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Evidence for male XO sex-chromosome system in *Pentodon bidens punctatum* (Coleoptera Scarabaeoidea: Scarabaeidae) with X-linked 18S-28S rDNA clusters

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In scarab beetle species of the genus *Pentodon,* the lack of analysis of sex chromosomes in females along with the poor characterization of sex chromosomes in the males, prevented all previous investigations from conclusively stating sex determination system. In this study, somatic chromosomes from females and spermatogonial chromosomes from males of *Pentodon bidens punctatum* (Coleoptera: Scarabaeoidea: Scarabaeidae) from Sicily have been analyzed using non-differential Giemsa staining. Two modal numbers of chromosomes were obtained: 2n = 20 and 19 in females and males, respectively. This finding along with other karyological characteristics such as the occurrence of one unpaired, heterotypic chromosome at metaphase-I and two types of metaphase-II spreads in spermatocytes demonstrate that a XO male/XX female sex determining mechanism – quite unusual among Scarabaeoidea - operates in the species investigated here. Spermatocyte chromosomes have also been examined after a number of banding techniques and fluorescent *in situ* hybridization with ribosomal sequences as a probe (rDNA FISH). The results obtained showed that silver and CMA_3 staining were inadequate to localize the chromosome sites of nucleolus organizer regions (NORs) due to the over-all stainability of both constitutive heterochromatin and heterochromatin associated to the NORs. This suggests that heterochromatic DNA of *P. b. punctatum* is peculiar as compared with other types of heterochromatin studied so far in other invertebrate taxa. By rDNA FISH major ribosomal genes were mapped on the X chromosome.

Key words: chromosomes, Coleoptera, FISH, ribosomal DNA, Scarabaeoidea, XO sex system

INTRODUCTION

In animals, sexual differentiation is generally controlled by morphologically differentiated chromosomes, designated XY and ZW in male and female heterogamety, respectively. However, sex types as the simple XO male/XX female and multiple $X_1.....X_nY$ male/$X_1X_1.....X_nX_n$ female have also been found.

According to Paulian (1988), different sex determining mechanisms occur in Coleoptera. These include the XY/XX type, frequently observed in these organisms, and the male XO type that is frequent in Cantharididae but more or less occasional in most of the remaining coleopteran groups.

As concerns Scarabaeoidea, the XY sex type has been reported in almost all of more than 200 species investigated representing all major groups. On the contrary, the male XO sex determining system has been found in a low number of species belonging to three Scarabaeidae subfamilies: Scarabaeinae (one species), Melolonthinae (four species) and Dynastinae (two species) (see Yadav et al. 1979). An exception is represented by the tribe Passalini, (family Passalidae) where this sex type has been

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described in all studied species (Virkki and Reyes-Castillo, 1972; Serrano et al., 1998).

Previous cytological studies have proposed two different systems of sex determination operating in the genus *Pentodon* (Dynastinae). These include the XY/XX type in *P. bispinifrons* (Joneja, 1960) and the male XO type in *P. sp*. and *P. punctatus* (= *P. bidens punctatum*: Endrödi, 1967; 1985) (Joneja, 1960; Salamanna, 1966). However, owing to the fact that these investigations were based exclusively on male chromosome numbers (chromosomes were not examined in females and description of chromosome morphology was not provided for the males), they are not sufficient to allow sex determining systems to be conclusively stated in *Pentodon* species.

In order to contribute to the resolution of the above problem and to widen karyological knowledge of the genus *Pentodon*, we analyse Giemsa stained mitotic chromosomes of both male and female specimens of *P. b. punctatum* from Sicily and present the chromosome patterns obtained after a number of banding techniques on the chromosomes in spermatocytes. Moreover, the aim of the present study is to analyse nucleolus organizer regions (NORs) using fluorescent *in situ* hybridization with ribosomal sequences as a probe (rDNA FISH), which have proved to be essential to conclusively map major (18S-28S) ribosomal loci in other scarab beetles species.

MATERIALS AND METHODS

A total of seven specimens (four males and three females) of the scarab beetle *P. b. punctatum* (Coleoptera Scarabaeoidea: Scarabaeidae: Dynastinae), identified according to Endrödi (1985), were collected from October 1999 to March 2003 in two geographically separated areas: Levanzo (Egadi islands, Trapani Province, north-western Sicily; two males and three females) and Polizzi Generosa (Palermo Province, north-western Sicily; two males).

