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Segregation of the univalent X chromosome in the wide-footed treehopper *Enchenopa latipes* (Say 1824)

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Abstract. In metaphase I, autosomal bivalents align on the metaphase plate, while naturally-occurring univalent sex chromosomes can display a number of different behaviours depending on cellular conditions. Here we describe the behaviour of the univalent X chromosome in the wide-footed treehopper *Enchenopa latipes* (Say 1824). We confirm the chromosome number and sex determination method for this species, and that males possess a univalent X chromosome. We show that the univalent X chromosome forms a bipolar attachment to the spindle in metaphase I, and then segregates intact toward one spindle pole in late anaphase I (long after autosomes have initiated poleward movement). Movement of the univalent toward one pole is associated with loss of microtubule connections toward the opposite spindle pole.

Keywords: chromosomes, bivalent, univalent, X chromosome, meiosis, Auchenorrhynca, *Enchenopa latipes*.

INTRODUCTION

The aim of meiosis is to divide the chromosome number of the cell by two, creating haploid gametes. Reduction of chromosome number requires the formation of bivalents. To form a bivalent, the DNA in each chromosome is replicated. The replicated chromosomes pair, and are held together through sister-chromatid cohesion. After DNA replication, the homologues or partner sex chromosomes connect and undergo recombination, completing construction of the bivalent (Moore and Orr-Weaver 1998). Sister kinetochores are fused together in meiosis I and act as a single attachment site, allowing one half-bivalent to attach to microtubules coming from one spindle pole (a syntelic attachment), while the homologous half-bivalent associates with the opposite pole (Moore and Orr-Weaver 1998). Fusion of sister kinetochores ensures that sister chromatids will not separate prematurely in anaphase I. In "typical" meiosis I, homologues are guaranteed to separate from one another because they are initially connected, and because sis-

ter kinetochores are fused together. Cells then undergo a second meiotic division, with the sister kinetochores now facing in opposite directions and associating with opposite spindle poles (an amphitelic attachment) in metaphase II, and separating sister chromatids in anaphase II (Moore and Orr-Weaver 1998). Initial formation of a bivalent is, in general, required for successful creation of four gametes, each with half of the chromosomes of the original parent.

Correct chromosome distribution depends on homologues linking with one another, but what if homologues fail to link? Or, what if there is no partner at all? A number of organisms have sex chromosomes that do not have a pairing partner for meiosis I, and thus remain univalent in the first meiotic division. While errors that create univalent autosomes lead to erratic chromosome behaviours for the univalent, such as frequent and rapid oscillations between spindle poles and a failure to align at the metaphase plate, naturally-occurring univalent sex chromosomes appear to have a set of characteristic, stable behaviours depending upon conditions in the cell (Fabig, et al. 2016; Rebollo et al. 1998; Nokkala 1986; Bressa et al. 2001; Rebollo and Arana 1995; Rebollo and Arana 1997; Ault 1984; Bauer et al. 1961; Dietz 1954; Dietz 1969; John 1990). In some cases, the univalent's sister kinetochores are fused together in meiosis I just like those of the autosomal bivalents nearby (Fabig et al. 2016; Ault 1984). In such systems, the univalent can form an attachment to a single spindle pole early in meiosis I and remain adjacent to that spindle pole through telophase I (Fabig et al. 2016). In other cases, the univalent has sister kinetochores facing in opposite directions. It aligns on the metaphase plate with the bivalents. In anaphase I, when the autosomal half bivalents separate from one another, the univalent remains at the center of the spindle, and following separation of the autosomes, the univalent moves intact to one spindle pole (Fabig et al. 2016). Still other behaviors have been observed in univalentsand are well described in Fabig et al. (2016).

Univalent X chromosomes are frequently seen in insects of the order Hemiptera, suborder Auchenorrhyncha. In fact, these insects are some of the first species in which an X chromosome was observed and realized to exhibit different behaviours than the other chromosomes in the cell. Very early work on one species in the suborder Auchenorrhyncha, the spittle bug *Philaenus spumarius*, described the X chromosome to be an "odd" chromosome that "lags behind the others but goes undivided to one pole" (Boring 1913).

