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**Transmission of parthenogenesis-inducing *Wolbachia* bacteria between  
two strains of the biocontrol agent *Eretmocerus mundus***

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## Abstract

The whitefly *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) is a severe pest worldwide because of its wide range of plant hosts. It causes direct feeding damage, is capable to transmit pathogenic viruses, and contaminates plants and fruit with honeydew. Chemical and biological control measures are the most frequently used measures to control this pest. Since chemical control is resulting in contamination and pest-resistance problems, the use of biological control has high priority. Research about increasing the potential of a commercially-used parasitoid species as *Eretmocerus mundus* Mercet (Hymenoptera: Aphelinidae) is, therefore, essential. One possibility might be the transfer of a parthenogenesis-inducing *Wolbachia* bacterium from a thelytokous strain (where females are originated from unfertilized eggs) to an arrhenotokous strain (where females are originated from fertilized eggs) of this parasitoid. Some articles mention that horizontal transmission of *Wolbachia* occurs in certain *Trichogramma* parasitoids in nature, and inter- and intra-specific horizontal transfer has appeared to be possible experimentally. In *E. mundus* physical elimination of supernumerary larvae has been reported in cases of superparasitism. Only the first instar *Eretmocerus* larva is equipped with mandibles that can perforate the host tissues. First instar larvae can spend a period varying from a few hours to three days outside the whitefly host, and a period of about one day inside the host. When there are two or more *E. mundus* larvae in contact with each other, physical attack can take place, both inside and outside the host. In future, successful horizontal transmission of *Wolbachia* and the consequent change in the reproductive system of parasitoids could increase their agronomic importance in biological control of pests.

The aim of this research was to get horizontal transmission of *Wolbachia* bacteria from a thelytokous population (Australian) of *E. mundus* to an arrhenotokous population (Spanish) of the same species. To accomplish the objective, experiments with first-stage larvae were done. The experiments consisted on putting one larva of *E. mundus* Australian population as *Wolbachia* donor over the mandible of one larva of *E. mundus* Spanish population as recipient in order to be eaten. After the experiments molecular analyses were performed on the larvae involved. Two experimental sets were done. In the first set of experiments, 61 pairs of larvae and 19 Spanish larvae as control were analyzed. Positive *Wolbachia* infection (16S) was identified in controls in the first set. An extra group of 25

Spanish adults were analyzed for *I6S* and some samples showed bands at the same level of *Wolbachia* positive control. Cycles in the PCR programming were adjusted to avoid false presence of bands and molecular analyses were done (twice) for 22 Spanish larvae this time. One Spanish larva remained positive for *Wolbachia* bacteria. These results annulated the experiments done in the first set. A new set of experiments were performed. Without presence of *Wolbachia* in the Spanish mothers, four Spanish larvae (from 73 horizontal-transmission experiments) resulted positive for *Wolbachia*. A rate as 5.47% of horizontal transmission was expected since previous studies in *Trichogramma* (Grenier et al. 1998; Cook & Butcher 1999; Huigens et al. 2004) mention *Wolbachia* is not easily transmitted by horizontal transmission. After almost all the analyses done, one question remains open: is there any chance *Wolbachia* can come from *B. tabaci*? A number of 49 samples of *B. tabaci* nymphs were analyzed for *I6S*. A-specific slight bands were visible. Sequencing for both *Wolbachia* isolates is suggested.

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## Introduction

### *Bemisia tabaci*

*Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) or commonly called sweet potato, tobacco or silverleaf whitefly (Figure 1) is one of the most severe and economically important pests worldwide in agriculture. It causes devastating yield decrements because of its wide range of plant hosts, high reproductive capacity, direct feeding damage, capability of transmitting pathogenic viruses, resistance to many insecticides, and honeydew contamination associated with fungal growth (Gerling 1990; Drost et al. 1998; Perring 2001; Brown 2007).



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**Figure 1.** *B. tabaci* adult on Poinsettia leaf.

Cock in 1993 mentions more than 500 plant species as host of this pest. Claridge et al. in 1997 and Perring in 2001 refer to *B. tabaci* as a classical cryptic species or species complex that has host-correlated races or biotypes, meaning similar morphological populations with different survival and development on different host plants. Both *B. tabaci* adults and nymphs feed exclusively on phloem sap, and adults are vectors of over 100 plant viruses (Cheek & Cannon 2003; Brown 2007). *B. tabaci* causes also phytotoxic disorders in its plant hosts with symptoms as silver colouring and vein-clearing of leaves, uneven ripening of fruit, and white stem streaking. In subtropical and tropical agriculture, *B. tabaci* is an important problem causing millions of dollars in lost (summarized by Brown et al. 1995).

