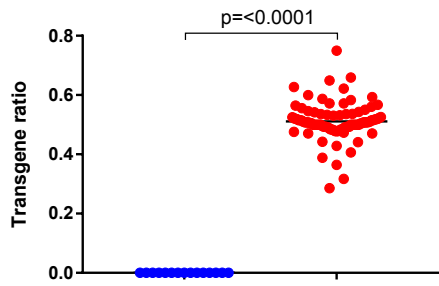
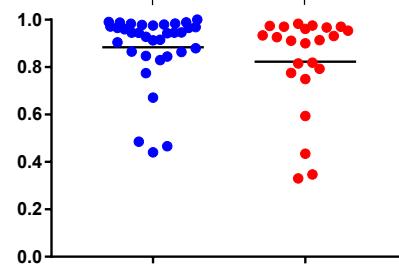
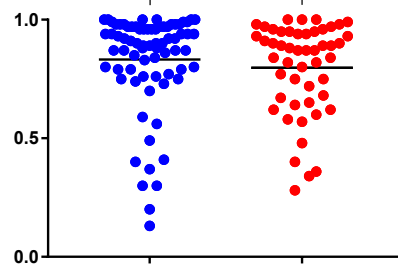
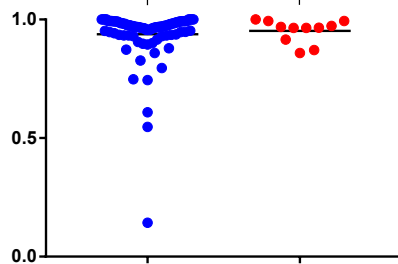
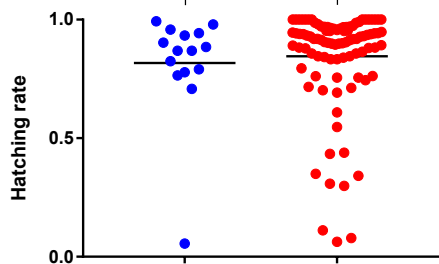
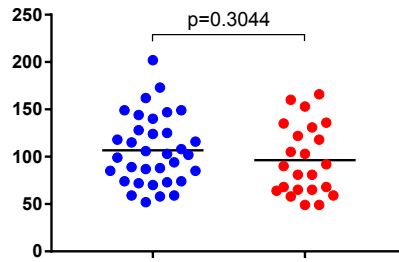
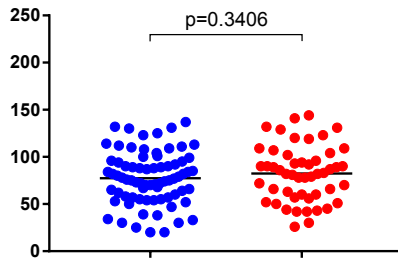
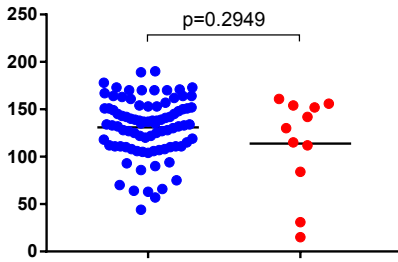
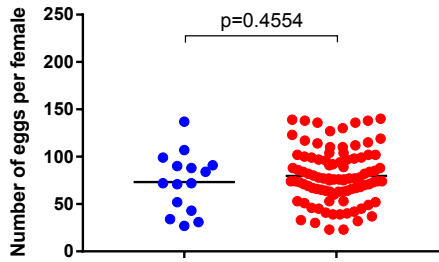
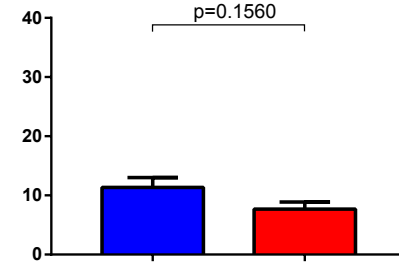
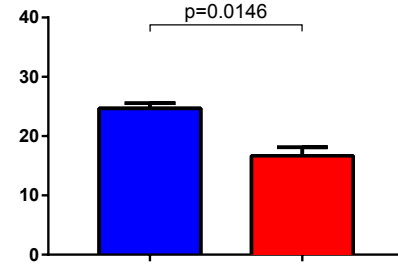
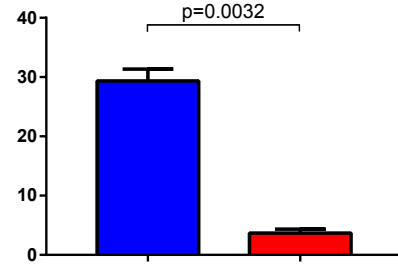
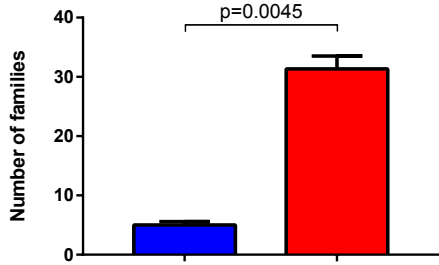
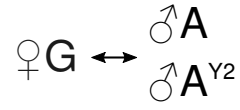
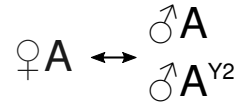
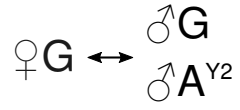
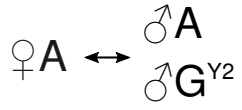
percent
repeat-masked



○ transcripts mapping to arabiensis contigs

▲ transcripts mapping to Y reference contigs (label)





♂A n=15 ♂G^{Y2} n=94

♂G n=88 ♂A^{Y2} n=11

♂A n=74 ♂A^{Y2} n=50

♂A n=34 ♂A^{Y2} n=23

chromosome	base pairs	total number of variants	biallelic SNPs w/o missing data	differentially fixed SNPs	
				number	%
X	24393108	1040639	583061	27	0.0046
2L	49364325	2180463	1464616	42	0.0029
2R	61545105	2233941	1573367	12	0.0008
3L	41963435	1702574	1159627	27	0.0023
3R	53200684	2480254	1653367	6	0.0004
Mt	15363	23	21	0	0
Unknown	42389979	749098	351870	115	0.0327
Y_unplaced	237045	4180	1902	677	35.5941