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Uniform distribution of satellite DNA variants on the chromosomes of tenebrionid species *Alphitobius diaperinus* and *Tenebrio molitor*

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The chromosomes of tenebrionid species *Alphitobius diaperinus* contain large blocks of pericentromerically located constitutive heterochromatin, as revealed by C-banding procedure. As previously reported, satellite DNA of this species is composed of two related monomeric units organized in three satellite subfamilies. In order to analyze the chromosomal location of the satellite DNA and the distribution of monomeric variants within it, and compare it with the distribution of monomer variants in *Tenebrio molitor* satellite DNA, the methods of in situ hybridization and restriction enzyme/nick translation were performed. Fluorescent in situ hybridization with the entire satellite DNA reveals the pericentromerically located signals on all chromosomes of the complement, coinciding with heterochromatic blocks. Results of fluorescent in situ hybridization with particular monomeric variants and of in situ restriction enzyme/nick translation show that monomeric variants are homogeneously dispersed within the entire satellite DNA. The spreading of satellite monomeric variants of the related species *T. molitor* within the pericentromeric heterochromatin of the entire complement, is demonstrated using the method of in situ restriction enzyme/nick translation. Although the complexity of organization of satellite DNAs is quite distinct in these two species, obtained results suggest similar efficiency of mechanisms of spreading and homogenization resulting in random chromosomal distribution of their satellite variants.

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Tandemly arranged highly repetitive DNA sequences or satellite DNAs are found in genomes of all higher eukaryotes and often comprise considerable portion of genomic DNA. Satellite DNAs are usually species-specific and can diverge significantly among related species. Despite their usual transcriptional inactivity and absence of coding functions, several possible roles are proposed for satellite DNAs, originating from their frequent connection with centromeric and telomeric regions of eukaryotic chromosomes (MIKLOS 1985; JOHN 1988; IRICK 1994).

Insect species belonging to the family Tenebrionidae (Coleoptera) have abundant satellite DNAs, which can build up to 50 % of the total genome, as in the case of *Tenebrio molitor* (PETITPIERRE et al. 1988). In species of this family analyzed so far, satellite DNAs are located in large blocks of constitutive heterochromatin in pericentromeric regions of all chromosomes (DAVIS and WYATT 1989; PLOHL et al. 1993; JUAN et al. 1993a; PONS et al. 1993; PLOHL and UGARKOVIĆ 1994a). Only in few cases can the satellite DNAs also be detected in telomeric regions of some chromosomes of the complement (JUAN et al. 1993b; UGARKOVIĆ et al. 1994). *Tenebrio molitor* is so far the best characterized representative from this insect family, at the molecular as well as at the cytogenetical level. Its satellite DNA is composed of tandemly arranged, highly conserved 142 bp long monomeric units (PETITPIERRE et al. 1988; DAVIS and WYATT 1989; UGARKOVIĆ et al. 1989). Monomer sequence variants, which are products of single base substitutions, are randomly distributed within the whole satellite DNA (PLOHL et al. 1992). Quite opposite to this simple organization, it was found that the satellite DNA of the species *Alphitobius diaperinus* is constituted of two related monomeric variants, dS1 and dS2 (123 and 126 bp, respectively), having 65.9 % average sequence homology. They are organized in repeating structures of higher order: dimer dS1-dS2 and trimer dS1-dS2-dS2, which furthermore create three satellite subfamilies (PLOHL and UGARKOVIĆ 1994b).

In this paper we report the chromosomal position of *A. diaperinus* satellite DNA as determined by fluorescent in situ hybridization (FISH); methods of in situ restriction enzyme/nick translation

