Trichogramma and its relationship with *Wolbachia*:

Identification of *Trichogramma* species, phylogeny,
transfer and costs of *Wolbachia* symbionts

Raul P. de Almeida

Promotor:	Prof. Dr. J. C. van Lenteren
	Hoogleraar in de Entomologie
Co-promotor:	Dr. Ir. R. Stouthamer
	Associate Professor,
	University of California, Riverside, USA
Promotiecommisie:	Prof. Dr. M.S.M. Sosef, Wageningen Universiteit
	Dr. J.A.J. Breeuwer, Universiteit van Amsterdam
	Dr. ir. J. Helder, Wageningen Universiteit
	Dr. P. de Jong, Wageningen Universiteit

Raul P. de Almeida

Trichogramma and its relationship with *Wolbachia*:

Identification of *Trichogramma* species, phylogeny, transfer and costs of *Wolbachia* symbionts

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op woensdag 21 januari 2004
des namiddags te half twee uur in de Aula.

Almeida, R.P. de (2004)

Trichogramma and its relationship with *Wolbachia*: Identification of *Trichogramma* species, phylogeny, transfer and costs of *Wolbachia* symbionts. Ph.D. Thesis.

Wagenignen University, The Netherlands.
With references – with summaries in English, Portuguese and Dutch.

ISBN: 90-5808-942-8

Table of Contents

Preface

Having this very long journey of four years in Wageningen, The Netherlands, that culminated in my Ph.D. defence, was certainly an exciting and worthwhile experience. This achievement brought me a new vision of the science world, although my pathway in research has been dated from the 80's.

The accomplishment of my goal was only possible, first of all, to Embrapa (Brazilian Agricultural Research Corporation)/PRODETAB that financed my Ph.D. at Wageningen University. I also would like to express my gratitude to Dr. Napoleão E. de M. Beltrão, former General Director of Embrapa Cotton (the Research Unit I belong to) and Dr. Antônio F.S.L. Veiga (UFRPE) for their support at the beginning of my application to post-graduation.

My first contact with Wageningen University came in 1998 when I wrote an email to Prof.dr. Joop van Lenteren (my promoter) telling him of my interest in applying to a Ph.D. position. He immediately indicated Dr. Richard Stouthamer as a possible supervisor. From the first exchange of emails I realised that my research with *Trichogramma* would continue in The Netherlands. My special thanks to Richard (my co-promoter) for his support in providing guidance, suggestions and criticism during the development of my Ph.D. course. I would like to thank my promoter for his valuable comments on my research.

At the Laboratory of Entomology many people came through my life. Their support and friendship will always be remembered. I cannot forget to say thanks to Americo Ciociola Jr. who first conducted my family from the airport to our flat at Abraham Kuyperstraat 23, De Nude. It was for sure the coldest day in our lives. My first contact with molecular biology techniques initiated with Americo's help. Thanks also to Bertha and Patrick for their

technical support. Their advice and suggestions in the practical use of the molecular techniques were very useful. To learn a little bit about horizontal transmission, was doubtless a great experience shared with Ties. His support was crucial in the final step of the thesis (printing time). I cannot forget Gilsang who also helped a lot in this period. He was a very nice friend as well. To attend the lunch meetings with the *Trichogramma* and Ph.D. Groups was doubtless an unforgettable experience. We learned a lot in our scientific moments.

To my officemates Ellis, Américo, Gladys and Tibor, my thanks for your friendship during my four years of Ph.D. Very nice talks about research and life took place during that time.

Moments of relaxation took place during very nice meetings at Wageningen city with my friends from Brazil, the Netherlands and other countries. I will never forget those lovely moments that we shared in a warm atmosphere. To all my family in Brazil for their support, even being so far away from Europe, but always present with their kind words and thinkings. Also to my Embrapa friends who never failed when I needed help.

I want to immensely thank my wife Viviane, my daughter Olívia, my son Victor and my stepson Bruno who came to the Netherlands to support my profissional wish. To my wife, my sincere acknowledgements for her love and friendship. *My beloved children*…you always gave me happiness during my stay in Wangeningen.

Finally, my warm thanks to my mother Edna that came with my family to the Netherlands at the beginning of our journey to share her sweetness, care and optimistic way of being with us. To my father Bernardo (*In memory*), my warm thanks for his advice, when we could talk about life (*The journey will be always forward*).

Thanks God.

Chapter

1

General introduction

Raul P. de Almeida

Chapter 1

GENERAL INTRODUCTION

Identification of *Trichogramma* species

The genus *Trichogramma* consists of a large number of species of the family Trichogrammatidae and is studied world wide. This tiny parasitoid of ca. 0.5 mm in length has been used with success in the control of pests of arable crops, orchards, forests and stored products (Kot 1964). Although ca. 180 species are known (Pinto 1998) the number of species in biological control programs is limited to five species (Li 1994).

Correct species identification is the first step for a successful biological control program. Identification of *Trichogramma* individuals remains difficult, requires specialized skills and is time consuming (Pinto & Stouthamer 1994). Their identification is mainly based on the male genitalic morphological characters (Nagarkatti & Nagaraja 1968, 1971). Many important species share similar genitalic structures and this has forced workers to continue relying on less dependable characters that often are intraspecifically variable and subject to phenotypic plasticity (Pinto *et al*. 1989; Pinto and Stouthamer 1994). In South America, taxonomic studies are relatively rare (Zucchi & Monteiro 1997), although many biological control programs have been developed. This situation has been improved recently, resulting in ten new *Trichogramma* species identified in Brazil (Querino & Zucchi 2003a, b).

An especially severe limitation of systematic studies is that *Trichogramma* females are virtually unidentifiable unless associated with males. This presents a particular problem for completely parthenogenetic forms in which males are not present at all, i.e. non-revertible parthenogenetic forms (Stouthamer *et al*. 1990a). A solution for this came out with a modern *Trichogramma* identification system based on the DNA sequence of the internally transcribed spacer 2 (ITS2) proposed by Stouthamer *et al.* (1999b).

The most common mode of reproduction in Hymenoptera is arrhenotoky where haploid males arise from unfertilised eggs and diploid females arise from fertilised ones. Thelytoky is another mode of reproduction in which females arise from unfertilised eggs (Luck *et al*. 1992) and is caused by the bacteria of the genus *Wolbachia*. This endosymbiont that belongs to the α-Proteobacteria, Rickettsiaceae family, infects numerous arthropods (Werren 1997) and was first described by Hertig (1936) in *Culex pipiens. Wolbachia* can induce cytoplasmic incompatibility (Cl), thelytoky (T) or feminization (F) in their host or increase the fecundity (Breewer & Werren 1990; Stouthamer *et al*. 1990b; Rousset *et al*. 1992; Vavre *et al*. 1999; Dyson *et al*. 2002).

In *Trichogramma*, seventeen species are reported as infected with *Wolbachia* symbionts. Until now, only two types of reproduction modification have been found: (1) thelytoky and (2) increasing of the fecundity (Table 1), and not cytoplasmic incompatibility and feminization. Induction of thelytoky is the most common reproduction modification as a result of *Wolbachia* symbiosis in *Trichogramma* species. Females are capable of producing daughters without mating. This manipulation of the host's reproduction enhances the transmission of *Wolbachia* to future generations because the bacteria are passed on vertically only from mothers to daughters. Males are dead ends for cytoplasmically inherited bacteria, i.e. they do not pass them on to their offspring (Huigens *at al*. 2000). Daughters are produced when parthenogenesis-inducing *Wolbachia* suppresses spindle formation during the anaphase of the first mitotic division, thus restoring diploidy by fusion of the two mitotic nuclei (Stouthamer 1977). However, that *Wolbachia* infection can be cured by antibiotic and heating treatment (Stouthamer *et al*. 1990a). A case in which thelytoky in *Trichogramma* is not curable and *Wolbachia* is not present at all in the host has been published by Stouthamer *et al*. (1990b) and Almeida & Stouthamer (2003) (Table 1).

Horizontal transfer has been predicted on the basis of phylogenies of *Wolbachia* and its

trichogrammatid hosts (Schilthuizen & Stouthamer 1997). Despite the wide variety of effects that different *Wolbachia* strains have on their hosts and their presence in a broad range of arthropods, there is less than 3% difference in the 16S rDNA of several *Wolbachia* strains (O'Neill *et al*. 1992; Stouthamer *et al*. 1993). To improve phylogenetic resolution, other *Wolbachia* genes have been analysed such as 23S rDNA and 16S rDNA (Rousset *et al*. 1992), the bacterial cell-cycle *ftsZ* gene (Holden *et al*. 1993; Werren *et al*. 1995); and the spacer-2 region (SR2) including 5S rDNA (Fialho & Stevens 1997; van Meer *et al*. 1998).

The *wsp* gene allowed Zhou *et al*. (1998) to classify the *Wolbachia* in two supergroups (A and B) and 12 groups. The supergroups correspond to the groups A and B previously defined according to the *ftsZ* sequences (Werren *et al*. 1995) or to the subgroups I and II according to the 16S sequences (Stouthamer *et al.* 1993).

Research on *Wolbachia* genes resulted in two new candidates for detailed phylogenetic studies: the groE-homologous operon showed more variation than *ftsZ* (Masui *et al*. 1997) but no large sequence data set exists of this gene and the *wsp* gene, coding for an outer membrane protein of *Wolbachia* (Braig *et al*. 1998) exhibiting higher variation than the *ftsZ* gene and an extensive sequence data-base was available (Zhou *et al*. 1998).

Unisexual wasps, biological control and *Wolbachia*

Aeschlimann (1990) has considered that unisexual forms of biological control agents may be highly favourable for the success of a biological control program, i.e. full use is made of the reproductive potential of a natural enemy of a pest, as all offspring is female. According to Stouthamer (1993) potential advantages for wasps that carry parthenogenesis-inducing *Wolbachia* are: (a) there are no males produced, which results in a higher population growth rate of the natural enemy; (b) the costs of a mass rearing program is less

because

Table 1. Cases of thelytoky and increase of the fecundity in *Trichogramma*

Trichogramma species	Reproductive modification	Reference
1. *T. atopovirilia*	+	Ciociola Jr. *et al.* (2001)
2. *T. bourarachae*	*	Girin & Boulétreau (1995) Vavre *et al.* (1999)
3. *T. brevicapillum*	+	Stouthamer *et al.* (1990a, b) Werren *et al*. (1995) Almeida & Stouthamer (*Chapter 4*)
4. *T. cacoeciae*	-	Stouthamer *et al*. (1990b) Almeida & Stouthamer (2003)
5. *T. chilonis*	+	Stouthamer *et al.* (1990a, b) Chen *et al.* (1992) Schilthuizen & Stouthamer (1997)
6. *T. cordubensis*	+	Cabello & Vargas (1985) Stouthamer *et al.* (1990b, 1993) Silva & Stouthamer (1996, 1997)
7. *T. deion*	+	Stouthamer *et al.* (1990a, b, 1993) Schilthuizen & Stouthamer (1997) Zhou *et al*. (1998) Van Meer *et al*. (1999)
8. *T. embryophagum*	+	Birova (1970) Stouthamer *et al.* (1990b) Pintureau *et al*. (2000) Almeida & Stouthamer (*Chapter 4*)
9. *T. evanescens* (*rhenana*)	+	Pintureau (1987, 2000) Stouthamer *et al.* (1990b)
10. *T. kaykai*	+	Stouthamer & Kazmer (1994) Schilthuizen & Stouthamer (1997) Schilthuizen *et al.* (1998)
11. *T. oleae*	+	Voegelé & Pointel (1979) Stouthamer *et al.* (1990b) Rousset *et al*. (1992) Louis *et al*. (1993)
12. *T. nubilale*	+	Schilthuizen & Stouthamer (1997) Van Meer *et al.*(1999)
13. *T. pintoi*	+	Wang & Zhang (1990)
14. *T. platneri*	+	Stouthamer *et al.* (1990a, b) Schilthuizen & Stouthamer (1997)
15. *T. pretiosum*	+	Orphanides & Gonzáles (1970) Stouthamer *et al.* (1990a, b) Pintureau *et al*. (2000) Almeida *et al.* (2001)
16. *T. sibericum*	+	Schilthuizen & Stouthamer (1997) Van Meer *et al.*(1999)
17. *T. semblidis*	+	Pintureau *et al.*(2000)
18. *T. telengai*	+	Sorokina (1987)

(+) Thelytoky= *Wolbachia* infection; (-) Thelytoky without *Wolbachia* infection; (*) Increase in the fecundity= *Wolbachia* infection.

only females, the agents that kill the host insect, are produced; (c) at low wasp population densities thelytokous females do not have to waste time in searching mates, which results in faster colonization; (d) asexual parasitoids may be able to depress a host population to a low level. In addition, infection of sexually reproducing parasitoids with thelytoky inducing *Wolbachia* can be potentially advantageous in biological control situations, because thelytokous parasitoids may produce more daugthers than normal reproducing parasitoids.

When infected females co-occur with uninfected individuals in populations, infected ones generally produce fewer offspring than uninfected conspecifics (Stouthamer & Luck 1993), when their life-time offspring production is determined in the laboratory. This negative influence of the infection appears to be less in those species where all individuals are infected.

Horizontal transfer of *Wolbachia*

The first case of successful horizontal transmission was shown by Grenier *et al*. (1998) in which purified *Wolbachia* from an infected species (*T. pretiosum*) were transferred by microinjection into "*in vitro"* developed pupae of an uninfected species (*T. dendrolimi*). Huigens *et al*. (2000) reported a natural intraspecific horizontal transmission by showing that uninfected wasp larvae acquired *Wolbachia* when they shared a common food source with infected ones, although the process by which uninfected *Trichogramma* larvae acquired *Wolbachia* remained unclear.

Cost of *Wolbachia* infection for the parasitoid

Wolbachia infection may inflict a physiological cost on the host due to the presence of large numbers of bacteria inside host tissues (Stouthamer *et al*. 1999a). Cases of *Wolbachia*

infection causing negative effects on host fitness have been shown (Hoffmann *et al*. 1990; Stouthamer & Luck 1993; Stouthamer *et al*. 1994; Horjus & Stouthamer 1995). On the other hand, cases with positive effects (Girin & Bouletreau 1995; Wade & Chang 1995; Stolk & Stouthamer 1996; Hariri *et al*. 1998; Wavre *et al*. 1999) or neutral effects on the fitness of infected females (Stouthamer *et al*. 1994; Giordano *et al*. 1995; Hoffmann *et al*. 1996) have also been detected.

RESEARCH AIMS

The first step in a biological control programme is the selection of correct species or strains of the natural enemy. This thesis project started with collecting *Trichogramma* from South America, aiming at a correct identification method based on rDNA sequencing using the internal transcribed spacer 2 (ITS2). Because some *Trichogramma* species may carry *Wolbachia* and only produce females, molecular techniques are the unique and reliable tool in identifying a single thelytokous female wasp, whereas the use of morphological traits for *Trichogramma* identification can only be used for males. In addition, *Wolbachia* phylogeny was performed with the purpose of classifying groups in the *Wolbachia* symbiont based on *wsp* sequences. Also, possible causes of *Wolbachia* horizontal transmission in *Trichogramma* species were addressed. Considering the intracellular bacteria *Wolbachia* as mainly vertically transmitted from mother to daughter through the egg cytoplasm and assuming horizontal transmission as a rare event, intraspecific and interspecific horizontal transmission experiments were performed. Finally, costs of *Wolbachia* infection on infected and cured *Trichogramma atopovirilia* were studied.

OUTLINE OF THE THESIS

The genus *Trichogramma* is represented by a large number of species in the Trichogrammatidae family. Species identification difficulties are mainly related to their small size and the lack of clear morphological features among the species. The discovery of male morphological characteristics was a great improvement in taxonomic studies, although it did not solve the cases in which only females are produced. With the use of antibiotic and heating treatment many cases could be solved due to the return to the reproductive mode by killing the *Wolbachia* bacteria inside *Trichogramma*. The use of a molecular technique based on rDNA sequencing of the internal transcribed spacer 2 (ITS2) region is used for *Trichogramma cacoeciae* identification, a species in which thelytoky is not revertible and considered as a result of some nuclear genetic factor. The presence of this species and its origin in South America is discussed (*Chapter 2*). With the same technique, seventeen native/introduced South American *Trichogramma* species are identified. Restriction analyses are performed allowing the construction of a molecular key for species identification based on the size of the ITS2 PCR product. Thelytoky in *Trichogramma* caused by *Wolbachia* infection was recorded, although most studied species reproduce sexually (*Chapter 3*).

For the construction of *Wolbachia* phylogenetic trees, different *Wolbachia* genes have been used. However, low divergence was found between *Wolbachia* strains. The *Wolbachia* clade was divided into two supergroups (A and B) instead of a division in groups as previously proposed (Werren *et al*. 1995; Zhou *et al*. 1998). Later, three other supergroups were designed (C, D and E). All *Wolbachia* that infect *Trichogramma* and cause thelytoky belong to supergroup B. Here, *Wolbachia* DNA is successfully detected by using the *wsp*

primers. Phylogenetic studies of *Wolbachia* are performed and different groups are distinguished (*Chapter 4*).

Phylogenetic relationships between *Wolbachia* and *Trichogramma* have revealed a clear discordance in the *Wolbachia* and *Trichogramma* phylogenies. The results of intra- and interspecific horizontal transmission experiments are evaluated when infected and uninfected *Trichogramma* larvae shared the same host egg. Under these conditions the horizontal transmission studies are analysed and investigated if they are a natural event. The fact that *Wolbachia* bacteria might be more easily horizontally transmitted in some *Trichogramma* species than others is discussed. (C*hapter 5*).

For several forms of thelytokous *Trichogramma* species a negative influence of the infection on their fitness has been reported (e.g. fecunditiy, longevity, survival, dispersal and parasitism). Thelytokous wasps and their arrhenotokous counterparts are also thought to differ physiologically, biologically and ecologically. Where there is a choice of either using thelytokous or arrhenotokous parasitoids in biological control projects we need to have information on the influence of *Wolbachia* on the parasitoid's behaviour, ecology and life-history. Thus, behaviour studies using infected and uninfected *Trichogramma* wasps are carried out to help designing the best biological control strategy. This study evaluates the influence of *Wolbachia* in the wasp's behaviour. Walking activity, walking speed and the components of behaviour of infected and unifected parasitoids are analysed (*Chapter 6*).

In the summarizing discussion (Chapter 7) the results are discussed in relation to one and another, conclusions are given and implications of the obtained results are discussed.

REFERENCES

Aeschlimann. J.P. 1990. Simultaneous ocurrence of thelytoky and bisexuality in Hymenopteran species, and its implications for the biological control of pests. *Entomophaga* 35: 3-5.

Almeida, R.P. de, A.I. Ciociola Jr. & R. Stouthamer 2001. *Wolbachia*-induced parthenogenesis: the first report in a Brazilian *Trichogramma pretiosum* population. *Proc. Exper. Appl. Entomol.* 12: 41-44.

Almeida, R.P. de & R. Stouthamer 2003. Molecular identification of *Trichogramma cacoecieae* Marchal (Hymenoptera: Trichogrammatidae): A new record for Peru. *Neotrop. Entomol.* 32: 269-272.

Birova, H. 1970. A contribution to the knowledge of the reproduction of *Trichogramma embryophagum. Acta Entomol. Bohemoslov.* 67: 70-72.

Braig, H.R., W. Zhou, S. Dobson & S.L. O'Neill 1998. Cloning and characterization of a gene conding the major surface protein of the bacterial endosymbiont *Wolbachia*. *J. Bacteriol.* 180: 2373-2378.

Breeuwer, J.A.J. & J.H. Werren 1990. Microorganism assoaciated with chromosome destruction and reproductive isolation between two insect species. *Nature* 346: 558-560.

Cabello, T. & P. Vargas 1985. Temperature as a factor influencing the form of reproduction of *Trichogramma cordubensis*. *Z. Angew. Entomol.* 100: 434-441.

Chen, B., R. Kfir & C.N. Chen 1992. The thelytokous *Trichogramma chilonis* in Taiwan. *Entomol. Exp. Appl.* 65: 187-194.

Ciociola Jr., A.C., R.P. de Almeida, R.A. Zucchi & R. Stouthamer 2001. Detecção de *Wolbachia* em uma população telítoca de *Trichogramma atopovirilia* Oatman and

Platner (Hymenoptera: Trichogrammatidae) via PCR com o Primer Específico *wsp*. *Neotrop*. *Entomol*. 30: 489-491.

Dyson, E.A., M.K. Kamath & G.D.D. Hurst 2002. *Wolbachia* infection associated with all female broods in *Hypolimna bolina* (Lepidoptera: Nymphalidae): evidence for horizontal transmission of a butterfly male killer. *Heredity* 88: 166-171.

Fialho, R.F. & L. Stevens 1997. Molecular evidence for single *Wolbachia* infections among geografic strains of the flour beetle *Tribolium confusum. Proc*. *R*. *Soc*. *Lond*. *B* 264: 1065-1068.

Giordano R., S.L. O'Neill & H.M. Robertson 1995. *Wolbachia* infections and the expression of cytoplasmic incompatibility in *Drosophila sechellia* and *D. mauritiana*. *Genetics* 40: 1307-13 17.

Girin, C. & M. Boulétreau 1995. Microorganism-associated variation in host infestation efficiency in a parasitoid wasp *Trichogramma bourarachae* (Hymenoptera: Trichogrammatidae). *Experientia* 52: 398-401.

Grenier S., B. Pintureau, A. Heddi, F. Lassablière, C. lager, C. Louis & C. Khatchadourian 1998. Successful horizontal transfer of *Wolbachia* symbionts between *Trichogramma* wasps. *Proc*. *R*. *Soc*. *Lond*. *B* 265: 1441-1445.

Hariri A. R., J.H. Werren & G.S. Wilkinson 1998. Distribution and reproductive effects of *Wolbachia* in stalk-eyed flies (Diptera: Diopsidae). *Heredity* 81: 254-260.

Hertig, M. 1936. The Rickecttsia *Wolbachia pipientis* (gen. Et sp. n.) and associated inclusions of the mosquito *Culex pipiens. Parasitology* 28: 453-490.

Hoffmann, A.A. & D.J. Clancy & J. Ducan 1996. Naturally-ocurring *Wolbachia* infection in *Drosophila simulans* that does not cause cytoplasmic incompatibility in *Drosophila melanogaster*. *Heredity* 76: 1-8.

Hoffmann, A.A. M. Turelli & L.G. Harshman 1990. Factors affecting the distribution of

cytoplasmic incompatibility in *Drosophila simulans*. *Genetics* 126: 933-948.

Holden, P.R. , J.F. Brookfield & P. Jones 1993. Cloning and characterization of the *ftsZ* homologue from a bacterial symbiont of *Drosophila melanogaster. Mol. Gen. Genet.* 240: 213-220.

Horjus M. & R. Stouthamer 1995. Does infection with thelytoky-causing *Wolbachia* in pre-adult and adult life stages influence the adult fecundity of *Trichogramma deion* and *Muscidifurax uniraptor*? *Proc. Exp. Appl. Entomol.* 6: 35-40.

Huigens, M.E., R.F. Luck, R.H.G. Klaassen, M.F.P.M. Maas, M.J.T.N. Timmermans & R. Stouthamer 2000. Infectious parthenogenesis. *Nature* 405: 178-179.

Kot, J. 1964. Experiments in the biology and ecology of species of the genus *Trichogramma* Westw. and their use in plant protection. *Ekol. Pol. Ser. A* 12: 243-303.

Li, L., 1994. Worldwide use of *Trichogramma* for biological control on different crops: a survey. In: E. Wajnberg & S.A. Hassan (eds), *Biological Control with Egg Parasitoids*. CAB International, Wallingford, pp. 37-53.

Louis, C., B. Pintureau & L. Chapelle 1993. Recherches sur l`origine de l`unisexualité: la thermothérapie élimine à la fois rickettsies et parthénogenèse thélytoque chez un Trichogramme (Hym., Trichogrammatidae). *C. Rendus Acad. Sci. Série III Sci. Vie*. 316: 27-33.

Luck, R.F., R. Stoutharner & L. Nunney, 1992. Sex determination and sex ratio patterns in parasitic hymenoptera. In: Wrench, D.L. & M.A. Ebbert (eds.), *Evolution and diversity of sex ratio in haplodiploid insects and mites*. Chapman and Hall, Inc., New York, pp. 442-476.

Masui, S., T. Sasaki & H. Ishikawa 1977. GroE-Homologous operon of *Wolbachia*, an intracelluar symbiont of arthropods: a new approach for their phylogeny. *Zool. Sci.* 14: 701-706.

Meer, M.M.M. van, F.J.M.P. van Kan & R. Stouthamer 1998. Space 2 region and 5S rDNA variation of *Wolbachia* strains involved in cytoplasmic incompatibility or sex-ratio distortion in arthropods. *Lett. Appl. Microbiol.* 28: 17-22.

Meer, M.M.M. van, J. Witteveldt & R. Stouthamer 1999. Phylogeny of the arthopod endosymbiont *Wolbachia* based on the *wsp* gene. *Insect Mol. Biol.* 8: 399-408.

Nagarkatti, S. & H. Nagaraja 1971. Redescription of some known species of *Trichogramma,* showing the importance of male genitalia as a diagnostic character. *Bull. Entomol. Res.* 61: 13-31.

Nagarkatti, S. & H. Nagaraja 1968. Biosystematic studies on *Trichogramma* species I. Experimental hybridization between *Trichogramma australicum, T. evanescens* and *T. minutum. CIBC Tech. Bull.*10: 81-96.

O'Neill, S.L., R. Giordano, A.M.E. Colbert, T.L. Karr & H.M. Robertson 1992. 16S RNA phylogenetic analysis of bacterial endosymbionts associated with cytoplasmatic incompatibility in insects. *Proc. Natl. Acad. Sci.* 89: 2699-2702.

Orphanides, G.M. & D. Gonzales 1970. Identity of a uniparental race of *Trichogramma pretiosum*. *Ann. Entomol. Soc. Am.* 63: 1784-1786.

Pinto, J.D. 1998. Systematics of the North American species of *Trichogramma* (Hymenoptera: Trichogrammatidae). Mem. Entomol. Soc. Wash. Allen Press Inc., Washington D.C. n. 22. 287p.

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

Pinto, J.D., R.K. Velten, G.R. Platner & E.R. Oatman, 1989. Phenotypic plasticity and characters in *Trichogramma*. *Ann. Entomol. Soc. Amer.* 85: 413-422.

Pintureau, B. 1987. *Systématique évolutive du genre Trichogramma Westwood (Hymenoptera, Trichogrammatidae) en Europe* - These Doctorat d´État, Univ. Paris VII, France, 311 pp.

Pintureau, B., S. Chaudier, F. Lassablière, H. Charles & S. Grenier 2000. Addition of *wsp* sequences to the *Wolbachia* phylogeny tree and stability of the classification. *J. Mol. Evol*. 51: 374-377.

Querino, R.B. & R.A. Zucchi 2003a. New species of *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) associated with lepidopterous eggs in Brazil. *Zootaxa* 163: 1-10.

Querino, R.B. & R.A. Zucchi 2003b. Six new species of *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) from a Brazilian forest reserve. *Zootaxa* 134: 1-11.

Rousset, F., D. Bouchon, B. Pintureau, P. Juchault & M. Solignac 1992. *Wolbachia* endosymbionts responsible for various sexual alterations of sexuality in arthropods. *Proc. R. Soc. Lond. B* 250: 91-98.

Schilthuizen, M. & R. Stouthamer 1997. Horizontal transmission of parthenogenesis-inducing microbes in *Trichogramma* wasps. *Proc. R. Soc. Lond. B* 264: 361-366.

Schilthuizen, M., J. Honda & R. Stouthamer 1998. Parthenogenesis-Inducing *Wolbachia* in *Trichogramma kaykai* (Hymenoptera: Tnchogrammatidae) originates from a single infection. *Ann. Entomol. Soc. Am.* 91: 410-414.

Silva, I.M.M.S. & R. Stouthamer 1996. Can the parthenogenesis-*Wolbachia* lead to unusual courtship behaviour in *Trichogramma? Proc. Exper. & Appl. Entomol.* 7: 27-31.

Silva, I.M.M.S. & R. Stoutharner 1997. To mate or not to mate, can sex pheromones be used as a taxonomic tool in *Trichogramma* spp.? *Proc. Exper. & Appl. Entomol.* 8: 41-46.

Sorokina, A.P. 1987. Biological and morphological substantiation of the specific distinctness of *Trichogramma telengai* sp. n. *Entomol. Rev*. 66: 20-34.

Stolk C. & R. Stouthamer 1996. Influence of a cytoplasmic incompatibility-inducing *Wolbachia* on the fitness of the parasitoid wasp *Nasonia vitripennis*. *Proc. Exp. Appl. Entomol*. 7: 33-37.

Stouthamer, R. 1993. The use of sexual versus asexual wasps in biological control. *Entomophaga* 38: 3-6.

Stouthamer, R. 1997. *Wolbachia*-induced parthenogenesis. In: O'Neill, S., J.H. Werren & A.A. Hoffmann (eds.), *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction*, Oxford University Press, New York, pp. 102-124.

Stouthamer, R. & D.J. Kazmer 1994. Cytogenetics of microbe-associated panthenogenesis and its consequences for gene flow in *Trichogramma* wasps. *Heredity* 73: 317-327.

Stouthamer, R. & R.F. Luck 1993. Influence of microbe-associated parthenogenesis on the fecundity of *Trichogramma deion* and *T. pretiosum*. *Entomol. Exp. Appl*. 67: 183-192.

Stouthamer, R., J.A.J. Breeuwer & G.D.D. Hurst 1999a. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol*. 53: 71-102.

Stouthamer, R., J.A.J. Breeuwer, R.F. Luck & J.H. Werren 1993. Molecular identification of microorganisms associated with parthenogenesis. *Nature* 361: 66-68.

Stouthamer, R., J. Hu, F.J.P.M. van Kan, G.R. Platner & J.D. Pinto 1999b. 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., S. Luko & F. Mak 1994. Influence of parthenogenesis *Wolbachia* on host fitness. *Norw. J. Agric. Sci*. 16: 117-122.

Stouthamer, R., R.F. Luck & W.D. Hamilton 1990a. Antibiotics cause parthenogenetic *Trichogramma* to revert to sex. *Proc. Natl. Acad. Sci. USA* 87: 2424-2427.

Stouthamer, R., J.D. Pinto, G.R. Platner & R.F. Luck 1990b. Taxonomic status of thelytokous forms of *Trichogramma. Ann. Entomol. Soc. Am.* 83: 475-581.

Vavre, F., C. Girin & M. Boulétreau 1999. Phylogenetic status of a fecundity-enhancing *Wolbachia* that does not induce thelytoky in *Trichogramma. Insect Mol. Biol.* 8: 67-72.

Voegelé, J. & J. Pointel 1979. *Trichogramma oleae*, n.sp. espèce de *Trichogramma* evanescens Westwood [Hym., Trichogrammatidae]. *Ann. Soc. Entomol. Fr.* 15: 643-648.

Wade M.J. & N.W. Chang 1995. Increased male fertility in *Tribolium confusum* beetles after infection with the intracellular parasite, *Wolbachia*. *Nature* 373: 72-74.

Wang, F. & S. Zhang 1990. *Trichogramma pintoi* (Hym.: Trichogrammatidae): deuterotoky, laboratory multiplication and field releases. *Les Colloq. INRA* 56: 155-157.

