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Germ line restricted B chromosomes in grasshoppers

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Original article

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Decades ago, the presence of extra chromosomes restricted to the male germ line in several grasshopper species was interpreted as recurrent polysomy, as experimental crosses suggested that the extra chromosomes were not transmitted from adult male parents to their embryo offspring. Under this hypothesis, polysomy was generated *de novo* through a nondisjunction for some chromosomes of the standard karyotype. In the current study, I test this hypothesis by analysing 17 families of tandem repeats (TRs) in two males of the grasshopper *Chorthippus parallelus*, which displays mosaicism for this kind of extra chromosome. According to the *de novo* polysomy hypothesis, the extra chromosomes should show the same FISH pattern for the TRs analysed as at least one of the A chromosomes. However, three TR families displayed patterns of FISH bands on the standard and extra chromosomes that ruled out the former as a possible source for the latter. Therefore, these extra chromosomes are best interpreted as B chromosomes restricted to the germ line, presumably present in both sexes, which are inherited as such and are not recurrently generated *de novo* from the A chromosomes.

Key words: Extra chromosomes, FISH, polysomy, tandem repeats

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Between 1963 and 1990, in the scientific literature on grasshopper cytogenetics, several cases of mosaic polysomy in the male germ line were reported (Hewitt 1963; Southern 1967; Hewitt and John 1968, 1970; John and Hewitt 1969; Peters 1981; Gosálvez and López-Fernández 1981; Camacho *et al*. 1981; Viseras and Camacho 1984, 1985; Talavera *et al*. 1990). In all cases, the extra chromosomes showed a size similar to some chromosomes of the standard set (i.e. A chromosomes), which was the main reason to consider them as cases of aneuploidy. However, they showed positive heteropycnosis (i.e. high condensation) during the first meiotic prophase, unlike the chromosome they supposedly were derived from, i.e. the same behaviour displayed by most of the known B chromosomes (Camacho 2005). Hewitt (1963), who reported the first case in *Chorthippus parallelus*, discarded the idea that the extra positively heteropycnotic chromosomes were B chromosomes, because the extra elements were unusually large and B chromosomes had not been reported in any *Chorthippus* species. However, B chromosomes larger than A chromosomes were later reported in grasshoppers (John and Freeman 1974), as well as in fish (Ziegler *et al*. 2003) and mammals (for a review, see Vujošević and Blagojević 2004).

The mosaicism described by Hewitt (1963) involved extra chromosomes similar in size to the longest acrocentric autosome (M4), and it implied

an intra-individual variation in the number of extra chromosomes, which was apparent between but not within the follicles from a same male, with some follicles carrying two extra chromosomes and other lacking them. The same kind of tetrasomic cells were observed by Southern (1967), but Hewitt and John (1968, 1970) as well as John and Hewitt (1969) reported a much more extensive variation ranging from trisomy to heptasomy in the Ashurst population, i.e. the same population where Hewitt (1963) first found this kind of chromosome variation. In this population, Hewitt and John (1968) found that 27 out of 101 males analysed were polysomic, four of which were full germ line tetrasomics for M4 (i.e. they carried two extra chromosomes in all the testis follicles analysed), whereas the remaining 23 individuals were mosaics. This showed the persistence of the M4 polymorphism across a number of years. As was previously observed by Hewitt (1963), all the M4 chromosomes in excess of two were regularly heteropycnotic from zygotene to diakinesis, and multivalents were very rare, especially those involving the standard M4 autosomes, with the exception of a diakinesis cell showing a positively heteropycnotic quadrivalent but no negatively heteropycnotic M4 bivalent, thus appearing to show a pairing between all four M4 chromosomes (as shown in Figure 23, Hewitt and John 1968). This was probably one of the main reasons to discard the possibility that the extra chromosomes were actually B chromosomes. As the researchers stated: 'Indeed were it not for their capacity to form multivalents they would almost surely be classified as supernumerary elements' (Hewitt and John 1968).

A few years later, Sannomiya (1973) reported the presence of extra chromosomes in the grasshopper *Atractomorpha bedeli*, which displayed many similarities with the M4 polysomy in *C. parallelus*. Specifically, they appeared to be restricted to the male germ line, as they were not visualised in the gastric caeca of both sexes or the ovariole cells, and showed a mosaicism also characterised by an intra-individual variation in number between follicles, but not within the follicles of the same male. The explanation for these observations also coincided with that proposed by Hewitt (1963), i.e. a mitotic non-disjunction of these extra chromosomes in the male germ line prior to follicle differentiation. Even so, Sannomiya (1973) suggested that the extra chromosomes found in *A. bedeli* were B chromosomes restricted to the male germ line.