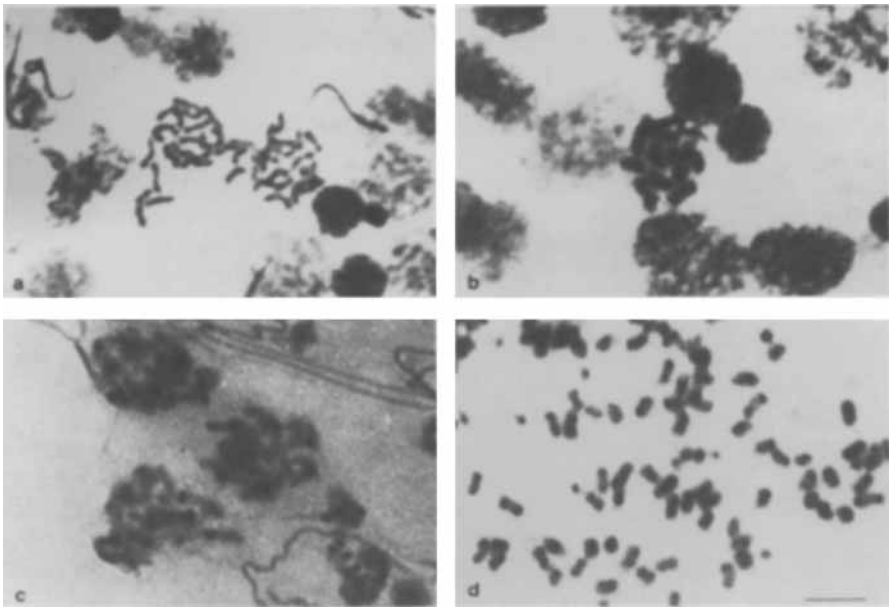

Fig. 1a–d. Chromosomes of *Alphitobius diaperinus* stained with Giemsa, in mitotic metaphase (**a**), and meiotic metaphase I (**b**). C-banding of *A. diaperinus* chromosome bivalents in the phases of pachytene (**c**) and meiotic metaphase I (**d**), with darkly stained heterochromatic regions. Bar = 5 μm.

(ISRE/NT) as well as FISH were used to determine the distribution and organization of particular monomeric units on the chromosomes of *A. diaperinus*. With the ISRE/NT we also defined the chromosomal arrangement of monomer sequence variants of the *T. molitor* satellite and compared obtained cytogenetical results with molecular characterization of their organization as obtained earlier (PLOHL et al. 1992).

Materials and methods

Chromosome preparations, C-banding and DAPI staining

Meiotic and mitotic chromosome spreads were obtained from male adult or larval gonads. The tissue was fixed in ethanol-acetic acid (3:1) for 1 h, squashed in 45 % acetic acid, and frozen in liquid nitrogen. Conventional staining was performed in 4 % Giemsa in phosphate buffer (pH 6.8) for 15 min. C-banding was done according to SUMNER (1972).

Staining of the chromosomes with fluorochrome DAPI was performed with 0.2 μg/ml DAPI in McIlvaine's phosphate buffer for 15 min (SUMNER 1990).

Fluorescent in situ hybridization

Chromosome preparations were pretreated with RNase A (100 μg/ml) in 2 × SSC for 1 h at 37°C and pepsin (100 μg/ml) in 10 mM HCl at 37°C for 10 min, dehydrated in an ethanol series, and denatured at 70°C for 2 min in 70 % formamide, 2 × SSC. Cloned *T. molitor* satellite monomer, *A. diaperinus* dS2 monomer, dS1-dS2 dimer, and dS1-dS2-dS2 trimer were labelled with biotin-16-dUTP (Gibco BRL) by nick translation. Hybridization was performed at 37°C overnight in a mixture which contained 10 ng/μl of labelled probe, 60 % formamide, 1.6 × SSC, 8 % dextran-sulfate, and 20 mM Na-phosphate. The hybridization probes for *A. diaperinus* chromosomes were the following: (1) the mixture of labelled dimer and trimer; (2) labelled dS2 monomeric unit; (3) labelled dimer with the addition of unlabelled monomeric dS2 unit in ten-fold concentration. Slides were washed 3 × 5 min in 50 % formamide, 2 × SSC at 37°C. Immunological detection was achieved using the avidin-FITC system with amplification (PINKEL