Here we report on our study of the behaviour of the univalent X chromosome in another member of the suborder Auchenorrhyncha, the treehopper, *Enchenopa latipes* (Say 1824). Halkka and Heinonen (1964) previously reported the karyotype and sex determination mechanism for the species to be 2N=19 in males with X0 (male)-XX (female) sex determination, but did not make any statement on the behaviour of the univalent X chromosome during meiosis. We confirm the previously-published report on chromosome number and sex determination mechanism, and use live-cell imaging and immunofluorescence staining to reveal that the X chromosome of *E. latipes* aligns with the autosomes in metaphase, forming an amphitelic attachment to the spindle. We also show that the X chromosome of *E. latipes* moves intact to one spindle pole after the autosomes have segregated, losing its connection to one spindle pole while retaining microtubule connections to the pole toward which it is moving. We also make conclusions about the conditions that lead to these characteristic behaviours.

MATERIALS AND METHODS

Collection and Identification

Adult *Enchenopa latipes* males were collected from a field site at the Bucknell University Farm (Lewisburg, PA). Treehoppers were identified and sexed according to Dietrich et al. (2001) and Kopp and Yonke (1973).

DNA Barcoding

DNA barcoding was done as described in the Carolina Biological Supply Company Using DNA Barcodes to Identify and Classify Living Things kit (Carolina 211385). Cytochrome c oxidase subunit 1 was amplified using the primers and PCR beads supplied by Carolina Biological Supply Company and sequenced at Genewiz using the M13forward and M13reverse primers. Sequence was analyzed using Sequencher v5.4.6 and trimmed to approximately 640 bp. Alignments were produced using ClustalOmega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Orcein Staining of Spread Chromosomes

Orcein stained chromosome spreads were prepared as described in Felt et al. (2017).

Living Cell Preparations

Testes were removed from the abdomens of *E. latipes* males and transferred to a culture chamber (Lin

et al. 2018) under a layer of Kel-F Oil #10 (Ohio Valley Specialty Company, Marietta, Ohio). Testes contents were spread thinly on a coverslip under oil, as described in Lin et al. (2018). Living meiosis I spermatocytes were imaged using a Zeiss inverted microscope equipped with a 100X 1.25 NA phase-contrast, oil-immersion objective and an Infinity 1 camera with Infinity Analyze software or a Nikon Eclipse TS100 microscope equipped with a 100X, 1.25 NA phase-contrast, oil-immersion objective and a Spot RT monochrome camera (Diagnostic Instruments Inc.) with Spot Basic 3.5.7 software.

Immunofluorescence

Fixation, immunostaining, and imaging of stained specimens were carried out as described in Felt et al. (2017).

RESULTS

DNA Barcoding

To confirm the identification of the insect specimens, we performed DNA barcoding analysis on one

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KF919639.1        ATTTTATTTTTGGTATATGATCTGGAATATTAGGGATAATAATAAGAATTATTATTCGAA  60
HM416189.1        ATTTTATTTTTGGTATATGATCTGGAATATTAGGAATAATAATAAGAATTATTATTCGAA  60
 MZ723494         ATTTTATTTTTGGTATATGATCTGGAATATTAGGGATAATAATAAGAATTATTATTCGAA  60
                  ********************************** ************************

KF919639.1        TTGAACTGAGTCAGCCGGGCCCTTTAATTCAAAATGACCAAATCTATAATACTGTAGTGA  120
HM416189.1        TTGAATTAAGTCAGCCGGGTCCTTTTATTCAAAATGACCAAATTTATAATACTGTAGTGA  120
 MZ723494         TTGAATTAAGTCAACCGGGTCCTTTTATTCAAAATGACCAAATTTATAATACTGTAGTGA  120
                  ***** * ***** ***** ***** ***************** ****************

KF919639.1        CTTCACATGCATTTATTATAATTTTTTTTATAGTTATACCCATTATAATTGGGGGATTTG  180
HM416189.1        CTTCACATGCATTTATCATAATTTTTTTTATAGTTATACCCATTATAATTGGGGGATTTG  180
 MZ723494         CTTCACATGCATTTATCATAATTTTTTTTATAGTTATACCCATTATAATTGGGGGATTTG  180
                  **************** ********************************************

KF919639.1        GAAATTGATTAGTACCATTAATAGTTGGAGCACCAGATATAGCTTTTCCTCGTCTTAATA  240
HM416189.1        GAAATTGACTAGTACCATTAATAATTGGAGCCCCAGATATAGCTTTTCCTCGTCTTAATA  240
 MZ723494         GAAATTGATTAGTACCATTAATAATTGGAGCCCCAGATATAGCTTTTCCTCGTCTTAATA  240
                  ******** ************** ******* ****************************

