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Abstract Molecular techniques were used to distinguish six *Trichogramma* species (Hymenoptera, Trichogrammatidae) collected from different ecological zones of Pakistan: *T. chilonis* (Ishii), *T. chilotraeae* (Nagaraja and Nagarkatti), *T. evanescens* (Westwood), *T. pintoi* (Voegele), *T. euproctidis* (Girault, 1911), *T. siddiqi* (Nasir and Schöller). Electrophoresis of PCR-amplified ribosomal DNA internal transcribed spacer 2 (ITS-2) followed by restriction endonuclease digestions was applied. The restriction enzyme analysis was carried out in order to obtain species specific banding patterns. To optimize the amplification and sequence reaction, two new primers were designed. The sequence of each species distinctly differed in length and in nucleotide composition. Thus, the results showed that this technique is a good tool to identify cryptic *Trichogramma* species, which are otherwise difficult to distinguish on the basis of morphological characters. Here we provide a dichotomous molecular key for *Trichogramma* species of Pakistan for easy and quick species identification.

Keywords ITS-2 rDNA · Polymerase chain reaction · Restriction analysis · *Trichogramma chilotraeae* · *T. evanescens* · *T. euproctidis* · *T. pintoi* · *T. chilonis* · *T. siddiqi*

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Introduction

The members of the genus *Trichogramma* (Hymenoptera: Trichogrammatidae) are difficult to distinguish because of their small size (<0.6 mm) and low interspecific morphological character diversity creating many identification problems (Nagarkatti and Nagaraja 1977, Smith and Hubbes 1986). For example in India, *T. australicum* Girault 1912 was erroneously referred to as *T. minutum* Riley 1871 or *T. evanescens* Westwood 1833 for nearly 50 years (Nagarkatti and Nagaraja 1968). Nagarkatti and Nagaraja (1968, 1971, 1973) showed the suitability of various characters of

the male genitalia as characters for identification of *Trichogramma* spp., and this character set became a standard in *Trichogramma* taxonomy (Pinto 1999). However, there are species/groups where even male genitalia show morphological homogeneity (Stouthamer et al. 1990; Pinto 1999). Furthermore the character set does not overcome the difficulties in identification of female individuals in particular if males are unavailable (Pinto and Stouthamer 1994) as is the case in completely parthenogenetic forms (Stouthamer et al. 1990). As sex ratios of field population of *Trichogramma* are often female biased (Pinto 1999), and all female populations are relatively common in this genus (Huigens and Stouthamer 2003; Vavre et al. 2004) reliable identification is troublesome.

To safeguard correct identification based on morphological characters other methods such as tests for mating incompatibility (Pinto and Viggiani 1991; Stouthamer et al. 1996; Glenn et al. 1997), and allozyme electrophoresis (Pintureau and Keita 1989; Kazmer 1991; Pinto et al. 1992, 1993) were explored and evaluated. Applying molecular biological tools such as the internally transcribed spacer 2 (ITS-2) of the nuclear ribosomal gene complex ensure a reliable identification of many *Trichogramma* species (Pinto and Viggiani 1991, Pinto et al. 1997; Silva et al. 1999; Stouthamer et al. 1999; Thomson et al. 2003; Huigens and Stouthamer 2003; Sumer et al. 2009; Sayed et al. 2011; Karimi 2012; Polaszek et al. 2012). There are however some exceptions e.g. *T. minutum* and *T. platneri*, that are morphologically identical (Pinto et al. 2003) and even do not differ in their ITS2 sequence (Stouthamer et al. 2000). These species can only be distinguished by the sequence of their COII gene (Borghuis et al. 2004).

