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Histological examination of the male gonad of hybrid specimens: *Microtus savii x M. brachycercus* (Rodentia-Arvicolinae)

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Abstract. *Microtus brachycercus,* previously described as a subspecies of *M. savii,* has a different karyotype and produces sterile male hybrids when crossed with *M. savii.* A histological examination of male gonads of hybrid specimens *M. savii x M. brachycercus* was carried out to assess causes of sterility. Differences in male hybrid sex chromosomes were shown to have a differential effect on fertility. Degenerative figures were present in cells where meiosis was not completed. A possible link between heterochromatin evolution and male hybrid sterility is discussed.

Key words. Rodentia, *Microtus,* speciation, hybrids, male gonad.

Introduction

M. savii (De Sélys Longchamps, 1838) and *M. brachycercus* (von Lehmann, 1961) are two different species within the morphologically homogeneus group of pine voles forming the "*Microtus savii* complex". Karyological analyses revealed striking differences in the sex chromosomes between these two species, but substantially identical autosomal G-banding patterns (Galleni et al. 1992, 1997). Both the X and the Y chromosomes of *M. brachycercus* exhibit a marked increase in their heterochromatic regions and a variation in the classes of heterochromatin amplified, as detected by C and DA/DAPI banding patterns (Galleni et al. 1992).

The F1 generation was obtained from crosses between specimens of *M. savii* and *M. brachycercus* without any reduction in the number of offspring, while the F2 was completely lacking. Backcrosses revealed male hybrid sterility, whereas the females were normally fertile (Galleni et al. 1994).

In the present paper we report the results of analyses performed by optical and TEM microscopy on the histological structure of male gonads in *M. savii* and *M. savii x M. brachycercus* hybrids.

Materials and methods

The four specimens included in this study were reared in the mammalian laboratory of the Dipartimento di Coltivazione e Difesa delle Specie Legnose, Sez. Entomologia Agraria of Pisa University. A specimen of *Microtus savii* (A1) was used as a control. Two specimens were obtained from crosses (M) *M. savii* x (F) *M. brachycercus* (A2 and A3) and the fourth specimen from crosses (M) *M. brachycercus* x (F) *M. savii* (A4).

The age of the specimens varied: A1 was 116 days old, A2 53 days old, A3 227 days old and A4 234 days old. As the maturity in these species is reached after about 50 days (Caroli & Santini 1996), all the specimens could be considered sexually mature.

For histological preparations testis were cut into small pieces (0.5 mm^3), fixed in a solution of 4 % paraformaldehyde in 0.1M phosphate buffer (pH 4), washed for 24 hours in fresh pho-

sphate buffer and then embedded into glycol metacrylate. Sections 0.5 μm thick were stained with methylene blue and toluidine blue.

For TEM observations, small pieces (0.5 mm^3) were fixed in Karnowsky (2 % paraformaldehyde, 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) for three hours and soaked in an OsO_4 solution (0.1 %) for a day. After dehydration in ethanol and propilene oxide, they were embedded into an Epon-Araldite mixture. Sections (50 nm) were stained with uranyl acetate and lead nitrate, and observed with a Siemens 102 electron microscope.

Results

The general morphology of A1 (control specimen) agreed with other descriptions of testis structure and seminiferous tubule organization in rodents (see Huckins 1971). The multi-stratified epithelium was well organized in a specific combination that progressed from spermatogonial cells to spermatozoa with typical scimitar-shaped heads (Fig.1). Spermatogenesis was greatly altered in hybrid specimens. Meiosis stopped completely at pachytene in A2 and A3 hybrids and no further stage was found in either animal while degenerative patterns appeared (Fig. 2 and Fig. 3). Sertoli cells and spermatogonia were present in all the hybrids without evident differences from the control but prophase I spermatocytes in A3 were markedly lower than in the other specimens.

A4 specimen presented different situations according to the tubule considered. While in some tubules a pattern similar to that of A2 and A3 hybrids was found, in others meiosis was completed and spermatids were visible (Fig. 4). Spermatids in advanced stage of spermiohystogenesis were observed in about 18.5 % of tubules.

Analyses carried out by TEM revealed a strong alteration of cytoarchitectonic in A2 and A3 spermatogonial cells and spermatocytes: chromatin was distributed in a homogeneous way without any structural feature to refer to as a clue of sexual body or synaptinemal complex formation, whereas a large number of vacuoli and secondary lysosomes were detected in the cytoplasm (Fig. 5). The number of cell organules was also lowered and axonema were absent in all the tubules observed. These wide degenerative events explain the specimens' sterility.