The life cycle of *B. tabaci*, according to Byrne et al. 1990 (cited by Ardeh et al. 2004), starts with egg stage followed by four nymphal stages and ending in adult stage for



female and male individuals. Adults live about 25 to 30 days, depending on the environment and the host plant. The female whiteflies oviposit on the underside of host plants leaves. Each female can lay more than 800 eggs. After hatching, the nymph in the first stage (called crawler) is able to move to find a convenient place to feed better and develop into the next three non-mobile nymphal stages. The pupae stage is characterized by the visible presence of the red eye-spots of the developing adults.

The worldwide spread of this pest is more serious nowadays for the increasing monoculture cropping, whitefly insecticide-resistance, and inadequate phytosanitary control in international arrival points (Oliveira et al. 2001).

The integrated pest management program of this pest is based on cultural and physical control, host-plant resistance, chemical and biological control. Preventive cultural practices and physical control such as natural or artificial barriers, intercropping, trap crops, and mulches are good tools in pest management. Host-plant resistance against *B. tabaci* is not well developed for cultivated plants. Due to the position of the whiteflies on the plant, the chemical control has application problems and it is not always effective. Moreover, the high cost of chemicals, contamination, and pesticide-resistance development are important limitations (summarized by Ardeh et al. 2004). Finally, pesticides interfere with biological pest controls inside IPM. Nowadays, biological control is getting more importance all over the world because it keeps the environment clean by effectively protecting crops against pests and producing pesticide-free food (van Lenteren 2000).

Some pathogenic fungi of insects e.g. *Verticillium*, *Paecilomyces* and *Beauveria bassiana* are used as myco-insecticides against *B. tabaci* with certain level of suppression and control in both greenhouse and field crops (Faria & Wraight 2001).

The main predators of *B. tabaci* belongs to Coccinellidae, Miridae and Anthocoridae (Heteroptera), Chrysopidae (Neuroptera), Phytoseiidae (Acari) and spiders (Araneae), summarized by Gerling et al. in 2001.

Since the 1920s, parasitoids from the family Aphelinidae have been used to control some pests in greenhouses, but only 25 years ago a large scale application has started (van Lenteren et al. 1997). In Europe, commercial use of biological control has had a very fast development during the past 30 years (van Lenteren et al. 1997). Research is focus on finding efficient natural enemies of whiteflies (Gerling 1990; Gerling & Mayer 1995; Gerling et al. 2001). For *B. tabaci*, Gerling et al. in 2001 list 34 species of *Encarsia* Foerster, 12 species of *Eretmocerus* Haldeman, one species of *Signiphora* Ashmead and *Methapycus* Mercet, and two *Amitus* Haldeman species. In North America and in the north

of Europe a mix of the parasitoids *Encarsia formosa* and *Eretmocerus eremicus* is used to control *B. tabaci*; meanwhile in the Mediterranean area a mix of *E. formosa* and *E. mundus* is used (van Lenteren 2000).

### *Eretmocerus mundus*

The parasitoid wasp *Eretmocerus mundus* Mercet (Figure 2) belongs to Hymenoptera: Aphelinidae. Aphelinidae are minute parasitic wasps (body length from 0.58 to 0.8 mm) that primarily attack whiteflies, armored scales, aphids and other Aphelinidae. This family includes more than six subfamilies. Aphelininae has four tribes: Aphelinini, Aphytini, Eutrichosomellini and Eretmocerini. *Aphytis*, *Aphelinus* and *Eretmocerus* are the most diverse and economically important of the 10 genera of Eretmocerini (Kim & Heraty, no year available).



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**Figure 2. Adult of *E. mundus*, Spain population.**

The biology of *E. mundus* has been studied by different authors (e.g. Tawfik et al. 1978; Sharaf & Batta 1985) in different host plants and under different conditions. Qiu et al. in 2004 studied comparatively the biology of *E. mundus*, among others, as parasitoid of *B. tabaci* on Poinsettia plants. The results were as follow: the juvenile development of *E. mundus* was 64 days at 15°C, 17 at 25 and 14 days at 32°C. Female adults in absence of host (fed with honey) presented a mean longevity of 55 days at 15°C, 25 days at 20°C and 6 days at 25°C; in the presence of hosts the variation was between 14 days at 15°C to 12 at 25°C. Females of *E. mundus* prefer to parasitize the third instar nymphs of *Bemisia*. A one-day old female parasitizes a daily mean of 20 hosts at 25°C and three or five days old

female parasitizes 4 at the same temperature. The same authors estimated the life-time parasitism of *E. mundus* of 11 days at 15°C and 43 days at 25°C.

