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Using internally transcribed spacer 2 sequences to re-examine the taxonomic status of several cryptic species of *Trichogramma* (Hymenoptera: Trichogrammatidae)

ZHENG-XI LI[1], LI ZHENG[2] and ZUO-RUI SHEN[1]*

[1]Department of Entomology, China Agricultural University, 2 Yuanmingyuan XiLu, Beijing 100094, P.R. China; e-mail: zxli@cau.edu.cn

[2]Present address: Institute of Dryland Crop Research, HeBei Academy of Agricultural and Forestry Sciences, Hengshui 053000, P.R. China

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Abstract. Mass releases of *Trichogramma confusum* Viggiani and *T. maidis* Pintureau & Voegele are widely used to control lepidopterous pests. They have long been considered to be the subspecies of *T. chilonis* Ishii and *T. brassicae* Bezdenko, respectively. To re-examine the taxonomic status of these closely related *Trichogramma* species, the internally transcribed spacer 2 (ITS2) of ribosomal DNA was used as a molecular marker to detect between-species differences. The ITS2 regions of 7 different *Trichogramma* species collected from China, Germany and France were sequenced and the inter-species distances were calculated. To quantify within-species sequence variation, the ITS2 regions of 6 geographical populations of *T. dendrolimi* Matsumura collected from across China were sequenced and compared. The results show that the ITS2 sequences of *T. confusum* and *T. maidis* are sufficiently different from those of *T. chilonis* and *T. brassicae*, respectively, that it is difficult to group them as cryptic species, whereas there are only minor differences between the *T. dendrolimi* populations. The ITS2 sequences identified in this study, coupled with 67 ITS2 sequences from a wide geographical distribution retrieved from GenBank, were then used for phylogenetic analyses. The results support previous records of minor within-species ITS2 sequence divergence and distinct interspecies differences. The cladograms show the *T. maidis* sequence clustered within *T. evanescens* Westwood, while the ITS2 sequences of *T. confusum* and *T. chilonis* are clustered in different branches. Taken together, these data suggest that *T. maidis* is not *T. brassicae*, but a cryptic or sibling species of *T. evanescens*; *T. confusum* and *T. chilonis* are not cryptic species but two closely related sister species.

INTRODUCTION

Identification and classification of *Trichogramma* species are difficult because of their small size. The closely related *Trichogramma* species or cryptic species complexes are extremely difficult or impossible to distinguish morphologically. For example, *T. confusum* and *T. maidis* have long been considered to be subspecies of *T. chilonis* and *T. brassicae*, respectively (Lin, 1994). Based on the examination of more than 10 thousand specimens on slides, mostly collected by sweeping from all over China, Lin (1994) described 39 genera and 128 species of the total of 142 species in 41 genera in China.

Trichogramma Westwood is the type genus of the Trichogrammatidae family, most species of which parasitize the eggs of lepidopterous pests. The larvae are morphologically indistinguishable, and the adults very difficult to differentiate. The identity of these egg parasitoids was based almost exclusively on the morphology of the male genitalia (Nagarkatti & Nagaraja, 1971; Nagarkatti & Nagaraja, 1977; Sorokina, 1993), but male wasps occur in very low proportions or are absent in natural populations (Aeschlimann, 1990). While the taxonomy of the genus *Trichogramma* is still being studied (Pinto, 1992; Pinto & Stouthamer, 1994; Neto & Pintureau, 1995), mass releases of these egg parasitoids for the biological control of crop pests have gained increasing attention worldwide. *Trichogramma* species are used to control over 20 pest species on corn, cotton, rice, sugarcane, vegetables and fruit trees. We used not only the native species, *T. dendrolimi*, *T. ostriniae*, *T. evanscens* and *T. confusum*, but also commercially available *Trichogramma* such as *T. brassicae* supplied by Biocare (Einbeck, Germany) and *T. maidis* produced by BASF (Valbonne, France) to control the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae) in the corn fields of HengShui in HeBei province, China. In Germany and France, *T. brassicae* is considered to be a strain of *T. maidis* (Hassan & Zhang, 2001).

With the extensive application of *Trichogramma* for biocontrol worldwide in the middle of 20th century, the identification of species and strains of *Trichogramma* became important (Smith & Hubbes, 1986). Quednau (1960) pointed out that only individuals reared in the same host at the same temperature could be differentiated. Lack of type specimens is a key factor affecting the accurate classification of *Trichogramma* species (Pang, 1999). For example, there is only one incomplete female specimen of *T. evanescens*. Pintureau & Voegele (1980) re-described *T. evanescens* when they described *T. maidis*

* To whom correspondence should be addressed: Tel: +86 (0)10 62893015; Fax: +86 (0)10 62817605; e-mail: ipmist@cau.edu.cn

and found that the previously described *T. evanescens* were actually *T. maidis*. Pintureau (1987) classified *T. maidis* as *T. brassicae* and Lin (1994) accepted this nomenclature. *T. chilonis* was first described by Ishii (1941) but there is no holotypes for verification. Nagarkatti & Nagaraja (1979) chose a male specimen mounted on a glass slide from the syntypes of *T. chilonis* described by Ishii (1941) as its lectotype. Meanwhile they classified *T. chilonis* as *T. confusum* (Viggiani, 1976). Lin (1994) agreed with this classification. However, *T. confusum* is common in China and it is essential to reconsider its taxonomic status according to Pang (1999). Viggiani's description of *T. confusum* was very simple and there are no holotypes for comparison, but there is a detailed figure of the male genitalia for reference. It seems incorrect to classify *T. confusum* as *T. chilonis* on the basis of this figure (Pang, 1999).

Many methods were used to discriminate sibling species of *Trichogramma* in addition to morphological comparisons (Pinto et al., 1997), such as allozyme analyses (Pinto et al., 1992, 1993; Pintureau, 1993) and reproductive compatibility tests (Pinto et al., 1991; Stouthamer et al., 1996, 2000a, b). Recently closely related or cryptic species were characterized using DNA-based methods (Landry et al., 1993; Vanlerberghe-Masutti, 1994; Sappal et al., 1995; Landais et al., 2000). Ribosomal DNA (rDNA) consists of several regions (genes and spacers) that evolve at different rates, among which the internal non-coding transcribed spacer (ITS) region usually evolves faster than the coding regions (Hoy, 1994). Many of the phylogenetic relationships between *Trichogramma* species deduced from ITS2 sequences were recorded by previous studies (Orrego & Silva, 1993; van Kan et al., 1996, 1997; Pinto et al., 1997; Schilthuizen & Stouthamer, 1997; Stouthamer et al., 1999; Chang et al., 2001; Pinto et al., 2002), which showed that the DNA sequence of the internally transcribed spacer (ITS2) of *Trichogramma* wasps could be used for species identification. Consistent differences occur among species, whereas the spacer sequences show little variation within species. As ITS2 sequences can be used to identify cryptic species, we used them to distinguish the proposed cryptic species of *Trichogramma*.