KF919639.1        ATATAAGATTTTGATTATTACCTCCATCAATCTTATTACTTCTATCTAGATCAGTGGTAG  300
HM416189.1        ATATAAGATTTTGATTATTACCTCCATCAATCTTATTACTTTTATCTAGATCAATGGTAG  300
 MZ723494         ATATAAGATTTTGATTATTACCTCCATCAATCTTATTACTTTTATCTAGATCAATGGTAG  300
                  ****************************************** *********** ******

KF919639.1        AATCAGGTGCAGGAACTGGATGAACAGTATACCCTCCTCTTTCTAGTAACATTGCTCATT  360
HM416189.1        AATCAGGTGCAGGTACTGGATGGACAGTATACCCCCCTCTTTCTAGTAATATTGCTCATT  360
 MZ723494         AATCAGGTGCAGGTACTGGATGGACAGTATACCCCCCTCTTTCTAGTAATATTGCTCATT  360
                  ************* ******** *********** ************** **********

KF919639.1        CTGGGGCTAGAGTAGATTTAGCTATTTTTTCTCTGCATTTAGCTGGTATTTCATCAATTT  420
HM416189.1        CTGGGGCTAGAGTAGATTTAGCTATTTTTTCTCTGCATTTAGCTGGTATTTCATCAATTT  420
 MZ723494         CTGGGGCTAGAGTAGATTTAGCTATTTTTTCTCTACATTTAGCTGGTATTTCATCAATTT  420
                  ********************************** ************************

KF919639.1        TAGGTGCAATTAATTTTATTACAACTATTATAAATATACGTTGTGATGAATTAAATATAG  480
HM416189.1        TAGGTGCAATTAATTTTATTACAACTATTATAAATATACGTTGTGATGAATTAAATATAG  480
 MZ723494         TAGGTGCAATTAATTTTATCACAACTATTATAAATATACGTTGTAATGAATTAAATATAG  480
                  ****************** ********************** ****************

KF919639.1        ATCGTCTTCCTTTATTTGTTTGGTCAGTAATAATCACAGCGGTTTTACTTTTATTGTCCC  540
HM416189.1        ATCGTCTTCCTTTATTTGTTTGGTCAGTAATAATCACAGCGGTTTTACTTTTATTATCCC  540
 MZ723494         ATCGTCTTCCTTTATTTGTTTGGTCAGTAATAATCACAGCGGTTTTACTTTTATTATCCC  540
                  ************************************************** ****

KF919639.1        TTCCCGTTTTAGCTGGTGCTATCACTATATTATTAACCGATCGTAATATAAATACTTCTT  600
HM416189.1        TTCCCGTATTAGCTGGTGCTATTACTATATTATTAACCGATCGTAATATAAATACTTCTT  600
 MZ723494         TTCCCGTATTAGCTGGTGCTATTACTATATTATTAACTGATCGTAATATAAATACTTCTT  600
                  ******* ************** ************** **********************

KF919639.1        TCTTTGATCCTTCTGGTGGAGGAGATCCTATTTTATACCAACATTTATTC      650
HM416189.1        TCTTTGATCCTTCTGGGGGAGGAGATCCCATTTTATACCAACATTTATTT      650
 MZ723494         TCTTTGATCCTTCTGGGGGGGGAGACCCCATTTTATATCAACATTTATTT      650
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Figure 1. ClustalOmega alignment of cytochrome oxidase 1 gene from *Enchenopa latipes* specimens. The top two sequences represent specimens with the closest identity to our specimen from two independent barcoding studies of *E. latipes* based on blastn analysis. Our specimen MZ723494 is 98.46% identical to sequence HM4161189.1 and 95.84% to sequence KF919639.1.

