OCCURRENCE AND MOLECULAR IDENTIFICATION OF THE DUTCH *TRICHOGRAMMA* SPECIES

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Summary
Little is known about distribution in the Netherlands of the minute, and difficult to identify parasitoid wasps of the genus *Trichogramma*. However, recently it has become possible to identify *Trichogramma* using molecular methods: i.e. size and sequence of the internally transcribed spacer 2 of the rDNA. The aim of this study was to collect *Trichogramma* species around Wageningen and identify them molecularly. *Trichogramma* were caught using trapcards containing eggs of *Mamestra brassicae* which were placed in different habitats. Wasps emerging from these eggs were used to establish isofemale lines. The identity of those lines was determined using the size and restriction patterns of the ITS2 region. Four species were found, *T. brassicae* and *T. evanescens* on cabbage, *T. cacoeciae* in arboreal habitats like forests and gardens and *T. semblidis* near the water and also on cabbage.

INTRODUCTION

Little is known about the distribution and occurrence of *Trichogramma* species (Hymenoptera; Trichogrammatidae) in the Netherlands. Pintureau (1987) names six species occurring in the Netherlands: *T. evanescens* (Noldus en van Lenteren, 1983), *T. brassicae* (Pak en van Heiningen, 1985), *T. semblidis* (Pak en van Heiningen, 1985), *T. cacoeciae* (capture by P. Grijpma, 1983), *T. embryophagum* (capture by P. Grijpma, 1984), *T. dendrolimi* (capture by G. Pak, 1983).

Much confusion exists about the taxonomy of *Trichogramma* (Pinto and Stouthamer, 1994). This is because the wasps are minute and have only a few characters that can be used for their identification. The best characters for their taxonomy are the male genitalia. All the species mentioned above are identified using morphological characters, however in several cases specifics on their identification are lacking.

Recently it has been shown that *Trichogramma* can also be identified using DNA characters. Species differ in the sequence and size of the ITS2 (internally transcribed spacer 2 of the ribosomal cistron). Using polymerase chain reactions (PCR) and in some cases followed by cutting the PCR-product with different restriction enzymes individual wasps can be identified. The ITS2 based identification is particularly useful for the identification of species that are thelytokous, i.e. all individuals are females that are capable of producing daughters without mating. In many cases thelytoky is caused by an infection with the bacterium *Wolbachia* (Stouthamer, 1997).

The aim of this study is to collect *Trichogramma* individuals around Wageningen, culture them in the laboratory, identify them using molecular techniques and determine if *Wolbachia* is present in thelytokous cultures.

MATERIALS AND METHODS

In the period of 26 May to 8 October 1999 several different locations near Wageningen were frequently sampled using trapcards. These trapcards consisted of a metal hairclip to which a paper strip is attached using transparent tape. At the end of this strip, a piece of filterpaper is attached with woodglue, containing 10-20 eggs of the noctuid moth, *Mamestra brassicae*. The eggs originated from our *Mamestra brassicae* rearing. In the rearing female moths attach their eggs to filter paper.

The cards were retrieved after 1-2 days, and inspected regularly in the laboratory to determine if the eggs had been parasitized by *Trichogramma* females in the field.

The cards were hung at the collecting site, usually in a row with 1 metre spacing, and the plant to which it was attached and the height were noted. When collecting them it was also noted from which cards the eggs were eaten by predators. While collecting the trapcards we also searched for naturally occurring eggs to take to the lab to determine if they are parasitized. We also collected parasitized clusters from cabbage fields.

The cards that still contained eggs were brought into the lab and put in separate glass vials. They were kept at 23 °C and a photoperiod of 14 hours. About 3 days after bringing them to the lab, the parasitized eggs turned black and each parasitized egg was isolated in a glass tube.

After emergence of the up to 3 *Trichogramma* from the egg, their sex was determined. If a male and a female emerged together, they were placed on eggs to start an isofemale line. If two females emerged together, they were separately put on eggs for 24 h. after which they were removed from the eggs and placed in a climate cabinet of 15 °C. This was done to allow the male offspring of this virgin female to emerge and subsequently mate with their mother so that an isofemale line could be initiated. Of each parasitized egg card, maximally four lines were kept for ITS2 analysis.

The most important collecting sites are: a vegetable garden where no insecticides were applied, cabbage fields, forelands along the river Rhine, deciduous forest, a fruit orchard , gardens and a ridge of oak trees surrounding a field that has been taken out of production 5 years earlier in the nature park "Planken Wambuis". Also see table 2.

For molecular identification five individuals of each line were ground up and their DNA was extracted using the Chelex method. After the DNA extraction a PCR reaction with ITS2-specific primers was carried out as described in Stouthamer et. al. (1999). The first step in identifying the *Trichogramma* specimen is to determine the size of the PCR product. Here considerable difference can be seen between some species. Additional characters can be gained when the ITS2 PCR product is cut with restriction enzymes. For some of the species reported from the Netherlands the ITS2 sequences are known and with that the restriction sites. On the basis of that information we chose the enzyme MSE1, because it can distinguish between all the species in a single cut. The restriction patterns of the Dutch species of *Trichogramma* are shown in table 1.

