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The utility of internally transcribed spacer 2 DNA sequences of the nuclear ribosomal gene for distinguishing sibling species of *Trichogramma*

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Abstract. The usefulness of the internally transcribed spacer 2 (ITS2) of the nuclear ribosomal gene complex is tested for providing taxonomic characters to identify *Trichogramma* species. The ITS2 sequences of a group of sibling species of the *T. deion/T. pretiosum* complexes were determined. A simple and precise identification key to the species of these assemblages was constructed using as taxonomic characters the size of the ITS2 and the difference in restriction length polymorphism of species with similarly sized ITS2. Individual wasps can be identified by amplification of their ITS2 with general primers, determining the size of the PCR product using standard agarose electrophoresis, followed in some species by a DNA-digestion with a restriction enzyme. Because this system works well for a number of closely related species we are hopeful that similar PCR-based identification can be extended to all species of the genus once their ITS2 sequences have been determined. 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, species specific primers were tested for the two most common species of these groups (i.e. *T. pretiosum* and *T. deion*). These primers can be used either as a direct identification tool or as a method to confirm the identification using the general key. The phylogeny of this group of wasps was also analyzed based on the ITS2 sequence.

Key words: Hymenoptera, identification key, phylogeny, Trichogrammatidae

Introduction

Trichogramma species (Hymenoptera: Trichogrammatidae) are minute wasps (<1 mm) that oviposit predominantly in eggs of Lepidoptera. Members of this genus are among the most widely used parasitoids in biological control programs (Smith, 1994), their use being virtually restricted to inundative release for the control of pestiferous Lepidoptera (Li, 1994). Unfortunately, species identification in this group is problematic. Diagnostic morphological

features are few and those that are useful assume specialized knowledge for interpretation (Pinto and Stouthamer, 1994). Additionally, specimens must be placed on slides for examination, a time consuming process that requires considerable experience. This 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. Because some, if not all, *Trichogramma* show strong habitat preferences (Nordlund, 1994), release of the correct species is critical for the success of control efforts.

The taxonomy of these wasps has historically been a problem because of their small size and lack of morphologically distinct characters. Initially, *Trichogramma* species were identified primarily by color and setation. Because of the plasticity of these traits Flanders and Quednau (1960) developed techniques, based on the ideas of Howard and Fiske (1911), that required rearing wasp species under carefully regulated environmental conditions before they could be identified. This method depended on the erroneous assumption that only six species of *Trichogramma* existed. With the number of known species of *Trichogramma* now approaching 200 (Pinto, 1999), this approach to identification has become impractical.

The modern taxonomy of *Trichogramma* started with the work of Nagarkatti and Nagaraja (1968, 1971) who discovered the usefulness of male genitalia for distinguishing species. This allowed unambiguous identification of many species. Unfortunately many important species share similar genitalic structure 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).

The use of male genitalic characters was indeed a large improvement, but it still requires specialized skills and is time consuming (Pinto and Stouthamer, 1994). An especially severe limitation is that female *Trichogramma* are virtually unidentifiable unless associated with males. This presents a particular problem for completely parthenogenetic forms in which males are not present at all, i.e. non-revertible parthenogenetic forms (Stouthamer et al., 1990). Thus various other methods have been proposed to further simplify the identification of *Trichogramma* species. Among these is the use of biochemical methods (Pintureau and Keita, 1989; Kazmer, 1991; Pinto et al., 1992, 1993). More recently DNA methods have been proposed using restriction length polymorphisms of the complete mitochondrial genome or RAPD PCR (Vanlerberghe-Masutti, 1994) and the DNA sequence of the internally transcribed spacer 1 (ITS1) of the ribosomal gene complex (Orrego and Agudelo-Silva, 1993).

Here we study the usefulness of ITS2 sequences as a general identification method for *Trichogramma* species. This spacer has proven useful

for distinguishing species of the genus *Nasonia* (Campbell et al., 1993). Before any character source can be proposed as appropriate for separating species, potential intraspecific variation must be taken into consideration. Also, the most useful characters are those that consistently separate similar species. Consequently, as an initial test of the ITS2 for *Trichogramma* identification, we determined its sequence for several collections of two complexes of closely related North American species. These consist of representatives of what we herein call the *Trichogramma deion* complex, composed of *T. deion*, *T. kaykai*, *T. sathon* and *T. pratti*, and the *T. pretiosum* complex, consisting of *T. pretiosum* and a form of undetermined status (*T. oleae*/*T. nr. pretiosum*). A representative of *T. interius* is used as an outgroup. The species status of the *T. deion* complex representatives and *T. pretiosum* has been substantiated using morphology, crossing compatibility and biochemical methods (Pinto et al., 1986, 1991, 1993, 1997). *Trichogramma deion* and *T. pretiosum* were themselves originally described as cryptic (Pinto et al., 1986), and the additional species now associated with each of them can be similarly designated based on morphology. As assemblages of minimally differentiated species they represent an ideal test for the value of the ITS2 sequences for identification. All species treated are reviewed in a recent revision of the North American *Trichogramma* (Pinto, 1999).