Werren, J.H. 1997. Biology of *Wolbachia*. *Ann. Rev. Entomol.* 42: 587-609.

Werren, J.H., W. Zhang & L.R. Guo 1995. Evolution and phylogeny of *Wolbachia*-reproductive parasites of arthropods. *Proc. R. Soc. Lond. B* 261: 55-63.

Zhou, W., F. Rousset & S.L. O'Neill 1988. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. *Proc. R. Soc. Lond. B* 265: 509-515.

Zucchi, R.A. & R.C. Monteiro 1997. O gênero *Trichogramma* na América do Sul. In: Parra, J.R.P. & R.A.Zucchi (eds.), *Trichogramma e o controle biolólgico aplicado*, FEALQ, Piracicaba, pp. 41-66.

Chapter

2

Molecular identification of *Trichogramma cacoeciae* Marchal (Hymenoptera: Trichogrammatidae): A new record for Peru

Raul P. de Almeida & Richard Stouthamer

Chapter 2

MOLECULAR IDENTIFICATION OF *Trichogramma cacoeciae* MARCHAL (HYMENOPTERA: TRICHOGRAMMATIDAE): A NEW RECORD FOR PERU

Abstract

Use of male genitalia for species identification was a great improvement in *Trichogramma* systematics, but not all species could be easily identified based on genitalial structure. In some cases, the lack of males (a thelytokous status of species that carry the *Wolbachia* symbiont) made *Trichogramma* identification impossible. This problem was solved via antibiotic and heating treatments for elimination of the bacteria and allowing the production of males. The only *Trichogramma* species reported in which thelytoky is not induced by bacterial infection is *T. cacoeciae*, so here another means of species identification is needed. This species was identified based on the ITS2 (Internal transcribed spacer 2) sequence, a modern technique that has been proved useful in providing a reliable identification of *Trichogramma* species. Here we report the first occurrence of *T. cacoeciae* in Peru and we discuss its distribution in South America.

2.1. Introduction

Trichogrammatids represent a large group of minute parasitic wasps that attack eggs of various insects, many of which are of economic importance (Nagarkatti & Nagaraja 1977). Their small size and the lack of clear morphological differences of species within each genus have made taxonomic studies difficult. This has resulted in many nomenclatorial problems (Nagarkatti & Nagaraja 1977, Smith & Hubbes 1986). The lack of easy identification has led to the unnoticed replacement of intended species in mass-rearings or the use of inappropriate species in the first place (Stouthamer *et al*. 1999). In India, *Trichogramma australicum* Girault was erroneously referred to as *T. minutum* Riley or *T. evanescens* Westwood for nearly 50 years (Nagarkatti & Nagaraja 1968). The importance of properly matching the correct *Trichogramma* species or strain to the appropriate pest situation has been discussed extensively (e.g. Kot 1979 and Voronin & Grinberg 1981). Rosen (1978) reported several cases of misidentification of natural enemies in initially unsuccessful biological control projects.

Studies of male genitalia by Nagarkatti & Nagaraja (1968, 1971) were a breakthrough and ushered in a new era of *Trichogramma* taxonomy. Unfortunately morphological traits for identifying females with the same level of confidence as males are unavailable (Pinto & Stouthamer 1994). Positive identification of thelytokous species is therefore difficult unless males can be obtained by rearing the species at higher temperatures (Nagarkatti & Nagaraja 1977) or treating them with antibiotics (Stouthamer *et al*. 1990a). When males are present in a very low proportion as found by Aeschlimann (1990) or in the case of completely parthenogenetic forms in which males are not present at all, i.e. non-revertible parthenogenetic forms (Stouthamer *et al.* 1990b), species identification remains a problem. An example of such a species is *T. cacoeciae* Marchal. The production of males is very rare

in this species (Pinto 1998) and their use is indispensable in *Trichogramma* taxonomy using morphological features.

Many other methods have been proposed for species identification after male morphological characters were discovered (Pintureau & Babault 1980, Pintureau & Keita 1989, Kazmer 1991, Pinto *et al.* 1992, 1993, Pintureau, 1993). The DNA sequence of the internal transcribed spacer regions (ITS-1 and ITS-2) have been used at species and intraspecific levels in the many groups of organisms (Carbone & Kohn 1993, Hsiao *et al.* 1994, Buckler *et al.* 1997). The usefulness of the internally transcribed spacer 2 (ITS2) of the nuclear ribosomal gene complex was shown in the identification of closely related species of the *T. deion* complex (Stouthamer *et al*. 1999).

This study aimed at the identification of *T. cacoeciae* Marchal based on the DNA sequence of the ITS2 region by using females for extracting the DNA.

2.2. Material and Methods

Trichogramma *sample, DNA Extraction, PCR Amplification and Electrophoresis*

Trichogramma cacoeciae was collected in *Cydia pomonella* eggs in Peru apple orchards in 1997. For DNA extraction five wasps were ground in 100 µl 5% Chelex-100 and 4 µl proteinase K (20 mg/ml) and incubated for at least 4 hours at 56^{o}C, followed by 10 min. at 95^{o}C. The PCR was performed in a total volume of 50 µl using a Techne thermocycler, 5 µl DNA template, 5 µl PCR-buffer, 1 µl dNTP's (each in a 10 mM concentration), 1 µl forward and reverse primers (ITS2-forward: 5'-TGTGAACTGCAG GACACATG-3' located in the 5.8S region of the rDNA; ITS2-reverse: 5'-GTCTTGCC TGCTCTGCTCTGAG-3' located in the 28S region of the rDNA; 0.14 µl SuperTAQ

polymerase (Sphaero-Q 5 units/µl) and 36.86 µl of sterile distilled water. The cycling program was 3 min. at 94°C followed by 33 cycles of 40 seconds at 94°C, 45 second at 53°C and 45 seconds at 72°C with 5 min. at 72°C after the last cycle. The size of the PCR product was determined using standard agarose gel (Stouthamer *et al*. 1999 with modifications). The *Wolbachia*-infected species *T. atopovirilia* (strain Tato-01) was used as positive control and uninfected *T. galloi* (strain Tgal-02) as negative control; both were collected in Brazil.

Cloning, Sequencing and Alignments

Following electrophoresis, PCR products were purified with a QIAquick PCR purification kit (Qiagen®). After the purification the PCR products were tied up to a Pgem-T® Vector (Promega), 2 µl of the ligation mix was transformed in the heatshock cells of DH5-α *Escherichia coli* and plated in a LB agar medium containing Ampicilin, X-GAL and IPTG. The plates were stored overnight at 37°C. The next day, white colonies were picked up with a sterile toothpick from the plates and placed into tubes containing 3.0 ml of LB liquid medium and 3µl Ampicilin and put to grow up overnight in a shaker set to 250 rpm at 37°C. To confirm that the correct piece of DNA had been cloned, a PCR reaction with a template extracted from the bacterial culture was added to 100 µl 5% Chelex-100 and incubated for 15 min. at 60°C followed by 5 min. at 95°C. The PCR was performed in a final volume of 50 µl. If indeed it was cloned the correct part of DNA, 850 µl of the bacteria culture was added to 150 ml of 87% glycerol and stored at –80°C. The rest of the culture was used in a QIAprep Miniprep kit (Qiagen®) to purify the plasmid, which was used for the sequencing in an Applied Biosystems automatic sequencer. *T. cacoeciae* was aligned manually using the ESEE 3.Os sequence editor (Cabot 1995).

Thelytoky in T. cacoeciae

To confirm whether the parthenogenesis in *T. cacoeciae* was not caused by *Wolbachia* infection, specific primers for DNA amplification of the *wsp* region were used: *wsp*-Forward primer 5'-TGGTCCAATAAGTGATGAAGAAAC-3' and *wsp*-Reverse primer 5'-AAAAATTAAACGCTACTCCA-3'. The cycling program was 3 min. at 94 ^{o}C followed by 40 cycles of 1 min. at 94^{o}C, 1 min. at 50^{o}C and 1 min. at 72^{o}C with 5 min. at 72^{o}C after the last cycle.

2.3. Results and Discussion

The molecular technique used for identifying *T. cacoeciae*, based on ITS2 sequence, proved to be reliable and solved the limitation of the morphological identification, which allows species identification by using males features only. The complete ITS2 sequence of *T. cacoeciae* (460 bp) has been deposited in Genbank (Accession number: AY166700).

Trichogramma cacoeciae has, among others, been recorded in eggs of *Prais oleae* Bernard (an insect pest of olive) in Greece and in *C. pomonella* (insect pest of apple trees) in the former USSR (Nagarkatii & Nagaraja 1977). This species is geographically distributed in Europe and is also known in the Pacific Northwest in North America (Pinto 1998). It has been considered genetically thelytokous (Stouthamer *et al*. 1990b; Pintureau 1994; Pinto 1998). Males are rare and treatment with antibiotics or elevated temperatures does not induce arrhenotoky. The morphology of the few available males is similar to two other Northwestern American species, *T. platneri* Nagarkatti and *T. californicum* Nagaraja & Nagarkatti, and all three are known to parasitize codling moth (*C. pomonella*) in California. Six replicate cultures of *T. cacoeciae* strain 101 from France, each with 50-100 individuals for ca. 30 generations produced only five males (Pinto 1998). The thelytokous

status of *T. cacoeciae* without carrying *Wolbachia* bacteria was confirmed based on the lack of DNA amplification by using specific primers (*wsp*) (Fig. 1).

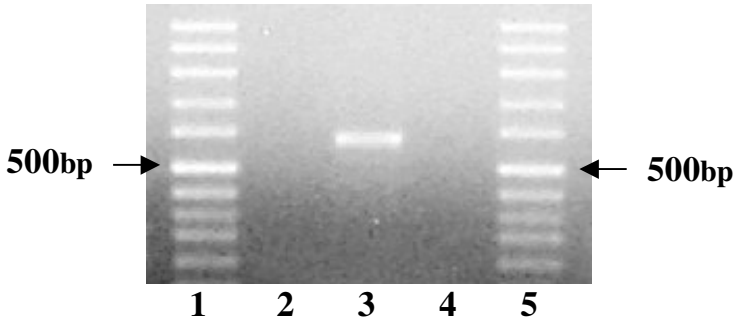

Figure 1. Gel electrophoresis of *wsp* PCR products. Lanes 1 and 5, low-molecular-weight markers; lane 2, *T. cacoeciae*; lane 3, *T. atopovirilia* (positive control); lane 4, *T. galloi* (negative control).

The identification of *T. cacoeciae* was confirmed via comparison with two other sequences from the GenBank with the following accession numbers: AF408653 and AF408654. This was the first report of *T. cacoeciae* collected in apple orchards on *C. pomonella* eggs in Peru. According to Dr. Mary Whu (personal communication) this species was collected in the Huarochiri Province at approximately 2500m altitude. Farms in that region of Peru cannot afford chemical application for insect pest control. This was the first time an egg parasitoid was collected in that area.

In Peru, exotic species of *Trichogramma* (*T. pintoi* Voegelé, *T. japonicum* Ashmead, *T. australicum* Girault, *T. atopovirilia* , *T. evanesces* Westwood, *T. dendrolimi* Matsumara and *T. embryophagum* Quednau) have been introduced since 1972. Thelytokous species have been detected in Peru (Whu & Valdivieso 1999) since 1976. However, the lack of males made the specific identification of *Trichogramma* impossible. Among the fifteen insect pests mentioned as host of *Trichogramma* spp., the commonly associated host to *T.*

cacoeciae, C. pomonella, was not found (Whu & Valdivieso 1999). The only case of *T. cacoeciae* introduction in Latin America reported in the literature was recorded in Argentina and Cuba by De Santis & Fidalgo (1994).

According to Dr. Bernard Pintureau (INRA/INSA, France), Dr. John Pinto (University of California, USA) Dr. Juan Carlos Monje (University of Hohenheim, Germany) and Dr. Sherif Hassan (BBA Institute, Germany) (personal communications) there is no information on the introduction of *T. cacoeciae* in Peru. Dr. Juan Carlos Monje (personal communication) reported, however, that *T. cacoeciae* was found in Chile and he assumes that this species may occur in fruit orchards in several South American countries. He also mentioned that it is possible that this species was accidentally introduced via the importation of apple varieties stock, and extensive collections are needed to clarify this situation. *T. cacoeciae* might also have been introduced under another species name as suggested by Dr. Roberto Antônio Zucchi and Dr. Ranyse B.Q. da Silva (ESALQ/USP, Brazil) (personal communication). Introduction of species incorrectly identified might be a real problem if the insect pest used as target for the biological control is not associated with the supposed *Trichogramma* species. According to Zucchi & Monteiro (1997) the preliminary knowledge of the distribution pattern of *Trichogramma* species on the American continent has also been complicated by species introduction without previous analysis of species already present. With new introductions for biocontrol the situation becomes even more uncertain, because species assumed to be indigenous for a particular region might in fact have been introduced.

Acknowledgments

The authors are thankful to Joop van Lenteren (Wageningen University, The Netherlands) for his valuable comments in the manuscript, and to Ranyse B.Q. da Silva (ESAQ-USP, Brazil) (*T. cacoeciae*), Americo I. Ciociola Junior (EPAMIG, Brazil) (*T. atopovirilia*) and Mary Whu (SENASA, Peru) (*T. galloi*) who have sent *Trichogramma* species. This research project was financially supported by Embrapa (PRODETAB).

References

Aeschlimann, J.P. 1990. Simultaneous occurrence of thelythoky and bisexuality in hymenopteros species, and its implications for the biological control of species. *Entomophaga* 35: 3-5.

Buckler IV, E.S., A. Ippolito & T.P. Holtsford 1997. The evolution of ribosomal DNA: divergent paralogues and phylogenetic implications. *Genetics* 145: 821-832.

Carbone, I. & L. Kohn 1993. Ribosomal DNA sequence divergence within transcribed spacer 1 of the Sclerotiniaceae. *Mycologia* 85: 15-427.

Cabot, E.L. 1995. "The Eyeball Sequence Editor (ESSE)," version 3.0s Dept. Biology, Univ. Rochester, Rochester, NY.

De Santis, L. & P. Fidalgo 1994. Catálogo de los himenópteros calcidoideos de América al sur de los Estados Unidos. Tercer Suplemento. *Aca. Nac. Agr. Vet. Bs. As.* 13. 154p.

Hsiao, C., N.J Chatterton., K.H. Asay & K.B. Jensen 1994. Phylogenetic relationships of 10 grass species: an assessment of phylogenetic utility of the internal transcribed spacer region in nuclear ribosomal DNA in monocots. *Genome* 37: 112-120.

Kazmer, D.J. 1991. Isoelectric focusing procedures for the analysis of allozymic variation

in minute arthropods. *Ann. Entomol. Soc. Am.* 84: 332-339.

Kot, J. 1979. Analysis of factors affecting the phytophage reduction by *Trichogramma* Westw. species. *Pol. Ecol. Stud.* 5: 5-59.

Nagarkatti, S. & H. Nagaraja 1968. Biosystematic studies on *Trichograrnrna* species. I. Experimental hybridization between *Trichogramma australicum* Girault, *T evanescens* Westw. and *T. minutum* Riley. *Tech. Bull. Corn. Inst. Biol. Contr.* 10: 81-96.

Nagarkatti, S. & H. Nagaraja 1971. Redescription of some known species of *Trichogramma* (Hym.. Trichogrammatidae), showing the importance of the male genitalia as a diagnostic character. *Bull. Entomol. Res.* 61:13-21.

Nagarkatti, S. & H. Nagaraja 1977. Bioystematics of *Trichogramma* and *Trichogrammatoidea* species. *Annu. Rev. Entomnol*. 22: 157-176.

Pinto, J.D. 1998. Systematics of the North American species of *Trichogramma* (Hymenoptera: Trichogrammatidae). Mem. Entomol. Soc. Wash. Allen Press Inc., Washington D.C. n. 22. 287p.

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

Pinto, J.D., G.R. Platner & C.A. Sassaman 1993. Electrophoretic study of two closely related species of North American *Trichogramma: T. pretiosum* and *T. deion* (Hymenoptera: Trichogrammatidae). *Ann. Entomol. Soc. Am.* 86: 702-709.

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

Pintureau, B. 1993. Enzymatic analysis of the genus *Trichogramma* (Hym.: Trichogrammatidae) in Europe. *Entomophaga* 38: 411-431.

Pintureau, B. 1994. Frequence and geographical distribution of thelytokous parthenogenesis in European species of *Trichogramma* (Hym.: Trichogrammatidae). *Norw. J. Agric. Sci. Suppl.* No. 16, p. 411.

Pintureau, B. & F.B. Keita 1989. Nouvelles données sur les estérases des Trichogrammes. *Biochem. Syst. Ecol.* 17: 603-608.

Pintureau, B. & M. Babault 1980. Comparaison des estérases chez 19 souches de *Trichogramma* (Hym., Trichogrammatidae) appartenant au groupe d'espèces *evanescens*. *Arch. Zool. Exp. Gén.* 121: 249-260.

Rosen, D. 1978. The importance of cryptic species and specific identifications as related to biological control. In: *Byosytematics in agriculture*. Beltsville Symposia in Agric. Res. 2, Osmun and Co., Allenheld, pp. 23-35.

Smith, S.M. & M. Hubbes. 1986. Strains of the egg parasitoid *Trichogramma minutum* Riley. I. Biochemical and biological characterization. *J. Appl. Entomol.* 101: 223-239

Stouthamer, R., R.F. Luck & W.D. Hamilton 1990a. Antibiotics cause parthenogenetic *Trichogramma* to revert to sex. *Proc. Natl. Acad. Sci. USA* 87: 2424-2427.

Stouthamer, R., J.D. Pinto, G.R. Platner & R.F. Luck 1990b. Taxonomic status of thelytokous forms of *Trichogramma* (Hymenoptera: Trichogrammatidae). *Ann. Entomol. Soc. Am.* 83: 475-581.

Stouthamer, R., J. Hu, F.J.P.M. van Kan, G.R. Platner & 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.

Voronin, K.E. & A.M. Grinberg, 1981. The current status and prospects of *Trichogramma* utilisation in the U.S.S.R.. In: J.R. Coulson (ed.), *Proceedings of the Joint American-*

Soviet Conference on the Use of Beneficail Organisms in the Control of Crop Pests. Entomolgical Society of America, College Park, Md., pp.49-51.

Whu, M. & L. Valdivieso 1999. Distribución y comportamiento de ocho especies de *Trichogramma* y *Trichogrammatoidea* (Hymenoptera: Trichogrammatidae) en el Perú. *Rev. Peru. Entomol.* 41: 61-68.

Zucchi, R.A. & R.C. Monteiro 1997. O gênero *Trichogramma* na América do Sul. In: Parra, J.R.P. & R.A.Zucchi (eds.), *Trichogramma e o controle biolólgico aplicado*, FEALQ, Piracicaba, pp. 41-66.

Chapter

3

ITS-2 sequences-based identification of *Trichogramma* species in South America

Raul P. de Almeida & Richard Stouthamer

Chapter 3

ITS-2 SEQUENCES-BASED IDENTIFICATION OF *Trichogramma* SPECIES IN SOUTH AMERICA

Abstract

ITS2 (Internal transcribed spacer 2) sequences have been used in systematic studies and proved to be useful in providing a reliable identification of *Trichogramma* species. This molecular technique was successfully used to distinguish among seventeen native/introduced *Trichogramma* species collected in South America. DNA sequences ranged in size from 379 to 632 bp. In eleven *T. pretiosum* lines *Wolbachia*-induced parthenogenesis was found for the first time. These thelytokous lines were collected in Peru (9), Colombia (1) and USA (1). A dichotomous key for species identification was built based on the size of the ITS2 PCR product and restriction analysis using three endonucleases (EcoRI, MseI and MaeI).

3.1. Introduction

Natural enemies of the family Trichogrammatidae are released worldwide against a number of lepidopterous pest on corn, rice, sugar-cane, cotton, vegetables and pines (Smith 1994; Li 1994; Grenier 1994). In the past, most successful biological control agents have been found through trial and error. Nowadays, there is an effort to predict the success of a *Trichogramma* strain before introductions are made (van Lenteren & Woets, 1988). Therefore, standard procedures to compare *Trichogramma* candidates for biological control have been proposed (Hassan 1994). According to Hassan (1995) before field releases are undertaken, a suitable *Trichogramma* strain of known qualities should be chosen. The effectiveness of *Trichogramma* in the field largely depends on its searching behaviour, host preference and tolerance to environmental conditions. However, correct identification of the *Trichogramma* species that is going to be tested, is a crucial step in any biological control program.

Despite the large improvement with the discovery of male genitalic morphology as species specific characters (Nagarkatti & Nagaraja 1968, 1971), the identification of these tiny insects remains difficult and requires specialized skills (Pinto & Stouthamer 1994). Their small size and lack of morphologically distinct characters has historically been a problem in taxonomic studies. Specimen must be prepared on slides for examination, a time consuming process that requires considerable experience (Platner *et al*. 1999). Unfortunately many important species share similar genitalic structures and this has forced workers to continue relying on less dependable characters that often are intraspecifically variable and subject to phenotypic plasticity (Pinto *et al*. 1989; Pinto & Stouthamer 1994).

To simplify the *Trichogramma* species identification, several methods have been proposed: (1) Allozymic analysis (Pintureau & Babault 1980, 1981, 1982; Pintureau &

Keita 1989; Kazmer 1991; Pinto *et al*. 1992, 1993; Pintureau 1993). Esterase electrophoresis has provided consistent results for differentiation of some *Trichogramma* species (Pinto *et al*. 1992, 1993; Silva *et al*. 1999). The limited variation at esterase loci only allows for the differentiation between a limited number of species (Richardson *et al*. 1986; Pinto & Stouthamer 1994). In addition the samples subjected to allozyme electrophoresis have to be kept at –70^{o}C to avoid the degeneration of the enzymes. (2) Reproductive compatibility tests (Nagarkatti & Nagaraja 1968; Pinto *et al*. 1991; Pintureau 1991) in which individuals of the unknown species are crossed with individuals of known species status. (3) The DNA sequence of ribosomal spacers (Landry *et al*. 1993; Orrego & Agudelo-Silva 1993; Sappal *et al*. 1995; Kan *et al*. 1996; Kan *et al*. 1997; Pinto *et al*. 1997). (4) RFLP's of complete mitochondrial genome has also been used for species differentiation (Vanlerberghe-Masutti 1994). Ribosomal DNA is present in all organisms and is composed of several regions (genes and spacers) that evolve at different rates (Hillis & Dixon 1991). The internal transcribed spacer regions (ITS-1 and ITS-2) have been used at species and intraspecific levels of many taxa for distinguishing the different forms (Carbone & Kohn 1993; Bowles & McManus 1993; Hsiao *et al*. 1994; Buckler IV *et al*. 1997). In insects these sequences have been often used for taxonomic purposes (Campbell *et al*. 1993; Hoy 1994; Kuperus & Chapto 1994; Vogler & DeSalle 1994; Stouthamer *et al*. 1999; Silva *et al*. 1999; Chang *et al*. 2001; Pinto *et al*. 2002).

Here we use the ITS2 sequences of *Trichogramma* species (native or introduced) from South America to develop an identification key. The advantage of this identification system over the morphology-based system is that non-specialists are able to quickly and cheaply identify individual specimens. In addition females can also be identified which is not possible in the morphologically based system (*Chapter 2*). Species found in South America are thelytokous, either because of infection with parthenogenesis inducing *Wolbachia* (*T.*

pretiosum and *T. atopovirilia*) (Grenier *et al*. 1998; Almeida *et al*. 2001; Ciociola Jr. *et al*. 2001a) or because of some nuclear genetic factor (*T. cacoeciae* Marchal) (Stouthamer *et al*. 1990; Almeida & Stouthamer 2003). Variation of ITS2 sequence within *Trichogramma* species is relatively small in comparison to the difference found between species and all morphologically distinct cryptic species are also distinguished by sequence differences (Stouthamer *et al*. 1999). ITS2 sequences within each species are very similar and there is no evidence for two or more gene families that differ substantially within the genome of a single individual, as has been found in other taxa (Vogler & DeSalle 1994).

3.2. Material and methods

Trichogramma *cultures*

Table 1 lists the *Trichogramma* species/lines studied here, including their origin, host insect, plant crop, collection date and status as native or introduced.

DNA Extraction, PCR Amplification and Electrophoresis

To extract DNA from the different species/lines we used five wasps that were homogenized in 100 µl 5% Chelex-100 and 4 µl proteinase K (20 mg/ml) and incubated for at least 4 hours at 56°C, followed by 10 min. at 95°C. The PCR was performed in a total volume of 50 µl using a Techne thermocycler, 5 µl DNA template, 5 µl 10x PCR-buffer, 1 µl dNTP's (each in a 10 mM concentration), 1 µl forward and reverse primers (ITS2-forward: 5'-TGTGAACTGCAG GACACATG-3' located in the 5.8S region of the rDNA; ITS2-reverse: 5'-GTCTTGCC TGCTCTGCTCTGAG-3'(Stouthamer *et al*. 1999) located in the 28S region of the rDNA; 0.14µl TAQ polymerase (5 units/µl) and 36.86 µl

Table 1. Trichogramma populations from South America

Species	Line Designiton	Origin	Host Insect	Plant Crop	Collection Date
T. acacioi Brun, Moraes & Soares, 1984	Taca-01	Botucatu-SP-Brazil	*Euselasia* sp.	-	-
T. atopovirilia Oatman & Platner, 1983	Tato-01	Sete Lagoas-MG-Brazil	-	Corn	-
	Tato-02	Colombia	-	-	-
T. brassicae Bezdenko, 1968	Tbra-01	Introduction from Germany	-	-	Dec., 1996
T. bruni Nagaraja, 1983	Tbru-01	Piracicaba-SP-Brazil	*Heliconius phyllis*	*Passiflora* sp.	
T. cacoeciae Marchal, 1927	Tcac-01	Huarochiri-Peru	*Cydia pomonela*	Apple	Oct., 1997
T. dendrolimi Matsumura, 1926	Tden-01	Introduction from Germany	-	-	Dec., 1996
T. esalqueanum Querino & Zucchi, 2003	Tesa-11	Piracicaba-SP-Brazil	*Helicornius erato phyllis*	*Passiflora* sp.	Oct., 1999
T. exiguum Pinto & Platner, 1983	Texi-01	Peru (different places)	*Diatraea saccharalis*	Sugar-cane, Corn	
			Helicoverpa zea	Corn	
			Erynis ello	Cassava	
			P. persimilis	Olive	
			Dione juno	*Passiflora sp.*	Mar., 2001*
	Texi-02	Colombia	-	-	-
T. fuentesi Torre, 1980	Tfue-01	Peru	*Diatraea saccharalis*	Sugar-cane, rice	
			Helicoverpa zea	Corn	
			Heliothis virescens, Anomis texana	Cotton	Dec., 1994*
T. galloi Zucchi, 1988	Tgal-01	Chiclayo-Peru	*Diatraea saccharalis*	Sugar-cane	Apr., 2000
T. iracildae Querino & Zucchi, 2003	Tira-12	Maceió-AL-Brazil	*Calpodes ethlius*	*Canna* spp	Apr., 2000
T. lasallei Pinto, 1998	Tlas-01	Lima-Peru	*Quinta cannae*	*Canna indica*	Mar., 2001
T. lopezandinesis Sarmiento, 1983	Tlop-01	Colombia	-	-	-
T. nerudai Pintureau & Gerding, 1999	Tner-01	Chile	*Rhyacionia buolina*	Pine forest	-
T. pintoi Voegelé, 1982	Tpin-01	Introduction from Chile	-	-	Jun., 1973
T. pretiosum Riley, 1879	Tpre-01	Chincha-Peru	*Diaphania nitidalis*	Pumpkins	Mar., 1992
	Tpre-02	Chancay-Peru	*Dione juno*	*Passiflora*	May, 1994
	Tpre-03	Lambayeque-Peru	*Diatraea saccharalis*	Corn	Jul., 1994
	Tpre-04	Tacna-Peru	*Palpita persimilis*	Olive	Apr., 1995
	Tpre-05	Moquengua-Peru	*Marasmia trapezalis*	Corn	Jul., 1996
	Tpre-06	Ica-Peru	Heliothis virescens	Cotton	Nov., 1996
	Tpre-07	Piura-Peru	*Alabama argillacea*	Cotton	Apr., 1997
	Tpre-08	Lima-Peru	*Diaphania nitidalis*	*Curcubita moschata*	Mar., 1997
	Tpre-09	Ayacucho-Peru	*Helicoverpa zea*	Corn	Nov., 1998
	Tpre-10	Colombia	*Neoleucinodes elegantalis*	-	-
	Tpre-11	USA	-	-	-
	Tpre-12	Jataizinho-PR-Brazil	-	Cotton	Feb., 2000
T. rojasi Nagaraja & Nagarkatti, 1973	Troj-01	Curitiba-PR-Brazil	*Anticarsia gemmatalis*	-	-

*Last collection date

of sterile distilled water. The cycling program was 3 min. at 94°C followed by 33 cycles of 40 seconds at 94°C, 45 second at 53°C and 45 seconds at 72°C with 5 min. at 72°C after the last cycle.

Cloning, Sequencing and Alignments

Following electrophoresis, PCR products were purified with a QIAquick PCR purification kit (Qiagen®). After the purification the PCR products were ligated into a Pgem-T® Vector (Promega), 2 µl of the ligation mix was transformed in the heat shock cells of DH5-α *Escherichia coli* and plated on a LB agar medium containing Ampicilin, X-GAL and IPTG. The plates were incubated overnight at 37°C. The next day, white colonies were picked up with a sterile toothpick from the plates and placed into tubes containing 3.0 ml of LB liquid medium and 3µl Ampicilin and put to grow up overnight in a shaker set to 250 rpm at 37°C. To confirm that the correct piece of DNA had been cloned, a PCR reaction with a template extracted from the bacterial culture was added to 100 µl 5% Chelex-100 and incubated for 15 min. at 60°C followed by 5 min. at 95°C. The PCR was performed with 5 µl of this template as described before. If indeed an ITS2 had been cloned, 850 µl of the bacteria culture was added to 150 µl of 87% glycerol and stored at –80°C. The rest of the culture was used in a QIAprep Miniprep kit (Qiagen®) to purify the plasmid, which was used for the sequencing in an Applied Biosystems automatic sequencer. *Trichogramma* sequences were aligned manually using the ESEE 3.0s sequence editor (Cabot, 1995).

Thelytoky in *Trichogramma*

For detection of thelytoky caused by *Wolbachia* infection, specific primers for DNA amplification of the *wsp* region of *Wolbachia* were used: *wsp*-Forward primer

5'TGGTCCAATAAGTGATGAAGAAAC-3' and *wsp*-Reverse 5'-AAAAATTAAAC GCTACTCCA-3' (Braig *et al*. 1998). These primers amplify 554bp of the *wsp* gene. The cycling program was 3 min. at 94°C followed by 40 cycles of 1 min. at 94°C, 1 min. at 50°C and 1 min. at 72°C with 5 min. at 72°C after the last cycle.

