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Sequence and Analysis of the Mitochondrial DNA Control Region in the Sugarcane Borer *Diatraea saccharalis* (Lepidoptera: Crambidae)

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ABSTRACT

This study aimed at the sequence and analysis of the mtDNA control region (CR) of the Diatraea saccharalis*. The genome PCR amplification was performed using the complementary primers to the flanking regions of* Bombyx mori *CR mitochondrial segment. The sequencing revealed that the amplified product was 568 bp long, which was smaller than that observed for* B. mori *(725 bp). Within the amplified segment, a sequence with 338 nucleotides was identified as the control region, which displayed a high AT content (93.5%). The* D. saccharalis *mtDNA CR multiple sequence alignment analysis showed that this region had high similarity with the Lepidoptera Cydia pomonella.*

Key words: *mtDNA Control Region; Diatraea saccharalis; Lepidoptera; sugarcane borer*

INTRODUCTION

A group of several Lepidoptera, primarily Noctuidae, Pyralidae and Crambidae, are the key pests in most of the world's sugar industries. The group includes the species that have a long evolutionary association with *Saccharum ssp*, as well as the species that have been spread by the humans. There are also many species that have only recently adapted to the feeding on the cultivated sugarcane (e.g. *Diatraea ssp;* Lange et al., 2004). Originally from the Asian Southeast, the sugarcane (*Saccharum ssp.*) is a monocot plant widely spread and economically important in many regions around the world. Thus, in the countries where the sugarcane culture is economically important, the pest, *Diatraea saccharalis* (Crambidae), is a target of the studies involving the biological control; moreover, this insect also attacks several other crops in the Gramineae family, including *Zea mays* L.; *Oryza sativa* L. and *Sorghum bicolor* L. (Reagan and Flynn, 1986).

The sugarcane borer larvae damage the plant in several ways reducing the total cane biomass, as well as the sugar quantity and quality. They build internal galleries in the sugarcane plants causing direct damages, resulting in the apical bud death, weight loss and atrophy. They also cause indirect damages such as the contamination by the yeasts that produce red rot in the stalks, resulting in yield loss in both sugar and alcohol (Macedo and Botelho, 1988). The genetic background of *D. saccharalis* is still largely unknown. Lange et al.,

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(2004) have reported the partial mitochondrial Cytochrome C Oxidase II gene and 16S rRNA gene sequences of six populations of *D. saccharalis.* These results have shown that the strains could be divided into two groups: Mexico/South America, and Caribbean/Southern USA. The differences could reflect two dispersals, one to the north and east, and one to the south that came from an original evolution on the grasses, perhaps the wild ancestor of the maize, in the southern Mexico. The molecular characterization of this insect is important for the development of different analyses such as the phylogenetic studies. Concerning the assessment of intra and interspecific variations, the analysis of the mitochondrial (mt) noncoding segment called Control Region (CR) has proven to be a powerful tool due to the high variability than other mitochondrial genome regions (Harrison, 1989; Mirol et al., 2002).

The mitochondrial genome of several insect species has been sequenced (http://amiga.cbmeg.unicamp.br). The complete sequence of mtDNA is known for some insect species such as *Drosophila yakuba* (Clary and Wolstenholme, 1985), *Apis mellifera* (Crozier and Crozier, 1993), *Anopheles quadrimaculatus* (Mitchell et al., 1993), *Anopheles gambiae* (Beard et al., 1993), *Cochliomyia hominivorax* (Lessinger et al., 2000), *Bombyx mori* (Lee et al., 2000) and *Bombyx mandarina* (Yukuhiro et al., 2002). The mtDNA CR, called D-loop in the vertebrates has been object of numerous functional studies, which have identified the transcription initiation sites for each strand and the main origin of replication (Clayton, 1982; Chang and Clayton, 1984). Several regulatory sequences have been identified in the CR of the vertebrate species and shown that this region contains H-strand origin, H-strand promoter, mitochondrial Transcription Factor I (mtTFI) binding site, besides and conserved sequence block that are involved in the replication and transcription of mtDNA (Han et al., 2003). The regulatory sequences involved in the initiation have not been identified in the invertebrates and the role of the CR in the replication initiation process is poorly understood (Saito et al., 2005).

The present study aimed at the sequencing and analysis of the mtDNA control region of *Diatraea saccharalis.* The genome PCR amplification was performed using the complementary primers to the mtDNA CR segment flanking regions of *Bombyx mori*.

