SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Molecular Tool for Identification of Closely Related Species of *Trichogramma* (Hymenoptera: Trichogrammatidae): *T. rojasi* Nagaraja & Nagarkatti and *T. lasallei* Pinto

AMÉRICO I. CIOCIOLA JR.[1], RANYSE B. QUERINO[1], ROBERTO A. ZUCCHI[1] AND RICHARD STOUTHAMER[2]

[1] *Depto. de Entomologia, Fitopatologia e Zoologia Agrícola, ESALQ/USP, Av. Pádua Dias, 11, CEP 13.418-900 Piracicaba, SP*
[2] *Dept. of Plant Sciences, Laboratory of Entomology, Wageningen University, P.O. Box 8031, 6700 EH, Wageningen, The Netherlands*

Neotropical Entomology 30(4): 575-578 (2001)

Técnica Molecular para a Identificação de Espécies Próximas de *Trichogramma* (Hymenoptera: Trichogrammatidae): *T. rojasi* Nagarkatti & Nagaraja e *T. lasallei* Pinto

RESUMO – O sequenciamento da região ITS2 (espaço interno transcrito) do rDNA foi utilizado para identificar duas espécies próximas de *Trichogramma*: *T. rojasi* e *T. lasallei*, esta última recentemente constatada no Brasil. O DNA das amostras foi extraído utilizando-se Chelex 100. O produto de PCR foi ligado ao vetor pGEM-T ® e ambos foram então acondicionados em placas de Petri para o crescimento de colônias brancas, o que constata a ligação correta entre o vetor e o DNA. A partir dessas colônias, uma pequena quantidade (10µl) foi purificada utilizando material específico e seqüenciada. As seqüências foram alinhadas pelo programa ESEE 3.0. Os *primers* específicos utilizados para a amplificação do rDNA da região ITS2 das espécies de *Trichogramma* estudadas foram eficientes. O sequenciamento das duas espécies diferiu em comprimento e posição dos nucleotídeos, mostrando que são distintas. Os resultados mostraram que esta é uma boa técnica para identificação de espécies crípticas de *Trichogramma*, difíceis de identificar quando são utilizados apenas caracteres morfológicos.

PALAVRAS-CHAVE: Insecta, região ITS2, controle biológico, rDNA, parasitóide.

ABSTRACT – The sequence of the ITS2 (internally transcribed spacer 2) region of the rDNA was used to identify two closely related *Trichogramma* species: *T. rojasi* and *T. lasallei*, the second species recently reported in Brazil. The DNA of the samples was extracted using Chelex 100. The PCR product was linked to a pGEM-T® vector and both were then placed into Petri dishes to grow white colonies, which demonstrate the correct ligation between the vector and the DNA. From these colonies, 10µl were purified and sequenced using a special kit, and the sequences aligned using the ESEE 3.0 software. Specific primers were efficient to amplify the ITS2 rDNA region of the Brazilian *Trichogramma*. The sequence of each species differed in length and in nucleotide position, showing that they are distinct. Thus, the results show that this technique is a good tool to identify *Trichogramma* cryptic species, otherwise difficult to identify when using only morphological characters.

KEY WORDS: Insecta, ITS2 region, biological control, rDNA, parasitoids.

Parasitoids of the genus *Trichogramma* are minute wasps (about 0,25mm in length) and are the most important egg parasitoids used in biological control programs against lepidopterous pests (Parra 1997). The taxonomy of this group is difficult due to its small size. Before the work done by Nagarkatti & Nagaraja (1971), who used the male genitalia of *Trichogramma* species for identification and started a modern taxonomy for this genus, the identification was made only using color and setation (Stouthamer *et al*. 1999). The use of the male genitalia to identify the almost 200 species of *Trichogramma* (Pinto 1998) is still a problem because some of them multiply by parthenogenesis. In addition to that, several populations are similar when the male genitalia is analysed. The ribosomal DNA (rDNA) has been reported as an important tool for taxonomic work since these ribosomal genes have been highly preserved throughout evolution (Orrego & Agudelo-Silva 1993, Hoy 1994, Pinto *et al*. 1997). The purpose of this work was to use the technique of

sequencing the ITS2 region of the rDNA recently developed by Stouthamer *et al*. (1999), for the first time used in Brazil, to identify two Brazilian *Trichogramma* cryptic species, *T. rojasi* and *T. lasallei*.