Molecular Key

A molecular key for separating *Trichogramma* species was constructed based on the size and the fragment profile following restriction with different restriction enzymes. Initially we determined ITS2 sequences of all species and lines, subsequently we first tried to distinguish the different species based on the size of the PCR product. Species with similar sized PCR products were distinguished by selecting restriction enzymes that would generate differently sized in restriction fragments. To predict the fragment sizes to be expected with different enzymes we use the program Webcutter 2.0 (Heiman 1997). Restriction of the PCR product was performed in a 10 µl volume (5 µl PCR product, 1 µl (10X) reaction buffer, 1 µl restriction enzyme and 3 µl distilled water). The mixture was incubated for 1 h at 37^{0}C. Digestions were checked by running them on a standard 1.5 % agarose gel. Three restriction enzymes were used (EcoRI, MseI and MaeI).

3.3. Results and Discussion

Seventeen native and introduced *Trichogramma* species collected in South America were identified based on ITS2 sequences. In total, thirty lines were studied (Table 2). Seventeen lines were recognised as arrhenotokous and thirteen as thelytokous. Twelve out of thirteen thelytokous lines studied here carry the *Wolbachia* symbiont. In *T. cacoeciae*,

Table 2. Reproduction mode, species group recognition, size of the ITS2 and PCR product (bp) and Genbank Accession number of the *Trichogramma* species

Line Designation	Reproduction Mode	Accession Number	ITS2 Product	PCR Product
Tner-01	Arrhenotoky	AY182756	632	746
Tpin-01	Arrhenotoky	AY182757	581	695
Tato-01	Thelytoky	AY182758	565	680
Tato-02	Arrhenotoky	AY182759	561	675
Taca-01	Arrhenotoky	-	559	674
Tbru-01	Arrhenotoky	AY187263	536	650
Troj-01	Arrhenotoky	-	524	638
Tira-12	Arrhenotoky	AY182760	522	636
Tlop-01	Arrhenotoky	AY182761	509	623
Tlas-01	Arrhenotoky	AY182762	485	599
Tesa-11	Arrhenotoky	AY182763	379	493
Tcac-01	Thelytoky	AY166700	460	574
Tgal-01	Arrhenotoky	AY182764	445	560
Tfue-01	Arrhenotoky	AY182765	437	553
Tpre-01	Thelytoky	AY182770	410	524
Tpre-02	Thelytoky	AY182771	410	524
Tpre-03	Thelytoky	AY182772	415	529
Tpre-04	Thelytoky	AY182773	410	524
Tpre-05	Thelytoky	AY184958	410	524
Tpre-06	Thelytoky	AY184959	410	524
Tpre-07	Thelytoky	AY187259	415	529
Tpre-08	Thelytoky	AY184960	410	524
Tpre-09	Thelytoky	AY184961	410	524
Tpre-10	Thelytoky	AY187260	412	526
Tpre-11	Thelytoky	AY187261	413	527
Tpre-12	Arrhenotoky	AY187262	412	526
Tbra-01	Arrhenotoky	AY182766	406	520
Tden-01	Arrhenotoky	AY182767	403	519
Texi-01	Arrhenotoky	AY182768	383	497
Texi-02	Arrhenotoky	AY182769	381	496

parthenogenesis was not caused by *Wolbachia* infection (Stouthamer *et al*. 1990; Almeida & Stouthamer 2003). For all other parthenogenetic lines, *Wolbachia* detection was possible by using specific primers of the *wsp* region. In all thelytokous species/lines no male was found, except for *T. atopovirilia* (culture form Brazil) and *T. pretiosum* (culture from the Entomology Lab., California University, Riverside-USA) in which few males were present.

In *T. atopovirilia* the presence of *Wolbachia* was recorded for the first time in Brazil by Ciociola Jr. *et al*. (2001a). In all *T. pretiosum* lines studied here *Wolbachia* infection was reported for the first time. In South America, *Wolbachia* infection has been reported in *T. pretiosum* from Uruguay (Grenier *et al*. 1998) and from Brazil (Almeida *et al*. 2001).

Complete ITS2 sequences have been deposited in GenBank (Table 2). *Trichogramma s*pecies were identified by (1) morphological identification: *T. atopovirilia*, line Tato-01, (Dr. Américo I. Ciociola Junior (EPAMIG, Brazil) (personal comunication), *T. lopezandinensis* and *T. fuentesi* (Dr. Ranyse B.Q. da Silva-ESALQ/USP, Brazil) (personal communication); (2) by comparison with ITS2 sequences from the GenBank: *T. pintoi*, *T. lasallei*, *T. cacoeciae*, *T. galloi*, *T. pretiosum*, *T. brassicae*, *T. dendrolimi* and *T. exiguum*; or (3) by comparison with sequences not published yet: *T. nerudai*. *T. rojasi* and *T. acacioi* lines were the same as those used by Ciociola Jr. *et al*. (2001b). Two new species from Brazil (*T. iracildae* and *T. esalqueanum*) sequenced here were recorded by Querino & Zucchi (2003).

An identification key was constructed for these species using as two characters of the PCR product its size and the restriction patterns generation using the three restriction enzymes EcoRI, MseI and MaeI (Table 3).

Currently thirty-six *Trichogramma* species are known in South America. Most species have been recorded from Brazil (24), followed by Venezuela (9), Colombia (7), Peru (6), Chile and Uruguay (5), Bolivia (4), Argentina (3), and Equator and Paraguay (2).

Table 3. Molecular key for *Trichogramma* species recognition based on the size of the PCR product and species-specific banding pattern

1.	Size of the PCR product < 620 bp	2
	Size of the PCR product > 620 bp	10
2.	Size of the PCR product < 550 bp	3
	Size of the PCR product >550 bp	7
3.	Size of the PCR product ≤ 500 bp	4
	Size of the PCR product > 550 bp	5
4.	PCR product cut by MseI ca. 280 and 191bp	*T. esalqueanum*
	PCR product not cut by MseI	*T. exiguum*
5.	PCR product cut by EcoRI	6
	PCR product not cut by EcoRI	*T. pretiosum*
6.	PCR product cut by MseI ca. 441 bp	*T. dendrolimi*
	PCR product cut by MseI ca. 411 bp	*T. brassicae*
7.	PCR product not cut by MseI	8
	PCR product cut by MseI	9
8.	PCR product cut by MaeI ca. 267 and 218 bp	*T. fuentesi*
	PCR product cut by MaeI ca. 182 and 113 bp	*T. galloi*
9.	PCR product cut by EcoRI ca. 346 and 228 bp	*T. cacoeciae*
	PCR product not cut by EcoRI	*T. lasallei*
10.	Size of the PCR product < 670 bp	11
	Size of the PCR product > 670 bp	14
11.	PCR product not cut by MseI	12
	PCR product cut by MseI	13
12.	PCR product cut by MaeI ca. 480 and 143 bp	*T. lopezandinensis*
	PCR product cut by MaeI ca. 517 bp	*T. iracildae*
13.	PCR product cut by MseI ca. 390 and 194bp	*T. rojasi*
	PCR product cut by MseI ca. 463 and 187bp	*T. bruni*
14.	Size of the PCR product > 700 bp	*T. nerudai*
	Size of the PCR product < 700 bp	15
15.	PCR product cut by EcoRI	*T. pintoi*
	PCR product not cut by EcoRI	16
16.	PCR product cut by MseI ca. 478 and 196 bp	*T. acacioi*
	PCR product cut by MseI ca. 345, 198 and 137 bp	*T. atopovirilia*

Trichogramma species have not been reported from French Guyana and Surinam. Six species have been reported as introduced in South America (Silva 2002).

ITS2 product size of the seventeen studied species ranged from 379 to 632 bp and sequences differ from each other consistently.

The usefulness of endonucleases analysis for distinguishing *Trichogramma* species was shown by Stouthamer *et al*. (1999), Silva *et al*. (1999) and Pinto *et al*. (2002). In our sample the size of the ITS2 product alone could not identify the species, but cutting the ITS2 product of the different species using only three restriction enzymes (EcoRI, MseI and MaeI) enabled us to identify all species (Table 3). How reliable are such molecular keys that are based in some cases on the sequence of only a few lines? The experience with other *Trichogramma* species is that the intraspecific variation in the ITS2 is rather limited. For instance the variation in the size of ITS2 was evaluated in *T. atopovirilia, T. exiguum* and *T. pretiosum.* The length of the complete sequences found for *T. pretiosum* lines all ranged from 410 to 415 bp (Table 3), for *T. atopovirilia* (561-565 bp) and for *T. exiguum* (381-383 bp). In general, variation was limited to the number of microsatellite repeat stretches found in the ITS2 sequences. The variation in number of microsatellite repeats seems to be common in ITS sequences, similar microsatellite variation was for instance also found in Eriophyid mites (Fenton *et al*. 1997). The range obtained here confirms the low intraspecific variation in the ITS2 length detected by Stouthamer *et al*. (1999) and Silva *et al*. (1999). According to Stouthamer *et al*. (1999) ITS2 can be used for species identification in *Trichogramma* because the sequence variation within species is small relative to the difference found between species. All morphologically distinct cryptic species are also distinguished by sequence differences, however in the morphologically indistinguishable species North American species *T. minutum* Riley and *T. platneri* Nagarkatti no consistent differences were found in their ITS2 sequences (Stouthamer *et al*.

2000). In this case the ITS2 did not improve upon the morphologically based system. A potential weakness of an ITS2 based molecular key as presented here is that in some cases the key relies on either a restriction of an ITS2 by a particular enzyme or the lack thereof. When no restriction digestion is found it is not immediately clear if the endonuclease worked or if the products lack the restriction site (Stouthamer *et al*. 1999). It is important to run along a positive control to assure that the restriction reactions work. Molecular laboratories with basic infrastructure can perform PCR amplifications followed by restriction digestions and DNA can be extracted from living, frozen, ethanol-preserved or dried material (Post *et al*. 1993; Kan *et al*. 1996; Ciociola *et al*. 2000). Just as in case of morphological keys, molecular keys will only be completely reliable once all species of a region are known.

This study together with previous studies (Stouthamer *et al*. 1999; Silva *et al*. 1999; Ciociola Jr. *et al*. 2001b) shows the utility of ITS2 sequences in *Trichogramma* identification. The molecular technique used in this study will be of a great utility in a near future for sequencing species already classified but not sequenced yet from South America. We also expect that with this technique new species will be soon discovered due to the high diversity of *Trichogramma* host insects in this region. The small number of species found until now is caused by the very limited activities to collect species of *Trichogramma*. In addition, most of the host species collected were restricted to insects of economic importance. Also there are only a few taxonomists specialised in identifying *Trichogramma* species using morphological features. Identification by the combination of morphological characters and molecular technique will be of extreme importance for a successful systematic analysis of this group.

Acknowledgments

The authors are thankful to Joop van Lenteren for his valuable comments on the manuscript, and to all those who sent *Trichogramma* species: Americo I. Ciociola Junior (EPAMIG-Brazil), Aristóbolo Lopez-Avila (CORPOICA-Colombia), Celso L. Hohmann (IAPAR-Brazil), Eduardo N. Botto (IMYZA (CNIA), INTA-Argentina), Mary Whu (SESANA-Peru), Ranyse B.Q. da Silva (ESAlQ-USP-Brazil), and Gary A. Platner (Universisty of California-Riverside-USA). This molecular biology research was financially supported by Embrapa (PRODETAB).

References

Almeida, R.P. de, A.I. Ciociola Jr. & R. Stouthamer 2001. *Wolbachia*-induced parthenogenesis: the first report in a Brazilian *Trichogramma pretiosum* population. *Proc. Exper. Appl. Entomol.* 12: 41-44.

Almeida, R.P. de & R. Stouthamer 2003. Molecular identification of *Trichogramma cacoecieae* Marchal (Hymenoptera: Trichogrammatidae): A new record for Peru. *Neotrop. Entomol.* 32: 269-272.

Bowles, J. & D. P. McManus 1993. Rapid discrimination of *Echinococcus* species and strains using a polymerase chain reaction-based RFLP method. *Mol. Biochem. Parasitol.* 57: 231-240.

Braig, H.R., W. Zhou, S. Dobson & S.L. O' neill 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial ensosymbiont *Wolbachia pipientis. J. Bacteriol.* 180: 2373-2378.

Buckler IV, E.S., A. Ippolito & T.P. Holtsford 1997. The evolution of ribosomal DNA: divergent paralogues and phylogenetic implications. *Genetics* 145: 821-832.

Cabot, E.L. 1995. "The Eyeball Sequence Editor (ESEE)," version 3.0s Dept. Biology, Univ. Rochester, Rochester, NY.

Campbell, B.C., J.D. Steffen-Campbell & J.H. Werren 1993. Phylogeny of the *Nasonia* species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed spacer (ITS2) and 28S rDNA sequences. *Insect Mol. Biol.* 2: 225-237.

Carbone, I. & L. Kohn 1993. Ribosomal DNA sequence divergence within transcribed spacer 1 of the Sclerotiniaceae. *Mycologia* 85: 15-427.

Chang, S.C., N.T. Hu, C.Y. Hsin & C.N. Sun 2001. Characterization of differences between two *Trichogramma* wasps by molecular markers. *Biol. Control* 21: 75-78.

Ciociola Jr., A.C., R.P. de Almeida & R. Stouthamer 2000. Does degradation of DNA in dead *Trichogramma* individuals lead to failure of their identification using PCR? *Proc. Exper. Appl. Entomol.* 11: 33-36.

Ciociola Jr., A.C., R.P. de Almeida, R.A. Zucchi & R. Stouthamer 2001a. Detecção de *Wolbachia* em uma população telítoca de *Trichogramma atopovirilia* Oatman and Platner (Hymenoptera: Trichogrammatidae) via PCR com o Primer Específico *wsp*. *Neotrop. Entomol.* 30: 489-491.

Ciociola Jr., A.C., R.A. Zucchi & R. Stouthamer 2001b. Molecular Key to seven Brazilian species of *Trichogramma* (Hymenoptera: Trichogrammatidae) Using sequences of the ITS2 region and restriction analysis. *Neotrop. Entomol.* 30: 259-262.

Fenton, B., G. Malloch & E. Moxey 1997. Analysis of eriophyid mite rDNA internal transcribed spaces sequences reveals variable simple sequence repeats. *Insect Mol. Biol.* 6:23-32.

Grenier, S. 1994. Rearing of *Trichogramma* and other egg parasitoids on artificial diets. In:

E. Wajnberg & S.A. Hassan (eds.), *Biological Control with Egg Parasitoids.* CAB International, Wallingford, pp. 73-92.

Grenier, S., B. Pintureau, A. Heddi, F. Lassablière, C. Jager, C. Louis & C. Khatchadourian 1998. Successful horizontal transfer of *Wolbachia* symbionts between *Trichogramma* wasps. *Proc. R. Soc. Lond. B* 265: 1441-1445.

Hassan, S.A. 1994. Strategies to select *Trichogramma* species for use in biological control. In: E. Wajnberg & S.A. Hassan (eds.), *Biological Control with Egg Parasitoids.* CAB International, Wallingford, pp. 55-71.

Hassan, S.A. 1995. Introduction to the "Effectiviness and Assessment" session. *Les Colloq. INRA* 73: 107-111.

Heiman, M. 1977. Webcutter 2.0. <http://www.firstmarket.com/cutter/cut2.html>. Accessed 2002 Jun 25.

Hillis, D.M. & M.T. Dixon, 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *Q. Rev. Biol.* 66: 411-453.

Hoy, M.A. 1994. *Insect molecular genetics. An introduction to principles and applications.* Academic Press, Inc. San Diego, California. 546 pp.

Hsiao, C., N.J. Chatterton, K.H. Asay & K.B. Jensen 1994. Phylogenetic relationships of 10 grass species: an assessment of phylogenetic utility of the internal transcribed spacer region in nuclear ribosomal DNA in monocots. *Genome* 37: 112-120.

Kan, F.J.P.M. van, I.M.M.S. Silva, M. Schilthuizen, J.D. Pinto & R. Stouthamer 1996. Use of DNA-based methods for the identification of minute wasps of the genus *Trichogramma. Proc. Exper. Appl. Entomol.* 7: 233-238.

Kan. F.J.P.M. van, J. Honda, J.D. Pinto & R. Stouthamer 1997. Molecular based techniques for *Trichogramma* identification. *Proc. Exper. Appl. Entomol.* 8: 59-62.

Kazmer, D.J. 1991. Isoelectric focusing procedures for the analysis of allozymic variation

in minute arthropods. *Ann. Entomol. Soc. Am*. 84: 332-339.

Kuperus, W.R. & W. Chapco 1994. Usefulness of internal transcribed spacer regions of ribosomal DNA in Melanopline (Orthoptera, Acrididae) systematics. *Ann. Entomol. Soc. Am.* 87: 751-754.

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

Lenteren, J.C. van & J. Woets 1988. Biological and integrated pest control in greenhouses. *Ann. Rev. Entomol.* 33: 239-269.

Li, L., 1994. Worldwide use of *Trichogramma* for biological control on different crops: a survey. In: E. Wajnberg & S.A. Hassan (eds.), *Biological Control with Egg Parasitoids.* CAB International, Wallingfor UK, pp. 37-53.

Nagarkatti, S. & H. Nagaraja 1968. Biosystematic studies on *Trichogramma* species. I. Experimental hybridization between *Trichogramma australicum* Girault, *T evanescens* Westw. and *T. minutum* Riley. *Tech. Bull. Corn. Inst. Biol. Contr.* 10: 81-96.

Nagarkatti, S. & H. Nagaraja 1971. Redescription of some known species of *Trichogramma* (Hym.. Trichogrammatidae), showing the importance of the male genitalia as a diagnostic character. *Bull. Entomol. Res.* 61:13-21.

Orrego C. & F. Agudelo-Silva 1993. Genetic variation in the parasitoid wasp *Trichogramma* (Hymenoptera, Trichogrammatidae) revealed by DNA amplification of a section of the nuclear ribosomal repeat. *Fla. Entomol.* 76: 519-524.

Pinto, J.D., D.J. Kazmer, G.R. Platner & C.A. Sassaman 1992. Taxonomy of the *Trichogramma minutum* complex (Hymenoptera: Trichogrammatidae): allozymic variation and its relationship to reproductive and geographic data. *Ann. Entomol. Soc.*

Am. 85: 413-422.

Pinto, J.D., A.B. Koopmanschap, G.R. Platner & R. Stouthamer 2002. The North American *Trichogramma* (Hymenoptera: Trichogrammatidae) parasitizing certain Tortricidae (Lepidoptera) on apple and pear, with ITS2 DNA characterizations and description of a new species. *Biol. Control* 23:134-142.

Pinto, J.D., G.R. Platner & C.A. Sassaman 1993. Electrophoretic study of two closely related of North American *Trichogramma: T. pretiosum* and *T. deion. Ann. Entomol. Soc. Am.* 86: 702-709.

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

Pinto, J.D., R. Stouthamer & 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. *Proc. Entomol. Soc. Wash.* 99: 238-247.

Pinto, J. D., R. Stouthamer, G.R. Platner & E.R. Oatman 1991. Variation in reproductive compatibility in *Trichogramma* and its taxonomic significance (Hymenoptera: Trichogrammatidae). *Ann. Entomol. Soc. Am.* 84: 37-46.

Pinto, J.D., R.K. Velten, G.R. Platner & E.R. Oatman 1989. Phenotipic plasticity and taxonomic characters in *Trichogramma*. *Ann. Entomol. Soc. Am*. 85:413-422.

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

Pintureau, B. 1993. Enzymatic analysis of the genus *Trichogramma* (Hym.: Trichogrammatidae) in Europe. *Entomophaga* 38: 411-431.

Pintureau, B. & M. Babault 1980. Comparaison des estérases chez 19 souches de

Trichogramma (Hym., Trichogrammatidae) appartenant au groupe d'espèces *evanescens*. *Arch. Zool. Exp. Gén.* 121: 249-260.

Pintureau, B. & M. Babault 1981. Caractérisation enzymatique de *Trichogramma evanescens* et de *T. maidis* (Hym., Trichogrammatidae); étude des hybrides. *Entomophaga* 26: 11-22.

Pintureau, B. & M. Babault 1982. Comparison des enzymes chez 10 souches de *Trichogramma* (Hym., Trichogrammatidae). *Les Colloq. INRA* 9: 31-44.

Pintureau, B. & F.B. Keita 1989. Nouvelles données sur les estérases des Trichogrammes. *Biochem. Syst. Ecol.* 17: 603-608.

Platner, G. R., R. K. Velten, M. Planoutene & J. D. Pinto 1999. Slide-mounting techniques for *Trichogramma* (Trichogrammatidae) and other minute parasitic Hymenoptera. *Entomol. News* 110: 56-64.

Post, R.J., P.K. Flook & A.L. Millest 1993. Methods for the preservation of insects for DNA studies. *Biochem. Syst. Ecol.* 21: 85-92.

Querino, R.B. & R.A. Zucchi 2003. New species of *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) associated with lepidopterous eggs in Brazil. *Zootaxa* 163: 1-10.

Richardson, B.J., P.R. Baverstock & M. Adams 1986. *Allozyme Electrophoresis. A handbook for animal systematics and population studies.* Academic Press Australia, Sydney. 410 pp.

Sappal, N. P., R.S. Jeng, M. Hubbes & F. Liu 1995. Restriction fragment length polymorphisms in polymerase chain reaction amplified ribosomal DNAs of three *Trichogramma* (Hymenoptera: Trichogrammatidae) species. *Genome* 38: 419-425.

Silva, I.M.M.S., J. Honda, F. van Kan, S.J. Hu, L. Neto, B. Pintureau & R. Stouthamer 1999. Molecular differentiation of five *Trichogramma s*pecies occurring in Portugal.

Biol. Control 16:177-184.

Silva, R.B.Q. 2002. Taxonomia do gênero *Trichogramma* Westwood 1833 (Hymenoptera: Trichogrammatidae) na América do Sul. Tese de doutoramento, Escola Superior de Agricultura Luiz de Queiroz-USP, Piracicaba. 214pp.

Smith, S.M. 1994. Methods and timing of releases of *Trichogramma* to control lepidopterous pest. In: E. Wajnberg & S.A. Hassan (eds.), *Biological Control with Egg Parasitoids.* CAB International, Wallingford, pp. 113-144.

Stouthamer, R., J. Hu, F.J.P.M. van Kan, G.R. Platner & 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, & J. D. Pinto 2000. ITS-2 sequences do not differ for the closely related species *Trichogramma minutum* and *T. platneri*. *Entomol. Exper. Appl.* 95: 105-111.

Stouthamer, R., J.D. Pinto, G.R. Platner & R.F. Luck 1990. Taxonomic status of thelytokous forms of *Trichogramma* (Hymenoptera: Trichogrammatidae). *Ann. Entomol. Soc. Am.* 83: 475-581.

Vanlerberghe-Masutti. F. 1994. Genetic identification and phylogeny of parasitic wasp (Hymenoptera: Trichogrammatidae) species by mitochondrial DNA RFLP and RAPD markers. *Insect Mol. Biol*. 3: 229-237.

Vogler, A.P. & R. De Salle 1994. Evolution and phylogenetic information content of the ITS-1 region in the tiger beetle *Cincidela dorsalis. Mol. Biol. Evol*. 11: 393-405.

Chapter

4

Phylogeny of the *Trichogramma* endosymbiont *Wolbachia,* an alpha-Proteobacteria (Rickettsiae)

Raul P. de Almeida & Richard Stouthamer

Chapter 4

PHYLOGENY OF THE *Trichogramma* ENDOSYMBIONT *Wolbachia*, AN ALPHA-PROTEOBACTERIA (RICKETTSIAE)

Abstract

Wolbachia endosymbionts are extensively studied in a wide range of organisms and are known to be transmitted through the egg cytoplasm to the offsping. *Wolbachia* may cause several types of reproductive modifications in arthropods. In *Trichogramma* species, parthenogenesis-inducing *Wolbachia* bacteria allow females wasps to produce daughters from unfertilised eggs and these bacteria are present in at least 9% of all *Trichogramma* species. Phylogenetic studies have led to the subdivision of the *Wolbachia* clade in five supergroups (A, B, C, D and E) and *Wolbachia* from *Trichogramma* belong to supergroup B. Here, using the *wsp* gene, four groups of *Wolbachia* that infect *Trichogramma* species were distinguished and the addition of a new group "*Ato*" was suggested due to the addition of *Wolbachia* from *T. atopovirilia*. Specific primers were designed and tested for the "*Ato"* group. Seventy-five percent of all evaluated *Wolbachia* strains from *Trichogramma* fell within "*Sib*" group.

4.1. Introduction

Wolbachia symbionts have been extensively studied in a wide range of organisms (Martin *et al.* 1973; Rigaud *et al*. 1991; Juchault *et al.* 1992; Sironi *et al*. 1995; Breeuwer & Jacobs 1996; Johanowicz & Hoy 1996; Bouchon *et al.* 1998). This bacterium was first described by Hertig (1936) in *Culex pipiens* and is estimated to be present in over 16% of insect species infected in Panama (Werren *et al*. 1995b), in 22% of British insects (West *et al*. 1998) and 19.3% of the temperate North American insects including the major orders Diptera, Coleoptera, Lepidoptera, Hymenoptera and Orthoptera (Werren & Windsor 2000).

Wolbachia infect the reproductive tissues of arthropods, are transmitted through the egg cytoplasm and cause several reproductive modification types (Werren 1997): (1) cytoplasmic incompatibility (CI) in insects (Laven 1951, 1967; Yen & Barr 1974; Barr 1980; Breeuwer *et al.* 1992; Breeuwer & Werren 1990; Giordano *et al.* 1995), isopods (Legrand & Juchault 1986; Rousset *et al*. 1992) and mites (Johanowicz & Hoy 1996; Tsagkarakou *et al.* 1996). Cytoplasmic incompatibility results in aborted karyogamy (O'Neill & Karr 1990) and occurs when infected males are crossed with females that are either uninfected (unidirectional incompatibility) (Yen 1975; Hoffmann *et al.* 1986) or infected with another bacterial variant (bidirectional incompatibility); (2) feminization in isopods where genetic males are converted into functional females (Rousset *et al.* 1992; Martin *et al.* 1973, 1994; Juchault *et al.* 1994); (3) the induction of complete parthenogenesis in some haplodiploid species (Stouthamer *et al*. 1990a, b; Zchori-Fein *et al*. 1992; Stouthamer *et al.* 1993; Arakaki 2001). Parthenogenesis-inducing *Wolbachia* bacteria allow infected female to produce offspring from unfertilized eggs due to a first mitotic division modification (Stouthamer & Kazmer 1994, Gottlieb *et al*.2002); (4) fecundity increase of the host for the egg parasitoid *Trichogramma bourarachae* (Girin & Boulétreau 1995; Vavre *et al*. 1999a) and (5) male-killing in a wide range of insects. This is a case in which inherited bacteria kill

male hosts during early development (Hurst *et al*. 1997; Dyson *et al*. 2002); (6) complete dependence on *Wolbachia* for egg development (Dedeine *et al*. 2001).

The phylogeny of *Wolbachia* has been studied using a number of different genes. (1) the 16S rDNA (O'Neill *et al.* 1992; Stouthamer *et al.* 1993); (2) the 23S rDNA (Rousset *et al*. 1992); (3) the bacterial cell-cycle *ftsZ* gene (Holden *et al.*1993; Werren *et al.* 1995a); (4) the spacer-2 region (SR2) which includes the 3' flanking sequences of 23S rDNA gene and the major part of 5S rDNA gene (van Meer 1999); (5) the groE-homologue (Masui *et al.* 1997); and (6) the *wsp* gene, that codes for an outer membrane protein of *Wolbachia* (Braig *et al.* 1998). Using the 16S rDNA gene O'Neill *et al.* (1992) and Stouthamer *et al.* (1993) showed less than 3% difference between *Wolbachia* strains despite different reproduction effects that they induce in their hosts. Rousset *et al.* (1992) reported that the phylogenetic trees produced by 23S rDNA and 16S rDNA were very similar and the phylogenetic resolution for the 23S was similar to that provided by 16S. Van Meer (1999) found a larger variation by using the SR2 in comparison to *ftsZ* gene, although the resolution was not improved because of its small size. Higher variation in groE-homologue operon than *ftsZ* was shown by Masui *et al.* (1997), but no large sequence data set exists of this gene. The *wsp* gene also exhibited higher variation than the *ftsZ* gene with an extensive sequence database available (Zhou *et al.* 1998). According to Bourtzis *et al*. (1998) the *wsp* gene appeared to result in a closer relationship between the phylogeny of *Wolbachia* and its reproductive modifications. All *Wolbachia* strains inducing no CI effect in *Drosophila* were able to rescue closely related strains that do induce CI.

Based on the *ftsZ* gene (Werren *et al.* 1995a) and wsp gene (Zhou *et al*. 1998) the *Wolbachia* clade was subdivided into two groups (A and B) and a maximum sequence difference of 15% was reported between groups, but this difference was relatively low within group A (3%) (Werren *et al*. 1995a). Zhou *et al.* (1998) distinguished twelve distinct

groups based on the grouping criterion of 2.5% sequence difference of the wsp. Van Meer *et al.* (1999) added one new *Wolbachia* group to the supergroup A and six groups to the supergroup B. The number of groups was increased to nineteen and three groups of *Wolbachia* that infect *Trichogramma* were recognised (Dei, Sib and Kay) (van Meer *et al*. 1999). Pintureau *et al*. (2000) studying *Wolbachia* in *Trichogramma* suggested the creation of a new group (Sem) for *T. semblidis* and to merge the groups Sib and Kay under the name Sib because after the addition of new hosts, these groups did not differ by 2.5% any longer.

Two other supergroups (C and D) were found in nematodes (Bandi *et al*. 1998). The most recent *Wolbachia* supergroup (E), based on the 16S rDNA, was found in Collembola (Vandekerckhove *et al*. 1999).

Though *Wolbachia* seems most often to be transmitted vertically, horizontal transmission has been suggested for *Nasonia* and their fly host *Protocalliphora* sp., because their *Wolbachia* strains, based on the *ftsZ* sequences, clustered together. The relatively low variation between *Wolbachia* strains within group A also suggests an extensive horizontal transmission (Werren *et al*. 1995a). In *Trichogramma*, Schilthuizen & Stouthamer (1997) showed that all *Wolbachia* strains were monophyletic using a phylogeny based on the *ftsZ* gene and cospeciation of host and symbiont was excluded as an explanation for that phenomenon because the phylogenetic trees of *Trichogramma* and their *Wolbachia* were not congruent. In addition, it was suggested that horizontal transmission sometimes occurs inside a common host egg of *Trichogramma.* Recently this hypothesis was confirmed (Huigens *et al.* 2000; This thesis= *Chapter 5*).

In this work the phylogeny of the parthenogenesis-inducing *Wolbachia* that infects *Trichogramma* was studied using the *wsp* gene.

4.2. Material and Methods

Host species of Wolbachia *symbiont*

Wolbachia *host species, line designation and origin are presented in Table 1.*

Trichogramma *and* Wolbachia *DNA Extraction, PCR Amplification and Electrophoresis*

Wolbachia DNA extraction was performed by grinding five *Trichogramma* females in 100 µl 5% Chelex-100 and 4 µl proteinase K (20 mg/ml), subsequently this mixture is incubated for at least 4 hours at 56°C, followed by 10 min at 95°C. PCR was performed in a total volume of 50 µl using a Techne thermocycler, 5 µl DNA template, 5 µl PCR-buffer, 1 µl dNTP's (each in a 10 mM concentration), 1 µl forward and reverse primers; 0.14 µl SuperTAQ polymerase (Sphaero-Q 5 units/µl) and 36.86 µl of sterile distilled water. DNA amplification was done using the specific primers of the *wsp* region (Braig *et al.* 1998): *wsp*-forward: 5'-TGGTCCAATAAGTGATGAAGAAAC-3' and *wsp*-reverse 5'-AAAAATTAAACGCTACTCCA-3'. The cycling program was 3 min at 94 °C followed by 40 cycles of 1 min. at 94°C, 1 min. at 50°C and 1 min. at 72°C with 5 min at 72°C after the last cycle.

