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Characterization and evolutionary dynamics of a complex family of satellite DNA in the leaf beetle *Chrysolina carnifex* (Coleoptera, Chrysomelidae)

Teresa Palomeque*, Martín Muñoz-López, José A. Carrillo & Pedro Lorite
Departamento de Biología Experimental, Área de Genética, Universidad de Jaén, 23071, Jaén, Spain;
Tel: +34-953-212156; Fax: +34-953-211875; E-mail: tpalome@ujaen.es
**Correspondence*

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Abstract

The present study characterizes the complex satellite DNA from the specialized phytophagous beetle species *Chrysolina carnifex*. The satellite DNA is formed by six monomer types, partially homologous but having diverged enough to be separate on the phylogenetic trees, since each monomer type is located on a different branch, having statistically significant bootstrap values. Its analysis suggests a common evolutionary origin of all monomers from the same 211-bp sequence mainly by means of base-substitution mutations evolutionarily fixed to each monomer type and duplications and/or deletions of pre-existing segments in the 211-bp sequence. The analysis of the sequences and Southern hybridizations suggest that the monomers are organized in three types of repeats: monomers (211-bp) and higher-order repeats in the form of dimers (477-bp) or even trimers (633-bp). These repetitive units are not isolated from others, and do not present the pattern characteristic for the regular tandem arrangement of satellite DNA. *In-situ* hybridization with biotinylated probes corresponding to the three types of repeats showed the pericentromeric location of these sequences in all meiotic bivalents, coinciding with the heterochromatic blocks revealed by C-banding, indicating in addition that each type of repeat is neither isolated from others nor located in specific chromosomes but rather that they are intermixed in the heterochromatic regions. The presence of this repetitive DNA in *C. haemoptera*, *C. bankii* and *C. americana* was also tested by Southern analysis. The results show that this satellite DNA sequence is specific to the *C. carnifex* genome but has not been found in three other species of *Chrysolina* occupying similar or different host plants.

Introduction

Satellite DNA (stDNA) is generally non-coding and is composed of tandemly repeated units which are clustered in thousands of copies throughout heterochromatic blocks. Concerted evolution of stDNA sequences usually leads to striking intraspecific similarities but marked interspecific divergences (Smith 1976). However, a low degree of concerted evolution leads to intraspecific divergences throughout units of a repetitive DNA family and may lead to the formation of a particular stDNA variant (Dover 2002). At the nucleotide level, variants show diagnostic mutations. High variability among stDNA repeat units with some genomes containing several subfamilies of stDNA has been described in several beetle species (Pons et al. 2002b, 2003).

Chrysomelidae (leaf beetles) is the second largest family of phytophagous beetles and one of the richest in species number within the order Coleoptera (Strong et al. 1984). The larvae and adults of the *Chrysolina* species are generally specialized phytophagous bee-

tles feeding on plants of eight families (Jolivet 1988) of which Lamiaceae (26% of all known) and Asteraceae (25%) are the most frequently selected. The genus *Chrysolina* is divided into 65 subgenera with more than 400 species having a worldwide distribution, except in South America, the Australian region, and Antarctica (Daccordi 1996). However, stDNA sequences have been studied only in the leaf beetles *Chrysolina americana* and *Xanthogaleruca luteola* (Lorite et al. 2001, 2002).

Phytophagous insects have often been used as a model from which to infer the evolutionary pathways leading to food specialization. The relationship between mitochondrial DNA phylogeny and the evolution of host-plant affiliation in the genus *Chrysolina* (Garin et al. 1999, Gómez-Zurita et al. 1999) and in the related genus *Timarcha* (Gómez-Zurita et al. 2000) has been studied. The relationship between karyological evolution and host-plant affiliation in the genus *Chrysolina* has also been analysed (Petitpierre 1975, 1981, 1999).

In a previous paper, we studied the stDNA from *Chrysolina americana* (Lorite et al. 2001). Hybridization experiments in other *Chrysolina* species showed the absence of this repetitive sequence in *C. bankii*, *C. carnifex* and *C. haemoptera*. We chose these species because they have different karyological characteristics with smaller chromosome numbers in *C. americana* and *C. bankii* (2n = 24 and 23, respectively) and higher chromosome number in the other two species (2n = 40) (Petitpierre 1975, 1981, 1999). In addition, each species is included in different and well-supported clades of the maximum-likelihood tree of the combined 16S and cytochrome oxidase subunit I mitochondrial genes (Garin et al. 1999). In the present paper, we report the cloning, characterization, and chromosomal location of apparently species-specific and complex stDNA from *C. carnifex* and we also test the possible presence of this repetitive sequence in *C. bankii*, *C. americana* and *C. haemoptera*.

Material and methods

Isolation of genomic DNA and cloning of the repetitive DNA

A population of the beetle *Chrysolina carnifex* (Coleoptera, Chrysomelidae) was collected in Seva (Barcelona, Spain). DNA was extracted from male and female adults. Genomic DNA was extracted according to the technique of Heinze et al. (1994). Isolated DNA was digested with restriction endonucleases (REs) according to the recommendations by the supplier, using 4 U/µg DNA. The digested DNA was analysed by electrophoresis on 2% agarose gels.

Repetitive DNA fragments obtained by digestion of genomic DNA with *Hae*III was eluted from the agarose gel and inserted into the pUC18 vector *Sma*I site. A portion of the eluted fragment was digoxygenin-labelled by random priming with the DIG system (Roche) and used as hybridization probes. Recombinants yielding positive hybridization signals were directly sequenced on both strands by the dideoxy sequencing method.

Southern-blot analysis of digested genomic DNA

Samples of 4 µg of genomic DNA were digested with *Hae*III according to the manual of the supplier. Digestion fragments were separated on a 2.5% agarose gel, and then blotted onto Hybond-N+ membranes (Amersham) under standard conditions. Southern hybridization was performed using 20 ng of labelled probe/ml. The probes were digoxygenin-labelled by random priming with the DIG system (Roche). Different probes were used: CCAH-4A (211-bp), CCAH-104 (477-bp) and a mix of CCAH-102 and -131(633-bp). The conditions used in Southern hybridizations were hybridization at 65°C and final wash in 2 × SSC at 65°C. Since the mean GC content for the stDNA of *C. carnifex* is about 37%, these conditions allow hybridization between DNA–DNA duplex sharing approximately 85% sequence homology (McClean 1998, Jolley-Souders 2001). Hybridization was detected with a DIG-detection kit (Roche).