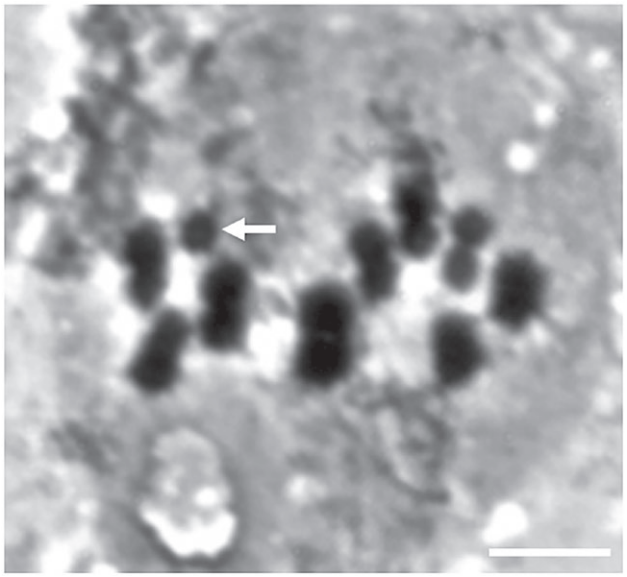

Figure 2. Orcein-stained chromosome spread generated from meiosis I spermatocyte of *E. latipes*. The spread shows 9 bivalents. X chromosome is indicated with arrow. Bar=5μm.

individual and submitted the sequence to Genbank. The sequence has accession number MZ723494. The partial Cox1 gene sequences were analysed using blastn and identified two sequences, one associated with KF919639, and a second associated with HM416189. The full sequence of MZ723494 was used in Clustal Omega (Madiera et al. 2019) to create the alignment (Figure 1). The MZ723494 isolate was 95.8% identical to the KF 919639 specimen and 98.5% identical to the HM416189 specimen (Figure 1).

Karyotype Analysis

Chromosome spreads from *E. latipes* were prepared and analysed to confirm chromosome number and sex determination mechanism. Spreads of testes contents from ten individuals were used to determine the chromosome number. *E. latipes* has a chromosome number of 2n=19 in males, with nine bivalents and one univalent X chromosome (Figure 2).

Sex Determination and Sex Chromosome Behaviour

Chromosome behaviour was observed in living metaphase I and anaphase I spermatocytes (Figure 3). In metaphase I, the univalent X chromosome aligned on the metaphase plate along with all of the autosomal bivalents (Figure 3; 0 min.). At anaphase I onset, the univalent X chromosome remained at the center of the spindle while the autosomes separated toward the spindle poles (Figure 3; 5, 15, 25 min.). By late anaphase I, the X chromosome moved to one side of the spindle, approaching the bulk of autosomal half bivalents (Figure 3; 45, 50 min.).

Immunofluorescence staining revealed microtubules associated with the X chromosome from both spindle poles in metaphase I spermatocytes (Figure 4A). Microtubule connections were also observed on both sides of the univalent X chromosome in early anaphase I (Figure 4B). In late anaphase I spermatocytes, the X chromosome had microtubules associated with one side of the univalent, but the other side had no apparent microtubule connections on the other side (Figure 4C and 4D). The X chromosome was located near the spindle pole with the microtubule connection in late anaphase I spermatocytes (Figure 4C and 4D), and was positioned on one side of the cleavage furrow (Figure 4D).

DISCUSSION

Our results confirm the results of Halkka and Heinonen (1964), with a chromosome number of 2n=19 in males and an XX (female)-X0 (male) sex determination mechanism. Our work also corroborates previous studies that reveal chromosome numbers between 2n = 18 and 2n = 22 for other species within the Membracidae family (of which *E. latipes* is a member), most of which have X0 (male)/XX (female) sex determination (Boring 1907; Halkka 1959; Halkka 1962; Tian and Yuan 1997; Bhattacharya and Manna 1973). As was previously observed, all males in this study have a univalent X chromosome that does not have a pairing partner in meiosis I.

The autosomes and the sex chromosomes of *E. latipes* all align on the metaphase plate in metaphase I (Figure 3; 0 min., Figure 4; metaphase). This demonstrates that the univalent X chromosome has a bipolar attachment to the spindle (reviewed in Fabig et al. 2016), that is confirmed through our immunofluorescence data (Figure 4A, 4B). Our observations of anaphase in living cells (Figure 3) and in fixed, stained specimens (Figure 4) revealed that segregation of the univalent X chromosome is delayed relative to the autosomes, and that movement of the X chromosome is associated with loss of microtubule connections to one spindle pole and retention of connections to the pole toward which the chromosome moves. Delayed or lagging segregation is frequently observed in cells that have bipolarly-attached univalent X chromosomes, including primary spermatocytes of other hemipteran insects, and the primary