MATERIAL AND METHODS

Insects

We established 12 iso-female lines, six from different *Trichogramma* species and six from geographical populations of another species (Table 1). *T. brassicae*, *T. maidis* and *T. embryophagum* (Hartig) were identified and provided by Sherif A. Hassen of the Federal Biological Research Center for Agriculture and Forestry, Institute for Biological Control, Heinrichstr, Darmstadt, Germany. *T. brassicae* was purchased from Biocare (Einbeck, Germany) and *T. maidis* from BASF (Valbonne, France). Six geographical populations of *T. dendrolimi* were collected from noctuid eggs on corn, cotton, rice or fruit trees in China. We selected *T. dendrolimi* for a within-species between-population study because the largest number of collections are available for this species, and it has the largest geographical distribution in China. The lines of *T. brassicae*, *T. maidis* and *T. embryophagum* were maintained in the Department of Entomology, China Agricultural University in Beijing. Other lines were maintained at the Biological Control Centre, Beijing Academy of Agriculture and Forestry Sciences, Beijing, China, by rearing *T. chilonis*, *T. confusum* and *T. dendrolimi* in the eggs of tussore worm, *Antheraea pernyi* Guérin-Méneville and the other lines in the eggs of the rice moth, *Corcyra cephalonica* (Stainton), at 20–25°C, RH 75–80% and 16L : 8D. Individual rearing was used to avoid linebreeding. Individual neonate wasps from different cultures were placed in sterile 1.5 ml tubes at –80°C for further analysis. Meanwhile, 10 freshly emerged wasps from each line were soaked in acetic acid prior to morphological identification. *Trichogramma* specimens were identified using the procedure of Lin (1994).

DNA isolation

DNA was extracted with Chelex-100 (Bio-Rad, California, USA) (Orrego & Silva, 1993) as follows: one previously frozen wasp was macerated in 50 µl Chelex-100 (5%) and 4 µl proteinase K (20 mg/ml) (Merck, Darmstadt, Germany) with a tissue grinder. The mixture was incubated at 56°C for 4 h, followed by 10 min at 95°C. The extracted genomic DNA was then used for PCR amplification.

TABLE 1. Strains, species, site and time of collection of the *Trichogramma* used in this study.

Strains*	Species	Collection site / time
con_JL	*T. confusum*	Changchun, Jilin, China, 1999
mai_FRA	*T. maidis*	BASF, Valbonne, France, 2000
bra_GER	*T. brassicae*	Biocare, Einbeck, Germany, 2000
eva_YQ	*T. evanescens*	Yanqing, Beijing, China, 1999
ost_JL	*T. ostriniae*	Changchun, Jilin, China, 1999
emb_GER	*T. embryophagum*	Biocare, Einbeck, Germany, 2000
den_CHA	*T. dendrolimi*	Chang'an, Shanxi, China, 1994
den _JL	*T. dendrolimi*	Changchun, Jilin, China, 1993
den _GZ	*T. dendrolimi*	Guangzhou, Guangdong, China, 1996
den _RH	*T. dendrolimi*	Renhe, Jilin, China, 1994
den _XZ	*T. dendrolimi*	Xuzhou, Jiangsu, China, 1994
den _YBL	*T. dendrolimi*	Yabuli, Heilongjiang, China, 1994

* The first 3 letters of species names suffixed with the corresponding strain names represent acronyms for strain designation.

PCR amplification of ITS2, cloning and sequencing

PCR was performed in 50 μl reaction volumes using a Hybaid thermocycler (Fisher Scientific Pte Ltd., Singapore) with 5 μl (10×) PCR buffer, 0.8 μl dNTP mixture (each in a 10 mM concentration), 0.5 μl forward and reverse primers (each in 0.25 μM), 2 μl genomic DNA, 0.2 μl Taq DNA polymerase (GibcoBRL, Eggenstein, Germany, 5U/μl), and 41 μl sterile water. The ITS2 region was amplified using the following primers: forward, 5'-TTCTCGCATCGATGAAGAACG-3' (ITSN2) located in the 5.8S rDNA; reverse, 5'-TCCTCCGCTTATTGATA TGC-3' (ITSB) located in the 28S rDNA (Amornsak et al., 1998). The PCR cycling program was 3 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C with 7 min at 72°C after the last cycle. PCR products were subjected to electrophoresis on a 1.5% (w/v) agarose gel. Gels were stained with ethidium bromide. Molecular weight standards (100 bp DNA ladder) were run along with the samples for reference. The target bands (approximately 600~650 bp) were then excised from the agarose gel and recovered using a QIAquick® DNA Purification kit (Qiagen, Hilden, Germany), and 4 μl of the final eluted solution (30 μl) were ligated into pGEM-T Vector System I (Promega, Madison, USA) following the protocol provided by the manufacturer. The ligated products were then transformed into *E. coli* DH5α competent cells (Life Technologies, Rockville, USA). The colonies containing an insert of correct size were checked by PCR using the same primers as described above. One to 3 positive clones from each line were selected and sent for sequencing (Sangon, Shanghai, China). To check the accuracy of the automatic sequencer, some clones were sequenced in two directions. Clones from different individuals of the same line were sequenced for a comparative analysis of the between individual (within line) sequence variation.

Alignment and sequence divergence analyses

The ITS2 regions sequenced in this study and additional 67 ITS2 sequences obtained from GenBank, representing a wide variety of *Trichogramma* species, were aligned in ClustalW (1.82) (Higgins et al., 1994). The sequences from GenBank are all previously published, except the sequence for *T. confusum* con_GZ (GenBank accession no. AY244461) (Table 2). This unpublished ITS2 sequence comes from an important geographical population of *T. confusum* in southern China. A fast pairwise alignment algorithm was chosen for global multiple alignment of ITS2 sequences. The alignment is progressive and considers the sequence redundancy. DNA Identity Matrix (Unitary Matrix) was selected to generate the alignment, which creates a positive score for a match, and a score of –10000 for a mismatch. The penalty for opening a gap is 10; the penalty for extending a gap is 0.05, and the gap separation penalty is 8. The end-unaligned sequences of the multiple alignments were carefully trimmed.