Computer analysis

Multiple-sequence alignment was performed using the CLUSTALW program. Nucleotide diversity and pairwise sequence divergence was estimated using the DnaSP program (Rozas & Rozas 1999). The nucleotide diversity Pi (JC) was calculated as the average number of nucleotide substitutions per site between two sequences with Jukes and Cantor's correction (Jukes & Cantor 1969). Sequence divergence was calculated as the average of nucleotide substitutions per site (Dxy value from DnaSP; Nei

1987, equations 10–20). Distance analysis was conducted with the program MEGA (Kumar et al. 1993). Trees were constructed by the neighbour-joining method (Saitou & Nei 1987) with bootstrap values on 500 replications. The sequence data were analysed and compared with the GenBank/NCBI DNA databases using the BLAST network service and the EMBL database using FASTA (Altschul et al. 1990, 1997, Pearson & Lipman 1998).

Chromosome preparation, DAPI staining, C-banding and fluorescence in-situ *hybridization (FISH)*

Chromosome spreads were made from adult male gonads as described in Juan et al. (1993). Staining of the chromosomes with DAPI fluorochrome was performed with 0.2 μg/ml in McIlvaine's phosphate buffer for 15 min (Sumner 1990).

C-banding was performed as described by Sumner (1972) with some modifications. Slides were treated with 0.2 N HCl at 23°C for 5 min, washed with water and incubated with 5% barium hydroxide at 60°C for 5 min. Then, slides were washed with water and with the 0.2 N HCl solution and incubated in 2 × SSC at 60°C for 1 h. Staining was performed with 10% Giemsa for 10 min.

FISH was performed using the same probes used for the Southern analysis. The probes were labelled with biotin-16-dUTP by nick translation (final concentration of 2 ng/μl, 50% formamide). The temperature used for hybridization was 37°C and post-hybridization washes were performed at 42°C in 50% formamide. These stringency conditions allow hybridization between DNA–DNA duplex sharing approximately 80–85% sequence homology (McClean 1998, Jolley-Souders 2001). Immunological detection was performed using the avidin-FICT/anti-avidin-biotin system with two rounds of amplification (Pinkel et al. 1986). The preparations were counterstained with propidium iodide.

Results

Characterization of the satellite DNA

Digestion of genomic DNA from *Chrysolina carnifex* with *Hae*III revealed the presence of a 200-bp band and other bands of different sizes although these were not exactly ladders (Figure 1). The bands of approximately 200, 400, 500 and 600 bp were cloned and sequenced. The clones obtained were termed CCAH clones (C = *Chrysolina*, CA = *carnifex*, H = *Hae*III).

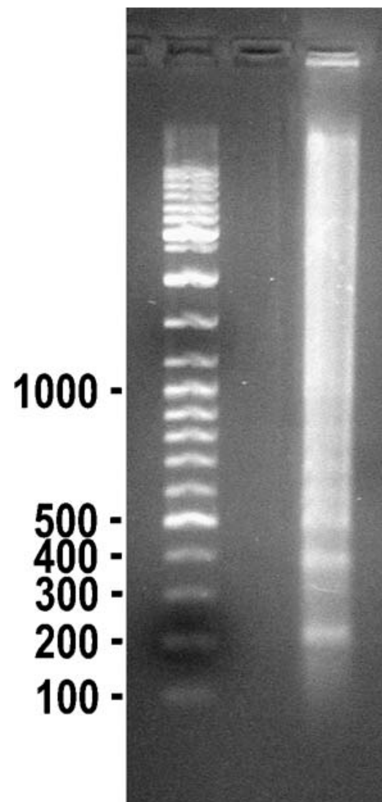

Figure 1. Electrophoretic separation in 2% agarose gel of *Chrysolina carnifex Hae*III restricted genomic DNA. The numbers on the left indicate the size of DNA fragments in bp.

The sequences corresponding to the different clones showed variable sizes as a result of the presence of different *Hae*III targets (Figure 2). The analysis of these sequences also showed great variability and the existence of six types of monomers. Some of these repeats are in the form of single monomers while others are arranged as higher-order repeats in the form of dimers and trimers, as explained in detail below. The comparison of sequencing results is summarized in Figure 2, where the best alignment of repeating units is presented. In Figure 2, the sequences designated 1, 2 etc. are monomeric units from the same clone. The consensus sequence of 211 bp, partially AT-rich (62.4%), is also shown in Figure 2. The sequences described here are not significantly similar

