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MOLECULAR CYTOGENETIC STUDY OF HETEROCHROMATIN IN SOME COLEOPTERAN INSECTS

Mirela BAUS LONČAR, Frane PAIĆ & Đurđica UGARKOVIĆ

Department of Molecular Biology, Ruđer Bošković Institute, Bijenička 54, P.O. Box 180, HR-10002 Zagreb, Croatia; phone: +385 1 4561197, fax:+385 1 4561177, ugarkov@.irb.hr

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Heterochromatin characteristics have been studied in *Leptinotarsa decemlineata* belonging to the leaf beetles (Chrysomelidae) and compared to the thoroughly analysed heterochromatin of the mealworm beetle *Tenebrio molitor*, representing darkling beetles (Tenebrionidae). C-banding reveals heterochromatin in pericentromeric regions of all chromosomes of both species; however heterochromatic blocks of *L. decemlineata* are significantly smaller in size. Digestion of *L. decemlineata* genomic DNA with 12 restriction enzymes reveals presence of bands characteristic for repetitive DNA, but again in a significantly lower amount than those found in genomic DNA of *T. molitor.* While *T. molitor* exhibits complete correspondence of restriction enzyme effects on naked DNA and mitotic chromosomes, *L. decemlineata* mitotic chromosomes are resistant to the digestion with restriction enzymes. The results show that two species have similar organization of heterochromatin on chromosomes but differ significantly in its amount. In addition, difference in accessibility to *in situ* restriction enzyme digestion indicates specificity of heterochromatin structure in the two species.

***Leptinotarsa decemlineata*, Chrysomelidae, *Tenebrio molitor*, repetitive DNA, heterochromatin**

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Proučavan je heterokromatin u krumpirove zlatice *Leptinotarsa decemlineata* kao predstavnice zlatnica (Chrysomelidae), te je uspoređen s detaljno opisanim heterokromatinom brašnara *Tenebrio molitor*, kao predstavnika crnokrilaca (Tenebrionidae). Metoda C-pruganja je pokazala prisutnost heterokromatina u pericentromernim područjima svih kromosoma obiju vrsta, iako je njegov udio znatno manji u krumpirove zlatice. Razgradnja genomske DNA krumpirove

zlatice sa 12 restrikcijskih enzima otkrila je prisutnost ponovljene DNA, ali opet u znatno manjoj količini u odnosu na brašnara. Iako kod brašnara *T. molitor* postoji podudarnost u razgradnji DNA i mitotskih kromosoma restrikcijskim enzimima, mitotski kromosomi krumpirove zlatice su otporni na razgradnju restrikcijskim enzimima. Rezultati pokazuju da obje vrste imaju podjednaku organizaciju heterokromatina na kromosomima unatoč velikoj razlici u količini. Pored toga, razlika u razgradnji mitotskih kromosoma restrikcijskim enzimima ukazuje na specifičnost heterokromatinske strukture u ovih vrsta.

***Leptinotarsa decemlineata*, Chrysomelidae, *Tenebrio molitor*, ponovljena DNA, heterokromatin**

Introduction

Chromosomes are composed of two types of domains, euchromatin and heterochromatin, known also as silent chromatin. While most of the genes are located in euchromatin, heterochromatic is gene poor and is in general transcriptionally silent. Heterochromatin usually surrounds functional chromosome structures such as centromeres and telomeres while smaller heterochromatic domains could be interspersed throughout the chromosome. After several decades of speculation it has become clear that heterochromatin plays a crucial role in proper sister-chromatin cohesion and chromosome segregation as well as in maintenance of the genome stability. It also plays a central role in the regulation of gene expression during development and cellular differentiation (Grewal & Moazed, 2003). The heterochromatic state is stably inherited through many cell divisions which take place under different developmental conditions. Since the heterochromatin contains few genes, the most of heterochromatic DNA is composed of highly repetitive noncoding DNA known as satellite DNA (Ugarković, 2005). Satellite DNA is in the form of tandem repeats organized in long arrays interspersed with transposons. The number of experimental data indicates that the repetitive nature of these elements is more important than the sequence itself for the heterochromatin establishment.

