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Infection by the microsporidium of Clado *Nosema/Vairimorpha* in pupal parasitoids

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Abstract: The sugarcane borer, *Diatraea saccharalis* is one of the hosts more used for parasitoid pupal multiplication in Brazil. The parasitoids pupal of *Trichospilus diatraeae* and *Palmistichus elaeisis* are generalist natural enemies with potential to suppress populations of diverse families of lepidopteran pests. The success in the utilization of these natural enemies in the field is directly related to the capacity of search of the host, this capacity might be affected by the presence of the pathogens. In this context, the aim of this essay was to detect the presence of intracellular parasites of Phylum Microsporidia. These pathogens may cause morphological and behavioral alterations. The presence of infection was verified by microscopy and was confirmed by amplification of region *small subunit (SSU)* of ribosomal RNA using universal *primers* for microsporidia of *Nosema* sp. The purified PCR products were submitted to sequencing, and the sequences that had been obtained were edited and aligned with the sequences in a Genbank database. In this way, it was possible to verify the presence of intracellular parasites in *T. diatraeae, P. elaeisis* and *D. saccharalis* pertaining to Clade *Nosema*/*Vairimorpha*. However, this is the first one report about detection of the microsporidia in the parasitoids *T. diatraeae* and *P. elaeisis*.

Key words: Biological control, microsporidium, pupal parasitoids, PCR.

INTRODUCTION

The parasitoids *Trichospilus diatraeae* (Cherian & Margabandhu) and *Palmistichus elaeisis* (Delvare & LaSalle) (Hymenoptera: Eulophidae) are natural enemies that can suppress populations of several families of lepidopteran pests of agricultural crops (Paron and Berti-Filho 2000) and forests (Pereira et al. 2011). In order, these natural enemies are generalists and effectives in suppressing populations of pest insects, therefore, they have the potential to be used in biological control programs (Rodrigues et al. 2013, Paron and Berti-Filho 2000).

The effective use of natural enemies in inundative releases depends of the selection of a physiologically suitable alternative host for mass production (Vinson and Iwantch 1980). The host must have high nutritional quality and be produced

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easily, efficiently and economically (Parra et al. 2002). The sugarcane borer, *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae) its stands out as a nutritionally appropriate host for parasitoid multiplication (Rodrigues et al. 2013). However, the quality control of the mass production of parasitoids, *T. diatraeae* and *P. elaeisis* must always be verified, because the presence of pathogens in the hosts can interfere in the development of the parasitoids and consecutively in the action of pest control in the field.

The occurrence of microsporids in the populations of *D. saccharalis* (Simões et al. 2015) may become a problem in the mass production of *T. diatraeae* and *P. elaeisis.* These microsporids are obligate intracellular parasitic fungi (Corradi and Keeling 2009). The spores are metabolically inactive and, when ingested, germinate in the lumen of the insect's midgut, injecting the infectious apparatus, composed of a polar tube that penetrates the cell membrane and allows the sporoplasm to be transferred to the host (Bigliardi and Sacchi 2001). In the host cell, the microsporids explore the organism to multiply, using organelles and metabolite products as substrate to complete the cycle (Dean et al. 2016). After the multiplication, new resistance spores are formed and released through cell lysis and excreted in insect feces (Gisder et al. 2011).

The parasites exploit and frequently manipulate the host inducing morphological and behavioral changes to increase transmission success (Kurze et al. 2016a). Infected populations commonly show increased time to complete the larval and pupal stages (Simões et al. 2012, Kermani et al. 2014), reduction in longevity of adults (Simões et al. 2012), decrease in the rate of emergence and fertility (Kermani et al. 2014), behavioral modifications in host search (Simões et al. 2012, Kermani et al. 2014), abnormal adult abdomen size (Simões et al. 2015), stress (Kurze et al. 2016b). The disease caused by these pathogens, in addition to affecting biological aspects of insects, has become an economic problem (Aroee et al. 2017), for example in bee hives causes reduction of honey production (Higes et al. 2008) and colony collapse disorder (Huang et al. 2007).

However, it is extremely difficult to morphologically distinguish between microsporids species by light microscopy because the spores are very similar. Then, the recognition of the species using molecular markers might be very helpful in the diagnosis and identification of microsporids. Thus, it is necessary to use molecular diagnostic tools by the PCR technique, which provides a more accurate result for the detection of microsporidial infection, because it allows detection of the parasite even at very low levels of infection (Ansari et al. 2017).

