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REPRODUCTIVE AND ELECTROPHORETIC COMPARISONS OF *TRICHOGRAMMA CALIFORNICUM* NAGARAJA AND NAGARKATTI WITH THE *T. MINUTUM* COMPLEX (HYMENOPTERA: TRICHOGRAMMATIDAE)

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Abstract.—*Trichogramma californicum* Nagaraja and Nagarkatti was compared with the *T. minutum* Riley complex, with which it is syntopic on codling moth and other tortricid pests. Reproductive crosses and allozymic electrophoresis at 14 loci were used to investigate the possibility of intermediates between them being due to interbreeding. A high degree of intraspecific variation was found for *T. californicum* in both investigations. No reproductive compatibility with *T. minutum* complex cultures was found, and three putatively distinct loci for *T. californicum* were discovered. The implications of these findings for the definition of *T. californicum* are discussed with reference to previous studies of the *T. minutum* complex.

Key Words: *Trichogramma,* taxonomy, interspecific comparisons

Trichogramma is the most important genus of egg parasitoids of Tortricidae in tree crops (Mills and Carl 1991), and is routinely released in augmentative biological control programs, although with mixed success (Falcon and Huber 1991, Smith 1996). By far the most commonly used species in augmentative control efforts of these pests in North America are the two species of the *T. minutum* complex, *T. minutum* Riley and *T. platneri* Nagarkatti. They are allopatric, with *T. minutum* occurring primarily east of 110°W longitude and *T. platneri* generally found to the west (Pinto 1999). Although these are the dominant naturally occurring egg parasitoids of tortricid pests in fruit orchards, at least nine other native *Trichogramma* species also occur on these hosts (Pinto et al., in prep.). One of these, *T. californicum,* is a western species occurring in sympatry with *T. platneri.*

Trichogramma californicum was described from specimens reared from eggs of the Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnogh), collected from Alturas, Modoc Co., in northeastern California (Nagaraja and Nagarkatti 1973). In addition to this lymantriid it has been recorded from eggs of several species of Lycaenidae, and two species of Tortricidae, including *Cydia pomonella* (L.), the codling moth, one of the primary hosts of the *T. minutum* complex (Pinto 1999). *Trichogramma californicum* is not a common species but its range does overlap broadly with *T. platneri* in the western United States (Pinto 1999), and both have been taken from codling moth on apple in Idaho and northern California. *Trichogramma californicum* and members of the *Trichogramma minutum* complex are similar morphologically, separated by minor differences in color and structure. Sympatry and the occurrence of at least limited character intermediacy suggested the possibility of interspecific hybridization between *T. californicum* and *T.*

Table 1. Collection localities and generation of cultures examined in electrophoretic and crossing studies.

Species	Acronym	Collection Site	Laboratory Generation Studied
T. californicum	CASB	San Bernardino Mts, CA	>55
	CAAD	Adin, Modoc Co., CA	>175
	CAYK	Yakima, WA	>55
T. minutum	MCVA	Chula Vista, San Diego Co., CA	>675
T. platneri	PRV1	Riverside (UCR Campus), CA	>475
T. exiguum	EXSL	Selma, AL	>500
	EXHN	Hendersonville, NC	>60

platneri in the west. Although reproductive crosses of *T. californicum* showed complete incompatibility with several similar species including *T. platneri* (Pinto 1999), these results were based on single crosses, each involving no more than 20 pairs in each direction.

This paper investigates the distinctness of *T. californicum* and members of the *T. minutum* complex with further reproductive compatibility tests and allozymic electrophoresis. A large number of crosses were performed between three lines of *T. californicum* and *T. platneri* to determine if cases of morphological intermediacy could be explained by a degree of reproductive compatibility. We also include the eastern North American *T. exiguum* Pinto and Platner in this study because of its similarity to the *T. minutum* complex and, in particular, to *T. californicum* (Pinto 1999). *Trichogramma exiguum* also is known from codling moth and other fruit tree tortricid pests. It has frequently been taken on these hosts at localities that also harbor *T. minutum* (Pinto et al., in prep.). The interspecific studies presented here are similar to those performed between members of the *T. minutum* complex (Pinto et al. 1991, 1992), and on the closely related species pairs *T. deion*/*T. pretiosum* (Pinto et al. 1993) and *T. deion*/*T. kaykai* (Pinto et al. 1997).

