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Germ cell restriction and regular transmission of an accessory chromosome that mimics a sex body in the zebra finch, *Taeniopygia guttata*

M. I. Pigozzi & A. J. Solari

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Mitotic and meiotic analysis with light and electron microscopy was performed in male and female zebra finches (*Taeniopygia guttata*). Somatic cells from bone marrow have a $2n = 80$ and show the usual sex chromosome mechanism of birds ZZ (male)/ZW (female). In the germ lines of both sexes, a single accessory chromosome was regularly present in all the cells examined from all the individual birds. In synaptonemal complex (SC) spreads of pachytene oocytes and spermatocytes, this accessory chromosome forms a single axis, but it behaves differentially in male and female meiosis. While this accessory chromosome is euchromatic in oocytes, it is strongly heterochromatic in spermatocytes. In pachytene spermatocytes, the accessory chromosome adopts a morphology strikingly similar to that of the XY body ('sex vesicle') of mammalian spermatocytes. This accessory chromosome is eliminated during male meiosis and forms a cytoplasmic dense body in young spermatids that shows strong fluorescence with DAPI. The presence of this germ line-restricted chromosome does not affect the behaviour of the ZW pair in oocytes, as the sex chromosomes pair regularly and show a localized recombination nodule. It is suggested that this accessory chromosome has transcriptional activity during oogenesis, and thus it is regularly transmitted through preferential segregation during female meiosis.

Key words: accessory chromosome, meiosis, pseudo-sex vesicle, synaptonemal complex

Introduction

The vertebrate class Aves has a large proportion – about 90% (Capanna *et al*. 1987) – of karyologically unknown species. Despite this fact, the presence of a ZZ (male)/ZW (female) sex-determining system has been verified in all the karyotyped species, which belong to almost all the orders of birds (De Boer 1984, Capanna *et al*. 1987). The behaviour of the avian sex chromosomes during female meiosis has been clarified since the discovery of partial synapsis of the Z and W chromosomes in the chicken (Solari 1977) and the demonstration of recombination nodule localization (Rahn & Solari 1986, reviewed in Solari 1993) and terminal chiasmatic restriction in the ZW pair at the lampbrush stage (Solovei *et al*. 1993). In male meiosis, the ZZ bivalent shows full synapsis and free recombination (Pollock & Fecheimer 1978, Kaelbling & Fechheimer 1983). In both sexes, the sex chromosomes are euchromatic during meiotic prophase, and no evidence exists on the presence of dosage compensation by sex chromosome inactivation, as seen in mammals (reviewed in Solari 1993).

During the course of a comparative study of the meiotic behaviour of the ZW pair in birds, a unique chromosomal constitution has now been discovered in the passeriform bird *T. guttata* (zebra finch). While somatic cells show a karyotype $2n = 80$, ZZ in males and ZW in females, germ cells in both sexes show a single additional accessory chromosome that is the largest component of the karyotype and that shows a differential behaviour in male and female meiosis. The accessory chromosome is euchromatic in oocytes and strongly heterochromatic in male germ cells, in which it mimics a 'sex body' similar to the heterochromatic XY pair of mammals (reviewed in Solari 1989, 1993). The synaptonemal complex analysis of oocytes and spermatocytes shows that along with the presence of regular ZW and ZZ pairs in females and males, respectively, the accessory chromosome shows a differential behaviour in each sex. This behaviour is independent of the sex bivalent. The accessory chromosome is eliminated at the end of meiosis in the male germ cells, while it is transmitted through females.

The presence of this germ-cell-restricted, accessory chromosome seems to be widely shared among different zebra finch strains, according to preliminary observations on the presence of this heterochromatic body in males (C.B. Gillies, unpublished observations). Additionally, the demonstration of partial synapsis and strict localization of a recombination nodule in the ZW pair of this bird extends to the large passeriform group the pattern of gonosomal behaviour that has been observed in other avian taxa.

M. I. Pigozzi and A. J. Solari (corresponding author) are at the Centro de Investigaciones en Reproduccion, Facultad de Medicina, (1121) Buenos Aires, Argentina. Fax: (+54) 1 963 8611; Tel: (+54) 1 963 8611; Email: cir@fmed.uba.ar.

Materials and methods

From a total of more than 20 birds used in this study, four adult males and four young females (from 3 to 5 days after hatching) were chosen for detailed electron microscopy and meiotic analysis. The synaptonemal complex spreads of oocytes and spermatocytes were made either with the microspreading method (Rahn & Solari 1986) or with a modified drying-down technique as described by Peters *et al.* (1997). The nuclei were recovered on glow-discharged slides coated with plastic film, washed in 0.4% Photoflo (Eastman, Kodak) adjusted to pH 8.0 with borate buffer and were then air dried. The preparations were stained with phosphotungstic acid (PTA) in ethanol (Moses 1977) or with silver nitrate (Howell & Black 1980). Cells were examined with a Siemens Elmiskop electron microscope, and length measurements were made with a Curvimetre (HCHB) on electron micrographs printed at magnifications ranging from 6000 to 20 000×. Statistical tests were performed using the Stata program (Computing Resource Center, LA, USA). Additional samples of testicular tissue were fixed in 2.5% glutaraldehyde in cacodylate buffer at pH 7.0, embedded in Maraglas and cut in thin and semi-thin (1-μm-thick) sections, the latter for light microscopy.

