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ITS-2 sequences-based identification of *Trichogramma* species in South America

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Abstract

ITS2 (Internal transcribed spacer 2) sequences have been used in systematic studies and proved to be useful in providing a reliable identification of *Trichogramma* species. DNAr sequences ranged in size from 379 to 632 bp. In eleven *T. pretiosum* lines *Wolbachia*-induced parthenogenesis was found for the first time. These thelytokous lines were collected in Peru (9), Colombia (1) and USA (1). A dichotomous key for species identification was built based on the size of the ITS2 PCR product and restriction analysis using three endonucleases (EcoRI, MseI and MaeI). This molecular technique was successfully used to distinguish among seventeen native/introduced *Trichogramma* species collected in South America.

Keywords: molecular markers, ITS-2 sequence, Hymenoptera, egg parasitoid.

Identificação molecular de espécies de *Trichogramma* na América do Sul via sequenciamento da região ITS-2

Resumo

Sequências do Espaço Transcrito Interno 2 (ITS2) têm sido utilizadas em estudos taxonômicos e sua utilidade constatada pela confiabilidade que o método confere à identificação das espécies de *Trichogramma*. Esta técnica molecular foi bem sucedida em distinguir dezessete espécies nativas e introduzidas de *Trichogramma*, coletadas na América do Sul. As sequências do DNAr variaram de 379 a 632 pb. Em 11 linhagens de *T. pretiosum* estudadas, o endosinbionte *Wolbachia* foi detectado pela primeira vez. Estas linhagens telítocas foram encontradas no Peru (9), Colômbia (1) e Estados Unidos (1). Uma chave dicotômica para identificação de espécies foi construída baseada no tamanho do produto da PCR do ITS2 e em análises de restrição utilizando-se três endonucleases (EcoRI, MseI and MaeI).

Palavras-chave: marcadores moleculares, sequências ITS-2, Hymenoptera, parasitóide de ovos.

1. Introduction

Natural enemies of the family Trichogrammatidae are released worldwide against a number of lepidopterous pest on corn, rice, sugar-cane, cotton, vegetables and pines (Smith, 1994; Li, 1994; Grenier, 1994). In the past, most successful biological control agents have been found through trial and error. Nowadays, there is an effort to predict the success of a *Trichogramma* strain before introductions are made (van Lenteren and Woets, 1988).

Therefore, standard procedures to compare *Trichogramma* candidates for biological control have been proposed (Hassan, 1994). According to Hassan (1995) before field releases are undertaken, a suitable *Trichogramma* strain of known qualities should be chosen. The effectiveness of *Trichogramma* in the field largely depends on its searching behaviour, host preference and tolerance to environmental conditions. However, correct identification of the *Trichogramma* species that is going to be tested, is a crucial step in any biological control program.

Despite the large improvement with the discovery of male genitalic morphology as species specific characters (Nagarkatti and Nagaraja, 1968, 1971), the identification of these tiny insects remains difficult and requires specialized skills (Pinto and Stouthamer, 1994).

Their small size and lack of morphologically distinct characters has historically been a problem in taxonomic studies. Specimen must be prepared on slides for examination, a time consuming process that requires considerable experience (Platner et al., 1999).

Unfortunately many important species share similar genitalic structures and this has forced workers to continue relying on less dependable characters that often

are intraspecifically variable and subject to phenotypic plasticity (Pinto et al., 1989; Pinto and Stouthamer, 1994).

