http://dx.doi.org/10.1590/1519-6984.14813 Original Article

Molecular identification of *Trichogramma* species from regions in Brazil using the sequencing of the ITS2 region of ribosomal DNA

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Received: August 13, 2013 – Accepted: March 19, 2014 – Distributed: May 31, 2015
(With 1 Figure)

Abstract

The objective of this work was the identification and differentiation of *Trichogramma exiguum* Pinto and Platner species, *T. pretiosum* Riley, and *T. galloi* Zucchi using sequences of the ITS2 region of ribosomal DNA. After extracting DNA from the studied species, a PCR reaction was performed, where the amplified samples were subjected to sequencing. The sequences obtained were submitted to a similarity search in GenBank (NCBI - National Center for Biotechnology Information) using the BLAST program, aiming to determine the similarity of these sequences with the species already deposited in the referenced database, and then multiple sequences were aligned using version 2.0 of the ClustalX program. According to the results of the multiple alignments of all sequences obtained, it was possible to observe the differences between the *T. pretiosum*, *T. galloi* and *T. exiguum* species. It was concluded that using the sequences of the ITS2 region of the ribosomal DNA was efficient in the differentiation of the studied *Trichogramma* species, which suggests a strong inter-specific variation among species.

Keywords: molecular systematic, microhymenoptera, parasitoid.

Identificação molecular de espécies de *Trichogramma* de regiões do Brasil utilizando o sequenciamento da região ITS2 do DNA ribossomal

Resumo

O objetivo deste trabalho foi realizar a identificação e diferenciação das espécies *Trichogramma exiguum* Pinto e Platner, *T. pretiosum* Riley e *T. galloi* Zucchi utilizando o sequenciamento da região ITS2 do DNA ribossomal. Após a extração do DNA das espécies estudadas, foi realizada a reação de PCR, onde as amostras amplificadas foram submetidas ao sequenciamento. As sequências obtidas foram submetidas à busca por similaridade no GenBank (NCBI – National Center for Biotechnology Information) por meio do programa BLAST visando-se determinar a similaridade destas com sequências das espécies já depositadas no referido banco de dados e em seguida foi feito o alinhamento múltiplo das sequências com o auxílio do programa CLUSTALX, versão 2.0. De acordo com os resultados do alinhamento múltiplo de todas as sequências obtidas, foi possível verificar as diferenças entre as espécies de *T.pretiosum*, *T. galloi* e *T. exiguum***.** Isto permitiu concluir que a utilização do sequenciamento da região ITS2 do DNA ribossomal foi eficiente na diferenciação das espécies de *Trichogramma* estudadas, o que sugere uma forte variação inter-específica entre as espécies.

Palavras-chave: sistemática molecular, micro-himenópteros, parasitoide.

1. Introduction

The use of *Trichogramma* species for biological control began with Flanders (1930), with work on mass multiplication of *Trichogramma* spp. in *Sitotroga cerealella* eggs Olivier, 1789 (Lepidoptera: Gelechiidae), a technique that rapidly dispersed in several countries (Navarro, 1998).

In South America, the use of *Trichogramma* species is still limited, mainly due to the high commercial production cost of parasitoids and the intensive use of pesticides, though the estimated area using these parasitoids is 1.2 million hectares (Van Lenteren and Bueno, 2003).

In Brazil, this parasitoid has been used on cotton, sugar cane, vegetables, corn, soybeans and tomatoes (Parra and Zucchi, 2004).

Due to the importance of its use, to succeed in a biological control program with the *Trichogramma* species, correct identification is essential, so one can check if they respond to the climatic conditions of the region and culture that they will be applied to (Borba et al., 2005).

The discovery of the male genitalia as a morphological characteristic in the identification of *Trichogramma* species was a major breakthrough in the taxonomy of these parasitoids (Pinto and Stouthamer, 1994). However, in some cases the identification is difficult due to the absence of males in thelytokous species infected by bacteria of the genus *Wolbachia*, the presence of cryptic species and the small size of the individual (Almeida and Stouthamer, 2003; Borba et al., 2005). A more recent method, molecular biology, has been widely used in the taxonomic identification of diverse groups of insects (Borba et al., 2005).