Mitotic chromosomes were obtained from marrow samples of all the examined individuals, after short-term cultures in RPMI-1640 medium, and added with colchicine at a final concentration of 0.05 μg/ml. Part of the testicular material and one of the ovaries were used for light microscope preparations, using the air-drying technique for meiotic cells (Evans *et al.* 1964); observations were made after routine and banding staining techniques. DAPI fluorescence was examined in a Leica DMRB microscope fitted with the corresponding combination of excitation and transmission filters.

Results

The mitotic karyotype

A modal number of $2n = 80$ was found in the micrographed metaphases from bone marrow cells of male and female individuals and the macrochromosomes (nos. 1–7) can be readily identified (Figure 1a & b). The Z chromosome is the fourth in size (6.1% of the haploid set) and has a submedian centromere and a secondary constriction in its long arm. The W chromosome is acrocentric and the ninth in size (r.l. = 2.5%). This W chromosome is strongly heterochromatic after C-banding. On the other hand, all the spermatogonial mitoses and the SC complements of oocytes and primary spermatocytes show a large accessory chromosome in addition to the somatic mitotic complement. In spermatogonial metaphases (Figure 1c), the accessory chromosome is found in all the spermatogonia as a single element that is significantly larger than pair no. 1 and has a submedian constriction, which is scarcely visible in many cells.

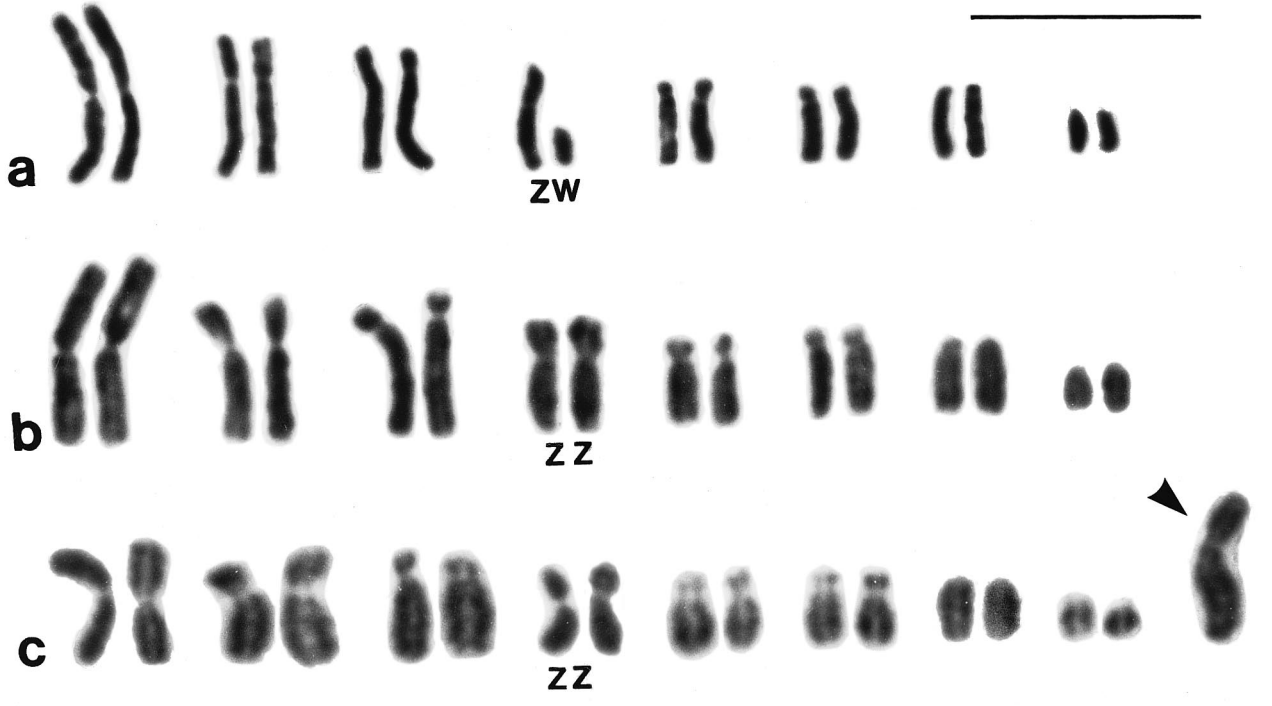

Figure 1. **a** Partial karyotype representing the first seven macrochromosomes and the Z and W chromosomes from bone marrow cells of female zebra finch. **b** The first seven macrochromosomes and the ZZ pair from bone marrow cells of male zebra finch. **c** The first eight macrochromosomes and the accessory chromosome (arrowhead) from a spermatogonial mitosis of a zebra finch. Bar = 10 μm.