Remarkably, eight years later, Peters (1981) reported a new case of germ line polysomy in the grasshopper *Atractomorpha similis*, a close relative to *A. bedeli*. The resemblances between both cases were quite high, including the absence of extra chromosomes in the somatic cells of both sexes and the inter- but not intra-follicular variation in number. This led the author to reinterpret the case in *A. bedeli* as one of polysomy instead of B chromosomes. In addition, Peters performed a series of controlled crosses in the laboratory and did not find extra chromosomes in the embryos analysed from those crosses. This finding suggested that the extra A9 chromosomes in this species were not transmitted from parents to offspring. In addition, the author performed artificial selection experiments for four generations and showed that 'germ line polysomy is a transmissible character, sensitive to both positive and negative selection'. This result, along with the absence of extra A9 chromosomes in the embryos, led him to suggest that 'the transmission of polysomy occurs through the agency of heritable factors which determine the probability of non-disjunction and thus the accumulation of a particular autosome during a specific series of mitotic divisions in the embryonic germ-line'.

This conclusion was a compelling one for the interpretation of subsequent cases of male germ line polysomy reported in the grasshoppers *Gomphocerus sibiricus* (Gosálvez and López-Fernández 1981), *Omocestus bolivari* (Camacho *et al.* 1981; Viseras and Camacho 1984) and *Chorthippus binotatus* (Talavera *et al.* 1990), as extra elements originated *de novo* in each male from a given A chromosome. In the case of *O. bolivari*, the extra chromosomes, reported as M4 autosome polysomy, showed exactly the same characteristics mentioned above for *C. parallelus*, *A. bedeli* and *A. similis*. In the latter species, Peters (1981) suggested that part of the extra A9 chromosomes could get lost as micronuclei, a fact that was also observed in *O. bolivari* (Viseras and Camacho 1984, 1985). Finally, the polysomy in *C. binotatus* showed essentially the same features as those observed in the remaining species (mosaicism, male germ line restriction and heteropycnosis), although in this case the extra chromosomes had a similar size to the X chromosome, which led Talavera *et al.* (1990) to suggest that they were extra X chromosomes. They performed 21 controlled crosses in the laboratory, two of which involved polysomic male parents, and all 22 embryo offspring analysed again lacked the extra chromosomes, suggesting that the polysomic chromosomes were not transmitted. Talavera *et al.* (1990) also observed the formation of microspermatids in polysomic males, which was more frequent in the testis follicles with odd rather than even numbers.

Following Peters, these authors claimed that the recurrence of these cases of polysomy might be due to a heritable tendency of a standard chromosome to non-disjunction during the development of the testes.

In the course of a recent analysis of the repetitive DNA in the grasshopper *C. parallelus*, Navarro-Domínguez *et al*. (2023) described 110 families of tandem repeats (TRs), 77 of which were analysed by FISH and 50 of them yielded FISH bands on the chromosomes. Among the males analysed in that paper, I found two males from a French population carrying extra chromosomes of a similar size and meiotic behaviour as those previously described in the English populations of this species (see above). The two males were mosaics, displaying some testis follicles with two (or rarely one) extra chromosomes and others lacking them. The high number of TR families displaying bands on the chromosomes after fluorescent *in situ* hybridisation (FISH) gave me the opportunity to investigate whether these extra chromosomes were the product of *de novo* polysomy in the male germ line, as was previously claimed (see above).

The antecedents concerning germ line polysomy in grasshoppers, described above, led to a hypothesis based on the *de novo* origin of a given A chromosome, during development, in those zygotes that inherited certain genetic factors able to trigger the non-disjunctions from becoming a standard chromosome into an extra element displaying mitotic instability and differential heteropycnosis. This hypothesis should meet the condition that the extra chromosomes, as well as the A chromosome from which the former were derived, should contain the same FISH band pattern for TR markers, because it is unlikely that they could display large differences after only a few mitoses. In the current study, I tested this condition by means of FISH for several TR markers in the grasshopper *C. parallelus*. The results for two mosaic males were conclusive in showing that the former condition was not met; thus, the polysomy hypothesis as an explanation for the male germ line mosaicism for extra chromosomes can be rejected.