In Pakistan, *Trichogramma* spp. are mass produced and these wasps are released especially in sugarcane and cotton (Shahid et al. 2007). Unfortunately, the species identity of the released wasps is often unknown as the morphological identification requires high skill levels to prepare slides (Platner et al. 1999) and to identify the subtle morphological characters (Pinto 1999). However, correct identification of *Trichogramma* spp. is crucial for successful biological control programs (Smith and Hubbes 1986; Stouthamer et al. 1999). The importance of properly matching the correct *Trichogramma* species or strain to the appropriate pest situation has been discussed extensively (Kot 1979; Voronin and Grinberg 1981). Incorrect identification may lead to release of unsuitable species, resulting in subsequent failure of biocontrol (Stouthamer et al. 2000; Rosen 1978; Wajnberg 1994). In some cases, the release of the wrong species in an area where another closely related species is present, can lead to a suppression of both native and introduced species in biological control programs (Stouthamer et al. 2000).

To evaluate the potential and when indicated the implementation of *Trichogramma* wasps for biocontrol of economically important insects, the *Trichogramma* fauna in different agro-ecosystems of Pakistan was recorded (Nasir et al. 2006). The preliminary identification of the wasps based on morphological traits revealed species previously not known to occur in the country.

Here, we provide characters to distinguish among six *Trichogramma* species collected from Pakistan based on their sequence information of ITS2 region of rDNA. This also includes one recently described species (Nasir et al. 2011), which is a sibling species to *T. chilonis* Ishii 1941. In addition, we developed a molecular identification key for the six species using the size of the ITS-2 PCR product, along with species specific fingerprints following restriction enzyme digestion.

Materials and methods

Trichogramma cultures

Thirteen iso-female lines belonging to six different species collected from different hosts and ecological zones of Pakistan were investigated. Characteristics and origin of the species and strains are given in Table 1. Cultures were maintained on *Sitotroga cerealella* eggs at 26 °C and 70 % R.H. The males were mounted on microscope slides using Canada balsam (Platner et al. 1999) and identified using the morphology of genitalia and posterior setal track of the hind wing (Pinto 1999). To consider the intra-specific variability among the selected species, six lines of *T. chilonis* and two of *T. evanescens* and *T. euproctidis* (Girault 1911) = *T. turkestanica* Meyer 1940 (Rohi and Pintureau 2003), respectively, were investigated.

DNA-extraction

DNA was extracted as described by Stouthamer et al. (1999) with some modifications described below: one

Table 1 Characteristic features and origin of investigated *Trichogramma* species and strains from Pakistan

S. no.	*Trichogramma* species	Sample/code no.	Collection locality with latitude and longitude	Host plant	Host
1	*T. chilotraeae*	QT-6	Quetta, 30°15′N 66°55′E	*Brassica oleracea*	*Pieris rapae*
2	*T. pintoi*	BNI-20	Booni, 36°26′N 72°36′E	*Malus domestica*	*Sitotroga cerealella* eggs used as bait
3	*T. evanescens*	CHT-1	Chitral, 35°50′N 71°56′E	*Solanum lycopersicum*	*Helicoverpa armigera*
4	*T. evanescens*	BNI-19	Booni, 36°26′N 72°36′E	*S. lycopersicum*	*H. armigera*
5	*T. euproctidis*	CHT-3	Chitral, 35°50′N 71°56′E	*S. lycopersicum*	*H. armigera*
6	*T. euproctidis*	SQG	Shaqar Gar, 32°15′N 75°90′E	*Saccharum officinarum*	Unknown
	T. siddiqi	ME 2	Murree 33°54′N 73°23′E	*S. lycopersicum*	*H. armigera*
8	*T. chilonis*	DIR	Dir, 35°08′N 71°59′E	*S. lycopersicum*	*H. armigera*
9	*T. chilonis*	KWL	Khanewal, 30°18′N 71°55′E	*Citrus sinensis*	*Papilio demoleus*
10	*T. chilonis*	NWB	Nawabshah, 6°14′N 68°23′E	*Brassica oleracea*	*Spodoptera litura*
11	*T. chilonis*	MWL	Mian wali, 32°38′N 71°28′E	*S. officinarum*	*Chilo infescatelus*
12	*T. chilonis*	TTS	T.T. Singh, 30°58′N 72°28′E	*S. officinarum*	*Mythemna* sp.
13	*T. chilonis*	GT	Gujrat, 32°40′N 74°02′E	*Ricinus communis*	*Euproctis lunata*

S. no. Sample number

to five frozen wasps were crushed with a pestle (grinder) in an 1.5 ml Eppendorf tube. 50 µl distilled water was added to these tubes followed by addition of 2 µl of proteinase K. All the Eppendorf tubes were then incubated overnight at 56 °C, followed by 10 min at 95 °C.

