SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Molecular Identification of *Trichogramma cacoeciae* Marchal (Hymenoptera: Trichogrammatidae): A New Record for Peru

RAUL P. DE ALMEIDA[1,2] AND RICHARD STOUTHAMER[3]

[1]*Centro Nacional de Pesquisa de Algodão,C. postal 174,Campina Grande, PB*
[2]*Lab. Entomology, Wageningen University, P.O. Box 8031, 6700EH, Wageningen, The Netherlands*
[3]*Dept. Entomology, University of California, Riverside, CA 92521, USA*

Neotropical Entomology 32(2):269-272 (2003)

Identificação Molecular de *Trichogramma cacoeciae* Marchal (Hymenoptera: Trichogrammatidae): Um Novo Registro Para o Peru

RESUMO - A história da taxonomia de *Trichogramma* teve um grande avanço com a descoberta dos caracteres morfológicos do macho. Entretanto, nem todas as espécies puderam ser facilmente identificadas. Em alguns casos, a identificação específica tornou-se impossível devido à ausência de insetos machos (condição das espécies telítocas infectadas por *Wolbachia*). Esse problema foi resolvido com a eliminação da bactéria por meio de tratamentos com uso de antibióticos e altas temperaturas. *T. cacoeciae* é a única espécie relatada até o momento cuja condição de telitoquia não é induzida por infeccção da bactéria *Wolbachia*, sendo necessário um novo método para identificação desta espécie. Resultados de alta confiabilidade têm sido obtidos na identificação específica de *Trichogramma* via sequenciamento da região ITS2 (Internal transcribed spacer 2). *T. cacoeciae* foi identificado com essa técnica e relatado pela primeira vez no Peru. A situação atual de *T. cacoeciae* na América do Sul é discutida.

PALAVRAS-CHAVE: Insecta, parasitóide, telitoquia, DNA ribosomal

ABSTRACT - Discovery of male morphological characters for species identification was a great improvement in *Trichogramma* systematic. However, not all species could be easily identified. In some cases, the lack of males (thelytokous status of species that carry the *Wolbachia* symbiont) made *Trichogramma* identification impossible. This problem was solved via antibiotic and heating treatments for elimination of the bacteria and allowing the production of males. The only *Trichogramma* species reported in which thelytoky is not induced by bacterial infection is *T. cacoeciae*, so here another means of species identification is needed. This species was identified based on the ITS2 (Internal transcribed spacer 2) sequence, a modern technique that has been proved useful in providing a reliable identification of *Trichogramma* species. Here we report the first occurrence of *T. cacoeciae* in Peru and we discuss its distribution in South America.

KEY WORDS: Insecta, parasitoid, thelytoky, ribosomal DNA

Trichogrammatids represents a large group of minute parasitic wasps that attack eggs of various insects, many of which are of economic importance (Nagarkatti & Nagaraja 1977). Their small size and the lack of clear morphological differences of species within each genus have made taxonomic studies difficult. This has resulted in many nomenclatorial problems (Nagarkatti & Nagaraja 1977, Smith & Hubbes 1986). The lack of easy identification has led to the unnoticed replacement of intended species in mass-rearings or the use of inappropriate species in the first place (Stouthamer *et al*. 1999). In India, *Trichogramma australicum* Girault was erroneously referred to as *T. minutum* Riley or *T. evanescens* Westwood for nearly 50 years (Nagarkatti & Nagaraja 1968). The importance of properly matching the correct *Trichogramma* species or strain to the appropriate pest situation has been discussed extensively (e.g. Kot 1979 and Voronin & Grinberg 1981). Rosen (1978) reported several cases of misidentification of natural enemies in initially unsuccessful biological control projects.

