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Satellite DNAs in Tenebrionid Species: Structure, Organization and Evolution

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The heterochromatic region of chromosomes contains highly repeated satellite DNAs with no or very few genes and is usually transcriptionally inactive. Its repetitive character has led to the suggestion that it is »junk DNA« with no utility, although some important inheritance functions have been mapped to it. Beetles from the family Tenebrionidae (Insecta, Coleoptera) contain a substantial amount of heterochromatin and satellite DNA in their genome and represent a suitable system for structural and organizational studies. Here, the data on the primary and higher order structure and organization of satellite DNAs from ten different tenebrionid species are presented, as well as on mutational processes effecting their evolution. Using these data, we have tried to determine conserved structural elements within satellites which can be important for maintaining the heterochromatin structure and compactness. Such analyses could help to define the structural components of the chromosome responsible for some heterochromatic functions, like chromosome pairing, centromere function and sister chromatid adhesion.

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INTRODUCTION

The eukaryotic genome is made up of two types of sequences: single copy DNA, or unique DNA, and repetitive DNA. Repetitive DNA consists of nucleotide sequences that occur several times in the genome either in tandem or in a dispersed fashion while unique DNAs do not repeat themselves. Repetitive DNA sequences create a substantial part of the eukaryotic genome, which varies over different taxa. In yeast, repetitive DNA builds up to 20% of the genome, in mammals up to 60% while in most plants this amount is higher than 80%. Highly repetitive sequences are divided into localized and interspersed ones according to their organization within a genome. Localized highly repetitive sequences or satellite DNAs are tandemly arranged and usually located in centromeric or telomeric regions of chromosomes.[1] In some species, they account for the majority of genomic DNA, like in the case of the kangaroo rat *Dipodomys ordii* and beetle *Tenebrio molitor* where satellite DNAs make about 50% of the genome.[2–4] The interspersed, highly repetitive sequences are found in introns, flanking regions of genes, intergenic and nongenic regions. According to the size of a repeating unit, they are classified into short interspersed repeated sequences or SINEs and long interspersed repeated sequences or LINEs.[5] Interspersed repetitive sequences, present in a moderate number of copies within a genome, include transposable elements, which are sequences capable of inserting copies of themselves into new genomic locations. Minisatellite and microsatellite sequences also belong to this class of moderately repetitive DNA. They contain arrays of short (2–5 bp-microsatellites, or about 15 bp-minisatellites) tandem nucleotide repeats and are found in euchromatic regions of the genome of vertebrates, insects, fungi and plants. In the euchromatic region of the human genome, nearly 30 000 microsatellite loci are detected, which vary considerably in size within a population. Satellite DNAs can also have repeating units similar in lengths to micro- or minisatellites or much longer (100 – more than 1 000 bp) but the main feature that distinguishes them from mini and microsatellites is their organization in long (up to 100 megabase) clusters, located in heterochromatin.[6]

Satellite DNAs are usually species specific and diverge significantly in the nucleotide sequence and copy number, even among very related species.[7] Despite their usual transcriptional inactivity and absence of coding function, some roles have been proposed for them relative to their centromeric and telomeric positioning on eukaryotic chromosomes. It was assumed that satellite DNA could play a role in the condensation of chromatin in the metaphase, as well as in the condensation of heterochromatin due to the defined secondary and tertiary structures.[8–10] The meiotic pairing in *Drosophila* has been shown to require heterochromatic homology and to be sensitive to the repeat number.[11,12] The increased pairing ability of repetitive sequences could be one of the reasons why most eukaryotes allow accumulation of tan-

dem repeated elements. This could be a partial explanation of the ubiquity of heterochromatin among eukaryotes. It has also been shown recently that human satellite DNA, which is located in centromeric regions of all human chromosomes, is a functional part of the centromere necessary for the regular segregation of chromosomes during mitosis.[13]

Within the animal kingdom the order Coleoptera (Insecta) is the largest, containing about 350 000 species, classified into 200 families. Among them is the family Tenebrionidae with about 25 000 species. Some members of the family are well known, common store product pests. The cytogenetical analysis performed on 200 species from the family revealed the conserved chromosomal number 2n being 18, 19 or 20 as well as a substantial amount of heterochromatin in all chromosomes, making up to 50% of the whole chromosome length.[14–16] Such a high amount of heterochromatin makes these species a very suitable model system for the study of the satellite DNA structure and organization, as well as the study of genetic processes affecting their evolution.

