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Chromosome fission associated with growth of ribosomal DNA in *Neodiprion abietis* (Hymenoptera: Diprionidae)

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The haploid complement consists of seven metacentric chromosomes in most diprionid species but has evolved to $n = 8$ by fission in *Neodiprion abietis*. This fission generated a small telocentric chromosome and a large pseudoacrocentric chromosome with a short arm carrying a satellite. *In situ* hybridization indicated that the location of the rRNA gene cluster corresponds to the whole short arm. This suggests that (i) the breaking point was located close to an rRNA gene cluster, and (ii) fission was associated with growth of rDNA. These results suggest rDNA as a preferential breaking point but with a role in the healing of naked chromosome ends.

Keywords: balsam fir sawfly; karyotype evolution; centric fission; ribosomal DNA; repeated DNA; *in situ* hybridization

1. INTRODUCTION

The Diprioninae sawflies are defoliating Hymenoptera occurring throughout the range of coniferous trees. These arrhenotokous insects constitute a small subfamily of *ca.* 100 species in eight genera. Twenty-seven species from five genera are cytogenetically known (reviewed by Rousselet *et al.* 1999*a*). The modal chromosome number is $n = 7$ and only a few deviants are known. Two species belonging to the genus *Gilpinia* show a chromosome number reduction ($n = 6$), while the genus *Diprion* seems to be characterized by a chromosome number doubling ($n = 14$) (Rousselet *et al.* 1999*a*).

The chromosome set in *Diprion* evolved from seven to 14 chromosomes by dissociation of metacentric chromosomes. The fission process was associated with the growth of pericentromeric heterochromatin, which converted telocentrics into acrocentrics. The short arm of the ancestral chromosome that carries the single nucleolar-organizing region (NOR) in a procentric location gave rise to a small chromosome with a short arm and a satellite, both of which carry ribosomal RNA (rRNA) genes (Rousselet *et al.* 1998, 1999*b*; Rousselet 1999). These results pose the question of interactions between karyotype evolution and genome organization in this family.

Constitutive heterochromatin blocks or rRNA gene clusters might constitute fragile sites and represent preferential breaking points for chromosomal rearrangements such as fissions (Hall & Parker 1995; Cerbah 1997). However, it may be that the presence of repeated DNA sequences at or close to the breaking point could allow stabilization of fission chromosomes (Imai 1991). Hall & Parker (1995) particularly focused on the possible role of ribosomal DNA (rDNA) in this context.

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A second case of chromosome number increase has been reported in the Diprioninae. Maxwell (1958) described the *Neodiprion abietis* haploid complement as having eight chromosomes but did not provide information on chromosome morphology. The objective of this study was to determine whether a single centric fission was responsible for karyotype differentiation in *N. abietis* and whether a chromosomal change involved the chromosome bearing rRNA genes.

2. MATERIAL AND METHODS

(a) *Source of animals and culture method*

Neodiprion abietis (Harris) is a pest species native to Canada and the USA. Most Nearctic diprionid species attack one or few *Pinus* species. However, *N. abietis* occurs on *Abies*, *Picea*, *Tsuga* and *Pseudotsuga*. The individuals used in this study were from Newfoundland (near Grand Lake). Late larvae were collected on *Abies balsamea* in July 1998 just before cocooning. Larvae and pre-pupae in cocoons were kept at 20 °C under a 14 L:10 D photoperiod. Pre-pupae were used for chromosome preparation two days after cocooning (early pronymphs).

(b) *Chromosome preparation*

Chromosome preparations were produced from gonads. Testes or ovaries were treated in 0.4 colchicine–phosphate-buffered saline solution for 15 min and then in hypotonic solution (1% sodium citrate) for 15 min before fixing in an ethanol–acetic acid mixture (3:1) for 30 min. The fixed tissue was then transferred to a drop of 60% acetic acid on a clean slide. Finally, the slide was placed on a warm hot-plate (45 °C) and the drop allowed to evaporate. Twenty individuals were successfully karyotyped. Mitotic chromosome observations were made on germ cells after staining in a 3% Giemsa solution (pH 6.8) for 17 min. Images were obtained using a JVC TK-1270 colour video camera and Image Grabber, then mounted using Adobe Photoshop and printed on a Kodak 8650 PS colour printer.