Studies of male genitalia by Nagarkatti & Nagaraja (1968, 1971) were a breakthrough and ushered in a new era of *Trichogramma* taxonomy. Unfortunately morphological traits

for identifying females with the same level of confidence as males are unavailable (Pinto & Stouthamer 1994). Positive identification of thelytokous species is therefore difficult unless males can be obtained by rearing the species at higher temperatures (Nagarkatti & Nagaraja 1977). When males are present in a very low proportion as found by Aeschlimann (1990) or in the case of completely parthenogenetic forms in which males are not present at all, i.e. non-revertible parthenogenetic forms (Stouthamer *et al.* 1990), species identification is still a problem.

Many other methods have been proposed for species identification after male morphological characters were discovered (Pintureau & Babault 1980; Pintureau & Keita 1989; Kazmer 1991; Pinto *et al.* 1992, 1993; Pintureau 1993). The DNA sequence of the internal transcribed spacer regions (ITS-1 and ITS-2) have been used at species and intraspecific levels in the many organisms groups (Carbone & Kohn 1993, Hsiao *et al.* 1994, Buckler *et al.* 1997). The usefulness of the internally transcribed spacer 2 (ITS2) of the nuclear ribosomal gene complex was shown in the identification of closely related species of the *T. deion* Pinto & Oatman complex (Stouthamer *et al*. 1999).

This study aimed at the identification of *T. cacoeciae* Marchal based on DNA sequence of the ITS2 region. In this species the production of males is rare (Pinto 1998) and their use indispensable in *Trichogramma* taxonomy using morphological features. Here *Trichogramma* females were used for extracting the DNA.

Material and Methods

***Trichogramma* Sample, DNA Extraction, PCR Amplification and Electrophoresis.** *T. cacoeciae* was collected in *Cydia pomonella* (L.) eggs in Peru apple orchard in 1997. For DNA extraction five wasps were ground in 100 ml 5% Chelex-100 and 4 ml proteinase K (20 mg/ml) and incubated for at least 4h at 56ºC, followed by 10 min. at 95ºC. The PCR was performed in a total volume of 50 ml using a Techne thermocycler, 5 ml DNA template, 5 ml PCR-buffer, 1 ml dNTP's (each in a 10 mM concentration), 1 ml forward and reverse primers (ITS2-forward: 5'-TGTGAACTGCAG GACACATG-3' located in the 5.8S region of the rDNA; ITS2-reverse: 5'-GTCTTGCCTGCTCTGCTCTGAG-3' located in the 28S region of the rDNA; 0.14 ml SuperTAQ polymerase (Sphaero-Q 5 units/ml) and 36.86 ml of sterile distilled water. The cycling program was 3 min. at 94ºC followed by 33 cycles of 40 seconds at 94ºC, 45 seconds at 53ºC and 45 seconds at 72ºC with 5 min. at 72ºC after the last cycle. The size of the PCR product was determined using standard agarose gel (Stouthamer *et al*. 1999 with modifications). The *Wolbachia*-infected species *T. atopovirilia* Oatman & Platner (strain Tato-01) was used as positive control and uninfected *T. galloi* (strain Tgal-02) as negative control; both were collected in Brazil.

Cloning, Sequencing and Alignments. Following electrophoresis, PCR products were purified with a QIAquick PCR purification kit (Qiagen®). After the purification the PCR products were tied up to a Pgem-T® Vector (Promega), 2 ml of the ligation mix was transformed in the heatshock cells of DH5-a *Escherichia coli* and plated in a LB agar medium containing Ampicilin, X-GAL and IPTG. The plates were stored overnight at 37ºC. The next day, white colonies were picked up with a sterile toothpick from the plates and placed into tubes containing 3.0 ml of LB liquid medium and 3ml Ampicilin and put to grow up overnight in a shaker set to 250 rpm at 37ºC. To confirm that the correct piece of DNA had been cloned, a PCR reaction with a template extracted from the bacterial culture was added to 100 ml 5% Chelex-100 and incubated for 15 min. at 60ºC followed by 5 min. at 95ºC. The PCR was performed in a final volume of 50 ml. If indeed it was cloned the correct part of DNA, 850 ml of the bacteria culture was added to 150 ml of 87% glycerol and stored at –80ºC. 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. *T. cacoeciae* was aligned manually using the ESEE 3.Os sequence editor (Cabot 1995).