Sequence divergence analyses at the levels of interspecies and intraspecies were conducted using MEGA version 2.1 (Kumar et al., 2001). For ITS2 sequence divergence analysis, a total of 27 groups were set up from 79 *Trichogramma* taxa (12 identified in this study and 67 retrieved from GenBank), i.e. 6 strains of *T. dendrolimi*, 11 of *T. deion*, 6 of *T. pretiosum*), 5 of *T. platneri*, 4 of *T. bourarachae*, 4 of *T. evanescens*, 4 of *T. cordubensis*, 3 of *T. alpha*, 3 of *T. turkestanica*, 3 of *T. kaykai*, 3 of *T. californicum*, 3 of *T. minutum*, 2 of *T. pratti*, 2 of *T. itsybitsi*, 2 of *T. brassicae* , 2 of *T. aurosum*, 2 of *T. sathon*, 2 of *T. sibericum*, 2 of *T. confusum*, 2 of *T. exiguum*, 2 of *T. cacoeciae*, 1 of *T. maidis*, 1 of *T. chilonis*, 1 of *T. ostriniae*, 1 of *T. embryophagum*, 1 of *T. oleae*, 1 of *T. nubilale*. The ITS2 sequence of *Nasonia vitripennis* (GenBank accession no. U02960) (Campbell et al., 1993), a species of Pteromalidae in the same superfamily Chalcidoidea as Trichogrammatidae, and the ITS2 of *Uscana semifumipennis* (GenBank accession no. U74608) (Doutt & Viggiani, 1968), a member of a related genus, were incorporated into the 79 *Trichogramma* ITS2 sequences for net between groups distance analysis. We chose 7 groups from the 27 groups for within group analyses, because each of them contained at least 4 members (strains), the largest numbers available. Net Between Groups and Within Groups methods in MEGA were used to compute average distances. The net average distance between two groups is given by:

$$d_A = d_{XY} - \frac{(d_X - d_Y)}{2}$$

where d_{XY} is the average distance between groups X and Y, and d_X and d_Y are the mean within-group distances. For each group, an arithmetic average is computed for all valid pairwise comparison. Distance algorithm Kimura 2-parameter (Kimura, 1980) in MEGA was used to compute genetic distances. The complete-deletion option and pairwise-deletion option were alternatively used. For the first option, sites containing missing data or alignment gaps are removed before the analysis begins; for the latter, sites containing missing data or alignment gaps are removed as the need arises during the analysis. Both transitions and transversions were included in the analyses, assuming that the substitution rates do not vary among sites.

Phylogenetic analyses

Phylogenetic analyses were based on the sequence alignments constructed by ClustalW with options and parameters as described above. Because different tree-building algorithms make different evolutionary assumptions, aligned sequences were evaluated by parsimony, maximum-likelihood and neighbour-joining methods. For parsimony, the branch-and-bound method of DNA parsimony algorithm, version 3.572c of PHYLIP (Felsenstein, 1993) was used. Bootstrapping was performed with the heuristic search option for 1000 replications. To construct maximum-likelihood trees, the fastDNAml program of PHYLIP (Olsen et al., 1994), based in part on Joseph Felsenstein's nucleic acid sequence Maximum Likelihood method (Felsenstein, 1993), was used with a transition / transversion ratio of 2.0. The neighbour-joining method (Saitou & Nei, 1987) of MEGA was used to construct a distance tree. The number of nucleotide substitutions per site was estimated by distance model Kimura 2-parameter (Kimura, 1980). Gaps or missing data were treated using Complete Deletion option in MEGA, which removed the sites that contain missing data or alignment gaps before the analysis begins. Both transitions and transversions were considered in the substitution analyses, assuming the substitution rates do not vary among sites. Distance method Kimura 2-parameter (Pairwise distances) was used, and bootstrapping of 1000 replications was performed to test the reliability of the putative tree. A total of 79 *Trichogramma* ITS2 sequences (12 identified in this study and 67 retrieved from GenBank) were used in the analyses, using *U. semifumipennis* as the outgroup based on previous phylogenetic work (Schilthuizen & Stouthamer, 1997).

RESULTS

ITS2 sequences and alignment

The 18 ITS2 sequences identified in this study were registered in GenBank with the accession numbers listed in Table 3. The registered sequences are complete ITS2 sequences containing no flanking sequences of 5.8S and 28S. The ITS2 sequences showed little difference in length between different individuals of the same line and

TABLE 2. Reference ITS2 sequences retrieved from GenBank for phylogenetic analysis.

Strains[a]	Species	GB ID[b]	Size(bp)[c]	References	Strains[a]	Species	GB ID[b]	Size(bp)[c]	References
dei_DLC1	*T. deion*	U76224	406	Pinto et al., 1997	dei_DPTL	*T. deion*	AF082826	408	Stouthamer et al., 1999
kay_KLC187	*T. kaykai*	U76229	469	Pinto et al., 1997	dei _DRIV	*T. deion*	AF082827	407	Stouthamer et al., 1999
kay_KSH1	*T. kaykai*	U76228	462	Pinto et al., 1997	dei _DRV1	*T. deion*	AF082824	404	Stouthamer et al., 1999
pre_PIRV	*T. pretiosum*	U76227	413	Pinto et al., 1997	dei _DSHE	*T. deion*	AF082825	404	Stouthamer et al., 1999
pre_PRV4	*T. pretiosum*	U76226	409	Pinto et al., 1997	kay_KDAN	*T. kaykai*	AF082821	478	Stouthamer et al., 1999
chi_Hawaii	*T. chilonis*	U74674	413	Schilthuizen & Stouthamer, 1997	sat_SAME	*T. sathon*	AF082815	438	Stouthamer et al., 1999
cor_Spain	*T. cordubensi*	U74675	416	Schilthuizen & Stouthamer, 1997	sat_SASO	*T. sathon*	AF082816	443	Stouthamer et al., 1999
dei _Irvine	*T. deion*	U74676	408	Schilthuizen & Stouthamer, 1997	pra_RDAN	*T. pratti*	AF082817	456	Stouthamer et al., 1999
dei _LC	*T. deion*	U74677	406	Schilthuizen & Stouthamer, 1997	pra_RSHE	*T. pratti*	AF082818	451	Stouthamer et al., 1999
dei _Menife	*T. deion*	U74678	398	Schilthuizen & Stouthamer, 1997	pre_PMES	*T. pretiosum*	AF082819	406	Stouthamer et al., 1999
dei _Pinyon	*T. deion*	U74679	404	Schilthuizen & Stouthamer, 1997	pre_PRV1	*T. pretiosum*	AF082820	413	Stouthamer et al., 1999
dei _Texas	*T. deion*	U74680	405	Schilthuizen & Stouthamer, 1997	alp_ACLE	*T. alpha*	AF408671	393	Pinto et al., 2002
nub_Nova	*T. nubilale*	U74600	409	Schilthuizen & Stouthamer, 1997	alp_ACOL	*T. alpha*	AF408672	397	Pinto et al., 2002
ole_Tunesi	*T. oleae*	U74601	399	Schilthuizen & Stouthamer, 1997	alp_ADRK	*T. alpha*	AF408673	395	Pinto et al., 2002
pla_Newcast	*T. platneri*	U74603	418	Schilthuizen & Stouthamer, 1997	aur_HCLE	*T. aurosum*	AF408668	382	Pinto et al., 2002
pla_River	*T. platneri*	U74602	418	Schilthuizen & Stouthamer, 1997	aur_HVER	*T. aurosum*	AF408667	385	Pinto et al., 2002
pre_Hawaii	*T. pretiosum*	U74604	411	Schilthuizen & Stouthamer, 1997	cac_CACA	*T. cacoeciae*	AF408654	465	Pinto et al., 2002
pre_Mexico	*T. pretiosum*	U74605	414	Schilthuizen & Stouthamer, 1997	cac_CACB	*T. cacoeciae*	AF408653	465	Pinto et al., 2002
sib_Rich	*T. sibericum*	U74606	443	Schilthuizen & Stouthamer, 1997	cal_CADI	*T. californicum*	AF408664	439	Pinto et al., 2002
sib_SIB	*T. sibericum*	U74607	443	Schilthuizen & Stouthamer, 1997	cal_CAYA	*T. californicum*	AF408663	447	Pinto et al., 2002
bou_Tb26-1	*T. bourarachae*	AF043626556		Silva et al., 1999	cal_XALS	*T. californicum*	AF408661	442	Pinto et al., 2002
bou _Tb26-2	*T. bourarachae*	AF043624553		Silva et al., 1999	exi_EXHE	*T. exiguum*	AF408669	380	Pinto et al., 2002
bou _Tb26-3	*T. bourarachae*	AF043625552		Silva et al., 1999	exi_EXSE	*T. exiguum*	AF408670	380	Pinto et al., 2002
bou _Tb27	*T. bourarachae*	AF043623553		Silva et al., 1999	its_ITBO	*T. itsybitsi*	AF408665	381	Pinto et al., 2002
cor_Tc13	*T. cordubensis*	AF043612416		Silva et al., 1999	its_ITBU	*T. itsybitsi*	AF408666	382	Pinto et al., 2002
cor_Tc14	*T. cordubensis*	AF043619416		Silva et al., 1999	min_MBUC6	*T. minutum*	AF408659	419	Pinto et al., 2002
cor_Tc15	*T. cordubensis*	AF043620416		Silva et al., 1999	min_MCOL	*T. minutum*	AF408658	420	Pinto et al., 2002
eva_Te5	*T. evanescens*	AF043616429		Silva et al., 1999	min_MMIN	*T. minutum*	AF408660	420	Pinto et al., 2002
eva_Te6	*T. evanescens*	AF043617435		Silva et al., 1999	pla_PLIR	*T. platneri*	AF408655	418	Pinto et al., 2002
eva_Te7	*T. evanescens*	AF043618438		Silva et al., 1999	pla _PLME	*T. platneri*	AF408656	418	Pinto et al., 2002
tur_Tt1	*T. turkestanica*	AF043615372		Silva et al., 1999	pla _PLWT	*T. platneri*	AF408657	419	Pinto et al., 2002
tur_Tt2	*T. turkestanica*	AF043614376		Silva et al., 1999	bra_BSSW	*T. brassicae*	AY163002	409	Thomson et al., 2003
tur_Tt4	*T. turkestanica*	AF043613376		Silva et al., 1999	con_GZ	*T. confusum*	AY244461	394	unpublished
dei_DEUR	*T. deion*	AF082823404		Stouthamer et al., 1999					