Materials and methods

Wasps and DNA purification

In this study we used wasps collected from different localities in North America. Wasps were collected as parasitized lepidopteran eggs; single mated females emerging from these field-collected eggs were used to start isofemale lines. Voucher specimens of all cultures are deposited in the collection of the Department of Entomology of the University of California, Riverside.

Origin of the examined cultures:

T. deion *complex*

T. deion – This species is distributed throughout western North America (Pinto et al., 1986). The following cultures examined represent a considerable portion of its known range: Portal, AZ [Arizona] (DPTL). Covelo, CA [California] (DCLO); Irvine, CA (DIRV); Last Chance Cyn. (Kern Co.), CA (DLC1); Marysville, CA (DMRY); Menifee Valley, CA (DMEN); Pinyon Mountains (Kern Co), CA (DPIN); Sheephole Mountains (San Bernardino

Co), CA (DSHE); Riverside, CA (two collections: DRIV, DRVI); Seven Pines (Inyo Co.), CA (DSVP); Eureka, NV [Nevada] (DEUR) and Sanderson, TX [Texas] (DTSN). DSVP is the culture from which the holotype of *T. deion* originated.

T. kaykai – The known distribution of this species is restricted to the Mojave Desert and the adjacent Sonoran Desert close to Palm Springs (Pinto et al., 1997). Cultures examined were from Walker Pass (KWPA), Last Chance Cyn. (KLCC = lc187), Sheephole Mountains (KSHE), and Danby (KDAN). KSHE represents the type locality of the species.

T. sathon – This secies occurs from southern California to western Texas. Cultures studied are from Socorro, NM (SASO) and Menifee Valley, CA (SAME). These cultures (as SOC and MNF, resp.), treated as *T. deion*, were incorporated in an earlier study of reproductive compatibility (Pinto et al., 1991).

T. pratti – This species has only been collected at four locations in the Mojave desert, two near Amboy, CA, one in Yucca Valley, CA, and one near Needles, CA. The cultures from near Amboy (Danby, CA (RDAN); Sheephole Mountains (RSHE)), were used for this study.

T. pretiosum *complex*

T. pretiosum – This common species is distributed throughout the Americas and also has been established in Hawaii and Australia (Pinto et al., 1986, 1993). Cultures examined for this study are as follows: Riverside, CA (PRV4) and (PRV1), Mesquite, NV (PMES), Nuevo Leon, Mexico (PNLE), Irvine, CA (PIRV) and Hawaii (PHAW).

T. nr. *pretiosum* – In North America this form is limited to one collection site, i.e. Mount Shasta, CA, but a morphologically similar and perhaps conspecific species is known from Europe as *Trichogramma oleae* (Voegelé and Pointel, 1979). Crossing studies have not been done between these collections. Both consist of parthenogenetic forms infected with *Wolbachia*, as reported for Mt. Shasta by Schilthuizen and Stouthamer (1997), and for *T. oleae* by Rousset et al. (1992) and Stouthamer et al. (1993). Cultures used here include Mt. Shasta, CA (NPSH) and Yugoslavia (NPOL = *T. oleae*) (Voegelé and Pointel, 1979).

Outgroup

T. interius – Although assigned to *T. platneri* by Pinto et al. (1992) this form is now considered a distinct species (Pinto, 1999). It is known from the southwestern USA and is morphologically similar to both *T. platneri* and *T. pretiosum*. The culture used here as an outgroup is from Mesquite, NV (IMSQ).

DNA-isolation
DNA was isolated from wasps that either were freshly killed by freezing, killed by freezing and stored for several years in liquid nitrogen, or killed and preserved in 100% ethanol. One to three wasps of each isofemale line were ground in 50 to 150 µl 5% Chelex-100 and 3 µl proteinase K (20 mg/ml) and incubated for at least 2 hours at 56 °C, followed by 10 min at 95 °C. The alcohol-preserved wasps were first shaken in 1 ml TAE for one hour prior to grinding in Chelex-100.

PCR amplification, cloning and sequencing
PCR was performed in 50 µl reaction volumes using a Hybaid thermocycler, 5 µl DNA template, 5 µl PCR-buffer, 1 µl dNTP's (each in a 10 mM concentration), 0.6 µl forward and reverse primer (10 ng), 0.1 µl SuperTth polymerase enzyme (5 units/µl), and 38 µl sterile distilled water. The ITS2 region was amplified using the following primers: forward, 5′-TGTGAACTGCAGGACACATG-3′ (ITS2-forward) located in the 5.8S rDNA; and reverse 5′-AATGCTTAAATTTAGGGGGTA-3′ (ITS2-reverse) located in the 28S rDNA. Primers used for the amplification of the ITS2 product were the same as those used by Campbell et al. (1993) for the phylogeny of *Nasonia* species. After using these methods for several months we changed the reverse primer to a location in the 28S rDNA closer to the 3′-end of the ITS2 to have a more specific amplification of *Trichogramma* ITS2 product. This primer has the sequence: 5′-GTCTTGCCTGCTCTGAG-3′ (ITS2rev-Trich). The PCR cycling program was 3 min at 95 °C followed by 33 cycles of 45 seconds at 92 °C, 45 seconds at 53 °C and 45 seconds at 72 °C with 3 min at 72 °C after the last cycle. PCR products of about 550 bp were electrophoresed and excised from the agarose gel. They where then frozen and freeze-squeezed. The liquid phase was alcohol precipitated, washed and ligated into a T-tailed vector (Amersham Life Science) and amplified in *Escherichia coli* cells. *E. coli* colonies containing an insert of the correct size were checked by PCR using the primers mentioned above and sent for automatic sequencing (373 DNA Sequencer Stretch, Applied Biosystems using a Prism Ready Reaction DyeDeoxy Terminator Cycle sequence Kit). To check the accuracy of the automatic sequencer, some clones were sequenced

