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Molecular Identification of a Parasitic Fly (Diptera: Tachinidae) from the Introduced Helicoverpa armigera (Lepidoptera: Noctuidae) in Brazil

Guerra WD[1], Guerra ALLD[2], Ribas LN[3], Gonçalves RM[4] and Mastrangelo T[4*]

[1]*Ministry of Agriculture, Livestock and Food Supply, Federal Agricultural Superintendence in Mato Grosso, 78115-000, Brazil*

[2]*Federal University of Mato Grosso, 78060-900, Brazil*

[3]*Associação dos Produtores de Soja e Milho do Mato Grosso (Aprosoja), 78049-908, Brazil*

[4] *Center for Molecular Biology and Genetic Engineering, State University of Campinas, 13083-875, Brazil*

[*]**Corresponding author:** Thiago Mastrangelo, Center for Molecular Biology and Genetic Engineering, State University of Campinas (UNICAMP), Av. Candido Rondon 400, 13083-875, Campinas, SP, Brazil, Tel: +55 19 3521 1141; E-mail: mastrangelo@cbmeg.unicamp.br

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Abstract

The African cotton bollworm, Helicoverpa armigera Hubner (Lepidoptera: Noctuidae) was detected in Brazil, triggering a major phytosanitary crisis during the 2012/2013 harvest. Despite its recent introduction, larvae were found being parasitized by indigenous dipterans with estimated parasitism rates even higher than 30% at some localities. Two of these flies from the municipality of Campo Verde, Central-East Mato Grosso, were sent to the State University of Campinas to be identified through mitochondrial DNA. Partial sequences of the mitochondrial cytochrome c oxidase subunit I gene were obtained and matched more than 99% with Archytas marmoratus Towsen (Diptera: Tachinidae) known sequences from BOLD database. The pairwise genetic distances (K2P and p-distance) between the two Brazilian specimens and the 14 other tachinid species were all higher than 4%. The findings of this study serve as first evidence that an indigenous species of tachinid exists in Brazil that may be a good biological agent for the exotic *H. armigera*.

Keywords: Archytas; Mitochondrial DNA; Biological control

Introduction

The African cotton bollworm, Helicoverpa armigera Hubner (Lepidoptera: Noctuidae), is the most widely distributed pest of the Heliothinae group [1]. It is a highly polyphagous pest of several economically significant crops. Worldwide, annual yield losses and control costs reach US$ 5 billion [2]. In China, the estimated losses in cotton reached US$ 1.3 billion in 1992 [3] and, in India, the losses with this pest alone exceeded US$ 627 million in the late 1980s [2]. H. armigera was not present in the Americas, but it was detected in Brazil during the 2012/2013 growing season [4-6] and rapidly triggered a major phytosanitary crisis. In western Bahia, infestations of H. armigera larvae reduced crop yields up to 35%, the number of pesticide applications increased by more than 15% and the costs in cotton yields doubled (from US$ 400 to US$ 800 per hectare) [6]. In the State of Mato Grosso, one of the largest soybeans producer states in Brazil, extensive economic losses were also registered [4,7].

Inspectors from the Ministry of Agriculture, Livestock and Food Supply (Ministério da Agricultura, Pecuária e Abastecimento, abbreviated MAPA) performed intensive surveys for natural enemies between April and June 2013 at farms from the main municipalities from the State of Mato Grosso. More than 200 Helicoverpa larvae collected from millet, cotton and beans, were taken to laboratory and allowed to pupate individually in plastic card boards. However, instead of the moths, many dipterans emerged, revealing an estimated parasitism rate up to 38.8% and 37.5% (larvae from the municipalities of Querência and Nova Xavantina, respectively) [8]. Some of the specimens that emerged were sent for taxonomic identification at the University of Brasília (UnB), but morphological keys gave as result the genus Archytas. Two specimens (Figure 1) that emerged from parasitized larvae collected from infested beans in the municipality of Campo Verde, Central-East Mato Grosso, were then sent to the State University of Campinas (UNICAMP), trying to obtain the species identification through mitochondrial DNA (mtDNA).