Material and Methods

***Trichogramma* Cultures**. Cultures of *T. rojasi* and *T. lasallei* were collected on soybeans at Rio Grande Farm, Curitiba county, Paraná state. The parasitoids were reared on *Anagasta kuehniella* (Zeller) (Lep.: Pyralidae) eggs, under 70±10% relative humidity, 25±2°C temperature and 14h photophase (Parra 1997).

Taxonomy of *T. rojasi* and *T. lasallei*. Samples of males of each species were mounted on microscope slides using Hoyer's medium (Rosen & DeBach 1979) and the identification of the two species were based on the morphology of the genitalia and on the posterior setal track of the hind wing (Pinto 1998).

DNA Extraction by Chelex-100. Five individuals from isofemale populations of each species were shaken in 100µl of TE, ground in 100 µl of 5% Chelex-100 and 4 µl of proteinase K (20 mg/ml) and incubated overnight at 56°C followed by 10 min. at 95°C.

PCR, Cloning and Sequencing the ITS2 Region of the rDNA. A PCR was performed in a total volume of 50 µl using a Techne thermocycler. For one reaction, 5 µl of DNA template (1,6 µg/ml) were used, with 45 µl of the PCR mix [5 µl of 10x PCR-buffer, 1 µl of dNTP's (each at 10mM), 1µl of the ITS2 forward primer (5'-TGTGAACTGGAGGACACATG-3') located in the 5.8s region of rDNA, 1 µl of the reverse primer (5'-GTCTTGCCTGCTGAG-3'), located in the 28s region of the rDNA, 0.14 µl of TAQ polymerase 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 modifications on the position of some nucleotides to be more specific to *Trichogramma*. The thermocycler program used was as follows: 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 purification, the PCR product was linked to a pGEM-T ® vector (Promega). The ligation (2 µl) mix was transformed in the heat shock cells off DH5-α *E. coli* and plated in a LB agar medium containing 3,5 ml of LB liquid medium with Ampicillin, X-GAL and IPTG and let to grow overnight in a shaker set at 250 rpm and 37°C. The bacterial culture (10 µl) was added to 100 µl Chelex 5%, and incubated during 15 min. at 60°C followed by extra 5 min. at 95°C. A PCR, with the final volume of 25 µl, was done to confirm if the correct DNA inserted had been cloned. When the reaction was positive, 850 µl of bacterial culture was added to 150 µl of 87% Glycerol and stored at -80°C. The remaining culture was used in a QIAprep Miniprep kit by Qiagen ® and sequenced on an Applied Biosystems automatic sequencer.

Alignment of Sequences. The *T. rojasi* and *T. lasallei* sequences were manually aligned using the ESEE 3.0s sequence editor (Cabot 1995), and sent to the Genbank with the acession numbers AF-282237 and AF-282239, respectively.

Results and Discussion

The use of five individuals from each *Trichogramma* population with Chelex-100 to grind the samples and extract the DNA gave enough PCR products to be cloned and sequenced.

The PCR product was enough to clone and sequence the ITS2 region of the rDNA. After sequencing it was found the length of the ITS2 region to be 453 bp (base pairs) for *T. lasallei* (TL-20) and 526 bp for *T. rojasi* (TR-23). By using the ITS2 region of the rDNA it was possible to distinguish these two closely related species, and several differences were found among the sequences showing that the species are distinct (Table 1).

Based on the taxonomic studies of *T. lasallei* and *T. rojasi* by Pinto (1998) and together with the sequence of each population studied *T. lasallei* was confirmed as a new record for Brazil.

The report of *T. lasallei* for Brazil is recent (Ranyse Barbosa Querino, personal communication), and this species is morphologically similar to *T. rojasi*, already found in the country. The two species are sympatric and were collected in the state of Paraná. The morphological characteres to differentiate these two species were discussed by Pinto (1998), where *T. lasallei* differs from *T. rojasi* primarily by the broad genital capsule, the more anterior position of the ventral process, the narrower and slightly longer setae on the male flagellum, the shorter anterior mesoscutellar setae, and the shorter posterior setal track of the hind wing. On *T. rojasi* the genital capsule is only ca. 0.3 as wide as long, the ventral processes are positioned at less than 0.1 the basal distance, and the posterior track of the hind wing consists of ca. 12 elongate setae and reaches the wing apex. By contrast, in *T. lasallei* the genital capsule is ca. 0.35 as wide as long, the ventral process is positioned at 0.2 the basal distance, and the posterior track of the hind wing is short, consisting of only 5-8 setae and does not approach the wing apex. As seen, there are small differences between *T. rojasi* and *T. lasallei* and, in some cases, the identification of these parameters is difficult (Pinto 1998). So, to clarify the identification of these two species, the sequence of their ITS2 regions of the rDNA can easily be done to confirm the taxonomic data. Sequences of the ITS2 region of the rDNA are used as data bank to compare with sequences already done with new samples not yet sequenced (Ciociola Jr. 2000). The sequences obtained in this work were sent to the Genbank and it is available on internet. As for *T. lasallei* and *T. rojasi*, differences were found in the size of the ITS2 region of the rDNA and also on the position of some nucleotides, thereby showing that they are different species (Table 1).