Materials and Methods

The present analysis is based on two mosaic males of the grasshopper *Chorthippus parallelus parallelus* collected at Arudy (France) (43º 06′ 01″N, 0º 26′38″W) (see Navarro-Dominguez *et al*. 2023). During the analysis of the FISH photographs obtained for the former paper, I found two mosaic males displaying extra chromosomes in addition to the A chromosomes (see the methods for the TR search and FISH analysis in that paper). One of the mosaic males (No. 1) was analysed for nine TR families (CpaTR001-148, CpaTR003-133, CpaTR005-130, CpaTR006-11, CpaTR007-21, CpaTR008-331, CpaTR009-172, CpaTR011-213 and CpaTR012-247), while the other (No. 2) was analysed for eight families (CpaTR022-239, CpaTR026-239, CpaTR038-203, CpaTR049-215, CpaTR061-27, CpaTR062-56, CpaTR075-45 and CpaTR077-16) (see the molecular and FISH characteristics of these families in Navarro-Domínguez *et al*. 2023). All of the A chromosomes in *C. parallelus* are easily identifiable on the basis of their size and/or morphology, and were classified by Hewitt (1963) into three length groups: long (L1-L3), medium (M4-M7) and short (S8), with the X chromosome being longer than the M group, the L chromosomes being meta- or submetacentric, and the remaining chromosomes being acrocentric. The extra chromosomes were therefore identified by comparing the chromosomes present in the extra-carrying and extra-lacking spermatocytes from the same mosaic male.

Results

The two males with germ line mosaicism showed the presence of the extra chromosomes to be acrocentric and of a size similar to the longest acrocentric A chromosomes, i.e. the M4 autosome or the X chromosome. The extra chromosomes closely resembled those previously described in this species (Hewitt 1963), although it is difficult to know whether both chromosomes, found in such distant populations (at France and England), represented the same biological phenomenon. The present research did not allow the frequency of mosaic males in the population to be analysed (Arudy, France), because it would have been necessary to cytologically analyse all the testis follicles from all the males collected. However, my purpose here was to test the *de novo* origin of the extra chromosomes and such information was not needed.

Three out of the 17 TR families analysed were crucial to test the *de novo* origin of the extra chromosomes, and all three were analysed in male No. 1. The first one, CpaTR012-247, showed distal FISH bands on all autosomes but not on the X and the extra chromosomes (Figure 1a), thus excluding the autosomes as possible polysomic A chromosomes that gave origin to the extra chromosomes. The second family, CpaTR007-21, also showed distal FISH bands