Two ways of reproduction are commonly present in Hymenoptera: arrhenotoky and thelytoky. The most common is arrhenotoky (Figure 3) where daughters develop from fertilized diploid eggs and sons from unfertilized haploid eggs. Thelytoky, on the other hand, daughters develop from unfertilized eggs and there are no males. *E. mundus* is one of the haplodiploid species within Hymenoptera that present the two modes. (Stouthamer & Kazmer 1994). *Wolbachia* is involved in many cases of thelytoky but not in all (Pannebakker 2004).



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**Figure 3. Couple of *E. mundus* (Spanish population). Each adult has a length of approximately 2 mm length.**

Qiu et al. in 2004 mention that the *Eretmocerus* species are excellent parasitoids and rebound their effectiveness at relative high temperatures and their high reproductive rate over a short period.

De Barro et al. in 2000 in Australia comparatively studied the biology of five parasitoids of *B. tabaci*: two species of *Eretmocerus*, Australian Parthenogenetic Form (APF) of *E. mundus* and *E. queenslandensis* and three of *Encarsia*. It was concluded that *E. mundus* is the most effective parasite amongst the 5 examined due to a higher rate of oviposition and higher parasitism (both *Eretmocerus* spp. without competition between each other) when whitefly density is rising. Furthermore, parthenogenesis is enhanced as the beneficial factor which confers effectiveness to APF *E. mundus*. Compared with other closely related species (included *E. mundus* from Murcia, Spain), APF of *E. mundus* does not present evidence for reduction in fertility (De Barro et al. 2000). According to the same

author, “*Eretmocerus* spp. comprise some of the most effective parasites of *B. tabaci* biotype B”.

#### *Wolbachia* bacteria

The genus *Wolbachia* belongs to the family Rickettsiaceae within Phylum Alpha-Proteobacteria. They are obligate intracellular bacteria (endosymbiont) found in vacuoles, gonadal cells and somatic tissues. *Wolbachia* bacteria are widespread in invertebrate species, such as insects (including parasitic Hymenoptera), mites, spiders, terrestrial crustaceans and nematodes and are estimated to occur in 16% of all invertebrate species (Werren et al. 1995(a); Cook & Butcher 1999; Jeyaprakash & Hoy 2000; Stouthamer 2003). Floate et al. in 2006 detected infections in 46% of the 105 tested species (species of arthropods of current interest on biocontrol in Canada). These bacteria are transmitted from infected mothers to their offspring. *Wolbachia* alter host sexuality or crossing compatibilities to increase their vertical transmission into their hosts. Four mechanisms of action are known: feminization (in terrestrial isopods), parthenogenesis induction (especially in parasitoid wasps), male-killing and cytoplasmic incompatibility (in arthropods) (Float et al. 2006).

Parthenogenesis induction (PI) *Wolbachia* is predominantly present in Hymenopterans with haplodiploid sex determination, i.e. *E. mundus* population from Australia (De Barro et al. 2000). It is not known if these bacteria can cause parthenogenesis outside of Hymenoptera (Werren 1997). The cytogenetic mechanisms of PI *Wolbachia* have been studied in *Trichogramma* spp. In unfertilized infected eggs, meiosis is normal but the first mitotic division is aborted. The chromosomes condense properly in prophase but fail to segregate in metaphase, resulting in diploidization of the nucleus and a female is developed. This mechanism is known as gamete duplication and results in homozygosity at all loci. Subsequent mitotic divisions appear to be normal. Infection with PI-*Wolbachia* in itself does not interfere with sexual reproduction: if infected eggs are fertilized, the PI-*Wolbachia* appears not to influence the chromosome behaviour in the infected egg and, in these fertilized eggs, the paternal chromosome set participates in the formation of the infected female offspring in *Trichogramma* (Stouthamer & Kazmer, 1994).

To identify *Wolbachia* isolates, specific DNA gene sequences are used such as 16S rDNA (partial small subunit ribosomal DNA), 23S rDNA, *fstZ* (cell division gene), *groEl*

(heat shock protein gene), *23S* and *wsp* (outer surface coat protein gene) (Werren et al. 1995(a) and 1997; Schilthuisen & Stouthamer 1997; Floate et al. 2006). *Wolbachia* (sequenced in its *16S* rRNA gene and *ftsZ* gene) is divided in two subgroups (A and B) according to its phylogenetic tree.

