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Molecular identification and phylogeny of parasitic wasp species (Hymenoptera: Trichogrammatidae) by mitochondrial DNA RFLP and RAPD markers

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Abstract

Mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) were tested for their ability to discriminate between seven species of minute parasitic wasps belonging to the genus *Trichogramma*. They proved to be reliable species-diagnostic molecular markers. Pairwise comparisons of the mtDNA restriction maps revealed considerable differentiation among the seven species. Percentage of common restriction sites ranged from 30% to 83%. Phylogenetic analyses performed either on the mtDNA nucleotide distance matrix or on the matrix of the restriction site-state generated a tree congruent with those based on allozymes and morphology.

RAPD procedures also revealed species-specific banding patterns and seem promising for a rapid and easy identification of *Trichogramma* species. Moreover, for some *Trichogramma* species, RAPD banding patterns might be informative of the phylogenetic relatedness.

Key words: parasitoid wasps, *Trichogramma*, mitochondrial DNA, RAPD, genetic divergence, phylogeny.

Introduction

Trichogramma are minute wasps, less than 1 mm in length, that are insect-egg parasitoids. The genus *Trichogramma* consists of about 150 described species. It is distributed worldwide and encountered in all terrestrial habitats. Many studies in applied biology have focused on *Trichogramma* because of their importance in biological control of lepidopterous pests in crops. Some *Trichogramma* species are mass-reared, commercialized and inundatively released in fields and therefore they are economically important. Theoretically, optimization of *Trichogramma* efficiency in biological control relies on the choice of the right species to be used to regulate the population of a given pest in a given habitat. However, in their review Pinto & Stouthamer (1994) pointed out that the systematics of the genus *Trichogramma* are confused. First, numerous species have been typologically described on the basis of subtle differences in morphological features. Secondly, many species were redescribed on the basis of a great variability in male genitalia, congruent with reproductive data (Nagarkatti & Nagaraja, 1971, 1977), although for some described species reproductive isolation is not complete (Pintureau, 1991). However, this classification does not include *Trichogramma* species that are completely parthenogenetic with no males and no diagnostic traits for females are available. Moreover, characters such as male genitalia may display high plasticity and adaptive convergence, such that their validity as evolutionary units may be controversial.

The detection of cryptic species requires rapid and reliable methods for characterization of *Trichogramma*. Specific markers are also necessary to determine the fate of the field-released *Trichogramma* with regard to endemic populations. No definitive study has been done on founder effects which could affect the establishment of a durable population. Some species were analysed with protein electrophoresis to discriminate between species, but intraspecific genetic variability was not always easily detectable (Pintureau, 1987, 1990, Pintureau & Keita, 1989; Pinto *et al.*, 1992, 1993; Pinto & Stouthamer, 1994, for a review). In addition to phenotypic and allozymic methods for characterizing *Trichogramma*, DNA-based methods can be used. DNA-based species-specific markers are unaffected by life stage or sex of *Trichogramma*. In a preliminary study, Vanlerberghe (1991a, b) recommended the analysis of mitochondrial DNA which has become a standard tool in evolutionary biology (Harrison, 1989, for a review), for both reconstructing the phylogeny of *Trichogramma* species and for monitoring the dispersion of the wasps.

The purpose of this paper is to examine the level of genetic differentiation at two types of presumptively selectively neutral molecular markers among seven recognized species of *Trichogramma*. Nineteen restriction enzymes

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Table 1. Number of restriction sites found in each *Trichogramma* species.

	T. evanescens	*T. brassicae*	*T. voegelei*	*T. cacoeciae*	*T. daumalae*	*T. semblidis*	*T. principium*
Bcl I	2	1	2	1	1	1	2
Bgl II	1	1	1	1	1	1	0
Cla I	1	1	2	1	1	1	0
Eco RI	3	3	3	5	3	5	1
Eco RV	2	1	2	3	3	2	2
Hind III	2	1	0	1	1	2	0
Kpn I	1	1	2	1	1	1	1
Pst I	1	3	3	2	2	2	1
Pvu II	1	0	0	1	1	1	1
Sac I	0	2	2	1	2	2	1
Sca I	1	1	2	0	0	0	1
Sfu I	2	2	2	2	3	2	1
Xba I	1	1	1	1	1	0	0
Xho I	1	1	1	1	1	1	0
Total	19	19	23	21	21	21	11

were used to examine the mtDNA fragment length polymorphism and to build the restriction maps of the seven species. The resulting mtDNA phylogeny is congruent with the phylogeny predicted from morphological and allozymic data previously studied. The conclusions drawn from the analysis of the mtDNA restriction maps of the seven *Trichogramma* species are compared to mtDNA data published on other hymenopteran and dipteran insects. The recently developed PCR-based method called RAPD (Williams *et al.*, 1990) or AP-PCR (Welsh & McClelland, 1990) has been employed for a more rapid identification of *Trichogramma* species. This technique reveals much variation

