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Multiple supernumerary chromosomes in the pseudogamous parthenogenetic flatworm *Polycelis nigra*: lineage markers or remnants of genetic leakage?

Timothy F. Sharbel, Laas P. Pijnacker, and Leo W. Beukeboom

Abstract: *Polycelis nigra* is a free-living simultaneous hermaphroditic flatworm that has amphimictic and pseudogamous parthenogenetic biotypes. Sexual individuals are always diploid ($2n = 16$) and pseudogamous parthenogens are polyploid (usually triploid). Two types of supernumerary chromosomes are found in parthenogens, those resembling autosomes ("A-like") and typical B chromosomes, both of which reach frequencies in populations of close to 100%. Experiments measuring the transmission rates of the B chromosomes indicated that they are potentially inherited via the male line, escaping expulsion by pseudogamous parthenogenesis. This study used the C-banding technique to demonstrate (*i*) that there is a single morphologically distinct B chromosome (B1) and (*ii*) that there are two "A-like" chromosomes that can be considered B chromosomes (B2 and B3) and which are not simple polysomics of one of the eight autosomes. As there is no genetic exchange between pseudogamous parthenogenetic lineages, two different individuals carrying a similar B morph must either have received it through common ancestry (a lineage marker) or have acquired it horizontally from another parthenogenetic lineage (leakage). C-banding further revealed intra-individual heteromorphy for band regions on chromosomes 5 and 8. This supports the karyotypic observation that oogenesis is preceded by premeiotic chromosome doubling followed by pairing of replicate homologues.

Key words: B chromosome, C-banding, heterochromatin, heteromorphy, pseudogamous parthenogenesis.

Résumé : Le *Polycelis nigra* est un vers plat hermaphrodite simultané non-parasitaire qui comprend des biotypes amphimictiques et parthénogénétiques pseudogames. Les individus sexués sont toujours diploïdes ($2n = 16$) et les individus parthénogénétiques pseudogames sont polyploïdes (habituellement triploïdes). Deux types de chromosomes surnuméraires sont présents chez les individus parthénogénétiques : ceux qui ressemblent aux autosomes (« A-like ») et des chromosomes B typiques. La fréquence de ces deux types de chromosomes peut atteindre près de 100% chez des populations. Des expériences mesurant le taux de transmission des chromosomes B indique qu'ils sont potentiellement transmis par la voie mâle, évitant ainsi l'expulsion par parthénogenèse pseudogame. Cette étude a employé la technique de révélation des bandes C afin de démontrer : (*i*) qu'il y a un seul chromosome B (B1) qui présente une morphologie distincte et (*ii*) qu'il y a deux chromosomes « A-like » qui peuvent être considérés comme étant des chromosomes B (B2 et B3) et non pas de simples copies supplémentaires d'un des huit autosomes. Comme il ne se produit pas d'échange génétique entre deux lignées parthénogénétiques pseudogames, deux individus portant un chromosome B semblable doivent l'avoir reçu d'un ancêtre commun (un marqueur généalogique) ou encore l'avoir acquis horizontalement en provenance d'une autre lignée parthénogénétique (fuite génétique). L'étude des bandes C a aussi fait ressortir la présence à l'intérieur d'un même individu de régions polymorphes sur les chromosomes 5 et 8. Cette observation vient renforcer les résultats d'observations caryotypiques qui suggèrent que l'oogenèse est précédée d'un doublement chromosomique préméiotique suivi de l'appariement d'homologues répliqués.

Mots clés : chromosome B, révélation des bandes C, hétérochromatine, polymorphisme, parthénogenèse pseudogame.

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Introduction

Parthenogenetic reproduction is typically accompanied by elevated ploidy in the animal kingdom. This enigmatic association, which has been convergently evolved in a number of unrelated taxa, implies that the two phenomena may be linked (Suomalainen et al.1987), although polyploidy itself does not necessarily induce parthenogenesis (Asker and Jerling 1992; Dufresne and Hebert 1994). Unlike stable polyploid sexual taxa that have undergone speciation through genome duplication, apomictic polyploid taxa often have dynamic and unstable genomes, with aneuploidy and mosaicism for both germ and somatic cell lineages being viable states (Richards 1973; Benazzi and Benazzi Lentati 1976; Thiriot-Quiévreux et al.1989).