[a]The first 3 letters of *Trichogramma* species names suffixed with the corresponding strain names represent acronyms for strain designation as described in Table 1 and Table 2.
[b]GB ID indicates GenBank accession numbers.
[c]ITS2 sizes were trimmed from the original sequences as shown in the sequence alignment.

TABLE 3. Length and GenBank accession numbers of ITS2 sequences identified in this study.

Strains[a]	Species	GB ID[b]	Length (bp)[c]	PCR product (bp)[c]
ost_JL	*T. ostriniae*	AF250559	451	641
con_JL	*T. confusum*	AF422845	405	595
den_JL	*T. dendrolimi*	AF227949	406	596
den_CHA	*T. dendrolimi*	AF453554	407	598
den_GZ-clone01	*T. dendrolimi*	AF453555	407	597
den_GZ- clone02	*T. dendrolimi*	AF453556	410	600
den_GZ- clone03	*T. dendrolimi*	AF453557	410	600
den_RH	*T. dendrolimi*	AF453559	403	594
den_XZ	*T. dendrolimi*	AF453560	403	593
den_YBL	*T. dendrolimi*	AF453561	403	593
emb_GER	*T. embryophagum*	AF453562	474	651
eva_YQ- clone01	*T. evanescens*	AF453563	431	621
eva_YQ- clone02	*T. evanescens*	AF453564	428	618
eva_YQ- clone03	*T. evanescens*	AF453565	431	622
bra_GER- clone01	*T. brassicae*	AF453566	409	599
bra_GER- clone02	*T. brassicae*	AF453567	406	596
mai_FRA- clone01	*T. maidis*	AF453568	437	625
mai_FRA- clone02	*T. maidis*	AF453569	433	623

[a] Different clones represent sequences from different individuals of the same line. Strain abbreviations are as described in Table 1.
[b] GB ID indicates GenBank accession numbers.
[c] Length (bp) represents ITS2 without flanking sequences. PCR products (bp) include flanking regions.

between populations of the same species (0~7 bases in complete ITS2 sequences), while ITS2 sequences between species revealed inconsistent divergence. For example, the ITS2 sequences of *T. embryophagum* and *T. dendrolimi* strain den_RH have a difference of 71 bases, whereas those between *T. dendrolimi* strain den_JL and *T. brassicae* strain bra_GER clone02 do not differ in sequence length. Twelve of these ITS2 sequences were aligned with 67 ITS2 sequences taken from GenBank in ClustalW (1.82). DNA Identity Matrix was used, and the data matrices are available at TreeBASE (http://www.treebase.org/treebase/index.html).

Within groups and net between groups average distances

Within groups or within species, the ITS2 sequences showed little variation (Table 4). No distances could be detected in *T. platneri* using the Complete Deletion option, while a distance of 0.005 was found using the Pairwise Deletion option. In all cases, within groups average distances were consistently smaller than 0.02.

Between groups or at the interspecies level, the ITS2 sequences showed much greater divergence than within groups (Table 5). The overall pairwise distance between *Trichogramma* species is 0.23 (n = 27 groups). The largest pairwise ITS2 distances between *Trichogramma* species were found between *T. bourarachae* and other *Trichogramma* species, ranging from 0.634 to 0.826. There was no divergence between *T. minutum* and *T. platneri*, while the ITS2 distance between *T. evanescens* and *T. maidis* was only 0.003. The ITS2 sequences indicate that the species in these 2 pairs of *Trichogramma* species are identical. There are other very closely related *Trichogramma* species, such as *T. alpha* / *T. aurosum* (0.020), *T. pretiosum* / *T. oleae* (0.017), *T. sathon* / *T. deion* = (0.026) and *T. cacoeciae* / *T. embryophagum* (0.027), all of which have an ITS2 distance of approximately 0.02, the highest value for within groups average.

TABLE 4. Within groups average distances (Kimura, 1980).

Groups	Complete deletion (No. of sites = 303; d ± S.E.)	Pairwise deletion (No. of sites = 682; d ± S.E.)
T. bourarachae (n = 4)	0.018 ± 0.005	0.016 ± 0.004
T. deion (n = 11)	0.013 ± 0.004	0.013 ± 0.003
T. pretiosum (n = 6)	0.010 ± 0.004	0.011 ± 0.003
T. platneri (n = 5)	0.000 ± 0.000	0.005 ± 0.002
T. evanescens (n = 4)	0.004 ± 0.003	0.008 ± 0.003
T. cordubensis (n = 4)	0.010 ± 0.004	0.013 ± 0.004
T. dendrolimi (n = 6)	0.005 ± 0.003	0.008 ± 0.003

Standard error (S.E.) estimated by bootstrap method (Replications = 1000).