Thelytoky in *T. cacoeciae*. To confirm whether the parthenogenesis in *T. cacoeciae* was not caused by *Wolbachia* infection, specific primers for DNA amplification of the *wsp* region were used: *wsp*-Forward primer 5'TGGTCCAATAAGTGATGAAGAAAC-3' and *wsp*-Reverse 5'-AAAAATTA AACGCTACTCCA-3'. The cycling program was 3 min. at 94ºC followed by 40 cycles of 1 min. at 94ºC, 1 min. at 50ºC and 1 min. at 72ºC with 5 min. at 72ºC after the last cycle.

Results and Discussion

The molecular technique used for identifying *T. cacoeciae*, based on ITS2 sequence, was proved to be reliable and solved the limitation of the morphological identification in which allows species identification by using males features only. Complete ITS2 sequence of *T. cacoeciae* (460 bp) has been deposited in Genbank (Accession number: AY166700).

T. cacoeciae has been recorded in eggs of *Prais oleae* Bernard (an insect pest of olive) in Greece and in *C. pomonella* (insect pest of apple trees) in the former USSR (Nagarkatti & Nagaraja 1977). This species is geographically distributed in Europe and is also known in the Pacific Northwest in North America (Pinto 1998). It has been considered genetically thelytokous (Stouthamer *et al*. 1990, Pintureau 1994, Pinto 1998). Males are rare and treatment with antibiotic or elevated temperatures does not induce arrhenotoky. The morphology of the few available males is similar to two other western North American species, *T. platneri* Nagarkatti and *T. californicum* Nagaraja & Nagarkatti, and all three are known to parasitize codling moth (*C. pomonella*) in California. Six replicate cultures of *T. cacoeciae* strain 101 from France, each with 50-100 individuals for ca. 30 generations produced only five males (Pinto 1998). The thelytokous status of *T. cacoeciae* without carrying *Wolbachia bacteria* was confirmed based on the lack of DNA amplification by using specific primers (*wsp*) (Fig. 1).

The identification of *T. cacoeciae* was confirmed via comparison with two other sequences from the GenBank with the following accession numbers: AF408653 and AF408654.

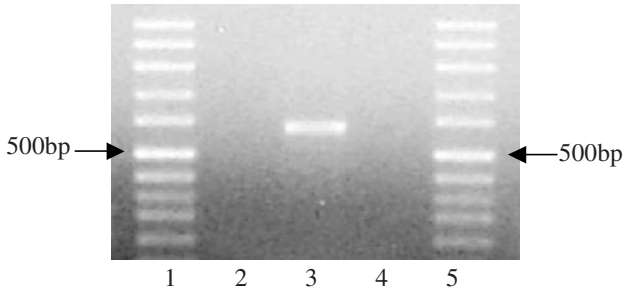

Figure 1. Gel electrophoresis of *wsp* PCR products. Lanes 1 and 5, low-molecular-weight markers; lane 2, *T. cacoeciae*; lane 3, *T. atopovirilia* (positive control); lane 4, *T. galloi* (negative control).

This was the first report of *T. cacoeciae* collected in apple orchards, on *C. pomonella* eggs in Peru. According to Dr. Mary Whu (personal communication) this species was collected in the Huarochiri Province at approximately 2500 m altitude. Farms in that region of Peru cannot afford chemical application for insect pest control. This was the first time an egg parasitoid was collected in that area.

In Peru, exotic species of *Trichogramma* (*T. pintoi* Voegelé, *T. japonicum* Ashmead, *T. australicum* Girault, *T. atopovirilia*, *T. evanescens* Westwood, *T. dendrolimi* Matsumara and *T. embryophagum* Quednau) have been introduced since 1972. Thelytokous species have been detected since 1976. However, the lack of males made the specific identification of *Trichogramma* impossible. Among the fifteen insect pests mentioned as host of *Trichogramma* spp., the commonly associated host to *T. cacoeciae, C. pomonella*, was not found (Whu & Valdivieso 1999). The only case of *T. cacoeciae* introduction in Latin America reported in the literature was recorded in Argentina and Cuba by De Santis & Fidalgo (1994).