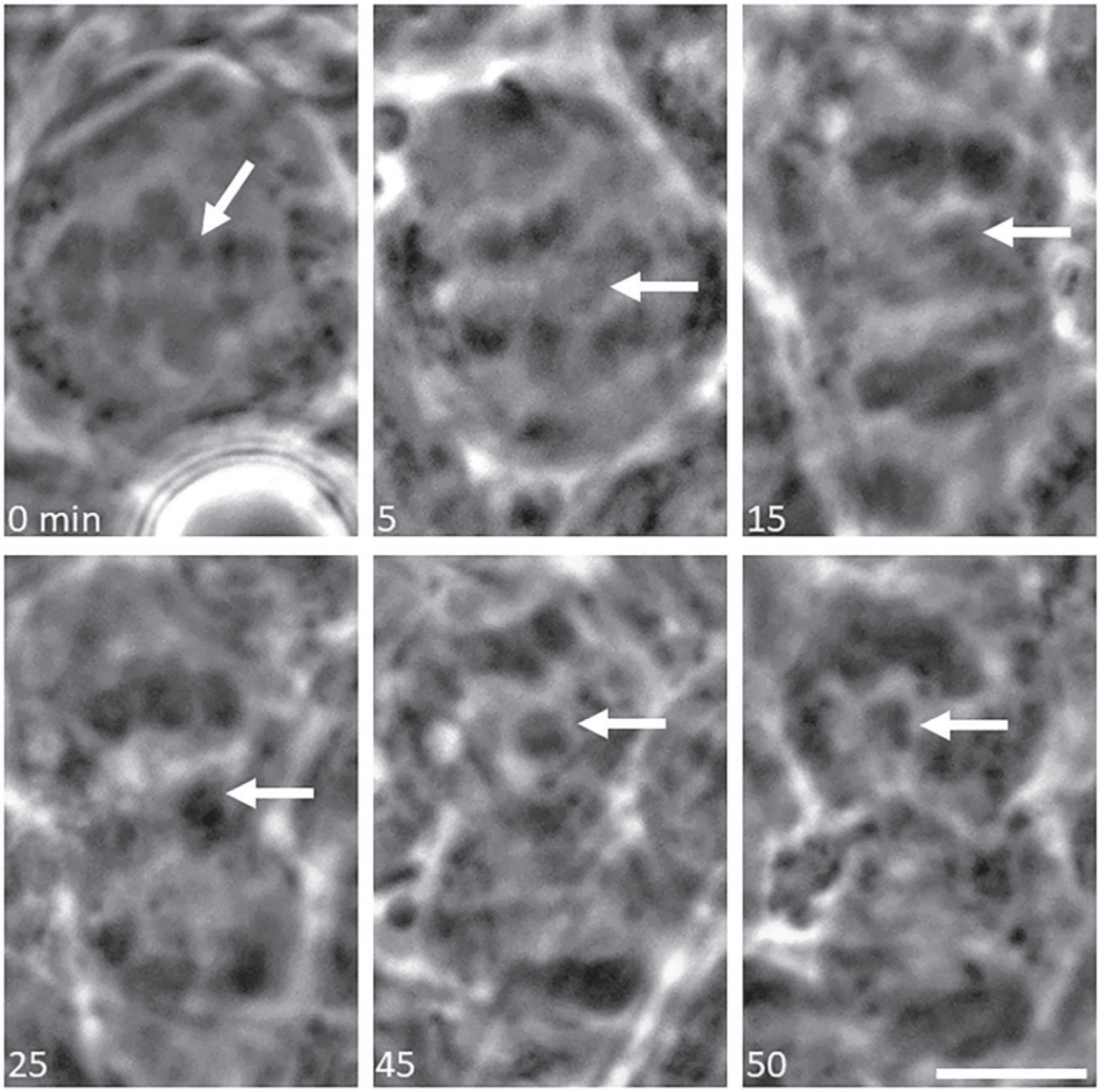

Figure 3. Delayed segregation of the intact univalent X chromosome. The X chromosome aligns with the autosomes on the metaphase plate (0 min) and remains at the centre of the spindle after the autosomal half bivalents have initiated segregation to their associated spindle poles (5, 15, 25 min). In late anaphase, the X chromosome moves intact toward the upper spindle pole. Bar=5μm.

spermatocytes in the male *Caenorhabditis elegans* (Fabig et al. 2020; Felt et al. 2017; Fabig et al. 2016; John and Claridge 1974; Rao 1956; Rebollo et al. 1998; Rebollo and Arana 1998).

