SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Molecular Key to Seven Brazilian Species of *Trichogramma* (Hymenoptera: Trichogrammatidae) Using Sequences of the ITS2 Region and Restriction Analysis

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Chave Molecular para Sete Espécies Brasileiras de *Trichogramma* (Hymenoptera: Trichogrammatidae) Utilizando o Sequenciamento da Região ITS2 e Análise de Restrição

RESUMO - Utilizando-se o sequenciamento da região ITS2 do DNA ribossomal juntamente com algumas enzimas de restrição, pôde-se construir uma chave molecular simples e precisa de algumas espécies brasileiras de *Trichogramma*. Esta chave é fácil de ser elaborada e resultados rápidos são obtidos na identificação desse pequeno parasitóide (0,25 mm). Usando-se essa metodologia, pode-se também verificar possíveis contaminações em criações de laboratório.

PALAVRAS-CHAVE: Insecta, DNA, enzimas, controle biológico.

ABSTRACT - Using the ITS2 sequences of the ribosomal DNA together with some restriction enzymes, a simple and precise molecular key to some Brazilian species of *Trichogramma* was created. This key is very easy to make and quick results can be obtained on the identification of this minute parasitoid (0.25 mm). The methodology presented is easily implemented and can be used to detect possible contaminations under laboratory rearings.

KEY WORDS: Insecta, DNA, enzymes, biological control.

In biological control programs using parasitoids of the genus *Trichogramma* (Hymenoptera: Trichogrammatidae), a very important step is the identification and the use of correct species to be released in the field. The identification of these wasps is difficult due to their size (0.25 mm in length) and only specialists can reliably identify specimens, using characteristics of the male genitalia. In the past, *Trichogramma* identification was based on body color and setation as morphological parameters. Later, Nagarkatti & Nagaraja (1971) found out the taxonomic use of male genitalic characteristics. This lead to an improvement of the identification of these minute wasps. Using this technique, around 200 species have been described (Pinto 1998). Due to the small size and few characters available, specific identification is still difficult, particularly when sibling species need to be distinguished. In addition, the specimen preparation for morphological identification is time consuming and requires much trainning. In this paper, the identification of this minute wasp was based on DNA sequences of the internally transcribed spacer (ITS-2). This technique can be applied by any lab with access to a PCR equipment and it allows a quick and precise *Trichogramma* species identification. At the species and intraspecific levels, the ITS1 and ITS2 regions have been often used as a taxonomic tool for insect identification (Campbell *et al.* 1993, Hoy 1994). The sequence and restriction analysis of the ITS2 rDNA has been described in recent studies as a tool for *Trichogramma* identification as well (Kan *et al*. 1996, Pinto *et al*. 1997, Stouthamer *et al*. 1999). Using this technique, it was possible to identify seven *Trichogramma* species and to elaborate a molecular key to these species recorded in Brazil.

Material and Methods

***Trichogramma* Cultures**. Populations of seven species (Table 1): *T. acacioi*, from Botucatu-SP, *T. galloi* Zucchi from

Copersucar Company, Piracicaba-SP, *T. pretiosum* Riley from several locations, *T. rojasi* Nagaraja & Nagarkatti and *T. lasallei* Pinto from Curitiba-PR, *T. bruni* from Piracicaba-SP, and one population of *T. atopovirilia* Oatman & Platner from Venezuela, were used to elaborate the molecular key. The population from Venezuela was used because no success on DNA extraction of one population of *T. atopovirilia* from Brazil was obtained.

of some nucleotides to be specific to *Trichogramma*. The termocycler program used was: 3 min. at 94°C, followed by 33 cycles of 40 seconds at 94°C, 45 seconds at 55°C and 45 seconds at 72°C, with 5 min. at 72°C after the last cycle.

The PCR product was purified with a QIAquick PCR purification kit (Qiagen®). After the purification the PCR product was tied up to a pGEM-T® vector (Promega), 2 µl of the Ligation mix was transformed in the heatshock cells of DH5-α *E. coli* and plated in a LB agar medium containing

Table 1. *Trichogramma* spp. lines used on the sequence of the ITS2 region of the rDNA.