several times. Restriction digestion was performed on 5 µl PCR product to which 3 µl water was added, 1 µl restriction buffer, as provided by the manufacturer, and 1 µl restriction enzyme. The reaction was incubated for 1 h at 37 °C.

Alignments and phylogenies

Sequences were aligned manually using the ESEE 3.0s sequence editor (Cabot, 1995). Phylogenetic parsimony analysis employed PAUP 3.1.1. (Swofford, 1993) using the branch and bound search algorithm with *Trichogramma interius* (IMSQ) as outgroup. Unpublished data (R.S.) on the overall phylogeny of many of the North American species show IMSQ consistently clustering basal to the *T. deion*-*T. pretiosum* complexes clade. A subset of the cultures itemized above for *T. deion*, *T. pretiosum* and *T. kaykai* were used for phylogenetic analysis. All cultures listed for *T. sathon*, *T. pratti*, and *T.* nr. *pretiosum* were incorporated. In the analysis, the aligned sequences were used with gaps treated as a fifth base and, alternately, as missing data. Initial parsimony analysis on unweighted characters was followed by successive approximations character weighting (Farris, 1989). Bootstrap values were based on 70–100 replications. GenBank accession numbers of the ITS2 sequences used in this study are: DCLO U76223, DEUR AF082823, DIRV U74676, DLC1 U76224, DMEN U74678, DMRY AF082822, DPIN U74679, DPTL AF082826, DRIV AF082827, DRV1 AF082824, DSHE AF082825, DSVP U76225, DTSN U74680, IMSQ U76230, KDAN AF082821, KLCC U76229, KSHE U76228, NPOL U74601, NPSH U74599, PHAW U74604, PIRV U76227, PMES AF082819, PNLE U74605, PRV1 AF082820, PRV4 U76226, RDAN AF082817, RSHE AF082818, SAME AF082815, SASO AF082816.

Species-specific primers

Species-specific primers were designed for the most common species of the two complexes, i.e. *T. deion* and *T. pretiosum*. These primers were chosen by identifying areas of maximal difference between the aligned sequence of the ITS2 of each species. In addition care was taken to choose the primers in such a way that they would not self-anneal. The *T. deion* specific primer (5′-CCTGATCGTTAACTGACAA-3′) anneals to the ITS2 of *T. deion* starting at position 290 ending at position 315 (DCOV). The *T. pretiosum* specific primer (5′AGAGCAAGAGATAGTGTGTGTGT-3′) anneals to the ITS2 of *T. pretiosum* starting at position 68 ending at position 90 (PIRV). The *T. deion* specific primer used with the ITS2 forward primer results in a PCR-product of approximately 404 bp, while the *T. pretiosum* specific

Table 1. Aligned sequences of the ITS2 of several lines of *Trichogramma deion.* Dots (.) indicate identity to the DRV1 sequence. Dashes (-) indicate insertions/deletions. Numbers indicate position in the aligned sequence

Deion line	ITS-2 sequence	
DRV1	GTTTATAAAAACGAACCCGACTGCTCTCTCTCTCTCTC----GCAAGAG--GAGAGAGAG	60
DEUR	.....----.....AA.....	
DMEN	.....----.....AA.--.....	
DTSN	.....----.....AA.....	
DRIV	.....TCTC.....AA.--.....	
DIRV	.....TCTC.....AA.--.....	
DSHE	.....A.....----.....AA.....	
DLC1	.....A.....----.....AA.....	
DPIN	.....------.....AA.....	
DPTL	.....----.....AA.--.....	
DSVP	.....------.....A-.....	
DMRY	.....------.....A-.....	
DCLO	.....----.....A-.....	
DRV1	CGTTGATCTGGGCGCTCGTG--TCGCTATCTCTTGCTGCTCTCTCAGGAGAGTAGTATAG	120
DEUR	.....--.....A.....	
DMEN	.....--.....	
DTSN	.....--.....A.....	
DRIV	.....--.....	
DIRV	.....--.....	
DSHE	.....--.....T.....	
DLC1	.....--.....	
DPIN	.....--.....	
DPTL	.....TG.....	
DSVP	.....--.....-.....	
DMRY	.....--.....-.....	
DCLO	.....--.....-.....	
DRV1	CAGTGTGCGCGACACGTCGCCTCAAACGAAACGCAAGAGAAAAGATGAATTAGCGTTCGT	180
DEUR	.....	
DMEN	.....	
DTSN	.....	
DRIV	.....A.....	
DIRV	.....	
DSHE	.....	
DLC1	.....	
DPIN	.....	
DPTL	.....	
DSVP	.....--.....G.....	
DMRY	.....--.....G.....	
DCLO	.....--.....G.....	

