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ITS2 as a molecular marker for the identification of *Diatraea saccharalis* and *D. flavipennella* and possible infection with *Cotesia* spp

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Genet. Mol. Res. 16 (3): gmr16039732
Received May 22, 2017
Accepted July 21, 2017
Published August 31, 2017
DOI http://dx.doi.org/10.4238/gmr16039732

ABSTRACT. In Brazil, the species *Diatraea flavipennella* and *D. saccharalis* play an important role in the sugar and alcohol agribusiness by causing many damages in sugarcane fields. The egg, larva, pupa, and adult stages are very morphologically similar between these species, and the identification can be confused. The internal transcribed spacer 2 (*ITS 2*) from ribosomal DNA has important features as evolutionary divergence. It is a good marker for species identification, participates in the rDNA processing, and has been applied in phylogenetic and population studies. This study aimed to make available a molecular marker to assist on the identification method of pests' species of *Diatraea* and to identify possible traces of *Cotesia* in the resistant host.

The DNA was extracted from the egg, larva, and adult samples. PCR amplicons were purified and sequenced. The sequences were analyzed in MEGA 5.01. The *ITS 2* length was 410 bp in *D. flavipennella* and 448 bp in *D. saccharalis*. The GC content was similar between the species. Three microsatellite loci were present in *D. saccharalis* and absent in *D. flavipennella*, contributing to differences in *ITS 2* length in the species. An additional 367-bp band was attributed to *Cotesia* spp. The differences among *ITS 2* from *D. flavipennella*, *D. saccharalis*, and *Cotesia* sp were sufficient to identify them on electrophoresis gel and sequencing. The presence of *Cotesia* sp traits in adult *D. flavipennella* showed possible host refractoriness, but further studies are necessary.

Key words: *Diatraea*; Internal transcribed spacer 2; Lepidoptera; Molecular identification

INTRODUCTION

In sugarcane fields (Saccharum complex) from Brazil, two Lepidoptera species highlight in a range of insect species that attack the sugarcane and bring many damages to the sugar-ethanol agribusiness. *Diatraea saccharalis* (Fabricius, 1794) and *D. flavipennella* (Box, 1931), most known as the little sugarcane moth borer, are widespread in the Brazilian territory, and can occur in other cultures as maize, sorghum, and rice (Hall et al., 1991; Bortoli et al., 2005; Araújo et al., 2010).

Diatraea saccharalis occurs throughout the Brazilian territory, while *D. flavipennella* is present in the states of Northeast and some states of the southeast. Freitas et al. (2006) described an overcoming in the potential plague of *D. flavipennella* over *D. saccharalis* in the State of Alagoas, where this overcoming is probably caused by the methods adopted for biological control.

Both species can occur at the same time in the sugarcane field, and all developmental stages can be found simultaneously through the year. In the egg stage, the morphology of both species is identical with white color in the early age, becoming orange and dark before hatching. The larval stage is very similar in both species with slight differences between them, *D. flavipennella* presents a brownish yellow head capsule while *D. saccharalis* presents a brownish head capsule, but in both, we observed a yellow body with brown spots in each segment. The pupa is elongated and slender, and yellowish brown to mahogany brown in color in both species. The adults are yellowish with black spots in the forewings in both species (Capinera, 2001; Freitas et al., 2006).

Due to high morphological similarities between the two species in all developmental stages, the identification by morphological characters is time-consuming and requires an expert in the morphological analysis of all stages. A quick identification of the two pests' species plays a meaningful role in the pest management in the sugarcane field, e.g., in the choice of the best control method: biological, chemical, or manual collection. Besides the chemical control method (using Triflumuron®, Lambdacyhalothrin® and Fipronil®), the manual collection, and the biological control method using *Metarhizium anisopliae* (Metsch) and *Beauveria bassiana* (Bals), the biological control method using *Cotesia flavipes* (Cameron) is the most applied in Brazil for *Diatraea* (Mendonça, 1996; Alves, 1998; Alves et al., 2008).

Cotesia Cameron (Hymenoptera: Braconidae: Microgastrinae) is a large genus with an estimated number of species comprising 1500-2500 and all species described until now are koinobiont endoparasitoids, and it is entirely associated with lepidopteran insects (Gupta et al., 2016). Previous studies show that *Cotesia* biocontrol is applied on two species of *Diatraea*, *D. grandiosella* and *D. saccharalis*, despite being highly specialized (Alleyne and Wiedenmann, 2001a; Kankare and Shaw, 2004). In Brazil, *C. flavipes* has been used on biocontrol of the two species included in this study: *D. flavipennella* and *D. saccharalis*, but no previous studies were performed to evaluate the response of *D. flavipennella* to *Cotesia* attack (Pinheiro et al., 2010).

