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SEX CHROMOSOME EVOLUTION IN THE POLYTYPIC SPECIES *PYCNOGASTER CUCULLATA*

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ABSTRACT

Pycnogaster cucullata (Charp.) is a polytypic species of Tettigonioidea having both X0 and neo XY populations. Moreover the neo XY populations are themselves of two distinct types referred to as neo XY_1 and neo XY_2.

A comparison between the X0 state and the two different neo XY systems, using C-banding and silver staining techniques, has served to clarify the differentiation of the two neo XY systems. In both cases, a centric fusion between the progenitor M_2 chromosome, the second largest autosome, and the X of the X0 form has been accompanied by loss of an active NOR in the neo Y, which is the direct derivative of the M_2. In both categories of neo XY sex bivalents, a secondary construction appears in the distal region of the X_L limb, which gives a silver reaction similar to that observed at the centromeres. The neo XY_2 system is distinctive in two respects in comparison with the neo XY_1. In the first place it has an additional C-positive block located distally in the neo Y. Additionally there is also a novel C-block in the proximal half of the X_R limb of the neo X. The neo XY_2 system thus provides the first unambiguous example of the "secondary heterochromatinization" of a sex chromosome system within a single species.

1. INTRODUCTION

To-date it has been assumed that the differentiation of those chromosomes that carry the sex determining genes must have resulted from a restriction of recombination between two originally homologous chromosomes. In theory this can be achieved either by genes which specifically suppress crossing-over, by chiasma localisation, or by a structural rearrangement in, or a heterochromatinization of, the sex bivalent. In a recent paper, for example, Grossman *et al.* (1981) indicated that differentiation of Z and W chromosomes in the American schistosomes (Trematoda) resulted from translocation of part of one homologous sex chromosome on to another with subsequent heterochromatinization of the W. In vertebrates with female heterogamety it has been concluded that the primary step in the differentiation of the W and the Z is the heterochromatinization of the W chromosome (Ray-Chaudhury *et al.*, 1971; Schmid *et al.*, 1979; Singh *et al.*, 1980). Mengden (1981), however, considers that we need to distinguish two categories of heterochromatinization in relation to sex chromosome differentiation: primary and secondary. With regard to secondary heterochromatinization only one example is known. This is the postulated "progressive heterochromatinization" of the Y-chromosome in the neo XY system of certain orthopterans (Saez, 1963; White, 1973); but the argument supporting this is based on a comparison between largely unrelated species, so that its validity remains to be demonstrated.

In a previous paper (Fernández-Piqueras *et al.*, 1982) we have described a polytypic species of Tettigonioid (*Pycnogaster cucullata*) with X0 and neo XY populations. We concluded that the neo XY forms had arisen by a centric fusion between the second largest M_2 autosome and the X of the X0 system. This was accompanied by loss in the neo Y of part of a C-band and the secondary constriction which characterizes the M_2, together with the appearance of a new secondary constriction in the distal region of the X_L limb of the neo X. In this paper, we describe a second neo XY system found in a population of *P. cucullata* from Morcuera which appears to have undergone additional secondary heterochromatinization in both X and Y chromosomes.

2. Material and methods

Individuals of *Pycnogaster cucullata* Charp. (Tettigonioidea, Orthoptera) have been studied from natural populations at Gredos (X0; 20 individuals), Truchas (neo XY_1; 70 individuals), and Morcuera (neo XY_2; 20 individuals).

Conventional acetic orcein squash preparations were used together with C-banding preparations (Sumner, 1972).

A silver technique described by Goodpasture and Bloom (1975) and Bloom and Goodpasture (1976) has also been employed though with some modifications. The silver method serves to detect both active NORs (nucleolar organizer regions) (Engel *et al.*, 1977; Hansmann *et al.*, 1978; Schmid *et al.*, 1977; Höfgartner *et al.*, 1979; Buys *et al.*, 1979) and centromeric regions (Buys and Osinga, 1980; Howell and Hsu, 1979). NORs may be distinguished by the fact that the amount of silver precipitate is variable during meiotic prophase, decreasing from zygotene to diakinesis. By constrast, at centromere regions the amount remains unchanged during all stages of meiosis. In *Pycnogaster* we found that a pretreatment with 2XSSC at 60°C for 15 min allows one to specifically identify centromeric regions.