Line	Number	Source/Collector	Host
T. pretiosum	TP-02	EPAGRI-SC/ Dirceu Pratissoli	-
T. pretiosum	TP-03	Quixadá-CE/ Raul P. Almeida	*Alabama argillacea*
T. pretiosum	TP-04	Alegre-ES/ Dirceu Pratissoli	*Tuta absoluta*
T. pretiosum	TP-05	V. N. Imigrante-ES/ Dirceu Pratissoli	*T. absoluta*
T. pretiosum	TP-06	Lavras-MG/ Lusinério Prezotti	*Helicoverpa zea*
T. pretiosum	TP-07	Piracicaba-SP/Lusinério Prezotti	*H. zea*
T. pretiosum	TP-10	Campo Novo dos Parecis-MT/Raul Almeida	*A. argillacea*
T. pretiosum	TP-11	Campina Grande-PB/Raul P. Almeida	Neuroptera eggs
T. pretiosum	TP-12	Campina Grande-PB/Raul P. Almeida	*Trichoplusia ni*
T. pretiosum	TP-13	Barreias-BA/Ivan Cruz	*Spodoptera frugiperda*
T. pretiosum	TP-15	CNPMS/EMBRAPA(área n. 1)/Ivan Cruz	*H. zea*
T. pretiosum	TP-16	CNPMS/EMBRAPA(área n. 2)/Ivan Cruz	*H. zea*
T. pretiosum	TP-17	Dourados-MS/Paulo DeGrande	*A. argillacea*
T. acacioi	TAC-18	Botucatu-SP/ Elisabete Bernardi	*Euselasia* sp.
T. galloi	TG-19	Piracicaba-SP/Copersucar	*Diatraea saccharalis*
T. lasallei	TL-20	Rio Grande Farm, Curitiba-PR/L.A. Foerster	*Anticarsia gemmatalis*
T. atopovirilia [1]	TA-21	Venezuela/Francisco Ferrer	-
T. bruni	TB-22	ESALQ/USP/João Cerignoni	*Heliconius phyllis*
T. rojasi	TR-23	Rio Grande Farm, Curitiba-PR/L.A. Foerster	*A. gemmatalis*

[1]Population provided by the Department of Entomology, Wageningen University, The Netherlands.

DNA Extraction by Chelex-100. Five individuals from each isofemale population, preserved in 100% ethanol, were shaken in 100 µl of TE before grinding. The wasps were ground with 100 µl of 5% Chelex-100 and 4µl of proteinase K (20mg/ml), incubated at least 6h at 56°C followed by 10 minutes at 95°C.

PCR, Cloning by *Escherichia coli* and Sequencing the ITS2 Region of the rDNA. The PCR was done in a total volume of 50 µl using a Techne thermocycler. For one reaction, 5 µl of DNA template were used, plus 45 µl of the PCR mix (5 µl of PCR buffer, 1µl of dNTP's each in a 10mM concentration), 1 µl of the ITS2 forward primer (5'-TGTGAACTGCAGGACACATG-3') located in the 5.8s region of rDNA, 1 µl of the reverse primer (5'-GTCTTGCCTGCTCTGAG-3'), located in the 28s region of rDNA, 0.14 µl of superTAQ polymerase (Sphaero-Q 5 units/µl and 36.86 µl of bidestiled and autoclaved water). The primers used to amplify the ITS2 region were the same used by Campbell *et al*. (1993) with sequence modification

Ampicillin, X-GAL and IPTG. The plates were stored overnight at 37°C. The next day, white colonies were picked up with a sterile toothpick from the plates and placed into tubes containing 3.5 ml of LB liquid medium and grown overnight in a shaker set to 250 rpm and 37°C. To confirm that the correct piece of DNA had been cloned, a PCR reaction with a template extracted from the bacterial culture was performed. Ten microliters of bacteria culture was added to 100 µl 5% Chelex and incubated for 15 min. at 60°C followed by 5 min. at 95°C. The PCR was performed in a final volume of 25 µl. If indeed an ITS-2 had been cloned, 850 µl of the bacteria culture was added with 150 ml of 87% glycerol and stored at –80°C. The rest of the culture was used in a QIAprep Miniprep kit (Qiagen®) to purify the plasmid, which was used for the sequencing in a Applied Biosystems automatic sequencer.

Alignment of the Sequences and Restriction Analysis. The sequences of the six *Trichogramma* species were aligned manually using ESEE 3.0s sequence editor (Cabot 1995).

The sequence sizes data and restriction analysis were used to elaborate a molecular key. The PCR products were digested with *Eco* RI, *Mse* I, *Pvu* I and *Nru* I in a total volume of 10 µl, and incubated at 37°C for 1h.

Results and Discussion

The five individuals used for DNA extraction were enough to obtain good PCR products (Fig. 1) for cloning and for sequencing. All the sequences (Table 1) were sent to the Genbank and have the accession numbers: AF282222-AF282239. Using the sizes of the PCR products (Fig. 1) with the enzymes *Eco* Rl, *Mse* l , *Pvu* I and *Nru* I (Table 2), all the species were distinguished in this work. The key made (Table 3) shows that the sequence size of the ITS2 region of the rDNA of some species of *Trichogramma* is useful and can be used as a tool to quickly identify species. It is important to know that this key fits well for the populations studied in this work. More samples are needed in order to construct a complete key in the future with all other *Trichogramma* species from Brazil. With a molecular key of all Brazilian species as well as all the sequences, it can be easily known if new species are occurring in Brazil just comparing the recorded sequences of each species. Currently, 14 species are recorded in Brazil (Zucchi & Monteiro 1997).