The internal transcribed spacer 2 (*ITS 2*) is a noncoding nucleotide region located inside the ribosomal DNA (rDNA) between the 5,8S and the 28S rRNA sequences. *ITS 2* plays an essential role in the maturation of the pre-ribosomal RNA as its secondary structure contained the cleavage sites for the pre-rRNA processing (Marinho et al., 2013). Previous studies showed that *ITS 2* is a good molecular marker to phylogenetic inferences among families, subfamilies, and especially as a molecular marker in lower taxonomic groups as genera and species. This nucleotide sequence has been analyzed in many families of Lepidoptera, and in all taxonomic levels, it was observed a variation in length and nucleotide composition of sequences. Therefore, *ITS 2* is an efficient barcode for lepidopteran insects and molecular phylogeny of *Cotesia* species (Kankare and Shaw, 2004; Barr et al., 2009; Wiemers et al., 2009; Wan et al., 2011a).

In this context, this study aimed to available a molecular marker to assist in a rapid and simple identification method of pests' species of *Diatraea* since the egg stage and to identify possible traces of *Cotesia* in the resistant host at the adult stage by visualization of *ITS 2* amplicons on an electrophoresis agarose gel. Besides, a sequencing of *ITS 2* of both *Diatraea* species was performed to determine the level of difference between them.

MATERIAL AND METHODS

Egg, larva, and adult achievement and DNA extraction

A total of 25 eggs, 20 larvae, and 30 adults belonging to the *Diatraea flavipennella* species from Laboratório de Patologia de Insetos (LPI)/UFRPE, Recife, PE, Brazil, were used. The individuals from LPI were collected at Usina Olho D'água located in Camutanga, Pernambuco. A total of 25 eggs, 25 larvae, and 40 adults belonging to the *D. saccharalis* species from Rede Interuniversitária para o Desenvolvimento do Setor Sucroalcooleiro (RIDESA), Carpina, PE, Brazil, were used. All individuals from these locations were obtained by breeding. Nine adult specimens of one *D. flavipennella* natural population were collected at Santa Rita, State of Paraíba, Brazil.

The methodology used for the egg, larva, and adult individuals followed Freitas et al. (2014). Briefly, the individuals were placed in 1.5-mL plastic tubes with 100 µL 5% Chelex® 100 (BioRad, Berkeley, CA, USA). Then, the eggs were macerated with a pestle until reach homogenization, and then they were kept for 1 h in a water bath at 54°C. Subsequently, the mixture was transferred to a plastic tube, and it was kept at 94°C for 30 min in the Multigene OptiMax Thermal Cycler TC 9610 (LABNET™, Edison, NJ, USA). Then, the solution was centrifuged at 13,000 rpm for 6 min, the supernatant was transferred to a 1.5-mL plastic tube, and stocked at -20°C. Finally, DNA quantification was carried out in NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Polymerase chain reaction (PCR)

For the amplification of the *ITS 2*, the primers NG02955 (5'-ATG AAC ATC GAC ATT TCG AAC GCA CA-3') and AB052895 (5'-TTC TTT TCC TCC GCT TAG TAA TAT GCT TAA-3'), previously tested in Crambidae species by Wan et al. (2011a), were used in our study. In general, 12.5 µL Mix Go Taq Colorless (Promega® Fitchburg, WI, USA), 7.5 µL nuclease-free water, 1.5 µL of each primer, and 2 µL template DNA were used. Then, the amplification reactions were carried out in the Multigene OptiMax Thermal Cycler TC 9610 (LABNET™) with the following cycle: initial denaturation at 95°C for 3 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min. The amplification products were analyzed by 3% agarose electrophoresis gel, which ran for 60 min at 120 V. Then, the amplicons were stained with Bluegreen (LGC Biotecnologia, São Paulo, SP, Brazil), and visualized in UV transilluminator.