Air drying and counterstaining in 1 per cent phosphate buffered (*p*H 6, 8) Giemsa for five min can be used additionally to define the outline of the chromosome.

3. Results

(i) *X0 system Gredos population*

As an addition to our previous findings (Fernández-Piqueras *et al.*, 1982), silver staining has allowed us to determine that there is a NOR region located in the M_2 autosome at a procentric site (fig. 1(c)). The secondary constriction is located close to the centromere in the short arm of the M_2 (see fig. 3).

(ii) *Neo XY_1 system Truchas population*

C-banding. We have already shown (Fernández-Piqueras *et al.*, 1982) that the neo X possesses a prominent procentric C-band with a minor band near the distal tip of the X_L limb (≡attached autosome for X0 state) (see also here figs. 2(a) and (b)). In some cases, as in fig. 2(a), there appears to

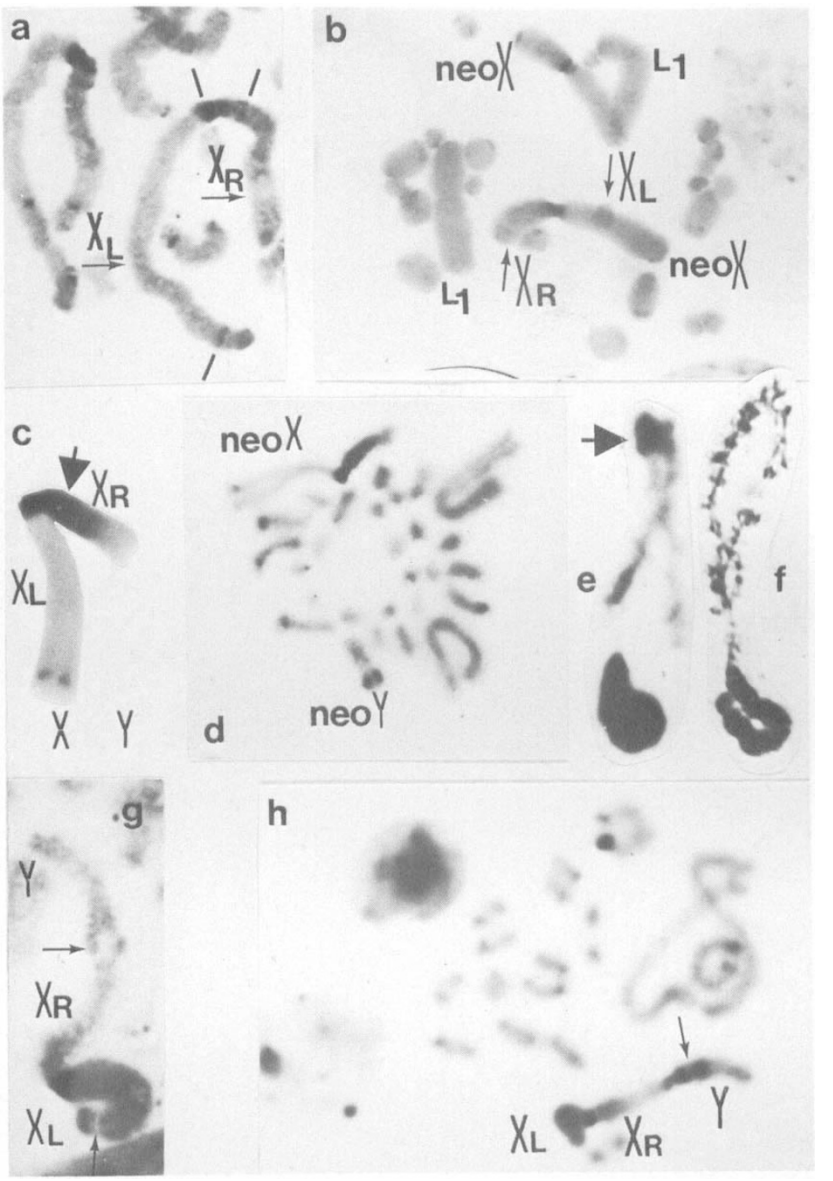

FIG. 2. C-banding and conventional stain. (a) C-banded neo X chromosome from a female of the neo XY_1 system, at mitotic prophase. Bars indicate C-bands. Note a small terminal "grey" C-banding areas in the distal extremities of both X_L and X_R arms. (b) C-banded mitotic metaphase from a female of the neo XY_1 system. (c) C-banded neo X and neo Y at mitotic prometaphase, from a male of the neo XY_2 system. Note a "grey" C-block in the proximal half of X_R limb (arrow). (d) C-banded mitotic prometaphase from neo XY_2. (e) An early prophase sex bivalent from neo XY_2, with conventional stain. Note a positive heteropycnotic region on the telomeric region of neo Y (arrow). (f) Idem from neo XY_1. Now, the mentioned above heteropycnotic region is absent. (g) C-banded neo XY_1 sex bivalent at diplotene. Note subterminal chiasma (arrow), a procentric C-band in the neo X and the distal secondary constriction of the X_L arm. (h) C-banded diplotene from neo XY_2 system. Note arrow indicates C-positive block on the neo Y (Y).

