

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

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Received: July 15, 2016 – Accepted: January 11, 2017 – Distributed: October 31, 2018

(With 1 figure)

Abstract

Wolbachia (Hertig) endosymbionts are extensively studied in a wide range of organisms and are known to be transmitted through the egg cytoplasm to the offspring. *Wolbachia* may cause several types of reproductive modifications in arthropods. In *Trichogramma* species, parthenogenesis-inducing *Wolbachia* bacteria allow females wasps to produce daughters from unfertilized 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 *Trichogramma atopovirilia* (Oatman and Platner). Specific primers were designed and tested for the “*Ato*” group. Seventy-five percent of all evaluated *Wolbachia* strains from *Trichogramma* fell within “*Sib*” group.

Keywords: symbiont, egg parasitoid, DNA sequencing, phylogenetic relationships.

Filogenia do endosimbionte *Wolbachia* em *Trichogramma*, an alpha-proteobacteria (Rickettsiae)

Resumo

Endosimbiontes do gênero *Wolbachia* (Hertig) são extensivamente estudados em uma ampla gama de organismos e são conhecidos por serem transmitidos via citoplasma do ovo hospedeiro para seu descendente. *Wolbachia* pode causar vários tipos de alterações reprodutivas nos artrópodes. Nas espécies de *Trichogramma*, a reprodução partenogenética induzida por *Wolbachia*, possibilita as fêmeas dos parasitoides a produção de fêmeas a partir de ovos não fertilizados e estas bactérias estão presentes em pelo menos 9% de todas as espécies de *Trichogramma*. Estudos filogenéticos têm levado a subdivisão do clado *Wolbachia* em cinco supergrupos (A, B, C, D and E). *Wolbachia* em *Trichogramma* pertence ao supergrupo B. Com o gene *wsp* foi possível se distinguir quatro grupos de *Wolbachia* que infectam *Trichogramma* e adicionar um novo grupo (*Ato*) devido a inclusão de *Wolbachia* detectada em *Trichogramma atopovirilia* (Oatman and Platner, 1983). *Primers* específicos foram construídos e testados para o grupo “*Ato*”. Setenta e cinco por cento de todas as linhagens de *Wolbachia* que infectam *Trichogramma* se enquadraram dentro do grupo “*Sib*”.

Palavras-chave: simbiote, parasitoide de ovos, sequenciamento de DNA, filogenia.

1. Introduction

Wolbachia (Hertig) (Rickettsiaceae) symbionts have been extensively studied in a wide range of organisms and are 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 and Windsor, 2000).

Wolbachia infects the reproductive tissues of arthropods, are transmitted through the egg cytoplasm and cause

several reproductive modification types: (1) cytoplasmic incompatibility (CI) in insects (Breeuwer and Werren, 1990; Breeuwer et al., 1992; Giordano et al., 1995), isopods (Legrand and Juchault, 1986; Rousset et al., 1992) and mites (Johanowicz and Hoy, 1996; Tsagkarakou et al., 1996).

Cytoplasmic incompatibility results in aborted karyogamy (O’Neill and Karr, 1990) and occurs when infected males are crossed with females that are either uninfected (unidirectional incompatibility) (Hoffmann et al., 1986) or infected with another bacterial variant (bidirectional

incompatibility); (2) feminization in isopods where genetic males are converted into functional females (Martin et al., 1994; Rousset et al., 1992); (3) the induction of complete parthenogenesis in some haplodiploid species (Almeida and Stouthamer, 2015; Almeida et al., 2010; Stouthamer et al., 1990, 1993). Parthenogenesis-inducing *Wolbachia* bacteria allow infected female to produce offspring from unfertilized eggs due to a first mitotic division modification (Stouthamer and Kazmer, 1994) and the genetic basis for the loss of female sexual function could be explained by a dominant nuclear effect (Russell and Stouthamer, 2011); (4) fecundity increase of the host for the egg parasitoid *Trichogramma bourarachae* (Pintureau and Babault, 1980; Vavre et al., 1999b) and (5) male-killing in a wide range of insects. This is a case in which inherited bacteria kill male hosts during early development (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 (Stouthamer et al., 1993); (2) the 23S rDNA (Rousset et al., 1992); (3) the bacterial cell-cycle *ftsZ* gene (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 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. Two other supergroups (C and D) were found in nematodes (Bandi et al., 1998). The *Wolbachia* supergroup (E),

based on the 16S rDNA, was found in Collembola (Vandekerckhove et al., 1999).

In *Trichogramma*, Schilthuizen and 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* and this hypothesis was confirmed by Huigens et al. (2000, 2004).

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

2. Material and Methods

2.1. 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 were 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' - AAAAATTAACGCTACT CCA-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.