PRIMARY STRUCTURE CHARACTERISTICS

Satellite DNAs have been detected by the restriction enzyme (RE) digestion of the total genomic DNA in ten species belonging to five different genera of the family Tenebrionidae (Table I). The most prominent bands obtained by these digestions have been cloned and the primary structure of the number of clones has been determined. These analyses show that all the species contain a substantial amount of satellite DNAs in a range between 17% in *Tribolium castaneum* to 50% as found in mealworm beetle *Tenebrio molitor*.[4,10,17–20] Most species contain a single satellite DNA, which is species specific and exhibits no sequence homology with any other satellite DNA. In *Tenebrio obscurus*, in addition to the species specific satellite DNA I having an abundance of 13%, another more abundant satellite DNA is present, which exhibits 80% sequence homology to satellite DNA from the related *Tenebrio molitor*.[21] Two different, unrelated satellite DNAs are also detected in *Misolampus goudoti*.[20] In *Tribolium madens* the main satellite builds up 30% of the genome, while the related, complex satellite II makes 5% of the total genomic DNA. A similar situation is found in *Alphitobius diaperinus* where satellite DNA is composed of three related repeat variants of similar length (Table I).

Despite the lack of sequence homology among satellite DNAs from different tenebrionid species, they can be classified into two groups according to the size of their basic repeating unit or satellite monomer (Table I). The first group comprises seven satellite DNAs of monomer lengths of about 100–160 bp. Their sequences are unique in the sense that they cannot be resolved into blocks of shorter repeats. Both satellites of *T. madens* and sat-

TABLE I

Characteristics of satellite DNAs from tenebrionid species

Species	Abundance of satellite (%)	No. of copies / hapl. genome	Monomer length (bp)	A + T content of satellite DNA (%)	Sequence variability (%)	Potential secondary or tertiary structures
1. Tenebrio molitor	50	$1.65 \cdot 10^6$	142	58.5	1.8	left superheli
2. Tenebrio obscurs	13	$1.77 \cdot 10^5$	344	67.7	4.6	no evident structure
	23	$7.61 \cdot 10^5$	142	64.1	4.1	left superhelix
3. Palorus ratzeburgii	40	$9.40 \cdot 10^5$	142	68.0	2.3	left superhelix
4. Tribolium confusum	40	$5.82 \cdot 10^5$	158	73.4	2.0	cruciforms
5. Tribolium castaneum	17	$8.76 \cdot 10^4$	360	73.0	3.3	cruciforms
6. Tribolium freemani	31	$4.05 \cdot 10^5$	166	70.5	3.0	cruciforms
7. Tribolium madnes	30	$3.16 \cdot 10^5$	225	74.0	4.1	–
	5	$2.37 \cdot 10^4$	711	70.0	2.3	cruciforms
8. Alphitobius diaperinus	25	$4.70 \cdot 10^5$	variant 123			
			variant 126	av. 49.2	3.7	cruciforms
			variant 128			
9. Misolampus goudoti	7	$1.20 \cdot 10^5$	196	64.3	2.4	–
	25	$6.81 \cdot 10^4$	1200	–	–	–
10. Palorus subdepressus	30	–	142	–	–	–

ellite I of *M. goudoti* can be added to this group despite their apparent larger monomer size. A search for internal subrepeats in these monomers shows that they are basically created from the unique sequence of an approximate length of 100 bp. This basic unit was duplicated and subsequently diverged by the action of mutational processes, creating complex satellite repeats of different size (225 bp, 711 bp, 196 bp), found in *T. madens* satellites and in *M. goudoti* satellite I.[20] The other group includes two satellites composed of unique sequences of about 350 bp. These are satellites of *T. castaneum* (360 bp) and *T. obscurus* satellite I (344 bp).[21] The *M. goudoti* satellite II with a monomer length of 1 200 bp has not been completely sequenced yet and it is not known if it can be classified into one of the groups.[20]

Satellite DNAs, due to their uniform nucleotide composition and often different G+C content relative to the rest of genomic DNA, can be separated from genomic DNA using the density gradient centrifugation. Most tenebrionid satellites have an A+T content higher than 64% (Table I), meaning that they are richer in A+T than the rest of the genome, which usually has an A+T content of about 60%. Such a composition is in accordance with the theory of neutral evolution predicting that freely evolving sequences which are not subjected to selective constraints would become A+T rich. However, there are exceptions, e.g. in the case of *T. molitor* satellite having a very similar nucleotide content to the rest of DNA and in *A. diaperinus* satellite DNA which has similar G+C and A+T contents.