In recent studies the internal transcribed region of space in ITS2 DNA has been used in sequencing for identification of *Trichogramma* species, differentiation of populations, as well as in the reconstruction of phylogenetic relationships between closely related species. This identification method is gaining recent importance in the study of cryptic species of *Trichogramma*, where the polymerase chain reaction is used to amplify these ITS spacers, via universal primers that bind to the highly conserved regions, it is possible to obtain the sequence and thus, the description of the species (Samara et al., 2008; Sumer et al., 2009).

Species particularly difficult to identify with the use of traditional taxonomic techniques may have their identities clarified using molecular sequencing techniques or sequence length polymorphism generated through the techniques of molecular markers (Borba et al., 2005).

The molecular data represents a valuable alternative to the taxonomy of *Trichogramma*, since many failures in biological control programs with *Trichogramma* were due, in part, to the misidentification of species (Querino and Zucchi, 2003). Thus, this work aimed to identify and differentiate species of *Trichogramma exiguum* Pinto and Platner, *T. pretiosum* Riley, and *T. galloi* Zucchi using sequences of the ITS-2 region of ribosomal DNA.

2. Material and Methods

2.1. Obtaining the sample Trichogramma species

Three species of *Trichogramma* were obtained for the study, *Trichogramma exigum* Pinto e Platner, 1983, from the municipality of Alegre – ES, *T. galloi* Zucchi, 1988 of Dourados – MS and *T. pretiosum* Riley, 1879 from Primavera do Leste – MT, all of the species were previously identified using morphological characteristics.

2.2. DNA extraction, amplification of ITS2 ribosomal DNA by chain reaction (PCR) and electrophoresis

Molecular studies were conducted in the laboratory of Biotechnology, Embrapa-Algodão in Campina Grande, PB and Molecular Biology Laboratory (LABIME), Universidade Federal da Paraíba, João Pessoa, PB.

The genomic DNA for each sample insect was extracted macerating five individuals of the same species in an Eppendorf tube along with 100 µl of Chelex resin (5%) and 4 µl of proteinase K, incubated at 56 °C bath for 4h, heated afterwards for 10 minutes at a temperature of 95 °C. This solution became the stock of genomic DNA from the insects and then stored in a freezer at 0 °C (Almeida, 2004).

The integrity and quality of the extracted genomic DNA were analyzed in a spectrophotometer (NANODROP ® Thermo Scientific 2000), the equipment provides the amount of DNA in ng/µL and the ratio of two optical densities read (260/280 nm) to verify the purity level of the sample.

The primers used to amplify the ITS-2 region were 5'TGTGAACTGC AGGACACATG3' (forward) and 5'GTCTTGCCTGCTCTGCTCTGAG3' (reverse) (Stouthamer et al., 1999). The PCR reaction was performed in a total volume of 50 µl using a thermocycler, 7 µl DNA template, 5 µl (10×) Taq assay buffer, 1 µl dNTP's (each in a 10 mM concentration), 1 µl forward and reverse primers (10 picomoles/ µl), 0,25 taq polymerase (1 U), 4 µl $MgCl_2$ and 30,75 µl of sterile distilled water. For the PCR reaction a negative control was used without any DNA and containing the remaining components. Amplifications were performed with a final reaction volume of 50 µl in a thermocycler programmed for 3 minutes at 94°C, followed by 33 cycles of 40 seconds at 94°C, 45 seconds at 53°C and 45 seconds at 72°C with a final extension of 5 minutes at 72°C. After the final extension, the specimens were stored at 40°C in the thermocycler until they were stored in a freezer at 0°C (Almeida, 2004).

For the analysis of the product reaction, an aliquot of 20 µl was removed and subjected to electrophoresis in 0.8% agarose gel in TAE buffer (Tris-Acetate-EDTA), with an electrophoretic run carried out at a constant voltage of 100V/75, stained with ethidium bromide for 20 minutes, visualized on a transilluminator. To determine the size of the PCR products, a 100 bp molecular weight marker was used (Promega, USA). For the PCR reaction a negative control was used without DNA and the remaining components.

2.3. Purification of ribosomal DNA fragments, PCR for sequencing the ITS2 of ribosomal DNA

The fragments of the amplified PCR product corresponding to 500 bp were cut from the gel with a scalpel and purified by a purification kit (DNA Gel Extraction Kit – Fermentas, USA) according to the protocol proposed by the manufacturer, so that sufficient quality and quantity for DNA sequencing could be obtained.