The SC karyotype of oocytes (see Figure 3) and spermatocytes shows a very good agreement between the relative lengths and the arm ratios of SCs and mitotic macrochromosomes nos. 1–7 from bone marrow cells (Figure 2), and there is not any extra element in somatic karyotypes that could match the additional element present in germ cells.

Sex chromosomes and the accessory axis in pachytene oocytes

The Z and W chromosomes pair regularly during early pachytene (Figures 2 & 4a). As pachytene advances, the Z axis shortens in a process of axial equalization, as previously described in other carinate birds (reviewed in Solari 1993) (Figure 4b). Synapsis of the Z and W axis

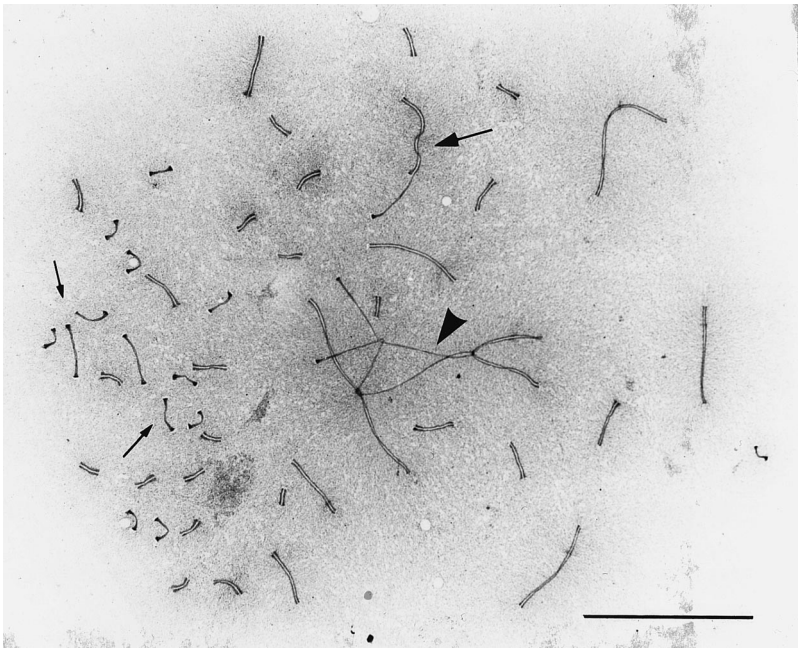

Figure 2. Electron micrograph of an oocyte from the youngest female. The ZW pair (arrow) is not yet equalized. The accessory axis (arrowhead) is asynaptic and is hooked on two autosomal SCs. Several microchromosomal axes are asynaptic (small arrows). Silver staining. Bar = 10 μm.

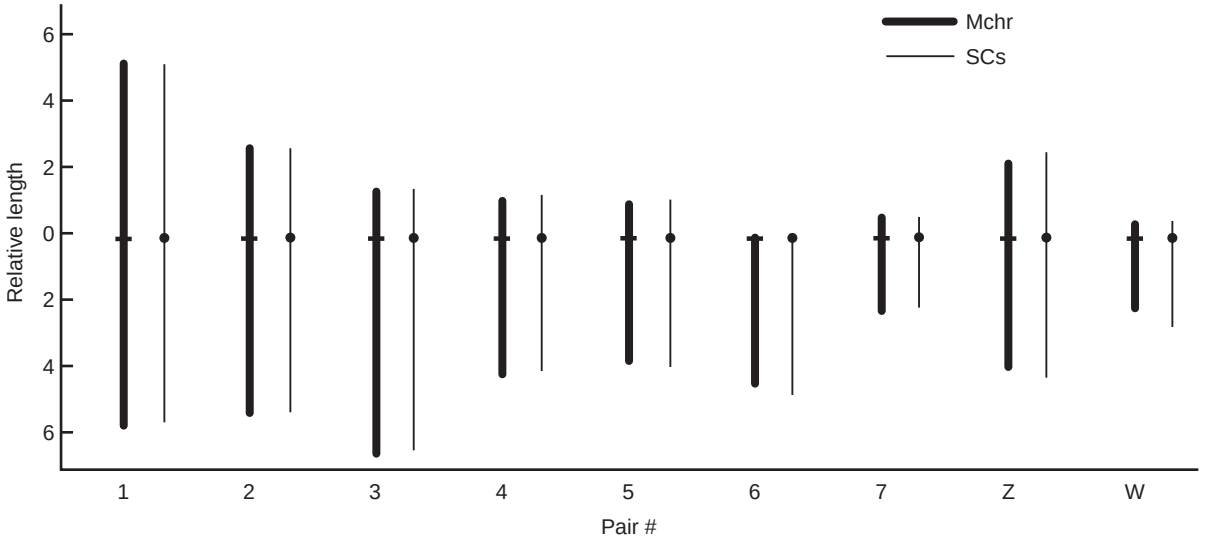

Figure 3. Comparative idiograms of mitotic chromosomes (thick lines) and synaptonemal complexes (thin lines) of zebra finches. Relative length (RL) and arm ratios are means of each population.