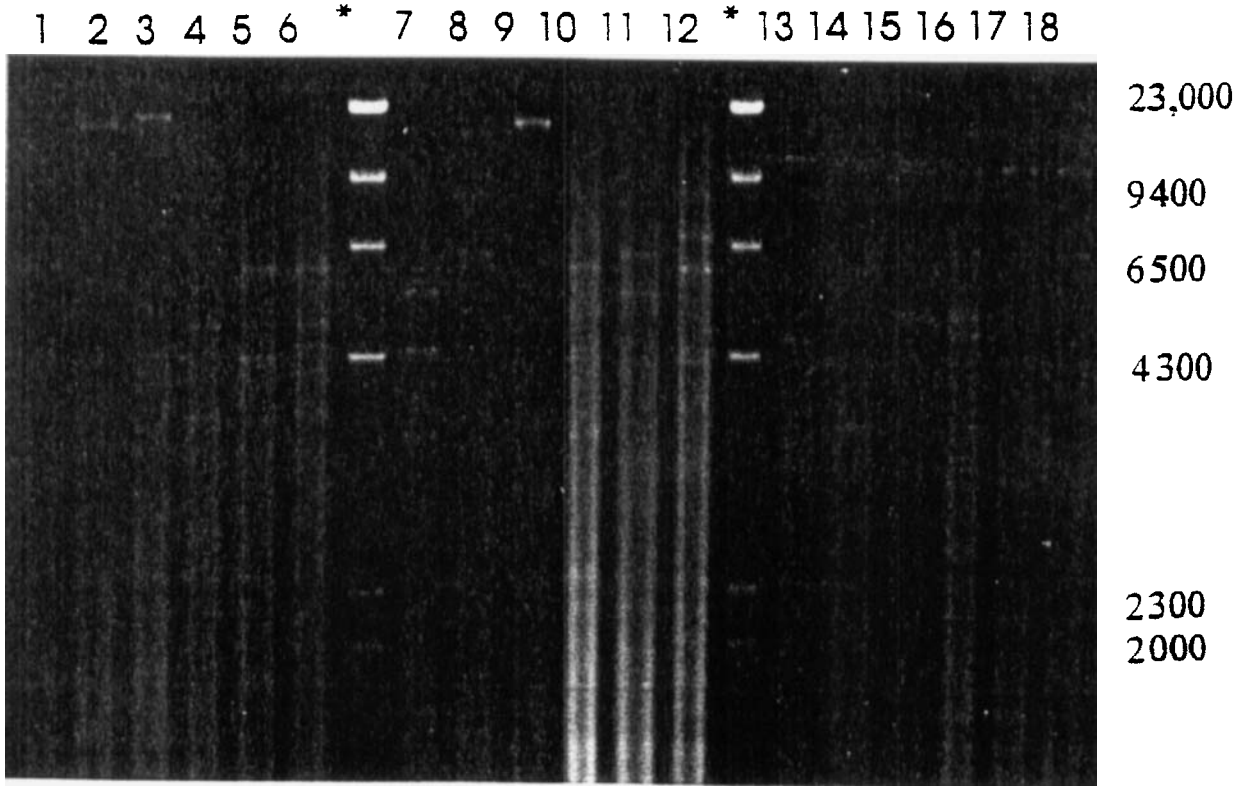

Figure 1. Restriction patterns for *Eco* RI–*Eco* RV, *Eco* RV–*Pst* I and *Eco* RI mtDNA digestions respectively for *T. principium* (lanes 1–3), *T. semblidis* (lanes 4–6), *T. daumalae* (lanes 7–9), *T. cacoeciae* (lanes 10–12), *T. brassicae* (lanes 13–15) and *T. evanescens* (lanes 16–18). Lanes marked * refer to *Hind* III restriction fragments of the Lambda phage DNA.

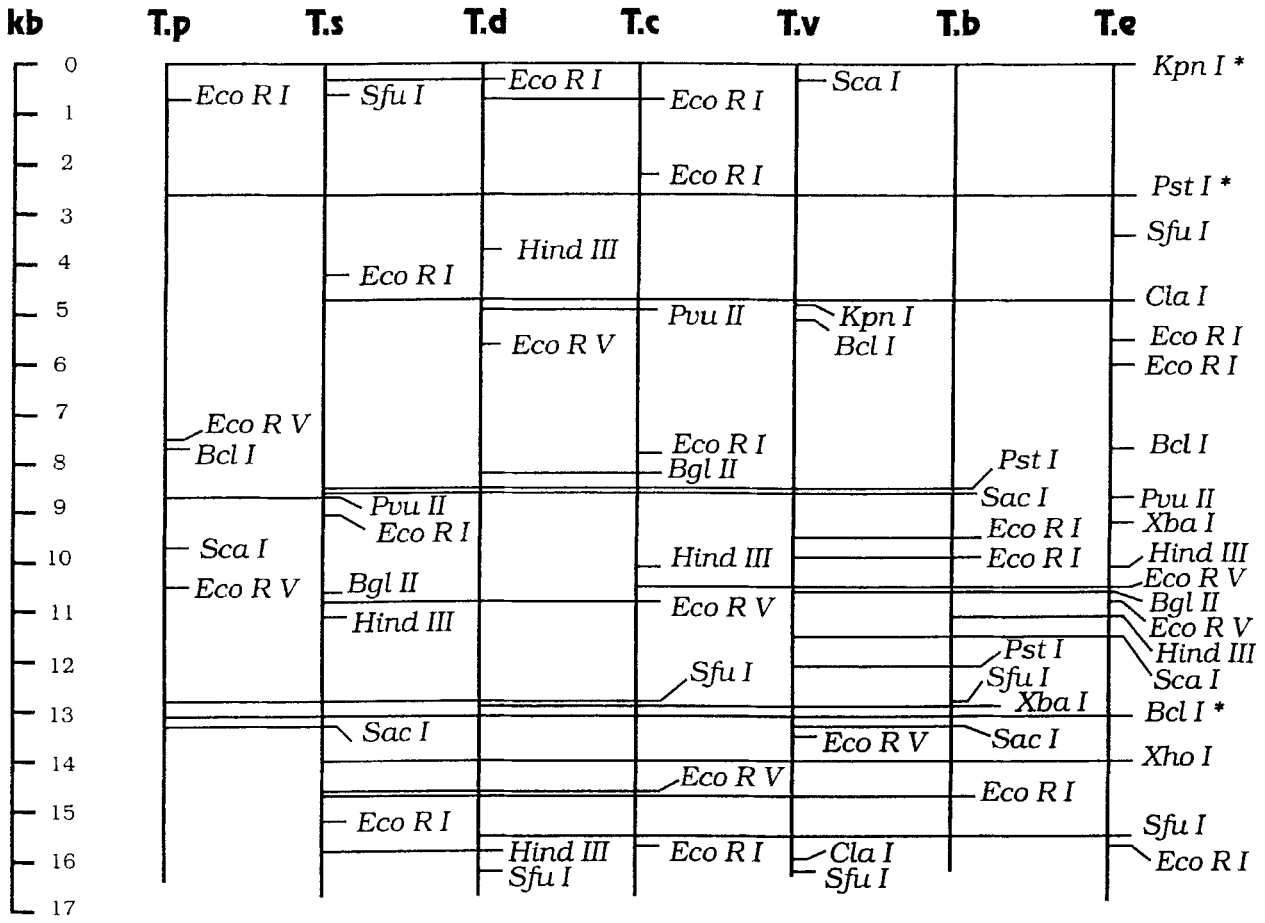

Figure 2. Cleavage site maps for mtDNA of the seven species of *Trichogramma*. Vertical lines show species restriction maps drawn to scale in kb and crossing horizontal lines show restriction sites shared by two or more contiguous restriction maps. Sites noted * are conserved in all species.