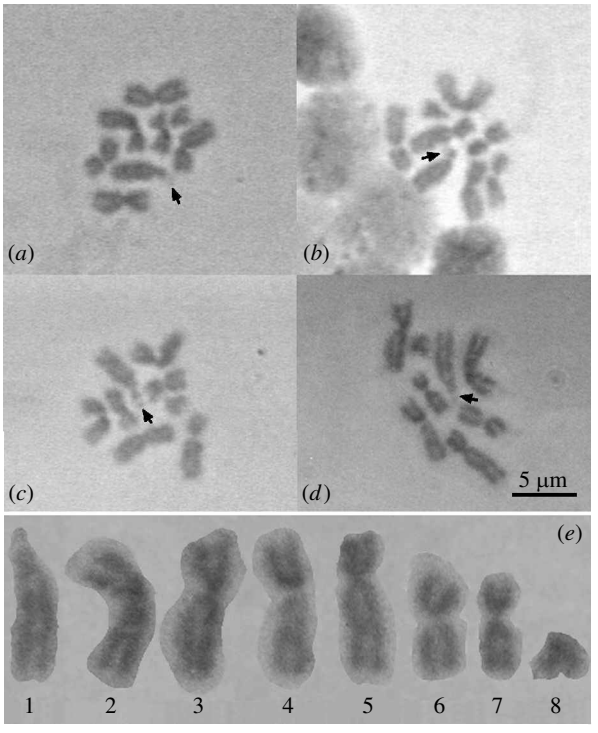

Figure 1. Mitotic chromosomes of *N. abietis*. (*a–d*) Giemsa-stained metaphase spreads showing the eight chromosomes of the male haploid complement (arrows indicate the short arm of chromosome 1 carrying a satellite). (*e*) Karyotype with two acrocentric chromosomes (chromosomes 1 and 8) and six metacentric chromosomes (chromosomes 2–6).

(c) In situ *hybridization with a biotinylated rDNA probe*

The rDNA probe used was Pyl2 cloned in pBr 322. It contains the 18S, 5.8S and 28S genes plus intergenic spacers of *Drosophila melanogaster*. The methods for the pre-treatment of slide preparations and hybridization followed the procedure described by Engels *et al.* (1986) with minor modifications. The hybridization mixture contained 35% formamide because of the heterologous nature of the probe. The probe was labelled with biotin-16-dUTP by nick translation and detected using extravidin-peroxidase and diaminobenzidine. Microscopy was performed using a Zeiss photomicroscope I and printed on a Kodak 8650 PS colour printer after staining in a 3% Giemsa solution. Photographs were taken with Fujicolor Reala ISO 100 film, then scanned and overlaid using Adobe Photoshop.

3. RESULTS

(a) *Chromosome complement*

The chromosome set consisted of eight chromosomes ($n = 8$ in males and $2n = 16$ in females) comprising six near metacentric and two acrocentric chromosomes (figure 1).

The metacentric chromosomes could be divided into two groups, which were characterized by their size. Four chromosomes were large and of similar size with centromere position from nearly median to clearly submedian (chromosomes 2–5, figure 1*e*) and two were of medium size, one with a median centromere and one with a submedian centromere (chromosomes 6 and 7, figure 1*e*).

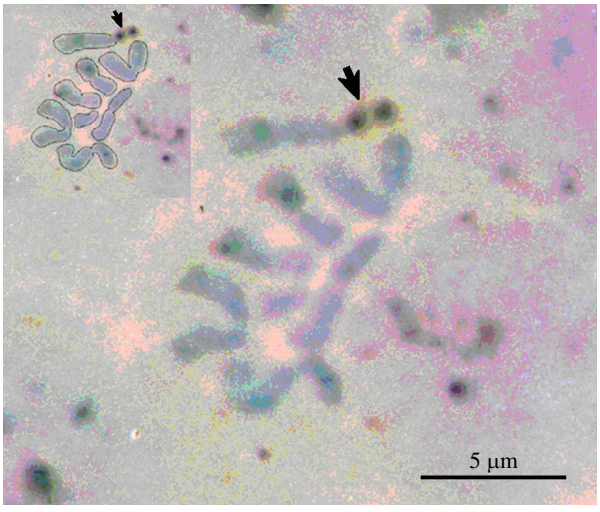

Figure 2. *In situ* hybridization with *Drosophila* rDNA probe to chromosomes of *N. abietis*. Haploid metaphase plate showing the site of hybridization (arrow) on the satellite and on the short arm of the large pseudoacrocentric chromosome (chromosome 1).

The acrocentric chromosomes were the shortest and largest ones in the complement (chromosomes 1 and 8, figure 1*e*). The former was telocentric and the latter corresponded to a pseudoacrocentric chromosome (see Imai 1991; Hirai *et al.* 1996) with a short arm bearing a satellite (figure 1). This satellite was hardly perceptible in most of the Giemsa-stained metaphase spreads and the chromosome could appear acrocentric (and slightly shorter than the four large metacentrics). Nevertheless, this pseudoacrocentric chromosome was subtelocentric in the strict sense (Levan *et al.*'s (1964) nomenclature).