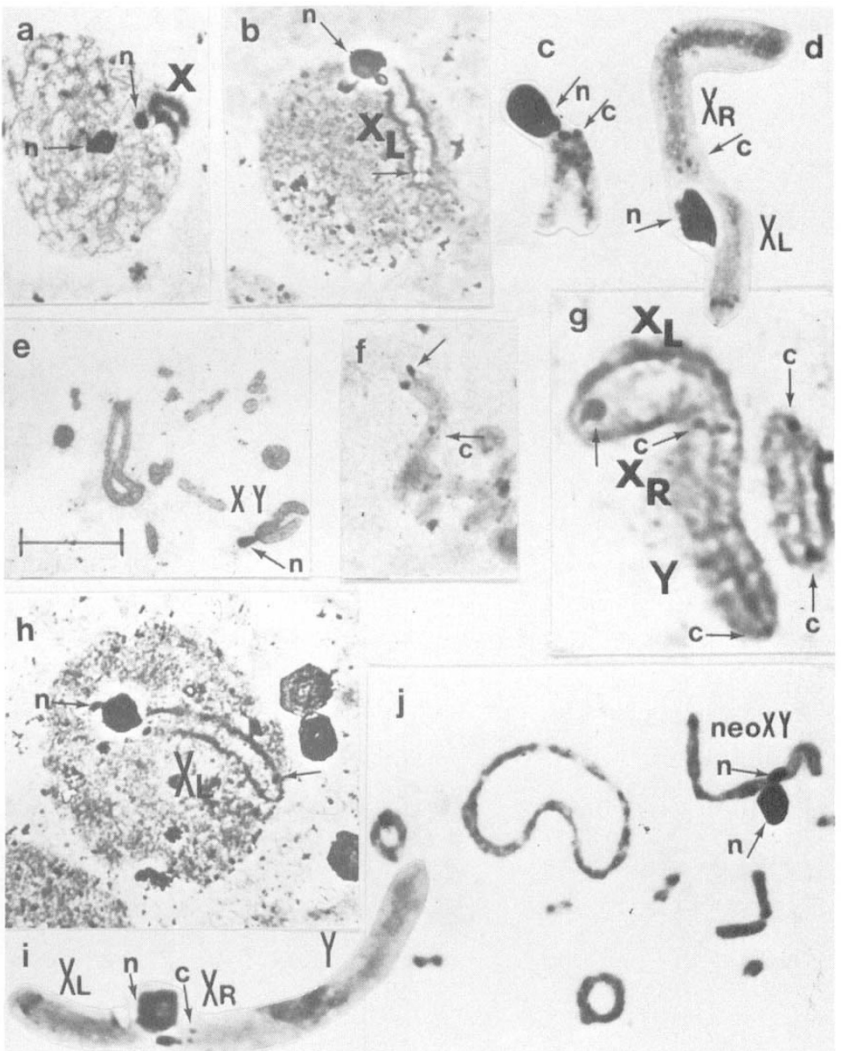

FIG. 1. Ag-staining. n, represents nucleolus; c, denotes centromeres. (a) Premeiotic nucleus from an X0 individual. (b) Premeiotic nucleus from the neo XY_1. The outline of the X_L is marked. Arrow indicates two silver spots on the distal secondary constriction of the X_L. (c) A pachytene M_2 bivalent from Gredos population. (d) An early pachytene neo XY_1 sex bivalent. (e) Diplotene from XY_1 state. Note reduced size of Ag-precipitate with regard to that in pachytene and premeiotic stages. (f) Partial metaphase I from XY_1. Note centromeres present in all chromosomes. (g) Idem after Giemsa staining. (h) Premeiotic nucleus from the neo XY_2. Arrow marks two silver spots on the distal secondary constriction of the X_L. (i) An early pachytene neo XY_2 sex bivalent. (j) Diplotene from XY_2 state.

[facing page 218