HIGHER ORDER STRUCTURES

The heterochromatic regions of chromosomes, where satellite DNAs are located, are the most condensed parts of chromosomes, which retain such a structure during most of the cell phases. It is also known that 50% more DNA is present per unit length of heterochromatic region than in the euchromatic region of the same size, meaning a denser package of satellite DNA. It is hypothesized that the satellite DNA higher order structure could be responsible for this effect of condensation either by inducing interaction with specific nonhistone proteins or by influencing nucleosome formation and positioning.[8,10,22]

In many satellite DNAs inverted repeats are very frequent, sometimes forming stable intrastrand dyadic structures.[23,24] The presence of such structures in segments of DNA can be experimentally proved by the method of gel electrophoresis.[25] Migration of such segments is slower in polyacrylamide gels in comparison with the migration of linear DNA of the same size. The same method is also applied to the study of curved DNA structures which are also characteristic of satellite DNA.[9,26,27] The curvature of DNA axis is a consequence of its primary structure. It was shown that stretches of (A)s or (T)s which appear periodically, on average each ten nucleotides,

in a phase with helical turn, can induce DNA curvature, although it is also detected in some DNA sequences without very prominent and regular A or T series.[9,26] In any case, such DNAs exhibit retardation during electrophoresis on polyacrylamide gels which depends on the position and number of bend centers in the analyzed fragment. The study of DNA curvature and prediction of its tertiary structure can also be approached by computer modeling, yielding curves which, in the position of bend centers and overall shape, closely resemble those obtained from electrophoretic data.[9,10,28] Most of the analyzed satellites have two bend centers in their repeat units.[9,10,29] If these centers cause bending in the same direction, the result is a helical structure. If, on the other hand, they induce bending in opposite direction, an »S« shaped structure is formed which does not exhibit electrophoretic retardation.[28] The presence of such unusual DNA structures can be also demonstrated by electron microscopy.[30]

Our analyses of tenebrionid satellites, using polyacrylamide gel electrophoresis and computer modeling reveal the presence of sequence induced DNA curvature in satellites from three different species: *T. molitor*, *T. obscurus* and *Palorus ratzeburgii* (Table I).[9,10,21] All three satellites have the same monomer length of 142 bp, *T. molitor* and *T. obscurus* satellites exhibit 80% sequence homology, while their homology with *P. ratzeburgii* satellite is insignificant. However computer modeling predicts tertiary structures of very similar geometry for all three satellites in the form of a left-handed superhelix. One turn of the helix has approximately 310 bp and a 30 nm pitch.[10] It seems to us very significant that satellite DNA from four different species (*Palorus subdepressus*, not sequenced) have identical monomer lengths and for three of them very similar higher order structures are predicted, despite sequence divergence. We term these satellites »142 bp family« of tenebrionid satellites and propose that they could be under selective constriction effecting conservation of their structural characteristics: monomer length and tertiary structure.[21] There is an assumption that curved satellite DNA structure codes for the protein binding function necessary for the organization of the higher order heterochromatin structure.[10,22] In the absence of DNA curvature, cruciform structures could perform a similar coding function causing specific protein binding.[24] However, there is a class of satellite DNA with no evident secondary or tertiary structures, some of them found also among tenebrionid satellites (Table I). For such satellites, we propose that some primary structure characteristics, like short stretches of a particular DNA sequence, could play a similar coding function as cruciforms and the curved helical structure. It has been shown recently that, in different avian centromeric satellites which have conserved monomer length of approximately 190 bp, some short DNA elements like tetra- and nonanucleotides are conserved at similar locations within the repeats of different satellites.[31] A 17 bp long sequence of human satellite DNA binds specifically a centromeric protein CENP-B[32], pointing to the fact that short DNA sequence elements are sufficient for a specific interaction. It seems that at

least three different types of condensation signals can be present in satellite DNA, inducing differences in heterochromatin condensation and the higher order structure. The existence of two abundant satellite DNAs in *T. obscurus*, one with a prominent curvature of the helix axis and the other with no evident higher order structure, indicates the presence of two different condensation mechanisms within a single genome.