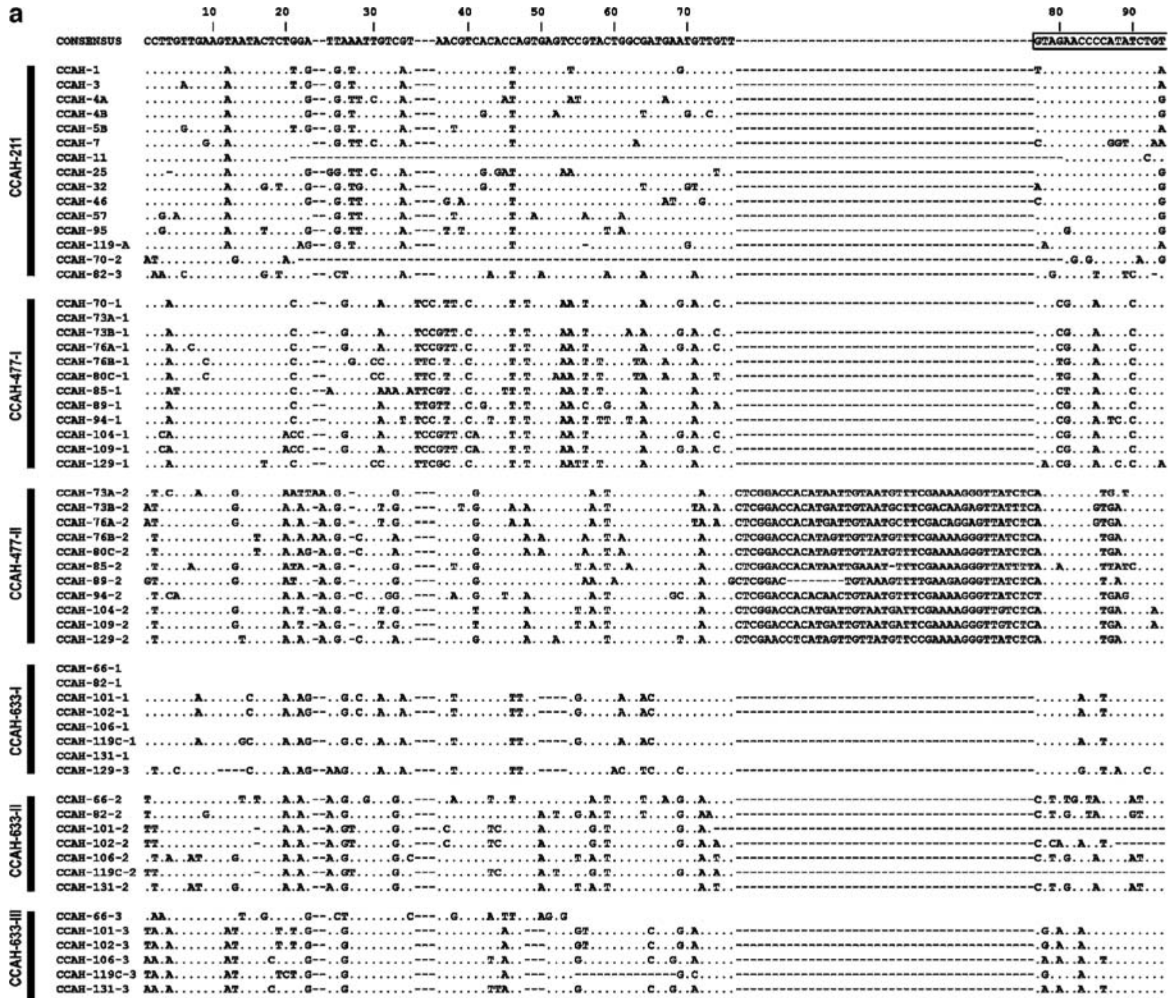

Figure 2. Multiple-sequence alignment of all sequenced clones from *Chrysolina carnifex* stDNA. The sequences designated 1, 2 etc. are monomeric units from the same clone. Monomer consensus sequence derived from them is also shown (consensus). The different monomer types are also shown. In the consensus sequence, the regions with homology to the 11- and 41-bp insertions are indicated by boxes.

to previously described sequences deposited in databases.

The phylogenetic tree constructed by the neighbour-joining method showed a topology in accordance with the aforementioned data. The six different types of monomers analysed are on different branches statistically supported by significant bootstrap values (Figure 3).

The first type of repeat is organized as single monomers. It is called the CCAH-211-bp type (Figure 2, upper panel). It includes the 13 clones sequenced from the 200-bp band (CCAH-1, -3, -4A, -4B, -5B, -7, -11, -25, -32, -46, -57, -95 and -119A) (GenBank accession nos. AJ937709–AJ937720, AM071489). All these clones are formed by a monomer of 211 bp, except the CCAH-25 and CCAH-119A monomers, which present a deletion of one nucleotide and CCAH-11 with one deletion of 60 nucleotides. The CCAH-70-2 and CCAH-82-3 monomers, from the CCAH-70 and CCAH-80 clones, respectively, are also included in this group. These two monomers are included in sequenced fragments larger than 200 bp. The nucleotide diversity (Pi) of the CCAH-211-bp monomer type is 0.20 ± 0.02. The nucleotide divergence (Dxy) between the CCAH-211-bp monomer type and the five others considered in this work is clearly greater than its

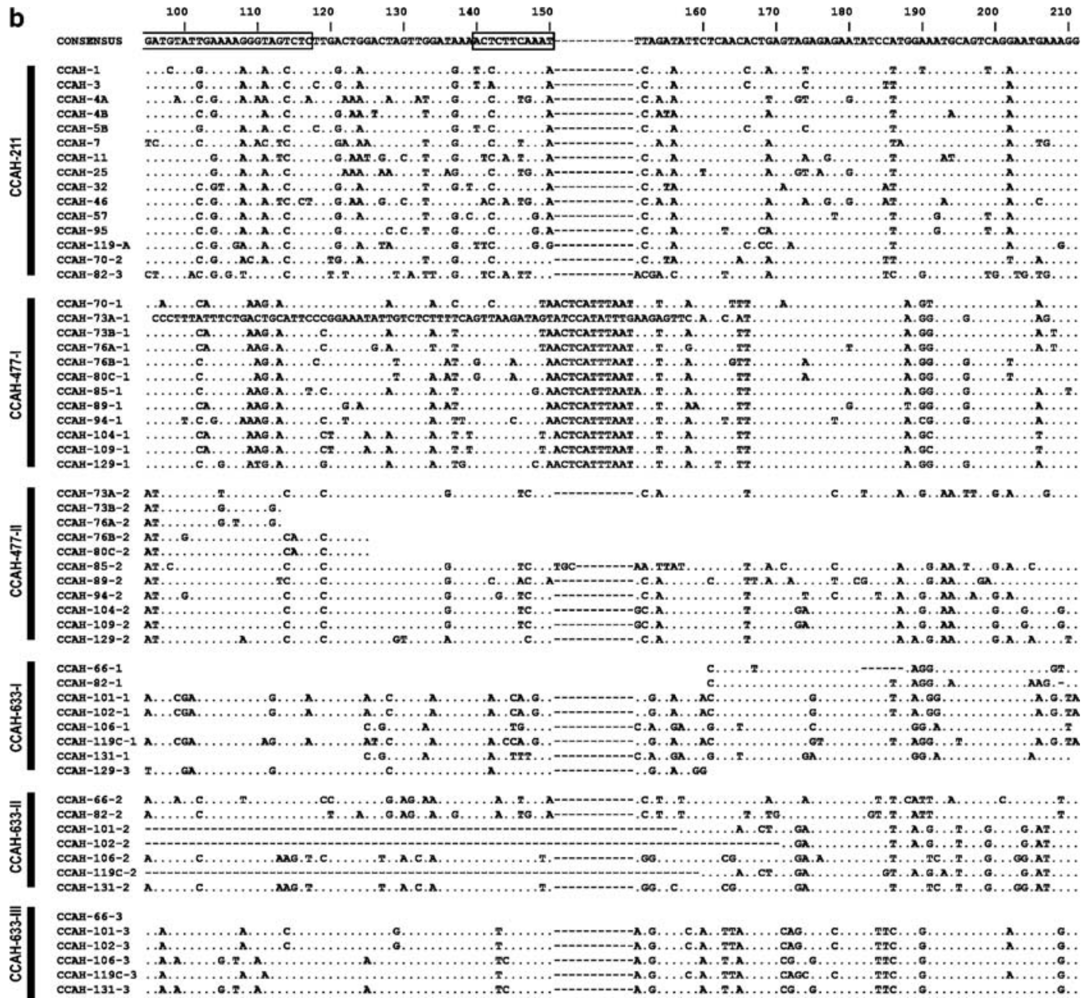

Figure 2. Continued.