Chromosomes were obtained by the air-drying method either from male gonads or female intestine epithelium after *in vivo* colchicine treatment, observed with a Leica microscope, photographed with a Kodak Ektacolor 800 ASA film and classified according to the criteria of Levan et al. (1964).

Giemsa and propidium iodide staining of untreated chromosomes were performed according to current methods, while characterization of nucleolus organizer regions (Ag-NORs) and heterochromatin was carried out as described by Howell and Black (1980) and Sumner (1972), respectively. For the staining of C-banded preparations, we used either the Giemsa dye or an antifade solution containing propidium iodide (5 μg/ml). The GC-specific CMA_3 and the AT-specific DAPI were employed following Schmid et al. (1983).

FISH was performed on fixed spermatocyte chromosomes as described by Colomba et al. (2000a) using a sea urchin (*Paracentrotus lividus*, Echinodermata) rDNA probe, consisting of the 18S rDNA. The use of a heterologous probe is justified by the fact that: (1) rDNA coding sequences for 'large' rRNAs are highly conserved in eukaryotes (Long and Dawid, 1980); and (2) the sea urchin (*P. lividus*) rDNA probe and rDNA probes obtained from other animals were successfully employed for rDNA mapping in invertebrate species of different phyla (i.e. López-León et al., 1999; Vitturi et al., 1999; Colomba et al., 2000a and 2000b). Nick translation labelling with digoxigenin was performed according to manufacturer's instructions (Roche). Slides were mounted in an antifade solution containing propidium iodide (5 μg/ml) and viewed under a Leica I3 filter set (BP 450–490, LP 515).

The systematic scheme adopted is that proposed by Lawrence and Newton (1995) for Scarabaeoidea, and by Endrödi (1985) for Dynastinae.

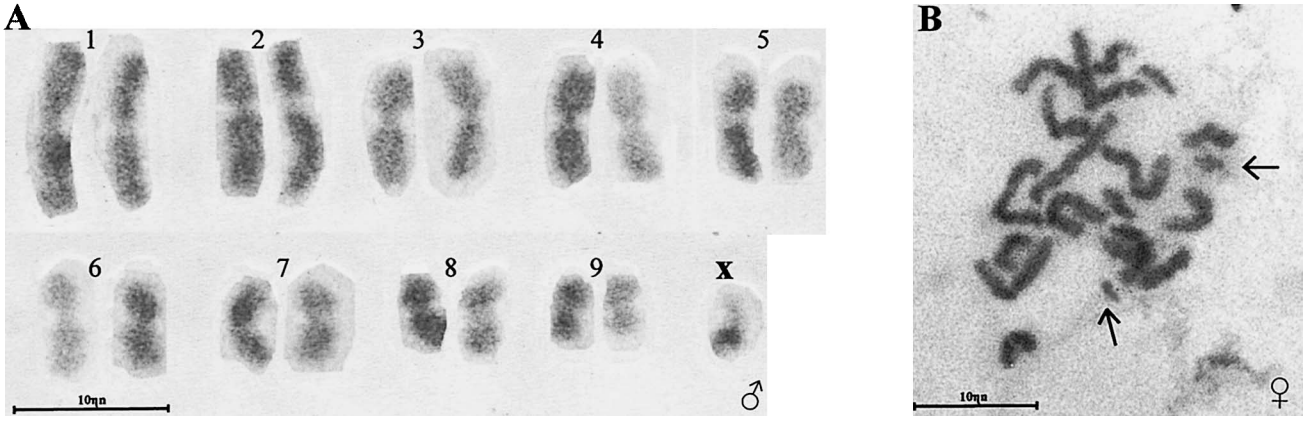

Fig. 1. **A** Karyotype of *Pentodon bidens punctatum* obtained from a Giemsa stained spermatogonial metaphase; **B** Giemsa stained female somatic metaphase spread (arrows indicate X chromosomes).

RESULTS

With respect to all techniques here employed, male specimens do not show any inter-populational differences. For this reason, the two samples are not further distinguished.