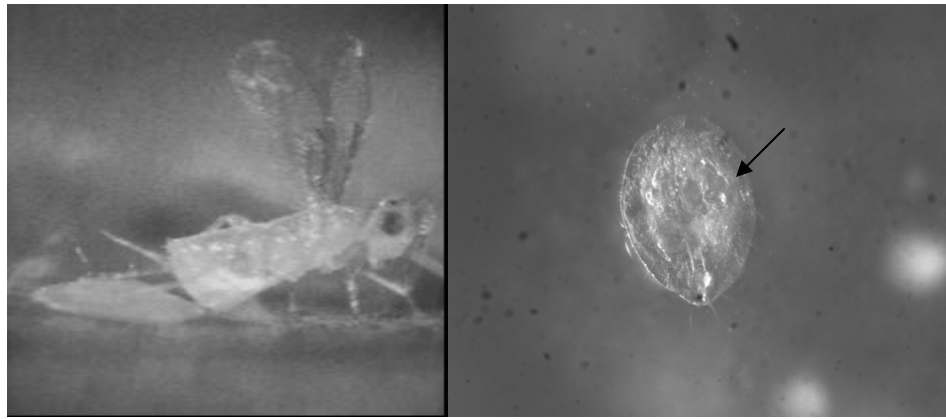
Levels of *Wolbachia* infection vary between genera and ‘higher’ taxonomic groups, between species, and between and within populations of a single species (Werren & Windsor 2000; Rokas et al. 2002). Multiple *Wolbachia* infections within individuals and genetic recombination between strains may occur due to the frequent occurrence in the same host (Warren 1997).

### *Interactions*

The three organisms involved in this research: *B. tabaci*, *E. mundus* and *Wolbachia* bacteria have close relationship between each other. Here we will see their interactions.

Hymenoptera of the genus *Eretmocerus* are solitary parasitoids of the whitefly *B. tabaci*. Oviposition by *E. mundus* lays its eggs under the 2<sup>nd</sup>- and the 3<sup>rd</sup>-instar nymphs of *B. tabaci* (Figure 4). It does not oviposit in the late 4<sup>th</sup> nymphal instar. After hatching, the larva enters the host and develops inside *B. tabaci* nymph. The life cycle of *E. mundus* consists on an egg (3 days at 27°C or 4 days at 25°C), three larval instars, a prepupa, a pupa and an adult. The first instar larvae hatch under *B. tabaci* nymphs and stay there until the hosts reach the preferred 4<sup>th</sup> instar stage for penetration (Gelman et al. 2005a and b; Urbaneja & Stansly 2004; Foltyn & Gerling 1985). Once it penetrates, the parasitoid larva finds itself surrounded by a capsule formed by the host (Appendix I) and moults to the second instar. The formation of this capsule is typical of the *Eretmocerus*-host association. As the larva grows and moults to the third instar, the walls of the capsule seem to be dissolved (Gerling et al. 1990); meanwhile the host turns into a pharate adult (Gelman et al. 2005). At 25°C the second and the third instars last about 4 days each, while the prepupal and pupal development last about 10 days in total (Foltyn & Gerling 1985).

Superparasitism in *Eretmocerus* wasps can be observed, although they are capable of discriminate between parasitized and unparasitized hosts. In cases of superparasitism, Gerling et al. in 1991 observed that several larvae stay outside the host but only one (occasionally two) penetrates. Nevertheless, only one wasp emerges from the host. Therefore during the first 4-8 days after the eggs are laid the elimination of the supernumerary larva or larvae take place.



M. Ardeh 2004

C. Castillo C.

**Figure 4.** Left picture, a female of *E. mundus* is ovipositing underneath a *B. tabaci* nymph. Right picture, *B. tabaci* nymph with a first-stage larva of *E. mundus* underneath.

The third component of the interaction is the parthenogenesis inducing (PI) *Wolbachia* bacteria which is capable of manipulating the chromosome behaviour of the Australian parthenogenetic form (APF) of *E. mundus* as it was previously explained.

New ideas have to be developed to improve the efficacy of biological control of *B. tabaci* by parasitoids. For instance, to maximize the potential of the already mass reared and released parasitoid *E. mundus* (Spanish population) (van Lenteren 2000). One of the possibilities is trying to infect it with parthenogenesis inducing *Wolbachia* bacteria (Stouthamer 1993; Stouthamer 2003).

#### *Horizontal transmission*

Vertical transmission is the primary mode of *Wolbachia* transmission, but most *Wolbachia* strains appear to have reached their host species via colonization (Warren 1997; Schilthuisen & Stouthamer 1997). The infection could pass from one insect to another when they share food source or after blood-blood contact, then superparasitism and elimination of supernumerary parasitoids are important for potential horizontal transmission of *Wolbachia* (Huigens et al. 2000). Huigens et al. in 2004 mentions uninfected immature wasps acquired *Wolbachia* while inside the host they were sharing the same host with infected ones, but not all of these newly infected females exhibited the parthenogenesis phenotype in their generations and the infection tended to be lost. In general, intraspecific horizontal transfer was more successful than interspecific transfer (closely related sympatric species).