be a region of differential C-banding in the proximal region of X_R which is somewhat reminiscent of the "grey" region that we describe later in the X_R of the neo XY_2 system. Additionally the X_L limb has an intermediate staining character during meiotic prophase after C-banding treatment, while the X_R limb, like the Y does not display any differential staining (fig. 2(g) for XY_1 and 2(h) for XY_2).

Ag-staining. An active NOR is located close to the centromere of the neo X, in the neo XY_1 system (figs. 1(d) and (e)). The precise spatial relationship between the centromere and the active NOR can be shown in prophase neo XY bivalents (fig. 1(d) and fig. 3). The Y chromosome of the neo XY_1 system does not show any NOR activity.

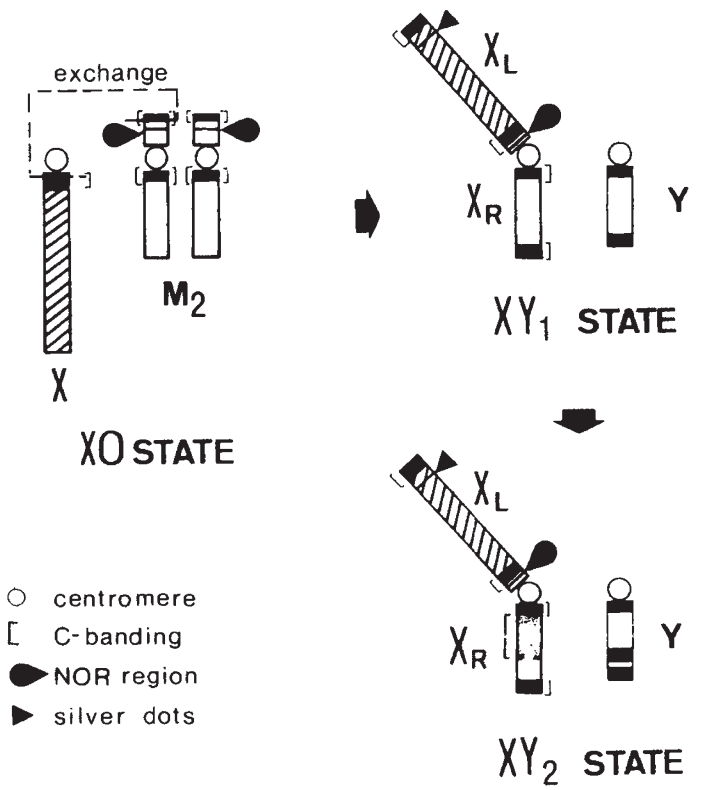

FIG. 3. A drawing to summarise the possible origin and evolution of the neo XY systems of this species.