Mapping of heterochromatin on chromosomes and mutual organization of heterochromatic and euchromatic regions what defines chromosomal organization is performed by a number of methods. The classical method of C-banding is based on strong alkali treatment of chromosomes which destroys euchromatin but leaves intact highly condensed heterochromatin (Sumner, 1972). The more recent methods are based on sequence characteristics of heterochromatic DNA.

This DNA is repetitive, composed of highly similar basic repetitive elements which are in a strong complex with a number of proteins including histones and heterochromatin specific proteins. Despite association with proteins, DNA endonucleases such as restriction enzymes which recognize specific DNA sequences, can digest heterochromatic DNA on chromosome preparation, leaving "gaps" in the chromosomal regions corresponding to heterochromatin. The method of *in situ* restriction enzyme digestion has been successfully used for mapping heterochromatin on chromosomes of different species (Mezzanote et al., 1983; Juan et al., 1991).

In this work we compare organization of heterochromatin on chromosomes of coleopteran species *Tenebrio molitor* and *Leptinotarsa decemlineata*, belonging to the families Tenebrionidae (darkling beetles) and Chrysomelidae (leaf beetles), respectively, by using C-banding and restriction enzyme digestion *in situ* and on naked DNA. Tenebrionid species are known to have conserved karyotype with haploid chromosome number n=10 for most of the 200 analysed species (Juan & Petitpierre, 1991). Different from Tenebrionidae, Chrysomelidae have variable karyotype with chromosomal number ranging from n=4-32. (Petitpierre et al.,1991). The higher rate of chromosomal evolution in Chrysomelidae relative to Tenebrionidae could be explained by the deme size theory, most of the former being trophic specialists whereas those of the latter are mostly trophic generalists.

Material and Methods

Chromosome preparation, C-banding and *in situ* digestion

Chromosomes were obtained from *T. molitor* male larvae and *L. decemlineata* male adults as described previously (Juan et al., 1991). C-banding was performed according to Sumner (1972). Slides were stained in 6% Giemsa in phosphate buffer at pH 6.8. All enzymes used for *in situ* digestion were purchased from Roche. Slides were incubated overnight at concentration 1U/µl in 50 µl of appropriate buffer at 37˚C in a humid chamber. After incubation, slides were washed in distilled water and stained in Giemsa as above.

DNA isolation and digestion

Genomic DNA was isolated from *T. molitor* larvae and *L. decemlineata* adults using the standard procedure. Restriction enzyme digestion was performed in the

appropriate buffer using 2U/ µg of DNA. The following restriction enzymes have been used: *Sau*3A, *Rsa*I, *Hinf*I, *Msp*I, *Sma*I, *Hae*III, *Sau*96I, *Dde*I, *Acc*I, *Alu*I, *Taq*I, *Eco*RI, *Dra*I, *Hpa*I, *Kpn*I, *Pst*I, and *Mnl*I. The digested DNA was analysed by electrophoresis in 1% agarose gel stained with ethidium bromide.

Results

C-banding and *in situ* restriction enzyme digestion

Mealworm beetle *Tenebrio molitor* has a typical tenebrionid karyotype with haploid chromosome number n=10 and sex-chromosome system "parachute" Xyp, shared by most of tenebrionids (Juan & Petitpierre, 1991; Figs. 1a,b). Previous C-banding studies revealed the presence of large heterochromatic blocks encompassing the pericentromeric regions of all chromosomes (Weith, 1985). It has been also shown that the heterochromatic blocks are composed of tandemly repeated satellite DNA which makes 50% of the whole *T. molitor* genomic DNA (Petitpierre et al., 1988; Davies & Wyatt 1989; Ugarković et al., 1989). Using restriction enzymes characteristic for this satellite DNA, digestion of heterochromatic regions on *T. molitor* chromosomes was obtained (Juan et al., 1991). C-banding performed on *T. molitor* chromosomes confirms the presence of large, darkly stained heterochromatic blocks clearly seen on chromosome bivalents