Polymerase chain reaction (PCR) and electrophoresis

Based on the GenBank sequences of *T. minutum* (U36235) and *T. chilonis* (AY167415, AY167418), the primers Trich-3 5′-GTG GAT CGA TGA AGA ACG-3′ and Trich-4 5′-GAT ATG CTT AAA TTC AGC GGG-3′ were designed to amplify the ITS-2 region (plus portions of the flanking regions 5.8S and 28S genes). PCR reaction was performed in 50 µl volumes containing 5 µl DNA template, 5 µl PCR buffer (10×), 1 µl dNTP's (10 mM), 0.6 µl forward and reverse primer each (20 pmol μl^{-1}), 2.5 µl Mg Cl2 (25 mM), 0.2 µl SuperTaq polymerase (Sphaero-Q, 5 units μl^{-1}) and 35.1 µl sterile water. The PCR cycling programme was 3 min at 94 °C, followed by 33 cycles of 45 s at 92 °C, 45 s at 53 °C, and 45 s at 72 °C, with 3 min at 72 °C after the last cycle. PCR products were electrophoresed on a 2 % agarose gel. Gels were stained using ethidium bromide. Molecular weight standards were run along with the samples for reference. Sterile water was used as a negative control lacking template DNA in all experiments.

Restriction analysis

For those PCR products of similar size, i.e. *T. chilotraeae* Nagaraja and Nagarkatti 1970 (QT-6), *T. evanescens* (BNI-19), *T. chilonis* (DIR) and *T. siddiqi* Nasir and Schöller 2011 (ME_2), two restriction enzymes Bsp119I and BseXI were selected to find specific differences. For the selection of these restriction enzyme we applied the program DNA strider 1.0 (Christian Marck) to DNA sequences. Restriction analysis was performed in a 20-µl volume, using 5–10 µl PCR product, 2 µl (103) reaction buffer, 2 µl BSA (1.0 mg ml^{-1}), 1 µl Bsp119I and BseXI (10 units μl^{-1}), and 5-10 µl distilled water. The mixture was incubated for 2 h at 37 °C. Samples were run on standard 2 % agarose gel to check digestions.

Sequencing and alignments

The lengths of the fragments derived from the ITS-2 region were determined on 2 % agarose gels. PCR conditions were optimized until all strains yielded a single PCR product which was purified using Prep-A-Gene DNA Purification kit (BIO-RAD) according to the manufacturer's instruction. The purified double-stranded PCR products were directly sequenced using

Amersham Thermo Sequenase kit (Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP, Amersham Pharmacia Biotech) and infra red-labeled primers, Trich3 IR (5′-GGA TCG ATG AAG AAC GCA GC-3′) and Trich4 IR (5′-GCT TAA ATT CAG CGG GTG-3′).

Primary nucleotide sequences were determined on a LI-COR 4000L automated DNA sequencer. The entire product was sequenced bi-directionally. Sequences were assembled and corrected using Sequencher 4.0.5 (Gene Codes Corporation, Ann Arbor, Michigan, USA). DNA sequences were aligned together with published sequence data (Table 2) and improved manually with Gene Doc, Multiple Sequence Alignment Editor and Shading Utility, Version 2.6.002.