MATERIALS AND METHODS

The Lepidoptera *D. saccharalis* was reared at 22ºC and treated with the artificial diet (Hensley and Hammond, 1968). The silk glands of 5º larval instars were dissected under Zeiss stereomicroscopy and stored in Eppendorf tubes at -20ºC in isopropyl alcohol. The DNA was extracted as described by Monesi et al., (1998). The PCR amplification was performed using the primers based in the regions that flanked the mtDNA control region segment of *Bombyx mori* (GenBank AF149768) with the forward primer (5'ATAACCGCAACTGCTGGCAC 3') on the 12S rRNA gene and reverse primer (5' TTGAGGTATGAGCCCAAAAGC 3') on the $tRNA^{Met}$ gene (Fig. 1). The set of the primers was constructed using the FAST-PCR software (version 3.5.30 by Ruslan Kalendar). The reaction was carried out in a 15 µl volume containing 40 ng of the template DNA, 12.5 mM of each primer, 2.5 mM of each dNTP, 1X PCR buffer, 1U of *Taq* DNA Polymerase (Invitrogen) and 0.6 mM of $MgCl_2$. The amplification cycle consisted of an initial denaturation step at 95°C for 10 min, followed by 35 cycles at 94°C for 30s; annealing at 58°C for 40s; extension at 72°C for 1 min and a final 10 min extension step at 72°C using a Mastercycler gradient (Eppendorf). The fragment was separated on 1.5% agarose gels and the amplified product, with approximately 570 bp long, was cloned into pDrive plasmid (PCR Cloning Kit Qiagen). Two positive clones, pDsCR1 and pDsCR2 were purified using the CTAB method (Del Sal et al., 1998). The sequencing of both strands was performed using DyEnamic ET Dye Terminator Kit (Amersham Bioscience) in automated DNA sequencer MegaBACE 1000 equipment with forward and reverse M13 vector primers. The consensus sequence, which matched for the two clones, was obtained from the forward and reverse sequences aligned with the BioEdit (Hall, 1999). The sequence reported in this paper has the following GenBank accession number AY818307. The BLASTN version 2.0.8 (Altschul et al., 1997) was used to identify the similar sequences in the database. The mtDNA CR nucleotide sequence was aligned using the ClustalW software (Thompson et al., 1994) set to default parameters, on the EMBL-EBI website.

RESULTS AND DISCUSSION

The mtDNA CR is particularly difficult to characterize because of its variable sequence and high AT contents, which tend to reduce the number of efficient annealing sites (Junqueira et al., 2004). The presence of a potential origin of replication or regulatory elements, long poly-A and poly-T stretches, fast-evolving primary sequences and structurally instable elements, such as the multiple repeats and sequences able to form the secondary structures, may increase the technical and methodological difficulties related to access the mtDNA CR sequence data (Azeredo-Espin and Lessinger, 2006).

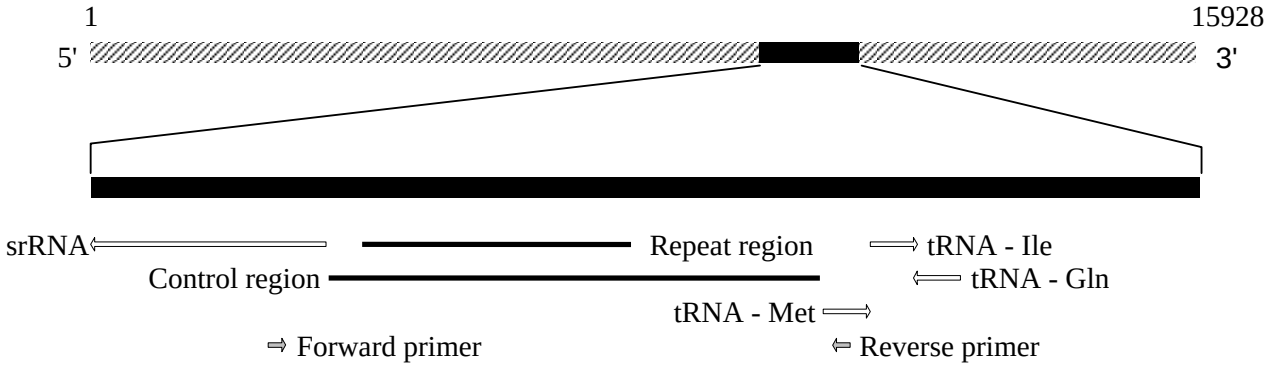

Figure 1 - *Bombyx mori* mtDNA genome map with 15928 base pairs (AF149768), the Control Region (CR) and the flanking sequences. The grey arrows show the localization of the forward and reverse primers constructed in this work.

The amplification and sequencing using the primers developed for *B. mori* were functional and efficient to amplify the control region of the *D. saccharalis* (Fig. 1). The mtDNA CR of *B. mori* was flanked by srRNA gene and $tRNA^{Met}$. The similar position on *D. saccharalis* mtDNA genome was essential for the amplification success and primers based on Bombycidae were transferable and reliable to amplify the Crambidae family. The *D. saccharalis* amplification product was ~ 570 bp long, which was smaller than that observed for *B. mori* (725 bp).