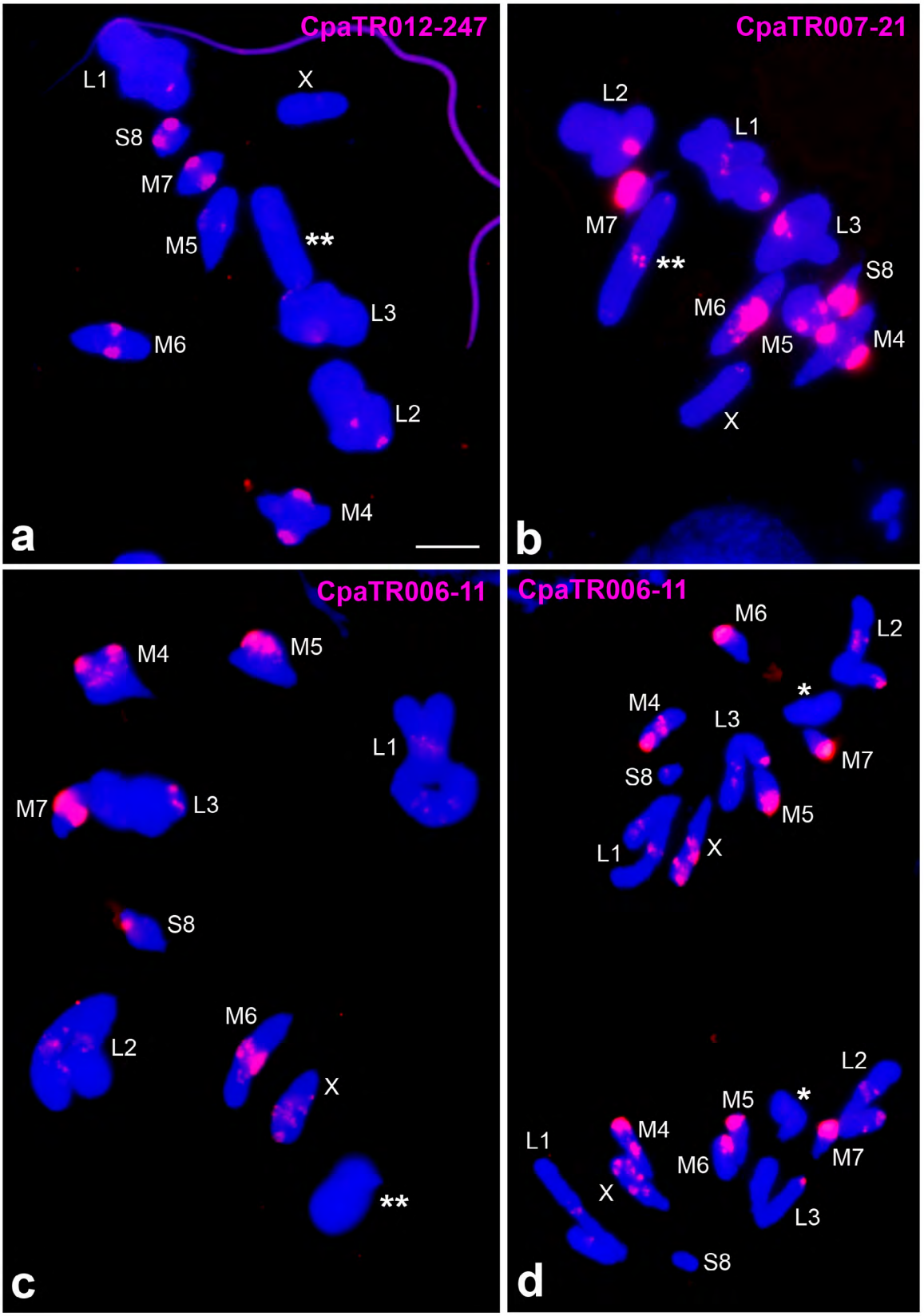

Fig. 1 Meiotic metaphase I (a-c) and anaphase II (d) spermatocytes from mosaic male No. 1 displaying FISH patterns for three TR families on both standard (L1-L3, Ma-M7, S8 and X) and extra chromosomes (one asterisk per extra chromosome). a) Note the presence of FISH bands for the CpaTE012-247 family on all autosomes (L1-S8) and the absence on X and the extra (**) chromosomes, which excludes all autosomes as a possible source for the *de novo* polysomy. b) Presence of CpaTR007-21 family on all chromosomes, including distal bands on the extra (**), whereas the X chromosome shows only a minute proximal band, which also excludes the X chromosome for the *de novo* origin for the recurrent polysomy. c and d) Presence of the CpaTR006-11 family on all A chromosomes and the absence on the extra bivalent (** in c) or the extra chromatids (* in d), thus excluding all A chromosomes as the source for the polysomy. Bar = 5 microns.

on all autosomes and the extra chromosome, as well as interstitial bands on two autosome bivalents, and a minute proximal band on the X and the extra chromosomes (Figure 1b). This was actually the only TR family to display FISH bands on the extra chromosomes, but their FISH pattern did not match those of any A chromosome. Finally, the CpaTR006-11 family displayed FISH bands on all the A chromosomes but not on the extra chromosomes (Figure 1c,d), thus ruling out the possibility that the extra chromosomes could have arisen *de novo* through aneuploidy for any A chromosome. Taken together, these observations were incompatible with the *de novo* origin of the extra chromosomes. Thus, the hypothesis of recurrent polysomy as an explanation for the extra chromosomes observed by us may be discarded.

In the mosaic male No. 2, the TR families analysed by FISH gave poorer information to test the *de novo* hypothesis, as only two of them (CpaTR026-239 and CpaTR077-16) were analysed by FISH on cells containing the extra chromosomes and these families showed FISH bands on only two (L1 and M6) (Figure 2a) or one (M6) chromosomes (Figure 2b), respectively. In addition, the cytological analysis of this male revealed the presence of spermatic micronuclei and microspermatids (Figure 2c,d).