(b) *rDNA localization*

Only the large acrocentric chromosome showed positive staining. The rDNA probe used hybridized to the short arm of the satellite and its carrier segment. These appeared as the dot-shaped structures in figure 2. The morphology of this chromosome only appeared clearly when the slides had been subjected to *in situ* hybridization (see figures 1 and 2).

4. DISCUSSION

The ancestral karyotype in the subfamily Diprioninae probably consists of seven metacentric chromosomes with one submetacentric/subtelocentric chromosome carrying rRNA genes situated close to the centromere on the short arm (Rousselet 1999; Rousselet *et al.* 1999*a*) (figure 3). In the North American, fir-feeding species *N. abietis* the only detectable chromosome change is a fission rearrangement, which is responsible for the haploid number increase from seven to eight.

The *in situ* hybridization experiment showed that one of the two fission products carries rDNA. The location of the rRNA gene cluster corresponds to the small short arm of the large acrocentric chromosome. This implies that the rDNA was located at or close to the breaking point (figure 3*a*).

Blocks of satellite DNA or rDNA sequences are often assumed to constitute preferential breaking points,

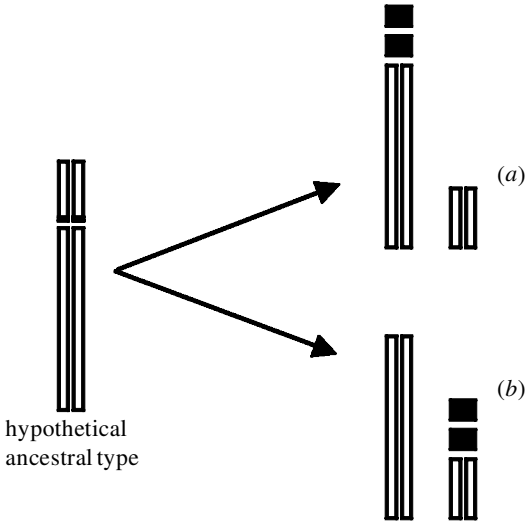

Figure 3. Hypothetical ancestral chromosome carrying an rRNA gene cluster with its fission products in (*a*) *N. abietis* and (*b*) *D. pini* (rDNA location in black).

particularly rRNA gene clusters (Hall & Parker 1995). Rapid chromosomal evolution in some vertebrate lineages may be driven by the activity of repetitive sequences (Wichman *et al.* 1991). In humans, the most common structural rearrangements are Robertsonian translocations involving the NOR-bearing chromosomes (e.g. Stahl *et al.* 1983; Choo *et al.* 1988). Breaking points may occur within the rDNA region or proximal to the rDNA within alphoid sequences (Cheung *et al.* 1990). At least 20 interchanges are known in maize, with a breaking point in the NOR itself (Philips *et al.* 1972). Karyotype differentiation in *N. abietis* also indicates this.

In situ hybridization also revealed the duplicate nature of the short arm of this large pseudoacrocentric chromosome (figure 2).

In higher hymenopteran taxa, such as ants and social wasps, the fission process is commonly associated with the growth of pericentromeric repeated DNA, giving rise to heterochromatic short arms (Imai 1991; Hirai *et al.* 1994, 1996; Imai *et al.* 1994; Hoshiba & Imai 1993). The satellite DNA and rDNA sequences are known to share the important property of self multiplication and accretion (e.g. Imai 1991; Hirai *et al.* 1994). According to these authors, fission events probably generate telomeric instability and an increase in repeated sequences in a saltatory fashion, which converts telocentrics into acrocentrics or pseudoacrocentrics. In the European, pine-feeding species *Diprion pini*, all the chromosomes result from centric fissions. Growth of pericentromeric constitutive heterochromatin is consistent with the chromosome morphology (Rousselet *et al.* 1998) and number of satellite DNA subfamilies found in this species (Rouleux-Bonnin *et al.* 1996).

The duplicate structure of rDNA in *N. abietis* is very similar to the NOR-bearing segments in *D. pini* (Rousselet *et al.* 1999*b*) (figure 3*b*). The chromosome arm maintaining an rRNA gene cluster is different in these two independent chromosome number increases, but the same amplification process has followed fission (figure 3).

According to Imai (1991), amplification could play a role in the stabilization of fission rearrangements. The appearance of functional telomeres and centromeres after centric fission is necessary. Imai (1991) proposed that the arms of pseudoacrocentric chromosomes, which elongated by addition of heterochromatin, should contain multiple 'dormant telomeres and centromeres'. In the ant genus *Myrmecia*, *'de novo* formation of centromeres' and the 'centromere shift', i.e. inactivation and reactivation of the centromeric activity, indeed suggest the presence of these multiple, dormant (or inactivated) centromeres in C-bands (Imai 1991).