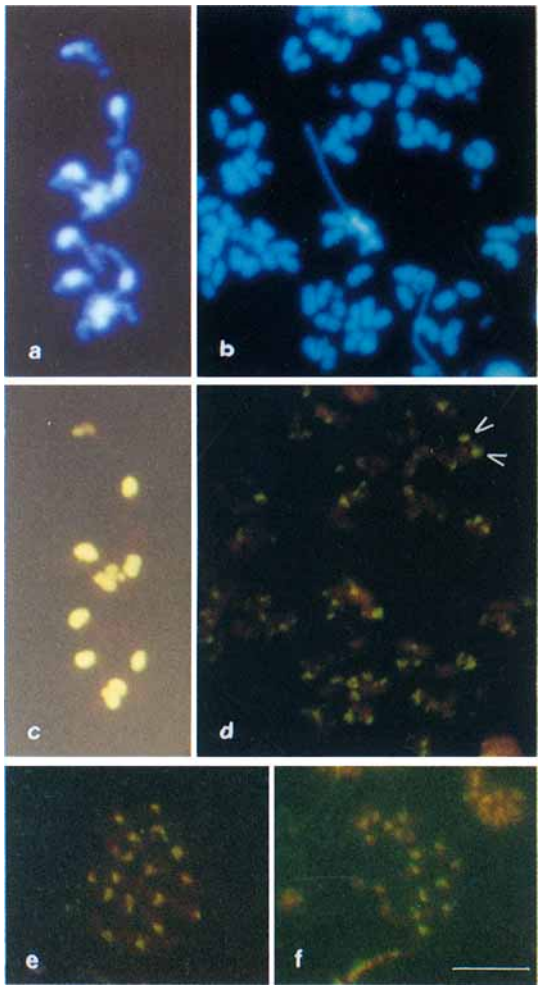

Fig. 2a–f. DAPI staining of *Tenebrio molitor* pachytene chromosomes (**a**) and *A. diaperinus* meiotic metaphase I chromosomes (**b**). FISH of *T. molitor* (**c**) and *A. diaperinus* (**d, e, f**) chromosomes with cloned satellites from each species. Mitotic prometaphase (**e**) and metaphase (**f**) chromosomes are hybridized with the cloned dS2 monomeric unit. For meiotic metaphase I chromosomes in (**d**) the mixture of cloned dS1-dS2 dimer and dS1-dS2-dS2 trimer is used as a hybridization probe. Chromosomes in (**c**), (**d**), (**e**), and (**f**) are counterstained with propidium-iodide, and yellow FITC regions correspond to hybridization signals. Arrowheads in (**d**) depict positions of centromeres on one of the bivalents. Bar = 10 μm in (a), (c), and 5 μm in (b), (d), (e), (f).

et al. 1986). The chromosomes were counterstained with propidium-iodide and photographed in a Zeiss Axiophot microscope using the appropriate filters for fluorescence.

In situ RE/nick translation

Chromosome preparations were incubated at 37°C for 30 min with 50 U of *BamH*I, *Kpn*I, *Ava*I or *Bgl*II, or with 20 U of *Hpa*I, in a total volume of 50 μl. The nick translation procedure was performed according to DE LA TORRE et al. (1991). Immunological detection of incorporated biotin-16-dUTP was as in the in situ hybridization procedure.

Results

Giemsa staining and C-banding

The males of *Alphitobius diaperinus* have a chromosome number of $2n = 19$ $(18 + X)$, chromosomes are very small, approximately 1-3 μm in mitotic metaphase and are mainly meta- or submetacentric. Conventional Giemsa staining procedure gives uniform staining along the mitotic and meiotic chromosomes (Fig. 1a and 1b), while C-banding of pachytene and meiotic metaphase bivalents results in large positive heterochromatic bands, located in pericentromeric regions of all chromosomes (Fig. 1c and 1d). The presence of large blocks of constitutive heterochromatin, located pericentromerically on all chromosomes of *Tenebrio molitor* and several other related species, has been previously reported (WEITH 1985; JUAN and PETITPIERRE 1989).

DAPI staining and fluorescent in situ hybridization

Spermatogonial meiotic chromosomes of *T. molitor* and *A. diaperinus* were stained with fluorochrome DAPI, which binds to A-T rich DNA and induces more intense fluorescence of such regions. Fig. 2a shows pachytene chromosomes of *T. molitor*, with bright pericentromeric regions corresponding to heterochromatic blocks. The A-T content of 58.5 % of this satellite DNA (PETITPIERRE et al. 1988; DAVIS and WYATT 1989; UGARKOVIĆ et al. 1989) enhances the intensity of DAPI binding in the pericentromeric areas.

Satellite DNA of *A. diaperinus* is composed of 50.8 % G–C base pairs (PLOHL and UGARKOVIĆ 1994b). Therefore, similar G-C and A-T content gives rise to uniform DAPI staining of whole chromosomes in meiotic metaphase I (Fig. 2b), making a distinction impossible between heterochromatic and euchromatic areas.