between the seven species. RAPD profiles are species-specific and reflect the phylogenetic relationships among some of the *Trichogramma* species.

Results

mtDNA RFLP

No differences in the restriction patterns were observed among the five to ten isofemale lines studied in each species. Forty isofemale lines of a *T. evanescens* population were analysed for their restriction polymorphism with *Eco* RI and *Eco* RV enzymes but no variation has been detected among them. In the same way, no polymorphism has been observed between the isofemale lines of two geographic strains of *T. cacoeciae* with any of the nineteen enzymes.

Table 1 gives the number of restriction fragments produced by digestion of mtDNAs of the seven *Trichogramma* species with fourteen of the nineteen restriction enzymes. No restriction site was detected for *Bam* HI, *Acc* I, *Ava* I, *Sma* I and *Nde* I. *Eco* RI produced the largest number of fragments in each species and gave species-specific pat-

Table 2. Nucleotide divergence in mtDNA of seven *Trichogramma* species. Along the diagonal: number of cleavage sites; above the diagonal: proportion of sites shared by two species; below the diagonal: mean number of nucleotide substitutions per site.

	T. evanescens	*T. brassicae*	*T. voegelei*	*T. cacoeciae*	*T. daumalae*	*T. semblidis*	*T. principium*
T. evanescens	**19**	0.53	0.44	0.45	0.35	0.45	0.40
T. brassicae	*0.107*	**19**	0.81	0.55	0.55	0.55	0.27
T. voegelei	*0.137*	*0.035*	**23**	0.50	0.50	0.50	0.30
T. cacoeciae	*0.133*	*0.100*	*0.116*	**21**	0.76	0.52	0.37
T. daumalae	*0.175*	*0.100*	*0.116*	*0.045*	**21**	0.57	0.37
T. semblidis	*0.133*	*0.100*	*0.116*	*0.108*	*0.093*	**21**	0.44
T. principium	*0.153*	*0.220*	*0.199*	*0.163*	*0.163*	*0.138*	**11**

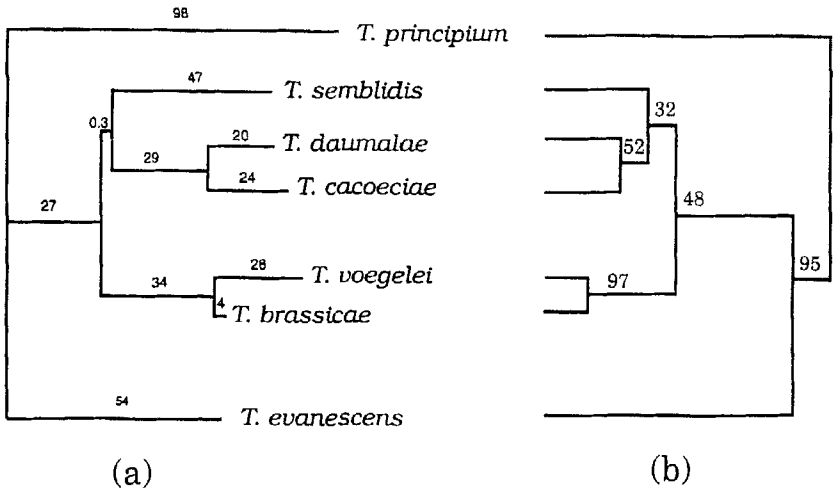

Figure 3. Phylogenetic networks: (a) distance method (Fitch), numbers refer to branch length; (b) parsimony method (Dolpenny) and bootstrap scores in percentages.

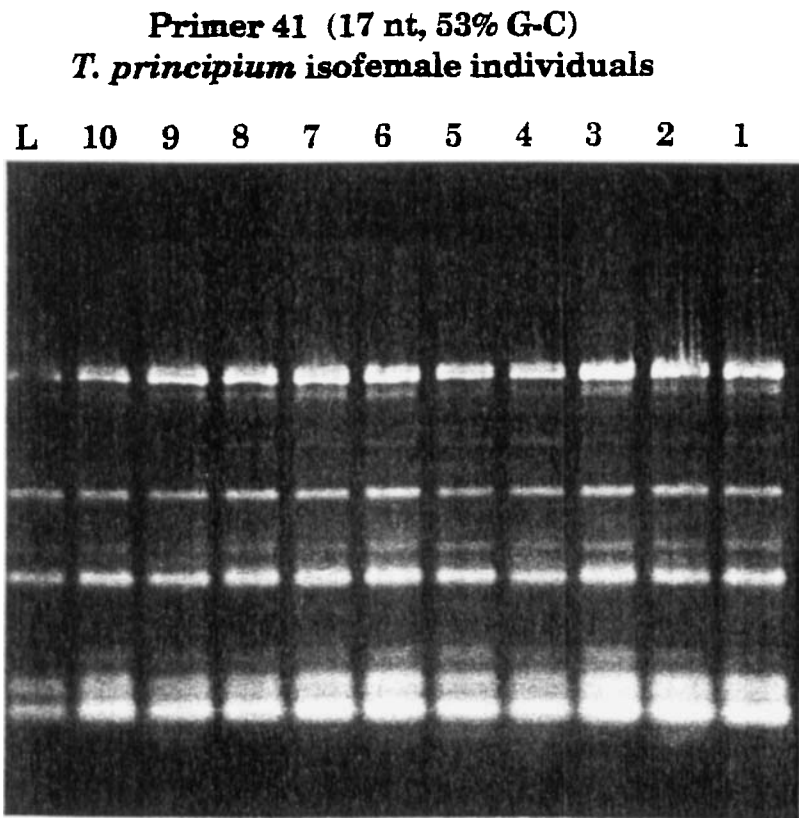

Figure 4. RAPD banding patterns with primer 41 (CCCTGGACGTCTACAAT) of ten individual wasps of the same isofemale line of *T. principium* as compared to lane L corresponding RAPD-PCR of 10 ng of genomic DNA extracted from pooling fifty individuals of the same isofemale line.