The hermaphroditic freshwater flatworm *Polycelis nigra* (Tricladida), has diploid sexual ($2n = 16$) and polyploid (typically $3n$ and sometimes $4n$) parthenogenetic biotypes that exist alone or in sympatry throughout Europe (Lepori 1950; Benazzi and Benazzi Lentati 1976). Polyploid individuals are often aneuploid and, depending upon the population, in addition carry morphologically variable B chromosomes (Lepori 1950; Canovai et al. 1985; Canovai 1989; Beukeboom et al. 1996; L.W. Beukeboom, T.F. Sharbel, and N.K. Michiels, submitted for publication[3]). Parthenogens are obligate pseudogamous outcrossers: sperm are exchanged between individuals during symmetrical copulation, but are required only to activate egg development. The paternal chromosome complement never enters the oocyte nucleus, but remains in the cytoplasm where it is degenerated and subsequently expelled just prior to the egg's first cleavage (Benazzi Lentati 1970). Sperm thus make no genetic contribution to offspring.

In a purely parthenogenetic ($3n$) population of *P. nigra* from Lago di Toblino in Northern Italy, we have identified two morphologically distinct supernumerary chromosomes that are carried by virtually all individuals (L.W. Beukeboom, T.F. Sharbel, and N.K. Michiels, submitted for publication).[3] The elements include a small metacentric B chromosome and a second putative aneuploid chromosome (an "A-like" chromosome) that cannot be differentiated from the autosomes (Fig. 1*a*). In a previous study, adult *P. nigra* were collected from Lago di Toblino, and the karyotypes of these individuals, and of their offspring produced in the laboratory, were determined in order to examine the origin and heritability of the smaller B chromosome (Beukeboom et al.1996). This analysis revealed that parental individuals which did not carry the B chromosome occasionally produced offspring which did carry a B chromosome. An intriguing interpretation of these results is that the B chromosome had been transmitted to the offspring from a B chromosome carrying sperm donor, having escaped the degenerative chromosomal processes of pseudogamous parthenogenesis. As the parental individuals used in this study were mated in the field, the paternal (i.e., the sperm donor's) karyotype was unknown, and thus paternal inheritance could not be unequivocally differentiated from *de novo* B-chromosome origin (Beukeboom et al.1996).

Given parthenogenetic reproduction, it can be theorized that many different types of B chromosomes exist, whereas according to the paternal inheritance hypothesis the spread of one or a few types of B chromosomes in a population is expected. If occasional genetic leakage in pseudogamous parthenogenetic crosses does occur, as is implied by the B-chromosome data (Beukeboom et al.1996), then the ubiquity of the second, "A-like" supernumerary element in parthenogenetic *P. nigra* may also be the consequence of paternal transfer. As with the possible explanations for the ubiquity of the standard B chromosome, the presence of one or a few "A-like" supernumerary elements would support the paternal leakage hypothesis, whereas different morphs may be the result of frequent origin from different autosomes. This distinction is important, because horizontal supernumerary chromosome transmission between parthenogens could have implications for the long-term evolution of their hosts, as it may represent a mechanism whereby genetic material is transferred between apomictic *P. nigra* lineages to give them the benefits of occasional or partial sex (Beukeboom and Vrijenhoek 1997).

Based on chromosome morphology alone, it has not been possible to determine whether the supernumerary elements are of one type, or whether the larger ("A-like") one has autosomal homology (Beukeboom et al.1996). This information is necessary in order to make any conclusions regarding their origin, proliferation, and transmission. Here, we report the results of metaphase chromosome C-banding, to elucidate the nature of the supernumerary chromosomes in pseudogamous parthenogenetic *P. nigra*.

