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A molecular key to the common species of *Trichogramma* of the Mediterranean region

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Abstract A molecular key for the identification of common *Trichogramma* (Hymenoptera: Trichogrammatidae) species found in agricultural settings around the Mediterranean is developed based on the sequence of the internal transcribed spacer 2 of the ribosomal cistron. Using the size of the ITS2 PCR product and restriction fragment length polymorphisms of the amplicon, ten *Trichogramma* species (*T. bourarachae* Pintureau and Babault, *T. brassicae* Bezdenko, *T. cacoeciae* Marchal/*T. embryophagum* Hartig, *T. cordubensis* Vargas and Cabello, *T. dendrolimi* Matsumura, *T. euproctidis* Girault, *T. evanescens* Westwood, *T. nerudai* Pintureau and Gerding, *T. oleae* Voegelé and Pointel, and *T. pintoi* Voegelé) can be distinguished.

Keywords *Trichogramma* · rDNA · Taxonomy · Internally transcribed spacer 2

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Introduction

Trichogramma (Hymenoptera: Trichogrammatidae) wasps are important biological control agents of lepidopterous insect pests. The genus *Trichogramma* belongs to the family Trichogrammatidae, which includes 80 genera and approximately 620 species (Pinto and Stouthamer 1994). These parasitic wasps are common natural enemies in agricultural areas surrounding the Mediterranean (Silva et al. 1999; Oztemiz 2007; Pintureau 1987). Thus, *Trichogramma* species attract significant interest, because of their potential role in biological control. A critical first step for the successful application of *Trichogramma* in biological control programs is choosing the best species to attack a target pest. Consequently, correct identification of these minute wasps is essential. However, *Trichogramma* species are difficult to identify because they are both very small and have few distinguishing morphological characters.

Furthermore, their morphology can be affected by environmental conditions such as host size and temperature (Pinto et al. 1989). Although Nagarkatti and Nagaraja (1971) successfully advocated the use of male genitalia as a character for the identification of *Trichogramma*, one important limitation of this technique is that females remain unidentifiable (Stouthamer et al. 1999). This is particularly troublesome because field population sex ratios of these wasps are often female biased (Pinto 1999), and all female populations are relatively common in this genus (Huigens and Stouthamer 2003; Vavre et al. 2004).

Since classical morphological methods are not always precise enough to differentiate micro-hymenopteran species to the species level (Landry et al. 1993), genetic means have become the "tools of choice" for the routine identification of *Trichogramma* species. Recent studies have focused on the use of the DNA sequence of internal transcribed spacer (ITS2) regions of nuclear rRNA for species identification. Nuclear rRNA consists of three highly conserved regions that code for the ribosomal RNA, separated by two spacer regions that are largely conserved within species, but vary substantially between species. The polymerase chain reaction (PCR) can be used to amplify these ITS spacers, using universal primers that bind in the highly conserved regions. Since these regions are so conserved, the primers work on a wide variety of taxa. Stouthamer et al. (1999) showed the usefulness of the ITS2 sequence as a general identification method for *Trichogramma*, and the sequence has also aided the description of at least one new *Trichogramma* species, *T. itsybitsi* Pinto and Stouthamer, (Pinto et al. 2002). For routine identification, inter-specific variation in ITS2 has been exploited, resulting in the development of several molecular keys for the identification of *Trichogramma* species occurring in: restricted areas (Silva et al. 1999; Ciociola et al. 2001b; Thomson et al. 2003); particular crops (Pinto et al. 2002); or simply as a method to distinguish closely related species (Borghuis et al. 2004; Chang et al. 2001; Ciociola et al. 2001a; Dang et al. 2005; Sappal et al. 1995; Stouthamer et al. 1999, 2000a, b).