Materials and Methods

Cultures.—Cultures from three geographically distant populations of *T. californicum* were available for study. They were initially identified using morphology, and this was supported by their nearly identical ITS2 ribosomal transcript sequences (R. Stouthamer, pers. comm.). All are assignable to Form A of this species as defined by Pinto (1999). We utilized our standard laboratory cultures of *T. minutum* and *T. platneri* (Pinto et al. 1991), and two cultures of *T. exiguum*. The origin of all cultures used is given in Table 1. Cultures were collected and maintained as detailed in Pinto et al. (1991). Each originated from a single mated female that emerged from a field-collected host egg, and was maintained in the laboratory at 21–27° C on irradiated *Trichoplusia ni* (Hübner) eggs. Slide-mounted vouchers of all cultures studied are on deposit in the collection of the University of California, Riverside, Department of Entomology Research Museum, and are labelled with the voucher code RB1 and numbers UCRC ENT 43850–43984.

Crosses.—The three cultures of *T. californicum* were crossed with each other, with standard cultures of *T. platneri* (PRV1) and *T. minutum* (MCVA) that were used in previous studies (Pinto et al. 1992, 1993), and with the Hendersonville (EXHN) culture of *T. exiguum* for a total of ten crosses. The PRV1 and MCVA standard cultures have each been shown to be reproductively compatible with numerous other conspecifics (Pinto et al. 1992, in prep.). Procedures used for crossing experiments are detailed in Pinto et al. (1991). A single cross between two cultures consisted of an equal number of heterogamic (males from the other culture) and homogamic (males from the same culture) replicates. Each replicate consisted of a single virgin male and a single virgin female in a 29.6 cc (8 dram) glass vial with many (40 or more) host eggs. The male and female progeny from each replicate were counted, and the mean sex ratio (msr) for the cross calculated as the percentage of female progeny. The number of heterogamic and homogamic replicates of each cross were designed to number from 12 to 20 each, but fewer were performed in some cases due to extremely poor viability of certain *T. californicum* cultures. A total of 296 pairs of *T. californicum* and *T. platneri* were crossed to increase the chance of detecting rare hybridization. This included an expanded number of heterogamic crosses conducted in both directions between PRV1 × CAAD and PRV1 × CASB, and 39 between PRV1 × CAYK (Table 2). For statistical analyses, however, only the first 20 replicates were compared with the homogamic replicates. For each cross, an additional 10 virgin females were placed individually into separate vials with host eggs but without males to confirm that cultures were arrhenotokous.

Reproductive compatibility of a cross in each direction is expressed as a percentage: 100% × msr (heterogamic combination)/msr (homogamic combination) (Fig. 1). In arrhenotokous *Trichogramma,* females hatch only from fertilized eggs, while males hatch from unfertilized eggs. The absence of female progeny indicates complete incompatibility. Relative degrees of compatibility were measured using the non-parametric Mann-Whitney U test to compare the mean sex ratio of the heterogamic crosses with that of the homogamic crosses (Sorati et al. 1996).

Electrophoresis.—A total of 14 enzyme systems were examined in the three cultures of *T. californicum,* the two reference cultures of *T. minutum* and *T. platneri,* and one of the two cultures of *T. exiguum* (Table 3). The enzyme systems, their Enzyme Commission numbers, and the abbreviations representing them in this paper are: aconitase (4.2.1.3) *Acon,* acid phosphatase (3.1.3.2) *Acp-2,* esterase (3.1.1.1) *Est-1,* fumarase (4.2.1.2) *Fum,* glyceraldehyde-3-phosphate dehydrogenase (1.2.1.12) *Gapd,* α-glycerol-phosphate dehydrogenase (1.1.1.8) α*Gpd-1* and α*Gpd-2,* glucose-phosphate isomerase (5.3.1.9) *Pgi,* glucose-6-phosphate dehydrogenase (1.1.1.49) *G6pd, B*-hydroxybutyrate dehydrogenase (1.1.1.30) *Hbdh,* hexokinase (2.7.1.1) *Hk,* isocitrate dehydrogenase (1.1.1.42) *Idh,* malate dehydrogenase (1.1.1.37) *Mdh-2,* malic enzyme (1.1.1.40) *Me,* phosphoglucomutase (2.7.5.1) *Pgm.* The same culture (EXHN) of *T. exiguum* used for crosses could not be used for all loci because of a shortage of available specimens. The scores of another *T. exiguum* culture from Selma, AL (EXSL) were substituted for *Est-1, Me,* and *Pgi.*

The electrophoretic analysis followed procedures reported in Pinto et al. (1992), originally detailed in Kazmer (1991). Whole females, four from each culture per run, were individually analyzed at each locus by isoelectric focusing in one or two layers of cellulose acetate membranes using a single blend of carrier ampholytes (8% pH 4–6.5 and 2% pH 3–10 Pharmalytes), and an effective gel length of 4.5 cm.