TABLE 5. Pairwise distances for ITS2 sequences between groups.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1. *T. bourarachae*	–	0.067	0.068	0.069	0.069	0.066	0.070	0.073	0.072	0.063	0.074	0.077	0.070	0.069	0.071	0.070	0.073	0.073	0.071	0.071	0.076	0.073	0.076	0.068	0.065	0.067	0.078	0.094	0.195
2. *T. alpha*	0.687	–	0.007	0.023	0.022	0.023	0.024	0.024	0.029	0.020	0.018	0.022	0.019	0.019	0.017	0.016	0.019	0.019	0.021	0.022	0.023	0.019	0.021	0.016	0.017	0.022	0.027	0.064	0.122
3. *T. aurosum*	0.671	0.020	–	0.024	0.023	0.024	0.024	0.025	0.031	0.020	0.017	0.020	0.018	0.019	0.017	0.015	0.019	0.019	0.020	0.020	0.022	0.019	0.021	0.016	0.017	0.022	0.027	0.065	0.130
4. *T. sathon*	0.684	0.167	0.178	–	0.008	0.012	0.013	0.020	0.035	0.022	0.026	0.027	0.027	0.026	0.026	0.025	0.024	0.023	0.026	0.027	0.030	0.024	0.024	0.025	0.024	0.029	0.028	0.086	0.104
5. *T. deion*	0.701	0.167	0.177	0.026	–	0.011	0.012	0.021	0.034	0.022	0.026	0.027	0.027	0.026	0.026	0.025	0.024	0.023	0.026	0.026	0.029	0.023	0.023	0.023	0.023	0.028	0.028	0.089	0.112
6. *T .pretiosum*	0.708	0.176	0.188	0.063	0.059	–	0.006	0.021	0.034	0.024	0.028	0.028	0.029	0.028	0.027	0.027	0.025	0.025	0.025	0.026	0.030	0.024	0.024	0.026	0.025	0.030	0.031	0.086	0.125
7. *T .oleae*	0.720	0.180	0.189	0.065	0.059	0.017	–	0.021	0.033	0.024	0.027	0.028	0.029	0.027	0.026	0.026	0.025	0.024	0.026	0.027	0.029	0.024	0.024	0.026	0.025	0.031	0.031	0.087	0.131
8. *T. kaykai*	0.780	0.197	0.201	0.158	0.147	0.162	0.151	–	0.033	0.024	0.032	0.033	0.033	0.029	0.029	0.029	0.030	0.029	0.028	0.029	0.032	0.029	0.027	0.028	0.025	0.032	0.032	0.087	0.136
9. *T. nubilale*	0.757	0.263	0.276	0.353	0.317	0.316	0.308	0.291	–	0.030	0.033	0.032	0.032	0.031	0.029	0.030	0.035	0.035	0.033	0.033	0.035	0.036	0.037	0.033	0.030	0.029	0.037	0.096	0.117
10.*T. itsybitsi*	0.635	0.138	0.133	0.168	0.163	0.178	0.182	0.197	0.270	–	0.021	0.023	0.023	0.022	0.021	0.022	0.021	0.021	0.023	0.023	0.025	0.021	0.021	0.021	0.020	0.021	0.026	0.079	0.110
11.*T. brassicae*	0.740	0.109	0.099	0.224	0.227	0.253	0.242	0.306	0.311	0.145	–	0.017	0.017	0.020	0.019	0.018	0.022	0.022	0.021	0.021	0.023	0.020	0.026	0.019	0.014	0.021	0.028	0.086	0.144
12.*T. sibericum*	0.826	0.169	0.137	0.239	0.244	0.270	0.277	0.325	0.310	0.171	0.118	–	0.010	0.017	0.018	0.020	0.025	0.024	0.023	0.024	0.025	0.021	0.023	0.023	0.016	0.025	0.032	0.085	0.160
13.*T. ostriniae*	0.788	0.132	0.119	0.229	0.238	0.265	0.268	0.312	0.293	0.158	0.113	0.039	–	0.016	0.017	0.019	0.025	0.025	0.023	0.023	0.024	0.022	0.024	0.022	0.015	0.023	0.031	0.079	0.139
14.*T. cacoeciae*	0.746	0.118	0.114	0.230	0.229	0.250	0.247	0.271	0.282	0.171	0.138	0.100	0.097	–	0.007	0.021	0.021	0.021	0.022	0.022	0.024	0.021	0.026	0.022	0.019	0.022	0.029	0.076	0.123
15.*T.embryophagum*	0.753	0.101	0.094	0.228	0.230	0.240	0.237	0.266	0.264	0.153	0.139	0.116	0.110	0.027	–	0.020	0.021	0.021	0.022	0.022	0.024	0.021	0.024	0.020	0.018	0.022	0.029	0.076	0.118
16.*T. californicum*	0.723	0.090	0.077	0.204	0.208	0.226	0.219	0.255	0.274	0.151	0.135	0.139	0.135	0.156	0.142	–	0.018	0.018	0.023	0.023	0.023	0.023	0.025	0.020	0.016	0.023	0.029	0.074	0.140
17.*T. minutum*	0.769	0.128	0.113	0.184	0.192	0.209	0.201	0.281	0.337	0.133	0.160	0.205	0.211	0.155	0.152	0.122	–	0.001	0.022	0.022	0.025	0.021	0.022	0.022	0.020	0.026	0.027	0.073	0.139
18.*T. platneri*	0.768	0.128	0.113	0.184	0.191	0.208	0.200	0.276	0.332	0.133	0.159	0.198	0.204	0.152	0.148	0.117	0.000	–	0.023	0.022	0.025	0.021	0.023	0.021	0.020	0.025	0.027	0.073	0.143
19.*T. evanescens*	0.765	0.148	0.137	0.218	0.209	0.212	0.222	0.257	0.305	0.168	0.152	0.204	0.199	0.162	0.162	0.171	0.171	0.171	–	0.001	0.012	0.017	0.023	0.025	0.016	0.021	0.030	0.075	0.149
20.*T. maidis*	0.774	0.151	0.139	0.226	0.217	0.219	0.230	0.264	0.305	0.173	0.155	0.211	0.205	0.166	0.164	0.173	0.171	0.172	0.003	–	0.012	0.017	0.023	0.025	0.017	0.021	0.030	0.075	0.144
21.*T. chilonis*	0.799	0.171	0.168	0.266	0.253	0.261	0.265	0.295	0.333	0.199	0.179	0.228	0.225	0.188	0.184	0.194	0.196	0.196	0.060	0.064	–	0.018	0.023	0.025	0.018	0.025	0.033	0.079	0.151
22.*T. confusum*	0.763	0.123	0.125	0.191	0.179	0.196	0.191	0.261	0.358	0.138	0.144	0.166	0.179	0.158	0.155	0.175	0.150	0.150	0.099	0.103	0.127	–	0.023	0.022	0.017	0.020	0.028	0.079	0.180
23.*T. exiguum*	0.770	0.143	0.142	0.202	0.187	0.205	0.198	0.253	0.325	0.144	0.202	0.172	0.193	0.213	0.191	0.199	0.173	0.175	0.165	0.169	0.177	0.159	–	0.026	0.022	0.025	0.030	0.073	0.131
24.*T. cordubensis*	0.686	0.092	0.085	0.202	0.190	0.221	0.215	0.265	0.336	0.154	0.126	0.177	0.169	0.165	0.150	0.146	0.167	0.162	0.179	0.182	0.193	0.148	0.199	–	0.019	0.024	0.032	0.084	0.163
25.*T. turkestanica*	0.634	0.106	0.093	0.183	0.181	0.201	0.208	0.219	0.267	0.128	0.065	0.094	0.090	0.130	0.119	0.097	0.126	0.129	0.096	0.100	0.123	0.100	0.164	0.135	–	0.018	0.029	0.078	0.114
26.*T. dendrolimi*	0.729	0.149	0.150	0.241	0.226	0.266	0.280	0.304	0.261	0.153	0.144	0.208	0.193	0.170	0.158	0.170	0.218	0.216	0.173	0.177	0.218	0.135	0.186	0.172	0.113	–	0.030	0.076	0.157
27.*T. pratti*	0.821	0.229	0.222	0.279	0.257	0.279	0.281	0.317	0.346	0.242	0.245	0.305	0.298	0.277	0.262	0.261	0.229	0.226	0.286	0.290	0.321	0.250	0.277	0.304	0.239	0.298	–	0.083	0.173
28.*Uscana*	1.017	0.667	0.673	0.904	0.882	0.860	0.873	0.933	0.969	0.772	0.862	0.854	0.834	0.773	0.780	0.755	0.764	0.765	0.757	0.763	0.785	0.791	0.747	0.871	0.780	0.781	0.855	–	0.186
29.*Nasonia*	1.496	1.080	1.100	1.020	1.065	1.139	1.177	1.226	1.088	1.024	1.203	1.270	1.213	1.104	1.093	1.154	1.198	1.204	1.199	1.188	1.210	1.326	1.148	1.216	1.038	1.180	1.328	1.266	–