The aim of this study was to develop a general molecular key to the common species known to occur in the agricultural areas of countries bordering the Mediterranean (see Table 1). The key was developed

Table 1 References to the occurrence of the common *Trichogramma* species found in agricultural areas in countries surrounding the Mediterranean

Country	Algeria	Egypt	France	Greece	Israel	Italy	Morocco	Portugal	Spain	Tunesia	Turkey	Greece	Former Yugoslavia
Trichogramma species													
bourarachae	4						8	3,10		4			
brassicae			12			7					6,7		7
cacoeciae/embryophagum	4	7	4		7	7	4		4	6		7	
cordubensis	8	4					8	3, 10	11				
dendrolimi			1				7				6		
euproctidis		4,9	9				9	3, 5, 10			6		
evanescens		2	7		7	3, 10	6						
nerudai								4					
oleae		7								4			7
pintoi					7			3, 10	7	7			

[1] Babi et al. (1984), [2] El-Hafez and El-Hafez (1995), [3] Gonçalves et al. (2006), [4] Herz et al. (2007), [5] Neto and Pintureau (1995), [6] Oztemiz (2007), [7] Pintureau (1987), [8] Pintureau and Babault (1988), [9] Rohi and Pintureau (2003), [10] Silva et al. (1999), [11] Vargas and Cabello (1985), [12] Voegelé et al. (1988)

using ITS2 sequences, and species can be distinguished using the size of the ITS2 PCR product alongside the restriction fragment length polymorphism (RFLP) "fingerprint" generated from this amplicon after digestion with particular restriction enzymes.

Materials and methods

Construction of molecular key for the identification of common *Trichogramma* found in the Mediterranean region

Eleven species of *Trichogramma* have been commonly collected in agricultural settings in countries surrounding the Mediterranean: *Trichogramma* species: *T. bourarachae* Pintureau and Babault, *T. brassicae* Bezdenko, *T. cacoeciae* Marchal, *T. cordubensis* Vargas and Cabello, *T. dendrolimi* Matsumura, *T. embryophagum* Hartig, *T. euproctidis* Girault, *T. evanescens* Westwood, *T. nerudai* Pintureau and Gerding, *T. oleae* Voegelé and Pointel, and *T. pintoi* Voegelé (Table 1). However, in this study we do not distinguish between *T. embryophagum* and *T. cacoeciae* since they do not differ in their ITS2 sequence, and the specific relationship between these two species remains ambiguous, although males of these species are distinguishable (Pintureau 2008), albeit that the males of *T. cacoeciae* are exceedingly rare (Stouthamer et al. 1990). For each of the ten species, published ITS2 sequences were retrieved from GenBank (see Table 2 for accession numbers). These sequences were then used to construct a molecular key to this particular assembly of species, based on the size of the ITS2 spacer and the pattern of RFLPs generated when the ITS2 PCR product is cut using different restriction enzymes. To determine how the different ITS2 sequences are cut by a suite of restriction enzymes (Table 2) we used the restriction map generator as implemented in the program BioEdit (Hall 1999).

For many species only the ITS2 sequences have been deposited in GenBank. However, when a PCR reaction with the ITS2 primers for *Trichogramma* is done an additional 113 bp are amplified (see Stouthamer et al. 1999). These additional bp are located in the 5.8S rDNA and in the 28S rDNA. These sequences are extremely conserved and do not differ within or between species. To determine the expected restriction patterns from PCR products, we add these sequences to the GenBanks "pure" ITS2 sequence before submitting them to the restriction map generator.

Based on the predicted restriction fragment sizes of the different species, the restriction enzymes *Mse*I, *Mnl*I and *Dra*I were chosen to produce a simple key to distinguish the ten species (Table 3). Although we are using a single accession number to initially construct the key, other deposited sequences for the same species can also be keyed out using the key. We checked the applicability of the key for all deposited sequences of the species present in GenBank (see Table 4). Finally this key was tested on PCR products obtained from specimens derived from other cultures of each species in the assemblage (see Table 5).