We have confirmed the previously-published chromosome number and sex-determination mechanism of the treehopper *Enchenopa latipes* (Halkka and Heinonen 1964). We have also shown that the univalent X chromosome aligns at the spindle equator in metaphase I alongside the bivalent autosomes, and forms a bipolar attachment to the spindle. We finally show that the univalent X chromosome moves intact to one of the spindle poles in late anaphase, after all of the autosomes have initiated segregation, by losing microtubule connections to one spindle pole and retaining connections to the pole toward which is moving.

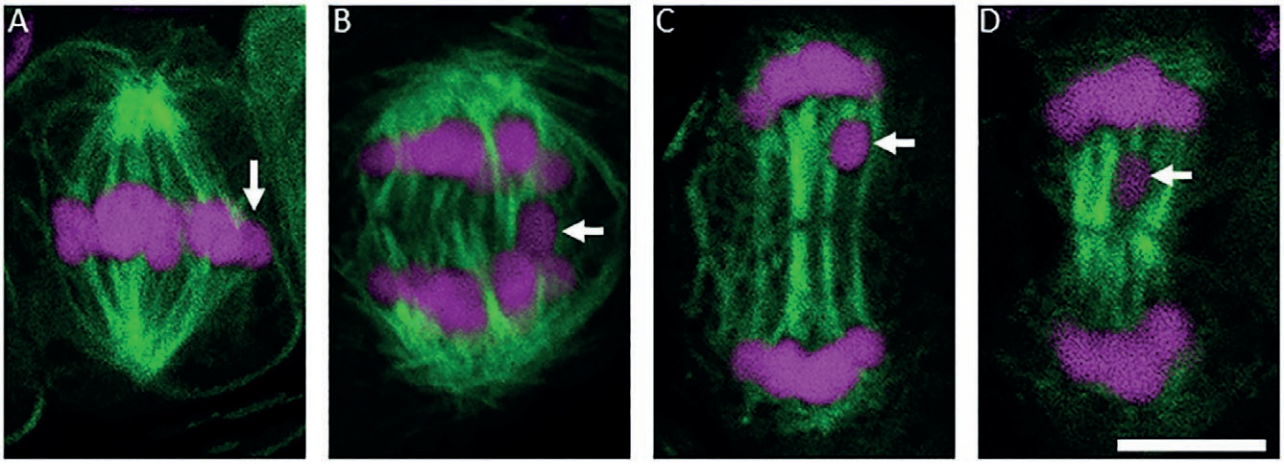

Figure 4. Segregation of the X chromosome in anaphase I *E. latipes* spermatocytes results from reduction and subsequent loss of microtubule connections on one side of the univalent. Immunofluorescence staining of microtubules (green) and DAPI staining (purple) of *E. latipes* spermatocytes in metaphase I (A), early anaphase I (B), and late anaphase/telophase I (C, D). In metaphase I, the X chromosome aligns on the metaphase plate, with microtubules connecting the univalent to both spindle poles. In early anaphase, the half bivalents move toward their associated poles while the X univalent remains at the centre of the spindle. It retains microtubule connections to both poles in early anaphase I (B). In late anaphase/telophase I (C, D), the X univalent only has microtubule connections to the spindle pole to which it is closest. Bar=5 μm.

Hemipteran insects like *E. latipes* have holocentric chromosomes in mitosis (Halkka 1959; Melters et al. 2012; Kuznetsova and Aguin-Pombo 2015). Hemipterans of the suborder Auchenorryncha (like *E. latipes*) appear to restrict kinetic activity of each bivalent so that bivalents behave as if they have localized kinetochores (Halkka 1959; Kuznetsova and Aguin-Pombo 2015). This allows one set of sister chromatids to move to one spindle pole while the homologous set moves to the opposite spindle pole in a traditional (non-inverted) meiosis (Melters et al. 2012). In our previous examination of the behaviour of univalent X chromosomes, we have found that systems that have holocentric chromosomes in mitosis, a non-inverted meiosis, and a univalent X chromosome show the same pattern of X-chromosome segregation in male meiosis I as we have observed in *E. latipes* (Fabig et al. 2016; Felt et al. 2017). This univalent-segregating behaviour is observed in different phyla of animals (Fabig et al. 2016; Felt et al. 2017; Fabig et al 2020), suggesting that the characteristics of the meiotic system, rather than phylogeny, dictate univalent behaviour in meiosis. The question for the future will be to find the mechanistic underpinnings for these characteristic chromosome behaviours.

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