corresponding nucleotide diversity (Table 1). In the phylogenetic tree, these sequences are on a branch statistically supported by significant bootstrap values (Figure 3).

The analyses of the fragments larger than 200 bp reveal certain differences in relation to the sequences analysed from the 200-bp band, showing the existence of higher-order repeats. The second type of repeat (dimer type, CCAH-477-bp) is represented by 12 clones (CCAH-70, -73A, -73B, -76A, -76B, -80C, -85, -89, -94, -104, -109 and -129) (GenBank accession nos. AJ937721–AJ937732) that are partial or complete dimeric repeats (Figure 2, middle panel). The incomplete dimers originated from a mutation that generates the presence of a new *Hae*III target in the second monomer. As an exception, clone CCAH-129 is made up of three monomer units, although one is incomplete. The 5′-end of clone CCAH-73A possesses a 74-bp sequence that shows no significant similarity to the cloned repetitive DNA. This fact suggests that the 74-bp sequence is unrelated flanking DNA. The monomers of these 12 clones could be grouped as two types, except for the CCAH-70-2 and CCAH-129-3 monomers. The first type could be formed by the first monomers of each dimer, and the second group by the second ones. Both groups are based on the differences in their sequences. The clearest difference is the existence of different insertions in each type of repeat. As an exception, the CCAH-70-2 and CCAH-129-3 monomers show no

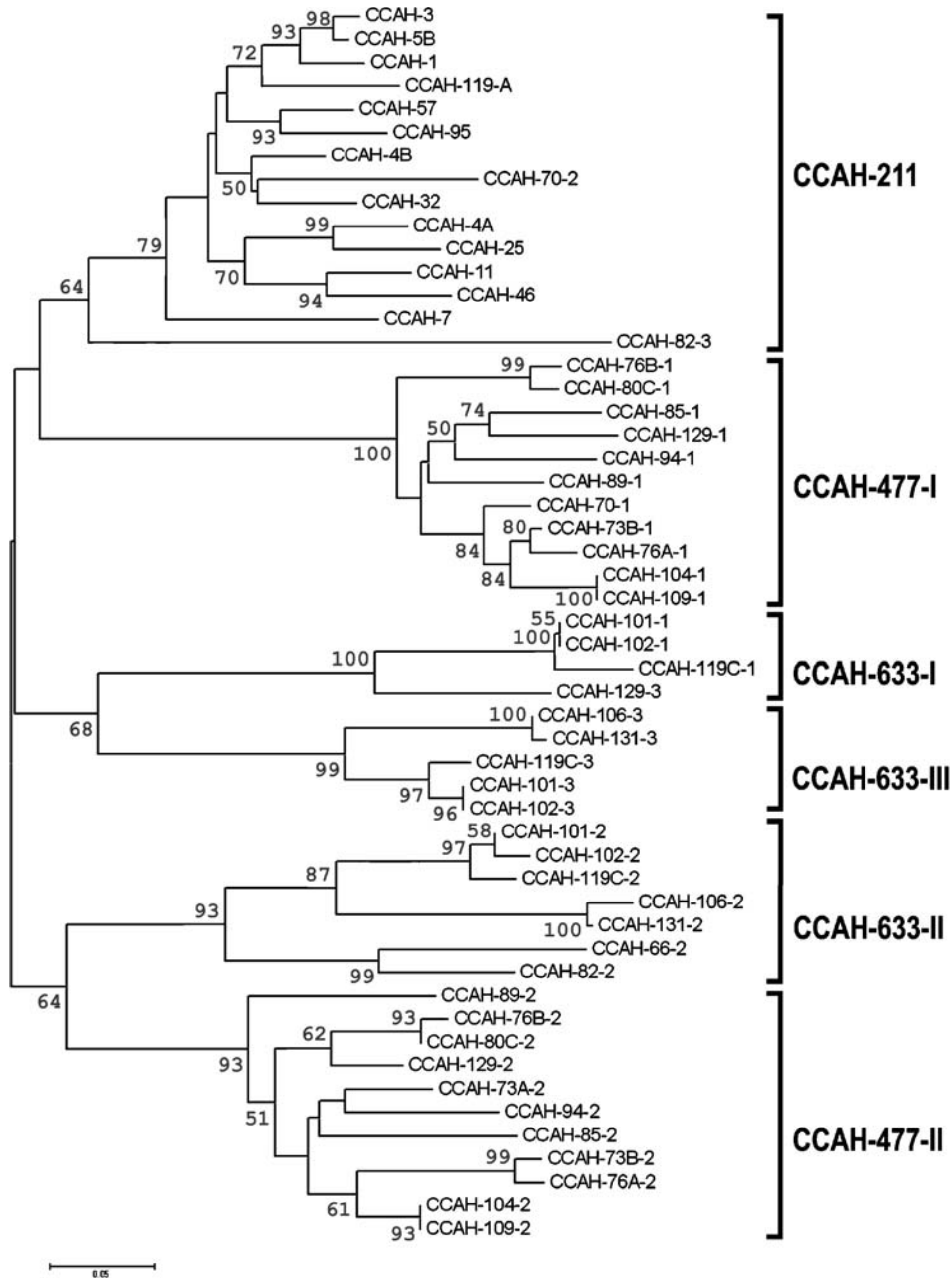

Figure 3. Dendrogram showing neighbour-joining tree of monomers from *Chrysolina carnifex* stDNA. The bar represents genetic distance d = 0.05 of pairwise comparisons calculated according to Kimura's 'Two-Parameter Method' (Kimura 1980). The numbers above and below the branches indicate bootstrap values for 500 replicates.