The silver precipitate which is found at the secondary constriction on the distal half of the X_L in the neo X is larger than that found at centromeric regions but it remains unchanged during meiosis (figs. 1(a), (f) and (g)), which suggests it may be of centromeric origin.

(iii) *Neo XY_2 system Morcuera population*

C-banding. A C-positive block is present at the distal region of the neo Y_2 but is not present either in its partner X_R or in the neo Y of the neo XY_1 system. This block consists of two C-bands (fig. 2(h)) but these

are not always resolvable when either C-banding (fig. 2(a), (c) and (d)) or conventional staining techniques (fig. 2(e)) are used.

A "grey" C-block, which occupies the proximal half of the X_R limb of the neo X in the neo XY_2 system, is found during mitotic prophase and prometaphase (figs. 2(c) and (d)). This C-block is not present during all stages of meiosis. As is also the case in the neo XY_1 system, the neo X possesses a procentric C-band and a C-positive secondary constriction towards the distal tip of the long arm. At meiosis, though not at mitosis, the X_L arm is characterised by intermediate C-staining.

In both neo XY systems the short X_R arm of the neo X is longer than its free partner neo Y (see fig. 2(c) for the neo XY_2 and Fernández-Piqueras *et al.* (1982) for the neo XY_1).

Ag-staining. No significant differences are evident between the sex chromosomes of XY_2 and XY_1 populations after silver staining. Thus, the neo X of the XY_2 system possesses an active NOR close to the centromere, denoted by two silver precipitates (nucleoli) with different sizes present around the proximal secondary constriction during meiotic prophase (fig. 1(j)). The centromere silver dots are located distal to this secondary constriction in the X_R arm (fig. 1(i)).

Two silver spots are also present at the distal secondary constriction on the X_L arm (fig. 1(h)). The neo Y again does not show any NOR activity (fig. 1(i) and (j)).

We include a drawing (fig. 3) to summarise the origin and evolution of the two neo XY systems in this species.

(iv) *Nature of the XY bivalents*

All of the 300 neo XY_2 bivalents examined had a single terminal association between the distal ends of the neo Y and the X_R arm of the neo X. Since both these regions are C-banding positive (fig. 2h) it is unlikely that this association represents a terminalised chiasma. Equivalent non-chiasmate associations have been observed in other orthopterans (John and King, 1977 and 1980).

Of 300 XY_1 bivalents analysed, 198 also showed a terminal association of the distal ends of the X_R and the Y. In the remainder, however, there was evidence of a subterminal chiasma (fig. 2(g)). These data suggest a possible difference in the mode of association of the X and Y chromosome in the two categories of neo sex systems.

4. Discussion

(i) *Progressive heterochromatinization*

According to White (1973) fifty species of grasshoppers are characterized by a neo XY mechanism. Of the six such systems that have been described in the Tettigonioidea, those of *Yorkiella picta*, *Polichne parvicauda*, *Caedicia marginata*, and probably, *Anabrus simplex*, are structurally unmodified. The two other systems show considerable modification from their presumed original state as result of an inversion and/or the development of allocycly in the proximal portion of the neo Y (Hewitt, 1979). Unlike the tendency of the neo Y chromosome to undergo secondary changes associated with its production, the neo X does not usually give

evidence of any marked changes. The acrocentric neo X of *Dichro plus silveiraguidoi*, however, is exceptional in this respect (Diaz and Saez, 1968).

When a comparative study is made between the two neo XY systems of *Pycnogaster*, two main differences can be observed (fig. 3). First, the development of a constitutive heterochromatic C-block in the distal region of the neo Y of the neo XY_2 which the equivalent neo Y of the neo XY_1 system does not possess. This heterochromatinization process is not accompanied by any size modification of the neo Y, because this is still shorter than the X_R as is also the case in the neo XY_1 (as it had been shown in a previous paper Fernández-Piqueras *et al.*, 1982). Second, the development of a proximal "grey" C-block of the X_R limb of the neo X in the neo XY_2 system which is not present in the neo X of the neo XY_1 system. This C-block is eupycnotic at all stages of both mitosis and meiosis with conventional procedures, but stains differentially during mitotic prophase and prometaphase after C-banding treatment (fig. 2(c) and (d)).