BIOSYS-1 (Swofford and Selander 1989, release 1.7) was used to analyze the data. Nei's (1972) genetic distances (*D*) were calculated with individual allozyme profiles as input. All specimens were homozygous at the loci examined, probably due to the fact that each culture was established from a single mated female and because of the large number of generations that each culture had undergone prior to study (Table 1).

Results

Crosses.—Results of the crossing studies are summarized in Fig. 1 and Table 2. Of the three crosses conducted among cultures of *T. californicum,* only that between

Table 2. Results of *Trichogramma* crosses conducted in this study.[1]

Heterogamic Crosses	No. Replicates	msr	Homogamic Crosses	No. Replicates	msr	*p*-value
CASB♂ CAAD♀	16	0.49	CAAD	16	0.62	0.2333
CAAD♂ CASB♀	8	0.67	CASB	8	0.43	0.0306*
CASB♂ CAYK♀	20	0	CAYK	20	0.59	—
CAYK♂ CASB♀	17	0	CASB	17	0.48	—
CAAD♂ CAYK♀	20	0	CAYK	20	0.68	—
CAYK♂ CAAD♀	20	0.19	CAAD	20	0.59	0.0001*
CASB♂ PRV1♀	20 (50)	0	PRV1	20	0.66	—
PRV1♂ CASB♀	20 (80)	0	CASB	20	0.66	—
CAAD♂ PRV1♀	20 (49)	0	PRV1	20	0.66	—
PRV1♂ CAAD♀	20 (78)	0	CAAD	20	0.33	—
CAYK♂ PRV1♀	19	0	PRV1	19	0.66	—
PRV1♂ CAYK♀	20	0	CAYK	20	0.59	—
CASB♂ MCVA♀	12	0	MCVA	12	0.65	—
MCVA♂ CASB♀	12	0	CASB	12	0.29	—
CAYK♂ MCVA♀	12	0	MCVA	12	0.70	—
MCVA♂ CAYK♀	12	0	CAYK	12	0.34	—
CASB♂ EXHN♀	12	0	EXHN	12	0.53	—
EXHN♂ CASB♀	12	0	CASB	12	0.49	—
CAYK♂ EXHN♀	12	0	EXHN	12	0.72	—
EXHN♂ CAYK♀	12	0	CAYK	12	0.48	—

[1] Numbers in parentheses indicate the actual number of replicates conducted in expanded crosses of *T. californicum* and *T. platneri,* with the accompanying number indicating the number of replicates used in the Mann-Whitney test. The *p*-value is that of the Mann-Whitney test, with those values significant at an α value of 0.05 indicated by an asterisk (*). Mean sex ratio (msr) is the average proportion of females in all replicates. See Table 1 for explanation of culture acronyms.

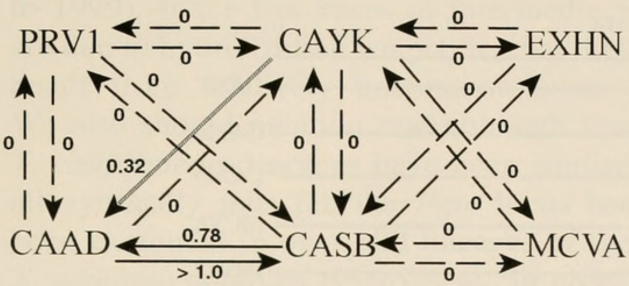

Fig. 1. Crossing results among cultures of *Trichogramma*. Solid arrows represent complete reproductive compatibility according to a Mann-Whitney U test. Dashed arrows represent complete incompatibility. Hatched arrows represent partial compatibility. Numbers along arrows represent level of reproductive compatibility for heterogamic cross relative to appropriate homogamic check cross. Arrows point to the parental female. See Table 1 for explanation of acronyms.