Alignment of Sequences. The sequences of the ITS2 region aligned showed, with the ESEE 3s program (Cabot 1995), that differences were found between the populations studied.

Table 1. Aligned sequences of the ITS2 region of rDNA from *T. lasallei* (TL-20) and *T. rojasi* (TR-23), showing in bold the differences between them.

```
Lines                          ITS2  sequence of rDNA

TL-20 GTTTATAAAAACTAACCCGACTG--------CTCATTTCC-----GAGCGTTGTTCT---
TR-23 GTTTATAAAAACTAACCCGACTGCTTTCTCTCTCTCTCTCTCTCTGAGACAGAGAGAGAA
      -------10+-------20+-------30+-------40+-------50+-------60+

TL-20 ----------GGGCGTTCGTCTCGATTTTAGAGAACGTCGCCTCAAACGATTAGCAAGAA
TR-23 ACGTTGATCTGGGTGTTCGTCTCGTCTT-AGAGAACGTCACCTTAAACGATTAGCAAGAA
      -------70+-------80+-------90+------100+------110+------120+

TL-20 AATAAAA-GATGAACTCGTGCTCGTCTTAGCTGGCGCGCGCGCCGACCGTCTGGAGGGAG
TR-23 AATAAAAAGATGAACTCGTGCTCGTCTTAGCTGGCGCGCGCGCCGACCGTCTGGAGGGAG
      ------130+------140+------150+------160+------170+------180+

TL-20 CTCGTTCGCGAGTTCTCGTTCGATCGTCGTGTTGCACGTGTCGAGTCCCGGAGCTCCTTG
TR-23 CTCGCTTGCGAGTTCTCGTTCGATCGTCGTGTTGCACGTGTCGAGTCCCGGAGCTGCTTG
      ------190+------200+------210+------220+------230+------240+

TL-20 CCTCGTTGCGAGAGCGGACCGACACTTCGCCGCACAATTAGTGCGTGTGAC-AACACTGC
TR-23 CCTCGTTGCGAGAGCGGACCGACACCTCGTATGTGTTATCATATATTATATGATGATATA
      ------250+------260+------270+------280+------290+------300+

TL-20 GAGGCGGACCTCTATTGTCCACGACGCGTTT--GCGAGCGTGCTCTCGTGCGTGCGTGCG
TR-23 AAATAATACGACAACGCGGCGAGCCGAACCTCTATTGTCCACGACTTGTAAGCGAACGTG
      ------310+------320+------330+------340+------350+------360+

TL-20 -----------------CGACGGCGATTCATTTCGACGTTGCGAGCGTCGCGTACGCGA
TR-23 CTCTCGTGCGTGCGTGCGCGACGGCGATTCATTTCGACGTTGCGAGCGTC----ACGCGC
      ------370+------380+------390+------400+------410+------420+

TL-20 ------CAAGGCTAGGACGGTACATTACATGGCACCGAGTTGCTCCTCGCTTAGGTGAGG
TR-23 GCTTGACAAGGCTAGGACGGTATATTACATGGCACGTAGGTACTTATTTTTTTTTTTCA
      ------430+------440+------450+------460+------470+------480+

TL-20 GT----------------------CATCTTTGTCGAAAATTTTC--TTGAT
TR-23 TATTTNATATAAAAAAAAAATTAACATCTTTGTNAAAAANTTTTTTGAT
      ------490+------500+------510+------520+------530+------540+
```