Cloning, Sequencing and Alignments

Following electrophoresis, PCR products were purified with a QIAquick PCR purification kit (Qiagen®). After purification the PCR products were ligated into a Pgem-T® Vector (Promega), 2 µl of the ligation mix was used to transform competent cells of DH5-α *Escherichia coli* using heat shock and plated in a LB agar medium containing Ampicilin, X-GAL and IPTG. The plates were stored overnight at 37°C. The next day,

Table 1. Host species, line designation, origin, GenBank accession numbers and references on the *Wolbachia* strains.

Host	Line Designation	Origin	Genbank Acces. Number	References
L. strialellus	-	-	AF020080	Zhou *et al.* (1998)
T. confusum	-	-	AF020083	Zhou *et al.* (1998)
T. bedeguaris	-	-	AF071915	Van Meer *et al*. (1999)
T. atopovirilia	Tato-01	Minas Gerais, Brazil	-	This study
T. brevicapillum	-	Mojave desert, CA, USA	-	This study
	M	Mojave River Forks	-	This study
T. cordubensis	28A63a	Divor, Portugal	-	This study
	-	-	AF245164	Pintureau *et al.* (2000)
T. dendrolimi	-	-	AB094397	Tagami & Miura (2002)
T. deion	TX	Sanderson, TX	AF020084	Zhou *at al*. (1998)
	SW436	Mojave desert, CA, USA	AF071925	Van Meer *et al*. (1999)
T. embryophagum	Uro3	Orumiyeh, Iran	AF245165	Pintureau *et al.* (2000)
T. evanescens	M36	Alpes-maritimes, France	AF245167	Pintureau *et al.* (2000)
T. kaykai	JT6-3	San Bernardino, CA, USA	AF071924	Van Meer *et al*. (1999)
	-	Last Chance Canyon, Kern Co, CA, USA	AF071927	Van Meer *et al*. (1999)
	B	-	AF071924	Van Meer *et al*. (1999)
T. nubilale	234	Nova Scotia, Canada	AF071926	Van Meer *et al*. (1999)
T. oleae	S2	Former Yugoslavia	AF245166	Pintureau *et al.* (2000)
T. pretiosum	Tpre-03	Lambayeque, Peru	-	This study
	Tpre-04	Tacna, Peru	-	This study
	Tpre-06	Ica, Peru	-	This study
	Tpre-09	Ayacucho, Peru	-	This study
	Tpre-13	Santa Catarina, Brazil	-	This study
	M	Nuevo Leon, Mexico	-	This study
	U	Uruguay	AF245163	Pintureau *et al.* (2000)
T. semblidis	Semv	Valbomme, Alpes-maritimes, France	AF145162	Pintureau *et al*. (2000)
T. sibericum	SIB	Canada	AF071923	Van Meer *et al*. (1999)

white colonies were picked with a sterile toothpick from the plates and placed into tubes containing 3.0 ml of LB liquid medium and 3µl Ampicilin and put to grow up overnight in a shaker set to 250 rpm at 37°C. To confirm that the correct gene had been cloned, a PCR reaction was done using a template extracted from the bacterial culture. This template extraction was done by adding 10 µl of the bacterial culture to 100 µl 5% Chelex-100, which was incubated for 15 min. at 60°C followed by 5 min. at 95°C. The PCR was performed using 5 µl of template in a final volume of 50 µl. If indeed the correct gene was cloned the culture was used in a QIAprep Miniprep kit (Qiagen®) to purify the plasmid, for the sequencing in an Applied Biosystems automatic sequencer.

Phylogenetic analysis

A total of 27 wsp sequences were used in this study (Table 1). *Wolbachia* sequences were aligned using the BioEdit sequence editor (Hall 1999). Analyses were performed using PAUP 4.0b2a (Swofford 1999) using heuristic search. Successive approximations weighting was done using the rescaled consistency index and a base weight of 1,000. Heuristic searches were performed (300 random replicate searches), bootstrap analysis was done with 300 replications. DNADIST program, version 3.5c was used to compute distance matrix from nucleotide sequences (Hall 1999). A DNADIST program (version 3.5c) from the BioEdit sequence editor was used to calculate the distance matrix of the *wsp* sequences.

4.3. Results and Discussion

Specific primers (*wsp*) (Braig *et al.* 1998) were successfully used for the amplification of the *Wolbachia* strains from the *Trichogramma* species studied here. The negative control

did not result in DNA amplification. *Wolbachia* phylogeny was built using *Wolbachia* strains from four *Trichogramma* species (*T. atopovirilia, T. pretiosum, T. cordubensis* and *T. brevicapilum*) sequenced in this study, combined with several other strains from the GenBank (Table 1). The *Wolbachia* strain that infects *T. atopovirilia* species was first reported from Brazil by Ciociola Jr. *et al.* (2001). All other *T. atopovirilia* collected so far from several countries (Mexico, El Salvador, Guatemala, Honduras, Colombia, and Venezuela) (Zucchi & Monteiro 1997; Pinto 1998) are not infected with the PI *Wolbachia*. Six *Wolbachia* strains from *T. pretiosum* were studied. The *Wolbachia* strain obtained from the line Tpre-13 of *T. pretiosum* was collected in Brazil (Almeida *et al.* 2001). Four lines (Tpre-03, 04, 06 and 09) were collected in Peru and one from Mexico (M). This was the first report of infected *Trichogramma* species with *Wolbachia* in Peru (*see Chapter 3*).

All *Wolbachia* strains studied here fell within the groups already known. Two *T. pretiosum* lines (Tpret-06 and Tpret-13) fell within the group of *T. deion* (*Dei*) and the three others lines fell within the merged group *Sib* (*Sib* + *Kay*) proposed by Pintureau *et al.* (2000). In addition, that proposal is in agreement with this study and was confirmed by including many other sequences from the GenBank database (Fig. 1). The *Wolbachia* strains that infect *T. cordubensis* and *T. brevicapillum* also belong to *Sib* group.

Inclusion of the *Wolbachia* from *T. cordubensis* in *Sib* group agrees with the results obtained by Pintureau *et al*. (2000). *T. brevicapillum* was also classified in the same group as *T. cordubensis* and its classification in the B group was shown by von der Schulenburg *et al*. (2000) using the *ftsZ* gene. *Wolbachia* from *T. dendrolimi* also fell in the *Sib* group.

Van Meer *et al*. (1999) and Pintureau *et al*. (2000) have found different strains of *Wolbachia* that infect *T. deion* species belonging to different groups (*Dei* and *Sib*). Here the same situation was found for *T. pretiosum.* The *Wolbachia* sequence obtained from *T.*

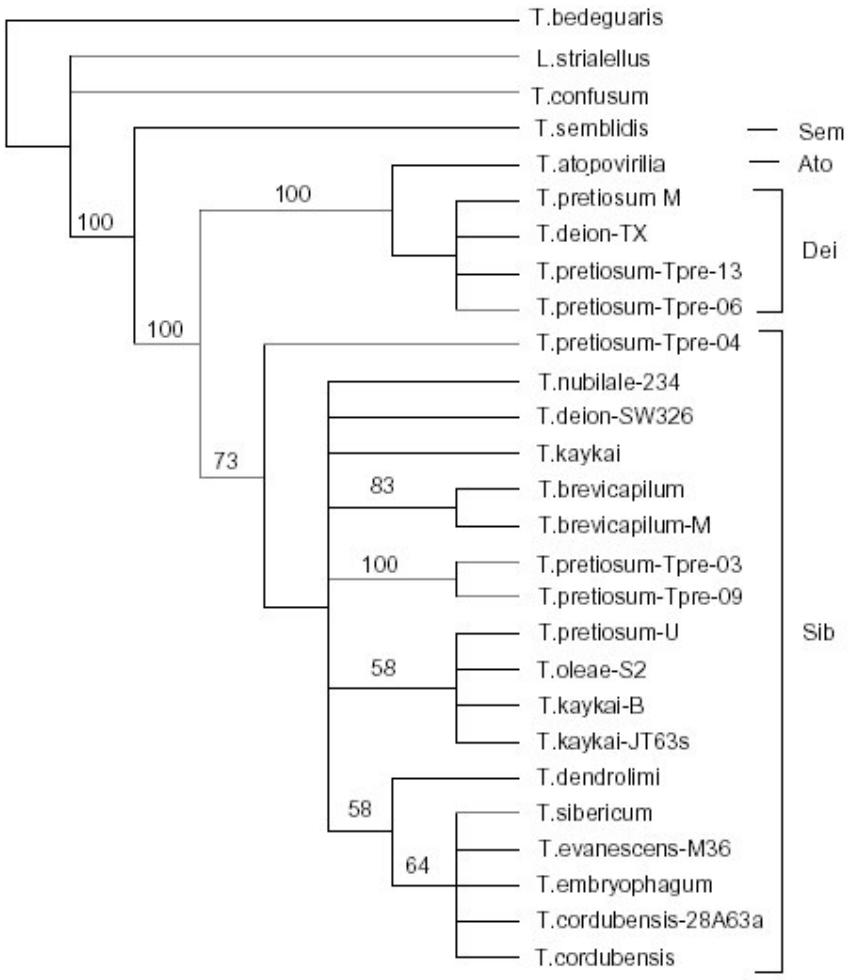

Figure 1. Phylogenetic relationships among *Wolbachia* strains from different *Trichogramma* hosts based on *wsp* gene. Heuristic searches were performed (300 random replicate searches) and Bootstrap values based on 1000 replicate searches with 2 random replicate per search.

atopovirilia did not fit in any existing groups (Table 2) using a threshold of 2.5% sequence of divergence (Zhou *et al*. 1998). The most closely related *Wolbachia* sequence from *T. atopovirilia* was *T. pretiosum* (line Tpret-13). With this finding the creation of a new *Wolbachia* group is proposed under the name "*Ato*". Thus, the inclusion of this group

Table 2. Percentages of *wsp* nucleotide sequence dissimilarities between different *Wolbachia* strains that infect *Trichogramma*

	1	2	3	4	5	6	7	8	9	10	11	12	13
2	10.92												
3	2.32	11.38											
4	8.06	19.36	9.37										
5	5.34	15.85	6.58	5.74									
6	6.39	17.11	7.65	6.80	1.35								
7	5.34	15.85	6.58	5.54	0.77	1.74							
8	5.14	15.62	6.38	5.34	0.57	1.54	0.19						
9	5.34	15.85	6.58	5.74	0.38	1.35	0.77	0.57					
10	7.19	18.54	8.91	4.92	5.13	6.17	4.93	4.72	5.13				
11	5.35	15.87	6.59	6.18	1.35	2.33	0.96	0.77	1.35	4.73			
12	5.14	15.62	6.38	5.34	0.57	1.54	0.19	0.00	0.57	4.72	0.77		
13	5.14	15.62	6.38	5.34	0.57	1.54	0.19	0.00	0.57	4.72	0.77	0.00	
14	5.34	15.85	6.58	5.74	0.38	1.35	0.38	0.57	0.38	5.13	1.35	0.57	0.57
15	5.81	16.51	7.07	6.22	0.77	1.75	1.16	0.97	0.77	5.59	1.75	0.97	0.97
16	5.34	15.85	6.58	5.74	0.38	1.35	0.38	0.57	0.38	5.13	1.35	0.57	0.57
17	4.94	15.85	6.58	5.74	0.38	1.35	0.77	0.57	0.38	5.13	1.35	0.57	0.57
18	5.34	15.85	6.58	5.74	0.38	1.35	0.38	0.57	0.38	5.13	1.35	0.57	0.57
19	5.34	15.85	6.58	5.74	0.38	1.35	0.38	0.57	0.38	5.13	1.35	0.57	0.57
20	6.37	17.57	8.06	3.51	4.12	5.15	3.92	3.72	4.12	1.35	3.72	3.72	3.72
21	6.37	17.57	8.06	3.51	4.12	5.15	3.92	3.72	4.12	1.35	3.72	3.72	3.72
22	6.78	18.06	8.48	3.90	4.52	5.55	4.32	4.12	4.52	1.74	4.12	4.12	4.12
23	5.55	16.39	7.23	7.21	2.33	3.33	2.72	2.52	2.33	6.15	3.33	2.52	2.52
24	5.55	16.15	7.23	7.21	2.52	3.53	2.92	2.72	2.52	6.15	3.53	2.72	2.72
25	5.34	15.85	6.58	5.74	0.38	1.35	0.77	0.57	0.38	5.13	1.35	0.57	0.57
26	9.13	18.60	10.43	10.22	8.24	9.33	7.82	7.61	8.24	10.01	8.27	7.61	7.61
27	5.97	16.10	7.23	6.17	1.35	2.33	0.96	0.77	1.35	5.55	1.55	0.77	0.77

1- *L. striatellus*; 2- *T. bedeguaris*; 3- *T. confusum*; 4- *T.atopovirilia*; 5-*T. brevicapillum*; 6-*T. brevicapillum* M; 7-*T.cordubensis*-28A63a; 8- *T.cordubensis*; 9- *T.deion*-SW326; 10- *T.deion*-TX; 11- - *T.dendrolimi*; 12- *T. embryophagum*; 13- *T. evanenscens*; 14- *T. kaykai* B; 15- *T. kaykai* JT6-3; 16- *T. kaykai*; 17- *T. nubilale*; 18- *T. oleae*; 19- *T. pretiosum*-U; 20- *T. pretiosum* M; 21- *T. pretiosum*-Tpre-13; 22- *T. pretiosum*-Tpre-06; 23- *T. pretiosumi*Tpre-03; 24- *T. pretiosum*-Tpre-09; 25- *T. pretiosum*-Tpre-04; 26- *T. semblidis*; 27- *T. sibericum*.

Table 2. Percentages of *wsp* nucleotide sequence dissimilarities between different *Wolbachia* strains that infect *Trichogramma*

	14	15	16	17	18	19	20	21	22	23	24	25	26
15	0.77												
16	0.00	0.77											
17	0.38	0.77	0.38										
18	0.00	0.77	0.00	0.38									
19	0.00	0.77	0.00	0.38	0.00								
20	4.12	4.57	4.12	4.12	4.12	4.12							
21	4.12	4.57	4.12	4.12	4.12	4.12	0.00						
22	4.52	4.97	4.52	4.52	4.52	4.52	0.38	0.38					
23	2.33	2.75	2.33	2.33	2.33	2.33	5.76	5.76	6.17				
24	2.52	2.95	2.52	2.52	2.52	2.52	5.76	5.76	6.17	0.57			
25	0.38	0.39	0.38	0.38	0.38	0.38	4.12	4.12	4.52	2.33	2.52		
26	8.24	8.32	8.24	8.24	8.24	8.24	9.15	9.15	9.58	9.35	9.35	7.82	
27	1.35	1.75	1.35	1.35	1.35	1.35	4.53	4.53	4.94	3.32	3.52	1.35	8.05

14- *T. kaykai* B; 15- *T. kaykai* JT6-3; 16- *T. kaykai*; 17- *T. nubilale*; 18- *T. oleae*; 19- *T. pretiosum*-U; 20- *T. pretiosum* M; 21- *T. pretiosum*-Tpre-13; 22- *T. pretiosum*-Tpre-06; 23- *T. pretiosumi*Tpre-03; 24- *T. pretiosum*-Tpre-09; 25- *T. pretiosum*-Tpre-04; 26- *T. semblidis*; 27- *T. sibericum*.

increased the number of groups of *Wolbachia* that infect *Trichogramma* species from three (Pintureau *et al*. 2000) to four.

Specific primers were designed for *Wolbachia* found in *T. atopovirilia*: ATOW-For, 5'-TGCAGCAAATAAAGACAAGGATA-3' and ATOW-Rev, 5'-CCAAAAGTGCC GTAAAGAACA-3'. A specific annealing temperature was used (66 ^{o}C). Confirmation of its specificity was done by DNA amplification of *T. atopovirilia*. Comparison was made with the closest *Wolbachia* found in *T. pretiosum* (line Tpre-13) showing that only *T. atopovirilia* DNA could be amplified. PCR products were loaded on 1% agarose gel stained with ethidium bromide. Differentiation of these two *Wolbachia* strains could also be done by restriction analysis using the endonucleases MboI and MboII (see *Chapter 5*). The use of the endonuclease MboI resulted in one cutting site and two restriction fragments (397 and 203 bp) for *T. atopovirilia* and two cutting sites and three restriction fragments (266, 203 and 131 bp) for *T. pretiosum*. With the enzyme MboII the cleavage of the DNA product resulted in two cutting sites and three restriction fragments (218, 204 and 76 bp) for *T. atopovirilia* and one cutting site and two restriction fragments (318 and 282 bp) for *T. pretiosum*.

Thelytoky caused by *Wolbachia* infection has been found in 17 (This thesis= *Chapter 1*) out of 190 described *Trichogramma* species (Pinto 1998; Querino & Zucchi 2003a, b). It results in all-female broods being usually caused by the cytoplasmically inherited bacterium *Wolbachia*, which induces gamete duplication in the haplodiploid organisms (Stouthamer & Kazmer 1994, Gottlieb *et al.* 2002). Parthenogenesis inducing *Wolbachia* infecting *Trichogramma* species are exclusively found in the B supergroup (Werren *et al*. 1995b; Zhou *et al*. 1998; van Meer *et al*. 1999; Pintureau *et al*. 2000; von der Schulenberg *et al*. 2000). Several other *Wolbachia* infections in *Trichogramma* have been found that belong to the supergroup A, among others the infection in *T. bourarachae* and *T. kaykai*

(LC110). All these group A *Wolbachia* sequences are very similar to the *Wolbachia* found in their laboratory host *Ephestia kuehniella*, which may indicate that these are either contaminations or are *Wolbachia* acquired from their hosts (van Meer *et al*. 1999; Vavre *et al*. 1999b).

All *Trichogramma* species and lines studied here are part of the B group. The *Wolbachia* strain found in *T. atopovirilia* is clearly distinct from all others and is therefore put in a new group (Table 2). The specific primers designed in this study will be very useful for distinguishing a possible similar *Wolbachia* found in *T. atopovirilia.* The utility of primer specificity in horizontal transmission studies where different hosts are used has been mentioned by Grenier *et al*. (1998).

Sequences studied here showed a maximum divergence of 10.22% between *Wolbachia* strains that infect *Trichogramma* (Table 2). The *Wolbachia* variants from different *Trichogramma* species still form a monophyletic clade when these new species and strains are added.

From all the *Wolbachia* strains that infect the *Trichogramma* species eleven belong to the same *Wolbachia* group (*Sib*), two fell within *Dei* group and other two were put individually in one group (*Ato* and *Sem*). Similarities in many *Wolbachia* sequences belonging to a same group as it has been found in *Sib* group (18 *Wolbachia* strains) for instance, suggest the possibility of horizontal transfer between different *Trichogramma* species. The fact that within *Trichogramma* species several *Wolbachia* variants were found indicates that different *Wolbachia* strains can adapt to specific hosts (Table 1). The cases where natural horizontal transfer was shown so far resulted of *Trichogramma* species from mixed population (Huigens *et al*. 2000; *Chapter* 5). When attempts of horizontal transfer were done using different *Trichogramma* hosts, as in the case of *T. atopovirilia, Wolbachia* transmission was not successful (*Chapter 5*). The lack of congruence between the

phylogenetic trees of the host species with the *Wolbachia* tree shown by Schilthuizen & Stouthamer (1997) indicates that horizontal transfer of *Wolbachia* must have occurred on an evolutionary time scale.

The occurrence of similar *Wolbachia* retrieved from unrelated hosts confirms the notion that *Wolbachia* are sometimes transmitted horizontally. However, in many cases it remains unclear how such transfers could have taken place, because the connection between the species is obscure (van Meer 1999). Intraspecific natural horizontal transmission in *Trichogramma* was reported by Huigens *et al*. (2000). Interspecific transfer has also been shown in this thesis (*Chapter 5*). *Wolbachia* transmission has been possible when infected larvae of *Trichogramma* share a common host with uninfected ones. However, the process by which uninfected *Trichogramma* larvae acquire *Wolbachia* remains unclear.

Acknowledgments

We thank to all those who sent *Trichogramma* species: Americo I. Ciociola Junior (EPAMIG-Brazil), Isabel M.M.M. Silva (Wageningen University), M.E. Huigues (Wageningen University), and Ranyse B.Q. da Silva (ESALQ-USP-Brazil). This phylogenetic study was financially supported by Embrapa (PRODETAB) and the Wageningen University Graduate School Production Ecology & Resource Conservation (PE&RC) (R.P.A).

References

Almeida, R.P. de, A.I. Ciociola Jr. & R. Stouthamer 2001. *Wolbachia*-induced parthenogenesis: the first report in a Brazilian *Trichogramma pretiosum* population. *Proc. Exper. Appl. Entomol.* 12: 41-44.

Arakaki, N., T. Miyoshi, H. Noda 2001. Wolbachia-mediated parthenogenesis in the predatory thrips *Fanklintothrips vespiformis* (Thysanoptera: Insecta). *Proc. R. Soc. Lond. B* 268: 1011-1016.

Bandi, C., T.J.C. Anderson, C. Genchi & M.L. Blaxter 1998. Phylogeny of *Wolbachia* in Filarial nematodes. *Proc. R. Soc. Lond. B* 265: 2407-2413.

Barr, A.R. 1980. Cytoplasmic incompatibility in natural populations of a mosquito *Culex pipiens. Nature* 282: 71-72.

Bouchon, D., T. Rigaud & P. Juchault 1998. Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. *Proc. R. Soc. Lond. B* 265: 1081-1090.

Bourtzis, K., S.L. Dobson, H.R. Braig & S.L. O'Neill 1998. Rescuing *Wolbachia* have been overlooked. *Nature* 391: 852-553.

Braig, H.R., W. Zhou, S.L. Dobson & S.L. O' Neill 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial ensosymbiont *Wolbachia pipientis. J. Bacteriol.* 180: 2373-2378.

Breeuwer, J.A.J. & G. Jacobs 1996. *Wolbachia*: intracellular manipulators of mite reproduction. *Exp. Appl. Acarol.* 20: 421-434.

Breeuwer. J.A.J., R. Stouthamer, D.A. Burns, D.A. Pelletier, W.G. Weisburg & J.H. Werren 1992. Phylogeny of cytoplasmic incompatibility microorganisms in the parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae) based on 16S ribosomal DNA sequences. *Insect Mol. Biol.* 1: 25-36.

Breeuwer. J.A.J. & J.H. Werren 1990. Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature* 346: 558-560.

Ciociola Jr., A.C., R.P. de Almeida, R.A. Zucchi & R. Stouthamer 2001. Detecção de *Wolbachia* em uma população telítoca de *Trichogramma atopovirilia* Oatman & Platner

(Hymenoptera: Trichogrammatidae) via PCR com o primer específico *wsp*. *Neotrop. Entomol.* 30: 489-491.

Dedeine, F., F. Vavre, Fleury, F., Loppin, B., Hochberg, M. E., Bouletreau, M. 2001. Removing symbiotic Wolbachia bacteria specifically inhibits oogenesis in a parasitic wasp. *Proc. Nat. Acad. Sci. USA* 98: 6247-6252.

Dyson, E.A., M.K. Kamath & G.D.D. Hurst 2002. *Wolbachia* infection associated with all-female broods in *Hypolimnas bolina* (Lepidoptera: Nymphalidae): Evidence for horizontal transmission of a butterfly male killer. *Heredity* 88: 166-171.

Giordano, R., S.L. O'neill & H.M. Robertson 1995. *Wolbachia* infections and the expression of cytoplasmic incompatibility in *Drosophiia sechellia* and *D. mauritiana. Genetics* 140: 1307- 1317.

Girin, C. & M. Boulétreau 1995. Microorganism-associated variation in host infestation efficiency in a parasitoid wasp *Trichogramma bourarachae* (Hymenoptera: Trichogrammatidae). *Experientia* 52: 398-401.

Grenier S., B. Pintureau, A. Heddi, F. Lassablière, C. lager, C. Louis & C. Khatchadourian 1998. Successful horizontal transfer of *Wolbachia* symbionts between *Trichogramma* wasps. *Proc. R. Soc. Lond. B*. 265: 1441-1445.

Gottlieb, Y., E. Zchori-Fein, J.H. Werren & T.L. Karr 2002. Diploidy restoration in Wolbachia-infected Muscidifurax uniraptor (Hymenoptera: Pteromalidae). *J. Invertebr. Pathol*. 81: 166-174.

Hall, T. 1999. Biological sequence alignment editor for Windows 95/98/NT (*BioEdit v5.0.9*. program). <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>.

Hertig, M. 1936. The Rickettsia *Wolbachia pipientis* (gen. et sp. n.) and associated inclusions of the mosquito *Culex pipiens. Parasitology* 28: 453-490.

Hoffmann. A.A, M. Turelli & G.M. Simmons 1986. Unidirectional incompatibility between

populations of *Drosophila simulans. Evolution* 40: 692-701.

Holden, P.R., J.F. Brookfield & P. Jones 1993. Cloning and characterization of a *ftsZ* homologue from a bacterial symbiont of *Drosophila melanogaster*. *Mol. Gen. Genet.* 240: 213-220.

Huigens, M.E., R.F. Luck, R.H.G. Klaassen, M.F.P.M. Maas, M.J.T.N. Timmermans & R. Stouthamer 2000. Infectious parthenogenesis. *Nature* 405: 178-179.

Hurst, G.D.D., L.D. Hurst & M.E.N. Majerus 1997. Cytoplasmic sex ration distorters. In: O'Neill, S., J.H. Werren & A.A. Hoffmann (eds.), *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction*, Oxford University Press, New York, pp. 125-154.

Johanowicz, D. L. & M.A. Hoy 1996. Molecular evidence for a *Wolbachia* endosymbiont in the predatory mite *Metaseiulus occidentalis*. *Ann. Ento. Soc. Am.* 89: 435-441.

Juchault, P., M. Frelon, D. Bouchon & T. Rigaud 1994. New evidence for feminizing bacteria in terrestrial isopods. *C. R. Acad. Sci.* 317: 225-230.

Juchault, P., T. Rigaud &. J.P. Mocquard 1992. Evolution of sex-determining mechanisms in a wild population of *Armadillidium vulgare* Latr. (Crustacea, Isopoda): competition between two feminizing parasitic sex factors. *Heredity* 69: 382-390.

Laven, H. 1951. Crossing experiments with *Culex* strains. *Evolution* 5: 370-375.

Laven, H. 1967. Eradication of *Culex pipiens fatigans* through cytoplasmic incompatibility. *Nature* 216: 383-384.

Legrand, J.J. & P. Juchault 1986. Rôle de bactéries symbiotiques dans l'intersexualité, la monogénie et la spéciation chez des Crustacés Oniscoïdes. *Boll. Zool.* 53: 161-172.

Martin, G., P. Juchault & J.J. Legrand 1973. Mise eu évidence d'un microorganisme intracytoplasmatique symbiote de l'Oniscoïde *Armadillidium vulgare* Latr. dont la présence accompagne l'intersexualité ou la féminisation totale des mâles génétiques de

la lignée thélygène. *C. R. Acad. Sci.* 276: 2312-2316.

Martin, G., S.G. Gruppe, M. Laulier, D. Bouchon, T. Rigaud & P. Juchault 1994. Evidence for *Wolbachia* spp. in the estuarine isopod *Spaeroma rugicauda* (Crustacea): a likely cytoplasmic sex ratio distorter. *Endocytobiosis Cell Res.* 10: 215-225.

Masui, S., T. Sasaki & H. Ishikawa 1997. GroE-Homologous operon of *Wolbachia,* an intracellular symbiont of arthropods: a new approach for their phylogeny. *Zool. Sci.* 14: 701-706.

Meer, M.M.M. van 1999. Phylogeny and host-symbiont interactions of thelytokous inducing *Wolbachia* in Hymenoptera. Ph.D. thesis, Wageningen Agricultural University, The Netherlands, 118 pp.

Meer, M.M.M. van, J. Witteveldt & R. Stouthamer 1999. Phylogeny of the arthopod endosymbiont *Wolbachia* based on the *wsp* gene. *Insect Mol. Biol.* 8: 399-408.

O'Neill, S.L. & T.L. Karr 1990. Bidirectional incompatibility between conspecific populations of *Drosophila simulans. Nature* 348: 178-180.

O'Neill, S.L., R. Giordano, A.M.E. Colbert, T.L. Karr & H.M. Robert son 1992. 165 RNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmatic incompatibility in insects. *Proc. Nat. Acad. Sci. USA* 89: 2699-2702.

Pinto, J.D. 1998. Systematics of the North American species of *Trichogramma* (Hymenoptera: Trichogrammatidae). Mem. Entomol. Soc. Wash. Allen Press Inc., Washington D.C. n. 22. 287p.

Pintureau, B., S. Chaudier, F. Lassablière, H. Charles & S. Grenier 2000. Addition of *wsp* sequences to the *Wolbachia* phylogeny tree and stability of the classification. *J. Mol. Evol.* 51: 374-377.

Querino, R.B. & R.A. Zucchi 2003a. New species of *Trichogramma* Westwood (Hymaneoptera: Trichogrammatidae) associated with lepidopterous eggs in Brazil.

Zootaxa 163: 1-10.

Querino, R.B. & R.A. Zucchi 2003b. Six new species of *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) from a Brazilian forest reserve. *Zootaxa* 134: 1-11.

Rigaud, T., C. Souty-Grosset, R. Raimond, J.P. Mocquard & P. Juchault 1991. Feminizing endocytobiosis in the terrestrial crustacean *Armadillidium vulgare* Latr. (Isopoda): recent acquisitions. *Endocytobiosis Cell. Res*. 7: 259-273.

Rousset, F., D. Bouchon, B. Pintureau, P. Juchault & M. Solignac 1992. *Wolbachia* endosymbionts responsible for various sexual alterations of sexuality in arthropods. *Proc. R. Soc. Lond. B*. 250: 91-98.

Schilthuizen, M. & R. Stouthamer 1997. Horizontal transmission of parthenogenesis-inducing microbes in *Trichogramma* wasps. *Proc. R. Soc. Lond B* 264: 361-366.

Schulenburg, J.H.G. von der, G.D.D. Hurst, T.M.E. Huigens, M.M.M. van Meer, F.M. Jiggins & M.E.N. Majerus 2000. Molecular evolution and phylogenetic utility of *Wolbachia ftsZ* and *wsp* gene sequences with special reference to the origin of Male-killing. *Mol. Biol. Evol.* 17: 584-600.

Sironi, M., C. Bandi, L. Sacchi, B. Di Sacco, B. Damiani & C. Genchi 1995. Molecular evidence for a close relative of the arthropod endosymbiont *Wolbachia* in a filarial worm. *Mol. Biochem. Parasitol.* 74: 223-227.

Stouthamer, R., J.A.J. Breeuwer, R.F. Luck & J.H. Werren 1993. Molecular identification of microorganisms associated with parthenogenesis. *Nature* 361: 66-68.

Stouthamer, R. & D.J. Kazmer 1994. Cytogenetics of microbe-associated panthenogenesis and its consequences for gene flow in *Trichogramma* wasps. *Heredity* 73: 317-327.