Table 1. Restriction sites for MSE1 for the expected species of which the ITS2 sequence is known

.Species	Size of ITS2 (bp.)*	MSE1 Restriction sites (bp)
T. brassicae	518	408 – 32 – 78
T. cacoeciae	576	198 – 378
T. evanescens	543	448 – 95
T. dendrolimi	518	440 – 78
T. semblidis	540	-

**. The sizes include the primer-binding-sites which are located just outside the ITS2. The actual size of only the ITS2 is the one given here minus 113 bp.*

The lines were also checked for *Wolbachia* infection by doing a PCR reaction using *Wolbachia* specific primers wsp (Zhou et. al., 1998) and ftsz (Holden et. al., 1993)

RESULTS

Of the six known species occuring in the Netherlands, four were found. These are *T. brassicae*, *T. cacoeciae*, *T. evanescens* and *T. semblidis*. *T. brassicae* was found in cabbage fields and in the vegetable garden. *T. semblidis* was found near a river as well as in a vegetable garden and in cabbage fields. *T. cacoeciae* was found in forest, fruit orchard and in gardens. *T. evanescens* was found in naturally occurring cluster in a cabbage field in Oosterhout (Gld.) (Coordinates: 184,600 x 432,050).

Table 2: parasitization percentages per habitat

Habitat	Location in Amersfoort coordinates	# cards	# parasitized	# predated	Percentage parasitization	Species	Plant
Vegetable garden	175,175 x 444,300	550	17	102	3,09%	*T. brassicae, T. semblidis*	Cabbage
Disease garden	173,150 x 442,875	300	1	122	0,33%	*T. cacoeciae*	Pine tree
Fruit orchard	173,050 x 442,950	140	1	55	0,71%	*T. cacoeciae*	Apple tree
Forest	178,200 x 447,875	255	3	130	1,18%	*T. cacoeciae*	Birch, Mountain Ash berry
Backyard		50	1	14	2,00 %	*T. cacoeciae*	Forsytia
Planken Wambuis	179,700 x 452,800	960	16	390	1,67 %	*T. cacoeciae*	Oak tree
Water edge	172,025 x 442,900	255	1	133	0,39%	*T. semblidis*	Reed
Other*		1264	0	454	0,00 %		
Total		3774	40	1400	1,06 %		

**. All habitats where no parasitization was found. These are: heath, arboretum, forelands, streetside and a flower edge.*

Overall parasitization was low, fig. 1 shows the parasitization percentages over time for the two sites that have been sampled continuously throughout the summer.

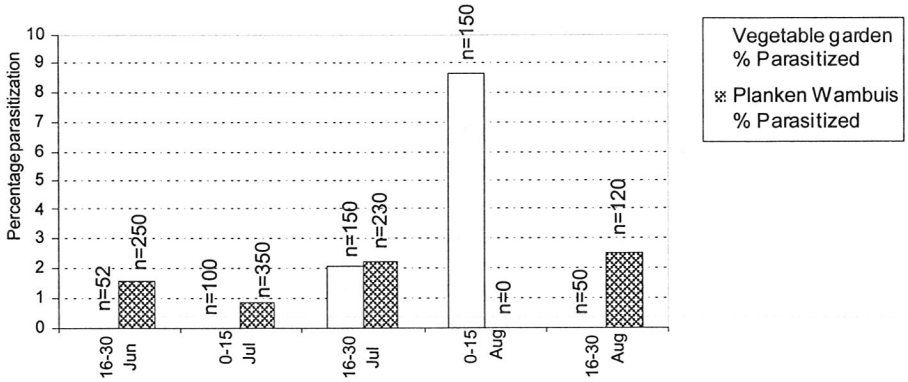

Figure 2. Parasitization percentages over time of the vegetable garden and Planken Wambuis.

A factor influencing the results is predation of the *M. brassicae* eggs. Predation percentages varied from 23 % in the vegetable garden to 52 % near the water. The average of all habitats is 37%.

Although thelytoky is found in *T. cacoeciae*, no *Wolbachia* was present according to the PCR results.

DISCUSSION

The overall parasitization percentage is quite low, despite the generally favourable weather during the summer of 1999. This could have different reasons. A first reason could be that population densities of *Trichogramma* are low in the Netherlands. It can also be the case that there are not enough suitable hosts, but that does not seem very likely because *Trichogramma* are not very host-specific, but more habitat-specific.

A second reason for the low parasitization percentage could be the method used to catch the *Trichogramma.* Maybe eggs attached to a paper card are more difficult to find than those attached to a leaf. Little is known about the searching behaviour of *Trichogramma* on a plant. Because of this the cards could be hung up at a suboptimal height or on the wrong side of leaves. Possibly the chemical cues emitted by our eggs were different from natural eggs because of the glue that was used or because a plant may send out chemical cues when eggs are attached to it. In addition we know very little about how *Trichogramma* distributes their searching effort over plants and how this may differ between species. Another reason could be that not all suitable habitats were checked or that *Mamestra brassicae* is not a suitable host for the species we did not find.

Predation could have had an influence on the parasitization. Eggs near predated eggs could be found unsuitable for parasitizing. Also it is possible that parasitized eggs have been predated. Nevertheless in natural situations predation also occurs, but it is possible that the eggs on the trapcard are easier to find for predators. In that case the observed predation percentage is higher than in the natural situation.

Some conclusions can be drawn about the habitat where the different species are found: *T. cacoeciae* is restricted to arboreal habitats. *T. brassicae* was found exclusively on cabbage, so it seems to be appropriately named. *T. semblidis* is known to be associated with water (pers. comm. Stouthamer), where it can even parasitize eggs of aquatic insects beneath the water surface. It is also found to parasitize Diptera (Pintureau et. al., 1990). *T. semblidis* was indeed found near the water but also in the vegetable garden and in cabbage fields, so it may have a wider range than was assumed. *T. evanescens* was found in a cabbage field, but only once so maybe it occurs in a different habitat more frequently.

No *Wolbachia* was found, so thelytoky must be caused by other factors (Stouthamer et. al., 1990).

The limited nature of this study must have resulted in an underestimation of the species present in the Netherlands. More studies are needed, using additional trapping methods and collections of wild host eggs. We expect the diversity of species to be larger because in the countries surrounding the Netherlands more species have been found.

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