terns (Fig. 1). *Kpn* I is diagnostic with two restriction sites on the mtDNA molecule of *T. voegelei*, whereas the other six species display only one *Kpn* I site on their molecule. *Cla* I discriminates *T. voegelei* with two sites and *T. principium* with no site from the other five species which have one site. Except for *Eco* RI, *Eco* RV, *Pst* I and *Sfu* I which yield at least two mtDNA restriction fragments, the other restriction patterns exhibit only one fragment in most of the species and are therefore not species discriminant.

The mean size of *Trichogramma* mtDNA molecule is 16,500±300 bp, based on summation of fragment lengths for *Eco* RI and several combinations of double digestions. This size is an estimate, because fragments smaller than 300 bp were hardly detectable. Nevertheless, fragment length differences ranging from 300 to 600 bp were observed repeatedly when single or double digest products from different species co-migrated. This suggests that mtDNA size variation may exist among *Trichogramma* species.

Linear mtDNA restriction maps of *Trichogramma* species were drawn and aligned extending from *Kpn* I site which is conserved in every species (Fig. 2). Likewise, *Pst* I

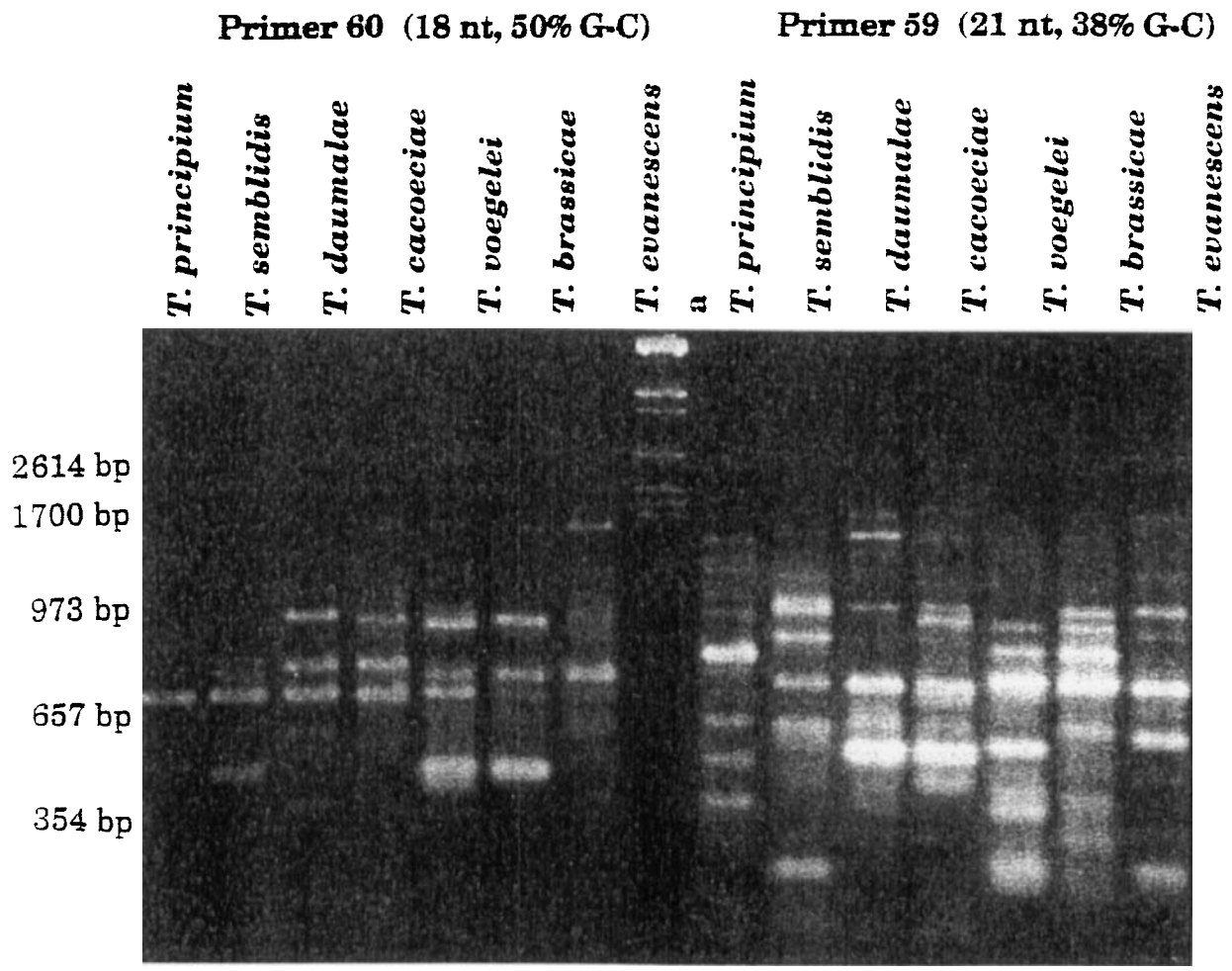

Figure 5. RAPD banding patterns for seven species of *Trichogramma* generated with primer 59 (GCTTTCGAATTCTTAAACACC) and primer 60 (CGCTTGTACTTCGACATG). Sizes in bp on the left refer to some of the *Cla* I restriction fragments of the Lambda phage DNA in lane a.