According to Dr. Bernard Pintureau (INRA/INSA, France), Dr. John Pinto (University of California, USA), Dr. Juan Carlos Monje (University of Hohenheim, Germany) and Dr. Sherif Hassan (BBA Institute, Germany) (personal communication) there is no information on the introduction of *T. cacoeciae* in Peru. Dr. Juan Carlos Monje reported, however, that *T. cacoeciae* was found in Chile and he assumes that this species may occur in fruit orchards in several South-America countries. He also mentioned that it is possible that this species was accidentally introduced via the importation of apple varieties stock and extensive collections are needed to clarify this situation (personal communication). *T. cacoeciae* might also have been introduced under another specific name as suggested by Dr. Roberto Zucchi and Dr. Ranyse B.Q. da Silva (ESALQ/USP, Brazil) (personal communication). Introduction of species incorrectly identified might be a real problem if the insect pest used as target for the biological control is not associated with the supposed *Trichogramma* species. According to Zucchi & Monteiro (1997) the preliminary knowledge of the distribution pattern of *Trichogramma* species in the American continent has also been limited by species introduction without previous analysis of species already present in some regions. With the introductions for biocontrol the situation now becomes more uncertain because species assumed to be indigenous for a particular region might in fact have been introduced.

Acknowledgments

The authors are thankful to Joop van Lenteren (Wageningen University, The Netherlands) for his valuable comments in the manuscript. To Ranyse B.Q. da Silva (ESALQ-USP, Brazil) (*T. cacoeciae*), Americo I. Ciociola Junior (EPAMIG, Brazil) (*T. atopovirilia*) and Mary Whu (SENASA, Peru) (*T. galloi*) who have sent *Trichogramma* species. This molecular biology research was financially supported by Embrapa (PRODETAB).

Literature Cited

Aeschlimann, J.P. 1990. Simultaneous occurrence of thelythoky and bisexuality in hymenopteros species, and its implications for the biological control of species. Entomophaga 35: 3-5.

Buckler IV, E.S., A. Ippolito & T.P. Holtsford. 1997. The evolution of ribosomal DNA: divergent paralogues and phylogenetic implications. Genetics 145: 821-832.

Carbone, I. & L. Kohn. 1993. Ribosomal DNA sequence divergence within transcribed spacer 1 of the Sclerotiniaceae. Mycologia 85: 15-427.

Cabot, E.L. 1995. "The Eyeball Sequence Editor (ESSE)," version 3.0s Dept. Biology, Univ. Rochester, Rochester, NY.

De Santis, L. & P. Fidalgo. 1994. Catálogo de los himenópteros calcidoideos de América al sur de los Estados Unidos. Tercer Suplemento. Acad. Nac. Agr. Vet. Bs. As. 13. 154 p.

Hsiao, C., N.J. Chatterton, K.H. Asay & K.B. Jensen. 1994. Phylogenetic relationships of 10 grass species: an assessment of phylogenetic utility of the internal transcribed spacer region in nuclear ribosomal DNA in monocots. Genome 37: 112-120.

Kazmer, D.J. 1991. Isoelectric focusing procedures for the analysis of allozymic variation minute arthropods. Ann. Entomol. Soc. Am. 84: 332-339.

Kot, J. 1979. Analysis of factors affecting the phytophage reduction by *Trichogramma* Westw. species. Pol. Ecol. Stud. 5: 5-59.

Nagarkatti, S. & H. Nagaraja. 1968. Biosystematic studies on *Trichogramma* species. I. Experimental hybridization between *Trichogramma australicum* Girault, *T. evanescens* Westwood and *T. minutum* Riley. Tech. Bull. Corn. Inst. Biol. Control 10: 81-96.