The centromere position of the chromosome generated from the short arm in *D. pini* and *N. abietis* does not correspond to the ancestral primary constriction, unlike the chromosome generated from the long arm (figure 3). Moreover, the breaking point in *N. abietis* does not correspond to the location of the primary constriction of the hypothetical ancestral NOR-bearing chromosome. Thus, each side of an rRNA gene cluster, whether corresponding to an ancestral centromere position or not, was able to give rise to a functional centromere. This observation can be explained by the existence of active and dormant centromeres on each side of the rRNA gene cluster with a centromere shift before the fission event. A secondary constriction occurs on the short arm near to the primary constriction in some diprionid species with $n = 7$ (J. Rousselet, unpublished data), which could support this hypothesis.

Chromosome stability depends on telomeres and their ability to protect ends from degradation, while also facilitating end replication (Zakian 1989). In order to produce stable chromosomes, centric fission must necessarily involve (i) *de novo* formation of telomeres, (ii) donor material, or (iii) the presence of telomeric sequences at the centromere region of the ancestral chromosome (Hall & Parker 1995).

In the first case, telomeric repeats could be added *de novo* onto non-telomeric sequences of broken ends by telomerase (Blackburn 1991; Werner *et al.* 1992). However, the presence of latent telomeres in the genome is a well-supported alternative. Chromosomes may have centromeres that overlap telomere sequences. Meyne *et al.* (1990) showed that telomere sequences commonly occur at non-telomeric sites in many vertebrates. The most frequent non-telomeric location is the pericentromeric regions. Telomeric instability following fission in ants could result in amplification of repeated DNA healing the chromosome end (Imai 1991). Meyne *et al.* (1995) suggested that latent telomeres in the form of short stretches of telomeric repeat sequences or similar sequences capable of priming telomerase could be present in the ant genome. This suggests a mechanism by which the naked end of fission products in *N. abietis* and *D. pini* could have recovered new functional telomeres.

It is interesting to note that, in *N. abietis*, the only broken chromosome was the rDNA-bearing one. Maggini *et al.* (1991) showed that repeat sequences from the ribosomal intergenic spacer hybridized to chromosome ends in the broad bean *Vicia faba*, while Salvadori *et al.* (1995) showed the presence of telomeric sequences scattered through the NOR in the eels *Anguilla*. Hall & Parker (1995) proposed that, in the plant *Hypochoeris radicata*, the origin and stabilization of a spontaneous fission involved transposition of rDNA from a donor chromosome into the centromeric region of the fissioned chromosome. Both

fission derivatives possess active NORs at their centric ends, which could provide telomeric sequences and stabilize the rearrangement. In human chromosomes, rDNA also appears to cap broken ends (Zankl & Huwer 1978). This suggests that either pericentromeric heterochromatin or ribosomal DNA could have provided telomeres to one of the fission products of *N. abietis*.

In conclusion, chromosome breakage involving rDNA should have an increased occurrence and a greater chance of viability. The array of factors that have been implicated in chromosomal evolution range from the population to the molecular level (e.g. White 1973; Redi *et al*. 1990; Wichman *et al*. 1991; Hewitt 1993; King 1993; Sites & Reed 1994; Ladevèze *et al*. 1998). Karyotype evolution is governed by the availability of new chromosomal rearrangements, their impact on the organism, their constraints on meiosis producing balanced gametes and the population structure. Nevertheless, chromosomal evolution is primarily controlled at the molecular level (Wichman *et al*. 1991). Genome organization that results in the repeated occurrence or better survival of a chromosomal mutation will increase the probability that this mutation will become fixed.

Even if the uniformity of number does not imply similarity of karyotype, the Diprionidae seem to be chromosomally conservative compared to other symphytan groups such as the extended family Tenthredinidae (Naito 1982; Westendorff *et al*. 1999) or the small family Pamphilidae (Battisti *et al*. 1998). The only known fission events involve either a breaking point close to rRNA gene clusters or breaking points in all the centromeric regions. Without ruling out coincidence, the fission associated with growth of rDNA in *N. abietis* also raises questions about the possible role of genomic factors in karyotype evolution.

N. abietis is a widely distributed species occurring from the east coast to the Rocky Mountains of North America. It is reported to consist of both host plant and phenological races (Knerer & Atwood 1973; Knerer 1993). There is no evidence of polytypy since Maxwell (1958) described both *Picea* and *Abies* races as having $n = 8$. The present study indicates the need for further population studies of *N. abietis* as well as studies of other Nearctic diprionid species.

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