Premeiotic and meiotic failures lead to hybrid male sterility in the *Anopheles gambiae* complex

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Abstract

Background: Hybrid male sterility contributes to speciation by restricting gene flow between related taxa at the beginning stages of postzygotic isolation. The genetic factors, cellular basis, and molecular mechanisms of hybrid male sterility are of considerable interest as they inform our understanding of both speciation and normal fertility function. However, we have limited knowledge about precise cellular phenotypes that come into play when fertility is first affected. Hybrids between closely related species of the *Anopheles gambiae* complex offer opportunities to identify spermatogenic errors that arise early during speciation.

Results: To investigate possible cellular causes of hybrid male sterility, we performed crosses between sibling species of the *An. gambiae* complex. Our results demonstrate that testes are

severely underdeveloped in hybrids between male *An. merus* and female *An. gambiae* or *An. coluzzii*. No meiotic chromosomes are identified and suppressed expression of germline-specific genes *β2-tubulin*, *Ams*, *mts*, and *Dzip1l* is observed in these hybrid males. However, testes have nearly normal morphologies and sizes but produce mostly nonmotile spermatozoa in hybrids from the reciprocal crosses. Using chromosome X- and Y-specific fluorescent probes, we followed the process of meiosis in each species and their F1 hybrids between female *An. merus* and male *An. gambiae* or *An. coluzzii*. Unlike for pure species, sex chromosomes in meiotic prophase I of F1 hybrids are largely unpaired and all chromosomes show various degrees of insufficient condensation. Instead of entering the reductional division in meiosis I, primary spermatocytes undergo an equational mitotic division producing abnormal diploid sperm. Meiotic chromosomes of some F1 hybrid individuals are involved in *de novo* genome rearrangements. Yet, the germline-specific genes *β2-tubulin*, *Ams*, *mts*, and *Dzip1l* express normally in these hybrid males.

Conclusions: Our study demonstrates that hybrid male sterility in the malaria mosquitoes involves two defects in reciprocal crosses: premeiotic arrest in germline stem cells and the failure of the reductional meiotic division in primary spermatocytes. We identified cytogenetic errors in hybrids that arise during the early stages of postzygotic isolation. This knowledge will inform the development of innovative mosquito control strategies based on population suppression by manipulating reproduction via genetic technologies.

Keywords: *Anopheles*, chiasmata, chromosome pairing, gene expression, hybrid male sterility, meiosis, microscopy, mitosis, mosquito, speciation, spermatogenesis.

Background

Hybrid male fertility is among the first phenotypes affected as the postzygotic isolation between species is being established [1]. Therefore, genetic factors, cellular basis, and molecular mechanisms of hybrid male sterility are of considerable interest, as they inform our understanding of both speciation and normal fertility function. Genetic loci responsible for hybrid male sterility in plants and animals are often being identified using quantitative trait loci (QTL) mapping [1-8]. In a variety of organisms, hybrid sterility can be caused by the genetic factors on the X chromosome or by epistasis between X-linked and autosomal alleles [4, 8-15]. Studies in animals commonly assess testis shape, size or weight, as well as sperm morphology, density or motility to define male sterility phenotypes. However, a cellular basis of hybrid male sterility is rarely investigated. Cytological studies of spermatogenesis in sterile hybrids indicate that multiple mechanisms of functional sterility are possible. Sterile hybrids between *Mus musculus domesticus* and *M. m. musculus* have spermatogenic arrest in early meiosis I with disrupted both homoeologous chromosome pairing and meiotic sex chromosome inactivation [16, 17], as well as reductions in spermatocyte and spermatid number, increased apoptosis of primary spermatocytes, and more multinucleated syncytia [18]. Studies of failed spermatogenesis in different *Drosophila* hybrids observed arrests at the premeiotic stage [19], reduced chromosome pairing and unequal chromosome segregation in meiosis [20, 21], characteristic spermiogenic arrests [19], spermatid abnormalities [22], and problems in sperm bundling and motility [23, 24]. Detailed analyses of cellular and developmental abnormalities in testes of various hybrid organisms could help better define the causes of infertility. Cytological studies of spermatogenesis in hybrids may link different sterility phenotypes to the early or advance stages

of postzygotic isolation. Moreover, identification of first-evolved cytogenetic errors in hybrids between closely related species could improve our understanding of speciation.

The *Anopheles gambiae* complex consists of at least eight morphologically nearly indistinguishable sibling species of African malaria mosquitoes [25, 26]. Genome-based estimations of the age of the *An. gambiae* complex vary from 1.85 [27] to as young as 0.526 million years [28]. Genomic introgression has been found prevalent in autosomal regions of several species indicating naturally occuring interspecies hybridization [27, 28]. Experimental crosses of species from the *An. gambiae* complex often produce sterile F1 hybrid males confirming Haldane's rule of sterility or inviability of the heterogametic sex [13, 29-31]. Early crossing experiments between members of the complex have found that hybrid male sterility is associated with various degrees of atrophies of testes and underdevelopment of sperms [30]. Examination of hybrid sterility in backcrosses of *An. gambiae* and *An. arabiensis* using an X-linked white-eye marker demonstrated a large effect of the X chromosome on male hybrid sterility [32]. The large X effect on hybrid sterility has been supported with a QTL mapping approach [13]. However, an introgression of the Y chromosome from *An. gambiae* into a background of *An. arabiensis* has shown no apparent influence on male fertility, fitness, or gene expression [29]. A recent study of hybridization in nature has shown that postmating isolation is positively associated with ecological divergence of *An. coluzzii*, *An. gambiae*, and *An. arabiensis* [33]. However, the cellular basis and molecular mechanisms of hybrid male sterility in mosquitoes are unknown. Because of the recent evolution and ease of hybridization, sibling species of the *An. gambiae* complex offer great opportunities to provide insights into mechanisms of speciation.

A recent World Health Organization report has shown that after a successful period of global malaria control, progress has stalled since 2015. There are still 219 million cases and 435,000 related deaths from malaria without a tendency to decrease [34]. In the 60s-70s, the sterile insect technique (SIT) was one of the first methods used to control the malaria-transmitting *Anopheles* mosquitoes as it aimed to interfere with their reproduction by introducing sterile males into natural populations. However, because of the low competitiveness of X-ray-irradiated males and the lack of institutional commitments, the traditional SIT largely failed in controlling malaria mosquitoes [35]. Novel genetic approaches based on CRISPR-Cas9 gene drives [36-40] show potential toward the generation of self-sustainable and species-specific mosquito control strategies. A better understanding of the normal male fertility function and mechanisms of naturally occurring hybrid male sterility will inform the development of novel SIT tools and implementation of pre-existing technologies for the control of malaria-transmitting mosquitoes.

Here, we investigate possible cellular causes of male sterility in hybrids between sibling species of the *An. gambiae* complex. We asked three specific questions: (i) Which cellular processes are involved in causing infertility in hybrid mosquito males? (ii) Are the defects leading to hybrid male sterility premeiotic, meiotic, or postmeiotic? and (iii) What cytogenetic errors trigger the spermatogenic breakdown? We demonstrate that hybrid male sterility in malaria mosquitoes involves two cellular defects in reciprocal crosses: premeiotic arrest in germline stem cells and the failure of the reductional meiotic division in primary spermatocytes. Our data suggest that the meiotic abnormalities in hybrid males stem from the unpairing of the sex chromosomes and

chromatin decondensation. Thus, our study identified first cytogenetic errors in hybrids that arise during the early stages of postzygotic isolation.

Results

Hybrid male sterility phenotypes at the cellular level

To obtain sterile males, we performed reciprocal crosses between *An. merus* MAF and *An. gambiae* ZANU or *An. coluzzii* MOPTI and MALI. Backcrossing of F1 males to parental females resulted in no progeny demonstrating sterility of F1 hybrid males. This result confirms Haldane's rule for the majority of interspecies crosses in the *An. gambiae* complex, except for crosses between *An. gambiae* and *An. coluzzii*: F1 females are fertile while F1 males are sterile [13, 29-31, 41, 42]. To investigate the developmental phenotypes involved in hybrid sterility, we dissected testes from adult males obtained from interspecies crosses and from pure species. Normal testes of pure species have a spindle-like shape (Fig. 1A). We found obvious asymmetry in morphology and sizes of testes when reciprocal crosses were compared. Hybrid males from crosses between female *An. merus* and male *An. gambiae* or *An. coluzzii* display normal-like reproductive organs (Fig. 1B). In contrast, F1 males from crosses between female *An. gambiae* or *An. coluzzii* and male *An. merus* show severely underdeveloped testes (Fig. 1C). We then tested if normal-like and underdeveloped testes of interspecies hybrids produce any sperm. In squashed testes of 2-day-old adults of pure species, large amounts of mature spermatozoa with long tails can be seen. After we crushed the testes, spermatozoa with vibrant motility escaped from the ruptures (Fig. 1A, Additional file 1: Movie S1). However, mature sperm or sperm motility hardly could be seen in squashed or crushed normal-like testes of 2-day-old adult

hybrids from crosses when *An. merus* was the mother. Instead, we see delayed spermatid differentiation, fewer spermatids, and mostly nonmotile spermatozoa with large heads and often two short tails growing from opposite ends of the head (Fig. 1B, Additional file 2: Movie S2). Only undifferentiated round cells could be seen in underdeveloped testes of hybrids from reciprocal crosses when *An. merus* was the father (Fig. 1C). Given the small size of the degenerate testes, these round cells may represent germline stem cells. Thus, neither normal-like nor underdeveloped testes of the interspecies hybrids produce mature motile spermatozoa.