“The *ftsZ* phylogeny clearly shows horizontal (intertaxon) transmission of *Wolbachia*. One A-*Wolbachia* in particular (designated Adm) shows extensive horizontal transmission. Different Adm isolates that are identical or nearly identical in *ftsZ* gene sequence can be found in hosts from the orders Coleoptera, Diptera, Hymenoptera, and Lepidoptera. Such bacteria are estimated to have diverged 0–1.6 million of years ago (MYA), whereas their respective hosts diverged over 200 MYA” (Werren et al. 1995(b), Hennig 1981)

To explain natural horizontal transmission, the analyses of particular cases are necessary. For instance, when *Wolbachia* phylogeny is compared to *Trichogramma* phylogeny, horizontal transmission during evolution is suggested due to their discordance. The parasitoid wasp *Nasonia* and its blowfly host (*Protocalliphora*) have B-*Wolbachia* strains with closed related phylogeny (Schilthuizen & Stouthamer 1997; Werren 1997; Grenier 1998).

Grenier in 1998 had successful horizontal transmission of *Wolbachia* by microinjection into in vitro developed pupae of *Trichogramma*. *Wolbachia* were still present in the recipient parasitoid wasps 26 generations after being transferred, but only partial induction of thelytoky was observed. Therefore, in *Trichogramma*, density of symbionts or symbionts-host interactions may be involved in the expression of parthenogenesis.

### *Impact*

Successful transfer of *Wolbachia* into commercial parasitoids to manipulate their reproductive system could increase the advantages for biological control (Grenier 1998). Benefits of parthenogenetic reproduction in wasps are highlighted by Stouthamer in 1993: first, thelytokous population will have a higher population growth rate and thus higher rates of parasitism in pests; second, they are likely to be better colonizers and establish more easily at low population densities as there is no need to find a mate (the encounter between sexes could be also very difficult in this small wasp, Ardeh 2004); and finally, there may be more cost effectiveness in mass rearing as production is not ‘wasted’ on males. Although fecundity of asexual females is usually lower than that of sexual conspecifics (van Meer et al. 1995), host-searching efficiency is more important than a high fecundity at low host densities (van Roermund & van Lenteren 1994).

## **Objective**

The aim of this research was to study whether horizontal transmission of *Wolbachia* bacteria is possible between larvae from an Australian, *Wolbachia* infected strain of *E. mundus* to a Spanish uninfected strain of the same species in cases of close contact.



## Materials and methods

### *Rearing*

*B. tabaci* was reared on Poinsettia (*Euphorbia pulcherrima* Willd. ex Klotzsch) in a greenhouse at 25°C, with 75% RH.

The thelytokous (*Wolbachia* infected) Australian population of *E. mundus* was reared on *B. tabaci* on Poinsettia plants in a climate chamber at 25°C. The arrhenotokous Spanish population was provided by Koppert Biological Systems.

### *Preparation for the experiments*

Poinsettia plants were exposed to *B. tabaci* adults for two days and kept isolated afterwards. Therefore, plants with whitefly nymphs of the same stage were obtained. Fourteen days after oviposition, leaves with 2<sup>nd</sup> and early 3<sup>rd</sup> instar nymphs were taken to the laboratory. Leaf discs were cut and placed with the back surface up onto 1% agar solution in plastic boxes. Each leaf disc had around 20 *B. tabaci* nymphs.

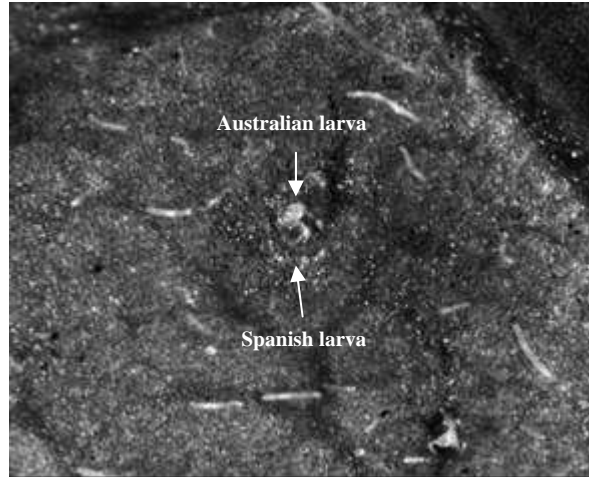
Pupae of *B. tabaci* parasitized by both *E. mundus* populations were isolated in glass tubes until emergency of adults. One Australian female adult was put on one leaf disc inside the mentioned boxes. For the Spanish population, after mating was observed, the fertilized female was left on other leaf disc. Females were allowed to lay eggs during 24 hours at 25°C. Four days after oviposition, the first instar larvae were expected to have hatched and be still outside the host.