type of insertion. The first type of monomer shows two insertions, one of 3 bp and another of 11bp. The insertion of 3 bp (36-nt position) is mainly TCC although, in some monomers, the last two bases change. The insertion of 11 bp (151-nt position) shows 100% identity in all of these monomers. The second monomer type presents a 41-bp insertion (77-nt position), averaging 3.8 nucleotide differences. In addition, some monomers show insertions and/or deletions of few nucleotides. The nucleotide diver-

sity (Pi) in the first group of monomers is 0.13 ± 0.01 and in the second one 0.15 ± 0.01. The nucleotide divergence between the two groups is clearly greater, Dxy = 0.60 ± 0.07. In addition to these differences, there are 27 fixed nucleotide differences between the two groups. The organizations of these dimers indicate that they could constitute a higher-order repeat unit of 477 bp, generated throughout the CCAH-211-bp sequences. We call these repeats CCAH-477-bp, formed by two different types of monomers: CCAH-477-I (the first monomers of the 477-bp sequences) and CCAH-477-II (the second monomers). In the phylogenetic tree, each type of monomer is also on a branch that is statistically supported by significant bootstrap values (Figure 3).

The insertions of greater size, characteristic of the CCAH-477-I and 477-II monomer types, could originate from duplication. The sequence consensus with 211 bp shows a region (position 140–150 nt) with 72.73% homology to the 11-bp insertion characteristic of the CCAH-477-I monomer type (Figure 2). This homology was found to be greater in some monomers, such as CCAH-73A-2, CCAH-85-2, CCAH-104-2 and CCAH-109-2, in which the homology with the 11-bp insertion was 81.8%. The sequence consensus also showed a region (position 78–117 nt) with 73.2% homology with the 41-bp insertion characteristic of the CCAH-477-II monomer type (Figure 2). This homology was also greater in such monomers as CCAH-82-2, in which the homology with the 41-pb insertion was 84.4%. A duplication process could also give rise to the small insertion of three pairs of bases characteristic of the CCAH-477-I monomer type.

The third type of repeat (trimer type, CCAH-633-bp) is represented by seven clones that were partial trimeric repeats (CCAH-66, -82, -106 and -131) or complete trimeric repeat (CCAH-101, -102 and -119C) (GenBank accession nos. AJ937733–AJ937737, AM071490 and AM071491). In the partial trimeric repeats, a new target for *Hae*III was generated in the first monomer (Figure 2, lower panel). We call these repeats CCAH-633-bp, formed by three types of monomer: CCAH-663-I (first monomers), CCAH-633-II (second monomers) and CCAH-633-III (third monomers). The nucleotide diversities (Pi) from CCAH-633-I, CCAH-633-II and CCAH-633-III (0.10 ± 0.04, 0.19 ± 0.03 and 0.09 ± 0.02, respectively) were smaller than the nucleotide divergences between the groups, and there were also fixed nucleotide differences (Table 1). As an exception, the CCAH-82-3 monomer could not be included in any of these three monomer types. The phylogenetic tree sample to CCAH-82-3 monomer on the same branch as the CCAH-211 monomer type and with a significant bootstrap value (Figure 3). In addition, the nucleotide distance (calculated using Kimura's two-parameter method; Kimura 1980) between the CCAH-82-3 monomer and that included in CCAH-211 monomer type was smaller than between CCAH-82-3 and the monomers included in the CCAH-633-I, 633-II and 633-III monomer types (data not shown).

The phylogenetic tree sample to CCAH-129-3 monomer including in the branch corresponding to the CCAH-633-I monomer type. The CCAH-129-3 monomer does not have the insertion characteristic of the CCAH-477-I and 477-II types of monomers. In addition, the nucleotide distance between CCAH-633-I and CCAH-129-3 was smaller than between CCAH-129-3 and the monomers included in the CCAH-477-I and 477-II monomer types (data no shown). For similar reasons, the CCAH-70-2 monomer is included in the CCAH-211 monomer type (Figure 2).

Organization of the satellite DNA and analysis of their presence among related species

The sequencing results of stDNA of *Chrysolina carnifex* appear to indicate the existence of mono-

Table 1. Nucleotide diversity in each monomer type (diagonal) and pairwise divergence values between monomer types.

	CCAH-211	CCAH-477-I	CCAH-477-II	CCAH-633-I	CCAH-633-II	CCAH-633-III
CCAH-211	0.20 ± 0.02					
CCAH-477-I	0.39 ± 0.05 (13)	0.13 ± 0.01				
CCAH-477-II	0.44 ± 0.05 (4)	0.60 ± 0.07 (27)	0.15 ± 0.01			
CCAH-633-I	0.53 ± 0.10 (8)	0.56 ± 0.11 (37)	0.43 ± 0.09 (16)	0.10 ± 0.04		
CCAH-633-II	0.37 ± 0.06 (2)	0.51 ± 0.08 (16)	0.28 ± 0.05 (4)	0.48 ± 0.12 (12)	0.19 ± 0.03	
CCAH-633-III	0.41 ± 0.07 (11)	0.44 ± 0.08 (37)	0.46 ± 0.08 (18)	0.39 ± 0.11 (24)	0.49 ± 0.11 (18)	0.09 ± 0.02

The number of fixed nucleotide differences is indicated in brackets.

mers and defined higher-order repeats in the form of dimers (CCAH-477-bp) or even trimers (CCAH-633-bp). This complex stDNA would therefore be formed by the repetition of these higher-order repeats which would be isolated from each other. However, the existence of different monomer types in a same clone could indicate that the different types of repeats are intermixed. For example, clone CCAH-129 includes a monomer of the CCAH-477-I type, another one of the CCAH-477-II type and third of CCAH-663-I type, as indicated above. Something similar happens with the clones CCAH-70 and CCAH-80.

For the determination of the organizational patterns of the different monomer types and their mutual arrangement, two analyses were made: cloning and sequencing of DNA fragments of high molecular weight and Southern hybridizations using as a probe each type of repeat.