The normal progress of meiosis in males of pure species

Before we investigate meiosis of interspecies hybrids, we provide the first description of chromosome behavior in meiosis of pure *Anopheles* species. The chromosome complement of *Anopheles* males consists of three chromosome pairs: two autosomes (2 and 3) and X and Y sex chromosomes. With the help of sex-chromosome-specific fluorescent probes, we followed the progress of meiosis in *An. gambiae*, *An. coluzzii*, and *An. merus*. The following DNA sequences were used as the probes for fluorescence *in situ* hybridization (FISH). Retroelement *zanzibar* is specific to the Y chromosome of *An. gambiae* and *An. coluzzii* but is absent in *An. merus*; 18S ribosomal DNA (rDNA) labels only the X chromosome in *An. gambiae* or *An. coluzzii* but labels both X and Y chromosomes in *An. merus*; and satellite AgY53B [43] labels both X and Y chromosomes in all three species [44] (Table 1). Fig. 2 shows normal activities of meiotic chromosomes in testes of *An. gambiae* ZANU. In primary spermatocytes, all homologous autosomes and sex chromosomes pair and display chiasmata in diplotene/diakinesis of prophase I, they align with each other at the cell equator in metaphase I, and then move from each other in anaphase I. In secondary spermatocytes, sister chromatids of each chromosome align with each

other at the cell equator in metaphase II and go to opposite poles of the cell during anaphase II. Meiotic divisions produce spermatids that contain a haploid set of autosomes and either a Y or X chromosome. *An. coluzzii* males show similar morphology and behavior of chromosomes during meiosis (Additional file 3: Figure S1). Because retroelement *zanzibar* is absent in *An. merus*, we used satellite AgY53B and 18S rDNA to label sex chromosomes in this species. Each of these probes hybridized with both X and Y chromosomes in *An. merus*, making discrimination between X and Y more difficult in this species. However, we could differentiate metaphase sex chromosomes by the euchromatic arm of the X chromosome and by the slightly larger distal heterochromatic block of the Y chromosome (Additional file 4: Figure S2). Heterochromatic parts of the X and Y chromosomes are relatively large and structurally similar in *An. merus* in comparison with *An. gambiae* or *An. coluzzii*. In the latter two species, the heterochromatic parts of the X and Y chromosomes are relatively small and substantially different from each other both in size and genetic content [44]. Despite these differences between *An. merus* and either *An. gambiae* or *An. coluzzii*, the activities of meiotic sex chromosomes in testes are similar in all three species.

Table 1. Labeled oligos and primer sequences used for FISH and RT-PCR.

Sequence name	**GenBank accession number**	**VectorBase gene ID**	**Labeled oligos and primer sequences**	**Reference to oligo design**
18S rDNA	AM157179	AGAP028978	F: AACTGTGGAAAAGCCAGAGC	This study
			R: TCCACTTGATCCTTGCAAAA	
zanzibar	KP878482		F: TTCTTCGATGTTGTGCTGGA	[44]
			R: ATGGAGAAACAGGGCAACAA	
			F: ATGCATGCTTGGATTCCTTC	

			R: GGTTTCTATGATCGCCTGGA	
			F: TTGGCATTCATCTGTCCAAA	
			R: GCACCCTTGATCTCATGTCA	
AgY53B	AY754156		F: CCTTTAAACACATGCTCAAATT	[43]
			R: GTTTCTTCATCCTTAAAGCCTAG	
AgY477-AgY53B junction region	AY754151		TTCTAAGTTTCTAGGCTTTAAGGATG AAGAAACCGACTATTC[Cyanine5]	This study
Contig_240	SRS667972, SRR1509742, SRR1508169		CAATAAATTTCCTTTTTAATGATGCA AAATCTACGTCTCTAGC[Cyanine3]	This study
Tandem-42345	ERX008245		GGCTTCATTTGGATGAAACATGGCCG TAACAACGATAATAGAT[Cyanine3]	This study
vasa	XM_314684	AGAP008578	F: TTCTGCTGAGGTGCTTAGCG	This study
			R: CGTCTCCGCTCATGTTTCCT	
β2-tubulin	XM_314718	AGAP008622	F: GTACGTGCCGGATCATTTCG	This study
			R: GGCCAGTTTGCAAATGCACTA	
Ams	FJ869235	AGAP029148	F: CATACGGGAGGTGAGGAAAT	[45]
			R: CCCCTTCATGCTTCATCTT	
mts	FJ869236	AARA006451	F: TGGGATCCAAATTATTTCGTG	
			R: CTGTTCGGTTCAACAATGGA	
Dzip1l	FJ869237	AGAP001165	F: GGCCAAAGTGATACAAATTGTTT	
			R: CGTTTCCAATAGGGACTTCG	
AgS7	L20837	AGAP010592	F: AGAACCAGCAGACCACCATC	[46]
			R: GCTGCAAACTTCGGCTATTC	

Meiotic failures in F1 males of interspecies hybrids

To determine possible cytogenetic mechanisms of hybrid male sterility, we analyzed chromosome behavior in testes of hybrids from interspecies crosses. Meiotic chromosomes were present in normal-like testes of hybrids from the ♀*An. merus* × ♂*An. coluzzii/An. gambiae* crosses and we identified important abnormalities of meiosis in these males (Fig. 3). In primary spermatocytes, homoeologous autosomes pair and form chiasmata in prophase I as in pure species, but X and Y chromosomes do not display chiasmata in diplotene/diakinesis of prophase I. We found this pattern consistent in all analyzed hybrid males. In hybrids, metaphase chromosomes are visibly longer than at the same stage in pure species, indicating insufficient chromatin condensation. Besides, homoeologous chromosomes in hybrids do not segregate during anaphase. Instead, sister chromatids move to opposite poles of the dividing cell. Because reductional division does not occur in hybrid males, both X and Y chromatids move to the same pole during anaphase. As a result, haploid secondary spermatocytes do not form in these males. Our FISH analysis demonstrated that each spermatid in testes of pure species normally contains either an X or Y chromosome. In contrast, we found that both X and Y chromosomes present in each spermatid of the hybrids (Additional file 5: Figure S3). Moreover, the abnormal spermatids are larger in size due to insufficient chromatin condensation and the double chromosome content. Thus, we discovered that chromosomes in normal-like testes of hybrid males start with a meiotic behavior in prophase and then switch to a mitotic behavior in anaphase. The equational division of primary spermatocytes results in dysfunctional diploid sperm in hybrids if *An. merus* is the mother. In contrast, degenerate testes of F1s from the reciprocal ♀*An. coluzzii/gambiae* × ♂*An. merus* crosses have only undifferentiated round germline stem cells. To visualize sex

chromosomes, we performed whole-mount FISH with labeled 18S rDNA (Cy3) and satellite AgY53B (Cy5) to mark the sex chromosomes of the hybrid (Additional file 6: Figure S4). Only interphase sex chromosomes in nuclei of germline stem cells are detected indicating that meiosis does not start in the underdeveloped testes of F1 hybrids if *An. merus* is the father. Thus, the premeiotic arrest in the degenerate testes is the reason for the lack of spermatids in these hybrids.

Chromosomal and molecular abnormalities in interspecies hybrids

Here, we performed quantitative analyses of chromatin condensation and the X-Y chromosome pairing in pure species and their hybrids. We also tested if germline-specific genes express in sterile male hybrids from the reciprocal crosses. To determine the extent of chromatin condensation in normal-like testes of interspecies hybrids in comparison with pure species, we measured the lengths of metaphase chromosomes (Additional file 7: Table S1). The results of a statistical analysis with a two-sample pooled *t*-test show that chromosomes in F1 hybrids are typically longer than chromosomes in *An. coluzzii*, *An. gambiae*, or *An. merus* (Fig. 4A, Additional file 8: Figure S5). For example, chromosomes of the *An. merus* origin in a hybrid background always show significant ($P<0.001$) elongation by at least 1.3 times in comparison with pure *An. merus*. The X chromosome of *An. merus* suffered the most serious undercondensation in hybrids exceeding the length of the X chromosome in the pure species background by 1.6-1.9-fold.

In our cytogenetic study of interspecies hybrids from the crosses ♀*An. merus* × ♂*An. coluzzii/An. gambiae*, the X and Y chromosomes do not show pairing or chiasmata in diplotene/diakinesis of prophase I (Fig. 3). We hypothesized that sex chromosome pairing is affected in the early

prophase I when individual chromosomes cannot be distinguished by direct visualization. To analyze the X-Y chromosome pairing at the pachytene stage of prophase I, we performed a whole-mount FISH and examined spatial positions of the X- and Y-specific fluorescent signals in confocal optical sections of nuclei in testes of pure species and their hybrids (Additional file 9: Figure S6). The *An. merus* X chromosomes were labeled with 18S rDNA (Cy3) and a satellite from Contig_240 (Cy3), while the *An. coluzzii* MOPTI Y chromosomes were labeled with satellite AgY53B (Cy5) and satellite AgY477-AgY53B junction region (Cy5). We recorded the number of nuclei with X and Y fluorescent signals colocalized versus X and Y fluorescent signals located separately (Fig. 4B). The results of a statistical analysis with a two-sample pooled *t*-test demonstrate that X and Y chromosomes pair in more than 90% of primary spermatocytes in the *An. coluzzii*, while they pair in less than 30% of primary spermatocytes in F1 hybrids of ♀*An. merus* × ♂*An. coluzzii* (Fig. 4C).

To test if premeiotic or meiotic failures are associated with the misexpression of germline-specific genes, we analyzed the presence of the postmitotic germline transcripts *Ams*, *mts*, *Dzip1l* [45], and *β2-tubulin* [47, 48] in F1 hybrid males (Table 1). The reverse transcription polymerase chain reaction (RT-PCR) results show that these genes express at similar levels in the reproductive tissues of *An. coluzzii* and F1 hybrids from the ♀*An. merus* × ♂*An. coluzzii* MOPTI cross (Fig. 4D). However, *Ams*, *mts*, *Dzip1l*, and *β2-tubulin* are strongly down regulated in gonads of the ♀*An. coluzzii* MOPTI × ♂*An. merus* F1 hybrids, supporting our observation that meiosis does not occur in these hybrids. In contrast, a pre-meiotic gene, *vasa* [49] (Table 1), expresses at similar levels in reproductive tissues of all hybrids and pure species, indicating that germline stem cells present even in degenerate testes of interspecies hybrids.

Besides the abnormalities that affect all progeny of the ♀*An. merus* × ♂*An. coluzzii/An. gambiae* crosses, chromosomes of some hybrid individuals display structural abnormalities. In several cases, chromosomes were involved in *de novo* genome rearrangements (Fig. 5). For example, a large fragment of the *An. merus* X chromosome, including the rDNA locus, was translocated to the 2L arm of *An. coluzzii* in the male hybrid from the ♀*An. merus* × ♂*An. coluzzii* MALI cross (Fig. 5A). In addition, we detected a duplication of a chromosomal segment involving the rDNA locus within the X chromosome of *An. merus* in the ♀*An. merus* × ♂*An. gambiae* ZANU cross (Fig. 5B). These rearrangements were not observed in pure species, and their occurrence in F1 hybrid males suggests an increased genome instability as a result of interspecies hybridization. In addition, the *An. merus* X chromosomes in the hybrid karyotype can be involved in formation of chromatin bridges during the abnormal anaphase (Additional file 10: Figure S7). Finally, we noticed that heterochromatic regions of hybrid males have elevated fragility compared with heterochromatic regions of pure species males. When squashed chromosome preparations are made, pericentromeric heterochromatin of meiotic chromosomes in the ♀*An. merus* × ♂*An. coluzzii* MOPTI hybrids, unlike pure species, can be stretched in prophase and uncoiled in metaphase due to normal mechanical pressure (Additional file 11: Figure S8), suggesting malfunctioning of heterochromatin proteins.