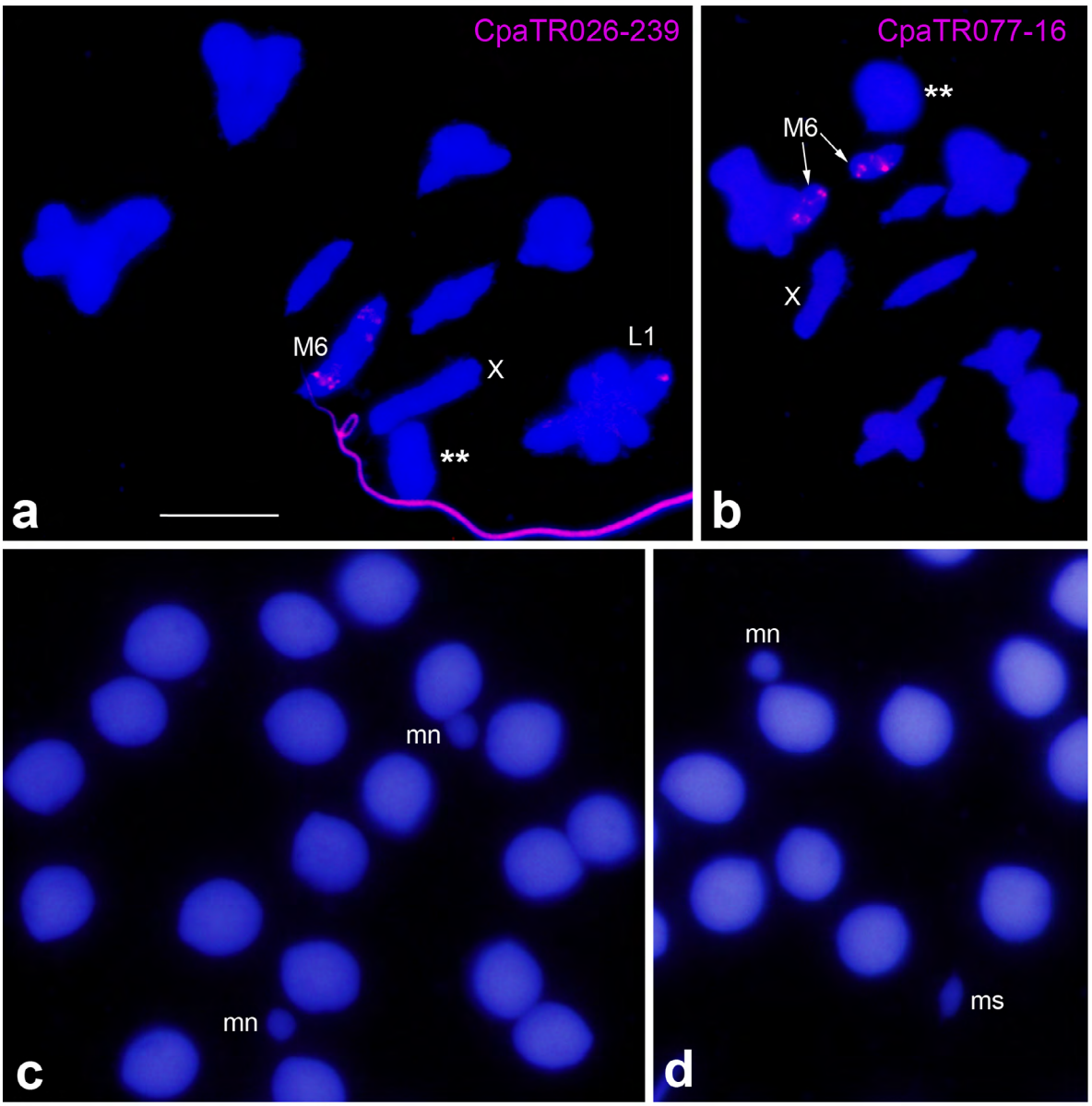

Fig. 2 Meiotic metaphase I (a, b) and micronuclei (mn) and microspermatids (ms) (c, d) from mosaic male No. 2. Note in a) the presence of FISH bands for CpaTR026-239 on the L1 and M6 chromosomes and its absence on the extra bivalent (**). Note in b) the presence of FISH bands for CpaTR077-16 on the M6 chromosomes and its absence on the extra bivalent (**). Bar = 5 microns.