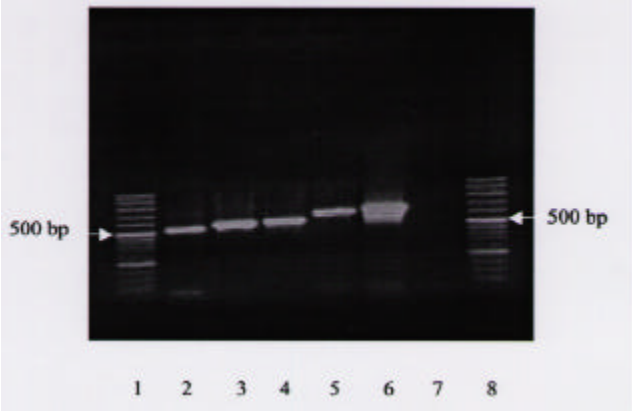

Figure 1. Agarose gel showing PCR products plus flanking regions of 89 bp of the 5.8S and 24 bp of the 28S rDNA genes, of five Brazilian *Trichogramma* species: Lines 1 and 8: molecular markers, Line 2: *T. pretiosum* (TP-07), Line 3: *T. galloi*, Line 4: *T. lasallei*, Line 5: *T. rojasi*, Line 6: *T. acacioi*, Line 7: negative control.

Sequences of the ITS2 region of the rDNA presented in this paper show their importance for identification of *Trichogramma* species, which were previously identified only by morphological characteristics. Using the sequence of the ITS2 region, closely related species of *Trichogramma* can also be distinguished (Stouthamer *et al.* 1999). *Trichogramma* identification using RAPD-PCR (Vanlerberghe-Masutti 1994) has several problems when compared to what is presented in this work. In RAPD there are problems of transportability and at least 50 individuals are required to obtain good results. The technique used in this study is relatively cheap and gives quick results not only on *Trichogramma* identifications, but it also allows to monitorate laboratory rearings for possible contaminations. Another advantage of this technique is that in field parasitism surveys, a quick rate of parasitism on collected host eggs can be determined by PCR (Amornsak *et al.* 1998). Morphological data are extremely important when combined with molecular work. Both taxonomy data and sequences of the ITS2 region of the rDNA can give a precise identification of *Trichogramma* species, and it will be an improvement for the use of this parasitoid in biological control programs against several lepidopterous pests. To make a complete key from Brazilian *Trichogramma* species more samples must be collected, which will be done by comparing the sequences presented in this work with the taxonomic data of a new sample.

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Table 2. Size (number of nucleotides) of the ITS2 region, plus flanking regions of 89 bp of the 5.8S and 24 bp of the 28S rDNA genes, and the position of restriction sites generated by the enzymes *Eco* RI, *Mse* I, *Pvu* I and *Nru* I, belonging to some species of *Trichogramma*.

Strains	Size of the ITS2 region of rDNA	*Eco* RI	*Mse* I	*Pvu* I	*Nru* I
T. acacioi	668	-	106	217	-
T. atopovirilia	669	-	699	-	-
T. pretiosum	522-528	-	-	-	-
T. galloi	556	192	-	-	-
T. lasallei	566	566	-	-	161
T. rojasi	639	639	-	-	639
T. bruni	662	-	98	204, 363	-

Table 3. Molecular key to seven Brazilian species of *Trichogramma* based on the PCR product plus flanking regions and the enzymes *Eco* RI, *Mse* l, *Pvu* I and *Nru* I.

1. PCR product bigger than 660 bp*	2
1' PCR product smaller than 660 bp	3
2. PCR product cut by the enzyme *Mse* I	4
2' PCR not cut by the enzyme *Mse* I	*T. atopovirilia*
3. PCR product bigger than 550 bp	5
3' PCR product between 522-528 bp	*T. pretiosum*
4. PCR product cut once by the enzyme *Pvu* I	*T. acacioi*
4' PCR product cut twice by the enzyme *Pvu* I	*T. bruni*
5. PCR product cut by the enzyme *Eco* RI	*T. galloi*
5' PCR product not cut by the enzyme *Eco* RI	6
6. PCR product cut once by the enzyme *Nru* I	*T. lasallei*
6' PCR not cut by the enzyme *Nru* I	*T. rojasi*

* bp = base pairs

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