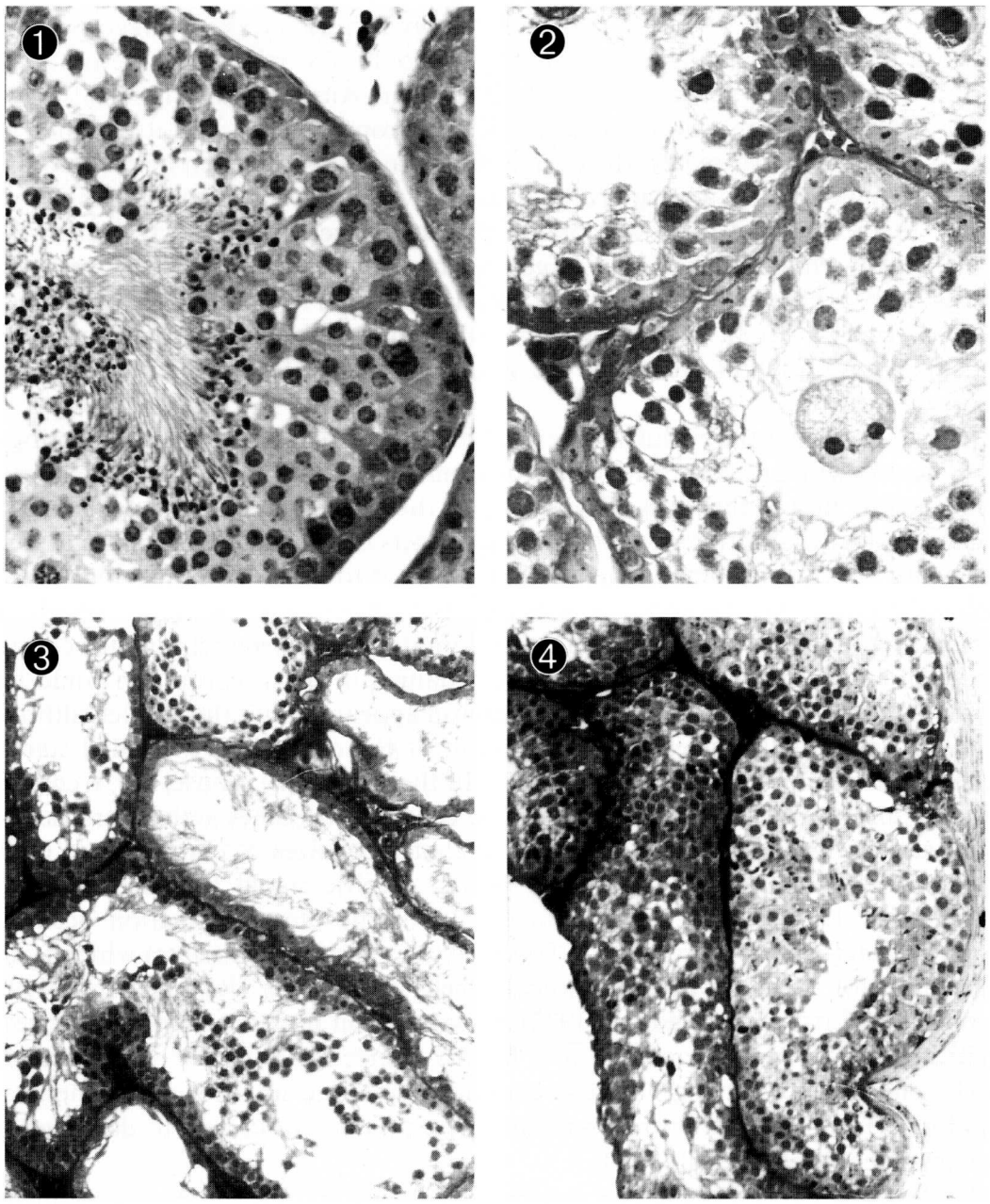
In A4 specimen alterations in acrosome formation took place during spermiohystogenesis and the axonema was unaffected. No difference was observed as regards the control in the area where meiosis proceeded successfully. However the few mature spermatozoa showed clear alterations in acrosomial morphology (Fig. 6). Oligospermia and spermatozoa alterations are factors leading to sterility in this specimen.