In view of the importance of *T. diatraeae* and *P. elaeisis* in biological control programs applied to several pests of agricultural and forestry importance, the objective of this work was to verify the presence of microsporids belonging to the Clado *Nosema/Vairimorpha* in parasitoids *T. diatraeae* and *P. elaeisis* and the host *D. saccharalis.*

MATERIALS AND METHODS

HOST AND PARASITOID REARING

Larvae and pupal of the host *D. saccharalis* were obtained from mass rearing of biofactory. The parasitoids *T. diatraeae* and *P. elaeisis* and the host *D. saccharalis* were kept in stock at the Laboratório de Controle Biológico de Pragas Florestais (LCBPF) da Faculdade de Ciências Agronômicas da Universidade Estadual Paulista (FCA / UNESP). The identification of the parasitoids was confirmed by the taxonomist Dr. Valmir A. Costa, the specimen vouchers of the parasitoids were deposited in the Entomophagous Insects Collection "Oscar Monte", from the Biological Institute, based in Campinas, State of São Paulo, Brazil. The identification of the host was confirmed by Dr. Roberto Antonio

Zucchi, the specimen vouchers were deposited in the Coleção Entomológica do Departamento de Proteção de Plantas da Faculdade de Ciências Agronômicas da Universidade Estadual Paulista (FCA / UNESP), Botucatu, Estado de São Paulo, Brazil. Pupal of *D. saccharalis* were transferred to polyvinyl chloride (PVC) cages (20 cm diameter by 20 cm height), closed with fabric at the bottom and with *voile* tissue at the top. The interior of these cages was coated with sulphite paper as a substrate for oviposition. The collected eggs were sterilized with formaldehyde (0.5%) and copper sulphate (17%) and inoculated into glass tubes (2 cm x 8 cm) containing artificial diet proposed by King and Hartley (1985). From 4 until 7 larvae were placed in each tube until reaching third instar. In this phase, the larvae were fed with 1 cm^3 of artificial diet and placed in a plastic container (1.5 cm x 5 cm) until reaching the pupal stage (Fonseca et al. 2015). The parasitoids were reared in pupal of *D. saccharalis* aged 48 to 72 hours. Host pupal were offered to the parasitoids, which were packed in glass tubes (2.5 cm in diameter and 8.5 cm long), sealed with voile-type tissue and fed with pure honey, for parasitism up to 72 hours. The insects were kept in an air-conditioned chamber at 25 ± 2 ºC, relative humidity of 70 ± 10% and a 12-hour photophase until adult emergence that occurs around the 15th to the 18th day (Zaché et al. 2010).

PREPARATION OF SMEARS

Diatraea saccharalis larvae of third instar with an apparent symptom of microsporidia infection and adults of pupal parasitoids were used to make smears and identify spores of microsporids. The insects were killed by freezing and, later, a cross-section with scalpel was carried out in the abdomen of the same. The extravasation of the intestinal contents was placed on a glass slide and a drop of saline (0.85% NaCl) added. Maceration of the larvae was performed on a microscopic slide and the excess body content of the insects was removed with forceps. The slides were fixed in methanol for ten minutes and then air dried. Spore staining was performed with 50% diluted Giemsa solution for 30 minutes. The slides were washed in tap water to remove excess dye and air dried. The smears were visualized with a phase contrast microscope on the 1000x objective (Zeiss Primo Star) (Alves et al. 1998).

GENOMIC DNA EXTRACTION AND PCR

Samples of *D. saccharalis*, *T. diatraeae* and *P. elaeisis* with confirmed identification were obtained from stock for evaluation of the presence of spores of *Nosema* spp., *Palmistichus elaeisis* and *T. diatraeae* adults and third instar larvae of *D. saccharalis* were stored in the freezer at -20 °C. After thawing, a caterpillar was randomly selected for genomic DNA extraction and standardization of the polymerase reaction in chain (PCR). The caterpillar was macerated and later the larger parts of the insect's body were removed 80 µl of 10% Chelex solution and 8 µl of 20 mg / mL proteinase K were added to the body contents in microtube and homogenized. Then the microtube containing the sample was placed in a thermal block at 95 °C for 20 minutes to release the DNA from the cells. Chelex protects DNA from the effects of heating used to release DNA from cells by sequestering divalent heavy metal ions from enzymes that could damage the structure of the molecule (Walsh 1991). The same procedure was used to extract parasitoids, however, a pool of 50 macerated and homogenized individuals was used with 80 µl of 10% Chelex solution and 8 µl of 20 mg / mL proteinase K in microtube and then placed in a thermal block at 95° C for 20 minutes. The protocol for DNA extraction was performed using Chelex 100® resin (Coombs et al. 1999). Then, a mini-spin centrifugation was performed and the supernatants were collected for PCR.