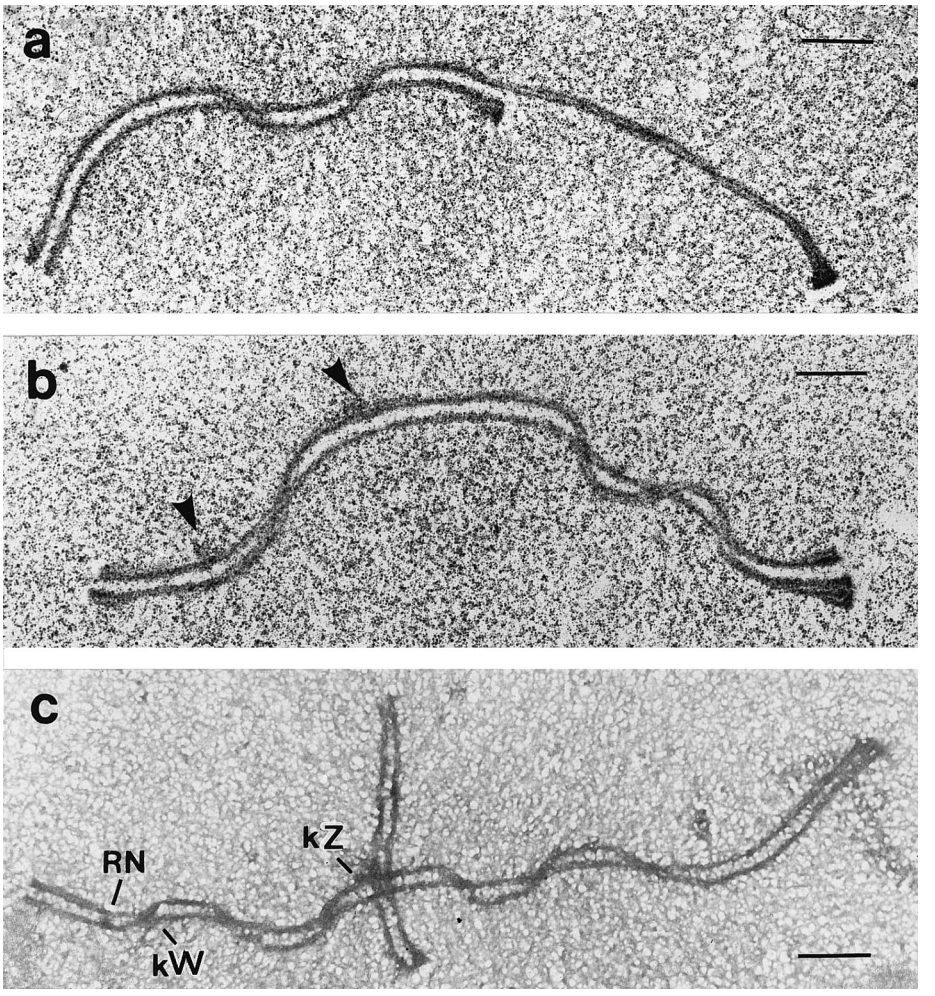

Figure 4. a Higher magnification of the ZW pair in an oocyte. Silver staining. **b** A ZW pair from an older female showing axial adjustment (equalization) of the Z axis. Arrowheads: kinetochores. Silver staining. **c** An equalized ZW pair showing the localized recombination nodule (RN) in the initial SC segment. kW, kZ: kinetochores. PTA staining. Bars = 1 μm.

is arm specific, as it always begins by the short arm of the W and the short arm of the Z, as can be shown by the kinetochore location (Figure 4b). A single, strictly localized recombination nodule (RN) is regularly found at a mean distance of 0.87 μm from the earlier synaptic termini (Figure 4c).

An accessory, single axis was present in all the oocytes examined ($n = 100$) (Figure 2). In the oocytes from the youngest female, this accessory axis was mainly asynaptic (Figure 2). On the other hand, the accessory axis was partly or completely self-paired in the majority of the oocytes coming from the oldest female (Figure 5), in which pachytene was more advanced as judged from the general adjustment (equalization) of the Z and W axes. Among the younger oocytes with an asynaptic accessory axis, many showed

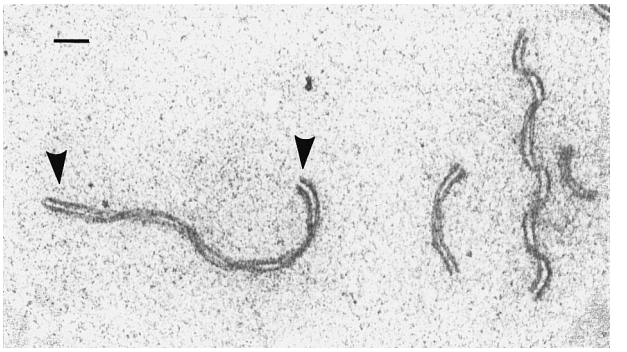

Figure 5. The accessory axis (arrowheads) in an oocyte from the older female, showing self-synapsis. Silver staining. Bar = 1 μm.

three to seven asynaptic microchromosomes (Figure 2), while in the older oocytes with a self-paired accessory axis there were no asynaptic microchromosomes.