Table 2 Predicted restriction fragment sizes when the different PCR products of the ITS2 are cut by different restriction enzymes

	GenBank accession #	Size PCR product	*Mse*I	*Mnl*I	*Dra*I
T. euproctidis	AF043614	489	489	82,137,270	489
T. brassicae	AY182766	519	32,75,412	82,129,308	413,106
T. oleae	U74601	512	512	82,139,140,151	512
T. dendrolimi	AF453560	516	74,442	56,68,76,82,109,125	516
T. cordubensis	AF043619	529	529	57,82,94,296	529
T. evanescens	AF043617	548	94,454	82,169,297	548
T. cacoeciae	AF408654	578	204,374	578	578
T. pintoi	AY182757	694	4,15,34,126,170,345	82,120,180,312	48,646
T. bourarachae	AF043624	666	50,616	82,125,459	49,617
T. nerudai	AY182756	745	7,8,120,125,485	82,166,497	745

Table 3 Molecular key to the common *Trichogramma* species found in the Mediterranean area based on the ITS2 PCR product and restriction fragments after digestion with the restriction enzymes *Mnl*I, *Mse*I and *Dra*I

1	PCR product smaller than 500 bp	*euproctidis*
	PCR product larger than 500 bp	2
2	PCR product smaller than 550 bp	3
	PCR product larger than 550 bp	7
3	*Mse*I does not cut PCR product	4
	*Mse*I cuts PCR product	5
4	PCR product restricted with *Mnl*I has a largest band >270 bp	*cordubensis*
	Largest band 150 bp	*oleae*
5	*Dra*I does not cut PCR product	6
	*Dra*I cuts PCR product in two parts, the largest band ~400 bp	*brassicae*
6	PCR product restricted with *Mnl*I has a largest band >300 bp	*evanescens*
	PCR product restricted with *Mnl*I has a largest band <170 bp	*dendrolimi*
7	PCR product 570–590 bp	*cacoeciae/embryophagum*
	PCR product larger than 650 bp	8
8	PCR product ca 750 bp	*nerudai*
	PCR product smaller than 700 bp	9
9	PCR product restricted with *Mnl*I results in several bands with the largest ca 300 bp	*pintoi*
	PCR product restricted with *Mnl*I results in several bands with the largest ca 450 bp	*bourarachae*

Table 4 GenBank accession numbers of the ITS2 sequences of *Trichogramma* that were used to determine if the molecular key (Table 3) would identify the sequences to the correct species

Trichogramma sp.	GenBank accession numbers
T. bourarachae	AF043623, AF043625, AF043626, DQ137256, DQ389071, DQ389073, EU882011
T. brassicae	AF453566, AF453567, AY146635, AY146636, AY163002, DQ314611, DQ381280
T. cacoeciae	AF408653, AF453562, AY146642; AY146643, AY146644, AY166700, AY244465, DQ344044, EU547667, EU547668, EU547669, EU547670, EU547671, EU547672, EU882009, EU882010
T. cordubensis	AF043612, AF043620, DQ137257; DQ137258, DQ389074, U74675
T. dendrolimi	AB094398, AF227949, AF453554, AF453555, AF453556, AF453557, AF453559, AF453561, AF517576, AY182767, AY244464, AY328907, AY343056, AY343057, AY895013, DQ344045
T. euproctidis	AF043613, AF043615, DQ088061, DQ088062, DQ137263, DQ137264, DQ389076
T. evanescens	AF043616, AF043618, AF453563, AF453564, AF453565, AF453568, AF453569, AY146637, AY146638, AY146639, AY146640, AY146641, AY146645, AY326469, DQ088058, DQ088059; DQ137259, DQ137260, DQ381280, EU547673, EU547674, EU547675, EU547676, EU547677, EU882008, FJ436332, FJ436333, FJ436334, FJ436335
T. nerudai	AY182756, AY244467, DQ872853
T. oleae	EU547678, EU547679, EU882007
T. pintoi	AF043621, AF043622, DQ088052, DQ137261, DQ137262