2.2. 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, white colonies were picked with a sterile toothpick from the plates and placed into tubes containing 3.0 ml of LB liquid medium and 3 µl 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

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

Host	Line Designation	Origin	Genbank Acces. Number	References
<i>L. strialellus</i>	-	-	AF020080	Zhou et al. (1998)
<i>T. confusum</i>	-	-	AF020083	Zhou et al. (1998)
<i>T. bedeguaris</i>	-	-	AF071915	Van Meer et al. (1999)
<i>T. atopovirilia</i>	Tato-01	Minas Gerais, Brazil	-	This study
<i>T. brevicapillum</i>	-	Mojave desert, CA, USA	-	This study
	M	Mojave River Forks	-	This study
<i>T. cordubensis</i>	28A63a	Divor, Portugal	-	This study
	-	-	AF245164	Pintureau et al. (2000)
<i>T. dendrolimi</i>	-	-	AB094397	Tagami et al. (2001)
<i>T. deion</i>	TX	Sanderson, TX	AF020084	Zhou et al. (1998)
	SW436	Mojave desert, CA, USA	AF071925	Van Meer et al. (1999)
<i>T. embryophagum</i>	Uro3	Orumiyeh, Iran	AF245165	Pintureau et al. (2000)
<i>T. evanescens</i>	M36	Alpes-maritimes, France	AF245167	Pintureau et al. (2000)
<i>T. kaykai</i>	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)
<i>T. nubilale</i>	234	Nova Scotia, Canada	AF071926	Van Meer et al. (1999)
<i>T. oleae</i>	S2	Former Yugoslavia	AF245166	Pintureau et al. (2000)
<i>T. pretiosum</i>	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
<i>T. semblidis</i>	U	Uruguay	AF245163	Pintureau et al. (2000)
	Semv	Valbomme, Alpes-maritimes, France	AF145162	Pintureau et al. (2000)
<i>T. sibericum</i>	SIB	Canada	AF071923	Van Meer et al. (1999)

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.

2.3. 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.

3. Results

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* Oatman and Platner; *T. pretiosum* Riley, 1879; *T. cordubensis* Vargas and Cabello, 1985 and *T. brevicapillum* Pinto and Platner, 1978) sequenced in this study, combined with several other strains of the genders *Laodelphax*, *Tribolium*, *Torymus* and *Trichogramma* (= *T. dendrolimi* Matsumura, 1926; *T. deion* Pinto and Oatman, 1986; *T. embryophagum* Hartig, 1838; *T. evanescens* Westwood, 1833; *T. kaykai* Pinto and Stouthamer, 1997; *T. nubilale* Ertle and Davis, 1975; *T. oleae* Voegelé and Pointel, 1979; *T. semblidis* Aurivillius, 1897 and *T. sibericum* Sorokina, 1981), obtained from the GenBank (Table 1). The *Wolbachia* strain that infects *T. atopovirilia* species was first reported from Brazil by Ciociola Junior et al. (2001). All other

T. atopovirilia collected so far from several countries (Mexico, El Salvador, Guatemala, Honduras, Colombia, and Venezuela) (Pinto, 1998; Zucchi and Monteiro, 1997) 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).

According to Van Meer et al. (1999), three groups of *Wolbachia* that infect *Trichogramma* were recognised (Dei, Sib and Kay). 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.

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 (Figure 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.