To simplify the *Trichogramma* species identification, several methods have been proposed: (1) Allozymic analysis (Pintureau and Babault, 1980, 1981, 1982; Pintureau and Keita, 1989; Kazmer, 1991; Pinto et al., 1992, 1993; Pintureau, 1993). Esterase electrophoresis has provided consistent results for differentiation of some *Trichogramma* species (Pinto et al., 1992, 1993; Silva et al., 1999). The limited variation at esterase loci only allows for the differentiation between a limited number of species (Richardson et al., 1986; Pinto and Stouthamer, 1994). In addition the samples subjected to allozyme electrophoresis have to be kept at –70 °C to avoid the degeneration of the enzymes; (2) Reproductive compatibility tests (Nagarkatti and Nagaraja, 1968; Pinto et al., 1991; Pintureau, 1991) in which individuals of the unknown species are crossed with individuals of known species status; (3) The DNA sequence of ribosomal spacers (Landry et al., 1993; Orrego and Agudelo-Silva, 1993; Sappal et al., 1995; van Kan et al., 1996, 1997; Pinto et al., 1997); (4) RFLP's of complete mitochondrial genome has also been used for species differentiation (Vanlerberghe-Masutti, 1994). Ribosomal DNA is present in all organisms and is composed of several regions (genes and spacers) that evolve at different rates (Hillis and Dixon, 1991); (5) The internal transcribed spacer regions (ITS-1 and ITS-2) have been used at species and intraspecific levels of many taxa for distinguishing the different forms (Carbone and Kohn, 1993; Bowles and McManus, 1993; Hsiao et al., 1994; Buckler IV et al., 1997). In insects these sequences have been often used for taxonomic purposes (Campbell et al., 1993; Hoy, 1994; Kuperus and Chapco, 1994; Vogler and DeSalle, 1994; Stouthamer et al., 1999; Silva et al., 1999; Chang et al., 2001; Pinto et al., 2002; Honda et al., 2006; Dem'yanchuk et al., 2008; Kumar et al., 2009; Polaszek et al., 2011; Poorjavad et al., 2012).

Here, we use the ITS2 sequences of *Trichogramma* species (native or introduced) from South America to develop an identification key. The advantage of this identification system over the morphology-based system is that non-specialists are able to quickly and cheaply identify individual specimens. In addition females can also be identified which is not possible in the morphologically based system. Species found in South America are thelytokous, either because of infection with parthenogenesis inducing *Wolbachia* (*T. pretiosum* and *T. atopovirilia*) (Grenier et al., 1998; Almeida et al., 2001, 2010; Ciociola Junior et al., 2001a) or because of some nuclear genetic factor (*T. cacoeciae* Marchal) (Stouthamer et al., 1990; Almeida and Stouthamer, 2003). Variation of ITS2 sequence within *Trichogramma* species is relatively small in comparison to the difference found between species and all morphologically distinct cryptic species are also distinguished by sequence differences (Stouthamer et al., 1999). ITS2 sequences within each species are very similar and there is no evidence for two or more gene families that differ substantially within the genome of a single individual, as has been found in other taxa (Vogler and DeSalle, 1994).

2. Material and Methods

2.1. Trichogramma cultures

Table 1 lists the *Trichogramma* species/lines studied here, including their origin, host insect, plant crop, collection date and status as native or introduced.

2.2. DNA Extraction, PCR Amplification and electrophoresis

To extract DNA from the different species/lines we used five wasps that were homogenized in 100 µL 5% Chelex-100 and 4 µL proteinase K (20 mg/mL) and incubated for at least 4 hours at 56 °C, followed by 10 min. at 95 °C. The PCR was performed in a total volume of 50 µL using a Techne thermocycler, 5 µL DNA template, 5 µL 10x PCR-buffer, 1 µL dNTP's (each in a 10 mM concentration), 1 µL forward and reverse primers (ITS2-forward: 5' -TGTGAACTGCAG GACACATG-3' located in the 5.8S region of the rDNA; ITS2-reverse: 5' -GTCTTGCC TGCTCTGCTCTGAG-3' located in the 28S region of the rDNA (Stouthamer et al., 1999); 0.14 µL TAQ polymerase (5 units/µL) and 36.86 µL of sterile distilled water. The cycling program was 3 min. at 94 °C followed by 33 cycles of 40 seconds at 94 °C, 45 second at 53 °C and 45 seconds at 72 °C with 5 min. at 72 °C after the last cycle.