Preparations of *T. molitor* and *A. diaperinus* chromosomes were hybridized with biotinylated,

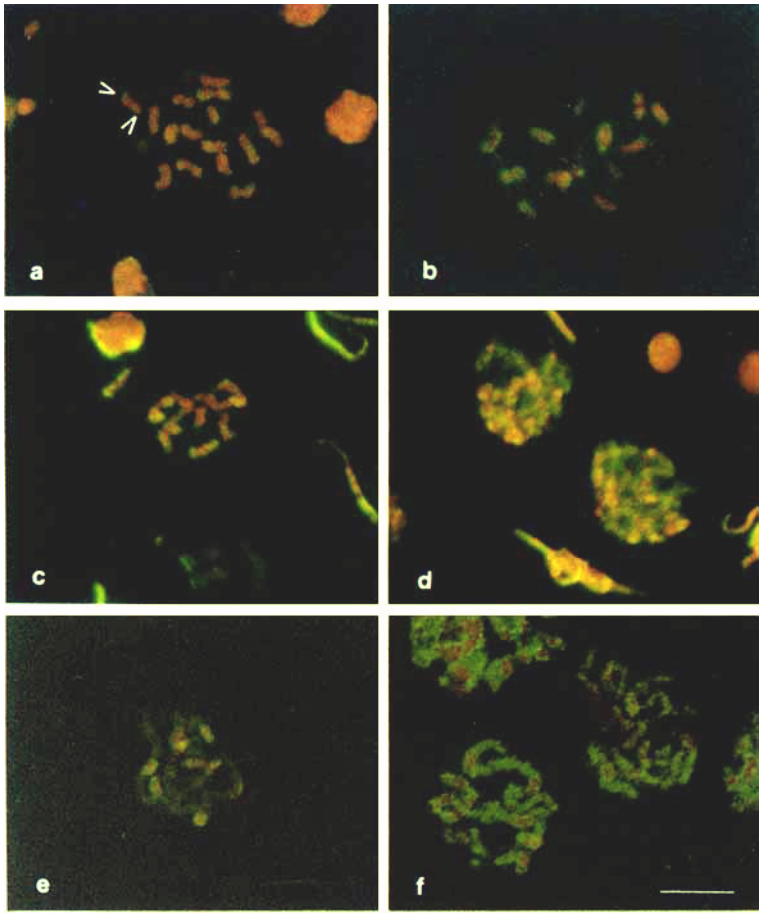

Fig. 3a–f. ISRE/NT of *Alphitobius diaperinus* meiotic chromosomes (**a–d**). Nicks on the chromosomes were induced with REs *Bgl*II in (**a**), (**b**), and with *Ava*I in (**c**), (**d**). Chromosomes are in meiotic metaphase I in the figures (**a**), (**b**), (**c**) and in meiotic prometaphase in (**d**). Arrowheads in (**a**) depict positions of centromeres on one of the bivalents. ISRE/NT of *Tenebrio molitor* pachytene bivalents after inducing nicks on the chromosomes with REs *Hpa*I (**e**) and *Bam*HI (**f**). Chromosomes are counterstained with propidium-iodide, while the regions with incorporated biotin-labelled nucleotides appear as bright FITC-signals. Bar = 5 μm in (a), (b), (c), (d), and 10 μm in (e), (f).

cloned satellite probes of the respective species. Results of FISH obtained on *T. molitor* chromosomes are in accordance with published data (DAVIS and WYATT 1989; JUAN et al. 1993b), showing the localization of satellite DNA in the pericentromeric regions of all 20 chromosomes (Fig. 2c). Fig. 2d shows meiotic metaphase bivalents of *A. diaperinus* hybridized with a probe constituted of the mixture of the cloned satellite dS1-dS2 dimer and dS1-dS2-dS2 trimer. Hybridization signals are positioned in centromeric areas, and coincide with regions of constitutive heterochromatin on all chromosomes of the complement.