Table 1. Continued

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DRV1    CTGGCTGGCGCGCGCGCTTACCGCTTGGAGAGTACTCGCGTGTGTAAAAGCGCGCGAGTG   240
DEUR    ............................................................A
DMEN    ............................................................A
DTSN    ............................................................A
DRIV    ............................................................A
DIRV    ............................................................A
DSHE    ............................................................A
DLC1    ............................................................A
DPIN    ............................................................A
DPTL    ...........................................................CA
DSVP    ..............................G.........................A
DMRY    ..............................G.........................A
DCLO    ..............................G.........................A

DRV1    CTTCCGATCGTTCTGCGTTCGAGTCCCGGAGCTTCTCGCCTCGTCGAGCAGCGGACCGAT   300
DEUR    ............................................................
DMEN    ............................................................
DTSN    ............................................................
DRIV    ..........N.................................................
DIRV    ............................................................
DSHE    ............................................................
DLC1    ............................................................
DPIN    ............................................................
DPTL    ............................................................
DSVP    ...........................................................C
DMRY    ...........................................................C
DCLO    .C..........................................................C

DRV1    TGTCAGTTAACGATCAGGCTCGTCCATGATTCGGTACACTAGAAAGGCGCGCGCGCGCGC   360
DEUR    ...........................................................--
DMEN    ........................................-.-.................
DTSN    ................G..........................................--
DRIV    ...........................................................--
DIRV    ....G........................................................
DSHE    ..........................................-..........--
DLC1    ..............................G.....................--
DPIN    ...........................................................--
DPTL    ...........................................................--
DSVP    CTG.........................................................--
DMRY    CTG.........................................................--
DCLO    CTG.........................................................--
```

Table 1. Continued

DRV1	TATTTTTTATA---AATAATGGCCAGCTCGAACAACAACGA-TCTTTTTTCTCGAT	416
DEUR	..........---.........-.....	
DMEN	.........----.........-.........-..A.-.......-.....	
DTSN	..........---.....G.....	
DRIV	..........---.....C.....G...N.....	
DIRV	..........---.......N.....G.....	
DSHE	........T.G---.....G.....	
DLC1	........T.G--A.T.....G.....	
DPIN	........T.tG--.....G.....	
DPTL	.....A..T.TAAT.....G.....	
DSVP	....--A.T.TT-T.....-.....	
DMRY	......A.T.TT-T.....-.....	
DCLO	......A.T.TT-T.....-.....	

primers result in a product of 179 bp. The ability to produce a product of these primers was tested with the following lines: PIRV, PRV4, PRV1, NPSH, DRV1, DLCC, DSVP, DPTL, SAME, SASO, RSHE, KWPA, KDAN. The PCR cycling program was 3 min at 94 °C followed by 33 cycles of 40 seconds at 94 °C, 40 seconds at 58 °C and 45 seconds at 72 °C with 5 min at 72 °C after the last cycle.

Results

DNA extraction and PCR-reaction