Number of groups – 29; number of taxa – 81; number of sites – 681; gaps/missing data – Pairwise deletion; distance method: Kimura 2-parameter (Net between group average). Standard errors were estimated by bootstrap method (replications – 1000). Average distances are shown below the diagonal while standard errors are shown above the diagonal.

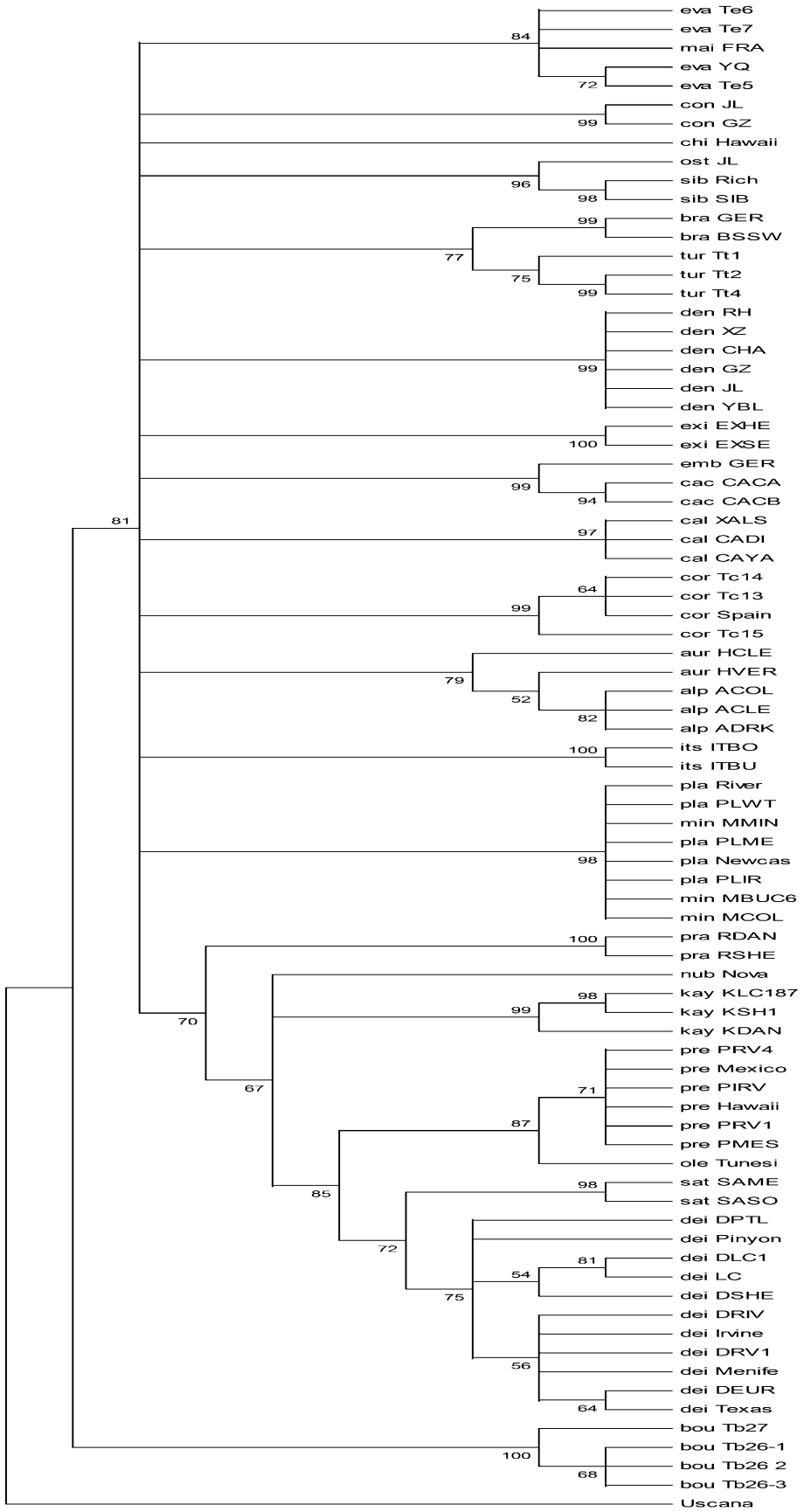

Fig. 1. Rooted NJ tree for 79 *Trichogramma* species based on Kimura 2-parameter distance model (Kimura, 1980). *U. semifumipennis* was chosen as outgroup. Bootstrap values for 1000 replicates are shown on the branches. Strain names are as described in Table 1 and Table 2.

Surprisingly, the distance between *T. confusum* and *T. chilonis* was 0.127, much greater than expected for within species distances. The overall average distance between *U. semifumipennis* and *Trichogramma* species, which could be regarded as an inter-genera ITS2-based distance, was 0.817, while the inter-family distance between *Nasonia vitripennis* and *Trichogramma* species was 1.177.