The sequencing reactions were performed in the Molecular Biology Laboratory (LABIME/UFPB). They were done in duplicate and inserted into 96-well microplates using the DNA Sequencing Kit – Big Dye Terminator Cycle Sequencing Ready ABI Prism, version 3 (Applied Biosystems, USA), in which the same primers ITS-R 2 were used.

The samples of PCR products were inserted in a thermocycler (Gene Amp PCR System 9700 appied

Table 1. Identification of three species of *Trichogramma*, by comparing the consensus sequence with sequences obtained from the GenBank database – NCBI.

Samples	Primer	Access number	Maximum identification	BLAST Species
T.pretiosum	ITS-2- R	DQ525178.1	95%	*T.pretiosum*
T. galloi	ITS-2- R	AY182764.1	91%	*T.galloi*
T. exiguum	ITS-2- R	AY182769.1	92%	*T. exiguum*

Biosystems, USA) using specific thermocycling (96°C/10 Sec–53°C / 20 sec –60°C / 1 min), samples remained at 4°C until they were removed for sequencing.

After amplification, 80 µl of isopropanol (75%) was added to the samples and left for 15 min at room temperature and then centrifuged at 1300 × g for 60 minutes. After centrifugation, the supernatant was discarded and the plates remained inverted for 5 minutes on absorbent paper. 150 µl of ethanol (70%) was added and centrifuged again at 1300 × g for 15 min.

The supernatant was then discarded and the plates were placed in a laminar flow hood for approximately 1 h protected from the light. Then the samples were re-suspended in 10 µl of Hi – Di formamide, heated to 95°C for 5 min in the thermal cycler, cooled on ice and subjected to sequencing using the ABI PRISM ® 3100 Genetic Analyses sequencer (Applied Biosystems, USA).

2.4. Aligning the sequences

The sequences were submitted to a search for similarity in GenBank (NCBI – National Center for Biotechnology Information) using the BLAST program (Altschul et al., 1990), aiming to determine the similarity to sequences already deposited in that database. Multiple alignments of the sequences were performed with the aid of the CLUSTALX program, version 2.0 (Larkin et al., 2007).

3. Results

The sequencing of *Trichogramma* species using the ITS2 of ribosomal DNA was carried out aiming at the identification of nucleotide sequences and confirmation of the studied species.

With the results of the search for similarities by BLAST tool in the GenBank database (NCBI – National Center for Biotechnology Information), it was found that they all correspond to the desired regions, consistent with the sequences of *Trichogramma* species, obtaining 95% similarity to *T. pretiosum*, *a value of 91% for T. galloi* and 92% for *T. exiguum* (Table 1).

The E values obtained from all aligned sequences with the sequences from the GenBank database were equal and close to 0 (zero), indicating a lower probability that these sequences were obtained by accident, confirming correct sequencing.

According to the results of multiple alignments of all the obtained consensus sequences, it was possible to detect differences between the species *T. pretiosum*, *T. galloi* and *T. exiguum* in regards to the presence or absence of nitrogenous bases and its distribution in the sequence. A small amount of gaps (spaces), were needed to align the sequences of samples, however for the analyses the 5' and 3' were removed to match the size of the sequences.

Table 2. Genetic distance matrix of ITS2 ribosomal DNA for three species of Trichogramma.

Species	*T. exigum*	*T. galloi*	*T. pretiosum*
T. exiguum	0		
T. galloi	0, 238	0	
T. pretiosum	0, 189	0, 215	0

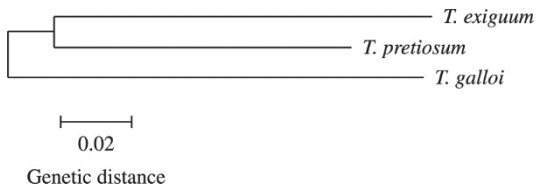

Figure 1. Dendrogram based on analysis of nucleotide sequences of ITS2 ribosomal DNA of three *Trichogramma* species using the neighbor-joining method.

Using the genetic distance matrix (Table 2), it can be seen that species *T. galloi* and *T. exiguum* were more genetically distant with 0.238 and *T. exiguum* and *T. pretiosum* were closer having a genetic distance of 0.189.

According to the dendrogram obtained from the genetic distance matrix using the neighbor-joining method, the presence of three groups can be verified: the first formed by *T. exigum*, the second by *T. pretiosum*, and the third, by *T. galloi* (Figure 1).