It is possible to identify closely related species of *Trichogramma* from Brazil using the size of the ITS2 region plus the flanking region. This technique has several advantages over RAPD because only few individuals are needed to extract the DNA and the specific primers for *Trichogramma* are already known. In RAPD many insects are necessary and several primers must be tested (Landry *et al*. 1993, Vanlerberghe-Masutti 1994). Another difficulty is that problems with transportability may occur (Stouthamer *et al*.1999). The ITS2 method has also advantages over allozyme markers because samples can be better preserved by storage in 100% alcohol or in liquid nitrogen. Furthermore, the ITS2 of the rDNA contains more options for species-specific characters than a single allozyme with at least 2-5 distinguishable alleles (Stouthamer *et al.* 1999). An additional advantage of this technique is that it is relatively unexpensive and gives quick results. It is important to know that the sequence of the ITS2 of the rDNA cannot be used alone to identify *Trichogramma* species. Morphology is very important and the samples must be analyzed in this respect before the molecular work is done. With the ITS2 sequence, it is also possible to check for possible contamination during laboratory rearings. The use of PCR for *Trichogramma* can enable entomologists to determine levels of parasitism under field conditions without delay (Amornsak *et al*. 1998). When the sequences of the species present in one country is already available, it is possible to determine if a new species is present or not by simply comparing the sequences of the populations under study. This technique is easy to be used but requires basic laboratory facilities. The use of this molecular tool may solve many problems with *Trichogramma* identification in Brazil and improves the adoption of biological control using this minute wasp.

Acknowledgments

The authors are thankful to CAPES and CNPq for the financial support, and Luis A. Foerster and Carolina L. Canete, from the Federal University of Paraná, for sending samples of *T. lasallei* and *T. rojasi* used in this work, as well as to Dr. José R. P. Parra (ESALQ/USP) for his valuable comments on this paper.

Literature Cited

Amornsak, W., G. Gordh & G. Graham. 1998. Detecting parasitised eggs with polymerase chain reaction and DNA sequence of *Trichogramma australicum* Girault (Hymenoptera: Trichogrammatidae). Aust. J. Entomol. 37: 174-179.

Cabot, E.L. 1995. ESEE V3.0s. Computer program distributed by the University of Rochester, Rochester, NY.

Campbell, B.C., J.D. Steffen-Campbell & J.H. Werren. 1993. Phylogeny of the *Nasonia* species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed spacer (ITS2) and 28S rDNA sequences. Insect Mol. Biol. 2: 225-237.

Ciociola Jr., A.I. 2000. Identificação de espécies de *Trichogramma* (Hymenoptera: Trichogrammatidae) utilizando o sequenciamento da região ITS2 do rDNA. Tese de doutorado, ESALQ/USP, Piracicaba 60p.

Hoy, M. 1994. Insect molecular genetics. An introduction to principles and applications. Academic Press, Inc. San Diego, California, 546p.

Landry, D., L. Dextrase & G. Boivin. 1993. Random amplified polymorphic DNA markers for DNA fingerprinting and genetic variability assessment of minute parasitic wasp species (Hymenoptera: Mymaridae and Trichogrammatidae) used in biological control programs of phytophagous insects. Genome 36: 580-587.

Nagarkatti, S. & H. Nagaraja. 1971. Redescription of some known species of *Trichogramma,* showing the importance of the male genitalia as a diagnostic character. Bull. Entomol. Res. 61: 13-31.

Orrego, C. & F. Agudelo-Silva. 1993. Genetic variation in the parasitoid wasp *Trichogramma* (Hymenoptera Trichogrammatidae) revealed by DNA amplification of a section of the nuclear robossomal repeat. Fla. Entomol. 76: 519-524.

Parra, J.R.P. 1997. Técnicas de criação de *Anagasta kuehniella*, hospedeiro alternativo para produção de *Trichogramma*, pp.121-150, In Parra, J.R.P. & R.A. Zucchi (eds.) *Trichogramma* e o controle biológico aplicado, 324p.

Pinto, J.D. 1998. Systematics of the North American species of *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae). Mem. Entomol. Soc. Wash. 22: 1-287.

Pinto, J.D., R. Stouthamer & G.R. Platner. 1997. A new cryptic species of *Trichogramma* (Hymenoptera Trichogrammatidae) from the Mojave desert of California as determined by morfological, reproductive and molecular data. Proc. Entomol. Soc. Wash. 99: 238-247.

Rosen, D. & P. Debach. 1979. Species of *Aphytis* of the World (Hymenoptera: Aphelinidae). Ser. Entomol. 17: 1-801.

Stouthamer, R., J. Hu., F. J.P.M. van Kan, G.R. Platner & J.D. Pinto. 1999. The utility of internally transcribed spacer 2 DNA sequences of the nuclear ribosomal gene for distinguishing sibling species of *Trichogramma* BioControl 43: 421-440.

Vanlerberghe-Masutti, F. 1994. Genetic identification and phylogeny of parasitic wasp species by mitochondrial DNA, RFLP and RAPD markers. Insect Mol. Biol. 4: 229-237.

Received 25/07/00. Accepted 10/09/01.