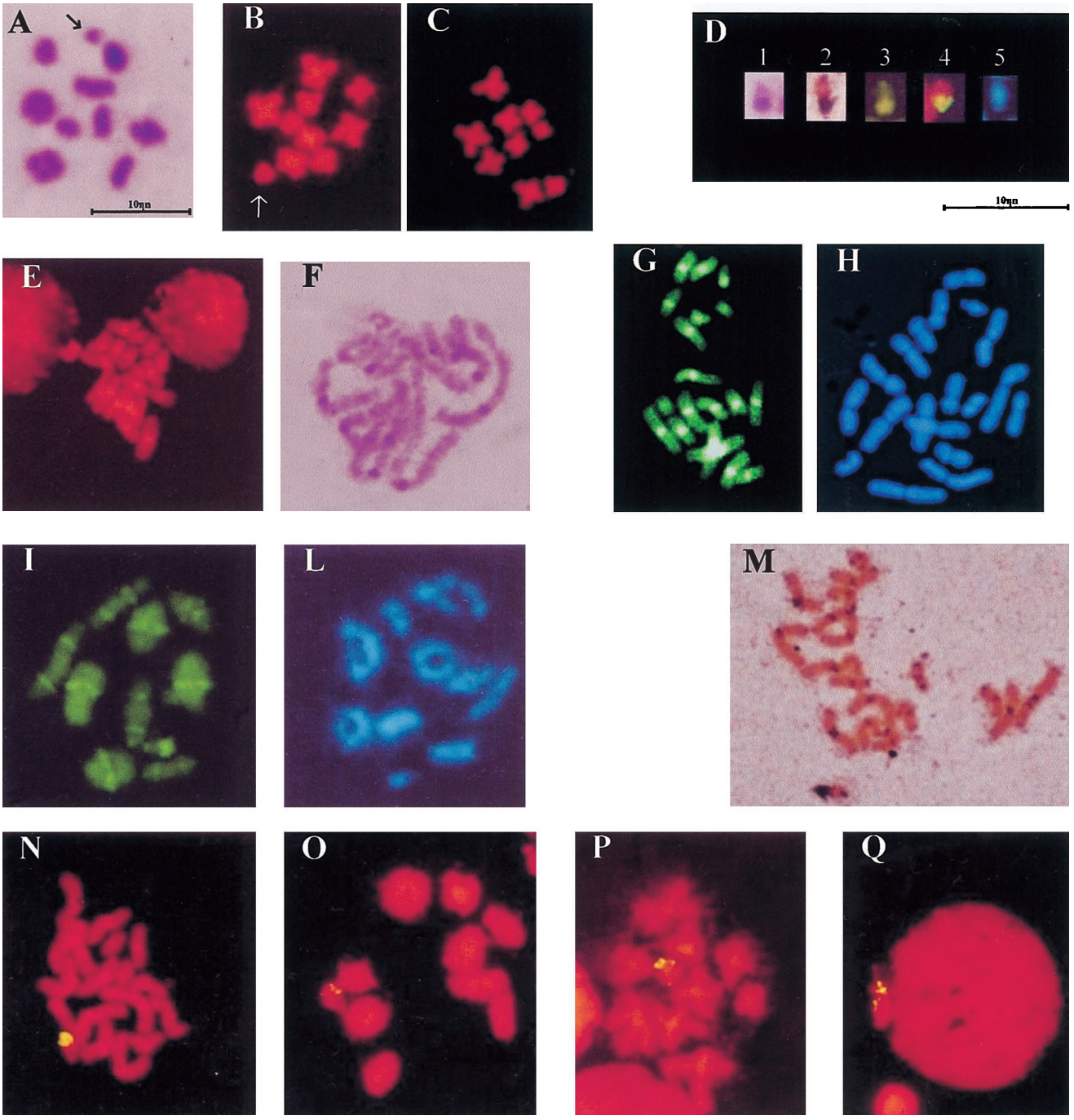

Fig. 2. **A** Giemsa stained metaphase-I chromosomes (arrow indicates the X chromosome); **B** Propidium iodide stained metaphae-II spread showing ten chromosomes (arrow indicates the X chromosome); **C** Propidium iodide stained metaphase-II spread showing nine autosomes; **D** X chromosome after C-banding (1), silver staining (2), CMA_3 staining (3), 18S rDNA FISH (4) and DAPI staining (5); **E** Propidium iodide stained spermatogonial metaphase chromosomes after C-banding; **F** Giemsa stained pachytene chromosomes after C-banding; **G** Spermatogonial metaphase chromosomes after CMA_3 staining; **H** Spermatogonial metaphase chromosomes after DAPI staining; **I** Metaphase-I bivalents after CMA_3 staining; **L** Metaphase-I bivalents after DAPI staining; **M** Spermatogonial metaphase chromosomes after silver staining; rDNA FISH of *P. b. punctatum*: **N** Spermatogonial metaphase plate, **O** Metaphase-I bivalents, **P** Metaphase-II chromosomes, **Q** Interphase nucleus.

Nondifferentially stained chromosomes and karyotyping All *P. b. punctatum* male specimens show the diploid number 2n = 19. Spermatogonial chromosomes are arranged according to length and centromere position into 9 autosome pairs plus an unpaired chromosome designated as X. Autosomes (pairs 1–9) are metacentric whereas the X chromosome, whose morphology is not easy to determine, appears as a small element with a large faintly stained terminal region (Fig. 1A). Somatic chromosome spreads from two out of three females, although of low quality, show that the diploid number is 2n = 20 and that the small chromosome, which is unpaired in males, is associated to its homologue (Fig. 1B).

In male specimens, diakinetic plates always have 10 elements including nine bivalents and one small unpaired chromosome - designated as h-element (h = heterotypic) - that, as in mitotic spreads, is distinct in its staining and size (Fig. 2A). Metaphase-II spreads consistently are of two types. The first type (A-metaphase-II) has ten elements always including the small X (Fig. 2B), while the second type (B-metaphase-II) consists of nine autosomes (Fig. 2C).