The small subunit (SSU) region of ribosomal RNA was amplified using universal primers for microspores of *Nosema* sp., (F) CACCAGGTTGATTCTGCT and (R) GTTACCCGTCACTGCCTTG, expected size of 222bp (Klee et al. 2006). The PCR reaction was prepared containing 12.5 µl of Gotaq Hot Start (Promega), with the reagents required for the reaction: 5 U / µl Taq, 100 µM of each dNTP and 25 mM $MgCl_2$, 5 µl of Nuclease Free Water (Promega), 1.25 µl of each primer [10 mM] and 5 µl of genomic DNA, totaling 25 µl per reaction. The polymerase chain reaction (PCR) was carried out in thermal cycler (Infinigen, model TC-96CG) with initial denaturation for 4 minutes at 95 °C, followed by 45 cycles with denaturation at 95 °C for 1 minute, followed by the annealing phase a 48 °C for 1 minute and extension at 72 °C for 1 minute and with a final polishing step for 4 minutes at 72 °C (Tay et al. 2005). The amplification products were visualized by 1% agarose gel electrophoresis, using a 100 bp marker (Norgen). The agarose gel was visualized and photographed in ultraviolet light transilluminator (Major Science).

SEQUENCING

Purifications of the PCR products were performed using the Norgen PCR Purification Kit (Qiagen, Cat # 14400), following the manufacturer's recommendations. Sequencing by Sanger DNA automatic sequencer (Model: ABI 3500 - Applied Biosystems) was performed at the Institute of Biotechnology (IBTEC - UNESP). The nucleotide sequences of SSU rRNA for the microsporide isolates obtained from *D. saccharalis* and the pupal parasitoids were analyzed using the Geneious v. 9.1.5 and compared to the database (GenBank, http://www.ncbi.nlm.nih.gov) for the identification of genetic similarity.

The alignment was performed using the Geneious v. 9.1.5, with the sequences obtained in this study added from other sequences of microsporidia obtained in GenBank. Phylogenetic analysis was done using the program MrBayes 3.2.2 (Huelsenbeck and Ronquist 2001). Bayesian analysis was performed for 30 million generations with sampling every 1000 generations. Each analysis consisted of four independent runs, each using four coupled Markov chains. The convergence of the race was monitored by finding the plateau in the probability score (standard deviation of divided frequencies <0.0015). The first 25% of each run was discarded for the estimation of a consensus topology and later probability for each node.

RESULTS AND DISCUSSION

INFECTION BY THE MICROSPORIDIUM OF CLADO *NOSEMA/VAIRIMORPHA* IN PUPAL PARASITOIDS

Microspores infect vertebrates and invertebrates, including insects, fish and mammals (Weiss 2001, Morsy et al. 2013, Liu et al. 2015). The presence of these pathogens has been widely discussed due to the increase of infection in several hosts (Emsen et al. 2016), a fact resulting from the biological and behavioral changes caused by the parasite (Simões et al. 2012, Kermani et al. 2014, Kurze et al. 2016b).

In this study, oval-shaped spores, resistance structure of intracellular parasites of Clado *Nosema*/*Vairimorpha* were isolated from the intestinal contents of *D. saccharalis* larvae (Fig. 1). In the slides of the body contents of parasitoids *T. diatraeae* and *P. elaeisis,* some parasite spores were visualized, but they did not quantified. This is possibly due to the lower degree of infection of the pathogen in the parasitoids (Bjørnson 2008) and/or the stage of development of the microspore in the host cell (Becnel et al. 2002).