4. Discussion

The *T. pretiosum*, *T. exiguum* and *T. galloi* species, morphologically similar, differ genetically with a high level of dissimilarity. The use of sequencing of ITS2 ribosomal DNA given here is efficient in the identification and differentiation of the species *T. pretiosum*, *T. exiguum* and *T.galloi*.

Similar results were obtained by Almeida (2004) and Ciociola Junior et al. (2001) to identify the species *T. galloi*, *T. exiguum*, *T. pretiosum* using the ITS 2 rDNA region.

Other authors have noted the advantage of using the ITS2 region of rDNA for species identification, and employed for the identification of *Trichogramma*, for example, Silva et al. (1999). In the same way Davies et al. (2006) used the ITS 2 region of rDNA to easily differentiate *T. pretiosum* species of *Trichogramma australicum* Girault.

This criterion was used by Espanã-Luna et al. (2008) who used the percentage of similarity between the ITS2 sequences of the ribosomal DNA to identify the species of *T. pretiosum* and *T. exiguum* among others.

Sequencing of the ITS2 ribosomal DNA is a relatively simple technique for the correct identification of the *T. pretiosum*, *T. exiguum* and *T.galloi* species. This method may be used in quality control evading possible contamination in the laboratory between the studied species. According to Almeida (2004) identification by combining morphological and molecular techniques are extremely important for a successful systematic analysis of this group. Molecular methods are important tools in the identification of which species are present in a given area when it is necessary to complement other methods of identification, and are also useful to determine the possible contamination or mixture of species in the laboratory, mass reproduction and dispersion studies and evaluation of parasitoids released in the field or native in a region Silva et al. (1999). Thomson et al. (2003) mentioned that the ITS2 region is useful to identify *Trichogramma* species, because the variation of intra-species sequences is relatively small compared to the variations between species, and also because the species have cryptic enough variation of the ITS2 region to be distinguished. It can be concluded that using the sequencing of the ITS2 region of the ribosomal DNA was efficient in the differentiation of the studied *Trichogramma* species, which suggests a strong inter-specific variation among species.

Acknowledgements

We would like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior for financial aid for the granting the scholarship of the author, the Brazilian Agricultural Research Company/Cotton Embrapa/CNPA, for access to the Biotechnology Laboratory and Research, Dra. Teresa Cristina Department of Molecular Biology, University Federal of Paraíba, for valuable technical assistance.

References

ALMEIDA, RP. and STOUTHAMER, R., 2003. Molecular identification of *Trichogramma cacoeciae* Marchal (Hymenoptera: Trichogrammatidae): a new record for Peru. *Neotropical Entomology*, vol. 32, no. 2, p. 269-272. http://dx.doi.org/10.1590/S1519-566X2003000200011.

ALMEIDA, RP., 2004. *Trichogramma and its relationship with Wolbachia: identifi cation of Trichogramma species, phylogeny, transfer and costs of Wolbachia symbionts*. Netherlands: Wageningen University. 142 p. Doctoral thesis.

ALTSCHUL, SF., GISH, W., MILLER, W., MYERS, EW. and LIPMAN, DJ., 1990. Basic local alignment search tool. *Journal of Molecular Biology*, vol. 215, no. 3, p. 403-410. http://dx.doi.org/10.1016/S0022-2836(05)80360-2. PMid:2231712

BORBA, RS., GARCIA, MS., KOVALESKI, A., OLIVEIRA, AC., ZIMMER, PD., CASTELO BRANCO, JS. and MALONE, G., 2005. Dissimilaridade genética de linhagens de *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) através de marcadores moleculares ISSR. *Neotropical Entomology*, vol. 34, no. 4, p. 565-569. http://dx.doi.org/10.1590/S1519-566X2005000400005.

CIOCIOLA-JUNIOR, AI., ZUCCHI, RA. and STOUTHAMER, R., 2001. Molecular key to seven brazilian species of *Trichogramma* (Hymenoptera: Trichogrammatidae) using sequences of the ITS2 region and restriction analysis. *Neotropical Entomology*, vol. 30, no. 2, p. 259-262. http://dx.doi.org/10.1590/S1519-566X2001000200008.