Discussion

Our present results clearly demonstrate that the extra chromosomes found in the testes of the two mosaic males of the grasshopper *C. parallelus parallelus* from the French population at Arudy did not originate *de novo*, through aneuploidy, from any member of the standard chromosome set. The extra chromosomes were acrocentric and similar to those of the X chromosome and the M4 autosome in size. I cannot rule out the possibility that they are the same chromosomes described by Hewitt (1963) as M4 polysomics, but this fact merits further research in English populations using the CpaTR006-11, CpaTR007-21 and CpaTR012-247 TR families.

In fact, the conclusion about the *de novo* origin of the extra polysomic chromosomes in several grasshopper species, reviewed in the introduction to this paper, suffers from several problems that were previously overlooked. The most important issue is that the presence of these chromosomes could have gone unnoticed in the embryos descended from polysomic males (Peters 1981; Talavera 1990), because the mitotic metaphases observed by these authors could all have been from somatic cells, while some of those embryos could have carried the extra chromosomes in germ cells that were not undergoing mitotic division at the moment of the analysis. Therefore, the conclusion that the sperm carrying the polysomic chromosomes do not transmit the extra chromosomes to the next generation could be wrong. In addition, the conclusion that the extra chromosomes described as cases of male germ line polysomy in the aforementioned species of grasshopper are absent in females has not properly been tested at the current time, as all the authors analysed mitotic cells of the gastric caeca or ovarioles, all of which were somatic cells. However, I cannot rule out the possibility that some females actually carry the extra chromosomes in their germ cells, and this could be tested by analysing the female meiosis, which is feasible in grasshoppers (Henriques-Gil *et al*. 1986). In fact, the crosses performed by Peters (1981) actually suggested the possibility that the extra chromosomes were also present in the female germ line. In his selection experiments for polysomy, the frequency of polysomic males sharply increased from 23% to 71% in only three generations, while in the selection against polysomy this frequency was reduced from 40% to 5% in only two generations. From these results, Peters (1981) estimated that the heritability of germ line polysomy was 0.65, and concluded that it is a transmissible characteristic through both sexes, which is sensitive to both positive and negative selection. On this basis, and after not observing the extra chromosomes in the embryos, the author suggested heritable factors as the determinants of recurrent polysomy. However, these observations were also compatible with these heritable factors being germ-line restricted B chromosomes present in both sexes.

In the case of *O. bolivari*, Viseras and Camacho (1984) wrongly concluded that the polysomy for the M4 autosome observed in the male germ line was absent from the female germ line, even though their ovary analysis was restricted to mitosis of the ovariole wall cells, which are actually somatic cells. In addition, these authors missed an important detail that suggested the extra chromosomes might not be homologous to the M4 autosome, because the extra chromosome failed to show nucleolar expression whereas the standard M4 showed a primary (frequently active) nucleolar organising region (NOR) close to the centromeric region. Of course, it was conceivable that the extra chromosomes could carry an inactive NOR, but it is still intriguing why the same chromosome region on the extra chromosome lacked a C-band present on the standard M4, which suggests some structural differences between the standard and extra M4 chromosomes, presumably similar to the presence/absence differences for TRs noticed in the case of the *C. parallelus* mosaics analysed here.

In *C. binotatus*, Talavera *et al*. (1990) followed Peters (1981) in concluding that the extra chromosomes were another case of polysomy, in this case for the X chromosome. A failure to visualise the extra chromosomes in the embryos descended from the two males carrying them led these authors to postulate that the ‘sperm containing E chromosomes is unfit for fertilization and that all offspring produced from polysomic males are derived from sperm lacking E chromosomes’. This conclusion would be wrong if all the mitotic cells analysed in those embryos corresponded to somatic cells. To overcome the problem of sex determination, in case the extra chromosomes were derived from the X chromosome, Talavera *et al*. (1990) speculated on the possibility that the extra X chromosome should be inactive during sex determination, and they even considered the alternative possibility that they actually were not additional X chromosomes.