2.3. Cloning, sequencing and alignments

Following electrophoresis, PCR products were purified with a QIAquick PCR purification kit (Qiagen®). After the purification the PCR products were ligated into a Pgem-T®Vector (Promega), 2 µL of the ligation mix was transformed in the heat shock cells of DH5-α *Escherichia coli* and plated on a LB agar medium containing Ampicilin, XGAL and IPTG. The plates were incubated overnight at 37 °C. The next day, white colonies were picked up with a sterile toothpick from the plates and placed into tubes containing 3.0 mL of LB liquid medium and 3 µL Ampicilin and put to grow up overnight in a shaker set to 250 rpm at 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 added to 100 µL 5%

Chelex-100 and incubated for 15 min. at 60 °C followed by 5 min. at 95 °C. The PCR was performed with 5 µL of this template as described before. If indeed an ITS2 had been cloned, 850 µL of the bacteria culture was added to 150 µL 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 an Applied Biosystems automatic sequencer. *Trichogramma* sequences were aligned manually using the ESEE 3.0s sequence editor (Cabot, 1995).

2.4. Thelytoky in Trichogramma

For detection of thelytoky caused by *Wolbachia* infection, specific primers for DNA amplification of the *wsp* region of *Wolbachia* were used: *wsp*-Forward primer 5'TGGTCCAATAAGTGATGAAGAAAC-3' and

Table 1. *Trichogramma* populations from South America.

Spécies	Line Designation	Origin	Host Insect	Plant Crop	Collection Date
T. acacioi Brun, Moraes and Soares, 1984	Taca-01	Botucatu-SP-Brazil	*Euselasia* sp.	-	-
T. atopovirilia Oatman and Platner, 1983	Tato-01	Sete Lagoas-MG-Brazil	-	Corn	-
	Tato-02	Colombia	-	-	-
T. brassicae Bezdenko, 1968	Tbra-01	Introduced from Germany	-	-	Dec., 1996
T. bruni Nagaraja, 1983	Tbru-01	Piracicaba-SP-Brazil	*Heliconius phyllis*	*Passiflora* sp.	-
T. cacoeciae Marchal, 1927	Tcac-01	Huarochiri-Peru	*Cydia pomonela*	Apple	Oct., 1997
T. dendrolimi Matsumura, 1926	Tden-01	Introduced from Germany	-	-	Dec., 1996
T. esalqueanum Querino and Zucchi, 2003	Tesa-11	Piracicaba-SP-Brazil	*Helicornius erato phyllis*	*Passiflora* sp.	Oct., 1999
T. exiguum Pinto and Platner, 1983	Texi-01	Peru (different places)	*Diatraea saccharalis*	Sugar-cane,	-
			Helicoverpa zea	Corn	-
			Erynis ello	Cassava	-
			P. persimilis	Olive	-
			Dione juno	*Passiflora P.*	Mar., 2001*
	Texi-02	Colombia	-	-	-
T. fuentesi Torre, 1980	Tfue-01	Peru	*Diatraea saccharalis*	Sugar-cane, rice	-
			Helicoverpa zea	Corn	-
			Heliothis virescens, Anomis texana	Cotton	Dec., 1994*
T. galloi Zucchi, 1988	Tgal-01	Chiclayo-Peru	*Diatraea saccharalis*	Sugar-cane	Apr., 2000
T. iracildae Querino and Zucchi, 2003	Tira-01	Maceió-AL-Brazil	*Calpodes ethlius*	*Canna* spp.	Apr., 2000
T. lasallei Pinto, 1998	Tlas-01	Lima-Peru	*Quinta cannae*	*Canna indica*	Mar., 2001
T. lopezandinesis Sarmiento, 1983	Tlop-01	Colombia	-	-	-
T. nerudai Pintureau and Gerding, 1999	Tner-01	Chile	*Rhyacionia buolina*	Pine forest	-
T. pintoi Voegelé, 1982	Tpin-01	Introduction from Chile	-	-	Jun., 1973
T. pretiosum Riley, 1879	Tpre-01	Chincha-Peru	*Diaphania nitidalis*	Pumpkins	Mar., 1992
	Tpre-02	Chancay-Peru	*Dione juno*	*Passiflora*	May, 1994
	Tpre-03	Lambayeque-Peru	*Diatraea saccharalis*	Corn	Jul., 1994
	Tpre-04	Tacna-Peru	*Palpita persimilis*	Olive	Apr., 1995
	Tpre-05	Moquengua-Peru	*Marasmia trapezalis*	Corn	Jul., 1996
	Tpre-06	Ica-Peru	*Heliothis virescens*	Cotton	Nov., 1996
	Tpre-07	Piura-Peru	*Alabama argillacea*	Cotton	Apr., 1997
	Tpre-08	Lima-Peru	*Diaphania nitidalis*	*Curcubita moschata*	Mar., 1997
	Tpre-09	Ayacucho-Peru	*Helicoverpa zea*	Corn	Nov., 1998
	Tpre-10	Colombia	*Neoleucinodes elegantalis*	-	-
	Tpre-11	USA	-	-	-
	Tpre-12	Jataizinho-PR-Brazil	-	Cotton	Feb., 2000
T. rojasi Nagaraja and Nagarkatti, 1973	Troj-01	Curitiba-PR-Brazil	*Anticarsia gemmatalis*	-	-