The difference in the heterochromatin higher order structure between closely related tenebrionid species *T. molitor* and *T. obscurus* was demonstrated by molecular-cytogenetic methods: chromosomal digestion *in situ* by REs and by *in situ* nick translation.[33,34] In the case of *T. molitor*, which contains a single, curved satellite DNA in centromeric heterochromatin, *in situ* digestion with REs having recognition sites in satellite monomers, resulted in complete removal of the heterochromatic region from the chromosomes.[33] In *T. obscurus,* where satellite DNA related to that from *T. molitor* is present in the centromeric region, together with another satellite DNA of a different primary and higher order structure (Table I), heterochromatin is completely resistant to digestion by REs having recognition sites in any of the satellites. Only a short pretreatment of *T. obscurus* chromosomes with proteinase K makes the heterochromatic regions accessible to RE digestion.[34] These simple experiments demonstrate that the heterochromatin higher order structure or conformation can also depend on the satellite DNA composition and differs even among congeneric species.

ORGANIZATION AND CHROMOSOMAL DISTRIBUTION

Satellite DNAs are characterized by a tandem arrangement in a head-to-tail order of monomer sequence units which form long segments of megabases size. Most tenebrionid satellites have well conserved monomer sequences and form no satellite subfamilies. An exception is a satellite DNA of *A. diaperinus* composed of three monomer variants organized in higher order repeating structures: dimer and trimer. The dimeric and trimeric units create three higher order satellite subfamilies: two of them contain either tandemly arranged dimers or trimers, while the third is composed of both types of repeats, mutually interspersed.[17]

In situ hybridization experiments reveal localization of tenebrionid satellites in centromeric, heterochromatic regions of all chromosomes.[18,19,21,34] The only exception is a *M. goudoti* satellite I which is not present on chromosome Y.[35] Besides the centromeric location, *T. obscurus* satellite I and *M. goudoti* satellite II are found in the regions of subtelomeric heterochromatin but in an essentially lower copy number.[34,35]

MUTATIONAL PROCESSES AND EVOLUTION

Satellite DNAs are not completely homogeneous but contain many variants that differ from the consensus sequence in nucleotide composition. This difference can be a result of nucleotide substitutions, insertions or deletions. In all analyzed tenebrionid satellites, insertions and deletions are very rare events e.g. in 22 randomly cloned *T. molitor* satellite monomers no deletions or insertions have been found.[4] Sequence variation is in the range between 1.8% to 4.6% relative to the consensus sequence (Table I) and is mainly a result of single point mutations, which are spread randomly within the sequence. This rate of sequence divergence is similar to that seen in other insect and vertebrate species.[36,37] Analysis of base substitutions reveals that, in some tenebrionid satellites, C→T and G→A transitions are predominant, particularly for *T. molitor* and *A. diaperinus* satellites which are relatively A+T poor and whose sequence tends to become A+T rich. This is less stressed in A+T rich satellites of *P. ratzeburgii, T. confusum* and *T. obscurus,* which exhibit a very similar tendency of G,C and A,T residues to substitutions.[18,21] Very often, the same nucleotide substitution occurs in several clones of a satellite monomer at the same position in the sequence. We propose that such substitutions do not occur by two or more independent mutational events but result from a single point mutation which is further spread throughout the satellite probably by a mechanism of gene conversion. The effect of gene conversion is more evident in the *A. diaperinus* satellite, where exchange of longer DNA segments (30 bp and 46 bp, respectively) between two clones is observed. There is also indication that a process of replication slippage has taken part in the formation of satellite units together with recombination responsible for creation of a new satellite unit.[17] These results show that mutational processes can differ significantly even among related species. While in most tenebrionid satellites single point mutations are major mutational events, the *A. diaperinus* satellite is affected by additional processes like replication slippage, recombination and gene conversion. The result is a quick divergence of *A. diaperinus* satellite repeats and creation of three groups of satellite variants (Table I) with relatively low mutual sequence homologies between 65 and 81%.[17]

The monomer unit of the *T. madens* satellite I is created by duplication of the basic repeat subunit, approx. 100 bp long, probably by the process of unequal crossing-over. The result is a complex monomer sequence composed of two related 70 % homologous halves. In the *T. madens* satellite II monomer unit of 711 bp, built up of basic 100 bp subunits, inversion of the second subunit within the monomer has occurred, probably by recombination. This generates a very long inverted repeat which can form a stable intrastrand dyadic structure (Table I).