A molecular key for the identification of *Trichogramma* species, based on the size and the fragment profile was constructed. First ITS-2 sequences of all species and lines were determined and then the different species were distinguished based on the size of the PCR product. In order to separate the species having similar sized PCR products, restriction enzymes were selected using the programme Webcutter 2.0, which is a web-based restriction analysis tool. These sequences were then used to construct a molecular key based on the size of the ITS2 spacer and using two restriction enzymes Bsp119I (AsuII) and BseXI (BbvI) for the identification of *Trichogramma* species.

Results

PCR of ITS-2 rDNA, followed by restriction analysis, using the endonucleases Bsp119I and BseXI allowed the identification of six *Trichogramma* species namely *T. chilonis* (DIR), *T. euproctidis* (SQG and CHT-3), *T. evanescens* (BNI_19 and CHT_1), *T. chilotraeae* (QT-6), *T. siddiqi* (ME 2), and *T. pintoi* (BNI-20) (Table 3). On the basis of the restriction analysis a molecular key could be constructed (Table 4). PCR amplification followed by agarose gel electrophoresis resulted in a single band of DNA product for all the six wasps species tested (Fig. 1). Based on the size in base pairs (bp) of ITS-2 rDNA PCR products, three groups could be distinguished: (1) - *T. euproctidis* (551 bp), (2) - *T. chilonis* (ca. 592 bp), *T. siddiqi* (ca. 603 bp), *T. chilotraeae* (ca. 604 bp), *T. evanescens* (ca. 609 bp) and (3) - *T. pintoi* (ca. 750 bp).

Trichogramma species having similar sized PCR products, i.e. *T. chilotraeae*, *T. evanescens*, *T. euproctidis*, *T. siddiqi* and *T. chilonis* were distinguished by restriction enzyme analysis using two endonucleases i.e. Bsp119I (AsuII) and BseXI (BbvI). Restriction digests using endonuclease Bsp119I yielded a distinct pattern for *T. chilotraeae* from the remaining three species i.e. *T. euproctidis*, *T. siddiqi* and *T. chilonis* (Fig. 2a), while endonuclease BseXI differentiated among *T. evanescens, T. siddiqi* and *T. chilonis* (Fig. 2 b). Based on the length of the complete ITS-2 sequences of four *T. chilonis* lines (DIR etc.), two of *T. euproctidis* lines (CHT-3 etc.) and two of *T. evanescens* lines (CHT-1 etc.), showed low intraspecific variability.

To confirm the identification of *Trichogramma* species the sequences were compared with the identified ITS-2 sequences of rDNA from GenBank (Table 2). The comparison showed that *T. chilonis* (DIR) and *T. pintoi* (BNI-20) were similar to *T. chilonis* and *T. pintoi* in GenBank accession numbers AY244461, AF422845, AY167415 and AF043622, and AF043621, respectively. The two strains of *T. euproctidis* (CHT-3 and SQG) were similar to *T. turkestanica* = *T. euproctidis* available in GenBank (AF043613, AF043615, JF415945 & JF415946) and were also similar to each other. Similarly, *T. evanescens* (BNI-19 and CHT-1) were similar to each other and to *T. evanescens* available in GenBank (AF043616, AF043617, JF415940, JF415942 and JF415944).

Discussion

This study showed that the ITS-2 provides an excellent method for separating closely related species of *Trichogramma,* for example under the present study three closely related species i.e. *T. evanescens*, *T. euproctidis* (Rohi and Pintureau 2003) and *T. chilotraeae* were difficult to distinguish morphologically, but were easily separated on the basis of their sequence differences. Similarly, *T. siddiqi* was distinguished from *T. chilonis* because of consistent differences in their sequences, though only minor differences were found in their morphology (Nasir et al. 2011). Moreover, the DNA characters allowed identification of female *Trichogramma* spp. which was not possible using external morphological characters.