Discussion

The effects of chromosome rearrangements occurring with addition or deletion of heterochromatin are still under debate (for a wide revision and discussion of the relationships between chromosome evolution and speciation see King 1993 and Jablonka & Lamb 1988, 1991, for the peculiar aspect of sex chromosomes and speciation). Miklos (1974) argued that chromosomal pairing is not affected when the amount and position of the centromeric heterochromatin is altered and also when their homology is disturbed. Patton and Sherwood (1982) found out that there was a lack of substantive data to support heterochromatin as an agent of speciation by their analysis on the *Thomomis bottae* complex. Also King (1987) and John (1988) denied a strict relationship between variation in heterochromatin content and repro-

ductive barriers. However, contrasting results emerge from other works. Solari & Ashley (1977) considered the absence of XY pairing and synaptonemal complex formation in the sand rat, *Psammomys obesus,* to be a consequence of heterochromatin addition to both ends of the X chromosome.

Male specimens of *Nesokia indica* with polymorphic X and Y chromosomes differing in amounts of heterocromatin, are sterile or exhibit reduced fertility. Cryptic-



Figs 1—4: Seminifera tubules of *Microtus savii* (A1; upper left), hybrid specimens from crosses between (M) *M. savii* and (F) *M. brachycercus* (A2 and A3; upper right and lower left) and from crosses between (M) *M. brachycercus* and (F) *M. savii* (A4; lower right).

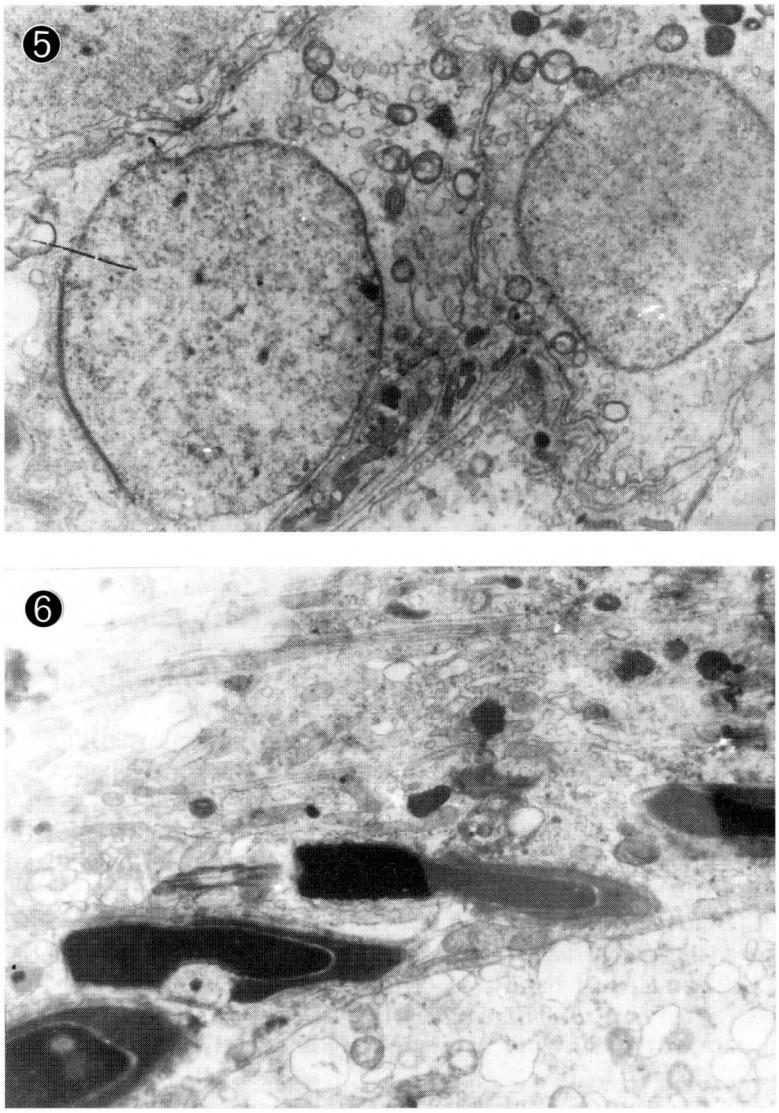
coding DNA sequences possibly interspersed in the heterochromatin and lost during rearrangements are also thought to affect male hybrid fertility (Juyal et al. 1989).

Finally, two morphologically similar species of the Indian pigmy field mouse (*Mus boodoga* and *Mus dunni*) have shown identical euchromatic G-banded regions within their genomes but marked differences in their X and Y C-bands. The acquisition of heterochromatin by sex chromosomes seems to have determined the evolutionary differentiation of these sympatric species (Sharma et al. 1990).