The evolution of satellite DNAs is mainly determined by the processes of gene conversion and unequal crossing-over, the result of which is se-

quence homogenization. This means that the satellite variants created by some mutational processes are spread within the satellite, which is termed »homogenization«, and further spread to other individuals in the population, the process called »fixation«. This process of horizontal spreading of a mutation, which is manifested within an individual, is called concerted evolution.[1]

In most satellites, the variants created by particular mutations remain clustered, creating satellite families, often chromosome specific.[38–40] Statistical analyses of long segments of human satellite III also give evidence that the mutations are not randomly positioned but in a correlated fashion with long segments of »intact« repeats and clustered mutations.[41] In contrast to such organization, sequence variants of the *T. molitor* satellite, which are products of single base substitution in a consensus sequence, are completely randomly spread within the satellite DNA and uniformly distributed on all chromosomes.[42] The same type of organization is characteristic of three *A. diaperinus* satellite variants which are also equally and homogeneously distributed in heterochromatic regions of all chromosomes. This means that, in the case of tenebrionid beetles, the process of interchromosomal spreading is faster than the mutation rate and intrachromosomal homogenization, indicating that unequal crossing over and gene conversion occur very frequently among non-homologous chromosomes. The high frequency of these events is probably influenced by the presence of long and uninterrupted stretches of highly homologous satellite DNA on all chromosomes within each tenebrionid species.

CONCLUSIONS

1. In ten analyzed tenebrionid species, satellite DNA is present in large amount, making 20% to 50% of the genomic DNA and is located in pericentromeric heterochromatin. In most species, a single satellite DNA is found, except in *T. obscurus*, *T. madens* and *M. goudoti* where two abundant satellites are present, both located in pericentromeric regions while one of them can also be detected in subtelomeric heterochromatin.

2. According to the size of the basic repeating unit, satellites are classified in two groups: the first with a basic unit of 100–160 bp, and the second with an approximate monomer length of 350 bp. Satellites from three different species have identical monomer lengths of 142 bp, exhibit sequence induced DNA curvature with two bend centers and computer modeling predicts their tertiary structures in the form of left helices of very similar geometry. We propose that such DNA structures code for a protein binding function necessary for the maintenance of heterochromatin conformation and compactness. In the absence of DNA curvature, intrastrand dyadic structures, or short specific DNA sequences, could perform the same function.

3. The methods of *in situ* RE digestions and *in situ* nick translation demonstrate the difference in the heterochromatin higher order structure between congeneric tenebrionid species, which probably depends on the satellite DNA composition and its interaction with heterochromatic proteins.

4. The satellite sequence variability is between 2–5%, mostly due to single nucleotide substitutions. In addition, effects of gene conversion, replication slippage and recombination are evident in some of the satellites, resulting in the appearance of subfamilies. Satellite variants as well as subfamilies are randomly distributed within the whole satellite DNA and uniformly spread on all chromosomes. This indicates a high frequency of interchromosomal gene conversion and unequal crossing over.

5. The analyzed satellites are species specific and can represent a diagnostic characteristic for the given species.

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SAŽETAK

Satelitske DNA vrste iz porodice Tenebrionidae: struktura, organizacija i evolucija

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Heterokromatinsko područje kromosoma sadrži visoko ponavljajuću satelitsku DNA i vrlo mali broj gena a obično je transkripcijski neaktivno. Na osnovi tih svojstava je pretpostavljeno da je heterokromatin beskorisna, otpadna DNA iako su unutar njega mapirane neke nasljedne funkcije. Kukci iz porodice Tenebrionidae (Insecta, Coleoptera) sadrže u genomu znakovitu količinu heterokromatina i satelitskih DNA i zbog toga predstavljaju pogodan sustav za proučavanje njihove strukture i organi-

zacije. U ovom radu iznosimo podatke u svezi primarne strukture i organizacije kao i strukture višeg reda satelitskih DNA iz deset vrsta porodice Tenebrionidae. Također se osvrćemo na mutacijske procese koji utječu na njihovu evoluciju. Korištenjem ovih podataka pokušavamo odrediti sačuvane strukturne elemente unutar satelitskih DNA koji mogu biti važni za održavanje heterokromatinske strukture i gustoće. Ove analize mogu pomoći u utvrđivanju strukturnih dijelova kromosoma odgovornih za neke heterokromatinske uloge kao što su sparivanje kromosoma, funkcija centromere i prianjanje sestrinskih kromatida.