CAAD and CASB showed full bidirectional compatibility. Although the Mann-Whitney test indicated a significant difference between the homogamic check and the cross involving CAAD males and CASB females, the direction of significance was that of more females in the heterogamic cross. This difference is likely due to the generally poor viability of *T. californicum*. There was incomplete unidirectional compatibility between CAYK males and CAAD females as indicated by the Mann-Whitney test. Only 10 of the 20 CAAD females produced female progeny in this heterogamic cross, whereas 19 of the 20 CAAD females produced daughters in the homogamic check. The cross between CASB and CAYK was incompatible in both directions.

Table 4. Nei genetic distances (*D*) among cultures of *Trichogramma* examined electrophoretically.

Culture	CASB	CAYK	MCVA	PRV1	EXHN/ EXSL
CAAD	0.470	0.693	0.693	0.693	0.981
CASB	—	0.470	0.693	0.470	0.981
CAYK		—	0.981	0.693	1.386
MCVA			—	0.470	0.693
PRV1				—	0.625

None of the interspecific crosses yielded female progeny, including the relatively large number of replications between *T. californicum* and *T. platneri*. This is consistent with an earlier cross between CAAD and a collection of *T. platneri* from Cow Head Lake (Modoc Co.), CA (Pinto 1999). It is not known whether interspecific matings occurred or not.

Electrophoresis.—Of the 14 loci examined, eight showed variation (Table 3). No usable results were obtained with *Acon, Fum, Gapd, Hbdh, Hk,* or *Mdh*. The *T. californicum* cultures differed from those of the other species at three loci, *Acp-2, G6pd* and *Pgm,* although each of these loci was variable among cultures of *T. californicum* as well. Nei's genetic distance was calculated for the cultures analyzed in this study (Table 4), and the distances plotted in a phenogram using UPGMA clustering (Fig. 2). The distances and phenogram are intended as numerical and visual representations of the results, and should not be in-

Table 3. Allelic designation of 8 loci for the cultures examined.[1]

Species	Culture	*Acp-2*	α*Gpd-1*	*Est-1*	*G6pd*	*Idh*	*Me*	*Pgi*	*Pgm*
T. californicum	CAAD	D	A	C	C	B	A	A	B
	CASB	C	A	B	C	B	A	A	D
	CAYK	C	A	B	D	A	A	A	B
T. minutum	MCVA	A	A	null	B	B	A	A	C
T. platneri	PRV1	B	A	B	B	B	A	A	A
T. exiguum	EXHN	E	B	—	A	B	—	—	E
	EXSL	—	—	A	—	—	A	A	—

[1] Relative distances traveled for electromorphs at each locus expressed as a ratio of distance between edge of cathode and homomeric band to entire gel length in alphabetical order of allelic designation: *Acp-2* (0.42, 0.51, 0.58, 0.60, 0.62), α*Gpd-1* (0.22, 0.33), *Est-1* (0.07, 0.11, 0.20), *G6pd* (0.09, 0.22, 0.42, 0.47), *Idh* (0.58, 0.64), *Me* (0.49), *Pgi* (0.51), *Pgm* (0.11, 0.27, 0.29, 0.33, 0.58).

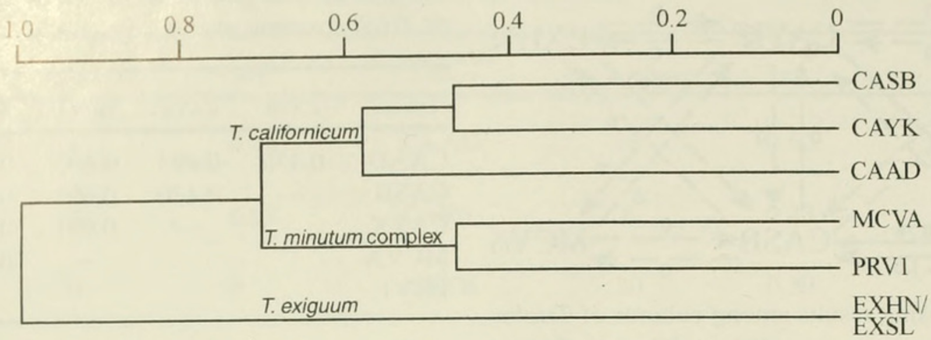

Fig. 2. Phenogram (UPGMA clustering) of Nei's genetic distances among cultures of *Trichogramma* examined electrophoretically. See Table 1 for explanation of acronyms.

terpreted phylogenetically. The phenogram does show the three cultures of *T. californicum* to be nearer to each other than to the cultures of the other species, but it should be noted that CASB is as near to PRV1 in the distance matrix as it is to either of the other homospecific cultures (Table 4).