No typical kinetochore is recognized in the accessory axis, and the chromatin surrounding this axis has the same degree of packing as that surrounding autosomal SCs (Figures 2 & 5). The lack of any heteropycnotic element was also evident in Giemsa-stained oocytes examined with light microscopy (not shown).

The ZZ synaptonemal complex and the accessory chromosome axis in spread pachytene spermatocytes

In all the spermatocytes examined with the electron microscope, the SC complement has the same autosomal sets as the oocytes, a ZZ pair and prominent body composed of differentially packed chromatin enclosing a single, asynaptic axis (Figure 6). The degree of chromatin packing of the accessory chromosome is variable and in many cells is as tight as to mask – partly or completely – the axial element. A mean length of 14.9 μm was recorded for those accessory axes that were not masked by chromatin. The ZZ bivalent is the fourth in length and has a regular, complete SC with submedian kinetochore (Figure 6).

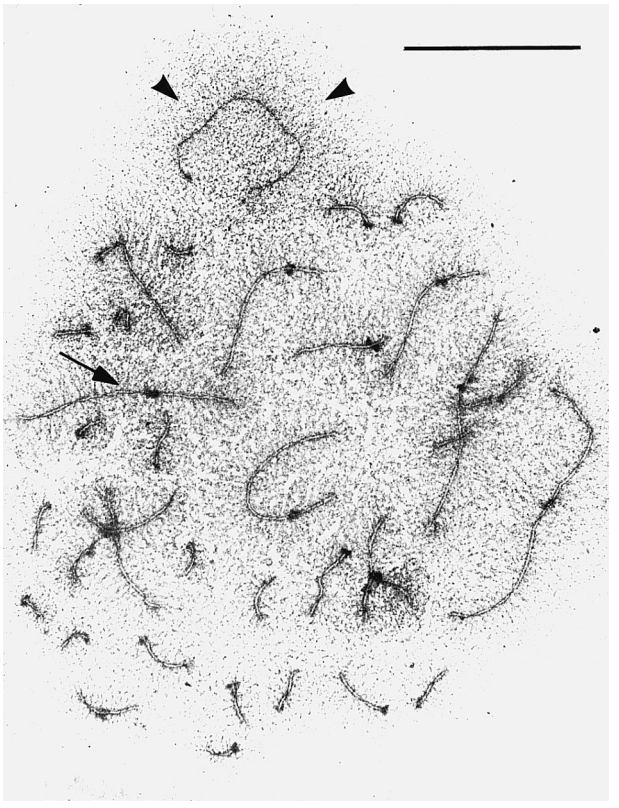

Figure 6. Electron micrograph of a pachytene spermatocyte of a zebra finch. A prominent peripheral body has the accessory axis inside (arrowheads). The ZZ bivalent (arrow) is the fourth in size and has a submedian kinetochore. Silver staining. Bar = 10 μm.

Behaviour of the accessory chromosome during spermatogenesis

As already mentioned, all the spermatogonial mitoses observed with the light microscope showed a very large accessory chromosome that was typically heterochromatic after Giemsa staining (Figure 1c). At early stages of prophase spermatocytes (lepto–zygotene), a prominent heteropycnotic body composed of a heavily stained, coiled filament is observed. During pachytene stage, this filament is more condensed and forms an oval, dense body (Figure 7a), often with a non-stained inner gap that strongly resembles the 'XY body' (Solari 1974) or 'sex vesicle' found in mammalian spermatocytes at pachytene.

At metaphase I, this 'pseudo-sex vesicle' is no longer observed, but a large univalent is present along with the normal complement (Figure 7b). At this stage, the accessory chromosome is negatively heteropycnotic and often has a fuzzy outline (Figure 7b). In metaphase II, the accessory chromosome cannot be identified as the largest element among the macrochromosomes, but a round, dense body, 2–3 μm in diameter, was found associated with 40% of the micrographed divisions II (Figure 7c). This round body was homogeneously stained with Giemsa, although in some cases it showed a fibrilar appearance. In semi-thin sections of testes, a similar, round body was observed in the perinuclear cytoplasm of about 40% of the secondary spermatocytes and also in a significant number of young spermatids with round nuclei. The DNA content of this body was assessed using DAPI fluorescence in air-dried spreads. As expected from a DNA-containing structure, these round bodies have a strong DAPI fluorescence and are clearly differentiated from the nuclei of late spermatids and the elongated and wavy sperm heads (Figure 8a). A prominent oval body with DAPI fluorescence is also observed as the 'pseudo-sex vesicle' in pachytene spermatocytes (Figure 8b).

Discussion

The present observations extend the knowledge about the synaptic pattern of the ZW pair to one species of the order Passeriformes. This behaviour is characterized by: (a) delayed, partial synapsis of the gonosomal axes at early pachytene; (b) progressive adjustment of the axial lengths; and (c) presence of a strictly localized RN near the synaptic end. This pattern has been shown in the ZW pairs of all previously examined carinate birds (reviewed in Solari 1993). Thus, the conserved nature of this behaviour in this avian subclass (Solari 1993, Solari & Pigozzi 1993) is strongly supported by the findings in *T. guttata*, as the order Passeriformes is considered the more evolved group in avian phylogeny and it is one of its latest branchings (Sibley & Alhquist 1990, Cooper & Penny 1997).