Microscopy is a cheap and routine technique for the identification of microsporids, however, it can be laborious and requires knowledge to detect the spores. In addition, the pathogen may be present in only a few individuals when the prevalence

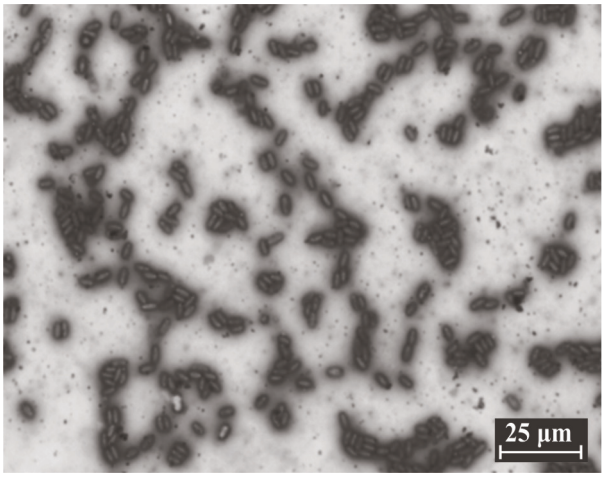

Figure 1 - Image of the smear stained with Giemsa. Spores of the Clado *Nosema/Vairimorpha* isolated from the intestinal contents of *D. saccharalis* viewed under a phase contrast microscope (1000x). Scale bar = 25 µm.

is low, a fact that makes it difficult to diagnose the infection (Bjørnson 2008). Although it is considered reliable for detection, it is not sufficient for a precise identification of these parasites, since the morphological structures of some pathogens are very similar, resulting in a high risk of errors in diagnosis (Ansari et al. 2017).

The PCR reaction revealed by amplification of ribosomal RNA from microsporids that the host populations of *D. saccharalis* and parasitoids *T. diatraeae* and *P. elaeisis* are infected by intracellular parasites. The SSR rRNA sequence has been widely used as a molecular marker to estimate phylogenetic relationships between microsporidia by having highly conserved gene. However, this gene can not be used to distinguish closely related species (Canning et al. 1999, Tsai et al. 2003).

The results suggest that the isolates are closely related to the species of *Vairimorpha* sp., with which it shares 100% identity of the SSU rRNA gene (222 bp) of these sequences in Nucleotide BLAST, and the species of the genus *Vairimorpha* forms a clade with *Nosema* spp. and *Rugispora istanbulensis* (Fig. 2). However, the genera *Nosema* and *Vairimorpha* can not be separated into different clades using molecular analyzes (Tsai et al. 2003, Ku et al. 2007).

The occurrence of microsporids in the populations of *D. saccharalis* and parasitoids *T. diatraeae* and *P. elaeisis* may become a problem in mass production and in biological control programs. Pathogens of this group can remain latent without causing visible symptoms in hosts (Bjørnson 2008). This can be perpetuated for several generations through the possibility of vertical transmission, when infection is transmitted from parents to offspring and horizontal when it occurs through contact between individuals, which may be favored by the confinement environment of mass productions (Dubuffet et al. 2013). Recent studies have shown a significant effect of the breeding laboratories' ambient temperature on the intensity of the endoparasites *Nosema* spp. in bees. Spores find temperatures reasonably regulated during their life stages inside the host, but when exposed to the outside temperature, they exhibit a differential sensitivity to temperatures during the transmission to new hosts (Gisder et al. 2017, Retschnig et al. 2017).

Parasitoids massively multiplied, are usually contaminated when the larvae feed on the hemolymph or infected tissues, resulting in a variety of changes (Simões et al. 2012). Depending on the degree of infection in populations of natural enemies, changes in the duration of the juvenile phase, reduced survival, altered flight behavior and host search may occur. These symptoms were reported in *C. flavipes* (Simões et al. 2012), *Cotesia vertalis* (Haliday) (Hymenoptera: Braconidae) (Kermani et al. 2014), *Macrocentrus grandii* Goidanich (Hymenoptera: Braconidae) (Andreadis 1982), *Phytoseiulus persimilis* (Acari: Phytoseiidae) (Bjørnson and Keddie 1999) and *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) (Bjørnson et al. 2013). Thus, it is characterized the need for detailed investigation of the interaction between infection by this microsporid in hosts *D.*