The results for *Pgm* were compared with those reported for several cultures of the *T. minutum* complex (Pinto et al. 1992) using the results for MCVA and PRV1 as standards of comparison. Other than crossing unknowns with standard cultures, this locus provides the only means of separating most collections of *T. platneri* and *T. minutum* from one another. The electromorphs for *T. californicum* were different from all known *Pgm* electromorphs in the *T. minutum* complex and in *T. exiguum* (Table 3). The data for *Acp-2* and *G6pd* could not be compared with information from the prior study, but they are tentatively assumed to be diagnostic loci for *T. californicum.*

Discussion

The crossing and electrophoretic results provide no basis for explaining the morphological intermediacy previously found between *T. californicum* and *T. platneri.* Every one of the almost 300 reproductive pairings between the two species was negative. Also, the two species have distinct allozymic profiles, as well as different ITS2 sequences (van Kan et al. 1996). Speculation on causes other than gene flow for this intermediacy is premature and the basis of reproductive incompatibility between the species remains unknown.

The failure to detect reproductive or molecular intermediacy between *T. californicum* and *T. platneri* in this study, of course, could be explained by limited sampling. Although we failed to find hybridization in the numerous heterospecific pairings, the individuals of *T. californicum* and *T. platneri* crossed represent few isofemale lines (three and two, respectively). More extensive sampling would be useful. Utilization of isofemale lines unfortunately is necessary in *Trichogramma* studies to insure that all replicates are homospecific (Pinto et al. 1992). The preferable approach of utilizing unrelated individuals for replications is precluded by the presence of heterospecifics at most collection sites (potentially in the same host egg) coupled with problems of identification. In *Trichogramma,* females can not be identified unless associated with males, and slide-mounted material is required for male identification. Genetic variation could be better estimated by utilizing a larger number of isofemale lines, but this alternative is not straightforward in uncommon species such as *T. californicum.*

The limitations of our sampling procedure notwithstanding, it should be noted that the species of *Trichogramma* studied reproductively thus far indicate that the magnitude of morphological difference separating *T. californicum* and the *T. minutum* complex, as minor as it is, does correlate well with reproductive incompatibility (Pinto 1999). If the few cases of intermediacy are due to hybridization we predict that they result from relatively uncommon events. We also should mention that although few *T. californicum* lineages have been studied allozymically thus far, the *Pgm* locus has been examined in over 100 lineages of the *T. minutum* complex (Pinto et al., in prep). In all cases, the alleles at this locus in both species of the complex are distinct from those reported here for *T. californicum.*

Both crossing and electrophoretic results indicate a high degree of intraspecific variation in *T. californicum* as compared to that found in certain other species of *Trichogramma* (Pinto et al. 1992, 1993). The greatest Nei's distance found among the three cultures of *T. californicum* (0.693) is far greater than distances reported in all species of *Trichogramma* analyzed to date (Pinto et al. 1992, 1993), including that found in *T. minutum* (0.486). In fact, the least distance between cultures of *T. californicum* (0.470) is greater than the greatest distance between cultures of all other species previously examined except *T. minutum.* These allozymic differences are not predicted by the bidirectional reproductive compatibility between the Adin and San Bernardino cultures of *T. californicum,* or the partial compatibility between the Adin and Yakima cultures. They also are not predicted by known morphological or ITS2 sequence similarity. ITS2 sequences are useful in separating all morphologically distinctive species examined thus far (Stouthamer et al. 1999), but are nearly identical in the three *T. californicum* cultures (Stouthamer, pers. com.). The degree of reproductive disjunction within *T. californicum,* however, is not completely without precedence in *Trichogramma.* Pinto et al. (1991) found similar levels of incompatibility among cultures of *T. deion.* They also reported one-way incompatibility and reduced two-way compatibility in cultures currently assigned to *T. minutum.*