*Last collection date.

wsp-Reverse 5' –AAAAATTAAACGCTACTC CA-3' (Braig et al., 1998). These primers amplify 554bp of the *wsp* gene. The cycling program was 3 min. at 94 °C followed by 40 cycles of 1 min. at 94 °C, 1 min. at 50 °C and 1 min. at 72 °C with 5 min. at 72 °C after the last cycle.

2.5. Molecular key

A molecular key for separating Trichogramma species was constructed based on the size and the fragment profile following restriction with different restriction enzymes. Initially we determined ITS2 sequences of all species and lines, subsequently we first tried to distinguish the different species based on the size of the PCR product. Species with similar sized PCR products were distinguished by selecting restriction enzymes that would generate differently sized in restriction fragments. To predict the fragment sizes to be expected with different enzymes we use the program Webcutter 2.0 (Heiman, 1997). Three restriction enzymes were used (EcoRI, MseI and MaeI).

3. Results

Seventeen native and introduced *Trichogramma* species collected in South America were identified based on ITS2 sequences. In total, thirty lines were studied (Table 1). Seventeen lines were recognised as arrhenotokous and thirteen as thelytokous. Twelve out of thirteen thelytokous lines studied here carry the *Wolbachia* symbiont. In *T. cacoeciae*, parthenogenesis was not caused by *Wolbachia* infection (Stouthamer et al., 1990; Almeida and Stouthamer, 2003). For all other parthenogenetic lines, *Wolbachia* detection was possible by using specific primers of the *wsp* region. In all thelytokous species/lines no male was found, except for *T. atopovirilia* (culture form Brazil) and *T. pretiosum* (culture from the Entomology Lab., California University, Riverside-USA) in which few males were present.

Complete ITS2 sequences have been deposited in GenBank (Table 2). *Trichogramma* species were identified by (1) morphological identification: *T. atopovirilia*, line Tato-01, (Dr. Américo I. Ciociola Junior - EPAMIG, Brazil, personal comunication), *T. lopezandinensis* and *T. fuentesi* (Dr. Ranyse B.Q. da Silva-ESALQ/USP, Brazil, personal communication); (2) by comparison with ITS2 sequences from the GenBank: *T. pintoi*, *T. lasallei*, *T. cacoeciae*, *T. galloi*, *T. pretiosum*, *T. brassicae*, *T. dendrolimi* and *T. exiguum*; or (3) by comparison with sequences not published yet: *T. nerudai*. *T. rojasi* and *T. acacioi* lines were the same as those used by Ciociola Junior et al. (2001b). Two new species from Brazil (*T. iracildae* and

Table 2. Line designation, reproduction mode, genbank accession number of the *Trichogramma* species and size of the ITS2 and PCR product (bp).