Amplicon purification and sequencing

The amplicon purification was performed using the Wizard® SV Gel and PCR Clean-Up System kit (Promega®), following the manufacturer's instructions. Subsequently, purified amplicon sequencing was carried out in the ABI 3500 automated sequencer (Applied Biosystems, Cleveland, OH, USA). Then, the sequences were evaluated using the programs Pregap4 v 1.5 and Gap4 incorporated into the Staden Package (Staden, 1996), using Phred value equal to 30 as a quality cutoff. Similarity searches in GenBank were performed using BLASTn (Altschul et al., 1990). The sequences were aligned in MEGA 5.05 by the Muscle method (Edgar, 2004; Tamura et al., 2011). The microsatellite mining was performed in Microsatellite Repeats Finder available online (Bikandi et al., 2004). The sequences were deposited in GenBank database (http://www.ncbi.nlm.nih.gov/) under the following accession numbers: KT288209, KT288210, KT288211, KT288212, KT288213, KT288214, KT288215, KT288216, KT288217, KT288218, KT288219, KT288220, KT288221, KT288222, KT288223, KT288224, and KT288225.

RESULTS

The *ITS 2* amplicons visualized on the electrophoresis gel from the eggs, larvae, and adults of *D. flavipennella* showed about 580 bp in length, while *D. saccharalis* amplicons showed about 610 bp in length (Figure 1). The adult specimens of *D. flavipennella ITS 2* sequences from the State of Paraíba showed about 580 bp in length, perhaps three of them showed an additional band with approximately 400 bp in length (Figure 2). These two additional amplicons were also purified and sequenced.

Five (three from larvae and two from adults) and four (all samples from adult individuals) *ITS 2* sequences were obtained from *D. flavipennella* by breeding and natural populations from the State of Paraíba, respectively. Seven (six from larvae and one from the adult) *ITS 2* sequences were obtained from *D. saccharalis*. The sequences ranged from 575 to 613 bp. We aligned the *ITS 2* sequences with the GenBank using BLASTn (Altschul et al., 1990), and observed 101 bp from 5,8S rDNA and 60 bp from 28S rDNA included in these amplicons. After the identification of the rDNA sequences, they were removed from the

analysis. The additional DNA band observed on the electrophoresis gel of the *D. flavipennella* population from Paraíba showed 367 bp and matched with *ITS 2* sequences from *Cotesia* available in GenBank.

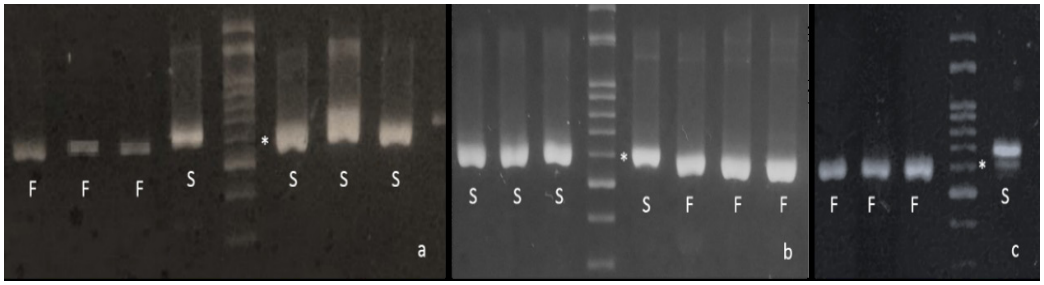

Figure 1. Electrophoresis gel showing length differences among *ITS 2* amplicons of *Diatraea flavipennella* and *D. saccharalis* from three developmental stages: egg (a), larvae (b), and adult (c). *Lane F*: *D. flavipennella*, and *lane S*: *D. saccharalis*. Asterisk indicates a 600-bp band.

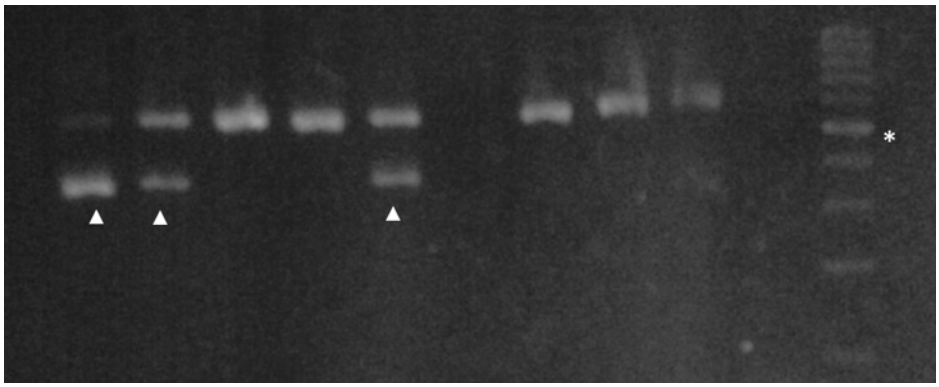

Figure 2. Electrophoresis gel showing an additional band of about 400 bp found in the PCR using *ITS 2* primers in *Diatraea flavipennella* individuals. Arrowheads indicate the additional bands and asterisk indicates a 500-bp band.