Considerable morphological variation within *T. californicum* already has been noted and the species was divided into two forms, A and B, on this basis (Pinto 1999). The two are broadly sympatric in California but Form B is known only from museum specimens. Within Form A, populations from Baja California and western Texas also have been identified as morphological outliers (Pinto 1999). Crossing and molecular studies are needed to determine their relationship to the cultures investigated here. Clearly, *Trichogramma californicum* remains a highly variable and poorly understood entity. It may constitute a unit of variation similar to or greater than the *T. minutum* complex where the two component species also are morphologically similar but reproductively incompatible. As in *T. californicum,* these reproductive units (*T. minutum* and *T. platneri*) are electrophoretically distinct (Pinto et al. 1992) but do not differ in ITS2 sequence (Stouthamer et al. 2000). However, species status for *T. minutum* and *T. platneri* also has been supported by clear-cut reproductive incompatibility and distinct geographic distributions. The geography of reproductive incompatibility and allozymic variation in *T. californicum* is unknown and any proposal to subdivide the species without more extensive sampling is premature.

Acknowledgments

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Literature Cited

Falcon, L. A. and J. Huber. 1991. Biological control of the codling moth, pp. 355–369. *In* van der Geest, L. P. S., and H. H. Evenhuis, eds. Tortricid Pests. Their Biology, Natural Enemies and Control. Elsevier: Amsterdam.

Kazmer, D. J. 1991. Isoelectric focusing procedures for the analysis of allozymic variation in minute arthropods. Annals of the Entomological Society of America 84: 332–339.

Mills, N. J. and K. P. Carl. 1991. Parasitoids and predators, pp. 235–252. *In* van der Geest, L. P. S., and H. H. Evenhuis, eds. Tortricid Pests. Their Biology, Natural Enemies and Control. Elsevier: Amsterdam.

Nagaraja, H. and S. Nagarkatti. 1973. A key to some New World species of *Trichogramma* (Hymenoptera: Trichogrammatidae), with descriptions of four new species. Proceedings of the Entomological Society of Washington 75(3): 288–297.

Nei, M. 1972. Genetic distance between populations. American Naturalist 106: 283–292.

Pinto, J. D. 1999 ["1998"]. Systematics of the North American species of *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae). Memoirs of the Entomological Society of Washington No. 22, 287 pp.

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

Pinto, J. D., G. R. Platner, and C. A. Sassaman. 1993. Electrophoretic study of two closely related species of North American *Trichogramma*: *T. pretiosum* and *T. deion* (Hymenoptera: Trichogrammatidae). Annals of the Entomological Society of America 86: 702–709.

Pinto, J. D., R. Stouthamer, and G. R. Platner. 1997. A new cryptic species of *Trichogramma* (Hymenoptera: Trichogrammatidae) from the Mojave Desert of California as determined by morphological, reproductive and molecular data. Proceedings of the Entomological Society of Washington 99: 238–247.

Pinto, J. D., R. Stouthamer, G. R. Platner, and E. R. Oatman. 1991. Variation in reproductive compatibility in *Trichogramma* and its taxonomic significance (Hymenoptera: Trichogrammatidae). Annals of the Entomological Society of America 84: 37–46.

Smith, S. M. 1996. Biological control with *Trichogramma*: Advances, successes, and potential of their use. Annual Review of Entomology 41: 375–406.

Sorati, M., M. Newman, and A. A. Hoffmann. 1996. Inbreeding and incompatibility in *Trichogramma* nr. *brassicae*: Evidence and implications for quality control. Entomologia Experimentalis et Applicata 78: 283–290.

Stouthamer, R., J. Hu, F. J. P. M. van Kan, G. R. Platner, and J. D. Pinto. 1999. The utility of internally transcribed spacer 2 DNA sequences of the nuclear ribosomal gene for distinguishing sibling species of *Trichogramma*. BioControl 43: 421–440.

Stouthamer, R., Y. Gai, A. B. Koopmanschap, G. R. Platner, and J. D. Pinto. 2000. ITS-2 sequences do not differ for the closely related species *Trichogramma minutum* and *T. planteri*. Entomologia Experimentalis et Applicata 95: 105–111.

Swofford, D. L. and R. B. Selander. 1989. BIOSYS-1. A computer program for the analysis of allelic variation in population genetics and biochemical systematics. Release 1.7. Urbana, IL (Software).

van Kan, F. J. P. M., I. M. M. S. Silva, M. Schilthuizen, J. D. Pinto, and R. Stouthamer. 1996. Use of DNA-based methods for the identification of minute wasps of the genus *Trichogramma*. Proceedings of the section Experimental and Applied Entomology of the Netherlands Entomological Society (N.E.V.) 7: 233–237.