The sequenced fragment from *D. saccharalis* had 568 bases pairs (AY818307) and the mtDNA CR was identified as composed by 338 bp. In relation to the bases composition, the sequence for *D. saccharalis* mtDNA CR presented 93.5% A+T nucleotides (A = 42.4%, C=4.7%, G = 1.8%, T = 51.1%). This region is called A+T rich since it presents between 84-96% of these nucleotides in insects (Zhang and Hewitt, 1994). The BLASTN of the *D. saccharalis* amplified product indicated the homology with *B. mori* and Chinese *B. mandarina* among other Lepidoptera species. For the ClustalW alignments, only the mtDNA CR sequences from *B. mori* (AF149768), *B. mandarina* (Chinese AY301620; Japanese AB070263) and the apple pest *Cydia pomonella* (AF527392) were used (Fig. 2). The higher identity (76%) was observed between *C.pomonella* and *D. saccharalis* mtDNA CR regions.

Although the *D. saccharalis* mtDNA CR size was similar to the one described for most Lepidoptera (Taylor et al., 1993), it was the shortest among the control regions analyzed in this study. The length variation and stretches of the repetitive and non-repetitive sequences in the Lepidoptera mtDNA CR were described for *Epirrita autumnata*, which presented an mtDNA CR with 1075 bp (Snall et al., 2002). The mtDNA CR from *B. mandarina* (Japanese) presented 746 bp, *B. mori* 498 bp, *B. mandarina* (Chinese) 483 bp, *C. pomonella* 432 bp and *D. saccharalis* 338 bp, Fig. 2. The sequence variation within the insect's mtDNA CR can be clustered into three categories: variable number of nucleotides in the polynucleotide runs, nucleotide substitutions and insertions/deletions of the taxa specific tandem repeats ranging in the size from 150 to 750bp. The large mtDNA CR of Japanese *B. mandarina* could be given its initial sequence composed by the AT stretches that was not present in other Lepidoptera analyzed in this study.