Phylogenetic analyses

Three different tree-making methods were used: Maximum Parsimony (MP), Neighbour-Joining of distances (NJ) and Maximum Likelihood (ML). All three methods gave similar results. A NJ tree was reconstructed for 80 taxa with *U. semifumipennis* as the outgroup (the sum of branch length, SBL = 2.2065). Different distance models were used and all produced similar phylogenetic topologies. The bootstrapped NJ tree is shown in Fig. 1. A search for the most parsimonious trees was performed by branch-and-bound analysis. A total of 58 most-parsimonious trees were found, which required a total of 2683 steps in each site (CI = 0.784566, RI = 0.581250, number of informative sites = 309). A bootstrapped search generated the 50% majority-rule tree in Fig. 2 and yielded 19 nodes with strong support (>90%). When evaluated by maximum likelihood (jumble 10×, global rearrangement, randomized input order), the ITS2 datasets produced 24068 ML trees (total weight of positions in analysis = 708; transition / transversion ratio = 2; transition / transversion parameter = 1.4765). The extended majority rule consensus tree (Fig. 3) clearly supported the phylogenetic relationship deduced by using the distance method and parsimony.

In general, all phylogenetic trees generated showed that the ITS2 sequence of *T. maidis* clustered within *T. evanescens* but not in the same group as *T. brassicae*, while *T. confusum* and *T. chilonis* sequences clustered in different branches.

DISCUSSION

As expected, our studies show that within group or intraspecies divergence is significantly smaller than between groups or interspecies divergence. The ITS2 sequences clearly separated *T. maidis* and *T. confusum* from *T. brassicae* and *T. chilonis*, respectively. The 6 populations belonging to *T. dendrolimi* formed a distinct and unique clade, and *T. maidis* is always in the same branch as *T. evanescens* populations in the topologies obtained using different methods. On the basis of the distance data, we concluded that *T. confusum* is not a subspecies of *T. chilonis*, and *T. maidis* is not *T. brassicae* but a cryptic or sibling species of *T. evanescens*. Our results provide the first molecular evidence of the taxonomic status of these previously proposed cryptic species complexes.

The utility of ITS2 as a means of identification was tested on the *T. deion* (Pinto & Oatman) and *T. pretiosum* (Riley) complexes (Stouthamer et al., 1999). This indicated it could be used for species identification in *Trichogramma*, because the sequence variation within species was minor relative to the differences found between species and all the morphologically distinct cryptic species were distinguished by sequence differences. As shown in our topologies, the populations of *T. deion* and *T. pretiosum* clustered as separate groups. Stouthamer et al. (2000) used ITS2 to separate *T. minutum* (Riley) and *T. platneri* (Nagarkatti), two North American species that cannot be distinguished morphologically (Pinto, 1999), but as no species-specific sequence differences were found the authors suggested that both species had recently diverged from a common ancestor. In all three trees presented in this paper, *T. minutum* and *T. platneri* always cluster together. However, as *T. minutum* and *T. platneri* are reproductively incompatible (Nagarkatti, 1975; Pinto et al., 1991), the general correlation between sequence variation and reproductive compatibility is complicated, because the biological species concept is based solely on the fact that they are reproductively compatible. However, as reproductive incompatibility is often associated with differences in morphology and ITS2 sequence structure in many *Trichogramma* species that have been investigated, the taxonomic status of *T. minutum* and *T. platneri* is questionable. In the case of *T. maidis* and *T. brassicae*, our experiments indicate they are reproductively incompatible (data not shown), and based on this and their ITS2 variation, we conclude they are reproductively isolated species. As for *T. maidis* and *T. evanescens*, they can successfully mate but do not produce offspring. In the future we shall focus on making additional crosses and molecular studies, including studies on mitochondrial DNA (mtDNA). Although the taxonomic position of cryptic species of *Trichogramma* is still disputable, we suggest that *T. maidis* is a cryptic species of *T. evanescens*, because their ITS2 sequences are nearly identical and in the phylogenetic trees *T. maidis* is embedded in the *T. evanescens* group with a bootstrap support of 84% in NJ tree or 79% in MP tree. Insects are notorious for evolving morphologically similar sibling species, so the above needs to be confirmed by additional mating studies and the gathering of other data.

It is important to note that the species status of *T. confusum* is so uncertain that Chinese research workers decided to vote on its relationship with *T. chilonis* at the 1999 National Symposium on *Trichogramma* (Nai-Quan Lin, pers. commun.), which confirmed that the taxonomic status of *T. confusum* is disputable. Because no crossing experiments have been made, it is difficult to apply the biological species concept. However, the diagnosable differences or genetic distance between *T. confusum* and *T. chilonis* (0.127 compared with <0.02 within species) and their positions in the trees indicate they are closely related sister species but not cryptic or sibling species.

The potential use of the ITS2 sequence for identifying *Trichogramma* species depend on sound morphological studies, because traditionally species are morphologically based. In addition to single rearing, the samples (lines) used here were all tested for consistency using hundreds of independent specimens, so the chance of mixing the lines was very unlikely. It should be noted that the