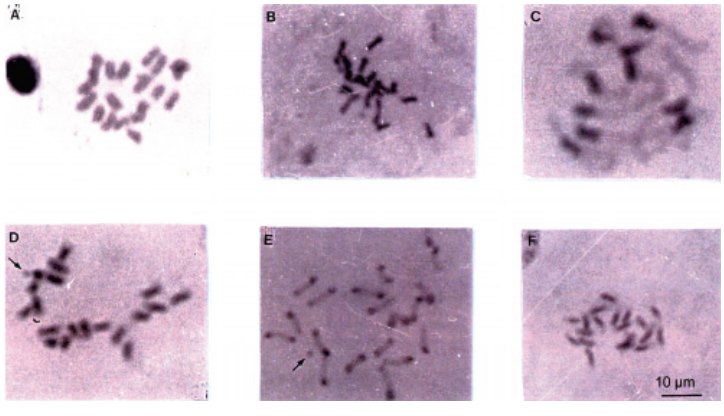

Figure 1. Chromosomes of *Tenebrio molitor*: mitotic metaphases stained with Giemsa (a,b); C-banding in pachytene (c) and in mitotic metaphase (d); *in situ* digestion of mitotic metaphases with *Eco*RI (e) and *Alu*I (d). Bar represents 10 µm; y chromosome is marked with arrow.

in pachytene (Fig. 1c), while on condensed chromosomes in mitotic metaphase the heterochromatin encompasses the most of the chromosome length (Fig. 1d). Digestion of metaphase chromosomes with restriction enzyme EcoRI, which has recognition site in highly abundant *T. molitor* satellite DNA, reveals specific digestion of chromosomal regions corresponding to the heterochromatic blocks (Fig. 1e). Euchromatic regions however, due to the relatively low frequency of restriction sites for *Eco*RI remain mostly undigested. The mirror image is obtained after digestion with restriction enzyme *Alu*I which cuts frequently euchromatic DNA, while its restriction sites are almost absent in heterochromatin (Fig. 1f).

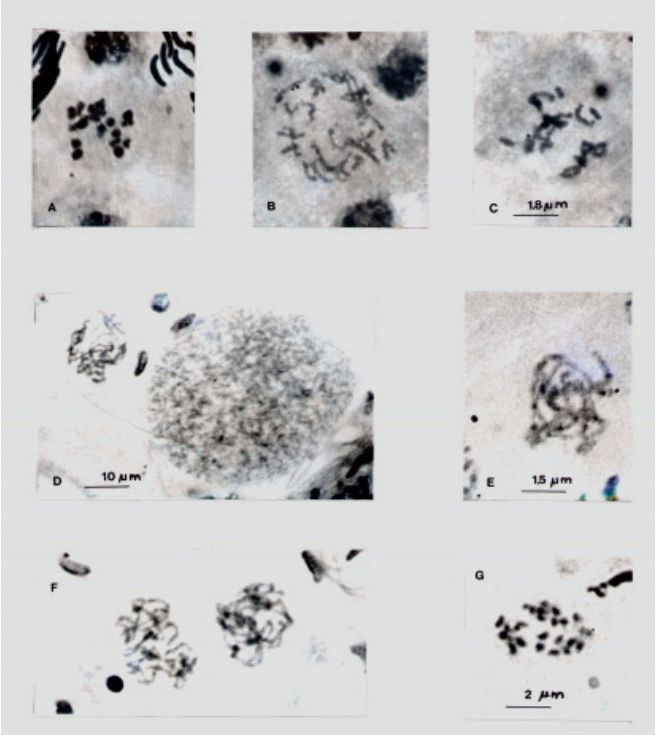

Figure 2. Chromosomes of *Leptinotarsa decemlineata*: meiotic metaphase I (a,c) mitotic prophase (b), stained with Giemsa; C-banding on endopolyploid nucleus (d) pachytene (e,f) and mitotic metaphase chromosomes (g). Bar represents 1.8 µm in (a), (b), (c) and 2 µm in (f), (g).