It is interesting to note that based on the study of morphological characters, the *Trichogramma* strains

Table 2 Sequences used from GenBank for comparison of the *Trichogramma* species recorded from Pakistan

S. no.	*Trichogramma* spp.	Sequence	Base pairs	Accession no.
1	*T. euproctidis*	Complete ITS-2	376	AF043613
2	*T. euproctidis*	Complete ITS-2	372	AF043615
3	*T. euproctidis*	Partial ITS-2	377	JF415945
4	*T. brassicae*	Complete ITS-2	375	AY163002
5	*T. brassicae*	Complete ITS-2	411	AY326470
6	*T. brassicae*	Complete ITS-2	406	AY182766
7	*T. nr. brassicae*	Complete ITS-2	422	AY163005
8	*T. nr. brassicae*	Complete ITS-2	422	AY163005
9	*T. evanescens*	Complete ITS-2	435	AF043617
10	*T. evanescens*	Complete ITS-2	429	AF043616
11	*T. evanescens*	Partial ITS-2	432	JF415940
12	*T. evanescens*	Partial ITS-2	430	JF415942
13	*T. evanescens*	Partial ITS-2	433	JF415944
14	*T. chilonis*	Complete ITS-2	394	AY244461
15	*T. chilonis*	Complete ITS-2	410	AF422845
16	*T. chilonis*	Partial ITS-2	1158	AY167418
17	*T. chilonis*	Partial ITS-2	1155	AY167415
18	*T. pintoi*	Complete ITS-2	555	AF043621
19	*T. pintoi*	Complete ITS-2	582	AF043622
20	*T. pintoi*	Complete ITS-2	581	AY244469
21	*T. exiguum*	Complete ITS-2	383	AY182768
22	*T. australicum*	Complete ITS-2	318	AY244469
23	*T. ivelae*	Complete ITS-2	455	AY244468
24	*T. dendrolimi*	Complete ITS-2	510	AB094398
25	*T. pretiosum*	Complete ITS-2	381	AY163009
26	*T. pretiosum*	Complete ITS-2	536	AY187263
27	*T. nubilale*	Complete ITS-2	413	TNU74600
28	*T. deion*	Complete ITS-2	409	TDU74680
29	*T. cordubensis*	Complete ITS-2	416	AF043619
30	*T. cordubensis*	Complete ITS-2	416	AF043620
31	*T. pintoi*	Complete ITS-2	582	AF043622

S. no. Sample number

with code numbers CHT-3, SQG and BNI-19 were identified as *T. brassicae* using the determination keys of Pintureau (1982, 1991) and Pinto (1999). These identifications were further verified by Dr. Pintureau. However, sequence analysis showed differences among these three strains, i.e. BNI-19 was similar to *T. evanescens* (AF043617, AF043616, JF415940, JF415942 and JF415944, see Table 2), while CHT-3 and SQG were more similar to *T. euproctidis* available in the GenBank (AF043613, AF043615, JF415945 and JF415946) rather than *T. brassicae*. This suggests that parts of the morphological data previously published for *T. brassicae* might refer to other species of *Trichogramma*. Moreover, this indicates that some of the ITS-2 sequences in GenBank are associated with the wrong species name. Similar misidentifications have also been reported by Sumer et al. (2009), who observed that two sequences under accession numbers AF453568 and AF453569 are listed as *T. maidis* (= *T. brassicae*), but were sequences of *T. evanescens*.

One reason for the observed mismatching of species name and sequence information deposited might be the