When all the weaknesses of the recurrent polysomy hypothesis mentioned above are taken together, I believe it is possible that the extra chromosomes reported as cases of polysomy by several authors, including myself, might instead be

germ line restricted B chromosomes. The apparent absence of the extra chromosomes in the embryos descended from polysomic males can be explained if the embryo cells analysed were all somatic, and the extra chromosomes could still have been hidden in the few germ cells existing at the stages that were analysed. In this case, the heritable factors suggested for *de novo* polysomy could actually be the germ line restricted B chromosomes themselves.

In fact, the extra chromosomes found as mosaic polysomy in the grasshopper testes of several species closely resemble the case of the germ line restricted chromosomes (GRC) found in the zebra finch *Taeniopygia gutatta* (Pigozzi and Solari 1998). GRCs are extremely widespread among songbirds, as Torgasheva *et al*. (2019) found them in all 16 species they analysed, opening up the possibility for a common descent of the GRC in this group of birds. Likewise, recurrent polysomy has been reported in several related grasshoppers within the Gomphocerinae subfamily, including *Chorthippus parallelus* (Hewitt 1963; Southern 1967; Hewitt and John 1968, 1970), *C. binotatus* (Talavera *et al*. 1990), *Omocestus bolivari* (Camacho *et al*. 1981; Viseras and Camacho 1984) and *Gomphocerus sibiricus* (Gosálvez and Lopez-Fernandez 1981). In fact, they could be even more frequent, as their mosaic nature and germline restriction make these chromosomes difficult to find.

Another resemblance between the mosaic extra chromosomes in grasshoppers and the GRC of songbirds is their high condensation at the early first meiotic prophase and later expulsion during the anaphase-telophase stages in the form of a dense micronucleus. This has been shown in songbirds (Pigozzi and Solari 1998, 2005; Kinsella *et al*. 2019) and probably represents a pathway to avoid the GRC paternal transmission, although it is not completely achieved (Pei *et al*. 2022). Likewise, in grasshoppers, the cases reported as male germ line polysomy usually show micronuclei and microspermatids (see the Introduction and Figure 2c,d), which are also frequent in many B chromosomes (Camacho *et al*. 2004). In addition, it has been demonstrated that the micronuclei contain repetitive DNA sequences specific to B chromosomes, suggesting that they represent a pathway to B chromosome elimination and are part of the host's defence against parasitic chromosomes (Cabrero *et al*. 2017).

The main difference between the GRC in songbirds and that of grasshoppers is that, in the former, all individuals carry the extra chromosome (Pigozzi and Solari 2005; Itoh *et al*. 2009), whereas it is only present in some individuals in the case of grasshoppers (see the Introduction section). Nonetheless, the female-biased biparental transmission in songbirds is reminiscent of the biparental transmission found by Peters (1981) for the A9 polysomy in the grasshopper *A. similis*, so that the polymorphic status in grasshoppers might represent the ancestral stage for songbirds.

Taken together, the present results mean that the hypothesis of recurrent polysomy can be rejected in the case of the *C. parallelus* analysed here. I would also call into question previous conclusions about other cases described as recurrent polysomy in grasshoppers, including some reported by myself. Alternatively, I would suggest that the extra chromosomes found in those cases were, in fact, germ line restricted B chromosomes, as previously described by Sannomiya (1973) in *A. bedeli*. This is a conclusion that could be extended to all cases previously reported as male germ line polysomy in grasshoppers (see the Introduction section). The hypothesis that they are B chromosomes can be further tested in at least three ways, following the steps of GRC research in songbirds and of B chromosomes in general: i) by analysing the grasshopper female meiosis (Henriques-Gil *et al*. 1986); ii) by searching for repetitive DNA sequences specific to the extra chromosomes and analysing their presence among the genomic sequences obtained from ovaries; and iii) by determining the gene content (and sequence) of the extra chromosomes (as in Kinsella *et al*. 2019) and comparing it to that of the A chromosomes. The best material to test the GRC hypothesis in grasshoppers is the Ashurst population of *C. parallelus* in England, where about 27% of males carried extra chromosomes in the testes (Hewitt and John 1970). The use of the TRs reported by Navarro-Dominguez *et al*. (2023) could serve as a rapid test to solve the polysomy-GRC conundrum.

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Author Contributions

Research concept and design, Collection and/or assembly of data, Data analysis and interpretation, Writing the article, Critical revision of the article, Final approval of article – J.P.M. Camacho.

Conflicts of Interest

The author declares no conflict of interest.

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