The simple procedure we used for extracting template DNA by adding 5% Chelex to grind the wasps was sufficient to give enough PCR-product to clone it into a plasmid. Using PCR directly on *E. coli* colonies to check whether they contained an insert gave some false positive results, but if we diluted our overnight cultures 40 times in Chelex and handled them the same way as the wasp DNA, no false positive results occurred.

Within species sequence variation

Within species the ITS-2 sequences showed little variation. Most of the variation was restricted to the number of microsatellite repeats. To illustrate the level of variation within the sequences of a species the complete sequences of the ITS2 of all *T. deion* lines is shown in Table 1. We chose *T. deion* because of all species studied the largest number of collections was available for this

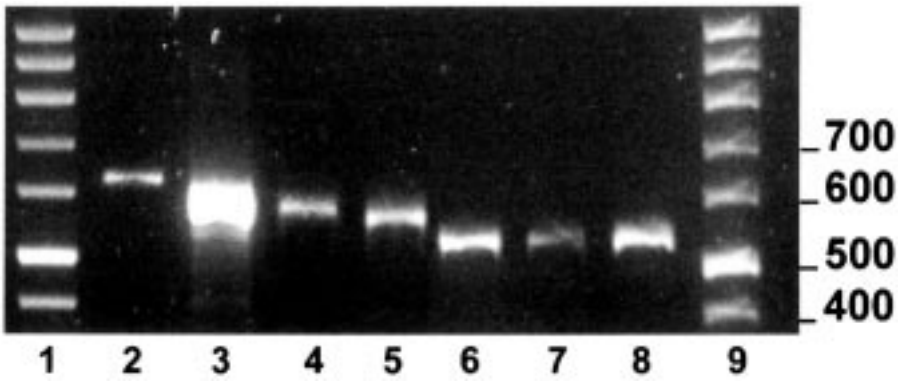

Figure 1. Gel showing the PCR-products of the ITS-2 plus flanking regions for the species of *the T. deion/T. pretiosum* complexes. Lane 1: Low ladder size standard, 2: *T. interius*, 3: *T. kaykai*, 4: *T. pratti*, 5: *T. sathon*, 6: *T. pretiosum*, 7: *T. oleae*, 8: *T.deion*, 9: Low ladder size standard.

species from a large geographical area ranging from Texas to California. Variation in the other species is similar to that found in *T. deion.*

Species differences

The ITS2 sequences of all studied species in the *T. deion/pretiosum* complexes differ from each other consistently. Within the *T. deion* complex the species differ in size of the ITS2 (Figure 1), resulting in differences in the PCR product size (i.e. 89 bp of 5.8S rDNA + size of ITS2 + 24 bp of the 28S rDNA). That of *T. kaykai* is the largest with a size of ca. 580 bp, while *T. deion* has the smallest PCR product with a size of 510–520 bp. Diagnostic differences among species can also be found using the restriction enzyme Mse1, EcoR1 and MaeII (Figure 2 and Table 2). These differences in size of the PCR product and the restriction length polymorphisms allow the construction of a key to species of these two complexes (Table 3).

Specific primers for T. deion *and* T. pretiosum

The specific primers for *T. deion* and *T. pretiosum* worked well (results not shown). The *T. deion* primer never amplified any *T. pretiosum* line, and the *T. pretiosum* specific primer only amplified the *T. pretiosum* and the near *T. pretiosum* lines we tested. With the *T. sathon* lines tested and the *T. pratti* lines the *T. pretiosum* primer occasionally gave very weak bands of approximately 400 bp (*T. pratti*) and 500 bp (*T. sathon*) with molecular weights much larger than the diagnostic *T. pretiosum* band of 180 bp.

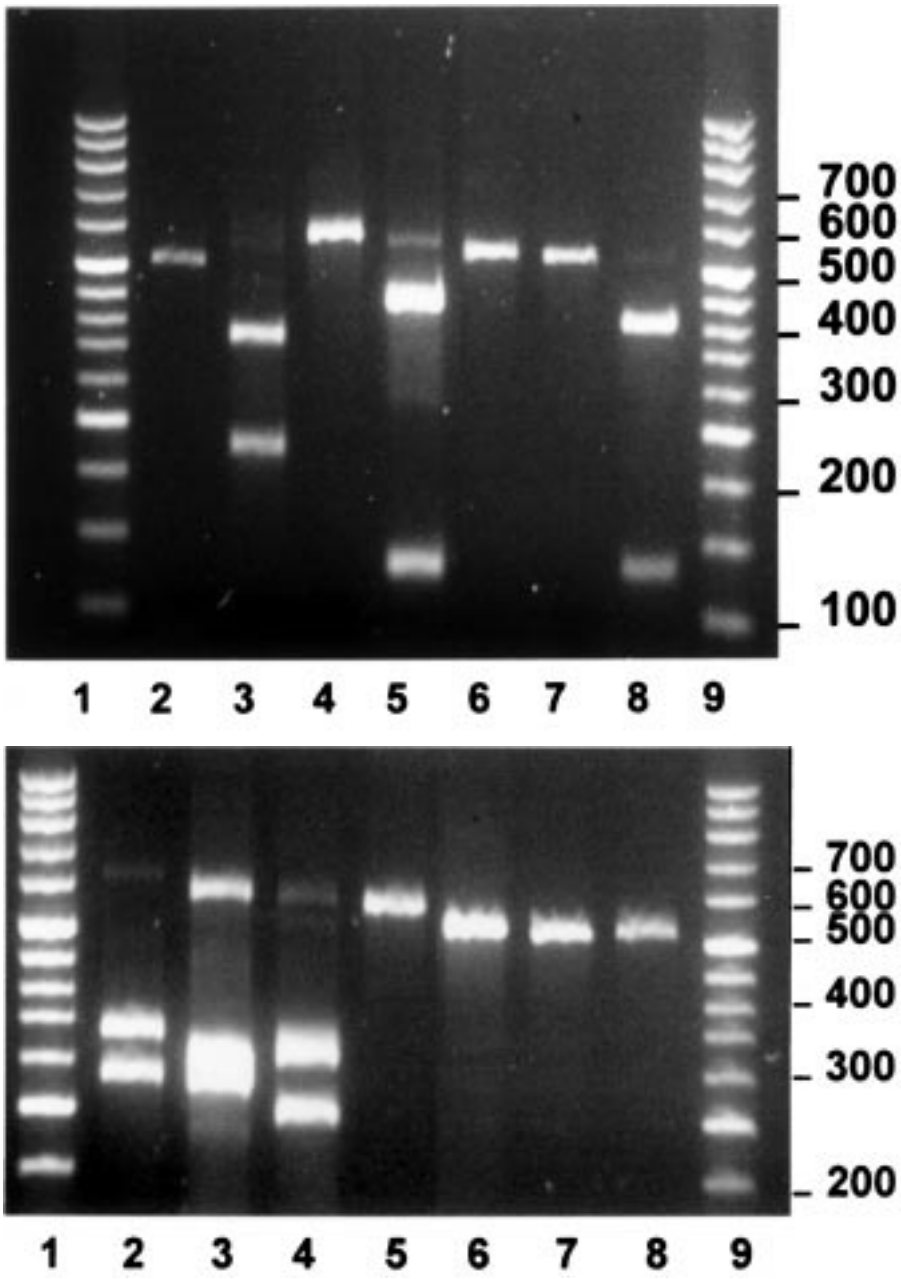

Figure 2. Gel showing the PCR-products of the ITS-2 plus flanking regions for the species of *the T. deion/T. pretiosum* complexes restricted with the enzyme Mse1 (A) and EcoR1 (B). Lane 1: low ladder size standard; 2: *T. interius*; 3: *T. kaykai*; 4: *T. pratti*; 5: *T. sathon*; 6: *T. pretiosum*; 7: *T. oleae*; 8: *T.deion*; 9: low ladder size standard.

Table 2. Size (in number of nucleotides) of the PCR product of the ITS2 and flanking regions of the 5.8S and 28S rDNA genes (using primers ITS2 forward and ITS2-rev Trich), and the restriction fragments generated by the restriction enzymes MaeII, MseI and EcoR1 of several species belonging to the *Trichogramma deion* and *T. pretiosum* complexes

	Size PCR product	MaeII	MseI	EcoR1
T. deion complex				
T. deion				
Seven Pines, CA	511	301,210	383,61,67	511
Eureka, NV	517	301,216	389,128	517
Covello, CA	515	303,212	385,63,67	515
T. kaykai				
Last Chance Canyon	582	315,267	359,223	312,279
Sheephole Mountains	575	315,260	352,223	575
T.sathon				
Menifee, CA	553	302,251	424,129	553
Socorro, NM	555	303,252	425,130	555
T. pratti				