Line Designation	Reproduction Mode	Acession Number	ITS-2 Product	PCR product
Tner-01	Arrhenotoky	AY182756	632	746
Tpin-01	Arrhenotoky	AY182757	581	695
Tato-01	Thelytoky	AY182758	565	680
Tato-02	Arrhenotoky	AY182759	561	675
Taca-01	Arrhenotoky	-	559	674
Tbru-01	Arrhenotoky	AY187263	536	650
Troj-01	Arrhenotoky	-	524	638
Tira-01	Arrhenotoky	AY182760	522	636
Tlop-01	Arrhenotoky	AY182761	509	623
Tlas-01	Arrhenotoky	AY182762	485	599
Tesa-11	Arrhenotoky	AY182763	379	493
Tcac-01	Thelytoky	AY166700	460	574
Tgal-01	Arrhenotoky	AY182764	445	560
Tfue-01	Arrhenotoky	AY182765	437	553
Tpre-01	Thelytoky	AY182770	410	524
Tpre-02	Thelytoky	AY182771	410	524
Tpre-03	Thelytoky	AY182772	415	529
Tpre-04	Thelytoky	AY182773	410	524
Tpre-05	Thelytoky	AY184958	410	524
Tpre-06	Thelytoky	AY184959	410	524
Tpre-07	Thelytoky	AY187259	415	529
Tpre-08	Thelytoky	AY184960	410	524
Tpre-09	Thelytoky	AY184961	410	524
Tpre-10	Thelytoky	AY187260	412	526
Tpre-11	Thelytoky	AY187261	413	527
Tpre-12	Arrhenotoky	AY187262	412	526
Tbra-01	Arrhenotoky	AY182766	406	520
Tden-01	Arrhenotoky	AY182767	403	519
Texi-01	Arrhenotoky	AY182768	383	497
Texi-02	Arrhenotoky	AY182769	381	496

T. esalqueanum) sequenced here were recorded by Querino and Zucchi (2003).

An identification key was constructed for these species using as two characters of the PCR product its size and the restriction patterns generation using the three restriction enzymes EcoRI, MseI and MaeI (Table 3). España-Luna et al. (2008) used two restricion enzymes (EcoRI and AluI) for distinguishing six *Trichogramma* species from each other in Mexico. Sumer et al. (2009) used three restricion enzymes (MnlI, MseI and DraI) for distinguishing six *Trichogramma* species from each other in agricultural settings around the Mediterranean.

Here, ITS2 product size of the seventeen studied species ranged from 379 to 632 bp and sequences differ from each other consistently. In our sample the size of the ITS2 product alone could not identify the species,

Table 3. Molecular key for Trichogramma species recognition based on the size of the PCR product and species-specific banding pattern.

1.	Size of the PCR product < 620 bp	2
	Size of the PCR product > 620 bp	10
2.	Size of the PCR product < 550 bp	3
	Size of the PCR product >550 bp	7
3.	Size of the PCR product ≤ 500 bp	4
	Size of the PCR product > 550 bp	5
4.	PCR product cut by MseI ca. 280 and 191bp	*T. esalqueanum*
	PCR product not cut by MseI	*T. exiguum*
5.	PCR product cut by EcoRI	6
	PCR product not cut by EcoRI	*T. pretiosum*
6.	PCR product cut by MseI ca. 441 bp	*T. dendrolimi*
	PCR product cut by MseI ca. 411 bp	*T. brassicae*
7.	PCR product not cut by MseI	8
	PCR product cut by MseI	9
8.	PCR product cut by MaeI ca. 267 and 218 bp	*T. fuentesi*
	PCR product cut by MaeI ca. 182 and 113 bp	*T. galloi*
9.	PCR product cut by EcoRI ca. 346 and 228 bp	*T. cacoeciae*
	PCR product not cut by EcoRI	*T. lasallei*
10.	Size of the PCR product < 670 bp	11
	Size of the PCR product > 670 bp	14
11.	PCR product not cut by MseI	12
	PCR product cut by MseI	13
12.	PCR product cut by MaeI ca. 480 and 143 bp	*T. lopezandinensis*
	PCR product cut by MaeI ca. 517 bp	*T. iracildae*
13.	PCR product cut by MseI ca. 390 and 194bp	*T. rojasi*
	PCR product cut by MseI ca. 463 and 187bp	*T. bruni*
14.	Size of the PCR product > 700 bp	*T. nerudai*
	Size of the PCR product < 700 bp	15
15.	PCR product cut by EcoRI	*T. pintoi*
	PCR product not cut by EcoRI	16
16.	PCR product cut by MseI ca. 478 and 196 bp	*T. acacioi*
	PCR product cut by MseI ca. 345, 198 and 137 bp	*T. atopovirilia*