Materials and Methods

Total DNA was extracted from the entire body of the two dry pinned specimens using the phenol:chloroform method, adapted for microcentrifuge tubes [9], resuspended in 100 µL of TE buffer and stored at -20 °C. The partial sequences of the mitochondrial cytochrome c oxidase subunit I gene (COI) were amplified by PCR using the primers COIF and COIR [10].

The PCR reactions were conducted separately with 25 ng of total DNA, 2.5 mM $MgCl_2$, 0.25 mg/mL of BSA, 100 µM dNTPs, 0.5 mM of each primer, 1.5 U of TaqDNA polymerase (Fermentas International Inc., Burlington, Canada), and 10xTaqBuffer for a final reaction volume of 25 µL. Amplification was carried out on a GeneAmpPCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA), with the following conditions: an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 45°C for 30 s, 70°C for 1:30 s, and ended with a 7 min final extension at 70°C . After amplification, 2 µL aliquots were analyzed by 1% agarose gel electrophoresis in 1x TAE (40 mM Tris-acetate, 1 mM EDTA).

Amplicons were purified with the IllustraGFX kit (GE Healthcare, Bucks, UK) and sequenced bidirectionally by the ABI3730xl DNA Analyzer sequencer (Applied Biosystems, Foster City, CA), with the same primers used for the PCR reactions. The sequences were

assembled into a contig for each specimen by using the Geneious 6.0.6 Software (Biomatters Ltd., Auckland, New Zealand) and aligned using the multiple sequences alignment algorithm implemented in Clustal Ω [11].

Figure 1: Pinned specimen of *Archytas marmoratus* that emerged from parasitized larvae of *Helicoverpa armigera* from the State of Mato Grosso, Brazil

Results

The two COI sequences obtained were blasted against GenBank database and checked in BOLD Identification System [12] and they matched 94-96% with Archytas spp. sequences from the GenBank but 99.3% with Costa Rican Archytas marmoratus Towsen (Diptera: Tachinidae) sequences from BOLD. According to BOLD [12], this identification is accurate unless there is a very closely allied congeneric species that has not yet been analyzed. Such cases, however, are rare. The sequences from the two Brazilian A. marmoratus were deposited in GenBank (KM099221, KM099222).

Partial sequences of the COI gene (a 480 bp fragment) of the two Brazilian A. marmoratus specimens were used for the estimation of interspecific genetic distances between them and the following 14 tachinids: Lydella thompsoni Herting (Diptera: Tachinidae) (AY649319), Pseudoperichaeta nigrolineata Walker (AY649320), Chrysotachina sp. (JQ575016), Leskia sp. (JQ574740), Lespesia sp. (JQ574724), Lespesia postica (JQ574654), Winthemia sp. (JQ575021), Siphona sp. (JQ574987), Genea sp. (JQ574980), Phytomyptera sp. (JQ576395), Archytas sp. (GU141891), Archytas sp. (JQ574993), Archytas basifulvus (BOLD: AAC5527), and Archytas marmoratus (BOLD: AAJ1909) (numbers in parentheses are the respective GenBank or BOLD Accession numbers). The species Trichogramma pretiosum Riley (Hymenoptera: Trichogrammatidae) (KF573406) and Helicoverpa armigera (GQ995232) were used as outgroups.

The pairwise genetic distances (K2P and p-distance) between the Brazilian specimens and the other species are presented in Table 1. The genetic distance between the two Brazilian A. marmoratus and the A. marmoratus from Costa Rica was only 1.1%, what suggests conspecificity when considering a 2-3% mtDNA COI sequence divergence as the threshold to discriminate the insect species [13,14]. On the other hand, the estimated divergence values between the Brazilian A. marmoratus and the other 14 tachinid species, T. pretiosum and H. armigera were all higher than 4% (Table 1).