Discussion

Cellular and molecular defects involved in hybrid male sterility

In this study, we performed the first detailed cytological analysis of spermatogenesis in pure species and hybrids of mosquitoes. Hybrid male sterility phenotypes in the *An. gambiae* complex

are clearly asymmetric between the reciprocal crosses (Fig. 6), as has been demonstrated earlier for mosquitoes and other animals [8, 20, 30, 50]. The observed cellular and molecular abnormalities suggest the following developmental scenarios in testes of the interspecies hybrids. In hybrids from crosses with *An. merus* as the father, mitotic divisions of germline cells are impaired, no meiosis occurred, and expression of postmitotic germline-specific genes is repressed (Fig. 4D). As a result, these hybrids have degenerate testes with arrested germline stem cells. Degenerate testes have also been observed in F1 hybrids between sibling species of the *An. albitarsis* [51] and the *An. barbirostris* [52] complexes. However, in hybrids from the reciprocal crosses with *An. merus* as the mother, mitotic divisions of germ cells occur as normally as in pure species. Moreover, germ cells in these hybrids go through meiotic prophase I as evidenced from our cytogenetic analyses (Fig. 3) and expression of the postmitotic germline genes (Fig. 4D). However, the downstream developmental processes in these hybrids are impaired. The abnormalities begin in meiosis I where homoeologous chromosomes fail to segregate. Testes of these hybrids show delayed spermatid differentiation, a smaller number of these cells, and eventual formation of immotile abnormal (including two-tailed) sperm with insufficient chromatin condensation (Fig. 6). Thus, we demonstrate that premeiotic and meiotic defects are involved in hybrid male sterility in malaria mosquitoes. This observation is at odds with the commonly accepted view that more often hybrid males suffer postmeiotic sterility problems [1, 2]. Although many postmeiotic defects seen in *Drosophila* hybrids are indeed related to problems in sperm bundling and motility [2, 19, 23], some sperm abnormalities may stem from meiotic failures. For example, our results demonstrate that such sperm abnormalities as nonmotility, two-tailed heads, and chromatin decompaction in sperm heads result from the impaired meiosis I. However, very few studies have been devoted to the detailed cytological

investigation of meiosis in interspecies hybrids, especially in dipteran insects. For the first time, Theodosius Dobzhansky described pronounced defects in meiosis I in sterile male hybrids from the ♀*D. pseudoobscura* × ♂*D. persimilis* cross [20, 21]. Also, a histological investigation of spermatogenesis in hybrid mice identified defects in meiosis I as a primary barrier to reproduction [18]. Additional studies of interspecies hybrids of various organisms should determine if the first meiotic division commonly fails when fertility is affected.

Cytogenetic mechanisms of hybrid male sterility

Our cytogenetic investigation of meiosis in pure *Anopheles* species revealed that the X and Y chromosomes normally pair tightly with each other throughout prophase I (Fig.s 2, 4BC, and Additional files 3, 4). Sex chromosomes in malaria mosquito males genetically recombine [44] highlighting an important difference from male meiosis in fruit flies [53-55]. As a result of the recombination, some mosquito species, such as *An. merus*, have homomorphic heterochromatic parts in their X and Y chromosomes (Additional file 4: Figure S2). Future sequencing and assembly of the heterochromatin in malaria mosquitoes using long reads may yield important insights into structural and functional organization of the sex chromosomes, as it has been demonstrated for *Drosophila* [56].

Unlike pure species, the meiotic prophase I in F1 mosquito hybrids shows cytogenetic anomalies—low percentage of the X-Y chromosome pairing and insufficient chromatin condensation (Fig. 7). Disruption of chromosome pairing and synapsis is frequently observed in interspecies hybrids of various organisms. Similar to the mosquitoes, pairing of X and Y chromosomes is more adversely affected than that of autosomes during the prophase I in male

hybrids between Campbell's dwarf hamster and the Djungarian hamster [57]. A recent work has clarified that the autosomes of male hybrids between these hamster species undergo paring and recombination as normally as their parental forms do, but the heterochromatic arms of the X and Y chromosomes show a high frequency of asynapsis and recombination failure [58]. Another study has demonstrated a high rate of synaptic aberrations in multiple chromosomes of male hybrids between two chromosome races of the common shrew [59]. It has been proposed that asynapsis of heterospecific chromosomes in prophase I may provide a recurrently evolving trigger for the meiotic arrest of interspecific F1 hybrids of mice [8, 16].

The normal mechanics of meiotic segregation require that homologous chromosome pairs with each other because crossovers ensure their proper orientation on the meiotic spindle [60]. All chromosomes must achieve synapsis in pachynema, complete DNA repair, and disassemble their synaptonemal complexes in diplonema as homologous chromosomes prepare to segregate [61]. The presence of chiasmata and tension exerted across homologs ensures that cells undergo reductional segregation [62]. Whenever it occurs, an asynapsis of chromosomes almost invariably triggers pachytene checkpoint and meiotic breakdown [63]. The genetic determinants of chromosome pairing and synapsis in both plants and animals are of great interest. In bread wheat, the *Ph1* locus has the largest effect on preventing homeologous pairing in meiosis [64]. In mice, a null mutation in the *PRDM9* gene causes chromosome asynapsis, arrest of spermatogenesis at pachynema, impairment of double-strand break repair, and disrupted sex-body formation [65]. The *PRDM9*-null phenotype resembles observed univalents and frequent X-Y dissociation in interspecies hybrids of mice [17]. In fact, infertility in male hybrids has been rescued with bacterial artificial chromosomes (BACs) carrying the *PRDM9* gene from a strain

with the “fertility” allele [66]. In mice hybrids, *PRDM9*-mediated meiotic arrest causes sterility by a mechanism which relies on the DNA binding properties of PRDM9 [67]. A recent study has demonstrated a genetic link between meiotic recombination and hybrid male sterility [12]. It has been suggested that within species there may be selection to maintain sequence homozygosity for meiosis genes because their divergence can lead to reproductive isolation through failures of double-strand break formation and synapsis in hybrids [64].

Our data from male hybrids of the crosses ♀ *An. merus* × ♂ *An. coluzzii/An. gambiae* suggest that meiosis is unaffected until diplonema when autosomes pair and form chiasmata. However, the downstream processes of meiosis in F1 hybrids are abnormal probably due to the failure of X and Y chromosomes to synapse. It is possible that the absence of chiasmata between the sex chromosomes causes failure of reductional segregation in the mosquito hybrids. Unlike hybrids of mice, primary spermatocytes in the mosquito hybrids do not undergo arrest in meiosis I. Instead, they continue dividing by mitosis, in which sister chromatids move to the opposite poles of the dividing cell (Fig. 7). This meiosis-mitosis switch is likely caused by the bi-orientation instead of the normal mono-orientation of sister kinetochores, in which tension across the centromere regions and proteins shugoshins plays a key role [53, 68, 69]. It is known that kinetochore-microtubules attachment errors are more commonly found in meiosis I than in mitosis [70, 71]. A mathematical model explains why kinetochore-microtubules attachment errors occur more frequently in the first meiotic division than in mitosis. The model suggests that the gradual increase of microtubules may help turn off the spindle assembly checkpoint in meiosis I leading to chromosome mis-segregation errors [72]. In mosquito hybrids, change in the orientation of sister kinetochores may be caused by reduced tension across the centromeres of

homologous chromosomes due to chromatin decompaction. Changes in chromatin condensation have been documented in interspecies hybrids of diverse groups of organisms [73, 74]. In a study of F1 hybrids between *Arabidopsis thaliana* and *A. lyrata*, the *A. thaliana* chromatin became more compact, whereas the *A. lyrata* chromatin became moderately less compact [74]. An intriguing spatial arrangement model suggests that that Y chromosome-linked variation may alter spatial position and packaging of other chromosomes in the nucleus [75]. Chromosome condensation and spatial position could also be affected in mosquito hybrids by improper function of condensins. A study of *Drosophila* male meiosis demonstrated that condensin II subunits, Cap-H2 and Cap-D3, are required to promote chromosome territory formation in primary spermatocyte nuclei. Moreover, anaphase I is abnormal in Cap-H2 mutants as chromatin bridges are formed between segregating heterologous and homologous chromosomes [76]. Thus, the interplay between the two phenotypes in mosquito hybrids—sex chromosome unpairing and chromatin decompaction—may result in failing a reductional meiotic division and proceeding to an equational mitotic division.