DAVIES, AP., LANGE, CL. and O'NEILL, SL., 2006. A rapid single-step multiplex method for discriminating between Trichogramma (Hymenoptera: Trichogrammatidae) species in Australia. *Journal of Economic Entomology*, vol. 99, no. 6, p. 2142-2145. http://dx.doi.org/10.1093/jee/99.6.2142. PMid:17195685

ESPANÃ-LUNA, MP., GONZÁLEZ-HERNÁNDEZ, A., ALVARADO-GÓMEZ, OG. and LOZANO-GUTIÉRREZ, J., 2008. Identificación molecular de especies crípticas de *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) de importancia agrícola en México. *Acta Zoológica Mexicana*, vol. 24, p. 1-14.

FLANDERS, SE., 1930. Mass production of eggs parasites of the genus *Trichogramma*. *Hilgardia*, vol. 4, p. 465-501.

LARKIN, MA., BLACKSHIELDS, G., BROWN, NP., CHENNA, R., MCGETTIGAN, PA., MCWILLIAM, H., VALENTIN, F., WALLACE, IM., WILM, A., LOPEZ, R., THOMPSON, JD., GIBSON, TJ. and HIGGINS, DG., 2007. Clustal W and Clustal X version 2.0. *Bioinformatics*, vol. 23, no. 21, p. 2947-2948. http://dx.doi.org/10.1093/bioinformatics/btm404. PMid:17846036

NAVARRO, MA., 1998. *Trichogramma spp. Produción, uso y manejo en Colombia*. Guadalajara de Buga: Impretec. 176 p.

PARRA, JRP. and ZUCCHI, RA., 2004. Trichogramma in Brazil: feasibility of use after twenty years of research. Neotropical *Entomology*, vol. 33, no. 3, p. 271-281.

PINTO, JD. and STOUTHAMER, R., 1994. Systematics of the Trichogrammatidae with emphasis on Trichogramma. In WAJNBERG, E. and HASSAN, SA. (Eds.). *Biological control with egg parasitoids*. Wallingford: CAB International. p. 1-36.

QUERINO, RB. and ZUCCHI, RA., 2003. New species of *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) associated with lepidopterous eggs in Brazil. *Zootaxa*, vol. 163, p. 1-10.

SAMARA, R., MONJE, JC., REINEKE, A. and ZEBITZ, CPW., 2008. Genetic divergence of *Trichogramma aurosum* Sugonjaev and Sorokina (Hymenoptera: Trichogrammatidae) individuals based on ITS2 and AFLP analysis. *Journal of Applied Entomology*, vol. 132, no. 3, p. 230-238. http://dx.doi.org/10.1111/j.1439-0418.2007.01245.x.

SILVA, IMMS., HONDA, J., VAN KAN, F., HU, J., NETO, L., PINTUREAU, B. and STOUTHAMER, R., 1999. Molecular differentiation of five *Trichogramma* species occurring in Portugal. *Biological Control*, vol. 16, no. 2, p. 177-184. http://dx.doi.org/10.1006/bcon.1999.0755.

STOUTHAMER, R., VAN KAN, FJPM., PLATNER, GR. and PINTO, JD., 1999. The utility ofinternally transcribed spacer 2 DNA sequences of the nuclear ribosomal gene for distinguishingsibling species of Trichogramma. *BioControl*, vol. 43, p. 421-440.

SUMER, F., TUNCBILEK, AS., OZTEMIZ, S., PINTUREAU, B., RUGMAN-JONES, P. and STOUTHAMER, R., 2009. A

molecular key to the common species of *Trichogramma* of the Mediterranean region. *BioControl*, vol. 54, no. 5, p. 617-624. http://dx.doi.org/10.1007/s10526-009-9219-8.

THOMSON, LJ., RUNDLE, BJ., CAREW, ME. and HOFFMANN, AA., 2003. Identification and characterization of *Trichogramma* species from south-eastern Australia using the internal transcribed spacer 2 (ITS-2) region of the ribosomal gene complex. *Entomologia Experimentalis et Applicata*, vol. 106, no. 3, p. 235-240. http://dx.doi.org/10.1046/j.1570-7458.2003.00029.x.

VAN LENTEREN, JC. and BUENO, VHP., 2003. Augmentative biological control of arthropods in Latin America. *BioControl*, vol. 48, no. 2, p. 123-139. http://dx.doi.org/10.1023/A:1022645210394.