Species compared			Kimura two-parameters (K2P)	Uncorrected sequence divergences (p-distance)
Archytas marmoratus from Brazil	vs	*Helicoverpa armigera* (GQ995232)*	24.4	18.4
		Lydella thompsoni (AY649319)	16.2	13.3
		Pseudoperichaeta nigrolineata (AY649320)	13.2	11.2
		Trichogramma pretiosum (KF573406)	37.4	24.9
		Chrysotachina sp. (JQ575016)	16.5	13.5
		Leskia sp. (JQ574740)	17.2	14.0
		Lespesia sp. (JQ574724)	14.4	12.1
		Lespesia postica (JQ574654)	13.8	11.6
		Winthemia sp. (JQ575021)	14.4	12.1
		Siphona sp. (JQ574987)	18.8	15.0
		Genea sp. (JQ574980)	14.4	12.1

		Phytomyptera sp. (JQ576395)	12.9	11.0
		Archytas sp. (GU141891)	7.5	6.8
		Archytas sp. (JQ574993)	6.9	6.3
		Archytas basifulvus (BOLD: AAC5527)	4.3	4.0
		Archytas marmoratus (BOLD: AAJ1909)	1.1	1.1

Table 1: Mitochondrial DNA COI pairwise genetic distances (%) for the Archytas marmoratus specimens collected in Brazil and other species, numbers in parentheses are the respective GenBank or BOLD Accession numbers.

A. marmoratus is a solitary larval-pupal parasitoid of several noctuid species and it is indigenous to the southern United States, Central America, and South America as far south as Chile [15,16]. In Brazil, there a few reports of tachinids parasitizing native pests [17-19]. The females of A. marmoratus are efficient in finding host larvae even at low densities [20] because they can sense the kairomones associated with the frass of the host [21]. Earlier instars of the corn earworm, Helicoverpa zea Boddie (Lepidoptera: Noctuidae), and the tobacco budworm, Heliothis virescens F. (Lepidoptera: Noctuidae), can be successfully parasitized by A. marmoratus, but the parasitoid survival is higher when the later instars are attacked [22]. The eggs laid by the females hatch immediately, releasing planidium-type maggots that penetrate the host larva and molt to second instar after host pupation. The maggots go under the pupa wing pads where they induce a secondary respiratory funnel and complete development, killing the host [22].

Previous studies have demonstrated that the mtDNA COI gene is very promising for the correct detection and discrimination of tachinid parasitoids, which are morphologically very similar [23,24]. Accurate identification of parasitoids is a critical step to the success of biological control programs both during the initial phases when candidate natural enemies are chosen and also during subsequent evaluations phases [25]. The findings of this study, therefore, serve as first evidence that an indigenous species of tachinid exists in Brazil that may be a good biological agent for the invasive H. armigera.

Many studies have suggested a role for A. marmoratus in Area-wide Integrated Pest Management (AW-IPM) through inundative releases, due to the high number of planidia produced by the females and technical feasibility of mass rearing, along with the efficiency of parasitism [20,26]. Gross [27] demonstrated that release rates of 370 to 860 female A. marmoratus per hectare could yield between 50 and 80% parasitism of late instar corn earworm larvae. After a pilot test, Proshold et al. [20] verified that 70% of the parasitized H. zea larvae collected from release fields was superparasitized. In the field tests performed by Carpenter & Proshold [28], nearly 23% of the corn earworm larvae were parasitized and 18% superparasitized, and the authors suggested that inundative releases would be most effective against the first generation to reduce the seasonal increase of H. zea populations.

The results of this study demonstrated that a molecular method was very useful for the accurate identification of an indigenous tachinid fly capable of parasitize field populations of the recently introduced H. armigera in Brazil. This data also shows the great potential of A. marmoratus as controlling agent for AW-IPM programs against this exotic pest in South America. Further investigations of this parasitoid in Brazil must be conducted to determine its geographical distribution, relative abundance and genetic diversity.

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