The CCAH-213 (898-bp), CCAH-223 (986-bp) and CCAH-234 (976-bp) clones were sequenced (GenBank accession nos. AM071492–AM071494). Their analysis revealed that all of them contain the three different types of repeats (Figure 4). CCAH-213 is a tetramer repeat that includes a CCAH-477-I; and a CCAH-477-II monomer types (477-bp dimer sequences), a CCAH-633-II monomer type (monomer including 633-bp trimer sequences), and a CCAH-211 monomer type (211-bp monomer sequences). The CCAH-223 clone is a pentamer repeat formed by CCAH-477-I and -477-II monomer types (477-bp dimer sequences), CCAH-633-I and -II monomer types (first and second monomers of the 633-bp trimer sequences) and a CCAH-211 monomer type (211-bp monomer sequences). Finally, the CCAH-234 clone, also a pentamer repeat, is composed of a CCAH-477-I monomer type (monomer including 447-bp dimer sequences), a CCAH-211 monomer type (211-bp monomer sequences), and CCAH-633-I, -II and -III monomer types (633-bp trimer sequences).

Southern hybridizations using each type of repeat were performed using as probe the CCAH-4A monomer repeat (CCAH-211-bp), the CCAH-104 dimer repeat (CCAH-477-bp) and the CCAH-102 and CCAH-131 trimer repeats (CCAH-633-bp). The two CCAH-102 and CCAH-131 repeats have been used together as a probe because all sequenced fragments with sizes about 633-bp have deletions of larger or smaller size. The results of the Southern hybridizations show that the typical ladder of repetitive DNA is not observed with any of the three probes (Figure 5). These hybridizations gave different results with each of the probes used. Although definite hybridization bands are visible with each probe, the results altogether appear to indicate the absence of the pattern characteristic for the regular tandem arrangement. Nevertheless, the fact that defined hybridization bands with about 211 bp, 477 bp and 633 bp are found using as probes the monomer, dimer and trimer repeats, respectively, suggests the existence of monomer units and defined higher-order repeats, as also suggested by the sequencing results presented for stDNA.

Southern hybridization experiments were performed to test the possible existence of a repetitive sequence similar to that cloned in *Chrysolina carnifex* in other species of the genus *Chrysolina*. Genomic DNA from *C. bankii*, *C. americana* and

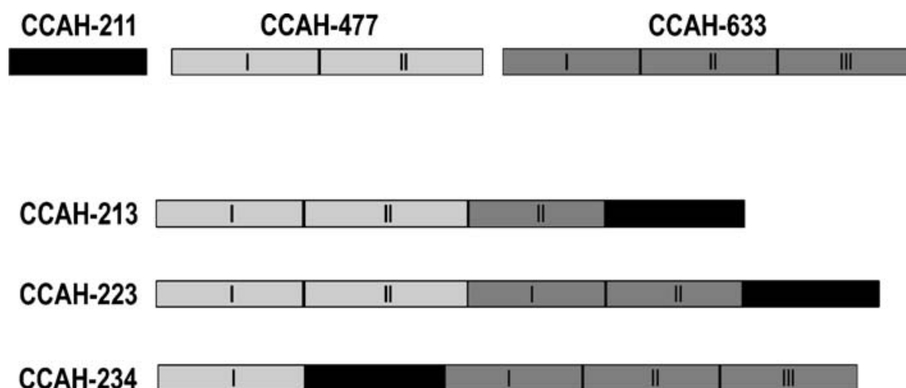

Figure 4. Schematic representation of three types of repeats found, monomers (211-bp) and defined higher-order repeats in the form of dimer (477-bp) and trimer (633-bp). Schematic representation of the organization of the CCAH-213, -223 and -234 clones.

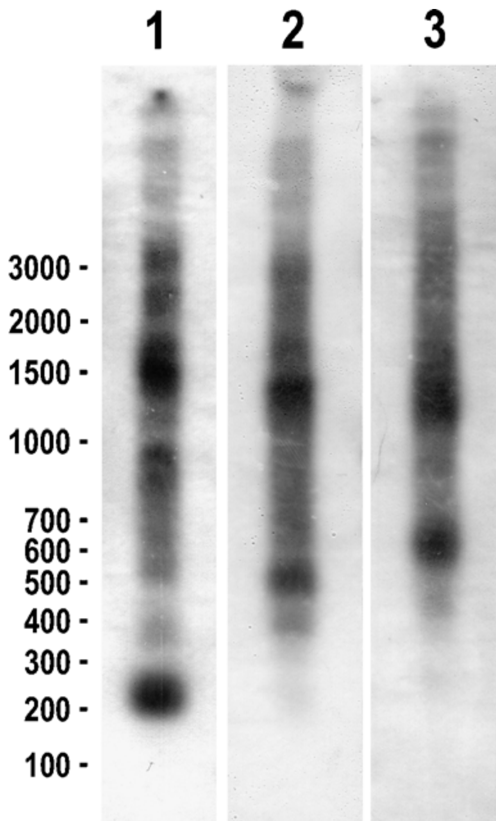

Figure 5. Southern-blot of *Hae*III restricted genomic DNA, fractionated in 2.5% agarose gel, using a probe CCAH-4A monomer repeat (CCAH-211-bp) (lane 1), a CCAH-104 dimer repeat (CCAH-477-bp) (lane 2) and CCAH-102 and CCAH-131 trimer repeats (CCAH-633-bp) (lane 3).The numbers on the left indicate the size of DNA fragments in bp.

C. haemoptera was cleaved with *Hae*III enzyme. The three types of repeats were used as probe and different stringency conditions were used. Interspecific sequence similarities were not detected between these species of *Chrysolina* by Southern analysis (data not shown).

DAPI staining, C-banding and FISH

Chrysolina carnifex has a diploid karyotype of 40 chromosomes, consisting of medium-sized acrocentric chromosomes, save a small Y-chromosome. This species shows 19 autosomal bivalents and the typical parachute-like system of Coleoptera male sex chromosomes, (Xy_p) (Petitpierre 1975, 1983).

Figure 6 shows male meiotic bivalents in metaphase I of *C. carnifex* uniformly stained with DAPI (Figure 6a) and the same metaphase I after C-banding (Figure 6b). C-banding procedure revealed the existence of large constitutive heterochomatic blocks in the pericentromeric regions of all autosomal bivalents. Also heterochromatic blocks are visible in the sexual pairs, in the X chromosome as well as in the minute Y chromosome.