Trichogramma samples

Table 5 lists the origin of the specimens used for DNA amplification and RFLP analysis (Figs. 1, 2, 3, 4). DNA was extracted from single wasps using the Chelex method. In short, wasps were ground in 60 µl 5% Chelex-100 and 2 µl Proteinase K (20 mg ml^{-1}), followed by an incubation for 1 h at 55°C, and finally by 10 min at 99°C. The ITS2 spacer was then amplified using PCR primers and conditions given in

Table 5 Collections of *Trichogramma* species used for PCR amplification and restriction digestion as illustrated in Figs. 1, 2, 3 and 4

Species	Strain designation	Origin	Date collected
T. euproctidis	PB (Pintureau)	Portugal	1991
T. brassicae	Bra 2 (Pintureau)	Moldavia	1973
T. oleae	S2 (Pintureau)	Yugoslavia	1972
T. dendrolimi	It18 (Pintureau)	Italy	1991
T. cordubensis	MB35 (Silva)	Portugal	1994
T. evanescens	A1 (Pintureau)	France	1989
T. cacoeciae	082090-1(Pinto)	USA	1990
T. nr. pintoi	92-25-4a (Pinto)	Russia	1992[a]
T. bourarachae	TP-41-E.G (Pintureau)	Egypt	2002
T. nerudai	TR (Pintureau)	Chili	1994

[a] Date that the culture was received at UC Riverside

Stouthamer et al. (1999). The size of PCR product was determined by electrophoresis on a 1% agarose gel stained with ethidium bromide. Restriction digestion of the PCR products were performed in 15 µl reactions containing 10 µl PCR product, 1× the appropriate buffer, and 10 units of the restriction enzyme *Mse*I, *Dra*I or *Mnl*I (New England BioLabs, Ipswich, MA, USA). *Mse*I and *Mnl*I reactions also contained 100 µg ml^{-1} BSA (NEB). Digests were incubated at 37°C for 2 h and then inactivated at 65°C for 20 min. Restriction products were visualized after electrophoresis on 2% agarose gels stained with ethidium bromide.

Results

In this assemblage of species, *T. euproctidis* is distinguished from all others by the small size of its ITS2 spacer (<500 bp; Tables 2, 3; Fig. 1). The remaining species can be separated into two groups based on the size of the ITS2 PCR product (Tables 2, 3; Fig. 1). Species with an ITS2 PCR product between 500 and 550 bp consist of *T. brassicae*, *T. oleae*, *T. cordubensis*, *T. evanescens* and *T. dendrolimi*. These species can subsequently be distinguished using the restriction enzymes *Mse*I, *Mnl*I and *Dra*I (see Figs. 2, 3, 4; Table 3). Species with an ITS2 product larger than 550 bp can be distinguished based on the size of the PCR product with *T. cacoeciae/embryophagum* at around 570–590 bp, *T. nerudai* at around 750 bp (see Fig. 1), and finally *T. pintoi* and *T. bourarachae* at around 660–695 bp. The latter pair can subsequently be distinguished using the restriction enzyme *Mnl*I (Fig. 4; Table 3). We also tested the key on the sequences deposited in GenBank for the species used in this key (See Table 4), to make sure that the restriction patterns we had chosen would apply to all the known sequences for these species, and indeed the different species can be identified unambiguously using the GenBank sequences.

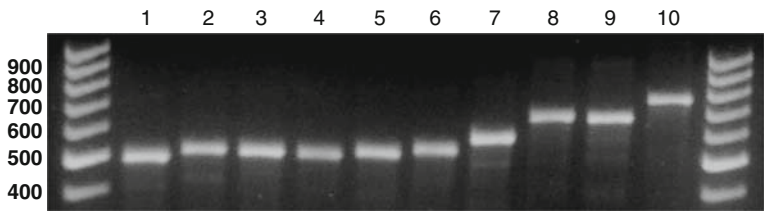


Fig. 1 Undigested ITS2 product, consisting of the ITS2 plus amplified parts of the 5.8 and 28S rDNA. Outer lanes contain size standards (GeneRuler™ 100 bp DNA Ladder, Fermentas Life Sciences, Glen Burnie, Maryland, USA). Lane 1–10 the amplified products, with 1: *T. euproctidis*, 2: *T. brassicae*, 3: *T. oleae*, 4: *T. dendrolimi*, 5: *T. cordubensis*, 6: *T. evanescens*, 7: *T. cacoeciae*, 8: *T. pintoi*, 9: *T. bourarachae* and 10: *T. nerudai*