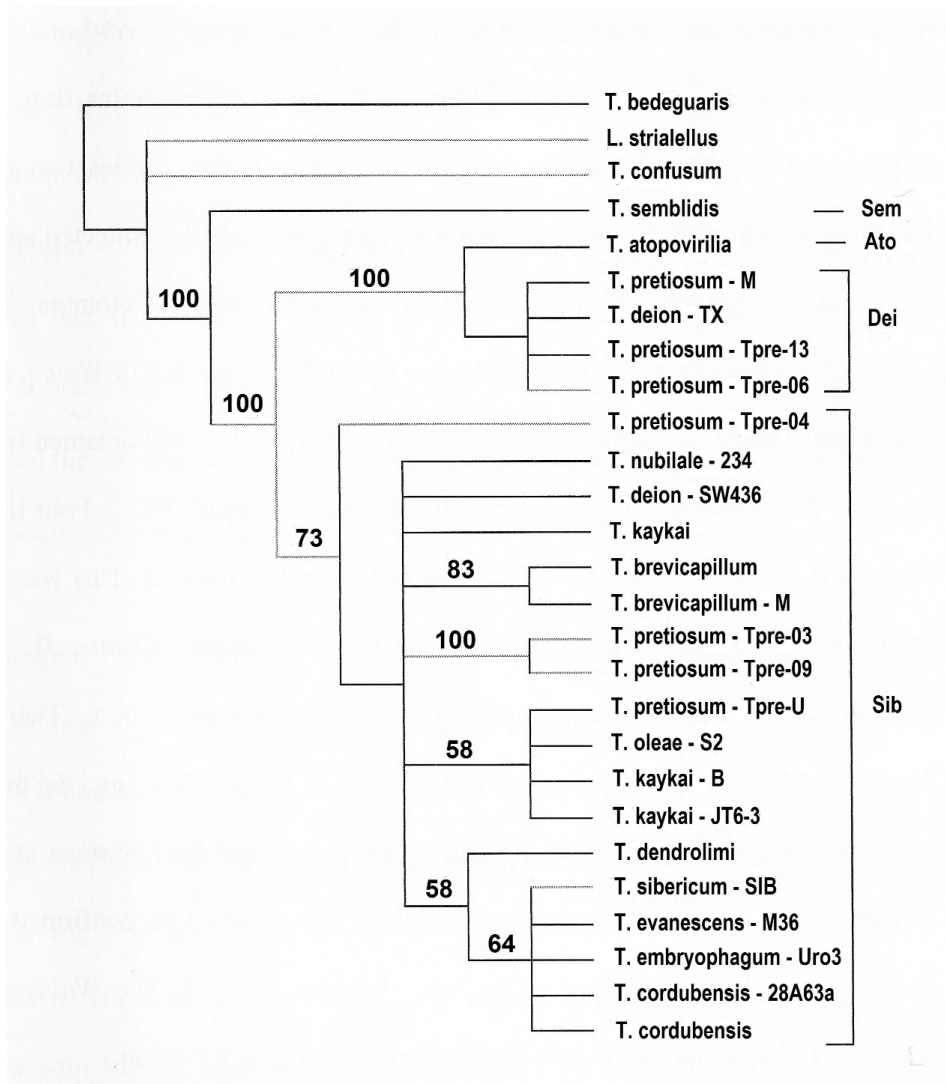


Figure 1. Phylogenetic relationships among *Wolbachia* strains from different *Trichogramma* hosts based on *wsp* gene. Heuristic searches were performed (300 random replicate searches) and Bootstrap values based on 1000 replicate searches with 2 random replicate per search.

4. Discussion

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. 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 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'-CCAAAAGTGCCGTAAAGAACA-3'. A specific annealing temperature was used (66 °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. 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*.

In 210 described *Trichogramma* species (Pinto 2006) thelytoky caused by *Wolbachia* infection has been found in 18 species. It results in all-female broods being usually caused by the cytoplasmically inherited bacterium *Wolbachia*, which induces gamete duplication in the haplodiploid organisms (Stouthamer and Kazmer, 1994).

Parthenogenesis inducing *Wolbachia* infecting *Trichogramma* species are exclusively found in the B supergroup (Pintureau et al., 2000; Poorjavad et al., 2012; Van Meer et al., 1999; Schulenburg et al., 2000; Werren et al., 1995b; Zhou et al., 1998). 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 *Ephesia kuehniella* (Zeller) (Lepidoptera: Pyralidae), which may indicate that these are either contaminations or are *Wolbachia* acquired from their hosts (Van Meer et al., 1999; Vavre et al., 1999a).

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, 2004). When attempts of horizontal transfer were done using different *Trichogramma* hosts, as in the case of *T. atopovirilia*, *Wolbachia* transmission was not successful (Huigens et al., 2004).

The lack of congruence between the phylogenetic trees of the host species with the *Wolbachia* tree shown by Schilthuisen and Stouthamer (1997) indicates that horizontal transfer of *Wolbachia* must have occurred on an evolutionary time scale. Watanabe et al. (2013) suggests studies on *Wolbachia* transfers between various host combinations to systematically understand the condition of *Wolbachia* required to express its phenotype, which would contribute to better understanding of *Wolbachia*-induced host manipulations.

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 (Huigens et al., 2004). *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.

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

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* - SW436; 10 - *T. deion*-TX; 11 - *T. dendrolini*; 12 - *T. embryophagum*; 13 - *T. evanescens*; 14 - *T. kaykai* B; 15 - *T. kaykai* ITG-3; 16 - *T. kaykai*; 17 - *T. kaykai*; 18 - *T. nubilale*; 19 - *T. pretiosum*-U; 20 - *T. pretiosum* M; 21 - *T. pretiosum*-Pre-13; 22 - *T. pretiosum*-Pre-06; 23 - *T. pretiosum*-Pre-03; 24 - *T. pretiosum*-Pre-09; 25 - *T. pretiosum*-Pre-04; 26 - *T. semblidis*; 27 - *T. sibiricum*.

Acknowledgements

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

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