Materials and methods

Chromosome preparation

Polycelis nigra triploids from Lago di Toblino (Northern Italy) were collected and transported back to the laboratory. Individuals were subsequently isolated in small containers set in a water flow system at 16°C, and cocoons were collected on a daily basis. Chromosomes were prepared from embryos extracted from developing cocoons. Two weeks after oviposition, cocoons were gently punctured with a needle and then placed in a 0.15% colchicine solution. The cocoons were left in the colchicine for 2–3 h and then transferred to a 1% sodium citrate solution for a further 20–25 min, after which they were fixed in cold (−4°C) Carnoy's solution (absolute ethanol – glacial acetic acid (3:1)) for a minimum of 2 h.

To make metaphase chromosome preparations, the cocoons were opened using forceps and needles, and the embryos were placed on an alcohol-cleaned microscope slide. The tissue was covered with a drop of 60% acetic acid and then chopped into pieces using fine needles. The resultant cell suspension was surrounded with Carnoy's solution, an additional drop of Carnoy's was placed on top, and the slide was then left to air-dry (Pijnacker and Ferwerda 1995).

C-banding

Air-dried slides were first incubated in 0.2 M HCl for 60 min and then rinsed with tap and demineralized water. The slides were incubated in freshly made 5% $Ba(OH)_2 \cdot 8H_2O$ for 15 min, rinsed in tap and then demineralized water, incubated in 2× SSC (1× SSC: 0.15 M NaCl plus 0.015 M sodium citrate) (60°C) for 30 min, and finally rinsed twice in demineralized water. The slides were stained with 2% Giemsa in freshly made Sörensen's buffer (55 mL 1/15 M Na_2HPO_4 plus 45 mL 1/15 M KH_2PO_4, pH 6.9) for 60 min at room temperature, rinsed with buffer and then demineralized water, air-dried, and finally made permanent with DePeX (Sumner 1972).

[3] L.W. Beukeboom, T.F. Sharbel, and N.K. Michiels. Reproductive modes, ploidy distribution, and supernumerary chromosome frequencies in Italian populations of the flatworm *Polycelis nigra* (Platyhelminthes: Tricladida). Submitted for publication.

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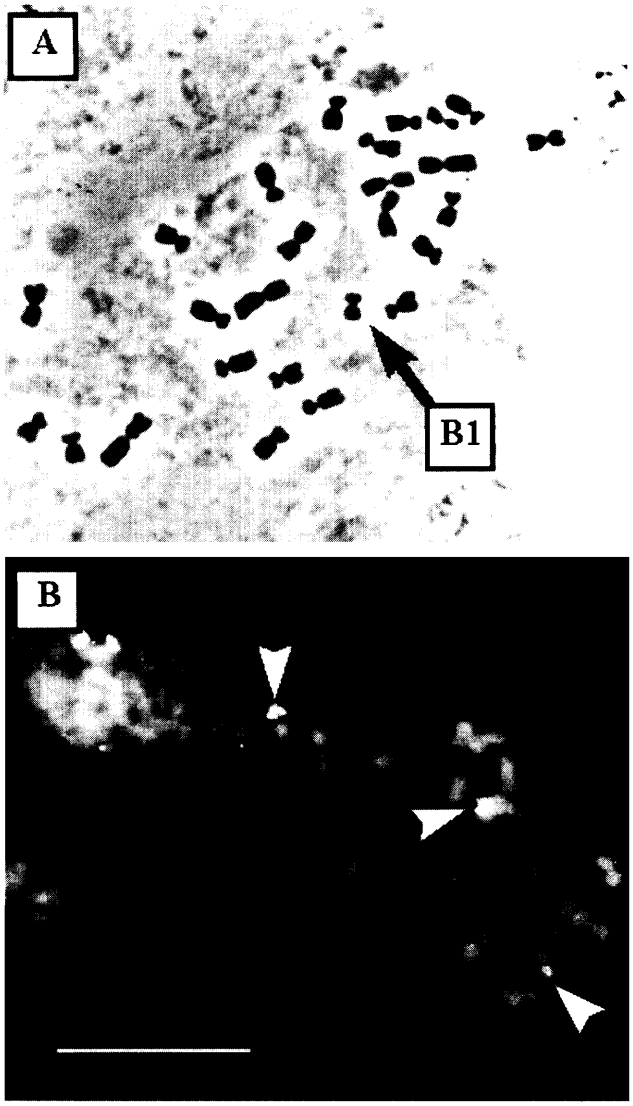
Fig. 1. (A) Normal metaphase spread of triploid *Polycelis nigra* showing a 25 + B karyotype. (B) FISH using a rDNA probe. Scale bar = 10 μm.