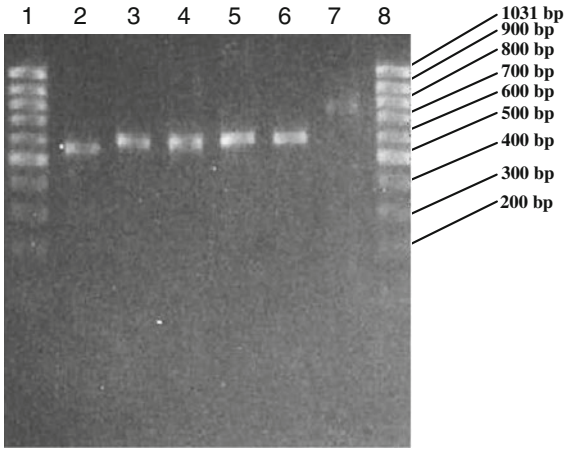

Fig. 1 Gel electrophoresis of ITS-2 PCR products. Lanes 1 and 8 100 bp ladder, (Fermentas), Lane 2 *T. euproctidis* (CHT-3), Lane 3 *T. chilotreae* (QT-6), Lane 4 *T. evanescens* (BNI-19), Lane 5 *T. siddiqi* (ME-2), Lane 6 *T. chilonis* (DIR) and Lane 7 *T. pintoi* (BNI-20)

fact that in the past *Trichogramma* species were not always identified by specialists. Moreover, due to development in the taxonomy of this genus the species name of taxa has been changed, e.g. *T. maidis* was synonymous with *T. brassicae* (Pintureau 1987) or the valid name of *T. turkestanica* was shown to be *T. euproctidis* (Rohi and Pintureau 2003). The length of the complete sequences for all the four *T. chilonis* lines, i.e. DIR, GT, TTS, and NWB ranged from ca. 552–560 bp, while the range for *T. euproctidis* was between ca. 512–514 bp and in case of *T. evanescens* the range was between ca. 531–559 bp. The low range of intra-specific variation obtained is in agreement to the range detected by Stouthamer et al. (1999) and Silva et al. (1999). In general, variation was limited to microsatellite repeat stretches found in the ITS-2 sequences. The variation in the number of microsatellite repeats seems to be common in ITS-2 sequences.

The construction of an identification key was possible using the PCR product size and the restriction patterns generated by the restriction enzymes BseXI and Bsp119I. ITS-2 product size of the six studied species ranged from ca. 551–750 bp and sequences differed from each other consistently (Table 4). The key now supplements the increasing number of molecular keys available for different geographic areas (Silva et al. 1999; Ciociola et al. 2001; Kumar et al. 2009).

The results of our study showed that the small amount of sequence variation within the species relative to that found between species, which allows ITS-2 to be used for species identification, is in agreement with the results by Stouthamer et al. (1999), Silva et al. (1999) and Pinto et al. (2002). However, one case of two North American morphologically indistinguishable species, i.e. *T. minutum* Riley and *T. platneri* Nagarkatti, is known where no consistent differences were found in their ITS-2 sequences (Stouthamer et al. 2000).

This work proved that molecular studies are a good tool for the identification of Pakistani *Trichogramma* species. However, for stable nomenclature and reliable comparison of species with GenBank information, it is important to include molecular data from the type strains in the GenBank if possible (e.g. the Moldavian strain of *T. brassicae*), or at least strains from the type locality. In case of *T. evanescens*, a