Stouthamer, R., R.F. Luck & W.D. Hamilton 1990a. Antibiotics cause parthenogenetic *Trichogramma* to revert to sex. *Proc. Natl. Acad. Sci. USA* 87: 2424-2427.

Stouthamer, R., J.D. Pinto, G.R. Platner & R.F. Luck 1990b. Taxonomic status of

thelytokous forms of *Trichogramma. Ann. Entomol. Soc. Am.* 83: 475-581.

Swofford, D. 1999. PAUP 4.0b2a. Computer program distributed by the Sinauer Associates, Inc. Publishers, Sunderland, Massachussets.

Tsagkarakou, A., T. Guillemaud, F. Rousset & M. Navajas 1996. Molecular identification of a *Wolbachia* endosybiont in a *Tetranychus urticae* strain (Acari: Tetranychidae). *Insect Mol. Biol.* 5: 217-221.

Vandekerckhove, T., S. Watteyne, A. Willems, J.G. Swing, J. Mertens & M. Gillis 1999. Phylogenetic analysis of the 16S rDNA of the cytoplasmic bacterium *Wolbachia* from the novel host *folsomia candida* (Hexapoda, Collembola) and its implications for wolbachial taxonomy. *FEMS Microbial. Lett.* 180: 279-286.

Vavre, F., C. Girin & M. Boulétreau 1999a. Phylogenetic status of a fecundity-enhancing *Wolbachia* that does not induce thelytoky in *Trichogramma*. *Insect Mol. Biol.* 8: 67-72.

Vavre, F., F. Fleury, D. Lepetit, P. Fouillet & M. Boulétreau 1999b. Phylogeny evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. *Mol. Biol. Evol.* 16: 1711-1723.

Werren, J.H. 1997. Biology of *Wolbachia. Ann. Rev. Entomol.* 42: 587-609.

Werren, J.H. & D.M. Windsor 2000. *Wolbachia* infection frequences in insects: evidence of a global equilibrium? *Proc. R. Soc. Lond. B* 267: 1277-1285.

Werren, J.H., D.M. Windsor & L.R. Guo l995a. Distribution of *Wolbachia* among neotropical arthropods. *Proc. R. Soc. Lond. B.* 262: 197-204.

Werren, J.H., W. Zhang & L.R. Guo 1995b. Evolution and phylogeny of *Wolbachia:* reproductive parasites of arthropods. *Proc. R. Soc. Lond. B* 261: 55-71.

West, S.A., J.M. Cook, J.H. Werren & C.J. Godfray 1998. *Wolbachia* in two insect host-parasitoid communities. *Mol. Ecol.* 7: 1457-1465.

Yen, J.H. & A.R. Barr 1974. Incompatibility in *Culex pipiens.* In: R. Pal & M. J. Whilem

(eds.), Elsevier, Amsterdam, pp. 97-118.

Yen, J.H. 1975. Transovarial transmission of Richettsia-like microorganisms in mosquitoes. *Ann. NY Acad. Sci.* 266: 152-161.

Zhou, W., F. Rousset & S.L. O'Neill 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. *Proc. R. Soc. Lond. B* 265: 509-515.

Zchori-Fein, E., R.T. Roush & M. Hunter 1992. Male production induced by antibiotic treatment in *Encarsia formosa,* an asexual species. *Experientia* 48: 102-105.

Zucchi, R.A. & R.C. Monteiro 1997. O gênero *Trichogramma* na América do Sul. In: Parra, J.R.P. & R.A.Zucchi (eds.), *Trichogramma e o controle biolólgico aplicado*, FEALQ, Piracicaba, pp. 41-66.

Chapter

5

Natural inter- and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps

M. E. Huigens, Raul P. de Almeida, P. Boons,
Robert F. Luck & Richard Stouthamer

Chapter 5

NATURAL INTER- AND INTRASPECIFIC HORIZONTAL TRANSFER OF PARTHENOGENESIS-INDUCING *Wolbachia* IN *Trichogramma* WASPS

Abstract

The intracellular bacterium *Wolbachia* is one of the most common symbionts in arthropods and, because of its manipulative effects on host reproduction, assumed to be an important factor in several evolutionary processes. These bacteria are mainly vertically transmitted from mother to daughter through the egg cytoplasm and horizontal transmission is generally assumed to be rare. Here, we show natural inter and intraspecific horizontal transfer of parthenogenesis-inducing (PI) *Wolbachia* between parasitoid wasps of the genus *Trichogramma*. Horizontal transfer was observed when infected and uninfected larvae shared the same host egg. This is the first report on interspecific horizontal transfer of *Wolbachia* between closely related sympatric species. Originally uninfected immature wasps could acquire *Wolbachia* inside the host egg but not all newly infected females also exhibited the parthenogenesis phenotype. In general, intraspecific horizontal transfer was more successful than interspecific transfer. *Wolbachia* could undergo vertical transmission in a new species but infection tended to be lost several generations. Our results have important implications to understand the evolution of *Wolbachia*-host associations.

5.1. Introduction

Prevalence of *Wolbachia* is estimated at 17-76% of the insect species (Werren *et al*. 1995a; Jeyaprakash & Hoy 2000). This endosymbiont has received much attention mainly because it has evolved several alterations of its host reproduction, thereby optimizing its vertical cytoplasmic inheritance. These alterations include cytoplasmic incompatibility (CI) (Yen & Barr 1971; Hoffmann *et al*. 1986; Breeuwer & Werren 1990; O 'Neill & Karr 1990), feminization (Rigaud *et al*. 1991), male-killing (Hurst *et al*. 1999) and parthenogenesis-induction (PI) (Stouthamer *et al*. 1990; Stouthamer *et al*. 1993).

It is generally assumed that vertically inherited symbionts cospeciate with their host but this is certainly not the case for *Wolbachia.* Phylogenetic evidence showed that horizontal transfer of these bacteria must have occurred in the course of evolution because closely related bacterial strains can be found in unrelated hosts (O 'Neill *et al*. 1992; Rousset *et al*. 1992; Stouthamer *et al*. 1993; Werren *et al*. 1995b). Similarly, micro-injection studies successfully transferred *Wolbachia* into naïve hosts both intra- and interspecifically (Boyle *et al*. 1993; Braig *et al*. 1994; Grenier *et al*. 1998). Recently, Fujii *et al*. (2001) were able to show that, after transfection, a single *Wolbachia* strain can induce different phenotypes in different hosts. In general it is however very difficult to maintain a high infection rate over many generations in newly infected lines (Pintureau *et al*. 2000b; van Meer & Stouthamer 1999; McGraw *et al*. 2002).

In nature, horizontal transfer can only occur when a donor and a recipient host are in close confinement, since *Wolbachia* is assumed not to survive outside host tissues. *Wolbachia* were first semi-naturally transferred in woodlice via blood-blood contact (Rigaud & Juchault 1995). Such transfer might occur in nature when individuals become injured during crowding. Close confinement is certainly the case in host-parasitoid

associations. Two phylogenetic studies revealed the possibility for more frequent horizontal transfers in such communities (Schilthuizen & Stouthamer 1997; Vavre *et al*. 1999). Heath *et al*. (1999) thereafter showed a natural host-parasitoid transfer from an infected host (*Drosophila simulans*, where *Wolbachia* induces CI) to a parasitoid wasp (*Leptopillina boulardi*). Frequent natural horizontal transfer between conspecifics was first shown in the parasitoid wasp *Trichogramma kaykai* (Huigens *et al*. 2000). When PI *Wolbachia*-infected and uninfected *T. kaykai* larvae share the same host, originally uninfected larvae can acquire the infection. Newly infected females thereafter produce daughters from their unfertilized eggs. It is however still unknown how common such horizontal transfer is. Here, we investigate intra- and interspecific natural horizontal transfer in several *Trichogramma* species.

Trichogramma wasps display a haplo-diploid mode of reproduction in which daughters (diploid) arise from fertilized eggs and sons (haploid) from unfertilized eggs. *Wolbachia* is known to induce parthenogenesis in at least 16 *Trichogramma* species (see *Chapter 1*) of 190 nominal species known (Pinto 1998; Querino & Silva 2003a, b). Females infected with PI *Wolbachia* produce daughters from both their fertilized and unfertilized eggs. In unfertilized infected eggs, a modification of the anaphase in the first mitotic division causes a doubling of the haploid set of maternal chromosomes, a process called gamete duplication (Stouthamer & Kazmer 1994). Such parthenogenetic reproduction can be cured after antibiotic treatment (Stouthamer *et al*. 1990).

Wolbachia in *Trichogramma* are unique compared to almost all other *Wolbachia*-host associations because "*Trichogramma Wolbachia"* cluster together in all phylogenetic trees based on several *Wolbachia* specific genes (Stouthamer *et al*. 1999a). Phylogenetic analysis of the *Wolbachia-Trichogramma* association revealed an obvious discordance between *Trichogramma* and *Wolbachia* phylogenies that is most likely explained by horizontal

transfer of *Wolbachia* (Schilthuizen & Stouthamer 1997). Such horizontal transfer might take place when different species use the same host egg (Huigens *et al*. 2000). Horizontal transfer can even result in double or triple infections when wasps infected with different *Wolbachia* oviposit in the same host egg. Multiple infections have not yet been described in *Trichogramma* but are known from several other host species, mainly associated with CI-*Wolbachia* (Werren 1997). Multiple infection opens the way for recombination between different *Wolbachia.* Such genetic exchange has been shown in the *Wolbachia* surface protein (*wsp*) gene of several strains of *Wolbachia* (Jiggins *et al*. 2001; Werren & Bartos 2001).

Here, we study natural intra- and interspecific horizontal transfer of PI *Wolbachia* in three situations: (1) superparasitism, in which larvae of an infected and an uninfected mother of a single species share the same host egg; (2) multiparasitism, in which larvae of an infected and an uninfected mother of different species share the same host egg; and (3) multiparasitism, but now when larvae of two infected mothers of different species share the same host egg.

5.2. Material and Methods

Trichogramma *cultures*

Intra- and interspecific horizontal transfer were attempted using iso-female lines of four *Trichogramma* species: *T. kaykai, T. deion, T. pretiosum* and *T. atopovirilia*.

T. kaykai and *T. deion* lines were initiated with wasps collected on eggs of the butterfly *Apodemia mormo deserti* in the Mojave Desert, CA, USA. In both species infected and uninfected females coexist. A study on the *ftsZ* gene of *Wolbachia* in the two species revealed that *Wolbachia* in *T. kaykai* most likely originates from a single infection

(Schilthuizen *et al*. 1998). The two infected (LC 19-1 and LC 10-1) and three uninfected *T. kaykai* lines (LC 105A, LC 19-1 cured and LC 110 cured) all originate from Last Chance Canyon, El Paso Mountains, Kern County, California. Of *T. deion* we used an infected (SW 436-1) and an uninfected line (SW 649) from Sidewinder Mnts, Kern County, California, an uninfected line (LC 151) from Last Chance Canyon, El Paso Mountains, Kern County, California and an infected line (223) initiated with wasps collected at Sanderson, Texas. The infected *T. pretiosum* line (Tpre-13) was collected in Santa Catarina, Brasil (host species unknown). The infected (Tato-01) and uninfected (Tato-02) *T. atopovirilia* were collected in Minas Gerais State, Brazil and in Colombia respectively (host species unknown). The infection status of the natural *T. pretiosum* and *T. atopovirilia* populations is unknown. *Trichogramma* lines were cultured on eggs of the moth *Ephestia kuehniella* for many generations before the experiments were conducted.

Intra- and interspecific horizontal transfer

Intraspecific horizontal transfer of *Wolbachia* was attempted by giving an infected (donor) and an uninfected (recipient) female of a single species the opportunity to oviposit in the same host egg. This superparasitism was carried out with three species *T. kaykai, T. deion* and *T. atopovirilia.* Here, *T. kaykai* was used as a control because horizontal transfer should occur in this species (Huigens *et al.* 2000 3).

Interspecific horizontal transfer was determined by allowing (1) infected *T. kaykai* and uninfected *T. deion*; (2) infected *T. deion* and uninfected *T. kaykai*; and (3) infected *T. pretiosum* and infected *T. atopovirilia* females the opportunity to parasitize the same host egg. The latter multiparasitism might result in females carrying two different *Wolbachia* strains, i.e. a double infection.

Test for horizontal transfer of Wolbachia

A moth egg, either *Trichoplusia ni* or *Mamestra brassicae*, both uninfected hosts, was offered to a female ('line A') and two hours later, we exposed the same egg to a second female ('line B'). The latter female was either a conspecific (superparasitism) or a congener (multiparasitism). In eggs of both lepidopteran species, a *Trichogramma* female usually lays a clutch of 2-4 eggs. In half the cases, a female from 'line A' was offered the egg first and in the other half the order was reversed. We observed and recorded the number of eggs oviposited in a moth egg by each female using behavioral criteria described by Suzuki *et al*. (1984). If only F1 females emerged from a super- or multiparasitized egg, we exposed these virgin F1 females individually to host eggs and recorded the sex of their progeny.

The F1 females were linked to their parental female using a molecular marker and, in the multiparasitism experiments, female body color. To determine the origin of the F1 females in the superparasitism combinations we used a microsatellite DNA repeat TTG 49 for *T. kaykai,* a TAC 47 microsatellite repeat for *T. deion* and specific primer for DNA amplification of the ITS2 region for *T. atopovirilia*. In infected *T. atopovirilia* one DNA fragment is amplified whereas in uninfected *T. atopovirilia* two fragments of different size are amplified. In the multiparasitism combinations, we could easily distinguish the F1 females of the different species by the female body color. Females of *T. kaykai* have a yellow body color and *T. deion* females are brown. The *T. pretiosum* and *T. atopovirilia* females used in the experiments are respectively yellow and black.

The F1 females from the recipient line were tested for the presence of *Wolbachia* by PCR using *wsp* primers (Braig *et al*. 1998). To confirm the horizontal transfer, amplified *wsp* genes were sequenced in the donor lines and in the newly infected females. The presence of daughters in the offspring of an F1 virgin originating from a recipient line indicated horizontal transfer of *Wolbachia* and subsequent PI.

In the test for double infection when infected *T. pretiosum* and infected *T. atopovirilia* larvae share the same host egg, the amplified *wsp* fragments of *Wolbachia* in both species were distinguished using the restriction enzymes MboI and MboII. A combination of the restriction patterns in F1 *T. pretiosum* or *T. atopovirilia* females confirms a double infection.

Molecular techniques

DNA extraction was performed using one wasp homogenized in 50 µl 5% Chelex-100 and 2 µl proteinase K (20 mg/ml) and incubated for at least 4 hours at 56°C, followed by 10 min at 95°C. PCR reactions were performed in a total volume of 25 µl using a Techne thermocycler, 2.5 µl DNA template, 2.5 µl 10x PCR-buffer, 0.5 µl dNTP's (each in a 10 mM concentration), 0.5 µl forward and reverse primers, 0.07 µl TAQ polymerase (5 units/µl) and 18.43 µl of sterile distilled water. Primers sequences and cycling programs were: (1) *wsp*-Forward primer 5'-TGGTCCAATAAGTGATGAAGAAAC-3' and *wsp*-Reverse 5'-AAAAATTAAACGCTACTCCA-3' (Braig *et al*. 1998). Cycling program: 3 min. at 94°C followed by 40 cycles of 1 min. at 94°C, 1 min. at 50°C and 1 min. at 72°C with 5 min. at 72°C after the last cycle; (2) TTG 49-forward primer 5'-GTAGTCTGGTTTTCGATTCCCA-3' and TTG 49-reverse primer 5'-TCCCCGACCTATCGATTTTCC-3' (Stouthamer unpublished). Cycling program: 5 min. at 94°C followed by 45 cycles of 1 min. at 94°C, 1 min. at 63°C and 1 min. at 72°C with 5 min at 72°C after the last cycle; (3) TAC 47-forward primer 5'-CTACGGCGACAATTGCCAC-3' and TAC 47-reverse primer 5'-CATCTTGGTC GAACCGAGCAG-3' (Stouthamer unpublished). Cycling program: 5 min. at 94°C followed by 30 cycles of 1 min. at 94°C, 1 min. at 65°C and 1 min. at 72°C with 5 min. at 72°C after the last cycle; and (4) ITS2-forward primer 5'-TGTGAACTGCAG

GACACATG-3' and ITS2-reverse primer 5'-GTCTTGCCTGCTCTGCTCTGAG-3' (Stouthamer *et al*. 1999b). Cycling program: 3 min. at 94°C followed by 33 cycles of 40 seconds at 94°C, 45 second at 53°C and 45 seconds at 72°C with 5 min. at 72°C after the last cycle. All PCR products were run on a standard 1.5 % agarose gel.

Cloning, sequencing and alignments of the *wsp* genes of *Wolbachia* in donor lines and in newly infected females were done. PCR products were purified with a QIAquick PCR purification kit (Qiagen®) and ligated into a Pgem-T® Vector (Promega). After transformation a PCR reaction was performed to confirm if a correct piece of DNA had been cloned. To purify the plasmid we used a QIAprep Miniprep kit (Qiagen®). Sequencing was performed in an Applied Biosystems automatic sequencer. *Wolbachia* sequences were aligned manually using the ESEE 3.0s sequence editor (Cabot 1995).

Restrictions of the *wsp* genes using the enzymes MboI and MboII were carried out to confirm double infection in *T. atopovirilia* and *T. pretiosum* F1 females after they shared the same host egg. The sizes of the different digestions products were estimated using the Webcutter 2.0 program (Heiman 1997). To perform a restriction of the *wsp* fragments, 10-µl volume (5 µl PCR product, 1 µl (10X) reaction buffer, 1 µl restriction enzyme and 3 µl distilled water) was used and incubated for 1 h at 37°C. The digestion products were run on a standard 1.5% agarose gel. In *T. pretiosum* the use of the enzyme MboI generated in two cutting sites and three restriction fragments (266, 203 and 131 bp) of the *wsp* fragment and one cutting site and two restriction fragments (397 and 203 bp) in *T. atopovirilia*. With MboII the restriction of the *wsp* fragment resulted in one cutting sites and two restriction fragments (318 and 282 bp) in *T. pretiosum* and two cutting sites and three restriction fragments (218, 204 and 76 bp) in *T. atopovirilia*. For both enzymes, a restriction of the amplified *wsp* product from DNA template that was a mix of infected *T. atopovirilia* DNA and infected *T. pretiosum* DNA clearly showed a combination of the restriction patterns of

both *wsp* fragments.

5.3. Results and Discussion

Intra- and interspecific horizontal transfer of PI *Wolbachia* both occurred but not always. Depending on the super- or multiparasitism combination, 0-39% of the females acquired an infection inside the host egg (Table 1). Sequencing of the *wsp* fragments confirmed that *Wolbachia* in the newly infected *T. kaykai* and *T. deion* lines originated from the donor lines. The horizontal transfer rate might be underestimated in our tests because of a bacterial density below the threshold value necessary for the detection by PCR with *wsp* primers in some of the recipient F1 females.

Intraspecific horizontal transfer of PI Wolbachia

Both originally uninfected *T. kaykai* and *T. deion* larvae acquired an infection after sharing the host egg with infected conspecifics. Subsequent PI is much more efficient in newly infected *T. kaykai* females than in newly infected *T. deion* females. In *T. kaykai* 39 % of the originally uninfected F1 females became infected inside the host egg (17 of 44 were tested positive with *wsp*) and 88% of them produced some daughters from their unfertilized eggs. These results are similar to previous work described by Huigens *et al*. (2000). In *T. deion* 36 superparasitized host eggs resulted in only 11 all-female F1 broods consisting of 17 potential newly infected females. Twenty–nine percent of these F1 females were infected (5 of 17) and 1 of these newly infected virgins produced a few daughters. Intraspecific horizontal transfer in *T. kaykai* and in *T. deion* occurred independent of the order in which two *Trichogramma* females were allowed to oviposit in the host egg (respectively $\chi^2_{0.05,1} = 0.021$; p=0.886; n = 37 for *T. kaykai* and $\chi^2_{0.05,1} = 2.21$; p=0.137; n =

11 for *T. deion*). Only in *T. atopovirilia* we could not detect intraspecific horizontal transfer: Forty-seven all-female broods resulted in 80 potential newly infected F1 females but none of them were infected or produced daughters as a virgin (Table 1).

Interspecific horizontal transfer of PI Wolbachia

Interspecific horizontal transfer occurred from *T. kaykai* to *T. deion* and vice versa. Only newly infected *T. kaykai* females exhibited the parthenogenesis phenotype. Of *T. kaykai* 19 % of the F1 females acquired *Wolbachia* from *T. deion* (17 of 89) and 29 % of them produced at

Table 1. Inter- and intraspecific horizontal transfer of PI *Wolbachia* in *Trichogramma* sp.

Donor species females[1]	Recipient Species	Host egg (Number)	Recipient females tested	Horizontal transfer[1]	PI in newly infected
T. kaykai	*T. kaykai*	M (37)	44	39%[a]	88%[b]
T. deion	*T. deion*	T (11)	17	29%[ab]	20%[a]
T. atopovirilia	*T. atopovirilia*	M (47)	80	0%[2]	0%[2]
T. kaykai	*T. deion*	M (21)	26	12%[ab]	0%[a]
T. kaykai	*T. deion*	T (12)	13	8%[ab]	0%[a]
T. deion	*T. kaykai*	M (35)	59	19%[b]	0%[a]
T. deion	*T. kaykai*	T (21)	30	20%[ab]	83%[b]
T. pretiosum	***T. atopovirilia***	M (30)	95	0%[2]	0%[2]
T. atopovirilia	***T. pretiosum***	M (30)	120	0%[2]	0%[2]

[1] Chi square test; [2] Data not statistically analyzed; M = *Mamestra brassicae* and T = *Trichoplusia ni*; Bold entries indicate infection

least one daughter. Only 10% (4 of 39) of the *T. deion* F1 females acquired the *Wolbachia* from *T. kaykai*, but none of newly infected virgin produced some daughters. The host egg species, *T. ni* or *M. brassicae,* did not affect the interspecific horizontal transfer, or the percentage of the F1 females that acquired an infection inside the host egg, from *T. deion* to *T. kaykai* ($\chi^2_{0.05,1} = 0.36$; p=0.436; n = 56) nor vice versa ($\chi^2_{0.05,1} = 0.25$; p=0.614; n = 33). Like in the intraspecific transfer in these two species, interspecific horizontal transfer from *T. kaykai* to *T. deion* occurred independent of the order in which two *Trichogramma*

females were allowed to parasitize the host egg ($\chi^2_{0.05,1}$ = 1.01; p=0.316; n = 33). However, horizontal transfer occurred significantly more from *T. deion* to *T. kaykai* when *T. deion* was the first female to oviposit ($\chi^2_{0.05,1}$ = 12.08; p<0.001; n = 56).

We did not find any evidence for double infection in infected *T. pretiosum* and *T. atopovirilia* F1 females after they shared the same host egg. Hundred-twenty *T. pretiosum* females only carried the '*pretiosum Wolbachia*' and 95 *T. atopovirilia* females only the '*atopovirilia Wolbachia'* (Table 1).

Subsequent vertical transmission after interspecific horizontal transfer

We determined vertical transmission of *Wolbachia* and PI in subsequent generations in two *T. kaykai* lines that were newly infected with a "*deion Wolbachia*". In both cases, we detect infection and PI in the F2 but thereafter the infection already seems to have been lost in the F3, the sex ratio (% females) decreased (Table 2), and in the subsequent generations PI could be observed any longer. In one line, 15 infected virgin F5 females only produced sons as well as 10 virgin F4 and 10 virgin F9 females in the other line (Table 2).

Table 2. Vertical transmission of *Wolbachia* and PI in subsequent generations in two newly infected *Trichogramma kaykai* lines carrying a "*deion Wolbachia*".

Donor Species	Recipient Species	Sex ratio (% females)				
		F2	F3	F4	F5	Virgin females
T. deion	*T. kaykai*	0.82	0.37	0.79	0.85	F5: 0.00 (n=15)
T. deion	*T. kaykai*	0.87	0.74	0.76	-	F4: 0.00 (n=10) F9: 0.00 (n=10)

Bold entries indicate infection

These results are, to our knowledge, the first to show a natural interspecific horizontal transfer of PI *Wolbachia*, thereby partly explaining discordances between *Wolbachia* and

Trichogramma phylogenies (Schilthuizen & Stouthamer 1997). Both intra- and interspecific transfer should also occur in nature in *Trichogramma,* since these wasps are host-generalists and multiparasitism occurs (Pinto 1998). This study confirms the frequent intraspecific horizontal transfer previously observed in *T. kaykai* (Huigens *et al*. 2000). The interspecific horizontal transfer of PI *Wolbachia* between the sympatric species *T. kaykai* and *T. deion* should occur in nature because the two species have been found together in a single host egg in the Mojave desert: e.g. at Randsburg road, Kern County, California 3% of the parasitized *Manduca sexta* eggs was multiparasitzed by both species (Huigens unpublished data).

We certainly do not always find successful horizontal transfer in our experiments. For horizontal transfer to be successful, *Wolbachia* first need to attain a high density in the newly infected female, infect the ovaries, and subsequently be transferred vertically and induce PI. Unsuccessful horizontal transfer is most likely due to an incompatibility between *Wolbachia* and the host's nuclear/cytoplasmic background (Heath *et al*. 1999; Vavre *et al*. 1999). *Wolbachia* might be unable to adapt to a new nuclear background when it is not confronted with a new set of nuclear genes very frequently. Certainly in *T. kaykai* and most likely also in *T. deion*, *Wolbachia* is frequently confronted with a new nuclear background. In *T. kaykai*, we find 6-26% of the females to be infected (Stouthamer *et al*. 2001) and in *T. deion* only 2 of 229 broods were the offspring of an infected female (Huigens 2003). We know that most infected *T. kaykai* females also mate with uninfected males in the population (Kazmer 1992; Huigens 2003). *Wolbachia* from populations in which there is frequent gene flow into the infected population are therefore selected to adapt relatively easy to new nuclear backgrounds. After horizontal transfer such PI *Wolbachia* can be transmitted vertically and induce PI in a new host whereas PI *Wolbachia* from fixed populations cannot adapt to such a new situation. The fact that both the '*deion Wolbachia'*

and the *'kaykai Wolbachia'* can be transmitted to another species supports this idea. The infected *T. atopovirilia* and *T. pretiosum* lines used in our experiments might be from a population fixed for the infection, i.e. all females in the population are infected, explaining why these *Wolbachia* are not easily transmitted horizontally. At least, they do not even rise up to densities detectable with the PCR method used. In the future, double infection resulting from infected females sharing the same host egg should be tested with *Wolbachia*-host associations where infected and uninfected individuals coexist, e.g. infected *T. kaykai* and infected *T. deion*.

Another explanation for the fact that we only observe interspecific horizontal transfer between *T. kaykai* and *T. deion* might be the host phylogeny. These two North American species are closely related (Schilthuizen & Stouthamer 1997; Pinto 1998) in contrast to *T. pretiosum* and *T. atopovirilia* (Pinto 1998; Almeida unpublished results). The same goes for the *Wolbachia*'s in the species. Horizontal transfer of PI *Wolbachia* between more distant host species most likely occurs in nature (Schilthuizen & Stouthamer 1997) but might be a rare event, undetectable in our experiments.

Bacterial density is important for successful horizontal transfer of PI *Wolbachia* (Grenier *et al*. 1998). *Wolbachia* density effects have previously been shown on CI expression in *Nasonia* and *Drosophila* (Breeuwer & Werren 1993; Karr 1994; Bourtzis *et al*. 1996). In this study, the fact that newly infected virgin females never produce exclusively daughters and the large variation in sex ratio of their offspring suggest a density effect (see also Huigens *et al*. 2000). We only tested for horizontal transfer in virgin recipient F1 females and therefore indirectly selected for high bacterial density. Insufficient bacterial density in newly infected virgins to induce PI immediately causes the *Wolbachia* not to be transmitted to subsequent generations. In future work we should test mated recipient F1 females. In that case fertilized eggs with a relatively low *Wolbachia* titer will become females, this will

allow the *Wolbachia* to attain a high enough titer to be transmitted and to express itself over the subsequent generations.

When we compare the PI *Wolbachia-Trichogramma* association with the feminizing (F) *Wolbachia*-isopod association, we see some clear similarities. Rigaud *et al*. (2001) described the same pattern of interspecific horizontal transfer of *Wolbachia* when they semi-naturally transferred F *Wolbachia* between two closely related isopod species. Genetic sons of newly infected females were successfully feminized but maintenance of infection and feminization in future generations remains to be studied. Interspecific transfer between phylogenetically distant isopod species was however unsuccessful. In both *Wolbachia*-host associations, *Wolbachia* seem to form a monophyletic group. This applies to *Trichogramma-Wolbachia* (Schilthuizen & Stouthamer 1997; *Chapter 4*) and also to most isopod-*Wolbachia*. The *Wolbachia* seem to have a common descent and the discordance between the *Wolbachia*- and host phylogenies shows that *Wolbachia* have shifted between species in both host groups (Schilthuizen & Stouthamer 1997; Bouchon *et al*. 1998; Cordaux *et al*. 2001). In both associations the host populations are not fixed for infection (Stouthamer 1997; Rigaud 1997) which should facilitate horizontal transfer as described above. Also, both *Trichogramma* wasps and isopods have a gregarious behavior that offers excellent opportunities for natural intra- and interspecific horizontal transfer. These factors together may be the cause of the relatively high rate and success of horizontal transfers in both *Wolbachia*-host associations.

In conclusion, intra- and interspecific horizontal transfers of *Wolbachia* should occur in nature between organisms that interact in close confinement. We may have underestimated (i) the rate of natural horizontal transfer of PI *Wolbachia* in *Trichogramma* due to our PCR detection method and (ii) the subsequent vertical transmission because we only detect PI in the cases where bacterial density is high in newly infected virgins. Subsequent vertical

transmission most likely limits the successful spread of newly acquired infections (Cook & Butcher 1999; Heath *et al*. 1999; Rigaud 1997, Pintureau *et al*. 2000b), which is also indicated by the loss of infection in two *T. kaykai* lines newly infected with a '*deion Wolbachia'*. Such unsuccessful horizontal transfer is most likely due to incompatibilities between *Wolbachia* and the host's nuclear/cytoplasmic background. Such incompatibilities clearly exist in nature, otherwise experimental horizontal transfers must be extremely easy to obtain and research has shown that this is generally not the case. Although subsequent vertical transmission after horizontal transfer may occur at a very low rate (almost undetectable in laboratory experiments), on an evolutionary time scale it might be frequent enough to explain the discordance between *Wolbachia*- and host phylogenies.

Acknowledgements

We thank Fabrice Vavre, Gilsang Jeong and the Entomology Ph.D. discussion group in Wageningen for discussions and ideas. Joop van lenteren provided useful comments on an earlier version of the manuscript. Patrick Verbaarschot is thanked for his assistance with the molecular work and Leo Koopman, André Gidding and Frans van Aggelen for culturing some of the wasp lines. We are also grateful to Américo I. Ciociola Jr. for sending *Trichograma atopovirilia* and *T. pretiosum* lines. This work was supported by Earth and Life Sciences Foundation (ALW) (M.E.H. & R.S.), Embrapa (Brazilian Agricultural Research Corporation)/PRODETAB (R.P.A) and the graduate school Production Ecology & Resource Conservation of the Netherlands (M.E.H. & R.P.A.).