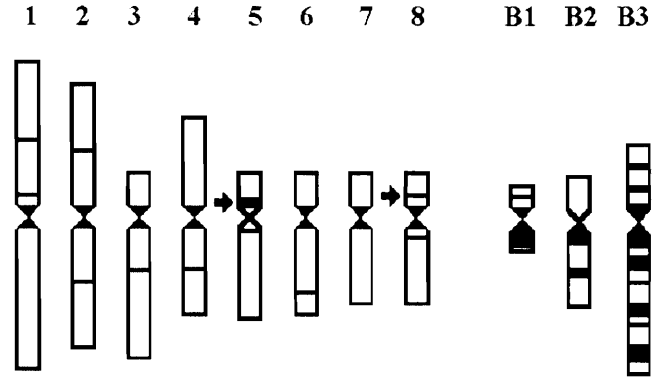
Fluorescent in situ hybridization

The clone VER17 (Yakura and Tanifui 1983), containing parts of the 18S, the 5.8S, and most of the 25S rDNA sequences and the internal transcribed spacer of *Vicia faba*, was used as a NOR-specific probe. The insert of the clone was labelled by nick translation with biotin-16-dUTP, and fluorescent in situ hybridization (FISH) was performed as described by Fuchs and Schubert (1995), using about 20 ng of labelled probe per slide. Biotin was detected using the FITC (fluorescein isothiocyanate)-streptavidin/anti-streptavidin system (Vector Laboratories). A fluorescence microscope with an appropriate filter system was used for signal detection and epifluorescence signals were recorded on Kodak Ektachrom film, ASA 400.

Results

The basic karyotype of *P. nigra* (n = 8) is characterized by 3 metacentric and 5 submetacentric chromosomes. As previously quantified (L.W. Beukeboom, T.F. Sharbel, and N.K. Michiels, submitted for publication, see footnote 3), triploid animals have typically demonstrated aneuploidy for 1 to 2 extra chromosomes that could not be differentiated from their nuclear counterparts ("A-like" elements), and in addition frequently had 1 or 2 true B chromosomes (Fig. 1*a*). The NOR site occurs on the short arms of chromosome 5, as is shown by FISH with the rDNA probe (Fig. 1*b*).

Fig. 2. Idiogram of C-banded chromosomes from triploid *Polycelis nigra* showing band distributions, heteromorphic autosomes 5 and 8 (arrows), and the 3 B-chromosome morphs. B2 and B3 never occurred in the same complement.



C-banding revealed centromeric and interstitial heterochromatin in 29 metaphase chromosome spreads from 17 triploid cocoons (Table 1). All autosomes except chromosome 5 showed centromeric banding (Figs. 2 and 3). Additionally, vague interstitial bands were observed on almost all autosomes. Bands of variable intensity were present on each of the short arms of chromosomes 5 and 8, the prior band corresponding to the NOR site (Figs. 1–3). Chromosomes 5 and 8 were heteromorphic for these two bands, revealing two, and sometimes 3 different autosomal homologue morphotypes within one nucleus (Figs. 2 and 3; Table 1).

The supernumerary chromosomes were easily distinguished in all preparations, as they showed significantly more intense C-banding relative to the autosomes (Figs. 2 and 3). All Type B1 chromosomes were identical, having a heteropycnotic arm and a euchromatic arm with an interstitial C-band (Figs. 2 and 3). At the population level, two different "A-like" elements could be distinguished, and each differed with respect to relative size and C-band distribution (Types B2 and B3; Figs. 2 and 3). The frequencies of Types B1, B2, and B3 were 72.4, 10.3, and 86.2, respectively (Table 1). Types B2 and B3 were never found together in the same nucleus.