### *Experiments*

Each experiment consisted in putting one *E. mundus* first instar larva from the thelytokous Australian population (infected with *Wolbachia*) in close contact with another larva from the arrhenotokous Spanish population (not infected). In particular we tried to place the infected larva in contact with the mouthparts of the uninfected one in order to get physical injury (Figure 5).

Each experiment was done with extreme caution, first observing the movement of the mandible of Spanish larva, second observing the movement of the *Wolbachia* infected

“victim”, and finally depositing the last one over the mandible of the Spanish larva. After 3-4 hours the two larvae were separated and isolated for DNA extraction. Disinfected pins were used to manipulate the involved larvae. Experiments were performed under stereomicroscope.



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**Figure 5.** Two larvae of *E. mundus* on a Poinsettia leaf. Experiments were done putting in close contact two larvae to obtain ingestion and horizontal transmission of *Wolbachia* bacteria.

### *Molecular analyses*

In the present research molecular analyses started with the DNA extraction from the larvae involved in the experiments. The primer *ITS2* was used for two reasons, first to be sure if it was possible to extract DNA from minute larvae (1 $\mu$ m), and second because is necessary to have PCR product from *ITS2* to proceed with the digestion of the samples. Digestion of the DNA was made with the enzyme *NRU I* to know the origin of the involved larvae (from Australia or Spain). The primer *16S* was used to find the presence of *Wolbachia* in the samples.

*DNA Extraction:* Each larva was ground in a 1.5 Eppendorf with a sterile glass rod. 50  $\mu$ l of Chelex®-100 (previously stirred) and 4  $\mu$ l of Proteinase K (20mg/ml) were added. Incubation at 56 °C for at least 6 (hours) followed. Eppendorf tubes were then centrifuged for one minute and heated for 10 minutes at 95°C in order to inactivate Proteinase K. After spinning for few seconds, the DNA extract was ready to be used in the PCR.

*Differentiation between E. mundus populations:* Polymerase Chain Reaction (PCR) were performed in 25  $\mu$ l volumes including, for each sample: 5  $\mu$ l PCR-buffer, 3.5  $\mu$ l of MgCl<sub>2</sub>,

0.5 µl of dNTP's (each in a 10 mM concentration), 0.5 µl of each forward and reverse *ITS2* primer, 0.125 µl of *Taq* DNA polymerase, 12.375 µl of sterile distilled water, and 2.5 µl of DNA templates. The PCR program is described in Table 1.

The PCR products (10 µl) were run in 1.5% agarose gel along with standard ladder (BIOTC) for 80 minutes and 70 volts. The *ITS2* fragment of *E. mundus* appears at about 450 bp. In this way we could make sure that the DNA extraction was successful. After that, in order to distinguish the two different populations, it was necessary to incubate in a stove at 37 °C, for at least 4 hours, 15 µl of the PCR products with 5 µl of a mixture containing 0.5 µl of “*Nru I*” enzyme, 0.2 BSA, 2 µl of buffer and 2.3 µl of distilled water. The enzyme cut the amplified DNA of the thelytokous population (to 330 and 120 bp) but not the one of the arrhenotokous population (450bp) (Ardeh et al. 2004).

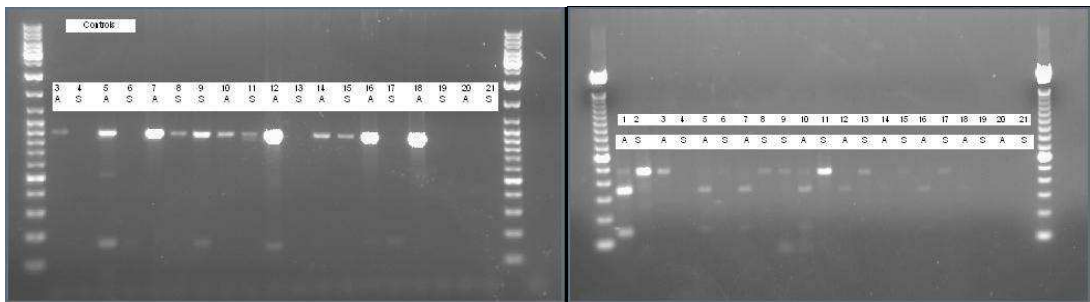
**Table 1. Sequences for *ITS2* primers and PCR-reaction program.**