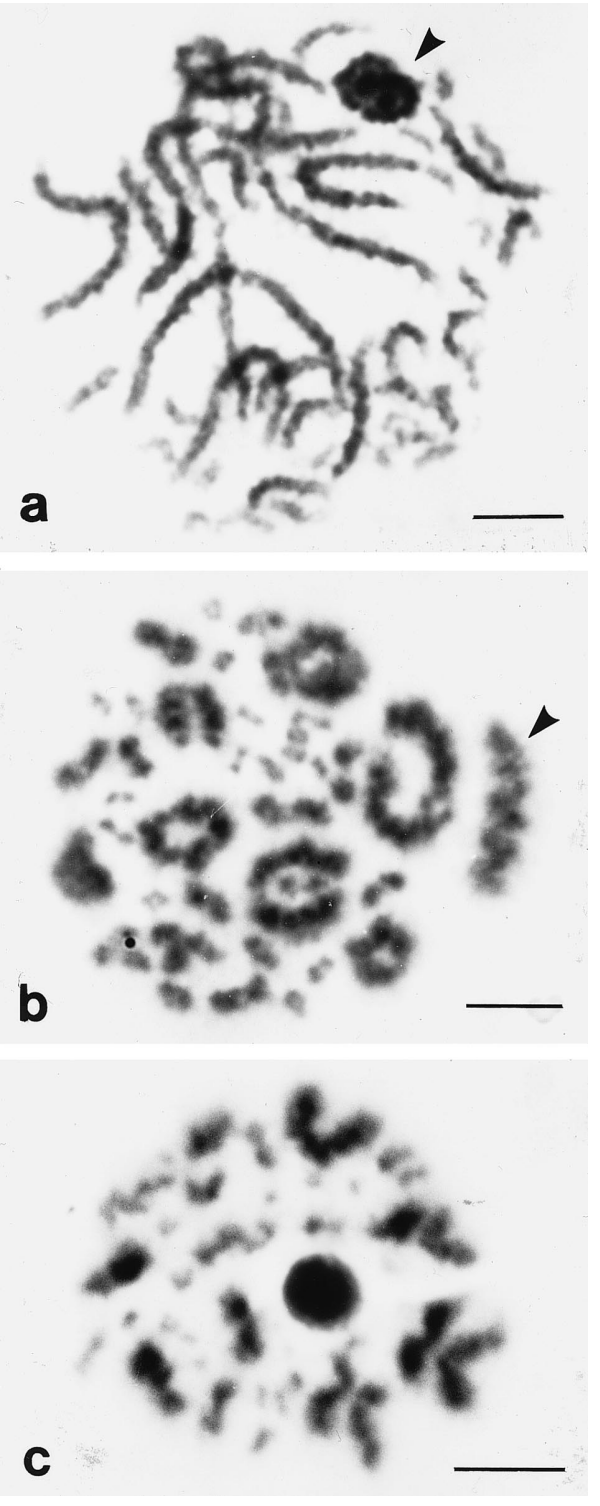

Figure 7. **a** Pachytene spermatocyte after Giemsa staining. The prominent, oval pseudo-sex vesicle (arrowhead) corresponds to the accessory chromosome. **b** First meiotic metaphase from zebra finch testis. A single, coiled and very large univalent (arrowhead) is present and is negatively heteropycnotic. **c** Second meiotic metaphase from zebra finch testis. A prominent round, dense body is present in about 40% of these M-II cells. Bar = 5 μm.