Discussion

B chromosomes

As is typical for B chromosomes in general (Jones and Rees 1982; Beukeboom 1994), the supernumerary chromosomes of *P. nigra* from Lago di Toblino are highly heterochromatic (Figs. 2 and 3). There appears to be only one type of the smaller typical B chromosome (B1). Contrary to our previous suspicions (Beukeboom et al.1996), this is not an isochromosome but instead has an asymmetrical heterochromatin distribution. The extra "A-like" chromosomes may also be considered B chromosomes, as they satisfy criteria that define them, i.e., they are dispensable, univalent, and largely heterochromatic (Jones and Rees 1982; Beukeboom 1994). We thus

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Table 1. B chromosomes and autosomal heteromorphy (presence (+), absence (–)) summarized for 17 triploid cocoons that were karyotyped and C-banded.

Cocoon	No. of metaphase spreads investigated	Heteromorphy		No. of B chromosomes		
		Chromosome 5	Chromosome 8	B1	B2	B3
1	1	+	+	2	0	1
2	1	–	–	0	1	0
3	1	+	–	0	0	1
4	2	–	–	1	1	0
5	1	–	–	1	0	1
6	1	–	+	0	0	1
7	1	–	–	0	0	1
8	2	–	–	1	0	1
8	1	–	+	1	0	0
9	1	+	+	1	0	1
9	1	+	–	1	0	1
10	1	+	–	0	0	1
10	1	+	–	1	0	1
11	1	+	+	1	0	1
11	1	–	–	1	0	1
11	1	–	–	0	0	1
11	1	+	–	0	0	1
11	1	+	+	0	0	1
12	1	+	+	1	0	1
13	1	–	–	1	0	1
13	1	+	–	1	0	1
14	1	+	+	0	0	1
15	1	–	–	1	0	1
16	1	+	+	1	0	1
16	1	+	–	1	0	1
17	1	+	+	1	0	1
17	1	+	–	1	0	1

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conclude that parthenogenetic *P. nigra* from Lago di Toblino have 3 different B-chromosome morphs: a smaller typical B chromosome (Type B1) and two larger ones (Types B2 and B3; Figs. 2 and 3; Table 1). The C-banding patterns reveal no homology between the larger B chromosomes and the autosomes, although we are still unable to infer whether B2 and B3 had ultimately been derived from the autosome set. If B2 and B3 had originated through nondisjunction, it is possible that they have subsequently lost homology with their autosomal progenitors through the accumulation of mutations (Charlesworth 1978; Green 1990; Rice 1994), but C-banding alone provides insufficient resolution to elucidate this possibility.

This study demonstrates that the smaller B chromosome (Type B1) can be found with either Type B2 or Type B3, and that Types B2 and B3 are never found together in the same nucleus (Table 1). The different B chromosomes are univalent, dispensable, and do not undergo synapsis with normal chromosomes during meiosis (T.F. Sharbel, personal observation), and thus constraints for the maintenance of chromosome structure and gene organization are expected to be lacking or relatively relaxed compared with autosomes. The stochasticity of the above processes implies that convergence in C-band morphotype is unlikely, at least with respect to Types B2 and B3, which are characterized by relatively complex banding patterns. Different individuals having the same B2 or B3 chromosome have thus attained this condition, either through common ancestry or via paternal inheritance of the elements (Beukeboom et al.1996), although we are unable to clarify this based upon these data alone. Similarly, the distribution of Type B1 may have been attained through the same two modes of transmission, and common ancestry would imply that Type B1 arose before Types B2 and B3, since individuals can have Type B1 alone or in combination with either of the other two types. Occasional paternal inheritance of B chromosomes would render their pattern of transmission reticulate, and therefore the various B-chromosome combinations would be uninformative with respect to their order of origination.