Banding analysis and rDNA FISH Figure 2D illustrates the X chromosome after banding experiments and rDNA FISH. The faint terminal region of this element is positively stained by C-banding (Fig. 2D, 1), silver (Fig. 2D, 2) and CMA_3 (Fig. 2D, 3) staining and FISH (Fig. 2D, 4), and negatively stained by DAPI (Fig. 2D, 5). Moreover, an additional, small, positive area can be seen after C-banding, silver impregnation and CMA_3 staining. This region, however, does not contain any ribosomal clusters because it is not labelled by rDNA FISH.

C-banding shows pericentromeric, positive regions in all chromosomes (Figs. 2E and F). Heterochromatin can also be distinguished from the euchromatin by its bright fluorescence after CMA_3 staining (Fig. 2G) and dull fluorescence after DAPI staining (Fig. 2H). The same type of reaction occurs in meiotic chromosomes (Figs. 2I and L). Silver staining shows argentophilic dots in correspondence of all heterochromatic blocks. Small silver positive areas also occur in correspondence of telomeric regions of few chromosomes (Fig. 2M).

The rDNA probe hybridizes only with the heterochromatic region of the X chromosome as shown by analysis of spermatogonial metaphases (Fig. 2N), metaphase-I bivalents (Fig. 2O) and A-metaphase-II chromosomes (Fig. 2P).

Analysis of FISH-treated interphase nuclei, confirms location of ribosomal genes on the X (Fig. 2Q). The marginal position of this chromosome and the asynchronized condensation of the X-chromatin with respect to the chromatin of autosomes, are the same as in other animal taxa.

DISCUSSION

XO/XX sex determining mechanism In *P. b. punctatum* from Sicily, counts of spermatogonial metaphase chromosomes have given the diploid number 2n = 19, as well as in *Pentodon punctatus* (= *P. bidens punctatum*) from Apulia (south-eastern Italy) (Salamanna 1966) and *P. sp*. from India (Joneja 1960), Moreover, counts of somatic chromosomes in two female specimens have given the value 2n = 20 in the present study. The occurrence of different modal numbers in the two sexes along with the finding of one unpaired, h-element at diakinesis and two types of metaphase-II in spermatocytes, allow to conclusively state that a XO male/XX female sex mechanism operates in the species examined here.