References

Bouchon, D., T. Rigaud. & P. Juchault 1998. Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. *Proc. R. Soc. Lond. B* 265: 1081-1090.

Bourtzis, K., A. Nirgianaki, G. Markakis & C. Savakis 1996. *Wolbachia* infection and cytoplasmic incompatibility in *Drosophila* species. *Genetics* 144: 1063-1073.

Boyle, L., S.L. O'Neill, H.M. Robertson & T.L. Karr 1993. Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila*. *Science* 260: 1796-1799.

Braig, H.R., H. Guzman, R.B. Tesh & S.L. O'Neill 1994. Replacement of the natural *Wolbachia* symbiont of *Drosophila simulans* with a mosquito counterpart. *Nature* 367: 453-455.

Braig, H.R., W. Zhou, S. Dobson & S.L. O'Neill 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia*. *J. Bacteriol*. 180: 2373-2378.

Breeuwer, J.A.J. & J.H. Werren 1990. Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature* 346: 453-455.

Breeuwer, J.A.J. & J.H. Werren 1993. Cytoplasmic incompatibility and bacterial density in *Nasonia vitripennis*. *Genetics* 135: 565-574.

Cabot, E.L. 1995. "The Eyeball Sequence Editor (ESEE)," version 3.0s Dept. Biology, Univ. Rochester, Rochester, NY.

Ciociola Jr., A.C., R.P. de Almeida, R.A. Zucchi & R. Stouthamer 2001. Detecção de *Wolbachia* em uma população telítoca de *Trichogramma atopovirilia* Oatman and Platner (Hymenoptera: Trichogrammatidae) via PCR com o Primer Específico *wsp*. *Neotrop. Entomol*. 30: 489-491.

Cordaux, R., A. Michel-Salzat & D. Bouchon 2001. *Wolbachia* infection in crustaceans: novel hosts and potential routes for horizontal transmission. *J. Evol. Biol.* 14: 237-243.

Cook, J.M. & R.J. Butcher 1999. The transmission and effects of *Wolbachia* bacteria in parasitoids. *Res. Popul. Ecol.* 41: 15-28.

Fujii, Y., D. Kageyama, S. Hoshizaki, H. Ishikawa & T. Sasaki 2001. Transfection of *Wolbachia* in Lepidoptera: The feminizer of the adzuki bean borer *Ostrinia scapulalis* causes male killing in the Mediterranean flour moth *Ephestia kuehniella*. *Proc. R. Soc. Lond. B* 268: 855-859.

Grenier, S, B. Pintureau, A. Heddi, F. Lassablière, C. Jager, C. Louis & C. Khatchadourian 1998. Successful horizontal transfer of *Wolbachia* symbionts between *Trichogramma* wasps. *Proc. R. Soc. Lond. B* 265: 1441-1445.

Heath, B.D., R.J. Butcher, W.G.F. Whitfield & S.F. Hubbard 1999. Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a natural occurring mechanism. *Curr. Biol.* 9: 313-316.

Heiman, M. 1977. Webcutter 2.0. <http://www.firstmarket.com/cutter/cut2.html>. Accessed 2002 Oct. 15.

Hoffman, A.A., M. Turelli & G.M. Simmons 1986. Unidirectional incompatibility between populations of *Drosophila simulans*. *Evolution* 40: 692-701.

Huigens, M.E., R.F Luck., R.H.G. Klaassen, M.F.P.M. Maas, M.J.T.N. Timmermans & R. Stouthamer. 2000. Infectious parthenogenesis. *Nature* 405: 178-179.

Hurst, G.D.D., F.M. Jiggins, J.H.G. von der Schulenburg, D. Bertrand, S.A. West, I.I. Goriacheva, I.A. Zakharov, J.H. Werren, R. Stouthamer & M.E.N. Majerus 1999. Male-killing *Wolbachia* in two species of insect. *Proc. R. Soc. Lond. B* 266: 735-740.

Jeyaprakash, A. & M.A. Hoy 2000. Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. *Insect Mol. Biol.* 9: 393-405.

Jiggins, F.M., J.H.G von der Schulenburg, G.D.D. Hurst & M.E.N. Majerus 2001. Recombinations confounds interpretations of *Wolbachia* evolution. *Proc. R. Soc. Lond. B* 268: 1423-1427.

Karr, T.L. 1994. Giant step sideways. *Curr. Biol.* 4: 537-540

Kazmer, D.J. 1992. *The evolution of reproductive strategies in Trichogramma and its relationship to biological control*. Ph.D. thesis, Department of Entomology, University of California, Riverside.

McGraw, E.A., D.J. Merrit, J.N. Droller & S.L. O'Neill 2001. *Wolbachia* density and virulence attenuation after transfer into a novel host. *Proc. Natl. Acad. Sci.* 99: 2918-2923.

Meer, M.M.M. van, & R. Stouthamer 1999. Cross-order transfer of *Wolbachia* from *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae) to *Drosophila simulans* (Diptera: Drosophilidae). *Heredity* 82: 163-169.

O'Neill, S.L. & T.L. Karr 1990. Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. *Nature* 348: 178-180.

O'Neill, S.L., R. Giordano, A.M.E. Colbert, T.L. Karr & H.M. Robertson 1992. 16S RNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl. Acad. Sci.* 89: 2699-2702.

Pinto, J.D. 1998. Systematics of the North American species of *Trichogramma* (Hymenoptera: Trichogrammatidae). *Mem. Entomol. Soc. Wash.* 22: 1-287.

Pinto, J. & R. Stouthamer 1994. Systematics of the Trichogrammatidae with emphasis on *Trichogramma*. In: E. Wajnberg & S.A. Hassan (eds.), *Biological Control with Egg*

Parasitoids. CAB International, Wallingford, pp. 1-36.

Pintureau, B., S. Chaudier, F. Lassablière, H. Charles & S. Grenier 2000a. Addition of *wsp* sequences to the *Wolbachia* phylogeny tree and stability of the classification. *J. Mol. Evol.* 51: 374-377.

Pintureau, B., S. Grenier, B. Boleat, F. Lassabliere, A. Heddi & C. Khatchadourian 2000b. Dynamics of *Wolbachia* populations in transfected lines of *Trichogramma*. *J. Invertebr. Pathol.* 76: 20-25.

Rigaud, T. 1997. Inherited microorganisms and sex determination of arthropod hosts. In: S.L. O' Neill, A.A. Hoffmann & J.H. Werren(eds.), *Influential passengers: inherited microorganisms and arthropod reproduction*, Oxford University Press, Oxford, pp. 81-101.

Rigaud, T. & P. Juchault 1995. Success and failure of horizontal transfer of feminizing *Wolbachia* endosymbionts in woodlice. *J. Evol. Biol.* 8: 249-255.

Rigaud, T., P.S. Pennings & P. Juchault 2001. *Wolbachia* bacteria effects after experimental interspecific transfers in terrestrial isopods. *J. Invertebr. Pathol.* 77: 251-257.

Rigaud, T., C. Souty-Grosset, R. Raimond, J. Mocquard & P. Juchault 1991. Feminizing endocytobiosis in the terrestrial crustacean *Armadillidium vulgare* LATR (Isopoda): recent acquisitions. *Endocytobiosis Cell. Res.* 7: 259-273.

Rousset F., D. Bouchon, B. Pintureau, P. Juchault & M. Solignac 1992. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proc. R. Soc. Lond. B* 250: 91-98.

Schilthuizen, M. & R. Stouthamer 1997. Horizontal transmission of parthenogenesis-inducing microbes in *Trichogramma* wasps. *Proc. R. Soc. Lond. B* 264: 361-366.

Schilthuizen, M., J. Honda & R. Stouthamer 1998. Parthenogenesis-inducing *Wolbachia* in *Trichogramma kaykai* (Hymenoptera: Trichogrammatidae) originates from a single infection. *Ann. Entomol. Soc. Am.* 91: 410-414.

Stouthamer, R. 1997. *Wolbachia*-induced parthenogenesis. In: S.L. O' Neill, A.A. Hoffmann & J.H. Werren (eds.), *Influential passengers: inherited microorganisms and arthropod reproduction*, Oxford University Press, Oxford, pp. 102-124.

Stouthamer, R. & D.J. Kazmer 1994. Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. *Heredity* 73: 317-327.

Stouthamer, R., R.F. Luck & W.D. Hamilton 1990. Antibiotics cause parthenogenetic *Trichogramma* to revert to sex. *Proc. Natl. Acad. Sci. USA* 87: 2424-2427.

Stouthamer, R., J.A.J. Breeuwer, R.F. Luck & J.H. Werren 1993. Molecular identification of microorganisms associated with parthenogenesis. *Nature* 361: 66-68.

Stouthamer, R., J.A.J. Breeuwer & G.D.D. Hurst 1999a. *Wolbachia pipientis*: Microbial manipulator of arthropod reproduction. *Ann. Rev. Microbiol.* 53: 71-102.

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

Suzuki, Y., H. Tsuji & M. Sasakawa 1984. Sex allocation and the effects of superparasitism on secondary sex ratios in the gregarious parasitoid, *Trichogramma chilonis* (Hymenoptera: Trichogrammatidae) *Anim. Behav.* 32: 478-484.

Vavre, F., F. Fleury, D. Lepetit, P. Fouillet & M. Bouletreau 1999. Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. *Mol. Biol. Evol.* 16: 1711-1723.

Weeks, A.R. & J.A.J. Breeuwer 2001. *Wolbachia*-induced parthenogenesis in a genus of phytophagous mites. *Proc. R. Soc. Lond. B* 268: 2245-2251.

Werren, J.H. 1997. Biology of *Wolbachia*. *Ann*. *Rev*. *Entomol*. 42: 587-609.

Werren, J.H. & J.D. Bartos 2001. Recombination in *Wolbachia*. *Curr*. *Biol*. 11: 431-435.

Werren, J.H., D. Windsor & L.R. Guo 1995a. Distribution of *Wolbachia* among neotropical arthropods. *Proc*. *R*. *Soc*. *Lond*. *B* 262: 197-204.

Werren, J.H., W. Zhang & L.R. Guo 1995b. Evolution and phylogeny of *Wolbachia* bacteria: Reproductive parasites of arthropods. *Proc*. *R*. *Soc*. *Lond*. *B* 250: 91-98.

Yen, J.H. & A.R. Barr 1971. New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens*. *Nature* 232: 675-678.

Chapter

6

Does *Wolbachia* infection affect *Trichogramma atopovirilia* behaviour?

Raul P. de Almeida, Joop C. van Lenteren & Richard Stouthamer

Chapter 6

DOES *Wolbachia* INFECTION AFFECT *Trichogramma atopovirilia* BEHAVIOUR?

Abstract

Unisexual *Trichogramma* forms have attracted much attention due to their potential advantages as biocontrol agents. Fitness studies have been performed and understanding the cost that *Wolbachia* may inflict on their hosts will help in deciding if *Wolbachia* infected (unisexual) forms are indeed better than sexual forms when used in biological control programs. The influence of *Wolbachia* on the foraging behaviour (including walking activity and speed) of *T. atopovirilia* is reported in this paper. Temperature strongly affected the walking activity of *T. atopovirilia* females, but *Wolbachia* infected and uninfected females differed in none of the behaviour components that were measured. Walking activity was highest at 25^{o}C and differed significantly from that at 20 and 15^{o}C. *Trichogramma* wasps hardly walked at 15^{o}C. Analysis of behaviour of females exposed to host eggs showed that wasps spent most of the time on drilling + ovipositing followed by host drumming and walking while drumming. The rate of parasitism and number of offspring did not differ significantly between infected and uninfected *Trichogramma* females. Implications of these findings for biological control are discussed.

6.1. Introduction

Selection of candidate species for biological control

In biological control, the proper selection of natural enemy strains and species is still an area of great concern. Although selection methods are available and various effective species have been identified, they can often not be effectively used in biological control programs, either because they are difficult to mass rear or because of poor quality after mass rearing (van Lenteren 2000, 2003). Current quality control guidelines consist mainly of characteristics that are easy to determine in the laboratory (van Lenteren 2000; van Lenteren *et al*. 2003). However, quality control should ensure good natural enemy performance in the field (van Lenteren 1991). Using adequate criteria to evaluate quality of parasitoids before they are released may help in improving the success of biocontrol (van Lenteren 2003). Good evaluation criteria allow for a choice between useless and potentially promising natural enemies (van Lenteren & Manzaroli 1999). Such a choice prevents the introduction of inefficient natural enemies. A list of criteria for pre-introductory evaluation of natural enemies includes seasonal synchronization with host, internal synchronization with host, climatic adaptation, no negative effects on other beneficial organism or non-target species, good rearing methods, host specificity, great reproductive potential, and good density responsiveness (van Lenteren & Manzaroli 1999). The importance of quality components for the mass production and use of *Trichogramma* wasps has been discussed by Bigler (1994). He identified the following major quality components, which partly overlap with the criteria proposed by van Lenteren & Manzaroli (1999): adaptability, habitat location, host location, host acceptance, host suitability, synchronization with host, density dependent properties, intrinsic rate of increase, laboratory rearing properties and quarantine and handling properties. Final selection of the appropriate *Trichogramma*

population to be released in the field is based on inter- and intraspecific variation, as well as on parasitoid quality (Smith 1996).

Recently Roitberg *et al*. (2001) has clarified the fitness concept of parasitoids by discussing its definition and the ways it can be measured. It should not be confused with vigour or quality (terms which are often used by biological control workers), although these may be indirect measures of fitness. Parasitoid fitness may be measured for testing evolutionary theory or for assessing ecological applications such as biological control. Body size, progeny survival, development rate, longevity and fecundity are the most used measurements of fitness.

Use of thelytokous wasps in biological control

For biological control the sex ratio produced by wasps is an important quality criterion, because only female wasps are the ones that are killing the pest insects through oviposition. For that reason it is often assumed that the use of completely parthenogenetic (thelytokous) wasps in biological control can be an advantage (Timberlake & Clausen 1924, Aeschlimann 1990, Stouthamer 1993). The cause of thelytoky in the majority of cases is an infection with parthenogenesis inducing bacteria such as *Wolbachia* (Stouthamer 1990) and Cytophaga like bacteria (Zchori-Fein, *et al*. 2001). Fitness studies have shown many cases of negative (Stouthamer & Luck 1993, Stouthamer *et al*. 1994; Horjus & Stouthamer 1995; Hoogenboom *et al*. 1998; Silva *et al*. 2000; Tagami *et al*. 2002); positive (Girin & Bouletreau 1995; Wade & Chang 1995; Vavre *et al*. 1999); or neutral (Stouthamer *et al*. 1994) effects of *Wolbachia* infection on *Trichogramma*. However, few studies have been done to evaluate biological control strategies using *Trichogramma* species infected with *Wolbachia*. Silva *et al*. (2000) tested the biocontrol performance of *Wolbachia* infected versus uninfected wasps in a greenhouse setting. That study showed that infected wasps

appeared as good as uninfected wasps in finding host patches. However, once a host patch had been found, infected females parasitized fewer eggs than uninfected females. In this case, like in other cases of thelytoky, a physiological cost such as a reduced fecundity on *Trichogramma* hosts (Stouthamer & Luck 1993) may be imposed because of the presence of a large number of *Wolbachia* bacteria inside host tissues (Stouthamer *et al*. 1999).

Wolbachia *infection and search efficiency of* Trichogramma

The searching behaviour of thelytokous *Trichogramma* species has hardly been studied. Important parameters for the determination of search efficiency are the walking activity and the walking speed (Suverkropp 1994). For *T. brassicae,* Suverkropp (2001) and Pompanon *et al*. (1994) found a walking activity, i.e. the fraction of total time spent walking, of 0.7-0.8 for females during the light period at 20-22^{o}C. Boldt (1974) used *T. evanescens* and observed that walking activity increased from 0.5-0.7 at 20^{0}C to 0.9 at 25^{0}C. At temperatures above 25^{0}C, the walking activity did not increase any further. However, a linear increase of the walking speed from 20 to 35^{o}C was found by Biever (1972) in *T. evanescens*. At 40^{o}C the *T. evanescens* wasps died. Suverkropp *et al*. (2001) also observed a significant effect of the temperature on *T. brassicae* walking speed. At 20^{o}C the average walking speed was twice as high as at 12^{o}C. Considerable variation in walking speed was found in *T. brassicae* between strains (Cerutti & Bigler 1991).

In an experimental study combined with simulation modeling of the foraging behaviour of *Encarsia formosa* Gahan, van Roermund (1995) showed that aspects such as leaf size, parasitoid walking speed and walking activity and, to a lesser extent, width of the parasitoids searching path and diameter of the immature host are the most important parameters in determining the encounter rate of the parasitoid with its host. Using *Trichogramma* wasps Pak (1988) studied similar traits as those studied in *E. formosa* to

evaluate the searching behaviour of female parasitoids.

In this study we determine the influence of carrying a *Wolbachia* infection by *T. atopovirilia* on its walking activity, walking speed and several other behaviour components.

6.2. Material and Methods

Trichogramma cultures and antibiotic treatment

Trichogramma atopovirilia Oatman & Platner (line Tato-01) was collected in Minas Gerais State, Brazil in corn. The originally *Wolbachia* infected *Trichogramma* females (Ciociola *et al*. 2001) were submitted to an antibiotic treatment, by mixing the honey that they use as a food source with 0.5% w/v rifampicin. This treatment was continued for three generations. Cured parasitoids were reared for several generations after the treatment ended before they were used in experiments. Thus, we could be sure that rifampicin residual effect would not affect the experimental results. Two tests were done to verify that the *Wolbachia* infection was truly cured: (1) twenty virgin females were allowed to lay eggs for one day. If only male offspring emerges the test indicates that the infection is no longer present, because under their normal mode of reproduction, i.e. arrhenotoky, males arise from unfertilized eggs (Stouthamer *et al*. 1990); (2) Testing for the presence of bacteria by doing PCR using *Wolbachia* specific primers (*wsp*) (Braig *et al*. 1998).

All behaviour experiments were done at 14:10 (L:D) and 75±10% R.H. using wasps that had been reared on *Ephestia kuehniella* Zeller eggs. The light phase started at 8:00 AM. All experiments were initiated with both infected and cured females that were 1 day old. To assure that the females were all of similar size we measured their hind tibia length (HTL) using an optical micrometer mounted in an eyepiece of a compound microscope.

Walking activity

Walking activity is defined as the fraction of females observed that were active at the moments of observation (Suverkropp *et al*. 2001). Walking activity was analysed at three temperatures (15, 20 and 25^{o}C). One day old infected and mated cured females *T. atopovirilia* wasps were placed into vials (75mm long and 10mm diameter) with a drop of honey; the vials were closed with cotton wool. The tubes with *Trichogramma* females were laid slightly inclined in the climate chambers. The experiment lasted for four days. The evaluation of each single individual was performed every 30 min. from 8:30 AM to 18:00 PM with a total of 20 observations per day. During each observation the wasps were checked to determine if they were moving, standing still or had died. Forty replicates were used for both infected and cured female wasps. All females used for the statistical analysis survived during the whole period of the experiment. Comparisons between walking activity of infected and cured females were done for each temperature studied. A completely random design was used in the data analysis. The data were submitted to an analysis of variance and the means were compared by Tukey test ($P \leq 0.05$ and $P \leq 0.01$).

Walking speed

Infected and cured females used for measuring the walking speed were one day old. To standardize the adult female size *Trichogramma* wasps were reared on *E. kuehniella*. A Student T-test ($P=0.05$) was used to determine if the size of the hind tibia length of infected and cured females were statistically the same. The walking speed was measured by placing individual female wasps in an arena (plastic Petri dish 5.5cm diameter) with wet cotton wool around it to avoid the wasps from escaping. Only when the wasps walked for at least 5 seconds we used the observation for inclusion in the calculation of the walking speed. Walking speed was recorded using a video camera (Panasonic GGTV), a video recorder

(Panasonic AG-6200) and colour video monitor (Panasonic TC 1470-y) connected to a computer. Video tracking and motion analysis were done using the program Ethovison (version 1.7 Noldus Information Technology). The experimental arena was illuminated from the underside using a circular fluorescent tube separated from the arena by a 1cm thick plexiglass plate to avoid heat build-up. For each treatment (infected and cured wasps) thirty replications were done and each female was used only once. The data were submitted to analysis of variance and the means compared using the Tukey test ($P \leq 0.05$).

Behaviour components

Trichogramma behaviour was studied in a climate room at 25°C. Wasps were released in an arena containing twenty-five *Mamestra brassicae* L. eggs that were placed in a square grid at a distance of 0.7 mm apart from each other. Twenty replications of infected and cured females were analysed. The following components of the wasp's behaviour were recorded: (1) Host contact (=the host egg is touched by the wasp's antennae); (2) Host drumming (=the female wasp drums on the host egg in a standing position or while walking); (3) Turning 360° (=in general, after the female wasp encounters the host egg, circular movements indicate acceptance for oviposition); (4) Drilling + ovipositing position (=female starts ovipositor penetration and adopts an oviposition posture); (5) Host feeding (=the wasp feeds on the host egg through a hole made by the ovipositor); (6) Walking while drumming (=the wasp walks and drums between a previous host egg encounter and the subsequent one); (7) Standing still while drumming (=while standing still the wasp drums her antennae); (8) Standing still (=the wasp stands still on the host egg or arena and is sometimes moving the antennae); (9) Preening (=the wasp preens body parts such as wings, legs, thorax, antennae etc.). During the behaviour experiment the oviposition of infected and uninfected females was measured by determining the number of parasitized host eggs

per female, the total number of offspring per female, the number of females and the offspring sex ratio. For the evaluation of *Trichogramma* behaviour a thirty min. observation (experimental unit) was made. The duration of each behaviour component was measured in seconds. Behaviour records started with the first host contact using a video cassette recorder (Panasonic AG-6200), a color monitor (Sony 20" Model No. PVM-2010QM) and a CCD Camera (Model CC-36) connected to a microscope (Zeiss). The hosts parasitized during the observation were allowed to develop and the mean number of eggs parasitized per parasitoid female, the total number of offspring per parasitoid female and the offspring sex ratio were determined. A completely random design was used to analysize the data. The data were submitted to analysis of variance ($P \leq 0.05$).

6.3. Results and Discussion

Curing of Wolbachia *infection*

After the infected females were fed on honey plus antibiotic in the first generation, male wasps were produced from the third day of oviposition onwards. The cured *T. atopovirilia* females have turned permanently to arrhenotoky from thelytoky after the third generation of antibiotic treatment. In all cases the virgin females from the cured line produced exclusively male offspring indicating that they were truly cured. Arrhenotoky was also confirmed using PCR and *Wolbachia* DNA amplification was not detected any longer.

Walking activity

Results of the mean walking activity are shown in Table 1 and 2. The temperature strongly affected the walking activity of *T. atopovirilia* and differences were highly

Table 1. Mean untransformed walking activity (± Standard error) of *T. atopovirilia* at different temperatures

Temperature (°C)	Walking activity[1] /day			
	1st	2nd	3rd	4th
15	0.00 ± 0.00 c	0.02 ± 0.00 c	0.03 ± 0.01 c	0.06 ± 0.01 c
20	0.18 ± 0.01 b	0.37 ± 0.02 b	0.56 ± 0.02 b	0.59 ± 0.02 b
25	0.92 ± 0.01a	0.93 ± 0.01a	0.93 ± 0.01a	0.93 ± 0.01a

[1]Means followed by the same letter are not significantly different by Tukey test (P≤0.01) of √(x+1)-transformed data

Table 2. Mean untransformed walking activity (± Standard error) at different temperatures, and walking speed (± Standard error) of *T. atopovirilia*

Reproduction mode	Walking activity[1]			Walking speed (cm/s)[2]
	15°C	20°C	25°C	
Thelytoky	0.03 ± 0.01a	0.44 ± 0.02a	0.94 ± 0.01a	0.35 ± 0.02a
Arrhenotoky	0.03 ± 0.01a	0.40 ± 0.03a	0.92 ± 0.02a	0.34 ± 0.03a

[1,2]Means followed by the same letter are not significantly different by Tukey test (P≤0.05) of √(x+1)-transformed data

significant (P≤0.01). The higher the temperature the higher the walking activity. Mean walking activity of infected and cured females wasps during the four days experiment are show in Figure 1 and 2. The daily walking activity patterns were similar for the infected and cured females at each of the temperatures studied. Walking activity was high the whole day at 25°C. At 20°C, walking activity was higher during the second part of the day. At 15°C, the female wasps were only active at the end of the period reaching maximum values

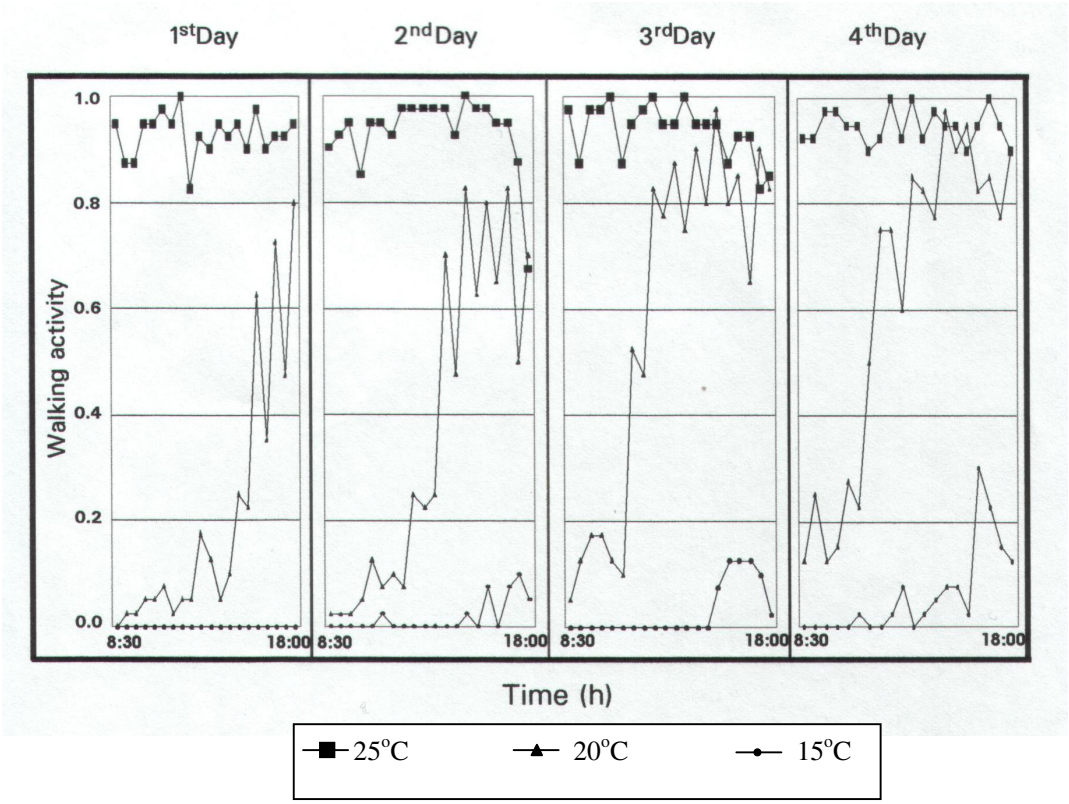

***Fig.** 1. Mean walking activity of infected T. atopovirilia females during 4 days at three temperatures.*

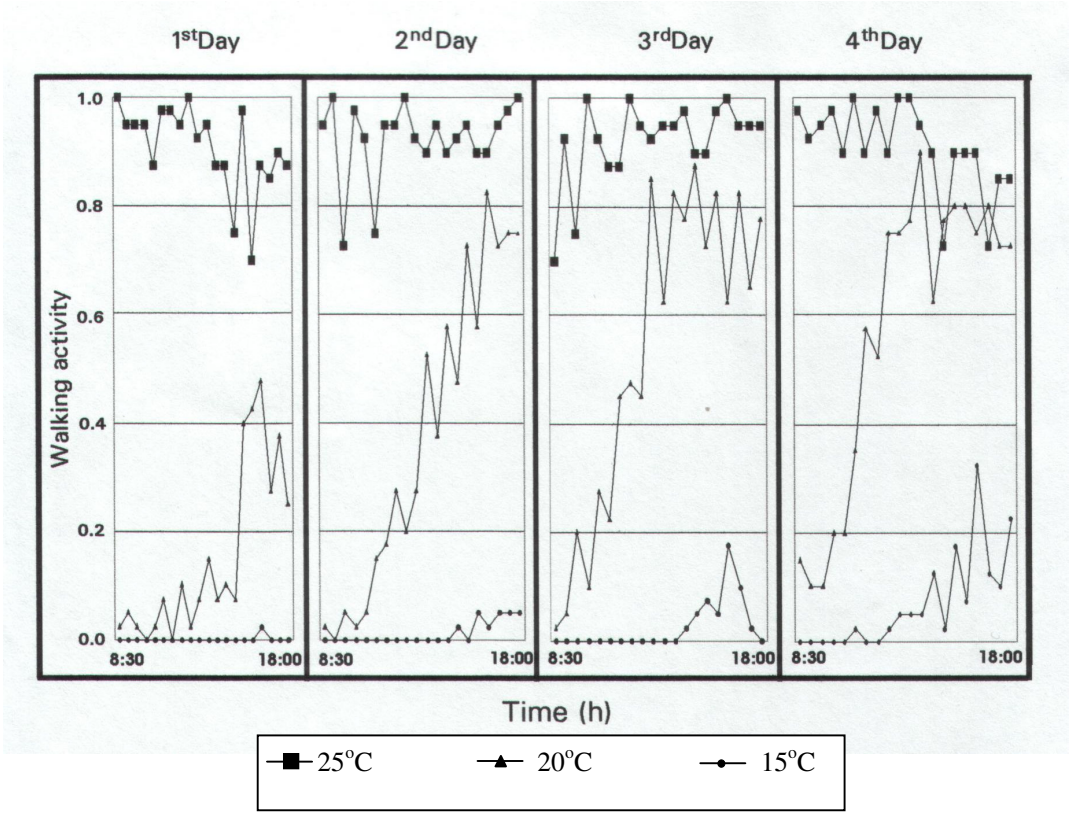

***Fig. 2**. Mean walking activity of cured T. atopovirilia females during 4 days at three temperatures.*

of less than 0.4. A higher walking activity was found in *T. brassicae* (Suverkropp *et al*. 2001) at 15°C than in *T. atopovirilia.* This fact can be explained because the latter species is well adapted to tropical climate and consequently more negatively affected by temperatures below 20°C, while *T. brassicae* is more adapted to low temperate conditions. For *T. brassicae* Suverkropp *et al.* (2001) found an opposite activity pattern with a higher activity at the first part of the day. During the first two days of the experiment the mean walking activity was lower than during the subsequent ones. The highest mean walking activity was observed at the fourth day of the experiment. Walking activity is not influenced by the reproductive mode of the females. This result was found by analysing the walking activity for each temperature averaged over all days. Infected and cured *T. atopovirilia* females responded similarly at all temperatures studied (Table 2).

Walking speed

Walking speed in *Trichogramma* females was not influenced by their infection status (Table 2). The mean hind tibia length of infected (0.148mm ± 0.004 mm) and cured (0.146 ± 0.005 mm) *T. atopovirilia* did not differ statistically (T-test; P=0.05).