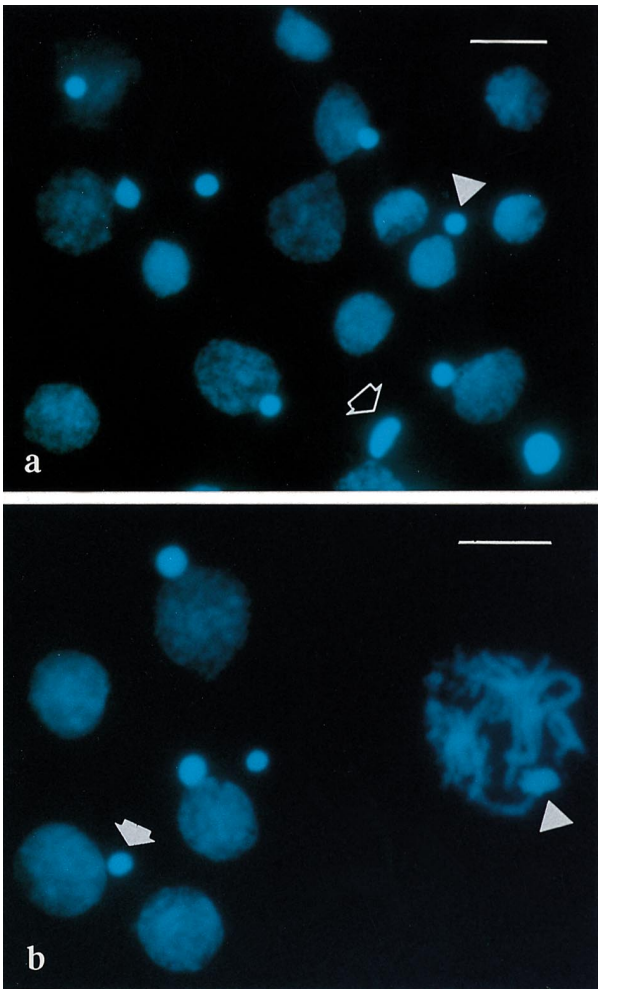

Figure 8. DAPI fluorescence in spreads from zebra finch testes. **a** The brightly fluorescent round body (arrowhead) associated with the nuclei of spermatids is present. Late spermatid nuclei and a sperm head (hollow arrow) are clearly differentiated. **b** DAPI fluorescence of the dense oval accessory chromosome (arrowhead) in a pachytene spermatocyte. The fluorescent round body (filled arrow) is associated to the round nuclei of early spermatids. Bar = 10 μm.

The presence of a recombination nodule near the synaptic termini in all the examined carinate birds has been interpreted as the existence of a short region of homology in the Z and W chromosomes (similar to the 'pseudoautosomal region' found in the XY pair of mammals), with a mandatory recombination event occurring at that place (Solari *et al*. 1988, Solari 1993). This hypothesis is supported by the regular association of the Z and W chromosomes during diplotene, when the bivalents adopt the lampbrush form (Solovei *et al*. 1993). Furthermore, this terminal association is clearly chiasmatic in *C. livia* (Solovei *et al*. 1993), and it is consistent with the presence of a RN near the telomeres in this species (M. I. Pigozzi & A. J. Solari, unpublished

observations). Molecular evidence about the existence of reciprocal recombination in the avian ZW pair is still lacking, as the sex-linked sequences isolated up to date do not map on the putative pseudoautosomal region (Ogawa *et al*. 1997, and references therein).

Presence and behaviour of an accessory chromosome in the germ line

As far as we know, no previous report is available on the presence of a B-chromosome in birds. However, this chromosome has exceptional features: it is invariably present as a single element in the germ line of individuals of both sexes, and it is absent in metaphases from bone marrow. As the stem cells from bone marrow give rise to several cell lineages, it is strongly suggested that this accessory chromosome is absent in other somatic tissues. This accessory chromosome seems to be transcriptionally active in the oocyte, as shown by the dispersed state of its chromatin – identical to that of the autosomes. The regular maintenance of this accessory chromosome in all the individuals suggests that this chromosome may have a functional role in the oocyte, and that this putative function is dispensable in somatic cells and in the male germ line. As the genome size in birds is the most conservative among vertebrates, (Tiersch & Wachtel 1991) a large additional chromosome would scarcely be tolerated if totally deprived of function or adaptive value. Although the functional role of this chromosome remains unknown, its presence is associated with the delayed synapsis of the smallest microbivalents in oocytes (see Results). Microbivalents are generally the first to complete synapsis (Solari 1977) and, furthermore, in the spermatocytes of *T. guttata* they do not show asynapsis, while the accessory chromosome is completely heterochromatic and presumably inactive. In fact, the heterochromatic B-chromosomes in the pachytene spermatocytes of the rodent *Apodemus peninsulae* are transcriptionally inactive, although they are highly variable in number (Ishak *et al*. 1991). Self-synapsis of the accessory axis at later pachytene stages in oocytes is similar to that observed in the univalent B-chromosomes of *Vulpes vulpes* (Switonski *et al*. 1987) and probably reflects the permissiveness for non-homologous pairing during these later stages (Moses *et al*. 1982, von Wettstein *et al*. 1984).

The dispensability of this accessory chromosome for the male meiosis is shown by the extreme chromatin condensation since the leptotene stage and finally by its elimination from the nuclei of secondary spermatocytes (see Results). The striking similarity of the accessory chromosome with the XY body of mammalian spermatocytes at pachytene (reviewed in Solari 1974, 1993) is probably due to the transcriptional inactivity shared by these two structures: both are associated with the nuclear envelope, and have a high degree of chromatin packing and inner axial structures. However, the XY body of mammalian spermatocytes is under the control of the X*ist* gene, which is restricted to mammals (reviewed in Solari 1993), while the mechanism underlying the chromatin packing of the accessory chromosome remains unknown.