The meiotic formula of *Leptinotarsa decemlineata* is 17+X0 for males, and 17+XX for females, respectively (Hsiao & Hsiao, 1983; Figs. 2a,b,c). C-banding reveals small heterochromatic blocks present on all chromosome in meiotic prophase of pachytene and careful inspection discloses their pericentromeric location (Figs. 2e,f). The heterochromatic blocks are equally dispersed over endopolyploid nuclei, exhibiting no aggregation (Fig. 2d). On highly condensed chromosomes during mitotic metaphase, darkly stained heterochromatin is clearly visible (Fig 2g). *In situ* digestion performed on metaphase chromosomes with few restriction enzymes which cut rarely within euchromatin such as *Eco*RI and *Pst*I did not disclose the presence of "gaps" corresponding to digested heterochromatic regions (not shown).

Restriction enzyme digestion of genomic DNA

To disclose the presence of tandemly repeated satellite DNA which is characteristic for heterochromatin, digestion of *L. decemlineata* genomic DNA was performed with 12 restriction enzymes and products were separated by electrophoresis on 1% agarose gels (Fig. 3a). The restriction profiles are in the form of smears encompassing higher size range for low frequency 6-cutters such as *Pst*I and *Eco*RI (lines 6, 13) as well as for *Msp*I, *Hae*III and *Sau*96I with G+C rich recognition sites (lines 5,7,8). As expected, frequent 4-cutters such as *Sau*3A,

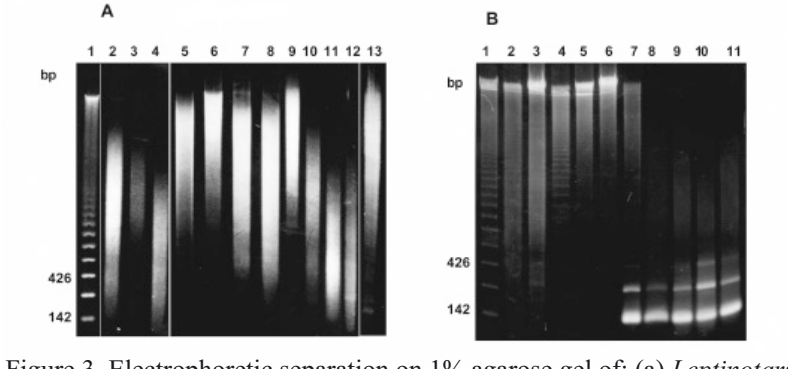

Figure 3. Electrophoretic separation on 1% agarose gel of: (a) *Leptinotarsa decemlineata* genomic DNA (10 µg) digested with *Sau*3A (2), *Rsa*I (3), *Hinf*I (4), *Msp*I (5), *Sma*I (6), *Hae*III (7), *Sau*96I (8), *Dde*I (9), *Acc*I (10), *Alu*I (11), *Taq*I (12), *Eco*RI (13).; and (b) *Tenebrio molitor* genomic DNA (10 µg) digested with *Alu*I (2), *Dra*I (3), *Hpa*I (4), *Kpn*I (5), *Pst*I (6), *Eco*RI (7), *Sau*3A (8), *Hinf*I (9), *Mnl*I (10), *Rsa*I (11). Line (1) represents size marker (multimers of 142 bp).

*Rsa*I, *Hinf*I, *Alu*I and *Taq*I (lines 2,3,4, 11,12) produce smears of lower size range. Distinct bands indicating presence of highly repetitive DNA are absent in most of the digests except in *Eco*RI where a faint band of approximate size of 250 bp is visible (line 13), as well as in *Taq*I digestion with a size of about 280 bp (line 12). A band of low intensity having approximate size of 300 bp is also detected in *Dde*I digest (line 9). These results indicate the presence of repetitive satellite DNA in *L. decemlineata* genomic DNA.