The mean walking speed obtained here was similar to that observed by Suverkropp *et al.* (2001) for *T. brassicae* at 20°C.

Behaviour and reproduction components

In all the behaviour and reproduction components we studied no statistical difference was found between infected and cured females (Table 3). Host contact, host drumming, drilling + ovipositing position and walking while drumming were the most common behaviour components observed during the thirty min. experiment. *Trichogramma* wasps spent most of the time on drilling + ovipositing (67%) followed by host drumming (16%) and walking while drumming (14%). Only 3% of the total time was spent on other

behaviours (host feeding, standing still while drumming, standing still and preening). Host feeding was a rare event, apparently because the female wasps had honey available to them from the time of emergence until the experiment started. Thelytokous and arrhenotokous females were equally efficient in the number of host eggs parasitized and in the number of offspring produced (Table 3). The sex ratio of infected females (100% females) differed, as was expected, significantly from that obtained from cured ones (65% females). Silva (1999) also found a higher production of daughters for the thelytokous females in comparison to the cured conspecific arrenotokous lines.

Table 3. Mean duration (s)[1] (± standard error) of behaviour components and number of parasitized hosts and offspring of infected and cured *T. atopovirila*

Component	Reproduction mode		F-test
	Thelytoky	Arrhenotoky	
Total host drumming[1]	284.50 ± 9.43	284.75 ± 30.73	0.00[ns]
Host drumming/host egg	26.75 ± 1.11	29.50 ± 4.48	0.35[ns]
Total drilling + ovipositing position[1]	1,240.25 ± 28.35	1,185.50 ± 18.70	2.60[ns]
Drilling + ovipositing position/host egg	124.50 ± 4.99	122.50 ± 5.87	0.07[ns]
Total walking while drumming[1]	210.75 ± 31.85	282.25 ± 34.81	2.30[ns]
Number of parasitized host eggs[1]	9.15 ± 0.25	8.95 ± 0.30	0.26[ns]
Total number of offspring[1]	21.60 ± 0.81	20.50 ± 0.77	0.98[ns]
Total number of females	21.60 ± 0,81	13.15 ± 0.82	54.34*
Sex ratio	1.00 ± 0.00	0.65 ± 0.04	77.77*

[1]During a thirty minute observation period

[ns]Means do not differ significantly by F-test (P≤0.05)

[*]Means differ significantly by F-test (P≤0.05)

Trichogramma wasps were able to easily find the host eggs individually distributed at a 0.7mm distance from each other in our experimental set-up. When released in the arena, the *Trichogramma* females immediately started searching for host eggs. After encountering a

host egg the *Trichogramma* wasp examined the egg by antennal drumming, followed by drilling through the eggshell with the ovipositor. Depending on the size of the host, one or more eggs are laid (Salt 1935; Klomp & Teerink 1962; Schimidt & Smith 1985a, b). In general, *T. atopovirilia* females laid 3-4 eggs per host egg in the first host egg encountered. In later hosts fewer eggs were laid. After withdrawal of the ovipositor, the wasps may feed on the host cytoplasm oozing from the eggshell puncture (host feeding).

T. atopovirilia wasps were rarely observed host feeding, standing still or preening. Sometimes when a female wassp was in a drilling activity, they would stop and start searching for another egg. These are most likely cases of host rejection during this phase of the parasitization process (Van Dijken *et al*. 1986). The parasitoid does not acquire the ability to discriminate hosts until a female has had an oviposition experience with an unparasitized host (Klomp *et al*. 1980). *Trichogramma* can discriminate between parasitized and unparasitized hosts (van Lenteren *et al*. 1978; van Lenteren 1981). In our experiments the females would sometimes encounter an already parasitized host egg two or three times, but these hosts were never superparastized.

Use of thelytokous wasps in biological control

The use of thelytokous strains of parasitoids and their potential advantages for biological control of insect pests has been discussed by Aeschlimann (1990) and Stouthamer (1993). It is known that infection with parthenogenesis inducing *Wolbachia* can have a negative impact on the lifetime offspring production of the wasps when this is measured under laboratory conditions where the wasps are supplied with food and have an unlimited number of hosts to parasitize (Stouthamer & Luck 1993; Stouthamer *et al*. 1994; Horjus & Stouthamer 1995; Hoogenboom *et al*. 1998). The impact may be such that infected females produce fewer daughters than uninfected females. This could be an argument against using

thelytokous lines in biological control. However the laboratory measurements of life time fecundity probably mean little for wasps in the field where we can assume that each wasp will find only a few hosts (Stouthamer & Luck, 1993). The advantage of using thelytokous forms for biological control stems from their ability to produce only daughters from all hosts parasitized. Here we show that indeed per host infected *T. atopovirilla* females produce more daughters than the uninfected females.

Our results show that *Wolbachia* infection does not negatively affect *T. atopovirilia* in the behaviours we tested. The situation where thelytokous females coexist with arrhenotokous ones is known as a mixed population. Infected females are able to mate and produce daughters sexually. The species used in this study (*T. atopovirilia*) probably originated from a fixed population (*Chapter 5*). All females in the population are infected. The theory in which the reduction of the negative impact of the symbiont on its host due to fixation of the infection (van Meer 1999) is consistent with the results obtained here.

The ability of infected females in producing more daughters when host availability is limited (Stouthamer & Luck 1993) may help improving biological control of an insect pest. When host eggs are limited the negative effect of *Wolbachia* infection on the total offspring production will not be important, most females will never lay all of their eggs. For *T. atopovirilia* the host egg availability was similar for both infected and cured female wasps and no difference in their searching ability was found indicating that the *Wolbachia* infection does not influence these traits, during the time frame and in the experimetal set-up of this study.

Based on our results we propose that the infected *T. atopovirilia* is a more suitable candidate for biological control programs than its conspecific arrhenotokous species, due to the production of only females, which leads to a higher population growth rate. Also, thelytokous *Trichogramma* can be reared at lower production costs (Stouthamer 1993).

Acknowledgements

We thank Américo Ciociola Jr. (EPAMIG, Brazil) who has provided *Trichogramma atopovirilia* used in this research. This research was supported by Embrapa (Brazilian Agricultural Research Corporation)/PRODETAB and the Wageningen University Graduate School Production Ecology & Resource Conservation (R.P.A).

References

Aeschlimann, J.P. 1990. Simultaneous occurrence of thelytoky and bisexuality in hymenopteran species, and its implications for the biological control of species. *Entomophaga* 35: 3-5.

Biever, K.D. 1972. Effect of temperatures on the rate of search by *Trichogramma* and its potential application in field releases. *Environ. Entomol.* 1: 194-497.

Bigler, F. 1994. Quality control in *Trichogramma* production. In: E. Wajnberg & S.A. Hassan (eds.), *Biological Control with Egg Parasitoids,* CAB International, Wallingford, pp. 93-111.

Boldt, P. E., 1974. Temperature, humidity, and host: effect on rate of search of *Trichogramma evanescens* and *T. minutum* Auctt. (not Riley, 1871). *Ann. Entomol. Soc. Am.* 67: 706-708.

Braig, H.R., W. Zhou, S. Dobson & S.L. O' Neill 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial ensosymbiont *Wolbachia pipientis. J. Bacteriol.* 180: 2373-2378.

Cerutti, F. & F. Bigler 1991. Methods for quality evaluation of *Trichogramma evanescens* used against the European Corn Borer. In: Bigler, F. (ed.), *Proceedings of the Fifth IOBC Global Working Group 'Quality Control of Mass Reared Arthropods',*

Wageningen, The Netherlands, pp. 119-126.

Ciociola Jr., A.C., R.P. de Almeida, R.A. Zucchi & R. Stouthamer 2001. Detecção de *Wolbachia* em uma população telítoca de *Trichogramma atopovirilia* Oatman and Platner (Hymenoptera: Trichogrammatidae) via PCR com o Primer Específico *wsp*. *Neotrop. Entomol.* 30:489-491.

Dijken, M.J. van, M. Kole, J.C. van Lenteren & A.M. Brand 1986. Host preference studies with *Trichogramma evanescens* Westwood (Hym.: Trichogrammatidae) for *Mamestra Brassicae*, *Pieris Brassica* and *Pieris rapae*. *J. Appl. Entomol.* 101: 64-85.

Girin, C. & M. Boulétreau 1995. Microorganism-associated variation in host infestation efficiency in a parasitoid wasp *Trichogramma bourarachae* (Hymenoptera: Trichogrammatidae). *Experientia* 52: 398-401.

Hoogenboom, A., IM.M.S. Silva, M.M.M. van Meer, M.M. Roskam & R. Stouthamer 1998. Quality assessments of *Wolbachia* infected versus non infected lines of *Trichogramma deion*. *Proc. Exper. Appl. Entomol.* 9: 99-104.

Horjus M. & R. Stouthamer 1995. Does infection with thelytoky-causing *Wolbachia* in pre-adult and adult life stages influence the adult fecundity of *Trichogramma deion* and *Muscidifurax uniraptor*? *Proc. Exp. Appl. Entomol.* 6: 35-40.

Klomp, H., B.J. Teerink & W.C. Ma 1980. Discrimination between parasitized and unparasitized hosts in the egg parasite *Trichogramma embryophagum* (Hym.: Trichogrammatidae): a matter of learning and forgetting. *Neth. J. Zool.* 30: 254-277.

Klomp, H., B.J. Teerink 1962. Host selection and number of eggs per oviposition in the egg-parasite *Trichogramma embryophagum* Htg. *Nature* 195: 1020-1021.

Lenteren, J.C. van 1981. Host discrimination by parasitoids. In: D.A. Norlund, R.L. Jones & W.J Lewis (eds.), *Semiochemichal, their role in pest control*, Wiley, New York. pp. 153-179.

Lenteren, J.C. van 1991. Quality control of natural enemies: hope or illusion. In: Bigler, F. (ed.) *Proceedings of the Fifth IOBC Global Working Group 'Quality control of mass reared arthropods',* Wageningen, The Netherlands, pp. 1-15.

Lenteren, J.C. van 2000. Measures of success in biological control of arthropods by augmentation of natural enemies. In: G. Gurr & S. Wratten (eds.), *Measures of Success in Biological Control*, Kluwer Academic Publishers, Dordrecht, pp. 77-103.

Lenteren, J.C. van 2003. Quality Control and Production of Biological Control Agents: Theory and Testing Procedures. CABI Publishing, Wallingford, UK, 327 pp.

Lenteren, J.C. van, K. Bakker & J.J.M. van Alphen 1978. How to analyze host discrimination? *Ecol. Entomol.* 3: 71-75.

Lenteren, J.C. van & G. Manzaroli 1999. Evaluation and use of predators and parasitoids for biological control of pests in greenhouses. In: R. Albajes, M.L. Gullino, J.C. van Lenteren & Y. Elad (eds.), *Integrated Pest and Disease Management in Greenhouse Crops*, Kluwer Academic Publishers, Dordrecht, pp.183-201.

Lenteren, J.C. van, H.W Nell & L.A.S. van der Lelie 1980. The parasite-host relationship between *Encarsia formosa* (Hymenoptera: aphelinidae) and *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae). IV. Ovipositon behaviour of the parasite, with aspects of the host selection, host discrimination and host feeding. *Z. Angew. Entomol.* 89: 442-454.

Lenteren,J.C. van, A. Hale, J.N. Klapwijk, J. van Schelt & S. Steinberg 2003. Guidelines for quality control of commercially produced natural enemies. In: J.C. van Lenteren (ed.), *Quality Control and Production of Biological Control Agents: Theory and Testing Procedures*, CABI Publishing, Wallingford, pp. 265-303.

Meer, M.M.M. van 1999. *Phylogeny and host-symbiont interactions of thelytokous inducing Wolbachia in Hymenoptera*. Ph.D. thesis, Wageningen University, The Netherlands, 118pp.

Pak, G.A. 1988. *Selection of* Trichogramma *for inundative biological control*. Ph.D thesis, Wageningen University, The Netherlands, 224 pp.

Pompanon, F.; P. Fouillet, M. Bouletreau 1994. Locomotor behaviour in females of two *Trichogramma* species: description and genetic variability. *Norw. J. Agric. Sci. Supplement* 16: 185-190.

Roermund, H.J.W. van 1995. Understanding biological control of greenhouse whitefly with the parasitoid *Encarsia Formosa.* Ph.D thesis, Wageningen University, The Netherlands, 243 pp.

Roitberg B.D., G. Boivin & L.E.M. Vet 2001. Fitness, parasitoids, and biological control: an opinion. *Can. Entomol.* 133: 429-438.

Salt, G. 1935. Experimental studies in insect parasitism. III. Host selection. *Proc. R. Soc. Lond. B*. 117: 413-435.

Schmidt, J.M. & J.J.B Smith 1985a. Host volume measurement by the parasitoid wasp *Trichogramma minutum*: the roles of curvature and surface area. *Entomol. Exp. Appl.* 39: 213-221.

Schmidt, J.M. & J.J.B Smith 1985b. The mechanism by which the parasitoid was *Trichogramma minutum* responds to host clusters. *Entomol. Exp. Appl.* 39: 287-294.

Silva, I.M.M.S. 1999. *Identification and evaluation of Trichogramma parasitoids for biological pest control*. Ph.D. thesis, Wageningen University, The Netherlands, 151 pp.

Silva, I.M.M.S., M.M.M. van Meer, M. M. Roskam, A. Hoogenboom, G. Gort & R. Stouthamer 2000. Biological control potential of *Wolbachia* infected (unisexual) versus uninfected (sexual) wasps: laboratory and greenhouse evaluation of *Trichogramma cordubensis* and *T. deion* strains. *BioControl* 10: 223-238.

Smith, S.M. 1996. Biological control with *Trichogramma*: Advances, successes, and potential of their use. *Annu. Rev. Entomol.* 41: 375-406.

Stouthamer, R. 1993. The use of sexual versus asexual wasps in biological control. *Entomophaga* 38: 3-6.

Stouthamer, R. & R.F. Luck 1993. Influence of microbe-associated parthenogenesis on the fecundity of *Trichogramma deion* and *T. pretiosum*. *Entomol. Exp. Appl.* 67: 183-192.

Stouthamer, R., J.A.J. Breeuwer & G.D.D. Hurst 1999. *Wolbachia* pipientis: microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol.* 53: 71-102.

Stouthamer, R., R.F. Luck & W. D. Hamilton 1990. Antibiotics cause parthenogenetic *Trichogramma* to revert to sex. *Proc. Natl. Acad. Sci. USA* 87: 2424-2427.

Stouthamer R., S. Luko & F. Mak 1994. Influence of parthenogenesis *Wolbachia* on host fitness. *Norw. J. Agric. Sci.* 16: 117-122.

Suverkropp, B. P. 1994. Landing of *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae) on maize plants. *Norw. J. Agric. Sci. Supplement* 16: 243-254.

Suverkropp, B.P., F. Bigler & J.C. van Lenteren 2001. Temperature influences walking speed and walking activity of *Trichogramma brassicae (*Hym., Trichogrammatidae). *J. Appl. Entomol.* 125: 303-307.

Tagami, Y., K. Miura & R. Stouthamer 2002. How does infection with parthenogenesis-inducing *Wolbachia* reduce the fitness of *Trichogramma*? *J. Invert. Pathol.* 76: 267-271.

Timberlake, P.H. & C.P. Clausen 1924. The parasites of *Pseudococus maritimus* in California. *Univ. Calif. Publ. Tech. Bull. Entomol.* 3: 223-292.

Vavre, F., C. Girin & M. Boulétreau 1999. Phylogenetic status of a fecundity-enhancing *Wolbachia* that does not induce thelytoky in *Trichogramma*. *Insect Mol. Biol.* 8: 67-72.

Wade M.J. & N.W. Chang 1995. Increased male fertility in *Tribolium confusum* beetles after infection with the intracellular parasite, *Wolbachia*. *Nature* 373: 72-74.

Wang, Z. & S.M. Smith 1996. Phenotypic differences between thelytokous and arrhenotokous *Trichogramma minutum* from *Zeiraphera Canadensis*. *Entomol*. *Exp*. *Appl*. 78: 315-323.

Zchori-Fein, Y. Gottlieb, S.E. Kelly, J.K. Brown, J.M. Wilson, T.L. Karr & M.S. Hunter 2001. A newly discovered bacterium associated with parthenogenesis and a change in host selection behaviour in parasitoid wasps. *Proc. Natl. Acad. Sci. USA* 98: 12555-12560.

Chapter

7

Summarizing discussion

Raul P. de Almeida

Chapter 7

SUMMARIZING DISCUSSION

Abstract

The interactions between the symbiont *Wolbachia* and its host, wasps of the genus *Trichogramma* are the main topic of this thesis. The *Trichogramma* species used in the studies were collected in South America. To determine their species identity a system was developed based on the sequence of the ribosomal spacer ITS-2. The phylogenetic relationship between the differenct *Wolbachia* was studied using the wsp gene. This study resulted in the recognition of a new subgroup of *Wolbachia*. The potential for intra- and interspecific transmission was studied experimentally, and showed that intra specific transmission is possible in some cases but the newly infected species cannot maintain the infection for many generations. In addition intra specific transmission was only possible in a single species, in none of the other species was horizontal transmission successful. The influence of the *Wolbahcia* infection on the behaviour of the wasps was studied in *Trichogramma atopovirilla*, where no negative impact of the infection was found on the behaviours studied.

1. Discussion

Trichogramma identification and distribution in South America

Species of the genus *Trichogramma* are among the most studied biological control agents. They are egg parasitoids of mainly Lepidoptera and belong to the family Trichogrammatidae. This family consists of approximately 650 described species in 80 genera worldwide (Pinto 1997). The genus *Trichogramma* contains ca. 190 species around the world (Pinto 1998; Querino & Zucchi 2003a, b) and has been collected from six biogeographic regions, the Palaearctic, Nearctic, Australian, Oriental, Neotropical and Afrotropical. This family includes the smallest of insects, ranging in length from 0.2 to 1.5 mm (Pinto & Stouthamer 1994).

In South America thirty-eight species have been recorded (Zucchi & Monteiro 1997; Silva 2002; Querino & Zucchi 2003a; Almeida & Stouthamer 2003) and in Brazil at least twenty five species have been found (Zucchi & Monteiro 1997; Silva 2002; Querino & Zucchi 2003a, b). *Trichogramma* species collected in South America (Table 1) were identified using molecular techniques based on DNA sequences of the ITS2 region of the ribosomal cistron. In addition, their distributions and reproduction type are presented. Seven species have been introduced in South America according to Whu & Valdivieso (1999) and Silva (2002). In this thesis *T. brassicae* is presented as a new record as introduced species. This species was thought to be *T. evanescens*, but molecular identification showed it to be *T. brassicae*. Silva (1999) reported this species as frequently misidentified. The controversial identification of *T. evanescens* has been reported by Kostadinov & Pintureau (1991). Nagarkatti & Nagaraja (1968) reported the case in which *T. australicum* was wrongly referred to as *T. evanescens* for approximately 50 years. The

Table 1. Distribution of *Trichogramma* species collected in South America and used for molecular identification in this thesis

Trichogramma Species	Distribution	Reproduction Mode	References
T. acacioi	Brazil[1,2]	Arrhenotoky	Zucchi & Monteiro (1997) Pratissoli & Fornazier (1999) Silva (2002)
T. atopovirilia	Brazil[1,2], Colombia[1,2], Peru[2] (Introduced), Venezuela[2]	Thelytoky and Arrhenotoky	Zucchi (1985) Zucchi & Monteiro (1997) Pinto (1998) Whu & Valdivieso (1999) Ciociola Jr. *et al*. (2001a) Silva (2002)
T. brassicae	Peru[1] (Introduced)	Arrhenotoky	This thesis (Chapter 3)
T. bruni	Bolivia[2], Brazil[1,2], Venezuela[2]	Arrhenotoky	Zucchi & Monteiro (1997) Silva (2002)
T. cacoeciae	Peru[1]	Thelytoky	Almeida & Stouthamer (2003)
T. dendrolimi	Peru[1,2] (Introduced)	Arrhenotoky	Whu & Valdivieso (1999)
T. esalqueanum	Brazil[1,2]	Arrhenotoky	Querino & Zucchi (2003a)
T. exiguum	Chile[2], Colombia[1,2], Peru[1,2], Uruguay[2]	Arrhenotoky	Zucchi & Monteiro (1997) Basso *et al*. (1999) Whu & Valdivieso (1999) Silva (2002)
T. fuentesi	Argentina[2], Peru[1,2]	Arrhenotoky	Zucchi & Monteiro (1997) Whu & Valdivieso (1999) Silva (2002)
T. galloi	Bolivia[2], Brazil[2], Paraguay[2], Peru[1,2], Uruguay[2]	Arrhenotoky	Zucchi (1988) Zucchi & Monteiro (1997) Basso *et al*. (1999) Silva (2002)
T. iracildae	Brazil[1,2]	Arrhenotoky	Querino & Zucchi (2003a)
T. lasallei	Brazil[2], Colombia [2], Peru[1,2], Uruguay[2]	Arrhenotoky	Foerster *et al*. (2000) Querino *et al*. (2000) Ciociola Jr. *et al*. (2001b) Silva (2002)
T. lopezandinesis	Colombia[1,2]	Arrhenotoky	Sarmiento (1993) Silva (2002)
T. nerudai	Argentina[2] (introduced), Chile[1,2]	Arrhenotoky	Pintureau *et al*. (1999) Silva (2002)
T. pintoi	Peru[1,2] (Introduced)	Arrhenotoky	Whu & Valdivieso (1999) Silva (2002)
T. pretiosum	Argentina[2], Bolivia[2], Brazil[1,2], Chile[2], Colombia[1,2], Equator[2], Paraguay[2],Peru[12], Uruguay[2], Venezuela[2]	Thelytoky and Arrhenotoky	Botto *et al*. (1991) Zucchi *et al*. (1991) Whu & Valdivieso (1999) Zucchi & Monteiro (1997) Basso *et al*. (1999) Almeida *et al*. (2001) Silva (2002)
T. rojasi	Argentina[2], Brazil[1,2], Chile[2]	Arrhenotoky	Nagaraja & Nagarkatti (1973) Zucchi & Monteiro (1997) Whu & Valdivieso (1999) Ciociola Jr. *et al*. (2001b) Silva (2002)

[1]This thesis; [2]Other literature sources

frequent misidentification of species represents a serious problem in biological control programs using *Trichogramma*.

In most cases, arrhenotoky was the common reproductive mode observed in the *Trichogramma* species studied in this thesis. Thelytokous reproduction was found in three species: *T.atopovirilia*, *T. pretiosum* and *T. cacoeciae* (Ciociola *et al*. 2001a; Almeida *et al*. 2001; Almeida & Stouthamer 2003). The first two cases are infected *Trichogramma* wasps and the detection of *Wolbachia* bacteria was possible by using PCR with the *Wolbachia*-specific *wsp* primers. In the eggs of infected females the meiosis is identical to that of uninfected females and at the end of the meiosis the pronucleus contains a singl haploid set of chromosomes. The restoration of the diploid number takes place in the first mitotic division. During the anaphase of the first mitotic division, the two identical sets of chromosomes do not separate and the net result of the first mitotic cycle is a single nucleus containing two copies of the same set of chromosomes. This stage is followed by normal mitotic divisions eventually resulting in female offspring that is completely homozygous at all loci. This cytogenetic mechanism is known as gamete duplication (Stouthamer & Kazmer 1994). The third thelytokous species was the record of *T. cacoeciae* in Peru. In this species *Wolbachia* is not involved in causing thelytoky (Stouthamer *et al*. 1990). This species presents a real problem in morphological identification, because males are rare and neither exposure to high temperatures or antibiotics leads to the reversion of the reproductive mode to arrhenotoky (Pinto 1998). So, molecular identification, in this case based on ITS2 sequences, was the only reliable technique in solving the limitation of identifying females. The discovery of *T. cacoeciae* in Peru, reinforces the need of a thorough taxonomic study of the South American *Trichogramma* species for a better knowledge of their distribution. Moreover, intensive collections in all kind of habitats,

aiming to identify new species, will prevent erroneous identification of introduced species as native ones.

The seventeen identified *Trichogramma* species were easily distinguished based on rDNA sequences. These results confirm previous studies done by Stouthamer *et al*. (1999), Silva *et al*. (1999) and Pinto *et al*. (2002). Species can be identified using the size of the PCR product for the ITS2 spacer and the restriction patterns generated after digestion with different endonucleases. However, the reliability of these molecular keys and also for morphology based keys will only be complete once all South America species are known.

The molecular technique used in *Chapters 2* and *3* shows its indispensable value in the identification of South American *Trichogramma* species. As a result, the status of introduced species will be better understood and new species can easily be identified. Classical taxonomic studies will be greatly helped by molecular methods, but strongly depend on an infrastructure of sound morphological studies (Stouthamer *et al.* 1999). In addition, this method is useful in the detection of potential contamination in laboratory rearing and in evaluation of performance of parasitoid strains following releases (Silva 1999). Furthermore, DNA sequences based on the same technique can be applied for phylogenetic studies (Schilthuizen & Stouthamer 1997; Stouthamer *et al*. 1999).

Phylogenetic trees and horizontal transmission

Wolbachia is a common and widespread group of bacteria found in reproductive tissues of arthropods. These bacteria are transmitted through the cytoplasm of eggs and cause several types of reproductive modification (Werren 1997). *Wolbachia* bacteria are α-Proteobacteria (Rickettsia) that were first described by Hertig (1936) in *Culex pipiens*, but now we know that they infect practically all orders of insects, Collembola, some nematodes and crustaceans (Werren & Windsor 2000).

Wolbachia infection in *Trichogramma* wasps causes gamete duplication in the haplodiploid organisms resulting in all-female broods (Stouthamer & Kazmer 1994) and it is found in ca. 9% of all *Trichogramma* species known. Another type of reproductive modification by *Wolbachia* was shown in *T. bourarachae* (Girin & Boulétreau 1995; Vavre *et al*. 1999a) in which infected individuals have an increased fecundity.

Several *Wolbachia* genes have been use to determine the phylogeny of the different *Wolbachia* strains. At present the best resolution is given by the sequence of the wsp gene (O'Neill e*t al*. 1992; Rousset *et al*. 1992; Holden *et al*. 1993; Masui *et al*. 1997; Meer *et al*. 1999) Currently, five supergroups can be distinguished. Initially, two major groups of *Wolbachia* where created (A and B) which diverged supposedly 58-67 millions years ago (Werren *et al*. 1995). Two other supergroups (C and D) were found in nematodes (Bandi *et al*. 1998). The newest one, supergroup E, was proposed by Vanderkerckhove *et al*. (1999) and found in Collembola. In the supergroup B, three groups of *Wolbachia* infecting *Trichogramma* are distinguished (Dei, Sib and Kay) (van Meer *et al*. 1999). A creation of a new group (Sem) and the merging of the two closely related groups Sib and Kay under the name Sib was suggested by Pintureau *et al*. (2000). In *Chapter 4* the creation of a new *Wolbachia* group under the name "*Ato*" was proposed increasing the number of groups of '*Trichogramma Wolbachia*' from three (Pintureau *et al*. 2000) to four. Specific primers (ATOW- forward and reverse) for *Wolbachia* found in *T. atopovirilia* were designed. All *Wolbachia* that infect the *Trichogramma* species studied here belong to the B group.

Horizontal transmission of *Wolbachia* has been suggested in arthropods. The indication that *Wolbachia* undergo horizontal transmission stems from the fact that similar *Wolbachia* are found in unrelated insect species (O'Neill *et al*. 1992). In *Nasonia giraulti* and their fly host *Protocalliphora* sp. horizontal transmission has also been suggested because their *Wolbachia* strains clustered together. The relatively low variation between *Wolbachia*

strains within group A also suggests extensive horizontal transmission (Werren *et al*. 1995). In many cases identical *Wolbachia* strains have been found in unrelated insect species. The situation is different in the Parthenogenesis Inducing *Wolbachia* of *Trichogramma*. Here all "*Trichogramma-Wolbachia*" form a monophyletic group. This could have been caused by co-speciation of the *Trichogramma* and its *Wolbachia* symbiont. However Schilthuizen & Stouthamer (1997) show that within the "*Trichogramma-Wolbachia*" clade extensive horizontal transmission must take place between the different *Trichogramma* species. Here (*Chapter 4*) the *Trichogramma Wolbachia* inducing parthenogenesis in *Trichogramma* still forms a monophyletic clade after the addition of new species and strains of *Trichogramma*. Similarities of *Wolbachia* sequences (e.g. 18 *Wolbachia* strains found in *Sib* group) suggest the possibility of horizontal transfer between different *Trichogramma* species.

Recently natural horizontal transmission was confirmed by Huigens *et al.* (2000). This takes place when uninfected wasp larvae acquired *Wolbachia* when they shared a host egg with infected larvae, although the process by which uninfected *Trichogramma* larvae acquire *Wolbachia* remains unclear. In *Chapter 5* inter- and intraspecific horizontal transmission are shown. These natural phenomena took place between two closely related species (*T. kaykai* and *T. deion*). However, not all the newly infected females exhibited parthenogenesis. Horizontal transmission was not successful when infected and uninfected *T. atopovirilia* shared the same host. Interspecific transmission was also unsuccessful between two infected *Trichogramma* species (*T. atopovirilia* and *T. pretiosum).* These species may have originated from populations fixed for the infection. In fixed populations we expect the opportunities for horizontal transmission to be limited, leading to a loss of this adaptation of the *Wolbachia*. Furthermore, incompatibility between *Wolbachia* and the new host nuclear/cytoplasmic background can lead to unsuccessful horizontal transmission limiting the spread of the host infection (Heath *et al*. 1999; Vavre *et al*. 1999b).

Are thelytokous species infected with the *Wolbachia* symbiont a suitable choice for a biological control program?

Choosing and applying criteria to evaluate and predict the performance of parasitoids is often proposed to improve the success of biocontrol programs. Guidelines for quality control of natural enemies now mainly consist of characteristics that are easily measured in the laboratory (van Lenteren 2000). However, quality control procedures should ensure the success of the natural enemy under field conditions (van Lenteren 1991). Quality components for the production and use of *Trichogramma* wasps have been discussed (Bigler 1994) and effective natural enemies might be selected through pre-introductory evaluation criteria (van Lenteren & Manzaroli 1999). One of these criteria is the reproduction capacity of natural enemies.

The advantage of using a particular mode of reproduction for the parasitoids may have a substantial impact on the success of a biological control program (Aeschlimann 1990 & Stouthamer 1993). Thelytokous parasitoids infected with *Wolbachia* have been suggested as good candidates for biological control programs and understanding the effect of the symbionts on *Trichogramma* may help in choosing the most appropriate thelytokous lines for use in biological control (Silva 1999). Two different infected groups have been distinguished in *Trichogramma*: fixed populations and mixed populations. In fixed populations infection is fully established so that only thelytokous females are present, and in mixed populations thelytokous females coexist with arrhenotokous ones. In mixed populations thelytokous females are still able to mate and can produce daughters sexually or parthenogenetically. In mixed populations, a potential cytoplasmic-nuclear conflict exists, but in fixed populations this conflict is absent. It is theorized that fixation of the infection results in a reduction of negative impact of the symbiont on its host (van Meer 1999).

The use of parthenogenetic or unisexual natural enemies has been viewed as a way to enhance the efficacy of biological control (Timberlake & Clausen 1924). It is expected that *Wolbachia* from fixed populations have co-evolved to some extent with their hosts which may have led to the loss of genes needed to function in other host species (*Chapter 5*). Van Meer (1999), Silva (1999) and Huigens (2003) have assumed that selection of infected *Trichogramma* lines that originate from fixed populations should be important in the future due to the reduced effect on the fitness in such populations.