The *D. flavipennella* and *D. saccharalis* sequences presented indels, which result in *ITS 2* length variation between the two species. The *ITS 2* loci present 410 bp in *D. flavipennella* and 448 bp in *D. saccharalis*. The *ITS 2* average nucleotide composition was similar between these two species: 30.3% thymine; 22.3% cytosine; 20% adenine, and 27.4% guanine. The average GC content was 48% in *D. flavipennella* and 51% in *D. saccharalis*. It was not observed any variability among individuals of the *D. flavipennella* species. Perhaps, among the *D. saccharalis* individuals, a 3-bp indel was observed. In *D. flavipennella* and *D. saccharalis ITS 2* sequences, the presence of microsatellites was observed, and there were slight differences in the microsatellite distribution (Table 1).

Among the *ITS2* DNA sequences from the two species *D. flavipennella* and *D. saccharalis*, we found four conservative DNA sites. After the 5.8S rDNA, we observed a 31-bp DNA sequence 5'-TTYTTCGAAACGGTGTTGACCGTTCTCCTCC-3' with a thymine in *D. flavipennella* and a cytosine in *D. saccharalis* at the third position of this sequence. An indel (guanine) at the fourth position in *D. saccharalis* was also observed. The second sequence was identical between

these two species with 21 bp, 5'-GAGTCGGTTTAAATATATTAC-3'. The third sequence has 42 bp, 5'-ACGCGTGTCGTTCTCGATATAGAAAGCGACTCTTTGTGGATG-3', also identical between these two species. The fourth conservative site is a DNA sequence with 127 bp in *D. flavipennella* and 131/133 in *D. saccharalis*; between these sequences, some indels and nucleotide changes were observed (see Figure 3).

Table 1. Microsatellite repeats found in *Diatraea flavipennella* and *D. saccharalis*.

SSR	Position		Repetition	
	D. flaviepnnella	*D. saccharalis*	*D. flaviepennella*	*D. saccharalis*
AT	59, 200, 238, and 257	61	3 and 4	3
AG	290	271 and 290	3	3
GT	70	243	3	6
AC	-	124	-	3
CGT	332 and 352	368	3 and 7	3
GCG	322	355	3	4
GAC	-	78	-	3
TCG	-	392	-	6

SSR: simple sequence repeats.

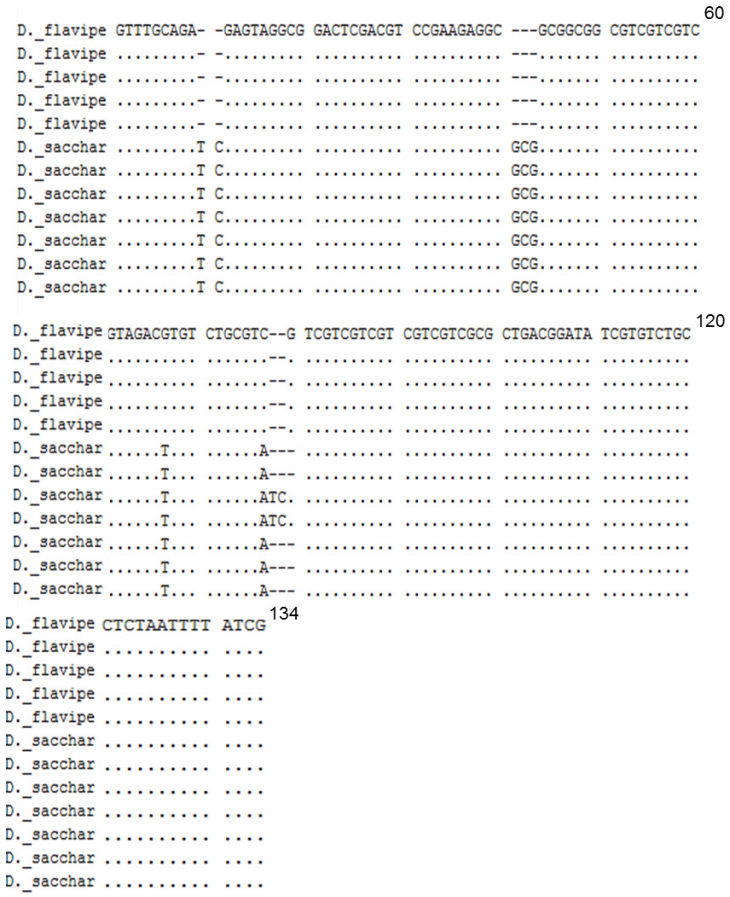

Figure 3. Alignment of *ITS 2* segment 4 from *Diatraea flavipennella* and *D. saccharalis.*