Danby, CA	569	280,162,127	569	313,256
Sheephole Mnts, CA	563	274,162,127	563	307,256
T. pretiosum complex				
T. pretiosum				
Riverside (4), CA	522	227,211,84	522	522
Irvine, CA	526	228,214,84	526	526
T. nr. *pretiosum*				
Mt. Shasta, CA	524	221,137,84,82	524	524
Oleae, Yugoslavia	524	215,131,84,82	524	524
T. interius				
Mesquite, NV	628	218,246,117,47	498,105,25	279,349

Table 3. Molecular key to the species of the *T. deion* and *T. pretiosum* complexes based on the ITS2 PCR product

1.	Size of the PCR product greater than 540 bp	2
	Size of the PCR product less than 540 bp	5
2.	PCR product not cut by EcoR1	3
	PCR product cut or partially cut by EcoR1	4
3.	Size of the PCR product 580 bp	*T. kaykai*
	Size of the PCR product 550 bp	*T. sathon*
4.	PCR product restricted with Mse1 gives 2 bands	*T. kaykai*
	PCR product not restricted by Mse1	*T. pratti*
5.	PCR product restricted with MseI gives 2 or 3 bands	*T.deion*
	PCR product restricted with MseI gives 1 band	6
6.	PCR product restricted with MaeII gives 2 visible bands	*T. pretiosum*
	PCR product restricted with MaeII gives 3 visible bands	*T. oleae*, *T.* nr *pretiosum*

Phylogeny of species in the T. deion *and* T. pretiosum *complexes*

Parsimony analysis using gaps as a 5th base resulted in three equally parsimonious trees each with a length of 626 (consistency index (CI) = 0.802; retention index (RI) = 0.845). Successive approximations weighting produced a single tree, topologically equivalent to one of the three trees following unweighted analysis. This cladogram, as well as the strict consensus tree of the three are shown in Figure 3. Analysis depicts all species analyzed as monophyletic, but only the *T. pretiosum* complex as monophyletic. *Trichogramma pratti* of the *T. deion* complex clusters peripherally to all other species examined, including those of the *T. pretiosum* complex. The remainder of the *T. deion* complex is monophyletic, showing *T. kaykai* and *T. sathon* as sister species, and both together as sister group to *T. deion.*

Initial analysis with the reduced character set (gaps = missing data) resulted in 480 equally parsimonious trees, each with a length of 178 (CI = 0.882; RI = 0.863). Successive approximations weighting produced 24 trees also of 178 steps. Strict consensus cladograms of both the unweighted and weighted trees are shown in Figure 4. Successive approximations shows the *T. deion* complex as monophyletic and the *T. pretiosum* complex as paraphyletic. It also renders *T. deion* itself as paraphyletic. All other species are monophyletic in both analyses. The unweighted consensus tree

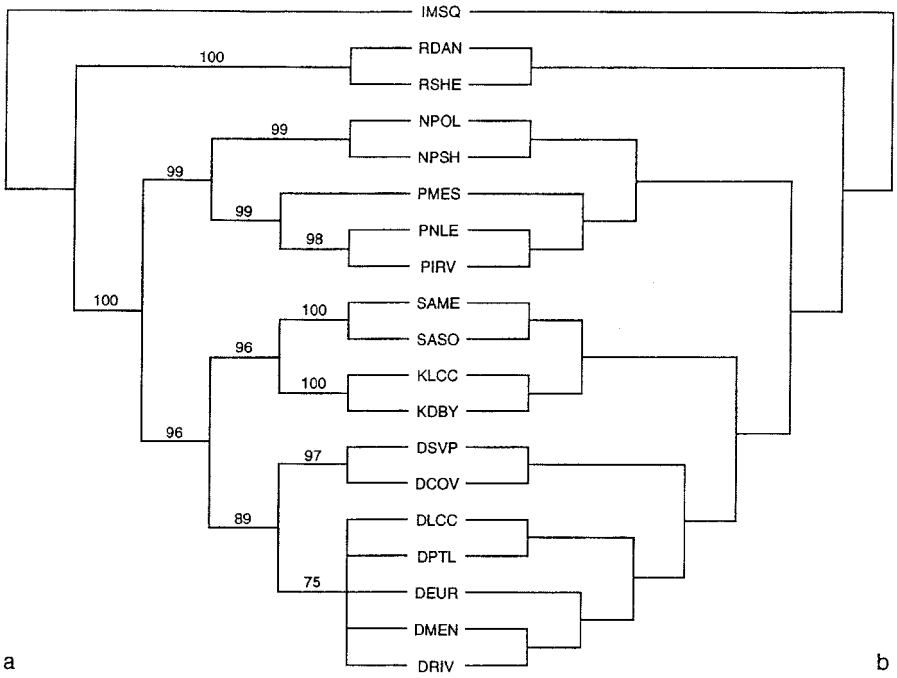

Figure 3. Cladograms from ITS2 analysis treating gaps as a 5th base. (a) Strict consensus tree with characters unweighted. Bootstrap values >50% (100 replicates) indicated above each branch. (b) Successive approximations tree. Species identity of cultures indicated by first letter of acronym. P (= *T. pretiosum*), N (= *T. nr. pretiosum*), D (= *T. deion*), K (= *T. kaykai*), S (= *T. sathon*), R (= *T. pratti*). P+N = *T. pretiosum* complex; D+K+S+R = *T. deion* complex.

divorces both *T. kaykai* and *T. pratti* from others in the *T. deion* complex. The peripheral position of *T. pratti* is in agreement with the prior analysis (Figure 3).

Discussion

ITS2 can be used for species identification in *Trichogramma* because (1) the sequence variation within species is small relative to the differences found between species and (2) all morphologically distinct cryptic species are also distinguished by sequence differences. Within each species the ITS2 sequences 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). Slight within individual variation is sometimes found in certain stretches of microsatellite DNA repeats. These microsatellite stretches are often also variable within a