and *Bcl* I cleave the seven molecules at invariant sites, respectively 2.6 kb and 13.1 kb from the *Kpn* I site. Mitochondrial nucleotide sequence divergence was calculated for all pairs of *Trichogramma* species from the fraction of shared restriction sites (Table 2), which ranged from 0.035 to 0.220 nucleotide substitution per site. From Table 2 it appears that the more closely related species are *T. brassicae* and *T. voegelei* on the one hand and *T. cacoeciae* and *T. daumalae* on the other; *T. principium* is the most divergent species. The relationships between the seven species of *Trichogramma* are represented by the phylogenetic network in Fig. 3(a). The Fitch option used to build this tree allows varying rates of evolution in the different lineages (no molecular clock) and produces an unrooted tree.

From the aligned maps in Fig. 2, forty-nine different restriction sites have been counted, out of which three are conserved in all species, nineteen are autapomorphic, and twenty-seven are informative. The maximum parsimony tree inferred from these discrete characters and the bootstrap values are shown in Fig. 3(b). The phylogenetic trees obtained by the two methods have an identical topology.

RAPD

RAPD produces a series of discrete DNA bands. Variations in intensity were not taken into account and only bands which were bright and reproducible were scored. Longer oligomers were as efficient as decamers in generating discriminant patterns. A screening of twenty individuals of the same isofemale line of *T. principium* was made to ascertain RAPD pattern uniformity in the line (Fig. 4). No polymorphism was detected among them so the genomic DNA extracted from pooling about fifty siblings exhibits a RAPD variant representative of the line. RAPD polymorphisms have been detected between the two geographic strains of *T. cacoeciae* (data not shown) and among the forty lines of the *T. evanescens* population (Vanlerberghe-

Masutti, 1994). However, the polymorphic bands are species-specific.

Each *Trichogramma* species has a distinctive RAPD pattern with each primer (Fig. 5). 217 bands ranging from 2500 to 200 bp were scored over the seven primers, which represents an average of 4.5 bands per primer per species. Pairwise genetic distances were not calculated because a whole set of bands appears to be species specific. Consequently, most of these bands are discriminant but not phylogenetically informative. However, great similarities in RAPD amplification products exist in two pairs of species. *T. brassicae* and *T. voegelei* display many bands in common when amplified with primers 02, 58 or 60, as well as *T. cacoeciae* and *T. daumalae* with primers 58, 59 and 60 (Fig. 5).

Discussion

mtDNA

The RFLP analysis of the mtDNA of the seven *Trichogramma* species revealed extensive variation. All of the species belong to clearly differentiated mitochondrial lineages. The restriction enzyme *Eco* RI can be considered as a good diagnostic taxonomic tool, because it generates species-specific patterns.

The size of the mitochondrial genome in *Trichogramma* averages 16,500 bp, which is well within the range of mtDNA length of other hymenopteran insects, such as *Apis* (Smith, 1988; Smith & Brown, 1988, 1990; Cornuet & Garnery, 1991) or the ant *Leptothorax acervorum* (Stille & Stille, 1992). As is the rule in the animal kingdom, the mitochondrial genome size of insects is between 15,000 bp and 20,000 bp, except for the bark weevil which is unusually large, 30,000–36,000 bp (Boyce *et al.*, 1989).

The forty-nine restriction sites which were scored from cleavage of the *Trichogramma* mtDNAs are equivalent to a 294 nucleotide sequence. This means that only 1.8% of the mitochondrial genome of *Trichogramma* has been investigated. Therefore the nucleotide divergences in Table 2 have to be considered in terms of relative rather than absolute values. The mean interspecific mitochondrial nucleotide divergence in *Trichogramma* is 12.6% and is equivalent to interspecific mtDNA divergence values estimated from RFLP data in other hymenopteran and dipteran genera. The nucleotide distance between two species of *Apis* averages 13% (Cornuet & Garnery, 1991) and is of the same order between species of the social wasp *Vespula* (Schmitz & Moritz, 1990). 2–3% mtDNA sequence divergence was found between four sibling species of the mosquito *Anopheles quadrimaculatus* complex (Mitchell *et al.*, 1992), whereas eleven in the genus *Aedes* share an average of only 34% of the fragments (Kambhampati & Rai, 1991). In the genus *Drosophila*, pairwise divergence between mtDNA sequences is also very variable (Hale & Beckenbach, 1985; Solignac *et al.*, 1986; Chang *et al.*, 1989; Gonzalez *et al.*, 1990; Pissios & Scouras, 1993).

The trees inferred from the distance method and the parsimony method (Figs 3a, 3b) have the same topology. Robustness of phylogenetic branching was statistically tested by bootstrapping (Fig. 3b). In ninety-five out of 100 bootstrap replicates, *T. principium* mtDNA lineage is branching out of the other six species. The node which branches *T. brassicae* and *T. voegelei* as the two most closely related species is very robust (97%), as expected from taxonomical data based on mating compatibility analysis (Pintureau, 1991). Even if the other bootstrap values are low, the mtDNA phylogenetic tree is consistent with the cladistic analysis of morphological and allozymic characters published by Pintureau (1987). These results suggest that there is no fundamental discrepancy between molecules and species evolution, as far as the mtDNA is concerned.

In the absence of data on an outgroup, the place of the root of the mtDNA lineages network cannot be ascertained. Attempts to align *Trichogramma* mtDNA restriction maps to the mtDNA map of the honeybee *Apis mellifera* L. established with forty-six restriction sites (Cornuet & Garnery, 1991) were not successful. The restriction maps of these two genera are too divergent to be compared with confidence and hence nucleotide divergence was not estimated. This is not surprising, because sequence data from the mtCO-II gene in several hymenoptera, honeybee, bumblebee, ant and wasp revealed that intergeneric distances reached more than 30% of nucleotide divergence (Garnery *et al.*, 1991; Liu & Beckenbach, 1992).