Evolution of spermatogenesis errors in interspecies hybrids

Reciprocal crosses in malaria mosquitoes and other organisms usually produce hybrids with sterility phenotypes of different degrees of severity [20, 30, 50]. This observation suggests that multiple stages of postzygotic isolation exist based on malfunction of the spermatogenesis. At the early stages of evolution, germline-specific genes still express normally and meiosis proceeds until metaphase I and then switches to an equational anaphase producing diploid sperm cells in hybrids. In this case, meiotic errors are characterized by the dramatically decreased pairing between sex chromosomes and insufficient chromatin condensation, which are seen in sterile

hybrids from the ♀*An. merus* × ♂*An. coluzzii/An. gambiae* crosses (Fig.s 3, 7). The observed incidental chromosome rearrangements in mosquito hybrids (Fig. 5) support the notion that genome instability is a common characteristic trait of hybrid incompatibility that may be associated with increased transposable element activity, ectopic recombination, and double-strand DNA breaks [77]. A recent work demonstrated that biogenesis of Piwi-interacting RNA (piRNA) is enhanced in testes of hybrids between *Drosophila buzzatii* and *D. koepferae*. The study argues that interspecies hybridization causes a genomic stress that can activate the piRNA response pathway to counteract transposable element deregulation [78]. Because piRNAs predominantly target long terminal repeats (LTR) retrotransposons in both *Anopheles* and *Drosophila* [79], future studies of the piRNA expression in mosquito hybrids may identify common and specific responses to the genomic stress in dipteran insect species. As species continue to diverge, meiotic errors become more prominent, and new hybrid phenotypes appear as has been seen, for example, in sterile male hybrids from the ♀*D. pseudoobscura* × ♂*D. persimilis* cross [20, 21]. At this stage of postzygotic isolation, an abnormal anaphase is characterized by unequal segregation of chromosomes resulting in spermatids with unbalanced chromosome content. The more advanced interspecies divergence of meiosis is manifested by the unpairing of the most chromosomes and by the malfunction of the abnormally elongated spindle in sterile male hybrids [20, 21]. The next stage of evolution is spermatogenic arrest in early meiosis I with disrupted homoeologous chromosome pairing, meiotic sex chromosome inactivation, and increased apoptosis of spermatocytes as seen in mice hybrids [16-18]. Interestingly, chromosome unpairing and asynapsis in male hybrids stand out as common spermatogenic phenotypes associated with sterility. It has been hypothesized that nongenic repetitive sequences, as the fastest diverging component of the genome, may facilitate asynapsis

in hybrids, thus, representing suitable candidates for a Dobzhansky-Muller incompatibility [16]. Findings in *Drosophila* hybrids demonstrate that rapid evolution of heterochromatin may indeed result in hybrid incompatibilities [73, 77, 80, 81]. Also, the centromere drive model has been proposed to explain how paired chromosomes at meiosis I can be subject to nondisjunction leading to infertility in *Drosophila* males [82, 83]. Accordingly, incompatibilities between rapidly evolving centromeric components of emerging species, such as co-evolving the CENP-A histone variant and its chaperone CAL1 [84], may account for species incompatibility between centromeric histones and for postzygotic reproductive isolation. Finally, spermatogenic abnormalities in hybrids can happen even before meiosis starts. A spermatogenic arrest at the premeiotic stage is characterized by the repression of germline-specific genes and by the lack of spermatocytes or spermatids in degenerate testes of hybrid males. These phenotypes have been observed in sterile hybrids from the ♀*D. mauritiana* × ♂*D. sechellia* cross [19] and in sterile hybrids from the ♀*An. coluzzii/An. gambiae* × ♂*An. merus* crosses (our study). Thus, cytological analyses of spermatogenesis in interspecies hybrids can associate sterility phenotypes with early or advance stages of speciation. Such studies of interspecies hybrids from diverse groups of organisms may highlight general patterns and mechanisms in the origin and evolution of postzygotic isolation.

Conclusions

Hybrid male sterility contributes to speciation by restricting gene flow between related taxa at the beginning stages of postzygotic isolation. Charles Darwin in the chapter "Hybridism" of his *Origin of Species* rightly argued that hybrid sterility ‘‘is not a specially endowed quality, but is incidental on other acquired differences [85].’’ Identification of the cellular and molecular

differences acquired during the early stages of postzygotic isolation between species is crucial to explaining both speciation and normal fertility function. Studies of sterility in male hybrids between recently evolved species offer opportunities to identify developmental errors that arise early in speciation. The cross between a female *An. merus* and a male *An. gambiae* or *An. coluzzii* produces sterile hybrid males with spermatogenic abnormalities in the first meiotic division. Our study uncovered differences in chromosome behaviors between pure *Anopheles* species and their hybrids. The obtained data suggest that the meiotic abnormalities in hybrid males stem from the unpairing of the sex chromosomes and chromatin decondensation. The meiotic abnormalities also include unpairing of sex chromosomes, chromatin decompaction, and, in some cases, *de novo* genome rearrangements. Therefore, these malaria mosquitoes represent a great new system for studying a genetic basis and molecular mechanisms of species incompatibilities at early stages of postzygotic reproductive isolation.

Knowledge of the mechanisms of reproductive isolation in *Anopheles* has important implications not only for evolutionary biology but also for malaria control. The success of malaria transmission highly depends on the rate of mosquito reproduction. The development of novel approaches to control the reproductive output of mosquitoes must include the understanding of how fertility is regulated. Determining the mechanisms of sterility in male hybrids between closely related species of mosquitoes can also empower the sterile insect technique. In this respect, our results shed new light on the cellular processes and possible mechanisms in spermatogenesis that first appear when fertility is affected.

Methods

Mosquito maintenance and crossing experiments

The laboratory colonies of *An. gambiae* ZANU (MRA-594), *An. coluzzii* MOPTI (MRA-763), *An. coluzzii* MALI (MRA-860), and *An. merus* MAF (MRA-1156) were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI). Authentication of the species was performed by a cytogenetic analysis and by PCR diagnostics [86, 87]. Mosquitoes were reared at 27±1°C, with 12-hour photoperiod and 70±5% relative humidity. Larvae were fed fish food, and adult mosquitoes were fed 1% sugar water. To induce oviposition, females were fed defibrinated sheep blood (Colorado Serum Co., Denver, Colorado, USA) using artificial blood feeders. To perform interspecies crosses, male and female pupae were separated to guarantee virginity of adult mosquitoes. We differentiated males and females at the pupal stage using sex-specific differences in the shape of their terminalia [88]. After the emergence of adults, crossing experiments were performed by combining 30 females and 15 males in one cage. Five days after random mating, the females were fed sheep blood. Two days later, an egg dish, covered with moist filter paper to keep the eggs from drying out, was put into the cage. Backcrosses of F1 males and parental females were done using a similar method. At least two blood meals were fed to females with three repeats of each crosses.

Male gonad and sperm observation

Male gonads were dissected and photographed using an Olympus SZ 61 stereoscopy microscope (Olympus, Tokyo, Japan) with an Olympus Q-Color 5 digital camera (Olympus, Tokyo, Japan). We observed the testes of 30 males and took pictures of the testes of five males from each cross.

For sperm observation, testes were mounted in 20 μl sperm assay buffer containing 4 mM KCl, 1.3 mM $CaCl_2$, 145 mM NaCl, 5 mM D-glucose, 1 mM $MgCl_2$, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) [89]. After gently covering testes with a coverslip, they were crushed, and sperm motility was observed under a phase contrast microscope BX 41 (Olympus, Tokyo, Japan). A movie of sperm motility for at least five males of hybrids from each cross and five males of pure species was recorded using a digital camera UC90 (Olympus, Tokyo, Japan).

Chromosome preparation

Testes with male accessory glands were dissected from male pupae and 0-12 hours-old adults in 0.075% potassium chloride (KCl) hypotonic solution on a frosted glass slide (Thermo Fisher Scientific, Waltham, MA, USA) under an Olympus SZ 61 dissecting microscope (Olympus, Tokyo, Japan). To observe meiotic chromosomes, male accessory glands and other tissues were removed, and only testes were left on the slide. Immediately after dissection, a drop of 50% propionic acid was added to the testes, and they were covered with a 22×22-mm coverslip (Thermo Fisher Scientific, Waltham, MA, USA). Preparations were tapped using the flat rubber end of a pencil and observed under an Olympus CX41 phase-contrast microscope (Olympus, Tokyo, Japan). The slides were frozen in liquid nitrogen, and a sharp razor was used to take off the coverslips. The preparations were placed in 50% ethanol chilled at -20ºC for a minimum of 2 hours. Later, serial dehydrations were performed in 70%, 90%, and 100% ethanol for 5 min each at room temperature (RT). Subsequently, the best preparations from at least 10 slides/individuals with desired meiotic stages were chosen for further studies.

DNA probe labeling

Six DNA probes were used for FISH in this study (Table 1): retroelement *zanzibar* specific to the Y chromosome of *An. gambiae* and *An. coluzzii*, 18S rDNA specific to the X chromosome of *An. gambiae* and *An. coluzzii* but hybridized to both X and Y chromosomes of *An. merus,* satellite AgY53B derived from the AgY53B/AgY477 satellite array and hybridized to X and Y chromosomes of all three species [44], satellite AgY477-AgY53B junction region specific to the Y chromosome of *An. gambiae* and *An. coluzzii*, a satellite from Contig_240 identified from *An. gambiae* sequencing data (SRS667972, SRR1509742, SRR1508169) [44] using the Redkmer pipeline [90] and specific to the X chromosomes of *An. coluzzii* and *An. merus*, and a satellite tandem-42345 [91] specific to autosomal pericentric heterochromatin in *An. coluzzii* and to pericentric heterochromatin of all chromosomes in *An. merus*. The 18S rDNA, AgY53B and *zanzibar* probes were labeled by Cyanine3 (Cy3) or Cyanine5 (Cy5) fluorochromes using PCR with genomic DNA as a template. Genomic DNA was extracted using DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany) from virgin males of *An. gambiae* ZANU or *An. coluzzii* MOPTI for labeling *zanzibar*, virgin males of *An. merus* MAF for labeling satellite AgY53B, and virgin females of *An. gambiae* or *An. coluzzii* for labeling 18S rDNA. Each 25 µl of a PCR mix consisted of 1-µl genomic DNA, 12.5-µl ImmoMix™ 2x reaction mix (Bioline USA Inc., Taunton, MA, USA), 1 µl of 10-µM forward and reverse primers, and water. PCR labeling was performed in the Mastercycler® pro PCR thermocycler (Eppendorf, Hamburg, Germany) starting with a 95°C incubation for 10 min followed by 35 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 45 sec; 72°C for 5 min, and a final hold at 4°C. Oligonucleotides with 3'-end-labeling by Cy5 or Cy3 fluorochromes (Sigma-Aldrich, St. Louis, MO, USA) were designed

based on sequences of the satellite AgY477-AgY53B junction region, satellite from Contig_240, and satellite tandem-42345.

FISH

FISH was performed as previously described [92, 93]. Briefly, slides with good preparations were treated with 0.1-mg/ml RNase at 37℃ for 30 min. After washing with 2×SSC for 5 min twice, slides were digested with 0.01% pepsin and 0.037% HCl solution for 5 min at 37°C. After washing slides in 1×PBS for 5 min at RT two times, preparations were fixed in 3.7% formaldehyde for 10 min at RT. Slides were then washed in 1×PBS and dehydrated in a series of 70%, 80%, and 100% ethanol for 5 min at RT. Then 10 μl of probes were mixed, added to the preparations, and incubated at 37℃ overnight. After washing slides in 1×SSC at 60℃ for 5 min, 4×SSC/NP40 solution at 37℃ for 10 min, and 1×PBS for 5 min at RT, preparations were counterstained with a DAPI-antifade solution (Life Technologies, Carlsbad, CA, USA) and kept in the dark for at least 2 hours before visualization with a fluorescent microscope.