DISCUSSION

The *ITS 2*, located between the 5.8S and 28S rDNA, has many valuable characteristics that can make it a good candidate for DNA barcode and, according Yao et al. (2010), one of them possesses sufficient variation among the sequences to discriminate species and needs to be conserved so that there is less variability within species than between species. The amplified *ITS 2* fragments visualized on electrophoresis agarose gel showed distinct sizes in each *Diatraea* species included in this study. This rDNA region has been used to identify many lepidopteran species and other insect orders without the need to be sequenced, but only comparing the amplicon sizes on agarose gel (Barr et al., 2009; Wan et al., 2011a; Sayed et al., 2013; Zomuanpuii et al., 2013). In the sugarcane field, in many cases, we only find pieces of the *Diatraea* larvae due to cuts in the sugarcane or eggs, but these situations are difficult to identify. Therefore, a molecular marker that allows the researcher or the professional who works with *Diatraea* control to perform a quick identification with a lower cost is very competitive.

ITS 2 sizes from *D. flavipennella* and *D. saccharalis* showed 410 and 448 bp, respectively, confirming the observed variation on an electrophoresis gel. The observed length variation between species could be due to insertions induced by many factors including the relative number and sizes of microsatellite repetitions, unequal crossing over, gene conversion, or genetic drift (Levinson and Gutman, 1987). More changes lead to bigger variations in the *ITS 2* sequence size that will be the effect on the secondary structure conservation (Kuracha et al., 2006).

Among the species that belong to the Family Crambidae analyzed until now, we could observe that the average size of the *ITS 2* region in that group is 445 bp, represented by the species, *Chilo hyrax* (488 bp), *C. supressalis* (484 bp), *Cnaphalocrocis medinalis* (447 bp), and *Maruca vitrata* (451 bp), while *ITS 2* in *O. nubilalis* is 390 bp (Margam et al., 2011; Wan et al., 2011a). The *ITS 2* region size is specific to each species in the family Crambidae, and this also happens in other families as Tortricidae, Noctuidae, Geometridae, and Lycaenidae (Barr et al., 2009; Wiemers et al., 2009; Wan et al., 2011a; Kvie et al., 2012).

In the *ITS 2* from both species, eight different microsatellite sequences were found. Among them, five (AT, GT, AG, GCG, and CGT) were present in both *Diatraea* species, but in some cases the repetition numbers were different. The dinucleotide AC and the trinucleotides GAC and TCG were only found in *D. saccharalis*; therefore, the insertion of these sequences in *D. saccharalis* or deletion of them in *D. flavipennella* may be contributed to differences in total sequence sizes. There was no intraspecific variation according to sequence length among *D. flavipennella* individuals from both populations, and in *D. saccharalis* two individuals showed an increase of three nucleotides. The sequence size homogenization among individuals, in the case of *D. saccharalis* and *D. flavipennella* by breeding, could be due to endogamy that is present in breeding. There was also no report about significant *ITS 2* size differences in some lepidopteran species: *Chilo* sp, *Cnaphalocrocis medinalis*, *Ostrinia* sp, *Spodoptera* spp, *Polyommatus* spp, and species from the families Geometridae, Lasiocampidae, Pieridae, Tortricidae, and Zygaenidae (Niehuis et al., 2006; Barr et al., 2009; Wiemers et al., 2009; Dincă et al., 2011; Margam et al., 2011; Wan et al., 2011a; Kvie et al., 2012). In other groups as Diptera, ten species belonging to genus *Anopheles*, from different geographic regions, did not also show intraspecific differences in nucleotide composition and sequence length. The authors attributed this phenomenon to a lack of environmental barriers and the concerted evolution that maintains the homogeneity of all rDNA copies that can lead to intraspecific homogeneity (Zomuanpuii et al., 2013). We found the same situation to both populations of

D. flavipennella, where there is no ecological barrier between populations from Pernambuco and Paraíba.

The GC content had low variation between the two species in this study. When we compare the value found in the *Diatraea* species with other species from Crambidae we can observe a similar GC content among them. *Cnaphalocrocis medinalis* and *M. vitrata* showed, respectively, 47.8 and 45.1% GC. However, *Chilo suppressalis* and *C. hyrax*, which are closely related to *Diatraea*, showed 57.2 and 55.2% GC; the higher values observed until now (Margam et al., 2011; Wan et al., 2011a). When we observe the GC content from other families, we can see a significant increase compared to Crambidae, as in Tortricidae (61%) and Noctuidae (58.6%), but this increase happens in both basal and advanced groups (Barr et al., 2009; Wan et al., 2011b). The GC content to Lepidoptera is considered comparatively high, and this can affect the energy of the secondary structure (Banerjee et al., 2007).