In order to check the distribution and chromosomal organization of *A. diaperinus* satellite variants dS1 and dS2, we performed FISH with cloned dS2 unit and separately with cloned dimeric unit (dS1-dS2) in the presence of huge excess of unlabelled dS2. Fig. 2e and 2f show mitotic prometaphase and metaphase chromosomes of *A. diaperinus* hybridized with the dS2 variant. Hybridization signals are positioned in pericentromeric areas of all 19 chromosomes, indicating the uniform distribution of the dS2 unit within the whole satellite DNA. Hybridization with labelled dimer blocked with dS2 monomeric unit reveals the distribution of dS1 identical to that of dS2, in peri-

centromeric regions of all chromosomes (results not shown).

In situ RE/nick translation

ISRE/NT was used as additional method for determination of monomer variant distribution within the satellite DNA. For inducing nicks on the chromosomes of *A. diaperinus*, REs *Ava*I, which recognizes only dS2 unit, and *Bgl*II, which preferentially cuts in dS1, were used. These two REs have a single restriction site in the particular monomeric unit.

Fig. 3a and 3b show meiotic metaphase bivalents of *A. diaperinus* after *Bgl*II/nick translation, with fluorescent signals visible along the centromeric regions of all chromosomes. Quite similar result was obtained after *Ava*I/nick translation (Fig. 3c and 3d), with incorporated labelled nucleotides in heterochromatic blocks of all chromosomes. Mild fluorescence visible in non-heterochromatic parts of chromosomes, originates from occasional restriction sites for these enzymes in euchromatic DNA. It can be concluded that both monomeric units dS1 and dS2 are present uniformly on all chromosomes of the *A. diaperinus* complement. Recognition sites for REs *Ava*I and *Bgl*II are characteristic for all three satellite subfamilies, and so the obtained results also indicate their random and uniform distribution.

REs *Bam*HI, *Kpn*I, and *Hpa*I were used to induce nicks on *T. molitor* chromosomes. The organization of *T. molitor* satellite DNA is much simpler than that of *A. diaperinus*. It is composed of just one type of monomeric unit, and the satellite monomer variants are defined by the existence of particular restriction sites. As previously described, *Hpa*I has restriction sites in approx. 50 % of satellite monomers (PLOHL et al. 1992). Such high frequency of *Hpa*I recognition sites in satellite DNA, in comparison with those in euchromatic DNA, results in incorporation of biotin-labelled nucleotides mainly in the pericentromeric regions of *T. molitor* chromosomes, while in the euchromatin the fluorescent signal is much weaker (Fig. 3e). Fluorescent signals appear equally distributed in pericentromeric heterochromatin of all 20 chromosomes, which means that *Hpa*I monomer variants are dispersed homogeneously within the entire satellite DNA. REs *Bam*HI (Fig. 3f) and *Kpn*I (data not shown) are present in a very small percentage of monomeric variants (0.5–1 %), and so the probability of their digestion is much higher in non-satellite parts of the genome. According to this, fluorescent signals are much more intense in euchromatic than in heterochromatic regions of the chromosomes. Nevertheless, the signals are present in the whole heterochromatin of all 20 chromosomes, so the distribution of *Bam*HI and *Kpn*I sequence variants is comparable to that of *Hpa*I variants. The results of ISNT after *Hpa*I, *Bam*HI, and *Kpn*I digestion point to the uniform and random distribution of these variants within the entire satellite DNA. Also, they confirm the expected frequency of distribution in eu- and heterochromatin based on the results of molecular analysis of *T. molitor* satellite DNA with the same REs (PLOHL et al. 1992).

Discussion

The insect family Tenebrionidae (Coleoptera) has several cytogenetical features well conserved in the sample of 200 species analyzed up to now (1 % of all described tenebrionids), which can therefore be regarded as characteristic for this family. The diploid chromosome number of $2n = 20$ is highly conserved, although the sex-determining systems can diverge in several ways (JUAN and PETITPIERRE 1991). Furthermore, C-banding technique showed the presence of large and compact blocks of constitutive heterochromatin, predominantly positioned pericentromerically on all chromosomes of the complement (JUAN and PETITPIERRE 1989). Our results reveal that such chromosomal organization of constitutive heterochromatin is also characteristic for *A. diaperinus*. All of 19 chromosomes appear to contain a considerable amount of positive C-banded material that occupies exclusively the pericentromeric regions of chromosomes. Similar ratio between eu- and heterochromatin was also observed for several other tenebrionid species (WEITH 1985; JUAN and PETITPIERRE 1989).