Cytogenetic analyses

Cytogenetic analyses of meiotic and mitotic stages and of behaviors of FISH-labeled chromosomes were performed on at least 10 male individuals of each pure species and of each hybrid. To visualize and photograph meiotic chromosomes after FISH, we used an Olympus BX61 microscope (Olympus, Tokyo, Japan) with a connected camera Olympus U-CMAD3 (Olympus, Tokyo, Japan). Lengths of well-spread 10 metaphase I chromosomes of each species and of each hybrid were measured using the ruler tool in Adobe Photoshop CS6 (Adobe Inc., San

Jose, CA, USA). Since the variances for both groups were equal ($s_{max}/s_{min} < 2$), a statistical two-sample pooled *t*-test was done with the JMP 13 software (SAS Institute Inc., Cary, NC, USA).

Whole-mount FISH and analysis of chromosome pairing

Testes from one-day-old adults of pure species and hybrids were dissected in 1×PBS solution and fixed in 3.7% paraformaldehyde in 1×PBS with 0.1% tween-20 (PBST) for 10 min at room temperature. After incubation with 0.1-mg/ml RNase for 30 min at 37 ºC, testes were penetrated with 1% triton/0.1M HCl in PBST for 10 min at room temperature. After adding labeled DNA probes, testes were incubated at 75 ºC for 5 min (denaturation) and 37 ºC overnight (hybridization). Later, testes were washed with 2×SSC and mounted with a DAPI-antifade solution (Life Technologies, Carlsbad, CA, USA). Testes from 6 individuals of pure species and of hybrids were scanned and analyzed. Visualization and z-tack 3D scanning were performed on the whole testes with an interval of 1.25 μm between two optical sections under a 63× oil lens of Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany). Based on the size of the testis cells and on the limitations of microscope imaging, we chose from the first to sixteenth optical layers with strong and clearly detected fluorescent signals to analyze sex-chromosome-pairing events. Specifically, cell nuclei at the early stages of meiotic prophase I from $4^{th}\pm1$, $8^{th}\pm1$, and $12^{th}\pm1$ optical layers were used to count paring events. A total of 418 and 489 nuclei at the early stages of meiotic prophase I were analyzed for paring and unpairing of the sex chromosomes in pure species and hybrids, respectively (Additional file 12: Table S2). The percentage of the numbers of cells with pairing and no paring of sex chromosomes was used to compare parental species and hybrids. Since the variances for both groups were equal ($s_{max}/s_{min} <$

2), a statistical two-sample pooled *t*-test was done using the JMP 13 software (SAS Institute Inc., Cary, NC, USA).

RNA extraction and RT-PCR

RNA was extracted from 40 abdomens (only distal segments that include testes) and from 20 carcasses of 1- to 2-day-old virgin adult males of *An. coluzzii* MOPTI, as well as of interspecies hybrids from crosses ♀*An. coluzzii* MOPTI × ♂*An. merus* and ♀*An. merus* × ♂*An. coluzzii* MOPTI using a Direct-Zol™ RNA MiniPrep Kit (Zymo Research, Irvine, California, US). cDNA for selected genes (Table 1) was generated in a 40-µl reaction that contained 8 µl of 5× first-strand buffer, 4 µl of 0.1-M dithiothreitol (DTT), 2 µl of 50-µM random hexamer primer (Thermo Fisher Scientific, Waltham, MA, USA), 1 µl of 25-mM dNTP mix solution (Thermo Fisher Scientific, Waltham, MA, USA), 1 µl of 40-U/µl RNAsin (Promega, Madison, WI, USA), 2 µl of 200-U/µl M-MLV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), 1ng to 2µg of RNA and water. After incubation at 42°C for 1h, the reaction was stopped by incubation at 95°C for 5 min. Two-step RT-PCR was performed to analyze the gene expression. Each 25-µl PCR mix consisted of 1-µl cDNA, 12.5-µl ImmoMix™ 2× reaction-mix (Bioline USA Inc., Taunton, MA, USA), 1 µl of 10-µM forward and reverse primers, and water. PCR was performed in the Mastercycler® pro PCR thermocycler (Eppendorf, Hamburg, Germany) starting with a 95°C incubation for 10 min followed by 35 cycles of 95°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec; 72°C for 10 min; and a final hold at 4°C. Amplification products were visualized in a 1-1.5% agarose gel.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Authors' contributions

IVS conceived the study. JL conducted mosquito maintenance and crosses. JL and IVS performed mosquito dissection and sperm motility assays. JL conducted FISH, microscopy, and RT-PCR. JL and IVS did data analysis. IVS led the writing of the manuscript with critical contributions from JL. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends

Fig. 1. Morphology and cell content of testes from 2-day-old adult males. (A) Whole testis, squashed testis, and mature spermatozoa in crushed testis from *An. coluzzii* MOPTI. (B) Normal-like whole testis, squashed testis, and immature spermatozoa and spermatids in crushed testis from an F1 hybrid of the cross ♀*An. merus* × ♂*An. coluzzii* MOPTI. (C) Underdeveloped whole testis, squashed testis, and undifferentiated cells in crushed testis from an F1 hybrid of the cross ♀*An. coluzzii* MOPTI × ♂*An. merus.* Vertical scale bars – 50 and 20 μm.

Fig. 2. Chromosome behavior during meiosis in testes of *An. gambiae.* X chromosomes are labeled with 18S rDNA (red), and Y chromosomes are labeled with retrotransposon *zanzibar* (green). Chromosomes are counterstained with DAPI (blue). Scale bar – 5 μm.

Fig. 3. Chromosome behavior during meiosis in testes of interspecies hybrids. (A) ♀*An. merus* × ♂*An. coluzzii* MALI. (B) ♀*An. merus* × ♂*An. coluzzii* MOPTI. (C) ♀*An. merus* × ♂*An.*

gambiae ZANU. X chromosomes are labeled with 18S rDNA (red), and Y chromosomes are labeled with retrotransposon *zanzibar* (green). Chromosomes are counterstained with DAPI (blue). Scale bar – 5 μm.

Fig. 4. Chromosomal and molecular abnormalities in interspecies hybrids. (A) Left panel: Lengths of metaphase chromosomes in *An. gambiae* ZANU males (filled boxplots) and in F1 hybrid males from the cross ♀*An. merus* × ♂*An. gambiae* ZANU (open boxplots). Right panel: Ratios of the lengths of chromosomes between F1 hybrids and *An. gambiae* ZANU. X and Y – sex chromosomes. 2 and 3 – autosomes. Statistical significance was assessed with a two-sample pooled *t*-test. (B) Confocal images of primary spermatocytes with FISH to demonstrate pairing and unpairing of X (red) and Y (green) chromosomes in *An. coluzzii* MOPTI males and F1 hybrid males from the ♀*An. merus* × ♂*An. coluzzii* MOPTI cross. (C) Percentage of the X and Y chromosome pairing in primary spermatocytes of six *An. coluzzii* MOPTI males and six F1 hybrid males from the ♀*An. merus* × ♂*An. coluzzii* MOPTI cross. Statistical significance was assessed with a two-sample pooled *t*-test. (D) Expression of meiotic (*Ams, mts, Dzip1l, β2-tubulin*) and pre-meiotic (*vasa*) germline-specific genes in testes of *An. coluzzii*, F1 hybrids of ♀*An. merus* × ♂*An. coluzzii* MOPTI (F1M×C), and F1 hybrids of ♀*An. coluzzii* MOPTI × ♂*An. merus* (F1C×M) analyzed by RT-PCR. *AgS7* – an endogenous control gene. R – reproductive tissues, B – rest of mosquito body.

Fig. 5. Genomic rearrangements of meiotic chromosomes in testes of interspecies hybrids. (A) A translocation of the fragment of the *An. merus* X chromosome to the *An. coluzzii* autosome 2 in a hybrid from the ♀*An. merus* × ♂*An. coluzzii* MALI cross. The red arrow points to the

translocated X-chromosome ribosomal locus labelled by 18S rDNA (red). (B) A duplication of the segment involving the rDNA locus within the *An. merus* X chromosome in a hybrid from the cross ♀*An. merus* × ♂*An. gambiae* ZANU. The red arrow indicates the new positions of the segment with the ribosomal locus labelled by 18S rDNA (red). Chromosomes are counterstained with DAPI.

Fig. 6. Schematic representation of testis development in pure species and reciprocal F1 hybrids of the *An. gambiae* complex.

Fig. 7. Scheme of male meiosis in pure species and interspecies hybrids of the *An. gambiae* complex. Unlike pure species, X and Y chromosomes in meiotic prophase I of hybrids are largely unpaired. Instead of a reductional division, primary spermatocytes in interspecies hybrids undergo an equational mitotic division leading to diploid nonmotile sperms.

Supporting Information

Additional file 1: Movie S1. Sperm motility in a crushed testis from a 2-day-old adult of *An. merus.*

Additional file 2: Movie S2. Sperm motility in a crushed normal-like testis from a 2-day-old adult hybrid of the cross ♀*An. merus* × ♂*An. coluzzii* MOPTI.

Additional file 3: Figure S1. Chromosome behavior during meiosis in testes of *An. coluzzii* MOPTI. X chromosomes are labeled with 18S rDNA (red), and Y chromosomes are labeled

with retrotransposon *zanzibar* (green). Chromosomes are counterstained with DAPI (blue). Scale bar – 5 μm.

Additional file 4: Figure S2. Chromosome behavior during meiosis in testes of *An. merus* MAF. Sex chromosomes are labeled with 18S rDNA (red) and satellite AgY53B (green). Chromosomes are counterstained with DAPI (blue). Scale bar – 5 μm.

Additional file 5: Figure S3. Sizes and sex chromosome content of spermatids in pure species and interspecies hybrids. (A) Spermatids from a 2-day-old adult of *An. coluzzii* MOPTI. (B) Spermatids from a 5-day-old adult F1 hybrid from the cross ♀*An. merus* × ♂*An. coluzzii* MOPTI. The X and Y chromosomes are visualized with 18S rDNA (red) and retrotransposon *zanzibar* (green), respectively. Chromatin is counterstained with DAPI (blue). The insets show magnified images of spermatids.

Additional file 6: Figure S4. Visualization of sex chromosomes in a degenerate testis of a hybrid adult from the cross ♀*An. coluzzii* MOPTI × ♂*An. merus*. (A) DAPI-stained male accessory glands and an underdeveloped testis of a one-day-old hybrid adult. (B) Whole-mount FISH of the degenerate testis with 18S rDNA (red) and satellite AgY53B (green) detects the interphase sex chromosomes. Chromatin is counterstained with DAPI (blue).