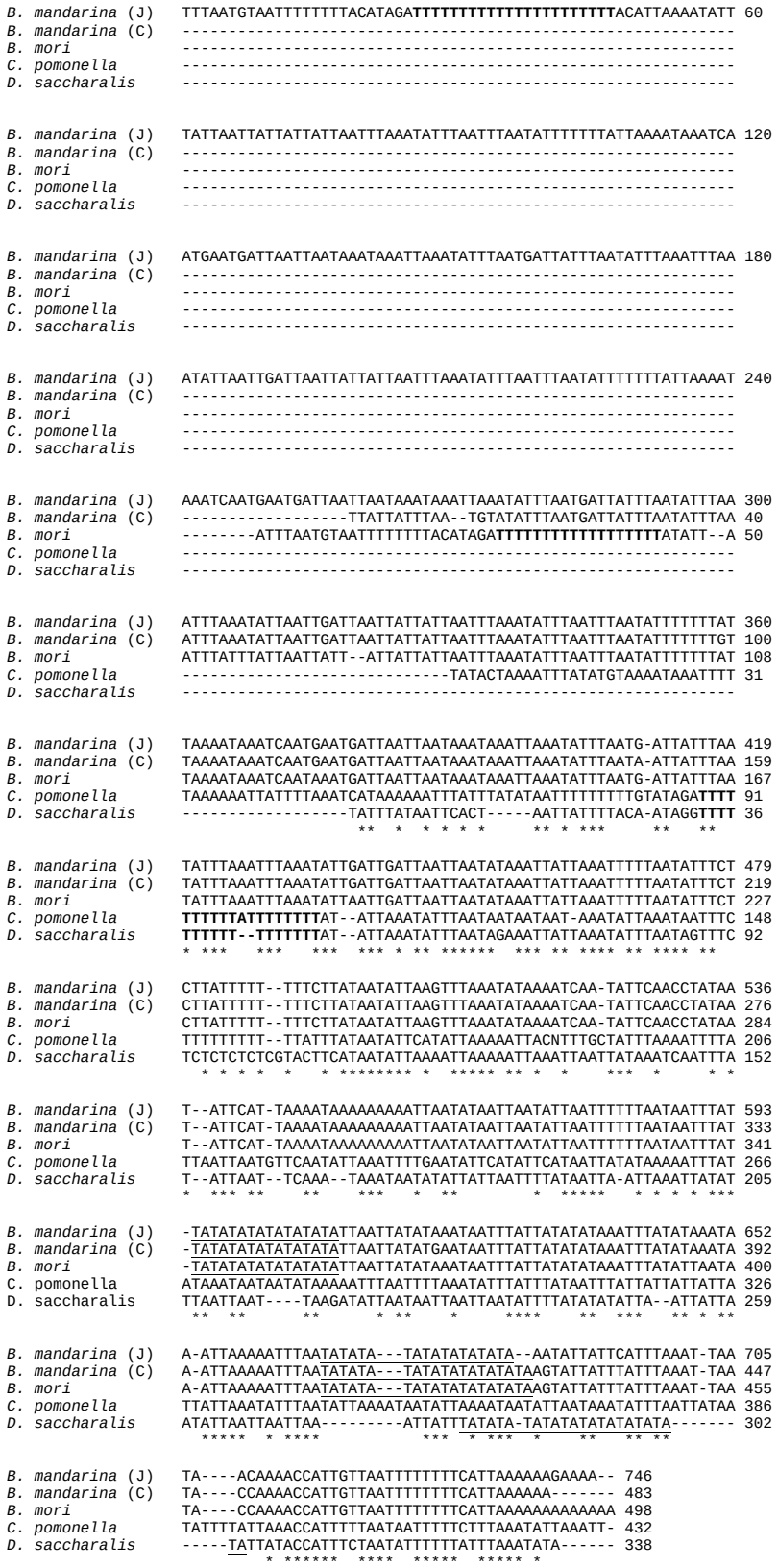

Figure 2 - The alignment amongst mtDNA CR sequences from Japanese *Bombyx mandarina* (J), Chinese *Bombyx mandarina* (C), *Bombyx mori*, *Cydia pomonella, and Diatraea saccharalis*. The poly T-stretches is in bold and TA motifs are underlined. *C. pomonella* and *D. saccharalis* mtDNA CR showing the greater identity between the sequences analyzed (76%).

The other variations were composed by the nucleotides deletions/insertions, and also in the number and length of the detected conserved regions as described elsewhere (Fig. 2, underline; review in Azeredo-Espin and Lessinger, 2006). The control region of *D. saccharalis* presented one conserved block of long polythymidine stretch (17 nucleotides; Fig. 2, bold).

Identical motif, with 18 thymidine nucleotides, was present at the same position in *C. pomonella* mtDNA CR sequence, but in this Lepidoptera the poly T stretch was separated by an Adenine (Fig. 2, bold). Except for the Chinese *B. mandarina*, the Japanese *B. mandarina* and *B. mori* contained a long polythymidine stretch, 22 and 18 nucleotides (Fig. 2, bold). However, the minimum length of the T-stretch indispensable for the mtDNA replication initiation is still unknown (Saito et al., 2005). The length of the T-stretch varies among the species of Diptera, Lepidoptera, Coleoptera and Hymenoptera; it is located immediately upstream from the L-strand origin in the mammalian mtDNA (Clayton, 1982). The precise mapping of the mtDNA replication origin was described for *B. mori*, *Triborium castaneum* and four *Drosophila* analyzed species: *D. yakuba*, *D. obscura*, *D. albomicans* and *D. virilis* (Saito et al., 2005). The localization of the T-stretch is expected also to compose the mtDNA CR replication promoter and can be a structural signal for the proteins recognition which is involved in the replication initiation in these species (Brehm et al., 2001).

D. saccharalis mtDNA CR sequence showed one large stretch $(TA)_{12}$ (Fig. 2, underlined). Two conserved TA motifs were noticed also on *B. mandarina* (Japanese and Chinese) and *B. mori* mtDNA CR sequences, but no large TA motifs were observed for *C. pomonella*. The conserved structural elements have been identified in both hemimetabolous and holometabolous insects, which reflected the functional importance of these motifs (Schultheis et al., 2002). The analyzed Lepidoptera Control Region demonstrated high nucleotide conservation around the (TA) dinucleotide repeats, which did not show a perfect alignment (Fig. 2), because *C. pomonella* did not show the (TA) dinucleotide repeats on their replication origin sequence.

In conclusion, the *D. saccharalis* mtDNA CR sequence could provide very informative data for the genetic variability study among the Lepidoptera species and it could also help the molecular studies regarding this important pest for the sugarcane production in Brazil.

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RESUMO

A broca da cana, *Diatraea saccharalis* pertence à família dos lepidópteros. A presença da larva pode ser extremamente destrutiva, chegando a inviabilizar a atividade canavieira, causando prejuízos consideráveis à agroindústria sucro-alcooleira. Atualmente a broca da cana vem sendo extinta da plantação por métodos de controle biológico, entretanto a evolução desses programas depende de maiores conhecimentos básicos da biologia molecular deste inseto. O estudo do segmento do genoma mitocondrial denominado região controle é amplamente utilizado em análises genéticas e filogenéticas em insetos. O objetivo desse trabalho foi sequenciar e analisar a região controle do genoma mitocondrial de *Diatraea saccharalis*. Esse segmento apresentou 338 nucleotídeos, menor que o observado em *Bombyx mori*, com conteúdo de 93,5% de A/T. As analises realizadas mostraram que *Diatraea saccharalis* apresenta 76% de similaridade com *Cydia pomonella*.

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