Fluorescence *in-situ* hybridization (FISH) techniques were carried out under stringency conditions similar to those used in the Southern hybridizations described. FISH with a biotinylated CCAH-4A (monomer repeat), with CCAH-104 (dimer repeat) and a mix of CCAH-102 and -131 (trimer repeat) as probes showed the location of the three types of repeats in all meiotic bivalents (Figure 6c, d & e, respectively). In metaphase I, where centromeres were co-orientated, hybridization signals are visible in pericentromeric regions. These results indicate that all types of repeats are located in the heterochromatic regions of all chromosomes and not isolated one from the other. In chromosomes that can be easily differentiated as the sexual bivalents a similar hybridization pattern can be observed when FISH is carried out with the three types of repeats (Figure 6f).

Discussion

The digestion of *Chrysolina carnifex* genomic DNA with *Hae*III has allowed the isolation and characterization of a very complex stDNA family. The stDNA has a consensus sequence of 211 bp that is partially AT-rich. Their AT richness (62.4%) is slightly lower when compared with the stDNA from other beetles, such as Tenebrionids, which have an AT content ranging from 64% to 74% (Ugarković et al. 1995), but similar to the AT content found in other leaf beetles, such as *Chrysolina americana* and *Xanthogaleruca luteola* (Lorite et al. 2001, 2002).

This stDNA showed great variability and the existence of six types of related monomers clearly separated on phylogenetic tree. Sequence analysis suggests a common evolutionary origin of all monomer from the same 211-bp sequence mainly by means of base-substitution mutations evolutionarily fixed to each monomer type and duplications and/or deletions of pre-existing segments in the 211-bp sequence. Analysis of the sequences and Southern hybridization results suggests that the monomers are organized

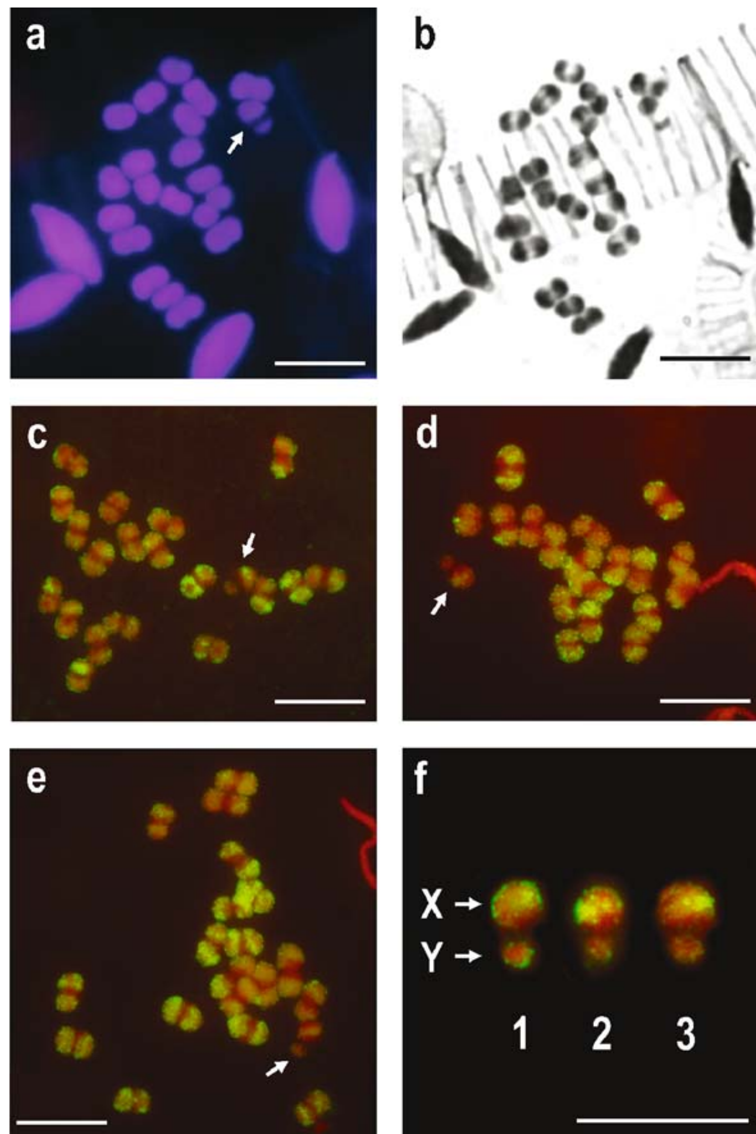

Figure 6. Male meiotic bivalents in metaphase I of *Chrysolina carnifex*stained with DAPI (**a**) and subsequently C-banded (**b**), showing the presence of large heterochromatic blocks in pericentromeric regions of all chromosomes. *In-situ* hybridization using the CCAH-4A repeats (**c**) the CCAH-104 repeats (**d**) and CCAH-102 and CCAH-131 repeats (**e**) as probes. The hybridization pattern shows the presence of these sequences in the pericentromeric regions of all bivalents (green-yellow). Chromosomes were counterstained with propidium iodide (red). (**f**) Selected sexual bivalents (Xyp) showing the *in-situ* hybridization with the three probes; CCAH-4A monomer repeat (CCAH-211-bp) (1), a CCAH-104 dimer repeat (CCAH-477-bp) (2) and CCAH-102 and CCAH-131 trimer repeats (CCAH-633-bp) (3). Arrows indicate the sexual bivalent. Bar = 10 μm.

into three types of repeats: monomers (211-bp) and higher-order repeats in the form of dimers (477-pb) or even trimers (633-pb).

It has been considered that repetitive sequences evolve by means of concerted evolution (reviewed in Ugarkovic & Plohl 2002). If the monomer is also the unit that undergoes concerted evolution, then dimers (two adjacent monomers) should randomly link monomers bearing different mutations, as has been described for example in the darkling beetle *Tenebrio molitor* (Plohl et al. 1992) and in most insect species. However, in other insects, such as the cave beetle *Pholeuon proserpinae glaciale*, two monomer types have been observed, type A and B; the dimer is

invariably composed of one monomer type A and one type B (Pons et al. 2003). Hence, this variation pattern suggests that the dimer is the repetitive unit undergoing concerted evolution.