Additional file 7: Table S1. Sizes of metaphase chromosomes in pure species and interspecies F1 hybrids of the *An. gambiae* complex.

Additional file 8: Figure S5. Increased lengths of chromosomes in testes of interspecies F1 hybrids. Left panel: Lengths of metaphase chromosomes in *An. coluzzii* MOPTI (filled boxplots) and in F1 hybrid males from the cross *An. merus* × ♂*An. coluzzii* MOPTI (open boxplots). Right panel: Ratios of the lengths of chromosomes between hybrids and pure species. X and Y – sex chromosomes. 2 and 3 – autosomes. Statistical significance was assessed with a two-sample pooled *t*-test.

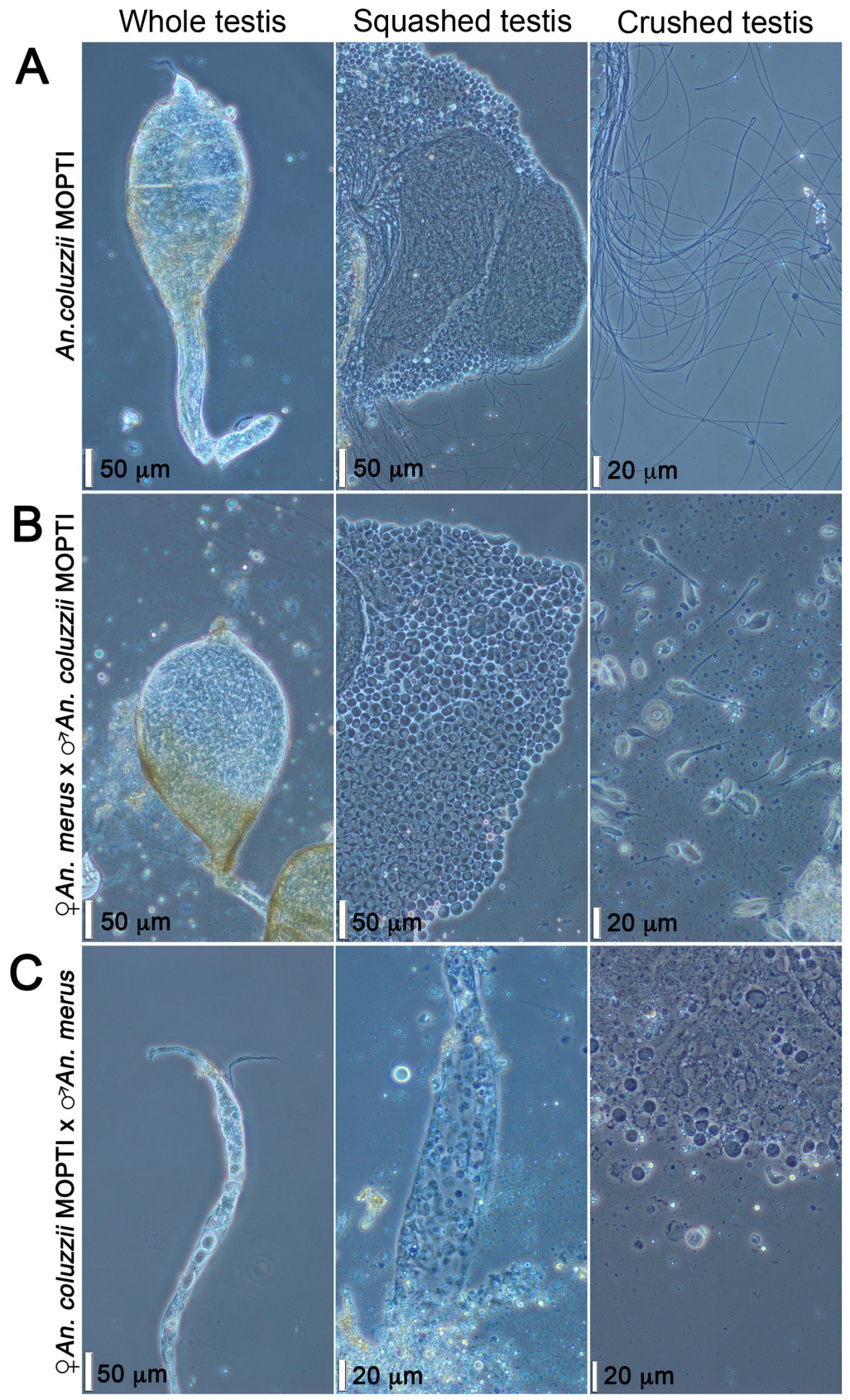
Additional file 9: Figure S6. Visualization of pairing between sex chromosomes in testes of a pure species and interspecies F1 hybrid. (A, B) Testis of a one-day-old adult of *An. coluzzii* MOPTI. (C, D) Testis of a one-day-old adult F1 hybrid of the cross ♀*An. merus* × ♂*An. coluzzii* MOPTI. Whole-mount FISH with 18S rDNA (Cy3), a satellite from Contig_240 (Cy3), satellite AgY53B (Cy5), and satellite AgY477-AgY53B junction region (Cy5) is performed to detect the X (red) and Y (green) chromosomes. Chromatin is counterstained with DAPI (blue).

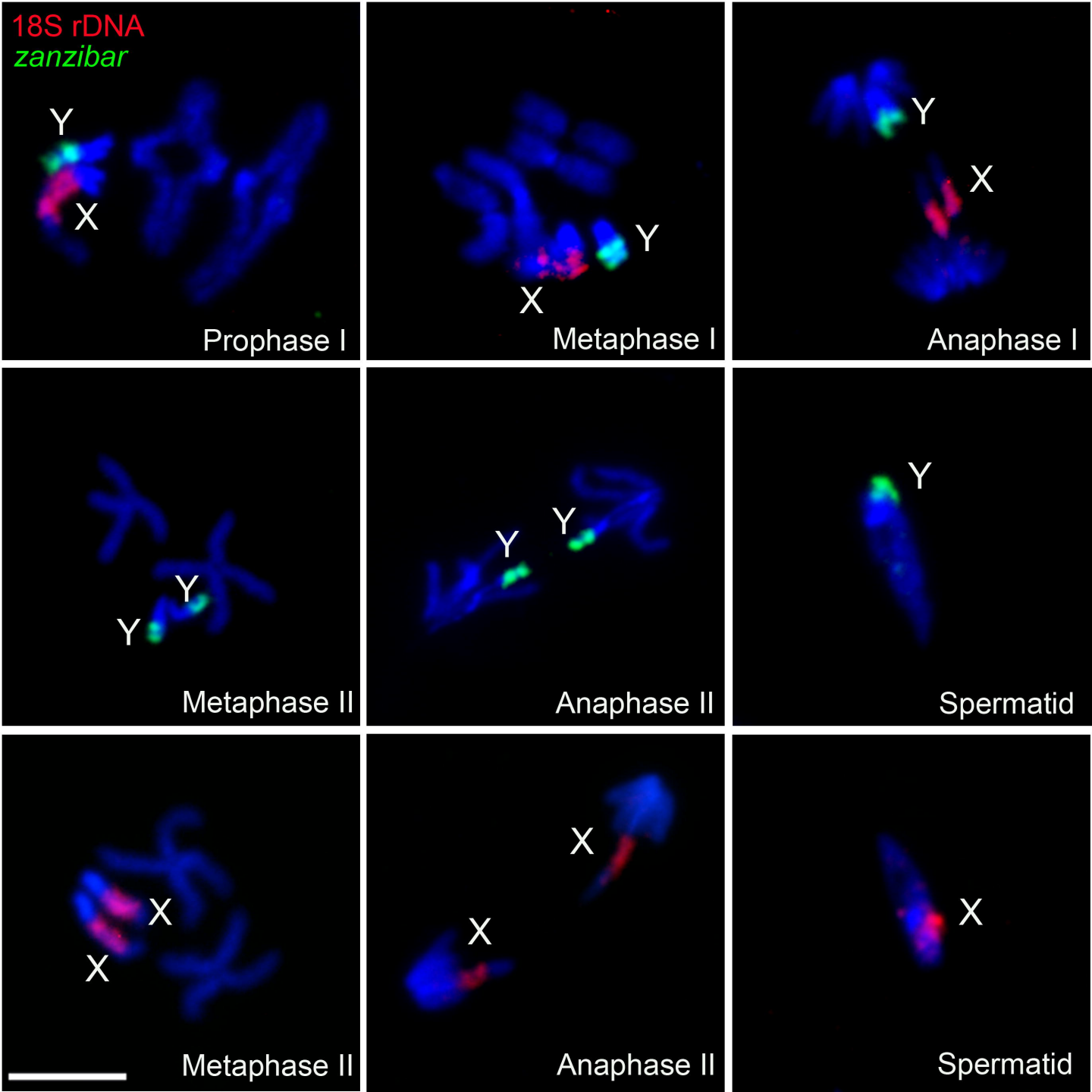
Additional file 10: Figure S7. Chromatin bridges formed by the *An. merus* X chromosomes during anaphase in primary spermatocytes of F1 hybrid males from the cross *An. merus* × ♂*An. gambiae* ZANU. The arrows show chromatin bridges between the X chromosomes.