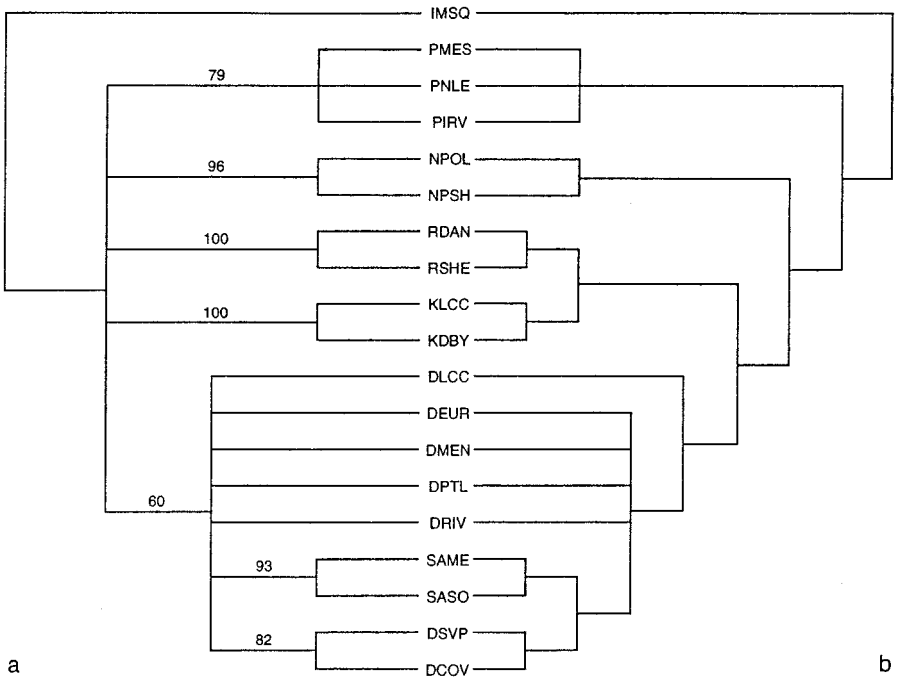

Figure 4. Cladograms from ITS2 analysis treating gaps as missing data. (a) Strict consensus tree with characters unweighted. Bootstrap values >50% (70 replicates) indicated above each branch. (b) Successive approximations consensus tree. See Figure 3 for species identity of the cultures.

species, for instance in the first section of TC repeats in the ITS2 of *T. deion*, starting at approximately position 25 of the spacer, three different types are found, i.e 6, 7 or 9 repeats (Table 1). Similar microsatellite variation has been found in eriophyid mites (Fenton et al., 1997). In *T. kaykai* some within individual variability exists because restriction analysis with EcoR1 gives only a partial digestion of the PCR product; in addition in some of the ITS2 sequences of this species the EcoR1 site is present while in others it is lacking.

The ability to create a PCR based key simply using this one spacer shows promise as a general identification system for *Trichogramma* species. Such a system would be a considerable improvement over the current method, which is dependent almost entirely on morphology. The main advantage of the DNA identification system over the morphological system is that it requires few specialized skills, is fast and relatively cheap. An important advantage of the ITS2-DNA method over allozymic methods of identification is that specimen(s) can be preserved and stored much more easily. Thus, for ITS2 identification they can be dried or stored in 100%

alcohol, whereas fresh material or material stored in liquid nitrogen is required for allozymic work. Also a single PCR product of ITS2 contains many more opportunities for species-specific characters than a single allozyme with at best 2–5 distinguishable alleles. The *Trichogramma* identification system based on RAPD PCR as used by Vanlerberghe-Masutti (1994) has the disadvantage over the system proposed here in that, firstly, it is based on RAPD PCR with the notorious problems of transportability, and, secondly, it requires 50 specimens of the wasp to be successful. The identification system based on mitochondrial DNA, also used by Vanlerberghe-Masutti (1994), is much more difficult, again because it relies on several thousands of specimens to acquire enough mitochondrial DNA.

Our key lacks a feature of positive identification. In other words, if no restriction digest is found, it is not immediately clear if the restriction reaction did not work or if the product indeed lacks the restriction site. A positive identification system could be generated by the design of primers that would result in a species-specific PCR product. Such specific primers have been developed for the most widespread and economically important species of this group: *T. deion* and *T. pretiosum*. These primers work well and may be used as a confirmation of the identification of these species. The availability of a primer for *T. pretiosum* is particularly valuable. Not only is this species commercially available and commonly used in biological control (Monje, 1995; O'Neil et al., 1998), but it is a common contaminant of other species commonly cultured in North America such as *T. platneri* (Pinto, pers. obs.).

A key restricted to a group of related species as presented here is of limited use unless one knows before hand that specimens belong to that assemblage. However, this appears to be a minor problem because the most common species of these complexes, *T. deion* and *T. pretiosum*, differ from other North American *Trichogramma* species in lacking a restriction site for EcoR1 in their ITS2. In any event, this potential drawback will hopefully be resolved shortly when work on a PCR-based identification key to all North American species groups is completed.