For the comparison, digestion of *T. molitor* genomic DNA with 10 restriction enzymes reveals the presence of highly abundant satellite DNA having basic monomer length of 142 bp in *Eco*RI, *Sau*3A, *Hinf*I, *Mnl*I and *Rsa*I digests (Fig. 3b, lines 7-11). Restriction enzymes such as *Alu*I, *Kpn*I and *Pst*I do not cut repetitive DNA significantly (lines 2, 5, 6), while *Dra*I and *Hpa*I produce bands of higher size range (lines 3,4). It has been previously shown that this satellite DNA makes around 50% of *T. molitor* genomic DNA (Petitpierre et al., 1988) and corresponds to heterochromatin blocks as is also revealed by digestion *in situ* with *Alu*I and *Eco*RI (Figs. 1e,f).

Discussion

The C-banding karyotypes of many tenebrionid species have shown highly conspicuous procentric heterochromatic blocks in all chromosomes (Weith, 1985; Juan & Petitpierre, 1989) while narrow telomeric bands are visible in some chromosomes of species *Misolampus goudoti* and *Tenebrio obscurus* (Pons et al., 1993; Ugarković et al., 1994). Molecular analyses revealed tandemly repeated satellite DNA as a major constituent of the heterochromatin in tenebrionids, comprising between 17% and 50% of the genomic DNA (Ugarković et al, 1995). Satellite DNAs have been also characterized in some species of Chrysomelidae. Satellite DNA comprises around 10% of genomic DNA in *Chrysolina americana* as well as in *Xantogaleruca luteola,* and coincides with pericentromeric heterochromatin (Lorite at a.l, 2001; 2003). Restriction profile of *L. decemlineata* genomic DNA also gives strong indication for the presence of satellite DNA in this species, in an amount comparable to those found in *C. americana* and *X. luteola*. This is significantly lower than the amount of satellites found in tenebrionid beetles, however it roughly corresponds to the lower amount of heterochromatin detected by C-banding on *L. decemlineata* chromosomes. This indicates that the prevalent component of heterochromatin in Chrysomelidae is tandemly repeated

satellite DNA. However, molecular characterization of repetitive DNA from *L. decemlineata* and other leaf beetles, followed by *in situ* hybridization, will give more reliable information about heterochromatin composition.

In situ restriction digestion of *L. decemlineata* chromosomes using enzymes *Eco*RI and *Pst*I did not reveal any banding pattern, although the *Eco*RI was shown to have recognition sites in *L. decemlineata* repetitive DNA producing distinct band of approximately 250 bp. The explanation for the absence of banding could be related to the organization of repetitive DNA which could be dispersed along chromosomes. In this case restriction enzymes cut repetitive DNA, but the DNA fragments remain attached to the rest of nondigested DNA, and no "gaps" are formed. The other possibility is that the *Eco*RI repetitive family is in the form of tandem repeats located in pericentromeric heterochromatin, but remains protected from the digestion due to some specific association to the chromosomal proteins. The resistance to *in situ* digestion of heterochromatic satellite DNA was thoroughly studied in the mealworm beetle *Tenebrio obscurus* (Ugarković et al, 1994). Only removal of proteins with proteinase K enabled *in situ* digestion of satellite DNA in this species. On the contrary, the mealworm beetle *T. molitor* represents a striking example of satellite DNA localization by in situ digestion and exhibits complete correspondence of the restriction enzymes effects on naked DNA and mitotic chromosomes (Juan et al., 1991; this work).

Conclusion

The heterochromatin has been studied in *L. decemlineata* using methods of C-banding and *in situ* restriction enzyme digestion combined with the analysis of repetitive DNA. The results show existence of repetitive DNA in the genomic DNA of *L. decemlineata* as well as the presence of pericentromerically located heterochromatric blocks on all chromosomes. Such a pattern of heterochromatin distribution is similar to that found in the mealworm beetle *T. molitor* although the amount of heterochromatin is significantly higher in the letter species. Besides, difference in the *in situ* restriction enzyme digestion between two species points to the species-specificity of the heterochromatin structure.

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