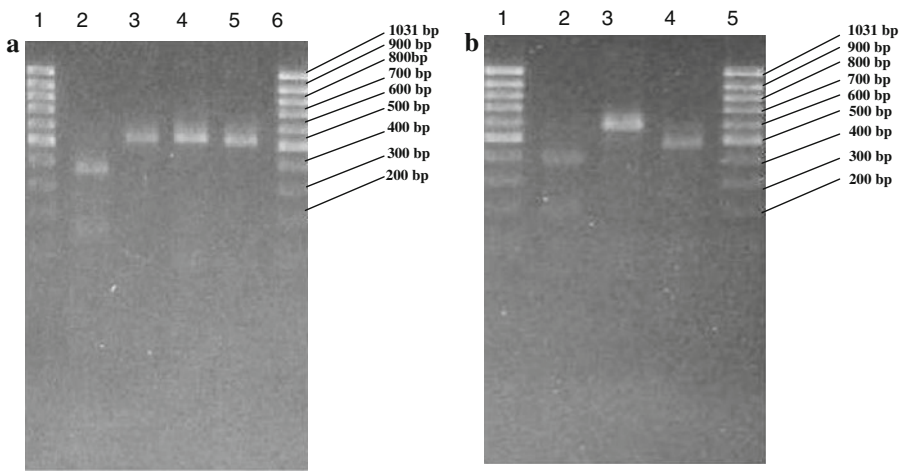

Fig. 2 Gel electrophoresis of restriction digestions by Bsp119I and BseXI endonucleases **a** Lanes 1 and 6 100 bp ladder, (Fermentas), Lane 2 *T. chilotraeae* (QT-6), Lane 3 *T. evanescens* (BNI-19), Lane 4 *T. siddiqi* (ME-2), Lane 5 *T. chilonis* (DIR). **b** Lanes 1 and 5 100 bp ladder, (Fermentas), Lane 2 *T. evanescens* (BNI-19), Lane 3 *T. siddiqi* (ME-2), Lane 4 *T. chilonis* (DIR)

Table 3 Sequences of the species and strains recorded from Pakistan submitted to NCBI Gen Bank

S. no.	Sample/code no.	*Trichogramma* spp.	Sequence	Base pairs	Accession no.
1	QT-6	*T. chilotraeae*	Complete ITS-2	565	DQ088051
2	BNI-20	*T. pintoi*	Complete ITS-2	667	DQ088052
3	DIR	*T. chilonis*	Complete ITS-2	553	DQ088053
4	GT	*T. chilonis*	Complete ITS-2	552	DQ088054
5	TTS	*T. chilonis*	Complete ITS-2	560	DQ088055
6	NWB	*T. chilonis*	Complete ITS-2	559	DQ088056
7	ME-2	*T. siddiqi*	Complete ITS-2	564	DQ088057
8	CHT-1	*T. evanescens*	Complete ITS-2	531	DQ088058
9	BNI-19	*T. evanescens*	Complete ITS-2	559	DQ088059
10	K-17	*T. chilonis*	Partial ITS-2	110	DQ088060
11	CHT-3	*T. euproctidis*	Complete ITS-2	512	DQ088061
12	SQG	*T. evanescens euproctidis*	Complete ITS-2	514	DQ088062
13	KWL	*T. chilonis*	Partial ITS-2	95	DQ088063

S. no. Sample number

Table 4 Dichotomous key for the identification of six Pakistani *Trichogramma* species based on PCR of ITS-2 rDNA, followed by restriction analysis, using the endonucleases Bsp119I and BseXI; bp = base pairs

1	PCR product < 600 bp	*T. euproctidis*	
	PCR product > 600 bp		2
2	PCR product < 650 bp		3
	PCR product > 650 bp	*T. pintoi*	
3	PCR product cut by Bsp119 ca. 376; 158 and 70 bp	*T. chilotraeae*	
	PCR product not cut by Bsp119		
4	PCR product cut by Bsex I ca. 506, 88, 3, 3 bp	*T. chilonis*	
5	PCR product cut by Bsex I ca. 388, 203 and 18 bp	*T. evanescens*	
6	PCR product (ca. 603 bp) cut by Bsex I ca. 585 and 18 bp	*T. siddiqi*	

neotype should be designated preferable from the type locality near London and molecular data should be included for the comparison and identification in future. It is also suggested that the GenBank data should contain information about the source of morphological identification and the natural geographical origin of the strain which will give more reliability of the GenBank information for the purpose of comparison.

The molecular identification method gave results which could be reproduced reliably. In future, this key will render identification easier for already known *Trichogramma* species from Pakistan. It can be used as a starting point for a dichotomous molecular key for *Trichogramma* species of the Oriental region as Sumer et al. (2009) have created a molecular key for the identification of *Trichogramma* species found around the Mediterranean by using ITS-2 of the ribosomal cistron. However, just as in the case of morphological keys, molecular keys will only be completely reliable once all species of a region are known. Therefore *Trichogramma*-workers should always be aware of possible additional species which might be newly recorded for Pakistan or species new to science.

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