Nagarkatti, S. & H. Nagaraja. 1971. Redescription of some known species of *Trichogramma* (Hym. Trichogrammatidae), showing the importance of the male genitalia as a diagnostic character. Bull. Entomol. Res. 61:13-21
.

Nagarkatti, S. & H. Nagaraja. 1977. Biosystematic of *Trichogramma* and *Trichogrammatoidea* species. Annu. Rev. Entomol. 22: 157-176.

Pinto, J.D. 1998. Systematics of the North American species of *Trichogramma* (Hymenoptera: -Trichogrammatidae). Mem. Entomol. Soc. Wash. Washington, Allen Press Inc., nº 22, 287p.

Pinto, J.D., D.J. Kazmer, G.R. Platner & C.A. Sassaman. 1992. Taxonomy of the *Tricho-gramma minutum* complex (Hymenoptera: Trichogrammatidae): allozymic variation and its relationship to reproductive and geographic data. Ann. Entomol. Soc. Am. 85: 413-422.

Pinto, J.D., G.R. Platner & C.A. Sassaman. 1993. Electrophoretic study of two closely related of North American *Trichogramma: T. pretiosum* and *T. deion.* Ann. Entomol. Soc. Am. 86: 702-709.

Pinto, J.D. & R. Stouthamer. 1994. Systematics of the Trichogrammatidae with emphasis on *Trichogramma*, p.1-36. In E. Wajnberg & S.A. Hassan (eds.), Biological control with egg parasitoids. Wallingford, CAB International, 286p.

Pintureau, B. 1993. Enzymatic analysis of the genus *Trichogramma* (Hym.: Trichogrammatidae) in Europe. Entomophaga 38: 411-431.

Pintureau, B. 1994. Frequence and geographical distribution of thelytokous parthenogenesis in European species of *Trichogramma* (Hym.: Trichogrammatidae). Norw. J. Agric. Sci. Suppl. nº 16: 411.

Pintureau, B. & F.B. Keita. 1989. Nouvelles données sur les estérases des Trichogrammes. Biochem. Syst. Ecol. 17: 603-608.

Pintureau, B. & M. Babault. 1980. Comparaison des estérases chez 19 souches de *Trichogramma* (Hym., Trichogrammatidae) apparte-nant au groupe d'espèces *evanescens.* Arch. Zool. Exp. Gén. 121: 249-260.

Rosen, D. 1978. The importance of cryptic species and specific identifications as related to biological control, p. 23-35. In Beltsville Symp. Agric. Res. 2, Osmun and Co., Allenheld.

Smith, S.M. & M. Hubbes. 1986. Strains of the egg parasitoid *Trichogramma minutum* Riley. 1. Biochemical and biological characterization. J. Appl. Entomol. 101: 223-239.

Stouthamer, R., J.D. Pinto. G.R. Platner & R.F. Luck. 1990. Taxonomic status of thelytokous forms of *Trichogramma* (Hymenoptera: Trichogrammatidae). Ann. Entomol. Soc. Am. 83: 475-581.

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.

Voronin, K.E. & A.M. Grinberg. 1981. The current status and prospects of *Trichogramma* utilisation in the U.S.S.R., p. 49-51. In J.R. Coulson (ed.), Proceedings of the Joint American-Soviet Conference on the Use of Beneficail Organisms in the Control of Crop Pests. Entomolgical Society of America, College Park, Md.

Whu, M. & L. Valdivieso. 1999. Distribución y comportamiento de ocho especies de *Trichogramma* y *Trichogrammatoidea* (Hymenoptera: Trichogrammatidae) en el Perú. Rev. Peru. Entomol. 41: 61-68.

Zucchi, R.A. & R.C. Monteiro. 1997. O gênero *Trichogramma* na America do Sul, p.41-66. In J.R.P. Parra & R.A. Zucchi (eds.), *Trichogramma* e o controle biológico aplicado. Piracicaba, FEALQ, 324p.

Received 30/10/02. Accepted 25/03/03.