The potential advantages of using the ITS2 sequence for identifying *Trichogramma* species notwithstanding, it must be stressed that the success of this method depends on an infrastructure of sound morphological studies. The differences in structure separating the cryptic species treated here may be relatively minor and difficult to communicate to non-specialists, but they were tested for consistency among hundreds of independent collections and thousands of specimens. Thus, the species concepts used in these complexes remain morphologically based. The sequence data reported has tested and

supported these concepts but it would have been logistically impractical to formulate them on the sequence data alone. We mention this because morphological and molecular data are sometimes thought of as alternative approaches to systematics; in most cases they are not.

ITS2 provides an excellent method for separating closely related species of *Trichogramma*, but additional work is required before determining its value for estimating phylogeny. Relationships based on incorporating gaps as a 5th base (Figure 3) show all species as monophyletic but treat one species of the *T. deion* complex (*T. pratti*) as the most remotely related member analyzed. This peripheral position for *T. pratti* is paralleled in the unweighted analysis when considering gaps as missing data (Figure 4a). This is not predicted by morphology which suggests an extremely close association with *T. deion*. The only dependable characters available for separating the two are relatively subtle differences in ovipositor length and length of the antennal setae in males. The genitalia, so variable within the genus at the species level (Pinto and Stouthamer, 1994), are virtually identical in these two (Pinto, 1999).

The weighted analysis treating gaps as missing data (Figure 4b) does bring *T. pratti* into a monophyletic *T. deion* complex as the sister group to its sympatriate *T. kaykai* which is more acceptable, but now treats *T. deion* as paraphyletic (as does unweighted analysis, Figure 4a). All other species are monophyletic. This analysis, although based on a considerably reduced character set, is included because we believe that paraphyly should not be immediately discounted for *T. deion*. It is one of the most common species in western North America, occurring in most habitats sampled thus far. The other three members of the complex are primarily restricted to arid habitats in southwestern North America. All are sympatric with *T. deion*. That one or more of them were derived from *T. deion* itself is a possibility deserving further investigation.

A recent phylogenetic analysis of the 68 species of North American *Trichogramma* based on morphology was unable to resolve relationships within a large group of 31 species which includes members of the *T. deion* and *T. pretiosum* complexes (Pinto, 1999). This group, containing most of the species of interest to biological control, is extremely homogeneous morphologically. The considerable ITS2 variation in the species groups studied here holds promise that this sequence can be useful in gaining a better appreciation of phylogeny in this important assemblage. However, a more advanced analysis using the secondary structure of the ITS2-RNA, may be needed to estimate species relationships (Schlötterer et al., 1994).

We can conclude that the ITS2 is useful both for identification and for studying the phylogeny of this group. The fact that this spacer enables us to

distinguish among closely related species makes it an especially promising candidate for systematics studies in this genus.

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