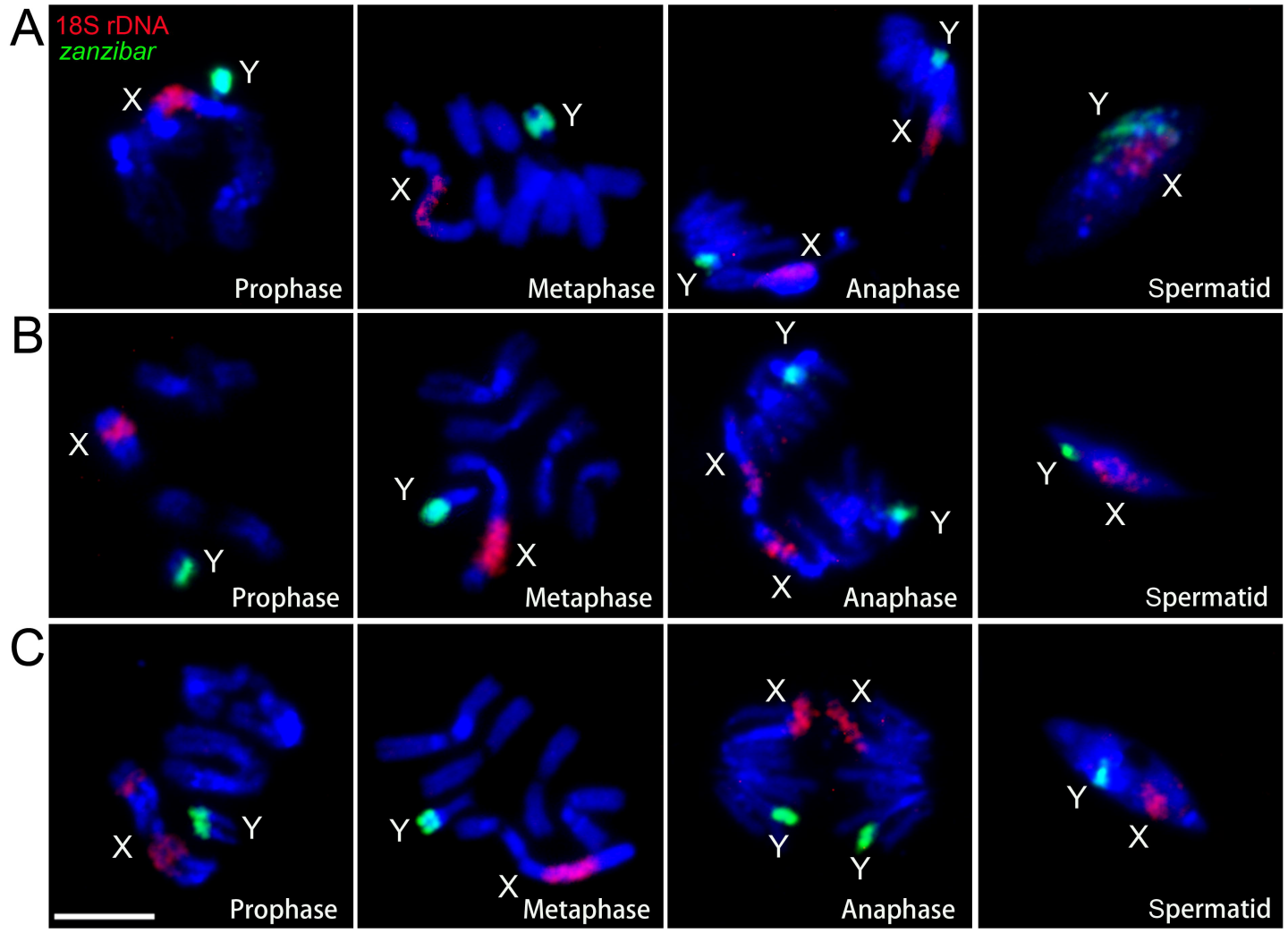
Additional file 11: Figure S8. Differences in condensation of heterochromatin between *An. coluzzii* MOPTI and F1 hybrid of the cross ♀*An. merus* × ♂*An. coluzzii* MOPTI. (A) A squashed preparation of meiotic chromosomes from *An. coluzzii* MOPTI male. (B) A squashed preparation of meiotic chromosomes from the ♀*An. merus* × ♂*An. coluzzii* MOPTI hybrid male. The *An. coluzzii* MOPTI Y chromosome is labeled by the satellite AgY477-AgY53B junction

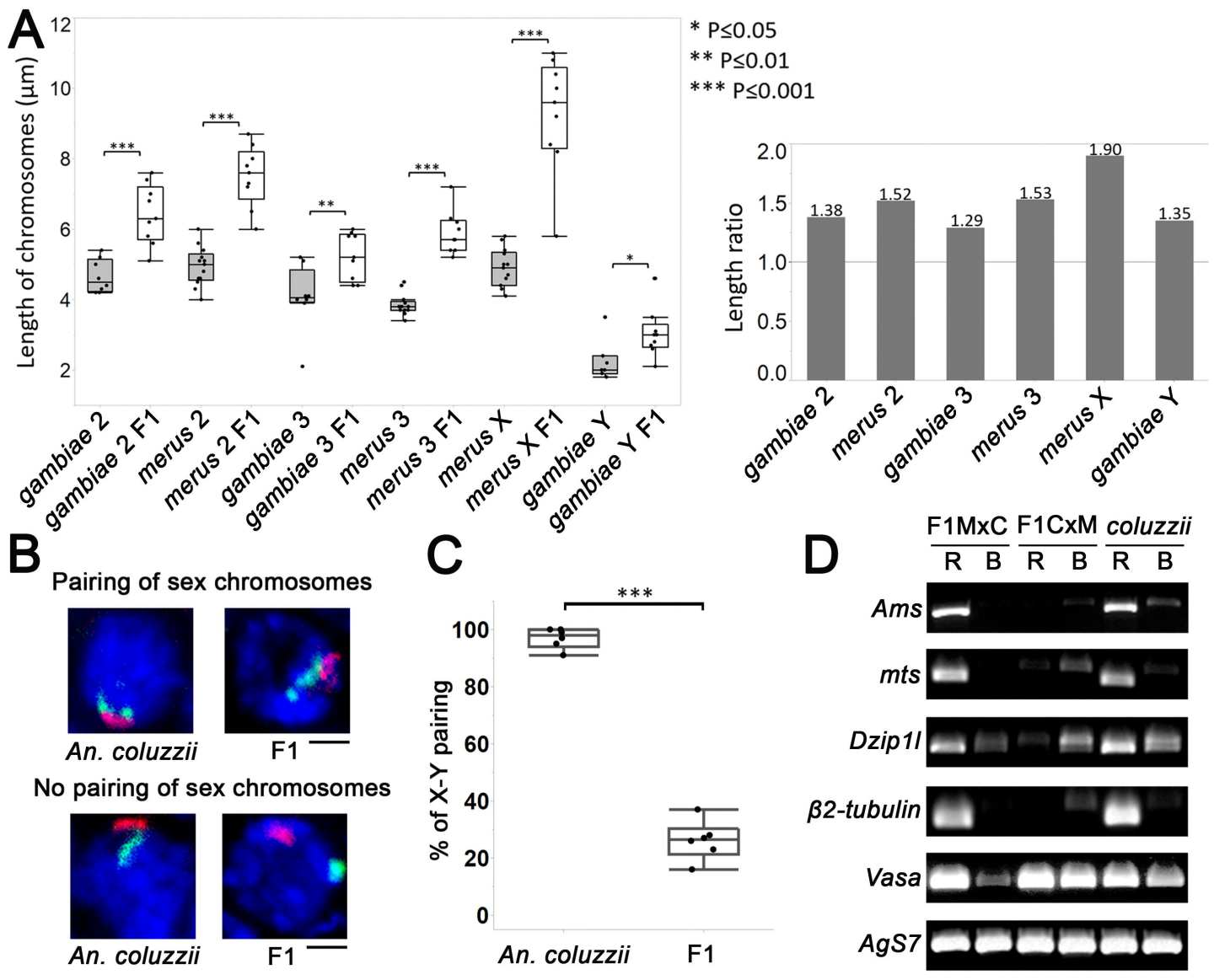
region (Cy5, red). The *An. merus* X chromosome and autosomes of both species are labeled by the satellite tandem-42345 (Cy3, green). The arrows point to the fibers of uncoiled heterochromatin on the grayscale inverted image. Chromatin is counterstained with DAPI (blue).

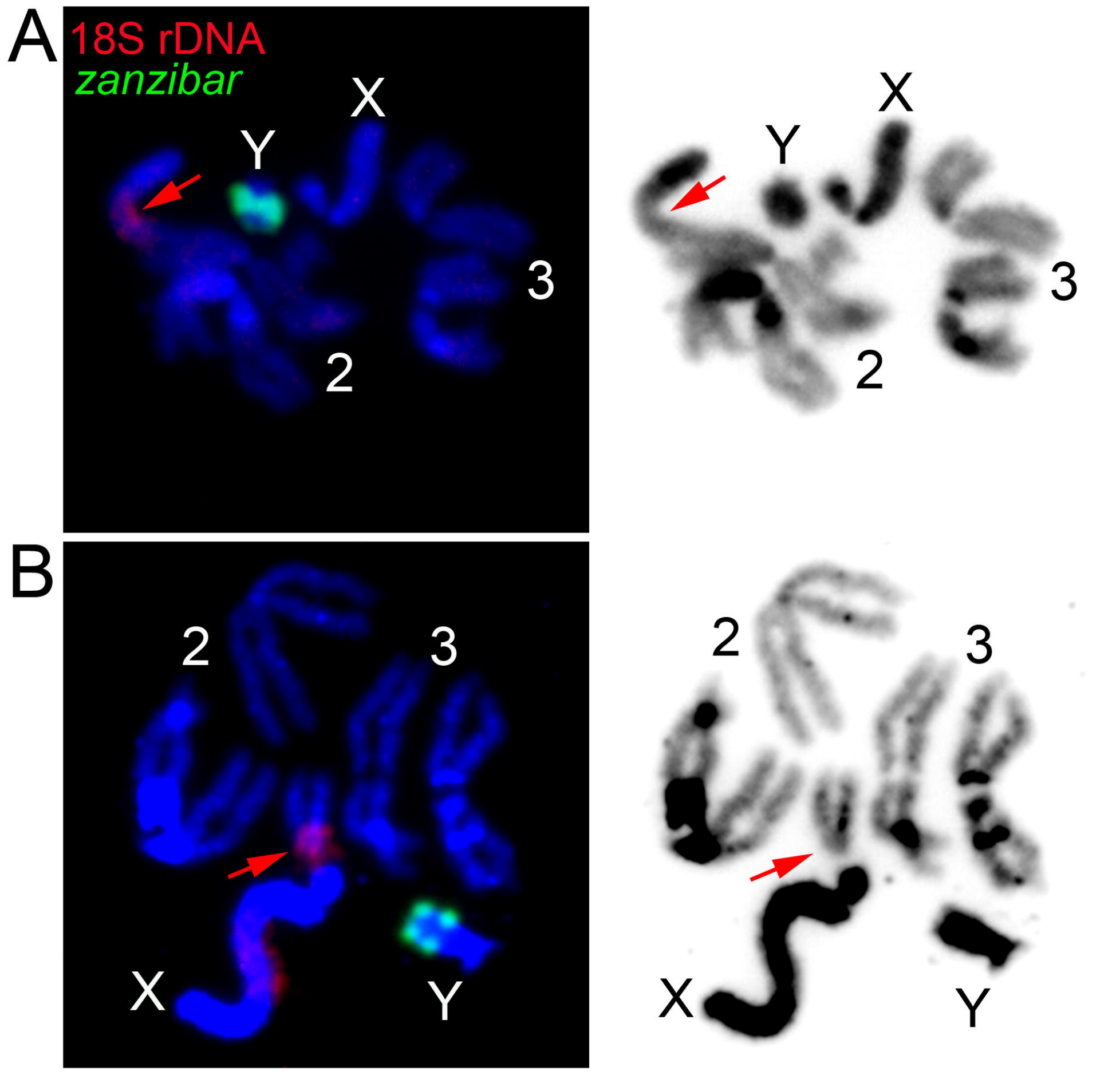
Additional file 12: Table S2. Numbers and percentages of primary spermatocytes with paired and unpaired sex chromosomes.