It is known that this type of sex determination is not rare within invertebrates. For example, it occurs in numerous species of locusts and grasshoppers (i.e. Cabrero et al., 1985), 25 species belonging to the family Neritidae (Mollusca: Prosobranchia: Archaeogastropoda) (Natarajan 1969; Komatsu and Inaba, 1982; Nakamura 1983; Vitturi and Catalano, 1988), and in two geographically separate populations of the periwinkle *Melarhaphe neritoides* (Mollusca, Caenogastropoda) (Vitturi et al., 1995). Within Scarabaeoidea, the results of this study along with data available for species so far investigated, show that the male XO sex chromosome system occurs in one tribe of Passalidae and seven species of Scarabaeidae belonging to three different subfamilies, i.e. one *Copris* (Scarabaeinae), three *Apogonia* (Melolonthinae) and one *Cephaloserica* (formerly Sericinae, at present Sericini, subfamily Melolonthinae), and two *Pentodon* (Dynastinae) species (Yadav et al., 1979). In Scarabaeidae the XO type has probably arisen independently. As shown by the fact that both XO and XY sex types operate within three not closely related subfamilies (i.e. Scarabaeinae, Melolonthinae and Dynastinae), the distribution of the XO type does not follow a phylogenetic pattern that has been proposed previously (Browne and Scholtz, 1999; Martín-Piera and López-Colón, 2000). Further data suggesting an independent origin of the XO sex type come from literature where it is reported that within Dynastinae the two sex types occur not only at a subfamily level involving different genera [i.e. *Pentodon* (XO) versus *Orycthes* (XY), *Ligyrodes* (XY) *Orizabus* (XY), *Eophileurus* (XY), *Phyllognathus* (XY) (Yadav et al., 1979) and *Enema pan* (XY) (Vidal and Giacomozzi, 1979)], but also at a genus level involving different species [i.e. *Pentodon b. p.* (XO) (Salamanna 1966; present paper) and *Pentodon sp.* (XO) versus *Pentodon bispinifrons* (XY) (Joneja 1960)]. Taking into account that, besides these considerations, in most scarab beetles showing the XY sex type the Y chromosome is a minute, spot-like body which can easily be lost, we conclude that, within Scarabaeoidea, the XO/XX sex determining mechanism can be interpreted as a true

homoplasy.

X-linked rDNA clusters As observed in the melolonthid *Phyllognatus excavatus* (Colomba 1998), the geotrupid *Thorectes intermedius* (Vitturi et al., 1999) and the scarabaeid *Gymnopleurus sturmi* (Colomba et al., 2000b) representing three different phyletic branches of the superfamily Scarabaeoidea, in *P. b. punctatum* as well, both constitutive and NOR-associated heterochromatins reacted positively to silver impregnation and CMA_3 staining. Such an extensive stainability of heterochromatin prevented detection of ribosomal sites in this species. Conversely, ribosomal sequences have conclusively been mapped by FISH which, for the first time in Scarabaeoidea, allowed to locate them on the X element. This result indicates that a twofold amount of ribosomal genes occurs in females (XX) of the species investigated here. It is difficult to find an answer to the question on the mechanism compensating the difference of the X-linked ribosomal DNA gene dosage between males and females as like as to compare the expression of the rDNA genes between male and female cells by Ag-NOR staining as shown in Fig. 2M. However, either intra-specific differences in the number and activity of rDNA clusters (i.e. Schmid 1982; Suzuki et al., 1990; Vitturi et al., 1999) or sex-specific difference in NOR number (i.e. Schmid et al., 1983) are not unusual in animals.

Due to the over-all heterochromatin stainability after application of silver and CMA_3 staining, two other implications of the present study deserve to be noted: (1) constitutive heterochromatin of *P. b. punctatum* constitutes a heterochromatin class that is peculiar as compared with other types of heterochromatin studied so far in invertebrates; and (2) as in scarab beetles studied so far, in *P. b. punctatum* as well, the "rDNA FISH" parameter represents the unique reliable tool for the physical mapping of major (18S-28S) ribosomal clusters.

In conclusion, data of this study surely provide the first detailed karyological information on the genus *Pentodon* which joined to future cytogenetic data on other species of Scarabaeoidea will help us to improve the knowledge of structural chromosome modifications that have occurred during evolution of the superfamily.

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