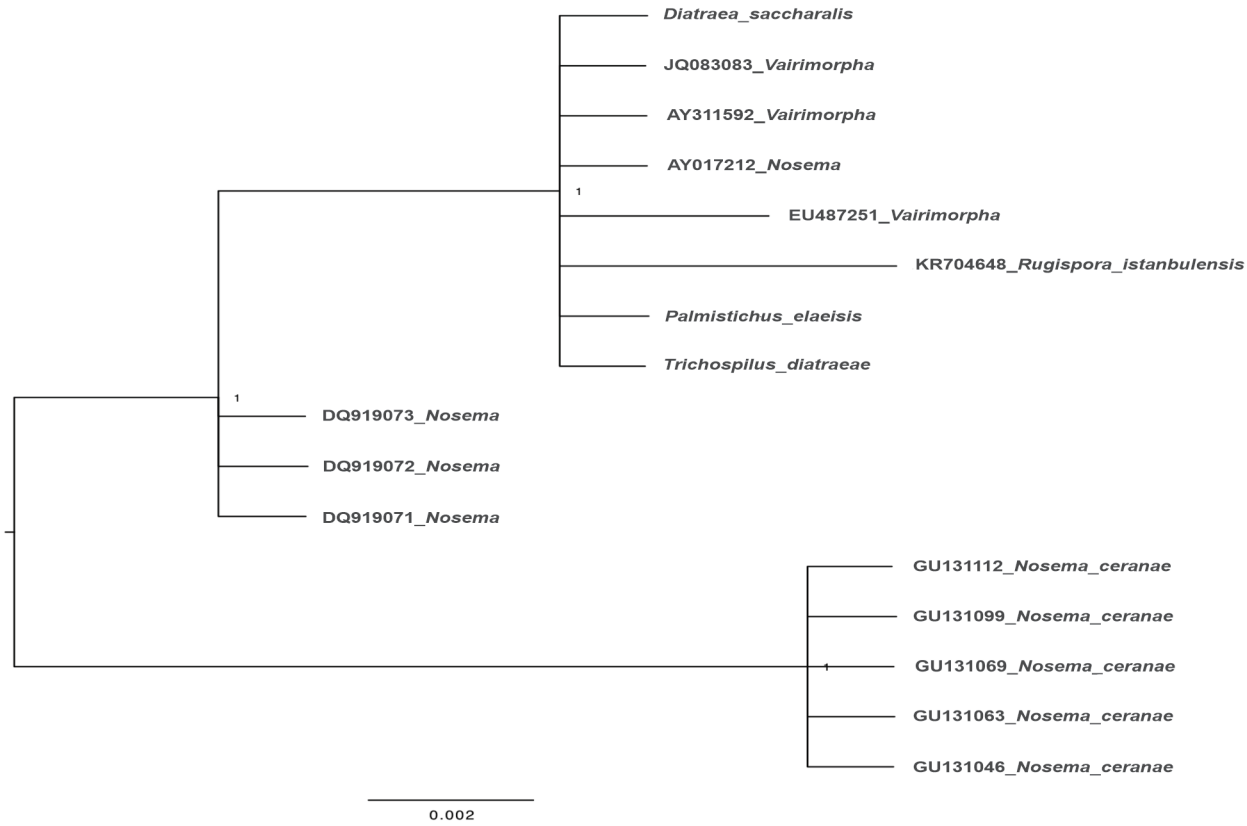

Figure 2 - Phylogenetic tree of the small subunit region (SSU) of ribosomal RNA from microsporidia isolated from *D. saccharalis* and from the parasitoid *T. diatraeae* and *P. elaeisis.*

saccharalis and pupal parasitoids, *T. diatraeae* and *P. elaeisis*, to verify the quality and improve the understanding of the real impact of this parasite in biology and in the search behavior of the host in the field.

CONCLUSION

We conclude that according to the morphological characteristics of the spores and molecular genetics, the presence of Clado *Nosema*/*Vairimorpha* microsporids in the pupal parasitoids *T. diatraeae* and *P. elaeisis* and in the host *D. saccharalis* was verified.

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AUTHOR CONTRIBUTIONS

João Paulo P. Paes, Vanessa R. de Carvalho and Amanda R. de Souza performed the experiment, analysed and interpreted the data, and drafted the manuscript. Carlos F. Wilcken and Regiane C.O. de Freitas Bueno planned the proposal and supervised the experiment, interpreted the data and were responsible for the revision of the manuscript.

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