This "grey" C-block appears to be consequence of a facultative heterochromatinization process, since it displays an intermediate staining and its presence is restricted to the sex chromosomes.

Differences in stain intensity in the sex chromosomes after C-banding have been reported in many cases (Hsu, 1971; Jalal *et al.*, 1974; Stock *et al.*, 1974; Vistorin *et al.*, 1977; Ryttmann *et al.*, 1979) including orthopterans (see for example Cardoso and Dutra, 1979). Whether the intermediate staining observed when sex chromosomes are C-banded is due to differences in DNA composition or to differences in the pattern of chromatin packing is not known.

The heterochromatinization process involving the X_R limb is quite different from that present in the Y_2 chromosome. The appearance of the C-block in the neo Y_2 presumably results from a secondary heterochromatinization process that has apparently involved euchromatin transformation of the type described by King (1980) since the Y_1 and Y_2 are similar in size.

Unlike the neo XY_1 system of *P. cucullata*, where a subterminal chiasma can often be demonstrated in the sex bivalent (fig. 2(g)), in the neo XY_2 system the two sex chromosomes are invariably associated terminally (fig. 1(j) and 2(h)). It is conceivable that the constitutive heterochromatinization of the neo Y_2 may have played a key role in this relationship, since such an event would be expected to lead to a novel mode of association between the X and Y. There are, however, a number of neo XY systems known where the X and the Y are regularly associated terminally but where there is no distal heterochromatinization of the neo Y. Consequently it is difficult to argue for a causal mechanism based on heterochromatinization without first defining the nature of the terminal association observed in many of the XY_1 bivalents of *Pycnogaster*.

The neo XY_2 system we have described here, appears to be the first convincing example of what Saez (1963) described as "progressive heterochromatinization" though with one significant difference. In Saez's original gradient of heterochromatinization hypothesis it was assumed that the Y become heterochromatic at its centric end first and this then spread progressively along the Y. In *P. cucullata*, however, the neo Y_2 has become heterochromatic at its distal end and it is the X which displays proximal heterochromatinization.

(ii) *Ag-staining*

Two other points of interest regarding the evolution of the neo XY sex chromosomes of *P. cucullata* merit comment. First the identification of the centromere and NOR positions on the M_2 and the neo XY bivalents following Ag-stain demonstrates that the centric fusion between the M_2 autosome and the X of the X0 system has probably been accompanied by the loss of the centromere region of the X and of the active NOR of the neo Y (figs. 1(d), (f) and (g)). Thus the centromere of the neo X is located on the eupycnotic X_R arm, close to the NOR region, occupying the same position it had on the progenitor M_2 autosome (compare figs. 1(c) and (d)). On the other hand, while both M_2 chromosomes in the X0 state have one active NOR, it has not been possible to demonstrate such activity in the derived neo Y.

The second issue relates to the presence of a secondary constriction on the distal half of the X_L limb of the neo X in both neo XY systems, but which is absent on the free X-chromosome in X0 state. Since the amount of silver precipitate remains unchanged at all stages of meiosis in this secondary constriction (compare figs. 1(a), (f) and (g)), we believe that this precipitate does not reflect any synthetic activity in that region. The question remains: what does it represent? It might be an atypical centromere (larger than the normal one) or some other structural protein. In the former case, the abnormal centromere could be derived by a pericentric inversion of the X-chromosome during the development of the neo XY system. We consider this interpretation very unlikely though the existence of a centromere in this region would be in the line with the fact that the two arms of the neo X do not always flex at first metaphase. Neocentric activity is certainly not unknown even in the univalent X of X0 orthopterans (John, 1976) and such activity could account for the absence of arm flexure.

It is clear that there is no uniform solution applicable to the evolution of all sex chromosome systems. Nevertheless, the neo XY_2 system of *P. cucullata* seems to provide some substantiation for regarding "progressive heterochromatinization" as one mechanism involved in the secondary sex chromosome differentiation of orthopterans.

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