D. flavipennella and *D. sacharalis ITS 2* sequence alignment showed four conserved sites that correspond to 53.6 and 49.1% of *ITS 2* sequences from *D. flavipennella* and *D. saccharalis*, respectively. These conserved sites are intercalated by variable regions. Even though they are highly conserved, these regions show differences that characterize each *Diatraea* species, as indels and base substitutions. Mai and Coleman (1997) presented the reason for this apparent alternation between conserved and variable sites. According to the authors, those conserved regions contribute to secondary structure helices, pairing where the RNA transcription begins, and this secondary structure leads the process.

Parasitic wasps that belong to *Cotesia flavipes* species are used as biological control of stem borers that attack some crops like sorghum, rice, maize, and sugarcane. *D. saccharalis*, *D. flavipennella*, *D. grandiosella*, and *O. nubilalis* are among the crambids affected by that parasitoid (Hall et al., 1991; Alleyne and Wiedenmann 2001a,b; Bortoli et al., 2005; Araújo et al., 2010). When the host is parasitized by *Cotesia* the host's immune system applies some defensive strategies to combat those parasitoids by humoral (production of antimicrobial peptides, reactive intermediates of oxygen and nitrogen, coagulation, or melanization) and cellular defenses (defenses mediated by hemocytes through phagocytosis, nodulation, and encapsulation) (Strand and Pech, 1995; Gillespie et al., 1997; Lowenberger, 2001; Vass and Nappi, 2001; Schmidt et al., 2001). However, parasitic wasps have some strategies to suppress the host's immune system to maintain the parasite integrity inside the host. The strategies involve maternal factors as ovarian proteins, and polydnavirus and embryonic factors as teratocytes.

According to our results, where we could identify the parasitoid in adults *D. flavipennella* using *ITS 2* marker, we can speculate that *D. flavipennella* have unknown mechanisms that dribble the immunosuppression caused by *Cotesia*. Rodríguez-Pérez et al. (2005), in a study with *C. flavipes* and *Manduca sexta*, observed that parasitism was unable to induce host immunosuppression in the non-habitual host. In habitual hosts, as *D. saccharalis*, parasitism induces host immunosuppression that is required to prevent encapsulation of *Cotesia* parasitoid (Alleyne and Wiedenmann, 2001b; White and Wilson, 2012). The status of the distribution of *D. flavipennella* and *D. saccharalis* in the northeast sugarcane fields supports the idea of host refractoriness by *D. flavipennella*. It was observed an inversion of species dominance where the prevalence of *D. flavipennella* was 97.67% over 2.33% of *D. saccharalis*, and the authors attributed that inversion to inefficient methods of biological control as *C. flavipes*, for example, that may favor *D. flavipennella* instead of *D. saccharalis* (Freitas et al., 2006).

In summary, we can conclude that *ITS 2* molecular marker is efficient to identify *D. flavipennella* and *D. saccharalis* in any developmental stage with accuracy by electrophoresis gel or sequencing. *ITS 2* was also used to identify traits of *Cotesia* spp parasitoid in adults *D. flavipennella*. With this information, we can speculate a reason that justifies the inversion of species dominance in the sugarcane field that is the host refractoriness of *D. flavipennella*, but further studies are required to prove that.

ACKNOWLEDGMENTS

We are grateful to Dr. Heidi Lacerda for her technical support in DNA sequencing. The authors also acknowledge Rede Interuniversitária para o Desenvolvimento do Setor Sucroalcooleiro (RIDESA) and the Platform for Genomics and Gene Expression of Universidade Federal de Pernambuco for the logistic and technical assistance. We are also grateful to Dr. Herbert Siqueira and Dr. Jacinto Marques for donating the *Diatraea* specimens used in this study. This study was supported by the Brazilian funding agencies Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and the Bioethanol Research Network of the State of Pernambuco (CNPq-FACEPE/PRONEM program, process #APQ-1452-2.01/10).

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