If we assume that nucleotide divergence values d are proportional to the divergence time, we can apply Nei's formula (1987, p. 41) $t(\text{Myr}) = d/k$, where k is the rate of divergence between two evolutionary lineages. If we assume that for *Trichogramma* mtDNA the rate of divergence is the same as for the whole mitochondrial genome of *Drosophila*, then it is close to 2% per million years (Myr) (DeSalle *et al.*, 1987). In this case, *T. principium* would have diverged from the other mtDNA lineages 8–10 Myr ago, whereas *T. brassicae* and *T. voegelei* split off from a common ancestor less than 1.75 Myr ago. However, these values must be considered as very rough estimates of divergence times, because no calibration of the mtDNA evolutionary rate in insects is supported by fossil evidence as in vertebrates. Calibration in *Drosophila* is also imprecise and Crozier *et al.* (1989) showed a higher rate of nucleotide substitution per site in the CO-I and CO-II genes in *Apis* than in *Drosophila*. Nevertheless, *Apis* and *Drosophila* lineages split off about 280 Myr ago (Crozier *et al.*, 1989) and no data have demonstrated that other hymenoptera are concerned by this increase of mtDNA evolutionary rate.

RAPD

Landry *et al.* (1993) submitted male specimens from three species of *Trichogramma* to a RAPD markers preliminary analysis: *T. dendrolimi* Matsumara, *T. pretiosum* Riley and *T. evanescens* Westwood. They reported that a great heterogeneity appeared in the RAPD patterns between the three species. The present study confirms this level of interspecific variability and it reveals species-specific RAPD banding patterns.

The level of similarity between banding patterns of the two species *T. brassicae* and *T. voegelei* and of the two species *T. daumalae* and *T. cacoeciae* (Fig. 5) suggests that RAPD might be used as an approach in searching for genetic proximity between taxa. This result contradicts the suggestion by Gawel & Bartlett (1993) that RAPD may not be useful in classifying organisms. These authors were led to this conclusion because, based on RAPD patterns, two biotypes of a whitefly, *Bemisia tabacci*, were no more closely related to each other than to other whitefly species, even in other genera.

The great advantage of RAPDs over other techniques for discriminating insect individuals is that it is fast, easy and efficient (for a review see Black, 1993). Application of molecular technologies allows the detection of diagnostic markers which are necessary to try to resolve ambiguities in taxonomical identification and systematics in the genus *Trichogramma*.

Experimental procedures

Isofemale lines of seven species of *Trichogramma* have been maintained in laboratory cultures on eggs of a substitution host, the flour moth *Ephestia kuehniella* (Lepidoptera: Pyralidae) for several years. The *Trichogramma* species submitted to experiments were: *Trichogramma evanescens* Westwood, *T. brassicae* Bezdenko, *T. voegelei* Pintureau, *T. cacoeciae* Marchal, *T. daumalae* Dugast & Voegelé, *T. daumalae* Aurivillius and *T. principium* Sugonjaev & Sorokina, and all were from a laboratory collection. All species, except *T. cacoeciae*, reproduce by arrhenotokous parthenogenesis; fertilized eggs (2n) produce females and unfertilized eggs (n) males; virgin females produce only male offspring. In contrast, the species *T. cacoeciae* reproduces by thelytoky; virgin females produce female offspring (2n) and males rarely occur. Thelytoky in *T. cacoeciae* is not induced by symbiotic microorganisms, because wasps cannot be reverted to arrhenotoky by antibiotic treatments or high temperatures (Stouthamer, 1991).

These seven particular species were chosen because they were well characterized, both morphologically and biochemically, by Pintureau (1987). For *T. brassicae*, *T. voegelei*, *T. daumalae*, *T. semblidis* and *T. principium* one geographic strain of each species and five to ten isofemale lines per strain were submitted to mtDNA RFLP and RAPD analysis. Two geographic samples of *T. cacoeciae* were analysed and forty lines from a natural population of *T. evanescens* were studied.

Mitochondrial DNA RFLP

The mitochondrial genome is generally only maternally inherited. Therefore, mtDNA was extracted from numerous offspring (200–300) of an isofemale line, as described by Powell & Zuniga (1983) and section III in Solignac (1991). Although the mtDNA preparation was crude, it was concentrated enough to visualize the mtDNA restriction fragments by staining the gel with ethidium bromide.

Aliquots of each mtDNA sample were digested with each of nineteen 6 bp restriction enzymes according to the manufacturer's recommendations (Boehringer Mannheim). Endonucleases used are listed in Table 1. Fragments were separated electrophoretically on 0.7% or 1.2% agarose gels in Tris–borate–EDTA buffer (Maniatis *et al.*, 1982). *Hind* III restriction fragments of Lambda phage DNA were used as molecular weight markers. No attempt was made to score fragments smaller than 300–500 bp. All the restriction sites for all the enzymes were mapped by single and double digestions of whole mtDNA molecules for each species.

Once the seven mtDNA maps were constructed, pairwise comparisons were used to compute the proportion of shared restriction sites S_{ij} between species i and j;

$$S_{ij} = 2m_{ij}/(m_i+m_j)$$

where m_{ij} is the number of restriction sites shared by the species i and j, m_i the total number of restriction sites in species i and m_j the total number in species j.

An estimate of the number of nucleotide substitutions per site between mtDNA sequences from species i and j was computed using the formula of Nei & Li (1979);

$$d_{ij} = (-\ln S_{ij})/r$$

where r is the number of nucleotides in the recognition sequence restriction enzymes, in this case, r is equal to 6.

Two different methods were used to reconstruct molecular phylogenies, distance and character-state (presence/absence of each restriction site) and both are available in the PHYLIP (Phylogeny Inference Package) program version 3.4 provided by J. Felsenstein (Department of Genetics, University of Washington, Seattle, WA 98195, USA). The FITCH program, a maximum likelihood method, was applied to the divergence matrix whereas the DOLPENNY program, a maximum parsimony method, was applied to the restriction site-state table.