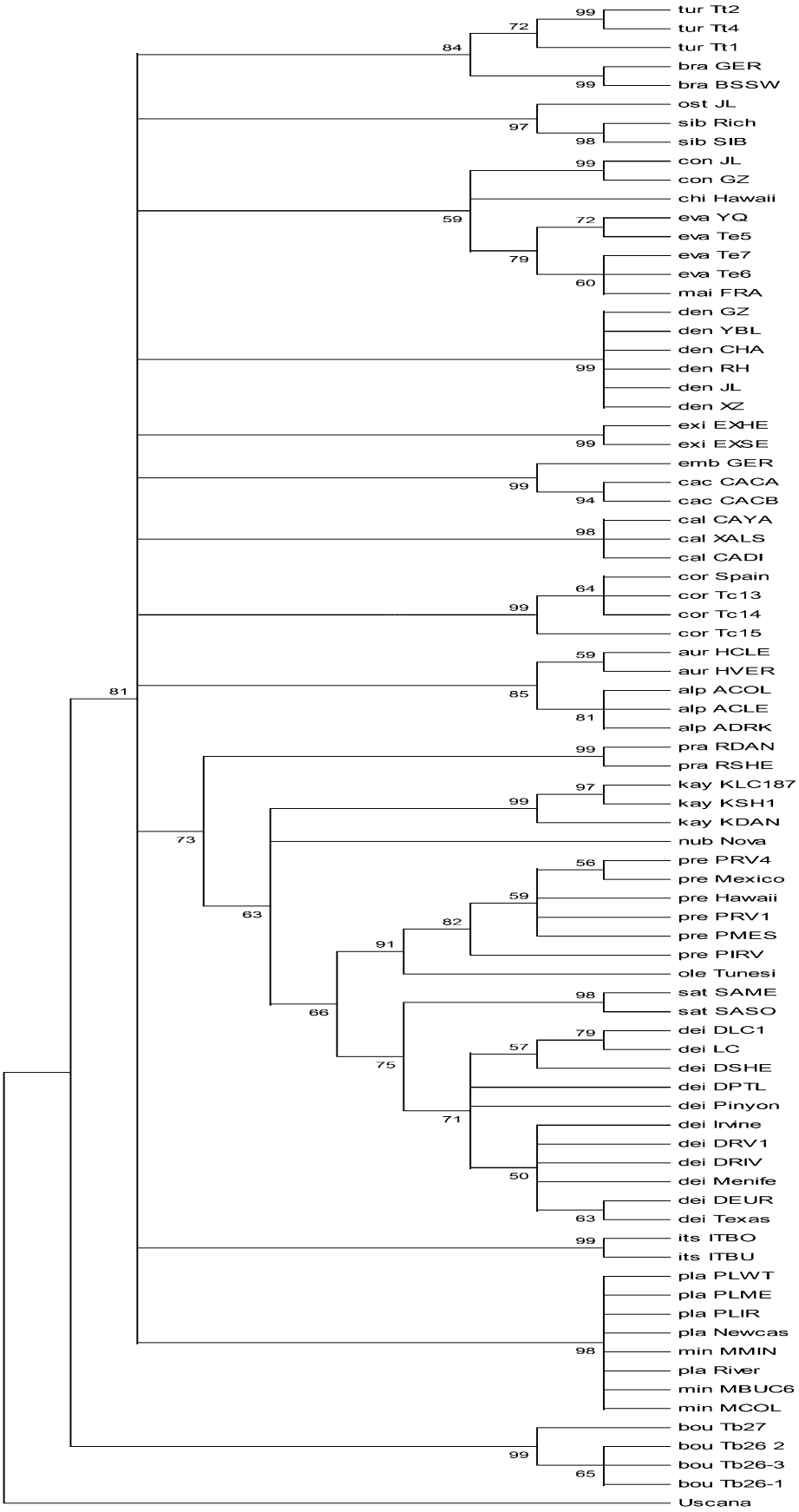

Fig. 2. MP consensus tree for 79 *Trichogramma* species using *U. semifumipennis* as outgroup. A total of 58 parsimonious trees were found, which required a total of 2683 steps in each site. Bootstrap values for 1000 replicates are shown on the branches. Strain names are as described in Table 1 and Table 2.

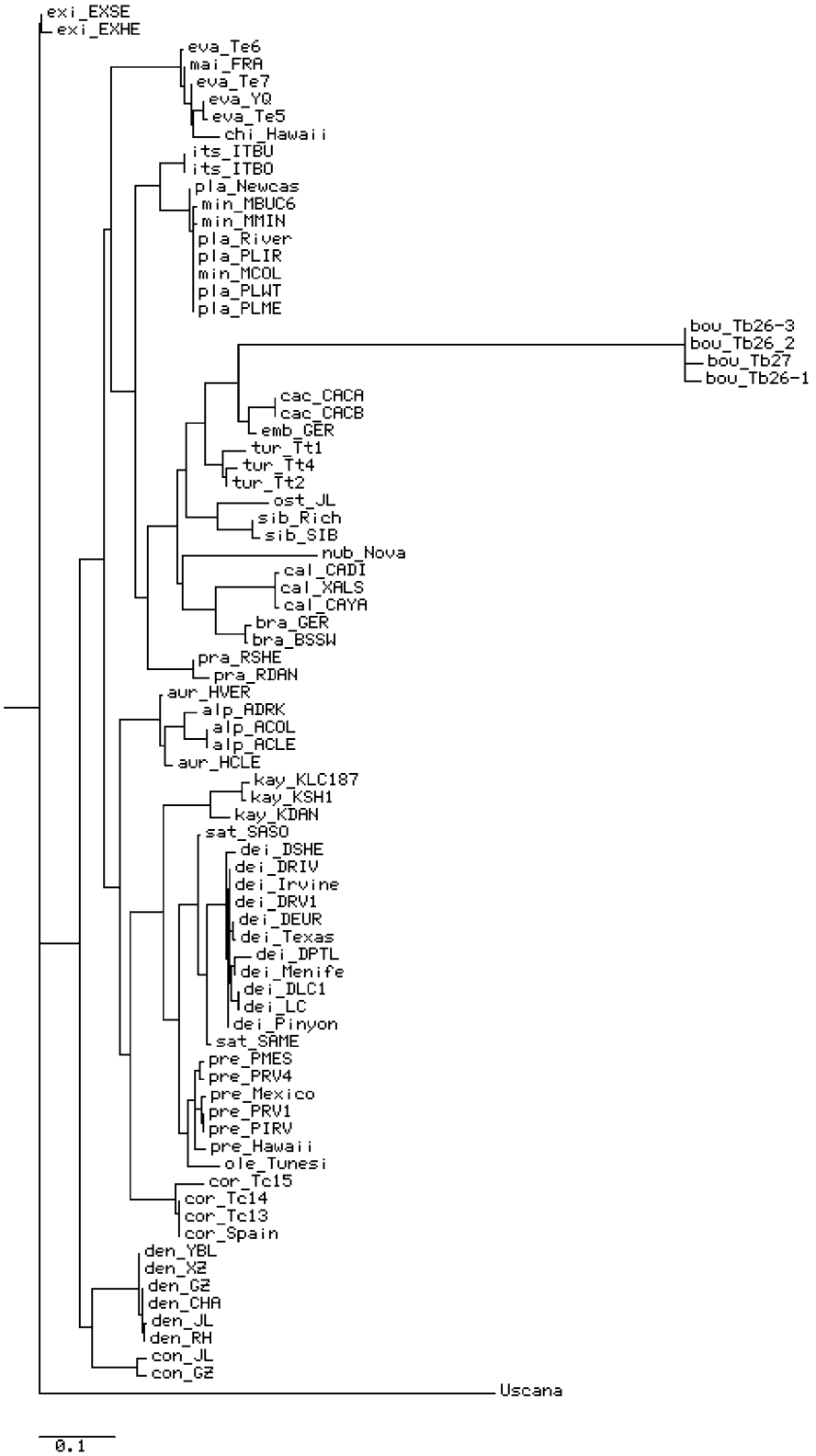

Fig. 3. The extended majority rule consensus ML tree for 79 *Trichogramma* species using *U. semifumipennis* as outgroup. A total of 24068 ML trees were examined using fastDNAml version 1.2.2 (Olsen et al., 1994). Total weight of positions in analysis = 708; transition / transversion ratio = 2; transition / transversion parameter = 1.4765. Strain names are as described in Table 1 and Table 2.

sequence data previously reported by different authors and used for phylogenetic analysis in this study all support the formerly discovered conclusions, namely minor within-species and distinct interspecies ITS2 sequence divergence. Moreover, based on comparatively large-scale sequence sampling, a baseline that can be regarded as a species border for delineating *Trichogramma* populations was determined, namely a distance value of approxi-

mate 0.02 calculated by the Kimura 2-parameter model. Can new taxa be erected on the basis of rDNA differentiation? Is it sufficient to have a distance of about 0.02 to define a new species? Such a baseline does not confirm the existence of a taxonomic relationship. This can only be done by performing crossing experiments as suggested by Pinto et al. (1991).

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