Primer	Sequence	Den-	Ann-	Ext-	Cycle
ITS2 Forward	TGTCAACTGCAGGACACATG	94°C	60°C	72°C	35
ITS2 Reverse	ATGCTTAAATTTAGGGGGTA	1 min	1 min	1.5 min	

*Determination of Wolbachia presence:* PCR reactions were performed in 25 µl volumes including: 5 µl of PCR-buffer, 1.5 µl of MgCl<sub>2</sub>, 0.5 µl of dNTP's (each in a 10 mM concentration), 0.5 µl of each forward and reverse *16S* primers, 0.125 µl of *Taq* DNA polymerase, 14.375 µl of sterile distilled water, and 2.5 µl of DNA templates. The PCR machine program was: 4 minutes at 94°C, 37 cycles composed by 30 seconds at 94°C, 45 seconds at 54°C and 1 minute at 72°C, and finally 5 minutes at 72°C. The DNA templates (10 µl) were run in 1.5% agarose gel along with standard ladder (BIOTC) for 80 minutes and 70 volts. The expected band size is between 900 and 1000 bp.

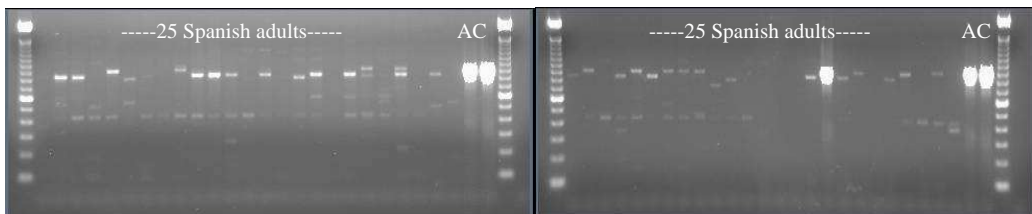
## Results and discussion

The aim of this research was to get horizontal transmission of parthenogenesis inducing *Wolbachia* bacteria from thelytokous larvae (Australian population) of *E. mundus* to arrhenotokous larvae (Spanish population) of the same species. Two set of experiments with first-stage larvae were done. In the first set, 61 pairs of larvae and 19 Spanish larvae as control were analyzed. Unfortunately, positive *Wolbachia* infection (using *16S* primers) was identified in some Spanish controls (Figure 6).



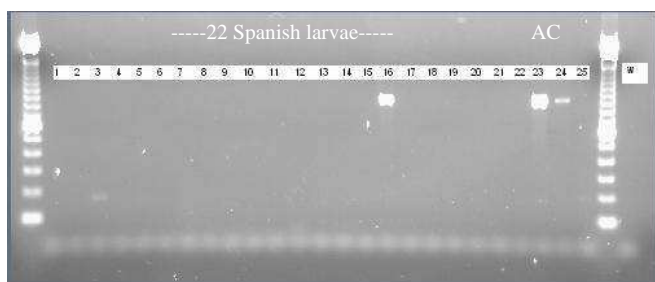
**Figure 6.** Left, PCR picture of *16S* analysis of Spanish (S) and Australian (A) larvae controls from the first set of experiments. Bands for *Wolbachia* infection are visible in Spanish larvae. Right, PCR picture of *ITS2* analysis of the same samples presented in the left picture. Here is confirmed the origin of the samples.

To reveal this non-expected *Wolbachia* positive infection was necessary to analyze more Spanish samples. A number of 25 Spanish adults were analyzed using the same primer *16S*. Bands appeared in some samples apparently at the same level of *Wolbachia* positive control (Figure 7 left). Contamination was thought as the answer of the problem and a new *16S* primer was used but bands still appeared. One band was as clear as the *Wolbachia* positive controls (Figure 7 right).



**Figure 7.** PCR pictures of *16S* analysis made for 25 Spanish adults and two Australian as *Wolbachia* infected control (AC).

Cycles in the PCR programme were adjusted<sup>1</sup> to avoid the possible false bands and molecular analyses were done (twice) for 22 Spanish larvae this time. Same results were obtained: one Spanish larva remained positive for *Wolbachia* bacteria (Figure 8). These results annulated the experiments done in the first set. A new set of experiments had to be done.