Whole testis
Squashed testis
Crushed testis
A
An.coluzzii MOPTI
50 µm
50 µm
20 µm
B
♀An. merus x ♂An. coluzzii MOPTI
50 µm
50 µm
20 µm
C
♀An. coluzzii MOPTI x ♂An. merus
50 µm
20 µm
20 µm


18S rDNA
zanzibar
Y
X
Prophase I
Y
X
Metaphase I
Y
X
Anaphase I
Y
Y
Metaphase II
Y
Y
Anaphase II
Y
Spermatid
X
X
Metaphase II
X
X
Anaphase II
X
Spermatid


A
18S rDNA
zanzibar
X
Y
Prophase
Metaphase
Anaphase
Spermatid
B
C


A
Length of chromosomes (µm)
12
10
8
6
4
2
***
***
**
***
***
*
gambiae 2
gambiae 2 F1
merus 2
merus 2 F1
gambiae 3
gambiae 3 F1
merus 3
merus 3 F1
merus X
merus X F1
gambiae Y
gambiae Y F1
* P≤0.05
** P≤0.01
*** P≤0.001
Length ratio
2.0
1.5
1.0
0.5
0.0
1.38
1.52
1.29
1.53
1.90
1.35
gambiae 2
merus 2
gambiae 3
merus 3
merus X
gambiae Y
B
Pairing of sex chromosomes
An. coluzzii
F1
No pairing of sex chromosomes
An. coluzzii
F1
C
***
% of X-Y pairing
100
80
60
40
20
0
An. coluzzii
F1
D
F1MxC
F1CxM
coluzzii
R
B
R
B
R
B
Ams
mts
Dzip1l
β2-tubulin
Vasa
AgS7


A
18S rDNA
zanzibar
X
Y
3
2
X
Y
3
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B
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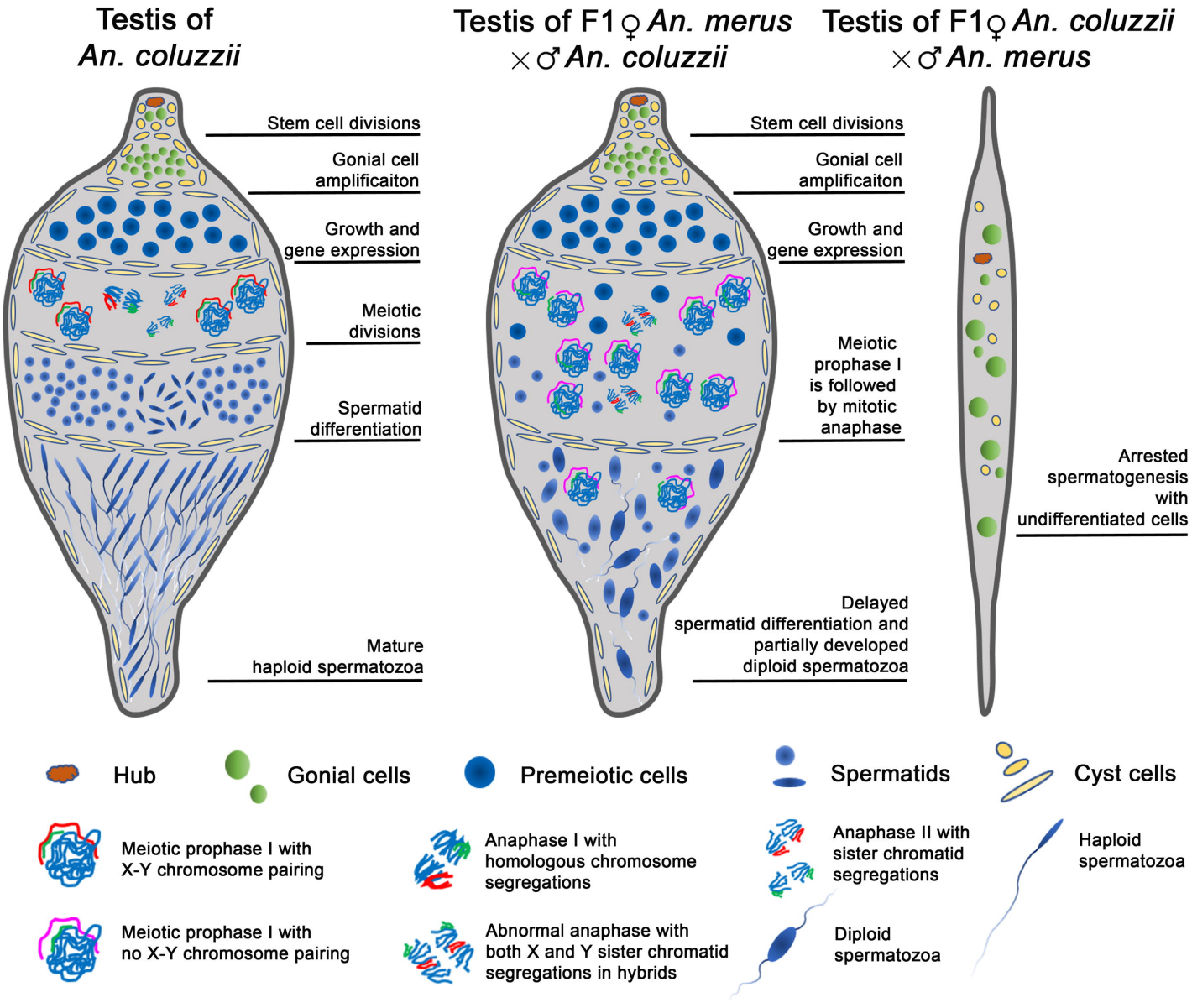
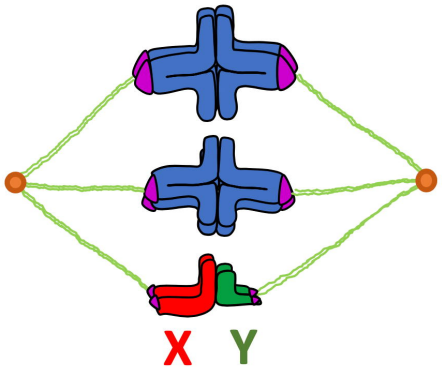
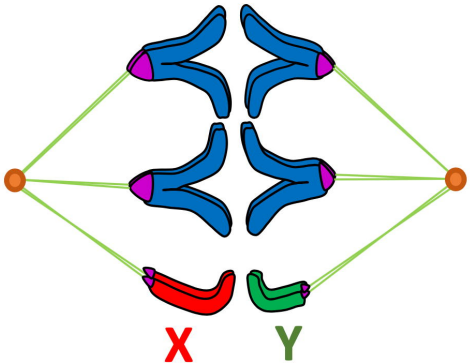
3
X
Y
2
3
X
Y


Testis of
An. coluzzii
Testis of F1 ♀ An. merus
× ♂ An. coluzzii
Testis of F1 ♀ An. coluzzii
× ♂ An. merus
Stem cell divisions
Gonial cell amplificaiton
Growth and gene expression
Meiotic divisions
Spermatid differentiation
Mature haploid spermatozoa
Stem cell divisions
Gonial cell amplificaiton
Growth and gene expression
Meiotic prophase I is followed by mitotic anaphase
Delayed spermatid differentiation and partially developed diploid spermatozoa
Arrested spermatogenesis with undifferentiated cells
Hub
Gonial cells
Premeiotic cells
Spermatids
Cyst cells
Meiotic prophase I with X-Y chromosome pairing
Meiotic prophase I with no X-Y chromosome pairing
Anaphase I with homologous chromosome segregations
Abnormal anaphase with both X and Y sister chromatid segregations in hybrids
Anaphase II with sister chromatid segregations
Diploid spermatozoa
Haploid spermatozoa

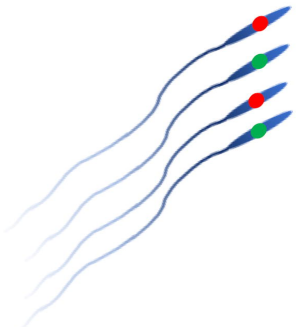
Normal male meiosis in pure species



Prophase I

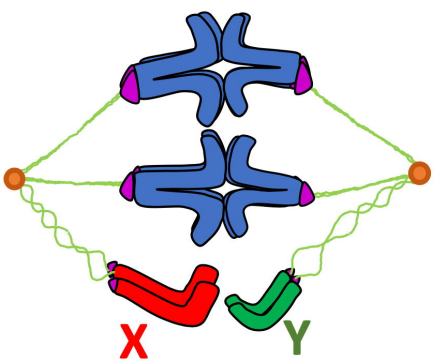


Anaphase I

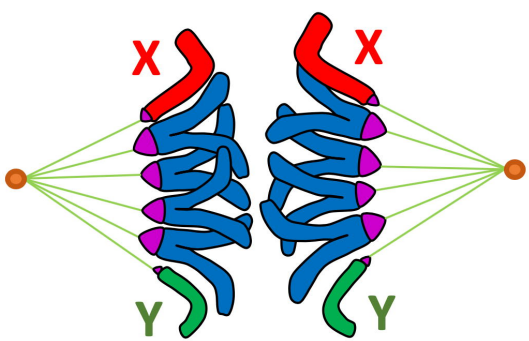


Normal sperm

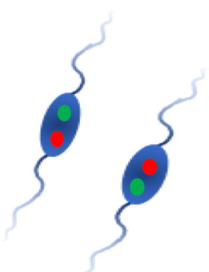
Failed male meiosis in F1 hybrids



Prophase I



Anaphase



Diploid sperm