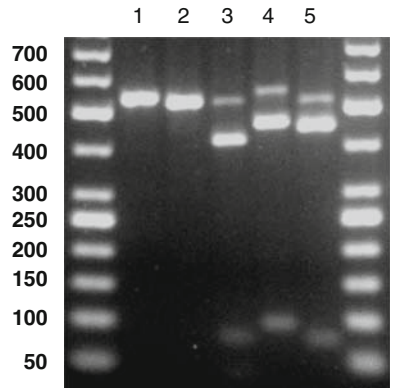


Fig. 2 *Mse*I digestion of PCR products consisting of the ITS2 plus flanking regions of the 5.8 and 28S rDNA Outer lanes contain size standards (GeneRuler^{TM} 50 bp DNA Ladder, Fermentas Life Sciences). Lane 1–5 the digested products, with 1: *T. cordubensis*, 2: *T. oleae*, 3: *T. brassicae*, 4. *T. evanescens* and 5: *T. dendrolimi*

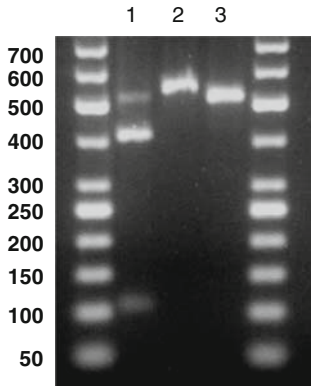


Fig. 3 *Dra*I digestion of PCR products consisting of the ITS2 plus flanking regions of the 5.8 and 28S rDNA Outer lanes contain size standards (GeneRuler^{TM} 50 bp DNA Ladder, Fermentas Life Sciences). Lane 1–3 the digested products, with 1: *T. brassicae*, 2: *T. evanescens*, 3: *T. dendrolimi*

Discussion

The molecular key given here provides a simple tool to help students of *Trichogramma* to precisely and rapidly identify the most common species found in the agricultural areas around the Mediterranean. The key was developed by using ITS2 DNA sequences deposited in GenBank, for each species initially a single sequence was chosen to represent that species. During the construction of the key several other sequences of the same species were checked to make sure that the RFLP pattern used in the key indeed was present in other sequenced specimen as well. Upon

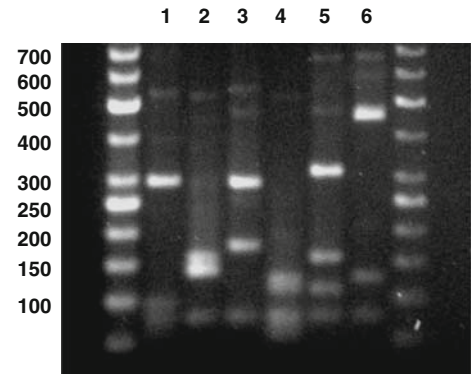


Fig. 4 *Mnl*I digestion of PCR products consisting of the ITS2 plus flanking regions of the 5.8 and 28S rDNA Outer lanes contain size standards (GeneRuler^{TM} 50 bp DNA Ladder, Fermentas Life Sciences). Lane 1–6 the digested products, with 1: *T. cordubensis*, 2: *T. oleae*, 3: *T. evanescens*, 4. *T. dendrolimi.* 5. *T. pintoi* and 6: *T. bourarachae*

completion of the key we tested the working of the key against all correctly deposited ITS2 sequences in GenBank. Some of the ITS2 sequences in GenBank are associated with the wrong species name. For instance two sequences under accession numbers AF453568 and AF453569 are listed as *T. maidis* (= *brassicae*) but are sequences of *T. evanescens*. These accessions were placed in the file with the correct species to determine if the species would be keyed out correctly. In addition we tested our key against a set of morphologically identified specimens obtained from the collections of the *Trichogramma* systematists Pintureau (Villeurbanne, France) and John Pinto (Riverside, California, USA).