In other organisms, stDNAs with a complex organization have also been described. Theoretical simulations on short tandem repeats indicate that the low ratios of unequal crossing over to mutation, common in heterochromatic regions, tend to form longer and complex repetitive units (Stephan & Cho 1994). For instance, in human chromosome 7, there are two higher-order repeats (HORs) based on divergent subfamilies of the ~171-bp alphoid monomer: a 6-monomer HOR and a dimer (Willard & Wayne 1987, Rudd & Willard 2004). These HORs show a certain sequence identity but, within a HOR, the monomers show substantial sequence divergence because they are composed of divergent subfamilies.

In stDNA from *Chrysolina carnifex*, the divergences between sequences of two different types of monomers are higher than their respective nucleotide diversities (within sequences of the same type of monomers). In addition, there are fixed nucleotide differences between the different monomer types. Both findings could indicate the beginning of a differentiation towards subfamilies or even families.

The stDNA from *C. carnifex* shows a variability and complexity much greater than does the stDNA from other *Chrysolina* species, such as *C. americana* (Lorite et al. 2001). It is not clear why some stDNA sequences have remained conserved for such long evolutionary periods while others have undergone dynamic nucleotide changes. This contrasting behaviour has been reported even between closely related species. The evolutionary dynamics of the PIM357 stDNA sequence from the Coleoptera genus *Pimelia* differ in species endemic to the Canary Islands with respect to species of the Iberian Peninsula (Pons et al. 1997, 2002a). In the stDNA from Iberian *Pimelia* species, the divergence (between sequences of two different species) is higher than the respective nucleotide diversity (within sequences of the same species). This does not apply to the stDNA of endemic Canary Islands species in which the nucleotide diversity is also higher than in Iberian species. These facts point to weaker homogenization processes in the stDNA from Canary Islands *Pimelia*. In one species endemic to the Canary Islands, *Pimelia radula*, the PIM357 stDNA has diverged, resulting in four different subfamilies arranged in different repeat units; two as monomers and two as dimers (Pons et al. 2002b). One of these repeat types is located only in three autosomal pairs. This compartmentalization could not totally explain this sequence differentiation since the other subfamilies are homogeneously dispersed in the heterochromatic regions of all chromosomes.

The results obtained in *C. carnifex* are different since all the data obtained in this study suggest that the different repetitive units are not isolated from others. This hypothesis is supported by the existence of different monomer types in the same clone and especially by the results of the study of DNA fragments of high molecular weight, which clearly suggest that the three types of repeats, monomers (211-bp), dimers (477-bp) and trimers (633-pb), are found together in the genome. In addition, and also in support of this hypothesis, no compartmentalization was discerned in the chromosomal distribution of each type of repeat in the genome of *C. carnifex*, as shown below.

Chrysolina carnifex has a diploid karyotype of 40 chromosomes. This species presents the most characteristic male sex-determining system of Coleoptera, the parachute-type Xy_p, an achiasmatic association of a generally metacentric X and a minute and mostly metacentric Y chromosome (Petitpierre 1975, 1983). These are held together from mid-prophase to the end of metaphase in the first meiotic division by the nucleolus and, at least in some cases, also by telomeric heterochromatin (Smith & Virkki 1978, Virkki 1984). All the species studied in the present work show this system except *C. bankii*, with an X0 system, the second most common male sex-determining system in Coleoptera. The X0 system could arise either from a loss of the Y chromosome or more often by its transfer to the autosomes (Smith & Virkki 1978, Virkki 1984).

C-banding selectively revealed the constitutive heterochromatin, generally enriched in highly repetitive DNAs such as the stDNA (Sumner 1972). The cytogenetic studies in chrysomelids are numerous, but information from C-banding analysis is very fragmentary. The analysed species present pericentromeric heterochromatin in most chromosomes and intercalary heterochromatin of variable amounts and locations in the sex chromosomes (Rożek et al. 2004). In *C. carnifex,* C-banding showed the existence of large pericentromeric heterochromatic blocks on the autosomes and on the X chromosome.

Also, a heterochromatic region is visible on the Y chromosome. The correspondence between stDNA location and pericentromeric heterochromatic blocks visible after the C-banding procedure have also been found in other beetles, such as Tenebrionid (Ugarković et al. 1995, Pons et al. 2002b). The FISH results, using each type of repeat as a probe, demonstrated that the stDNA is located in the heterochromatic regions of all chromosomes, indicating in addition that all types of repeats are not isolated one from the other. Satellite-DNA location in other Chrysomelid beetles also shows hybridization signals located in the pericentromeric regions (Lorite et al. 2001, 2002).

In summary, the data presented suggest that this complex stDNA is formed by six monomer types, partially homologous but divergent enough to be separate on the phylogenetic trees, since each monomer type is located on a different branch having statistically significant bootstrap values. The monomers are organized in monomer repeats and in definite higher-order repeats in the form of dimers or even trimers. Nevertheless, these repetitive units are not isolated from others and do not present the pattern characteristic for the regular tandem arrangement of stDNA.

In the genus *Chrysolina,* a correlation between the increase in the chromosome number and the adaptation to host plants has been suggested. The host-plant preferences fit well with the karyological data, since all 23- or 24-chromosome species feed on Lamiaceae while the species with higher chromosome numbers are associated with plants belonging to other families, such as Asteraceae (Petitpierre 1975, 1983). However, the data of the mitochondrial DNA phylogenies suggest the absence of parallel evolution of beetles and their host plants (Garin et al. 1999). The species studied here have 23–24 chromosomes (*C. bankii* and *C. americana,* respectively) or higher chromosome numbers (2n = 40 in *C. carnifex* and *C. haemoptera*). In addition, each of these species is included in different and well-supported clades of the maximum-likelihood tree of the combined 16S and cytochrome oxidase subunit I mitochondrial genes (Garin et al. 1999). Lorite et al. (2001) have studied the stDNA of *C. americana,* showing that it is specifically from this species. Similarly, the results presented here also demonstrate the existence of stDNA in *Chrysolina carnifex* and its absence in other species of the genus, even in such species as *C. haemoptera,* with similar chromosome numbers and similar host-plant selection. Further studies will provide new data on the utility of stDNA as a molecular marker for phylogenetic analysis as well as on its possible value for understanding the evolution of ecological associations in herbivorous insects.

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