but cutting the ITS2 product of the different species using only three restriction enzymes (EcoRI, MseI and MaeI) enabled us to identify all species (Table 3). For instance the variation in the size of ITS2 was evaluated in *T. atopovirilia, T. exiguum* and *T. pretiosum*. The length of the complete sequences found for *T. pretiosum* lines all ranged from 410 to 415 bp (Table 3), for *T. atopovirilia* (561-565 bp) and for *T. exiguum* (381-383 bp). In general, variation was limited to the number of microsatellite repeat stretches found in the ITS2 sequences.

4. Discussion

Distributed worldwide, *Trichogramma* are known with circa of 210 species (Pinto, 2006). In North America, Central America and South America are recorded respectively 60, 21 ad 41 species. Considering only the presence of native species in South America, Brazil has the largest number of known species (26) followed by Venezuela (13), Colombia (9), Peru (7), Uruquay (6), Chile (4) and Ecuador and Paraguay (2) (Zucchi et al., 2010; Querino and Zucchi, 2011).

In *T. atopovirilia* the presence of *Wolbachia* was recorded for the first time in Brazil by Ciociola Junior et al. (2001a). In all *T. pretiosum* lines studied here *Wolbachia* infection was reported for the first time. In South America, *Wolbachia* infection has been reported in *T. pretiosum* from Uruguay (Grenier et al., 1998) and from Brazil (Almeida et al., 2001).

The usefulness of endonucleases analysis for distinguishing *Trichogramma* species was shown by Stouthamer et al. (1999), Silva et al. (1999) and Pinto et al. (2002). How reliable are such molecular keys that are based in some cases on the sequence of only a few lines? The experience with other *Trichogramma* species is that the intraspecific variation in the ITS2 is rather limited. The variation in number of microsatellite repeats seems to be common in ITS sequences, similar microsatellite variation was for instance also found in Eriophyid mites (Fenton et al., 1997). The range obtained here confirms the low intraspecific variation in the ITS2 length detected by Stouthamer et al. (1999) and Silva et al. (1999). According to Stouthamer et al. (1999) ITS2 can be used for species identification in *Trichogramma* because the sequence variation within species is small relative to the difference found between species. All morphologically distinct cryptic species are also distinguished by sequence differences, however in the morphologically indistinguishable species North American species *T. minutum* Riley and *T. platneri* Nagarkatti no consistent differences were found in their ITS2 sequences (Stouthamer et al., 2000). In this case the ITS2 did not improve upon the morphologically based system. A potential weakness of an ITS2 based molecular key as presented here is that in some cases the key relies on either a restriction of an ITS2 by a particular enzyme or the lack thereof. When no restriction digestion is found it is not immediately clear if the endonuclease worked or if the products lack the restriction site (Stouthamer et al., 1999). It is important to run along a positive control to assure that the restriction reactions work. Molecular laboratories with basic infrastructure can perform PCR amplifications followed by restriction digestions and DNA can be extracted from living, frozen, ethanol-preserved or dried material (Post et al., 1993; van Kan et al., 1996; Ciociola Junior et al., 2000). Just as in case of morphological keys, molecular keys will only be completely reliable once all species of a region are known.

This study together with previous studies (Stouthamer et al., 1999; Silva et al., 1999; Ciociola Junior et al., 2001b) shows the utility of ITS2 sequences in *Trichogramma* identification. The molecular technique used in this study will be of a great utility in a near future for sequencing species already classified but not sequenced yet from South America. We also expect that with this technique new species will be soon discovered due to the high diversity of *Trichogramma* host insects in this region. The small number of species found until now is caused by the very limited activities to collect species of *Trichogramma*. In addition, most of the host species collected were restricted to insects of economic importance. Also there are only a few taxonomists specialized in identifying *Trichogramma* species using morphological features. Identification by the combination of morphological characters and molecular technique will be of extreme importance for a successful systematic analysis of this group.

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