In *chapter 6* walking activity, walking speed and behaviour components of infected and uninfected *T. atopovirilia* were studied. *Trichogramma* walking activity was affected by temperature (15, 20 and 25^{o}C) and was highest at 25^{o}C. Individual wasp showed high variability in the walking activity. However, no negative effect of the *Wolbachia* symbiont was found on *T. atopovirilia*. Also walking speed was not affected by the *Wolbachia* infection. The following components of *Trichogramma* behaviour were studied: (1) host contact; (2) host drumming; (3) ovipositing + drilling and; (4) walking while drumming. The number of parasitized eggs and offspring were also analysed. Results showed that *Wolbachia* did not have a negative influence on *T. atopovirilia* behaviour. Both infected and uninfected *Trichogramma* wasps behaved similarly. If the use of infected, unisexual *T. atopovirilia* does not result in any negative non-target effects, this infected strain should preferably been used in a biological control, because of the advantages of this mode of reproduction as described by Stouthamer (1993) clearly fit the unisexual *T. atopovirilla* form studied here.

Acknowledgements

I thank Richard Stouthamer and Joop C. van Lenteren for their valuable comments on the manuscript.

References

Aeschlimann, J.P. 1990. Simultaneous occurrence of thelythoky and bisexuality in hymenopteros species, and its implications for the biological control of species. *Entomophaga* 35: 3-5.

Almeida, R.P. de & R. Stouthamer 2003. Molecular identification of *Trichogramma cacoecieae* Marchal (Hymenoptera: Trichogrammatidae): A new record for Peru. *Neotrop. Entomol.* 32: 269-272.

Almeida, R.P. de, A.I. Ciociola Jr. & R. Stouthamer, 2001. *Wolbachia*-induced parthenogenesis: the first report in a Brazilian *Trichogramma pretiosum* population. *Proc. Exper. Appl. Entomol.* 12: 41-44.

Bandi, C., T.J.C. Anderson, C. Genchi & M.L. Blaxter 1998. Phylogeny of *Wolbachia* in filarial nematodes. *Proc. R. Soc. Lond. B* 265: 2407-2413.

Basso, C., B. Pintureau & G. Grille 1999. Taxonomic study of two *Trichogramma* species from Uruguay (Hymenoptera: Trichogrammatidae). *Bol. San. Veg. Plagas* 25: 372-382.

Bigler, F. 1994. Quality control in *Trichogramma* production. In: E. Wajnberg & S.A. Hassan (eds.), *Biological Control with Egg Parasitoids,* CAB International, Wallingford, pp. 93-111.

Botto, E.N., G.W.G. Sidorkewicz, D. Stilinovic, C. Greco & P. Carrizo 1991. Estudios biologicos sobre *Trichogramma pretiosum* Riley, nueva cita para la entomofauna Argentina. In: *Resumenes del II Congresso Argentino de Entomologia*, Córdoba, p. 181.

Ciociola Jr., A.C., R.P. de Almeida, R.A. Zucchi & R. Stouthamer 2001a. Detecção de *Wolbachia* em uma população telítoca de *Trichogramma atopovirilia* Oatman and Platner (Hymenoptera: Trichogrammatidae) via PCR com o primer específico *wsp*. *Neotrop*. *Entomol*. 30: 489-491.

Ciociola Jr., A.C., R.B. Querino, R.A. Zucchi & R. Stouthamer 2001b. Molecular tool for identification of closely related species of *Trichogramma* (Hymenoptera: Trichogrammatidae): *T. rojasi* Nagaraja & Nagarkatti and *T. lassalei* Pinto. *Neotrop*. *Entomol*. 30: 575-578.

Foerster, L.A., M.R.F. Avanci, J.M.R. Martin & C.L. Cañete 2000. Egg parasitoids of *Anticarsia gemmatalis* (Lep.; Noctuidae) on soybean in Brazil. In: *Proceedings of the XXI International Congress of Entomology*, Foz do Iguaçu, p. 385.

Girin, C. & M. Boulétreau 1995. Microorganism-associated variation in host infestation efficiency in a parasitoid wasp *Trichogramma bourarachae* (Hymenoptera: Trichogrammatidae). *Experientia* 52: 398-401.

Heath, B.D., R.J. Butcher, W.G.F. Whitfield & S.F. Hubbard 1999. Horizontal transfer of Wolbachia *between phylogenetically distant insect species by a natural occurring mechanism.* Curr. Biol. *9: 313-316.*

Hertig, M. 1936. The Rickettsia *Wolbachia pipientis* (gen. et sp. n.) and associated inclusions of the mosquito *Culex pipiens*. *Parasitology* 28: 453-490.

Holden, P.R., J.F. Brookfield & P. Jones 1993. Cloning and characterization of a *ftsZ* homologue from a bacterial symbiont of *Drosophila melanogaster*. *Mol*. *Gen*. *Genet*. 240: 213-220.

Huigens, M.E. 2003. *On the evolution of Wolbachia-induced parthenogenesis in Trichogramma wasps*. Ph.D thesis, Wageningen University, The Netherlands, 183 pp.

Huigens, M.E., R.F. Luck, R.H.G. Klaassen, M.F.P.M. Maas, M.J.T.N. Timmermans & R. Stouthamer 2000. Infectious parthenogenesis. *Nature* 405: 178-179.

Kostadinov, D. & B. Pitureau 1991. A possibility to discriminate females of three closely related species of *Trichogramma* (Hymenoptera: Trichogrammatidae) with special purpose analysis of the type of *Trichogramma evanescens* Westwood. *Ann. Soc. Ent. Fr.* 27: 393-400.

Lenteren, J.C. van 1991. Quality control of natural enemies: hope or illusion. In: Bigler, F. (ed.) *Proceedings of the Fifth IOBC Global Working Group 'Quality control of mass reared arthropods',* Wageningen, The Netherlands, pp. 1-15.

Lenteren, J.C. van, 2000. Measures of success in biological control of arthropods by augmentation of natural enemies. In: G. Gurr & S. Wratten (eds.), *Measures of Success in Biological Control*, Kluwer Academic Publishers, Dordrecht, pp. 77-103.

Lenteren, J.C. van & G. Manzaroli 1999. Evaluation and use of predators and parasitoids for biological control of pests in greenhouses. In: R. Albajes, M.L. Gullino, J.C. van Lenteren & Y. Elad (eds.), *Integrated Pest and Disease Management in Greenhouse Crops*, Kluwer Academic Publishers, Dordrecht, pp.183-201.

Masui, S., T. Sasaki & H. Ishikawa 1997. GroE-Homologous operon of *Wolbachia,* an intracellular symbiont of arthropods: a new approach for their phylogeny. *Zool. Sci.* 14: 701-706.

Meer, M.M.M. van 1999. *Phylogeny and host-symbiont interactions of thelytokous inducing Wolbachia in Hymenoptera*. Ph.D thesis, Wageningen University, The Netherlands, 118 pp.

Meer, M.M.M. van, J. Witteveldt & R. Stouthamer 1999. Phylogeny of the arthopod

endosymbiont *Wolbachia* based on the *wsp* gene. *Insect Mol. Biol*. 8: 399-408.

Nagaraja, H. & S. Nagarkatti 1973. A key to some new world species of *Trichogramma* (Hym., Trichogrammatidae), with descriptions of four new species. *Proc. Entomol. Soc. Wash*. 75: 288-297.

Nagarkatti, S. & H. Nagaraja 1968. Biosystematic studies on *Trichogramma* species. I. Experimental hybridization between *Trichogramma australicum* Girault, *T evanescens* Westw. and *T. minutum* Riley. *Tech. Bull. Corn. Inst. Biol. Contr*. 10: 81-96.

O'Neill, S.L., R. Giordano, A. M.E. Colbert, T.L. Karr & H.M. Robertson 1992. 16S RNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmatic incompatibility in insects. *Proc. Natl. Acad. Sci. USA* 89: 2699-2702.

Pinto, J.D. 1997. Taxonomia de Trichogrammatidae (Hymenoptera) com ênfase nos gêneros que parasitam Lepidoptera. In: J.R.P. Parra & R.A. Zucchi (eds.), *Trichogramma e o controle biológico aplicado*, FEALQ, Piracicaba, pp. 13-40.

Pinto, J.D. 1998. Systematics of the North American species of *Trichogramma* (Hymenoptera: Trichogrammatidae). *Mem. Entomol. Soc. Wash*. 22. 287pp.

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

Pinto, J.D., A.B. Koopmanschap, G.R. Platner & R. Stouthamer 2002. The North American *Trichogramma* (Hymenoptera: Trichogrammatidae) parasitizing certain Tortricidae (Lepidoptera) on apple and pear, with ITS2 DNA characterizations and description of a new species. *Biol. Control* 23: 134-142.

Pintureau, B., M. Gerding & E. Cisternas 1999. Description of three new species of Trichogrammatidae (Hymenoptera) from Chile. *Can. Entomol*. 131: 53-63.

Pintureau, B., S. Chaudier, F. Lassablière, H. Charles. & S. Grenier 2000. Addition of *wsp*

sequences to the *Wolbachia* phylogenetic tree and stability of the classification. *J. Mol. Evol.* 51: 374-377.

Pratissoli, D. & M.J. Fornazier 1999. Ocorrência de *Trichogramma acacioi* Brun, Moraes & Soares (Hym.:Trichogrammatidae) em ovos de *Nipteria panacea* Thierry-Mieg (Lep.: Geometridae), um geometrídeo desfolhador do abacateiro. *An. Soc. Entomol. Brasil* 28: 347-349.

Querino, R.B. & R.A. Zucchi 2003a. New species of *Trichogramma* Westwood (Hymaneoptera: Trichogrammatidae) associated with lepidopterous eggs in Brazil. *Zootaxa* 163: 1-10.

Querino, R.B. & R.A. Zucchi 2003b. Six new species of *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) from a Brazilian forest reserve. *Zootaxa* 134: 1-11.

Querino, R.B., R.A. Zucchi, A.I. Ciociola Jr., C.L. Cañete & L.A. Foerster 2000. Charcterization of three species of the genus *Trichogramma*. In: *Proceedings of the XXI International Congress of Entomology*, Foz do Iguaçu, p. 411.

Rousset. F., D. Bouchon, B. Pintureau, P. Juchault & M. Solignac 1992. *Wolbachia* endosymbionts responsible for various sexual alterations of sexuality in arthropods. *Proc. R. Soc. Lond. B*. 250: 9 1-98.

Sarmiento, M.C.E. 1993. Una nueva especie de *Trichogramma* (Hymenoptera: Trichogrammatidae) de los Andes de colombia. *Rev. Colomb. Entomol.* 19: 3-5.

Schilthuizen, M. & R. Stouthamer 1997. Horizontal transmission of parthenogenesis-inducing microbes in *Trichogramma* wasps. *Proc. R. Soc. Lond. B* 264: 361-366.

Silva, I.M.M.S. 1999. *Identification and evaluation of Trichogramma parasitoids for biological pest control*. Ph.D. thesis, Wageningen University, The Netherlands, 151 pp.

Silva, I.M.M.S., J. Honda, F. van Kan, S.J. Hu, L. Neto, B. Pintureau & R. Stouthamer 1999. Molecular differentiation of five *Trichogramma s*pecies occurring in Portugal.

Biol. Control 16: 177-184.

Silva, R.B.Q. 2002. *Taxonomia do gênero Trichogramma Westwood 1833 (Hymenoptera: Trichogrammatidae) na América do Sul*. Tese de Doutorado, Escola Superior de Agricultura Luiz de Queiroz-USP, Piracicaba. 214pp.

Stouthamer, R. 1993. The use of sexual versus asexual wasps in biological control. *Entomophaga* 38: 3-6.

Stouthamer, R., J. Hu, F.J.P.M. van Kan, G.R. Platner & 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. & D.J. Kazmer 1994. Cytogenetics of microbe-associated panthenogenesis and its consequences for gene flow in *Trichogramma* wasps. *Heredity* 73: 317-327.

Stouthamer, R., J.D. Pinto, G.R. Platner & R.F. Luck 1990. Taxonomic status of thelytokous forms of *Trichogramma* (Hymenoptera: Trichogrammatidae). *Ann. Entomol. Soc. Am.* 83: 475-581.

Timberlake, P.H. & C.P. Clausen 1924. The parasites of *Pseudococus maritimus* in California. *Univ. Calif. Publ. Tech. Bull. Entomol.* 3: 223-292.

Vandekerckhove, T.T.M, S. Watteyne, A. Willems, J.G. Swing, J. Mertens & M. Gillis 1999. Phylogenetic analysis of the 16S rDNA of the cytoplasmic bacterium *Wolbachia* from the novel host *Folsomia candida* (Hexapoda, Collembola) and its implications for wolbachial taxonomy. *FEMS Microbial. Lett.* 180: 279-286.

Vavre, F., C. Girin & M. Boulétreau 1999a. Phylogenetic status of a fecundity-enhancing *Wolbachia* that does not induce thelytoky in *Trichogramma*. *Insect Mol. Biol.* 8:67-72.

Vavre, F., F. Fleury, D. Lepetit, P. Fouillet, & M. Bouletreau 1999b. Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. *Molec. Biol. Evol.* 16: 1711-1723.

Werren, J.H. 1997. Biology of *Wolbachia. Ann. Rev. Entomol.* 42: 587-609.

Werren, J.H. & D.M. Windsor 2000. *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? *Proc. R. Soc. Lond. B* 267: 1277-1285.

Werren, J.H., D. Windsor & L.R. Guo l995. Distribution of *Wolbachia* among neotropical arthropods. *Proc. R. Soc. Lond. B* 262: 197-204.

Whu, M. & L. Valdivieso 1999. Distribución y comportamiento de ocho especies de *Trichogramma* y *Trichogrammatoidea* (Hymenoptera: Trichogrammatidae) en el Perú. *Rev. Peru Entomol.* 41: 61-68.

Zucchi, R.A. 1988. New species of *Trichogramma* (Hymenoptera: Trichogrammatidae) associated with sugar cane borer *Diatraea saccharalis* (F.) (Lepidoptera: Pyralidade) in Brazil. *Les Colloq. INRA* 43: 133-140.

Zucchi, R.A. 1985. *Taxonomia de espécies de Trichogramma (Hymenoptera: Trichogrammatidae) associados a algumas pragas (Lepidoptera) no Brasil*. Tese (Livre-Docência), Escola Superior de Agricultura Luiz de Queiroz-USP, Piracicaba. 77pp.

Zucchi, R.A. & R.C. Monteiro 1997. O gênero *Trichogramma* na América do Sul. In: Parra, J.R.P. & R.A. Zucchi (eds.), *Trichogramma e o controle biolólgico aplicado*, FEALQ, Piracicaba, pp. 41-66.

Zucchi, R.A., J.R.P. Parra & S. Silveira Neto 1991. *Trichogramma* species associated with some lepidopterous pests in Brazil. *Les Colloq. INRA* 53: 131-134.

Summary

Identification of *Trichogramma* individuals has long been very difficult. No reliable character was known for the identification of species in this genus, until the utility of male genitalia was recognized in 1968, some 135 years after the first *Trichogramma* was described (*T. evanescens* Westwood, 1833). However, the presence of completely parthenogenetic lines in this species remained an identification problem because the no reliable female characters exist that allowed identification. The discovery of *Wolbachia* as a cause for the complete parthenogenesis in this genus has allowed the identification of such thelytokous lines. Antibiotic treatment could revert *Wolbachia* infected thelytokous species to sexual ones, thus producing males and allowing their identification in the morphological system. However, the lack of morphological identification of *Trichogramma* females was only solved with the use of molecular techniques based on rDNA sequencing of the internal transcribed spacer 2 (ITS2) region. Here, this technique was proved to be a reliable tool for the identification of *T. cacoeciae*, a thelytokous species where the parthenogenetic reproduction is not due to *Wolbachia* infection. In this thesis we report the first record of this species in Peru (*Chapter 2*). This species is the only thelytokous *Trichogramma* known in which *Wolbachia* is not present. *T. cacoeciae* presence in South America is discussed. Identification of seventeen native/introduced *Trichogramma* species using rDNA sequences was done. A molecular key based on restriction analysis allowed identification of the species through the size of the PCR product and the generation of the restriction patterns. Thelytoky in *Trichogramma* species caused by *Wolbachia* collected in Peru, Colombia and USA were reported (*Chapter 3*). Phylogenetic analysis and comparison between DNA sequences of the *Wolbachia* wsp gene allowed for the recognition of new *Wolbachia* group "*Ato*" in the clade of *Wolbachia* that infect *Trichogramma* species. The construction of

Wolbachia phylogenetic tree showed four distinct groups. The similarities in the *Wolbachia* sequences for the studied groups suggested the possibility of horizontal transmission between *Trichogramma* species (*Chapter 4*). Natural inter- and intraspecific horizontal transfer of PI *Wolbachia* between wasps of the genus *Trichogramma* was shown. *Wolbachia* infection in uninfected *Trichogramma* females was possible when infected and uninfected immature wasps shared the same host egg. On the whole, intraspecific transfer tended to be more successful than interspecific transfer (*Chapter 5*). In *T. atopovirilia, Wolbachia* infection did not lead to any negative effect on the walking activity, walking speed or other behaviour components studied (*Chapter 6*). Finally, an overview of the most important outcomes and conclusions of this thesis is presented (*Chapter 7*).

Resumo

Dificuldades na identificação específica de *Trichogramma* tem se evidenciado desde há muito tempo. Caracteres confiáveis à sua identificação somente foram detectados com a descoberta da utilidade da genitália do macho, reconhecida em 1968, cerca de 135 anos após a descrição da primeira espécie (*T. evanescens* Westwood, 1833). Entretanto, devido à presença de linhagens partenogenéticas de *Trichogramma*, o problema da identificação permaneceu, em função de não haver caracteres confiáveis para descrição específica de indivíduos fêmea. A descoberta de *Wolbachia*, como a causa da reprodução partenogenética no gênero *Trichogramma* possibilitou a identificação das linhagens telítocas. O tratamento de espécies infectadas por *Wolbachia* por meio de antibióticos reverteu o modo de reprodução de assexuada para sexuada, possibilitando com isto, a produção de indivíduos machos e viabilizando a identificação via sistema de identificação morfológica. Contudo, a limitação da identificação morfológica de fêmeas de *Trichogramma* somente foi solucionada a partir da utilização de técnicas moleculares baseadas no sequenciamento do DNA ribossomal da região interna transcrita do espaço 2 (internal transcribed spacer 2-ITS2). Esta técnica da biologia molecular provou ser um instrumento confiável na identificação de *T. cacoeciae*, uma espécie telítoca, onde a reprodução partenogenética não é causada pela infecção de *Wolbachia*. A identificação de *T. cacoeciae* foi registrada pela primeira vez no Peru (*Chapter 2*). Esta espécie de *Trichogramma* é a única com reprodução partenogenética em que não há infecção por *Wolbachia*. A presença de *T. cacoeciae* na América do Sul é discutida. A identificação de dezessete espécies nativas e introduzidas de *Trichogramma* foi feita via seqüências do DNA ribossomal. Uma chave molecular com base na análise de restrição permitiu a identificação específica de *Trichogramma* através do tamanho do produto da reação em cadeia de polimerase (PCR) e da geração dos padrões de clivagem. Espécies telítocas de *Trichogramma*, infectadas por *Wolbachia*, foram coletadas

no Peru, Colômbia e USA (*Chapter 3*). Análises filogenéticas e comparação entre seqüências do DNA ribosssomal de *Wolbachia* (*wsp* gene) permitiu o reconhecimento de um novo grupo de *Wolbachia* denominado "*Ato*". Este grupo foi criado devido a *Wolbachia* que infecta *T. atopovirilia*. Com a construção da árvore filogenética de *Wolbachia* foi possível se distinguir quarto grupos. A similaridade entre as seqüências de *Wolbachia* em relação aos grupos estudados sugeriu a possibilidade de transmissão horizontal entre as espécies de *Trichogramma* (*Chapter 4*). A transmissão natural interespecífica e intraespecífica de *Wolbachia*, entre os indivíduos do gênero *Trichogramma*, foi comprovada em condições de laboratório. A infecção de *Wolbachia* em fêmeas não infectadas de *Trichogramma* foi possível quando larvas infectadas e não infectadas de *Trichogramma* compartilharam o mesmo ovo do hospedeiro. De modo geral, a transmissão intraespecífica de *Wolbachia* foi mais bem sucedida do que a transmissão interespecífica (*Chapter 5*). A infecção de *T. atopovirilia* não levou a nenhum efeito negativo sobre a atividade de caminhamento, a velocidade de caminhamento ou quaisquer outros componentes do comportamento estudados (*Chapter 6*). Finalmente, uma visão geral dos resultados mais importantes e conclusões da tese é apresentada (*Chapter 7*).

Samenvatting

Identificatie van *Trichogramma* individuen is lange tijd een lastige onderneming geweest. Voor de identificatie van soorten in dit genus was geen betrouwbaar morfologisch kenmerk bekend totdat de mannelijke genitaliën werden onderkend in 1968, 135 jaar na de eerste beschrijving van *Trichogramma* (*T. evanescens* Westwood, 1833). De aanwezigheid van parthenogenetische lijnen in deze soorten bleef echter een probleem omdat er geen vrouwelijke identificatie kenmerk bestond. De ontdekking van *Wolbachia* als de oorzaak van parthenogenese in dit genus maakte de identificatie van dergelijke thelytoke lijnen mogelijk. Behandeling met antibiotica zorgde voor de omvorming van *Wolbachia*-geïnfecteerde thelytoke lijnen tot sexuele lijnen en konden de mannetjes daarna morfologisch worden geïdentificeerd. Het gemis van morfologische identificatie van *Trichogramma* vrouwtjes werd opgelost met het gebruik van moleculaire technieken gebaseerd op het sequencen van rDNA van de Internal Transcribed Spacer 2 (ITS2) regio. Uit dit proefschrift is gebleken dat deze techniek een uiterst betrouwbare methode is voor de identificatie van *T. cacoeciae*, een thelytoke soort waarbij parthenogenese niet wordt veroorzaakt door een *Wolbachia* infectie. In dit proefschrift laten we de eerste beschrijving van deze soort in Peru zien (*Hoofdstuk 2*). Deze soort is de enige thelytoke *Trichogramma* waarbij *Wolbachia* niet aanwezig is. Daarnaast wordt *T. cacoeciae*'s aanwezigheid in Zuid-Amerika bediscussieerd. Verder is er een identificatie van zeventien inheemse/geïntroduceerde *Trichogramma* soorten uitgevoerd op basis van rDNA sequenties. Een moleculaire sleutel gebaseerd op een restrictie analyse maakte identificatie van deze soorten mogelijk. In een ander hoofdstuk zijn een aantal gevallen van *Wolbachia*-geïnduceerde thelytoky in *Trichogramma* soorten uit Peru, Colombia en de VS beschreven (*Hoofdstuk 3*). Uit een fylogenetische analyse van het *Wolbachia* gen, *wsp*, bleek dat er een

Wolbachia groep, genaamd "*Ato*", binnen het cluster van *Wolbachia* infecties in *Trichogramma* soorten. Een constructie van de fylogenetische stamboom van *Wolbachia* toonde vier verschillende groepen. De overeenkomsten in de *Wolbachia* sequenties van de bestudeerde groepen suggereert de mogelijkheid van horizontale transmissie van *Wolbachia* tussen *Trichogramma* soorten (*Hoofdstuk 4*). Natuurlijke inter- en intraspecifieke horizontale transmissie van PI *Wolbachia* tussen wespen van het genus *Trichogramma* is aangetoond in Hoofdstuk 4. Het met *Wolbachia* geïnfecteerd raken van ongeïnfecteerde *Trichogramma* vrouwtjes was mogelijk als geïnfecteerde en ongeïnfecteerde onvolwassen wespen hetzelfde gastheerei delen. Over het algemeen bleek intraspecifieke transmissie successvoller dan interspecifieke transmissie (*Hoofdstuk 5*). In *T. atopovirilia* leidde de *Wolbachia* infectie niet tot enige negatieve effecten op loopactiviteit, loopsnelheid en andere bestudeerde gedragingen (*Hoofdstuk 6*). Aan het einde van dit proefschrift is een overzicht van de belangrijkste vondsten en conclusies gepresenteerd (*Hoofdstuk 7*).

About the Author

Raul Porfirio de Almeida was born on June 15th, 1960 in Paudalho, Pernanbuco State (PE), Brazil. In 1965, his parents Bernardo Porfirio de Oliveira (*In memory*) and Edna Marinho Almeida de Oliveira decided to move to Recife, capital of Pernambuco. There, he attended the primary, secondary and high school. In 1980 he started Agronomy at the Federal Rural University of Pernambuco (UFRPE). His research training in the Entomology area started in the second year of his academic studies being sponsored by DAE (Departamento Acadêmico Estudantil)-UFRPE, an agreement between UFRPE/FIPEC/B.B and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). He obtained his BSc degree in February 1995. In the same year he was a research trainee at EPEAL (Empresa de Pesquisa Agropecuária do Estado de Alagoas), a project sponsored by CNPq/Embrapa (Brazilian Agricultural Research Corporation), for five months. From December 1986 to May 1987 he worked as agronomist at FAPEPE (Fundação de apóio a Pesquisa, Ensino e Extensão Rural de Pernambuco) and from July 1987 to June 1989 at SUEP, an agreement between CNPq/UFRPE/PDCT-NE/BID. His MSc degree in Phytossanity with specialisation in Entomology was obtained at the Federal Rural University of Pernambuco-UFPRE in March 1990. In January 1990 after a public examination, Raul began as Researcher at Embrapa / CNPA (National Center for Research on Cotton) where he started researching the use of *Trichogramma* in the biological control of cotton insect pests. In February 1999 he was sponsored by Embrapa/PRODETAB to carry out his Ph.D. at Wageningen University, Department of Plant Sciences, Laboratory of Entomology, in Wageningen, The Netherlands. His Ph.D. studies were completed on January 21st, 2004 with the defence of his thesis on *Trichogramma* and its relationship with *Wolbachia* symbionts.

Current Address:
Embrapa Cotton
Laboratory of Entomology
Rua Osvaldo Cruz 1143, Bairro Centenário
C.P. 174 - CEP 58.107.720
Campina Grande, PB, Brazil
Phone: ++ 55 83 3154314
Fax: ++ 55 83 3154367
E-mail: raul@cnpa.embrapa.br
URL: http://www.cnpa.embrapa.br

List of Publications (1999-2003)

Almeida, R.P. de 2000. Distribution of parasitism by *Trichogramma pretiosum* on the cotton leafworm. *Proc. Exper. Appl. Entomol.* 11:27-31.

Almeida, R.P. de 2000. Economic threshold for the cotton boll weevil (*Anthonomus grandis* Boheman) control. *Proc. Exper. Appl. Entomol.* 11:97-101.

Almeida, R.P. de 2000. Side effects of insecticides on the entomopathogenic fungus *metarhizium anisopliae* (Merch.) Sorokin. *Proc. Exper. Appl. Entomol.* 11:23-26.

Almeida, R.P. de 2001. Cotton insect pest control on a small farm: an approach of successful biological control using *Trichogramma*. *Proc. Exper. Appl. Entomol.* 12:81-84.

Almeida, R.P. de 2001. Effect of the population levels of *Aphis gossypii* on cotton agronomic traits and fibre quality. *Proc. Exper. Appl. Entomol.* 13:97-100.

Almeida, R.P. de, V.M.R.A. Almeida, S.J. Oliveira, R. Isidro, N.N.C. Andrade & N.F.A. Sousa 2002. Distribution and metric traits of *Stiphra robusta* (Orthoptera: Proscopiidae) ootecas and eggs. *Proc. Exper. Appl. Entomol.* 13:83-85.

Almeida, R.P. de & C.A.D. da Silva 1999. Manejo Integrado de pragas do Algodoeiro. In: Beltão, N.B.E de. O agronegócio do Algodão no Brasil. Brasília, SPI, 2, pp. 753-820.

Almeida, R.P. de & R. Stouhamer 2001. Crossing compatibility between Brazilian *Trichogramma pretiosum* strains. In: 53rd International Symposium on Crop Protection, Ghent, Ghent University, Annals, p.143.

Almeida, R.P. de & R. Stouhamer 2001. Temperature effect on the fitness and *Wolbachia* transmission in *Trichogramma pretiosum*. In: VII Simpósio de Controle Biológico, Poços de Caldas, Anais, p. 430.

Almeida, R.P. de, A.F.S.L. Veiga, D.N.D. Gomes & L.H.L. Batista, 2002. Lisfe history of *Pentilia sp.* and *Coccodophilus citricola* (Coleoptera: Coccinellidae) on the cactaceae scale insect. *Proc. Exper. Appl. Entomol.* 13:69-72.

Boeke S.J., A.A.C. Sinzogan, Almeida R.P. de, P.W.M.de Boer, G. Jeong, D.K. Kossou & J.J.A. van Loon 2003. Side effect of cowpea treatment with botanical insecticides on two parasitoids of *Callosobruchus maculatus*. *Entomol. Exp. Appl.* 108: 43-51.

Ciociola Jr., A.I., R.P. de Almeida & R. Stouthamer 2000. Does degradation of DNA in dead *Trichogramma* individuals lead to failure of their identification using ITS2 PCR. *Proc. Exper. Appl. Entomol.* 11:33-36.

Ciociola Jr., A.I., R.P. de Almeida, R.A. Zucchi & R. Stouthamer 2001. Detecção de *Wolbachia* em uma população telítoca de *Trichogramma atopovirilia* Oatman & Platner (Hymenoptera: Trichogrammatidae) via PCR com primer específico *wsp. Neotrop. Entomol.* 30:489-491.

Santos, R.C., O.L. Franco, R.P.de Almeida, R.G. Monnerat, R.G. & M.F. Grossi de Sá 2000. Influence of the soybean kunitz trypsin inhibitor on the oviposition, hatching and mortality of the cotton boll weevil (Anthonomus grandis). *Genet. Mol. Biol. Supplement* 23, p.548.

This thesis

Almeida, R.P. de, A.I. Ciociola Jr. & R. Stouthamer 2001. *Wolbachia*-induced parthenogenesis: the first report in a Brazilian *Trichogramma pretiosum* population. *Proc. Exper. Appl. Entomol.* 12:41-44.

Almeida, R.P. de & R. Stouthamer 2003. Molecular identification of *Trichogramma cacoecieae* Marchal (Hymenoptera: Trichogrammatidae): A new record for Peru. *Neotrop. Entomol*. 32: 269-272.

Almeida, R.P. de & R. Stouthamer. ITS2-based identification for South America *Trichogramma* species (*Submitted*).

Almeida, R.P. de & R. Stouthamer. Phylogeny of the *Trichogramma* endosymbiont *Wolbachia*, an alpha-Proteobacteria (Rickettsiae) (*Submitted*).

Almeida, R.P. de, J.C. van Lenteren & R. Stouthamer. Does *Wolbachia* infection affect *Trichogramma atopovirilia* behaviour? (*To be Submitted*).

Huigens, M.E., R.P. de Almeida, P. Boons, R.F. Luck & R. Stouthamer. Natural inter- and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps. *Proc. R. Soc. Lond. B* (*Accepted*).