The similarity of autosomal banding patterns and the striking differences in sex heterochromatin between *M. savii* and *M. brachycercus* karyotypes suggest that the variation of sex chromosome heterochromatin could also relate to the evolution of these two species. As results from DA/DAPI and Alu I bands, these differences could involve not only the amount of heterochromatin but also its quality (see Galleni et al. 1992). Moreover, the degree of heterogeneity between heterochromatic regions in male hybrids appears to be related to the level at which meiosis is interrupted. In specimens A2 and A3, obtained from crosses between (M) *M. savii* and (F) *M. brachycercus* and having a larger heterochromatin heterogeneity, spermatogenesis stops completely at the first meiotic division (Fig.3), while in A4 (M) *M. brachycerus* x (F) *M. savii* a few meiosis I elements and spermatozoa are found (Fig. 4). As A3 and A4 are about the same age, this difference cannot be due to gonad maturity.

Uncertainty about the evolutive role of chromosome rearrangements, occurring by addition or deletion of constitutive heterochromatin, mainly results from uncertainty about the function of this class of DNA within the genome. As a working hypothesis we can consider that different selective pressure acts on the heterochromatin of autosomes and sex chromosomes. This might be related to larger chromosomal conformational changes affecting sex chromosomes than autosomes during gametogenesis (Jablonka et al. 1991). However, in a general model where heterochromatin controls important functions during gametogenesis, changes of heterochromatin could lead to infertility. Sex chromosomes heterochromatin appears to suit this model, although in which ways is still unclear. The regulation of the condensed/expanded state of chromosomes could be a valid explanation. In the laboratory mouse, the Y chromosome is transcriptionally inactive in somatic cells, but becomes actively transcribed in the germ cells. A DNA binding protein that recognizes Y chromosome GATA repeats of Bkm (BBP) is supposed to be the specific signal for decondensation (Singh et al. 1994). This change parallels X chromosome inactivation occurring during spermatogenesis through XIST (X-inactive specific transcripts) which is also responsible for X inactivation in somatic cells of females (Richler et al. 1992). According to Lifshytz & Lindsley (1972) X inactivation in primary spermatocytes is a critical stage of spermatogenesis and factors interfering with this step negatively affect male fertility. Changes of the chromosomes state in the heterogametic sex would be efficient to prevent recombination events that could lead to damaged sex chromosomes (McKee et al. 1993).

An altered organization of XY pairing and/or formation of the synaptonemal complex due to heterogeneity in the heterochromatin content is an alternative hypothesis to explain male hybrid sterility. Meiotic studies on human (Faed et al. 1982; Bourrouillou et al. 1987; Guichaoua et al. 1992) and mice (Forejt 1979; Ashley



Figs 5—6: Degenerative patterns of (M) *M. savii* x (F) *M. brachycercus* spermatocytes (upper) and acrosomial alterations of (M) *M. brachycercus* x (F) *M. savii* spermatozoa (lower).

& Russel 1986; Setterfield et al. 1988) unfertile heterozygous carriers of autosomal rearrangements, showed that meiotic alterations depend on the failure of pairing between translocated chromosomes (asynapsis and heterosynapsis) and/or the association of these chromosomes with the XY body. This fact was, among others, widely analysed and connected with speciation in the dik-dik *Madoqua* (Ryder et al. 1989).

Whatever is the actual function of sex chromosomes heterochromatin and its role in the failure of male hybrid spermatogenesis, *M. savii* complex appears a good model for studying the links between the evolution of this kind of heterochromatin and cladogenetic events within this taxa. For this reason further analyses of genetic changes which accompanied species differentiation within the group would be very useful to obtain more information about its evolutionary history.

Zusammenfassung

Microtus brachycercus, zuvor als eine Unterart des *Microtus savii* beschrieben, weist einen unterschiedlichen Karyotyp auf und erzeugt bei Kreuzung mit *Microtus savii* sterile männliche Nachkommen. Um die Ursachen für die Sterilität herauszufinden, wurde eine histologische Untersuchung von männlichen Keimen von Hybriden *Microtus savii x M. brachycercus* durchgeführt. Es zeigte sich, daß sich Unterschiede bei den männlichen hybriden Geschlechtschromosomen jeweils unterschiedlich auf die Fruchtbarkeit auswirkten. Im Falle einer unvollständigen Meiose entstanden entartete Zellbilder. Eine mögliche Verbindung zwischen der heterochromatinen Evolution und der Sterilität männlicher Hybriden wird erörtert.

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