RAPD

Total genomic DNA was extracted from about fifty adults from a single isofemale line by a modification of the method of MacGinnis *et al.* (1983). Adults were thoroughly homogenized in 500 μl of NIB solution (0.1 M NaCl, 0.03 M Tris-HC1 pH 8.0, 0.01 M EDTA, 0.07% (v/v) beta-mercaptoethanol, 0.5% (v/v) Nonidet NP40). The homogenate was centrifuged for 1 min to pellet nuclei. The pellet was resuspended in 400 μl of HB solution (0.1 M NaC1, 0.2 M sucrose, 0.01 M EDTA) and membranes were lysed with 100 μl of LB (0.125 M EDTA, 0.5 M Tris-HC l pH 9.2, 2.3% SDS) for 30 min at 55°C, then DNA was phenol-extracted and ethanol-precipitated.

RAPD reactions were also carried out on single individuals whose DNA was extracted using a chelating resin, as described in Walsh *et al.* (1991). An adult parasitoid was placed into 1.5 ml microfuge tube and crushed with a flame-sealed Pasteur pipette. 20μl of a 5% (w/v) Chelex solution (Bio-Rad Laboratories) was

added to the specimen, heated at 56°C for 30 min then 100°C for 5 min, vortexed, centrifuged for a few seconds and stored at −20°C.

Amplification reactions were performed as reported by Williams *et al.* (1990), either with 10 ng of *Trichogramma* DNA or with 2 µl of Chelex supernatant and 0.5 unit of *Taq* DNA polymerase (Appligene). DNA was amplified by PCR in a ThermoJet thermal cycler (Equibio s.a., Angleur Belgium). Oligomers containing from seventeen to thirty nucleotides with 30–57% G-C content (kindly provided by D. Fournier and designed for other purposes) and a decamer served as primers for amplification reactions
(p02: ATGGATCCGC;
p20: TGACCCTCCAAGAAGGT;
p41: CCCTGGACGTCTACAAT;
p58: CCTCAGGTATTTGCCAAGGCTCCTGCAGAT;
p45: CTTATTTGGATTCTTTTGCT;
p59: GCTTTCGAATTCTTAAACACC;
p60: CGCTTGTACTTCGACATG).

As described in Welsh & McClelland (1990) and modified by Akopyanz *et al.* (1992), the cycling programme with each large primer consisted of three low-stringency amplification cycles of (94°C, 5 min; 40°C, 5 min ; 70°C, 5 min) and thirty high-stringency amplification cycles of 94°C, 1 min; 55°C, 1 min; 70°C, 2 min) followed by a final incubation for 10 min at 70°C. Products of amplification were electrophoresed in 1.4% agarose gels, stained with ethidium bromide, and photographed under UV light.

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References

Akopyanz, N., Bukanov, N.O., Westblom, T.U., Kreskovich, S. and Berg, D.E. (1992) DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acids Res* **20**: 5137–5142.

Black, W.C., IV (1993) PCR with arbitrary primers: approach with care. *Insect Mol Biol* **2**: 1–6.

Boyce, T.M., Zwick, M.E. and Aquadro, C.F. (1989) Mitochondrial DNA in bark weevils: size, structure and heteroplasmy. *Genetics* **123**: 825–836.

Chang, H., Wang, D. and Ayala, F.J. (1989) Mitochondrial DNA evolution in the *Drosophila nasuta* subgroup of species. *J Mol Evol* **28**: 337–348.

Cornuet, J.M. and Garnery, L. (1991) Mitochondrial DNA variability in honeybees and its phylogeographic implications. *Apidologie* **22**: 627–642.

Crozier, R.H., Crozier, Y.C. and Mackinlay, A.G. (1989) The CO-I and CO-II region of honeybee mitochondrial DNA: evidence for variation in insect mitochondrial evolutionary rates. *Mol Biol Evol* **6**: 399–411.

DeSalle, R., Freedman, T., Prager, E.M. and Wilson, A.C. (1987) Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian *Drosophila*. *J Mol Evol* **26**: 157–164.

Garnery, L., Vautrin, D., Cornuet, J.M. and Solignac, M. (1991) Phylogenetic relationships in the genus *Apis* inferred from mitochondrial DNA sequence data. *Apidologie* **22** 87–92.

Gawel, N.J. and Bartlett, A.C. (1993) Characterization of differences between whiteflies using RAPD-PCR. *Insect Mol Biol* **2**: 33–38.

Gonzalez, A.M., Hernandez, M., Volz, A., Pestano, J., Larruga, J.M., Sperlich, D. and Cabrera, V.M. (1990) Mitochondrial DNA evolution in the *obscura* species subgroup of *Drosophila*. *J Mol Evol* **31**: 122–131.

Hale, L.R. and Beckenbach, A.T. (1985) Mitochondrial DNA variation in *Drosophila pseudoobscura* and related species in pacific northwest populations. *Can J Genet Cytol* **27**: 357–364.

Harrison, R.G. (1989) Animal mitochondrial DNA as a genetic marker in population and evolutionary biology. *Trends Ecol Evol* **4**: 6–11.

Kambhampati, S. and Rai, K.S. (1991) Variation in mitochondrial DNA of *Aedes* species (Diptera: Culicidae). *Evolution* **45**: 120–129.

Landry, B.S., Dextraze, L. and Boivin, G. (1993) Random amplified polymorphic DNA markers for DNA fingerprinting and genetic variability assessment of minute parasitic wasps species (Hymenoptera: Mymaridae and Trichogrammatidae) used in biological control programs of phytophagous insects. *Genome* **36**: 580–587.

Liu, H. and Beckenbach, A.T. (1992) Evolution of the mitochondrial cytochrome oxidase II gene among 10 orders of insects. *Mol Phylogenet Evol* **1**: 41–52.