Our C-banding data share some similarities with the results obtained by Galleni et al. (1991) on the heterochromatin patterns of *P. nigra* from a more southern population in Monti Pisani. Individuals from that region had autosomes that were characterized by centromeric heterochromatin. We hypothesize that the described large interstitial band on the short arm of chromosome 3 likely corresponds to the NOR. In addition, Galleni et al. (1991) identified two B chromosomes that differed with respect to overall morphology and C-banding

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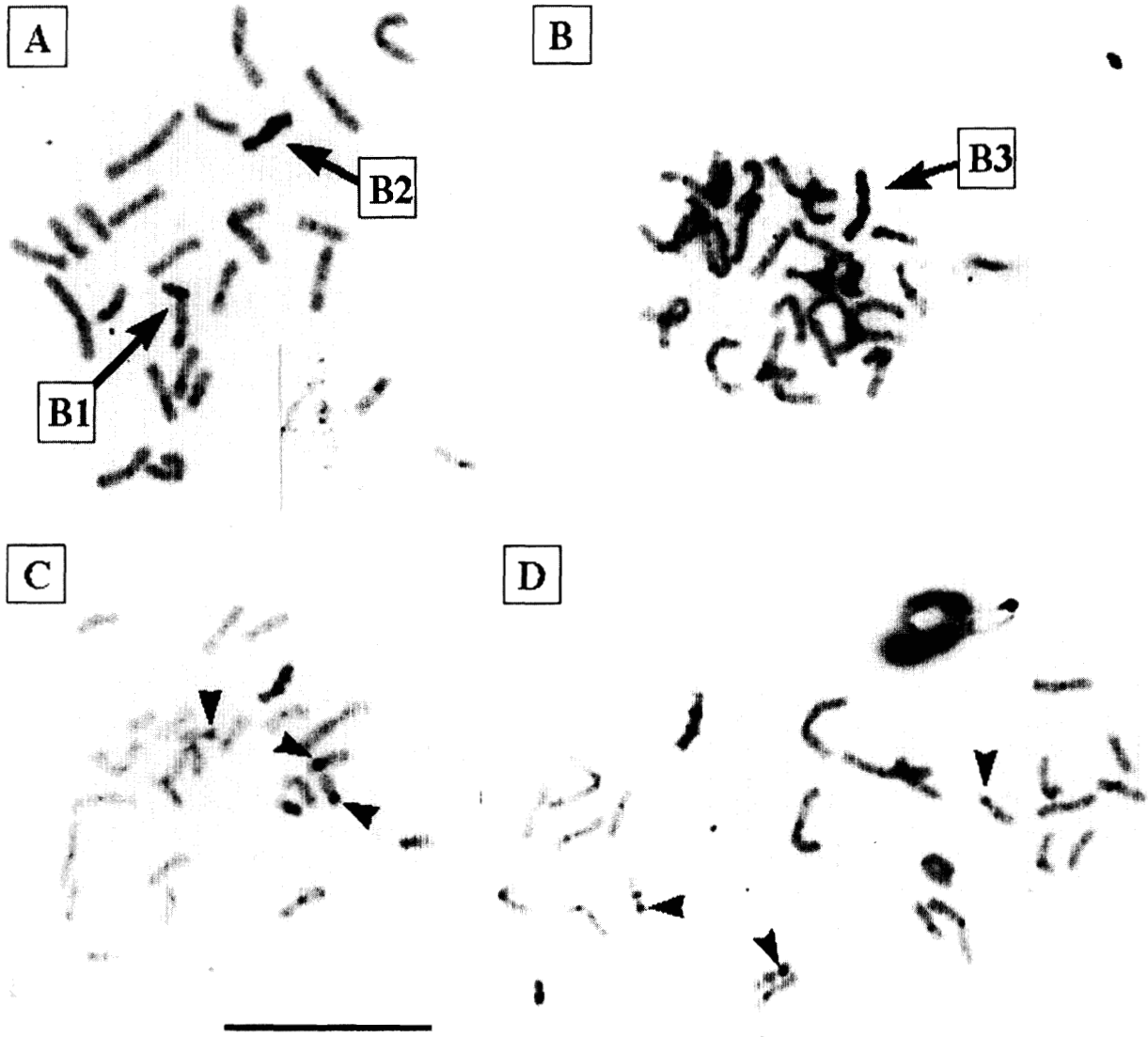
Fig. 3. C-banded *Polycelis nigra* chromosome spreads showing (A) chromosomes B1 and B2; (B) chromosome B3; (C, D) heteromorphic autosomes 5 (C; arrowheads) and 8 (D; arrowheads). Scale bar = 10 μm.