The chromosomal localization of satellite DNA has been reported so far for several tenebrionid species. For those among them containing only one highly abundant satellite DNA, its location coincides with pericentromeric heterochromatin of all chromosomes, like in *T. molitor* (JUAN et al. 1993b), *Tribolium confusum* (PLOHL et al. 1993), and *Tribolium freemani* (JUAN et al. 1993a). This type of chromosomal distribution also proved to be the case for *A. diaperinus*, having also a single satellite DNA which is organized in three subfamilies (PLOHL and UGARKOVIĆ 1994b). This

seems to be the main, although not exclusive, mode of satellite DNA distribution on the chromosomes within the family Tenebrionidae. In *Tenebrio obscurus* and *Misolampus goudoti*, which contain two different satellites, their chromosomal distribution is more complex. Besides the pericentromeric location on all chromosomes, hybridization signal of one of the satellites is also observed on subtelomeric regions on some of the homologues (JUAN et al. 1993b; UGARKOVIĆ et al. 1994). Similar distribution has been observed for satellite DNAs of some other insect species (ARNOLD and SHAW 1985; VALGEIRSDOTTIR et al. 1990; COHN and EDSTRÖM 1992).

The method of in situ RE/NT, originating from the commonly used method of DNA digestion with REs in situ, is very sensitive and useful for determining the chromosomal organization of satellite DNAs (ADOLPH 1988; DE LA TORRE et al. 1991). It could also enable the precise distinguishing among the satellite variants characterized by the presence of recognition sites for particular REs used to induce nicks on chromosomal DNA. To analyze distribution of satellite variants, we applied ISRE/NT to chromosomes of *A. diaperinus* and *T. molitor*, two related species with significant differences in organization of their satellite DNAs. *T. molitor* is a striking example of a species with highly conserved satellite DNA, composed of only one type of monomeric unit with average mutation rate of only 1.8 % (UGARKOVIĆ et al. 1989). Satellite DNA of *A. diaperinus* is also well conserved, its average mutation rate is 3.7 %, but its composition of two monomeric units and organization in three satellite subfamilies makes it much more complex than that of *T. molitor* (PLOHL and UGARKOVIĆ 1994b).

The results of ISRE/NT and FISH clearly show that the satellite monomeric variants of both species are completely randomly and uniformly distributed within the whole satellite DNA. Spreading of variants within the pericentromeric heterochromatin of the entire complement is observed and no clustering of any analyzed variant is detected. *A. diaperinus* and *T. molitor* satellite DNAs are thus, although organizationally quite distinct and diverse, very similar regarding their entire sequence homogenization and a process of intra- and interchromosomal spreading of variants within the entire chromosomal complement. It seems that the processes of spreading like gene conversion and unequal crossingover (DOVER 1986) are very efficient and much faster than average mutation rate, ensuring the equivalent distribution of newly originated variants within the chromosomes and between the homologous and nonhomologous chromosomes. Similar distribution of satellite variants has been observed in species from the genus *Peromyscus*. Four related satellite DNAs produced the same hybridization pattern on all chromosomes, supporting the idea of their concerted evolution (HAMILTON et al. 1992). Different from such random distribution, in a wide variety of higher eukaryotes satellite variants are clustered on particular chromosomes (ALTENBURGER et al. 1977; HORZ and ZACHAU 1977; WARBURTON and WILLARD 1990; HAAF et al. 1993; BORŠTNIK et al. 1994) or form chromosome-specific subsets which are described for many of the human chromosomes (CHOO et al. 1991).

The study of satellite DNA digestion in situ revealed the different factors affecting its ability to be cleaved by certain REs. It was proposed that heterochromatin conformation and satellite DNA-protein interaction could be important for the digestion pattern, which varies even between closely related species (JUAN et al. 1991; DE LA TORRE et al. 1991; UGARKOVIĆ et al. 1994). In the case of *A. diaperinus* and *T. molitor* chromosomes, satellite DNA digestion in situ proceeds with the same efficiency as in naked DNA. All tested REs cleave chromosomal DNA with predicted frequency in heterochromatic as well as in euchromatic regions, and so it can be concluded that the chromatin of these two species is composed in such a manner that does not affect DNA digestion in situ, making the ISRE/NT method reliable in the analysis of these species.

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