Mechanisms of maintenance and transmission of the accessory chromosome

Generally, B-chromosomes display a non-Mendelian inheritance as a consequence of accumulation mechanisms acting in the germ lines of both sexes (reviewed in Jones & Rees 1982). In *T. guttata*, the accessory chromosome is regularly found in the germ cells of both sexes, indicating a stable behaviour during mitotic divisions preceding meiosis, and its invariable presence in all the examined individuals shows its regular transmission from one generation to another. Thus, this chromosome is better defined as a germ-cell-restricted, accessory chromosome rather than a B-chromosome. No individuals were found carrying more than one accessory chromosome in the germ cells, as would be expected if this chromosome segregates randomly, and the gametes with and without the accessory chromosome were formed in equal proportions in both sexes. Given the large size of the accessory chromosome, severe constraints would be put to nuclear functions if more than one accessory chromosome occurred in one cell, particularly considering the small amounts of DNA found in birds compared with other taxa (Tiersch & Wachtel 1991). Furthermore, the deleterious effects of high numbers of B-chromosomes have been reported in several animal and plant species (Jones & Rees 1982). Hence, a mechanism must be working in *T. guttata* to prevent the accumulation of the large accessory chromosome, but at the same time ensuring its maintenance and transmission.

From the cytological evidence (see Results), it is suggested that the following events occur: (a) inactivation and elimination of the accessory chromosome in the male germ line; (b) preferential segregation during anaphase I in oocytes; and (c) elimination of the accessory chromosome from the somatic cells and its conservation in the germ line (Figure 9).

The inactivation of the accessory chromosome in the male germ line is indicated by the condensed chromatin at early meiosis. As an axis is built in the meiotic accessory chromosome, its two chromatids would move together to the same pole, and then secondary spermatocytes with and without the accessory chromosome would be expected to be present in the same amounts. Actual segregation in male meiocytes could not be verified because the accessory chromosome did not appear as a regular chromosome at metaphase II. The round heterochromatic body associated with metaphase II is considered to be a product of the inactive accessory element, leading to its elimination from male germ cells, because of the following evidence: (a) its chromatin constitution confirmed by DAPI staining and (b) its presence in metaphase II and later stages. The number of metaphases II carrying the round chromatin body is near the expected 50%, and the slight discrepancies with the expected value may be due to the disruption of the

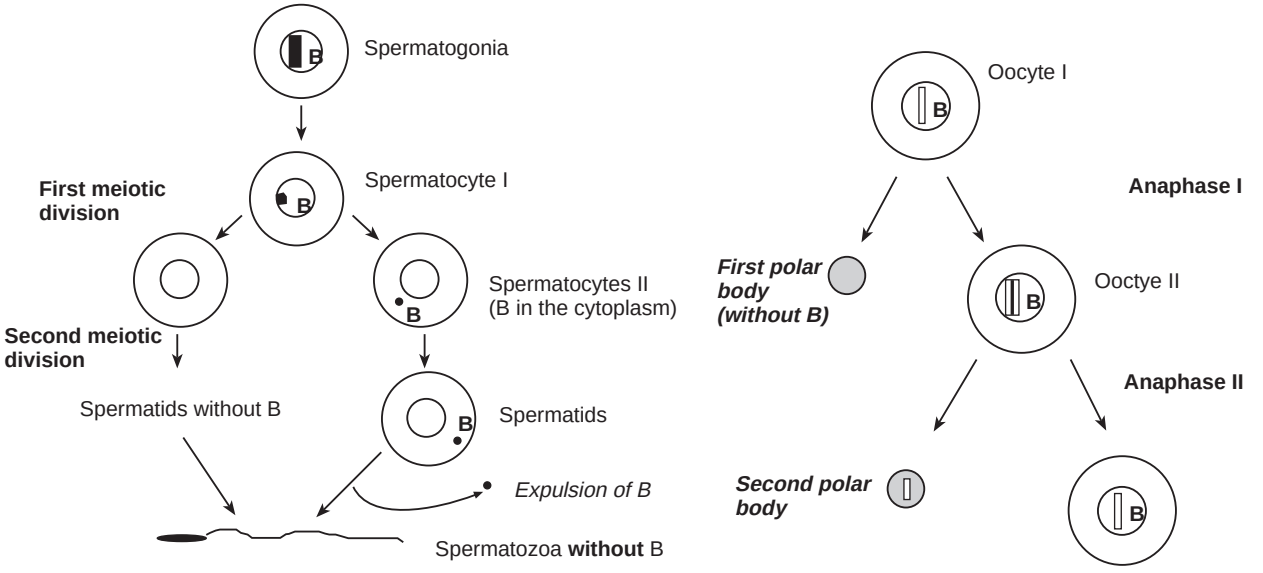

Figure 9. Schematic drawing of the mechanism of transmission of the accessory chromosome in the zebra finch.

cell nuclei in light microscopical preparations. The round body is confined to the cytoplasm (as seen in spermatocytes II and spermatids) and subsequently eliminated to give only spermatozoa without accessory chromosome.

In oocytes, the accessory chromosome seems to be fully active, and the regular formation of the accessory axis suggests the presence of normal sister-chromatid cohesion and the joined migration of the chromatids during anaphase I. Instead of the normal, random migration of the accessory chromosome either to the polar body or to the future oocyte nucleus (germinal vesicle), a preferential segregation to the oocyte-forming nucleus is assumed. Thus, only oocytes carrying an accessory chromosome would be originated. These oocytes when fertilized by spermatozoa without the accessory chromosome would restore the regular presence of the accessory chromosome in single dose, as actually observed in individuals of both sexes.

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