The association of the wrong species name with a sequence reflects the problem that has haunted *Trichogramma* research for a long time. Names associated with cultures often do not reflect the correct species. In the past species could only be reliably identified by a few specialists in *Trichogramma* morphology, but not all initial identifications were done by such specialists. Secondly even if cultures were initially identified correctly, contamination in the laboratory may have taken place. Many students of *Trichogramma* maintain collections consisting of several species.

As with all keys, if only a subset of the species included in this key are known to be present in a particular area, their identification can be further simplified and certain restriction digests can be avoided. An example of this may be the difference

between *T. bourarachae* and *T. pintoi*. As far as we are aware, this latter species has only been found commonly in tomato crops in Portugal. Similarly, *T. nerudai* may be limited to Portugal, since the natural distribution of *T. nerudai* is South America, and its presence in Portugal is unusual since no known releases of this species have taken place (Herz et al. 2007). Also in Portugal the species *T. brassicae* has not been found in tomato fields. And a much simpler key for those particular circumstance can be found in Silva et al. (1999). If the *Trichogramma* fauna of a particular crop or region is well known another approach to streamlining their identification would be the development of a species-specific multiplex PCR. In such a multiplex PCR, for each species a specific primer is designed, so that when combined in a single reaction with a complementary universal primer, all the species can be identified by the size of the resulting PCR product alone (Gariepy et al. 2005; Davies et al. 2006). Development of such multiplex primers is very feasible but the choice of primers depends on the species that are present.

As with all keys, one limitation of our method is that if species not included in the key are encountered, they cannot be identified unless characters such as the size of their ITS2 spacer and/or its RFLP pattern deviate from those of the included species. This could potentially result in "false" identifications and if such circumstances are suspected, the ITS2 spacer of the species would need to be sequenced in order to identify that species.

Finally, in addition to the use of our key for identifying field collected wasps, we suggest that this method may also be used for quality control purposes. Mass rearings of *Trichogramma* are particularly prone to contamination by other species. Contamination with different species is difficult to avoid, indeed in our own collection of *Trichogramma* cultures we always test the species identity of our cultures before we do experiments and more often than once we find that the culture has been contaminated by another species. Similarly we have tested the identity of collections of species used by other *Trichogramma* researchers and have often found that the *Trichogramma* name applied to the specimen of a particular collection are incorrect. Sometime this is caused by the changing insights in the species name of taxa, such as for instance *T. maidis* that is now called *T. brassicae* or *T. turkestanica* now called *T. euproctidis*. In other cases the name and the true species identity are not the same. In case of biological control applications this can lead to release of the wrong species and a failure of biological control. One particularly difficult case to notice is *T. minutum* and *T. platneri*, these two species are morphologically identical (Pinto et al. 2003) and even do not differ in their ITS2 sequence (Stouthamer et al. 2000a), specimens can only be distinguished by the sequence of their COII gene (Borghuis et al. 2004). The release of the wrong species in an area where the other is native can lead to a prolonged suppression of the population of both native and introduced species in biological control applications (Stouthamer et al. 2000b). The species sold through North American biocontrol producers in this complex are sometimes not identified correctly (Stouthamer unpublished). We have also found similar problems in the mid 1990s in *Trichogramma* sold in Europe for the control of the European Corn Borer (*Ostrinea nubilalis*). Using techniques similar to those given here it should be relatively simple to check on the correct species identification of wasps used in biological control. Students of *Trichogramma* and biological control workers alike should make sure that the species they report on working or releasing is indeed the correct species.

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