MacGinnis, W., Shermoen, A.W. and Beckendorf, S.K. (1983) A transposable element inserted just 5' to a *Drosophila* glue protein gene alters gene expression and chromatin structure. *Cell* **34**: 75–84.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, New York.

Mitchell, S.E., Narang, S.K., Cockburn, A.F., Seawright, J.A. and Goldenthal, M. (1992) Mitochondrial and ribosomal DNA variation among members of the *Anopheles quadrimaculatus* (Diptera: Culicidae) species complex. *Genome* **35**: 939–950.

Nagarkatti, S. and Nagaraja, H. (1971) Redescription of some known species of *Trichogramma* (Hym., Trichogrammatidae), showing the importance of the male genitalia as a diagnostic character. *Bull Ent Res* **61**: 13–31.

Nagarkatti, S. and Nagaraja, H. (1977) Biosystematics of *Trichogramma* and *Trichogrammatoidea* species. *Annu Rev Entomol* **22**: 157–176.

Nei, M. (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Nei, M. and Li, W.H. (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci USA* **76**: 5269–5273.

Pinto, J.D., Kazmer, D.J., Platner, G.R. and Sassaman, C.A. (1992) Taxonomy of the *Trichogramma minutum* complex (Hymenoptera: Trichogrammatidae): Allozymic variation and its relationship to reproductive and geographic data. *Ann Ent Soc Am* **85**: 413–422.

Pinto, J.D., Platner, G.R. and Sassaman, C. A. (1993) An electrophoretic study of two closely related species of north American *Trichogramma*: *T. pretiosum* and *T. deion* (Hymenoptera: Trichogrammatidae). *Ann Ent Soc Am* **86**: 702–709.

Pinto, J.D. and Stouthamer, R. (1994) Systematics of the Trichogrammatidae with emphasis on *Trichogramma*. In: *Biological Control with Egg Parasitoids* (Wajnberg, E. and Hassan, S.A., eds), pp. 1–36. CAB International, England.

Pintureau, B. (1987) Systématique évolutive du genre *Trichogramma* Westwood (Hym., Trichogrammatidae) en Europe. Thèse de Doctorat d'Etat, Université, Paris VII.

Pintureau, B. (1990) Polymorphisme, biogéographie et spécificité parasitaire des Trichogrammes européens. *Bull Soc Ent Fr* **95**: 17–38.

Pintureau, B. (1991) Indices d'isolement reproductif entre espèces proches de Trichogrammes (Hym., Trichogrammatidae). *Annls Soc Ent Fr* **27**: 379–392.

Pintureau, B. and Keita, F.B. (1989) Nouvelles données sur les estérases des trichogrammes (Hym., Trichogrammatidae). *Biochem Syst Ecol* **17**: 603–608.

Pissios, P. and Scouras, Z.G. (1991) Mitochondrial DNA evolution in the *Montium*-species subgroup of *Drosophila*. *Mol Biol Evol* **10**: 375–382.

Powell, J.P. and Zuniga, M.C. (1983) A simplified procedure for studying mtDNA polymorphisms. *Biochem Genet* **21**: 1051–1055.

Schmitz, J. and Moritz, R.F.A. (1990) Mitochondrial DNA variation in social wasps (Hymenoptera: Vespidae). *Experientia* **46**: 1068–1072.

Smith, D.R. (1988) Mitochondrial DNA polymorphisms in five old world subspecies of honeybees and in the new world hybrids. *Africanized Honeybees and Bee Mites* (Needham, G.R., Page, R.E., Delfinado-Baker, M. and Bowman, C.E., eds), pp. 303–312. Horwood, Chichester.

Smith, D.R. and Brown, W.M. (1988) Polymorphisms in mitochondrial DNA of European and Africanized honeybees (*Apis mellifera*). *Experientia* **44**: 257–260.

Smith, D.R. and Brown, W.M. (1990) Restriction endonuclease cleavage site and length polymorphisms in mitochondrial DNA of *Apis mellifera mellifera* and *A. m. carnica* (Hymenoptera: Apidae). *Ann Ent Soc Am* **83**: 81–88.

Solignac, M. (1991) Preparation and visualization of mitochondrial DNA for RFLP analysis. (Hewitt, G.M. *et al.* eds.) *Molecular Techniques in Taxonomy*, pp. 295–319. NATO ASI Series, Vol H57. Springer, Berlin.

Solignac, M., Monnerot, M. and Mounolou, J.C. (1986) Mitochondrial DNA evolution in the *melanogaster* species subgroup of *Drosophila*. *J Mol Evol* **23**: 31–40.

Stille, M. and Stille, B. (1992) Intra- and inter-nest variation in mitochondrial DNA in the polygynous ant *Leptothorax acervorum* (Hymenoptera: Formicidae). *Ins Soc* **39**: 335–340.

Stouthamer, R. (1991) Effectiveness of several antibiotics in reverting thelytoky to arrhenotoky in *Trichogramma spp. Trichogramma and Other Egg Parasitoid* (Wajnberg, E. and Vinson, S.B., eds), pp. 119–122. *Les Collogues de l'INRA* **56**.

Vanlerberghe, F. (1991a) Mitochondrial DNA polymorphism and relevance for *Trichogramma* population genetics. *Trichogramma and Other Egg Parasitoids* (Wajnberg, E. and Vinson, S.B., eds), pp. 123–126. *Les Colloques de l'INRA* **56**.

Vanlerberghe, F. (1991b) Mitochondrial DNA polymorphism discriminates *Trichogramma* species. *Insect Parasitoids, VIth European Workshop, Perugia, Italy. Redia* **94**: 407–409.

Vanlerberghe-Masutti, F. (1994) Detection of genetic variability in *Trichogramma* populations using molecular markers. *Norw J Agr Sci* Suppl. **16**: 171–176.

Walsh, P.S., Metzger, D.A. and Higuchi, R. (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* **10**: 506–513.

Welsh J. and McClelland, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* **18**: 7213–7218.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful genetic markers. *Nucleic Acids Res* **18**: 6531–6535.