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pattern from those described here. The variable morphology, biotype distributions, and heterochromatin patterns (this study; Galleni et al.1991) of the B chromosomes of *P. nigra* from northern and southern Italian populations strongly suggest independent B-chromosome origins in each region.

The three B-chromosome morphs of *P. nigra* are found in virtually all polyploid pseudogamous parthenogenetic individuals, while being completely absent from the diploid sexual biotype (L.W. Beukeboom, T.F. Sharbel, and N.K. Michiels, submitted for publication, see footnote 3). This is not reflective of genomic parasitism (Bell and Burt 1990), and thus the B chromosome biotype distribution may reflect the genomic processes involved in the transition from diploidy to polyploidy or from sexuality to parthenogenesis. Similar anomalous chromosome conditions have been documented previously in pseudogamous parthenogenetic polyploids, such as the marine clam *Lasaea* (Thiriot-Quiévreux et al. 1989) and the plant hopper *Ribautodlephax* (Den Bieman 1988), and therefore the establishment of polyploidy or parthenogenesis (or both) may occasionally induce chromosome fragmentation or nondisjunction. Although these cases have been interpreted as aneuploidy, our data suggest that the supernumerary elements are B chromosomes rather than polysomics. Because multiple origins of these pseudogamous parthenogenetic lineages are invoked (Den Bieman and Eggers-Schumacher 1987; O Foighil and Smith 1995), further characterization of the supernumerary elements would be informative with respect to the possibility of paternal inheritance in these systems.

Autosomal heteromorphy

The identification of interhomologue heteromorphy involving two different autosomes was an unexpected but interesting outcome of this study (Fig. 2). One of the variable sites involves an active rDNA polycistron, a commonly polymor-

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phic region (Liu et al. 1996). Based on C-banding alone, we are unable to determine whether the rDNA polymorphism involves expansion and contraction of the number of rDNA copies, or whether it represents high levels of rDNA spacer length polymorphisms (Richards 1989; Mellink et al.1994; Liu et al.1996). In addition, the characteristics of the variable site on chromosome 8 are unknown, but its heterochromatic nature does not necessarily imply genetic inertness (Mong-Huong et al. 1995). Regardless of the genetic function of the heteromorphic sites, the presence of the polymorphism itself may shed light on the chromosomal dynamics of oogenesis in pseudogamous parthenogenetic *P. nigra*.

Previous karyological studies have demonstrated that the triploid type of *P. nigra* possesses oogonial cells with 24 chromosomes, and that the oocytes at diplotene and metaphase I show 24 bivalents. Prophase of the first meiotic division occurs normally (Lepori 1950), and therefore it has been inferred that endomitotic doubling occurs just prior to meiosis, so that the triploid complement can be maintained (Lepori 1950; Benazzi Lentati 1970; Benazzi and Benazzi Lentati 1976). Following this, oocytes with 24 bivalents undergo normal reduction division to form two polar bodies, and the resultant mature egg possesses 24 univalents. Lepori (1950) suggested that the endomitotic doubling prior to meiosis was followed by meiotic pairing between replicate homologues, while Benazzi Lentati (1970) was not convinced of this.

If apomixis proceeds via mitotic (equational) chromosome duplication only, chromosomal homologues may evolve independently of one another to become heteromorphic (White 1973; William Birky 1996). In this way, different members of a particular homologue set could diverge with respect to one another, as they would be under no constraint to maintain synapsis (Rice 1994), with the upper boundary of tolerable chromosome change being set by nonfunctional mutation. The homologue polymorphisms of triploid *P. nigra* are indicative of differential synapsis between certain homologues or homologue regions during meiosis I. This may have resulted from a process analogous to equational duplication (i.e., the synapsis between replicate homologues subsequent to premeiotic doubling; Lepori 1950), which may have been initiated by the proximity of replicate homologues relative to sister homologues within the organized interphase nucleus (Imai et al.1986; Loidl and Länger 1993). Since differentiated homologues would always have a replicate partner with which to synapse (owing to premeiotic doubling), meiosis would remain undisturbed. The heteromorphic conditions of chromosomes 5 and 8 thus provide molecular support for premeiotic doubling during oogenesis.

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