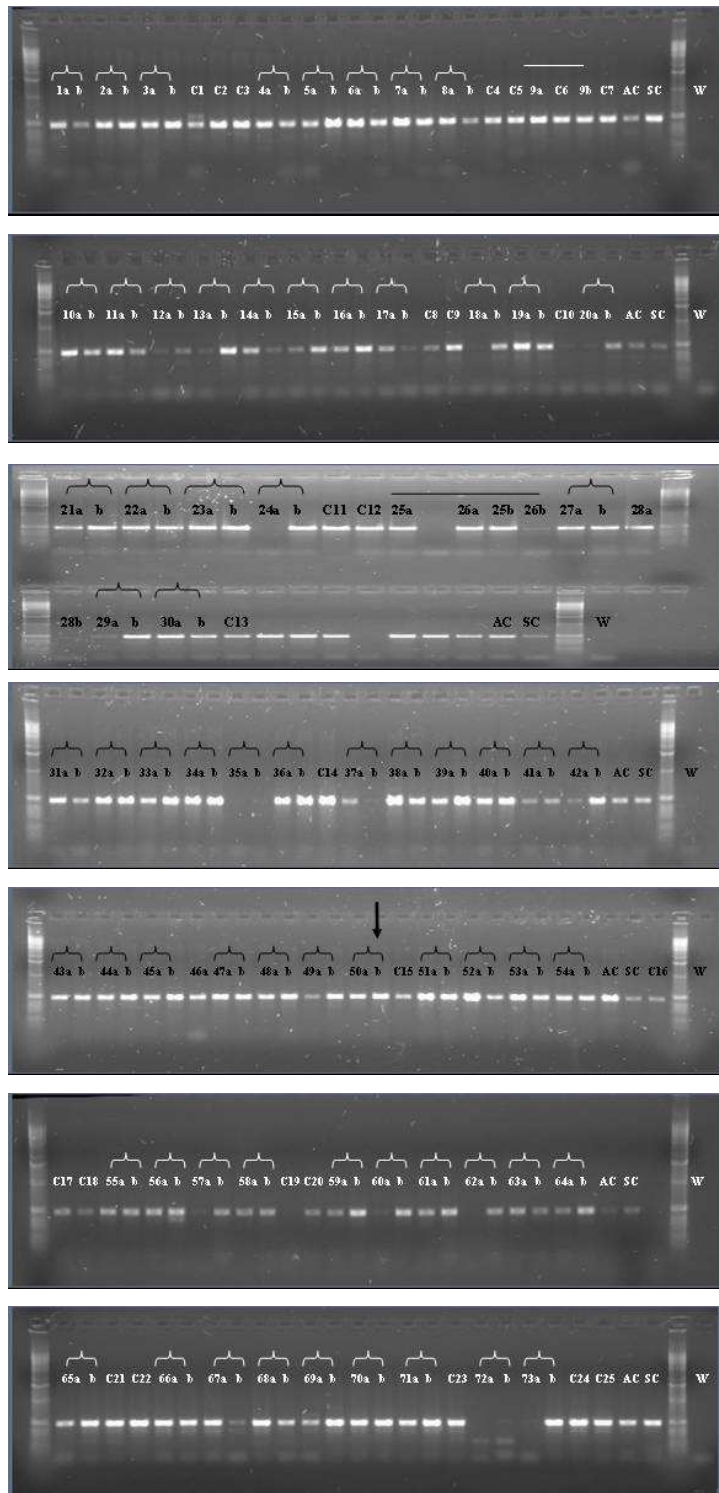
**Figure 8. PCR picture of *16S* analysis for 22 Spanish larvae and two Australian as *Wolbachia* infected control (AC). One sample (No. 16) was positive for *Wolbachia*.**

In the second set, 73 horizontal transmission experiments were performed in the same way as in the first set, but this time, instead of simply washing the rods with 95% alcohol, we autoclaved them every time before using them. Additionally, molecular analyses were performed also on the mothers of the Spanish larvae involved in the experiments. In this way, we could be sure that the Spanish larvae for the experiments were ‘clean’ of parental source of *Wolbachia*. Molecular analyses were done for 145 larvae (73 pairs) from the experiments (see Appendix II for summary of these results), for 25 Spanish (larvae) controls and for the Spanish mothers.

PCR amplification of an *ITS2* gene fragment was done for all the larvae from the experiments (Figure 9). From the 145 experimental larvae, only 10 did not present band. It represents the 93.1% of success. From the 25 control larvae, a similar successful percentage was obtained: 92%. The molecular methodology was developed for adult wasps but it was applicable also on larvae.

PCR amplification of a *16S* gene fragment was done for all the experimental larvae (Figure 10) to determine parthenogenesis-inducing *Wolbachia* infection. The results showed 61 infected larvae.

<sup>1</sup> PCR cycles showed in methodology.



**Figure 9.** PCR amplification of an *ITS2* gene fragment from the 145 larvae (one missed) involved in the 73 experiments plus 25 arrhenotokous larvae used as controls (C). Note: — = change in the order of the samples, AC=Australian control, SC=Spanish control, W=Water control.

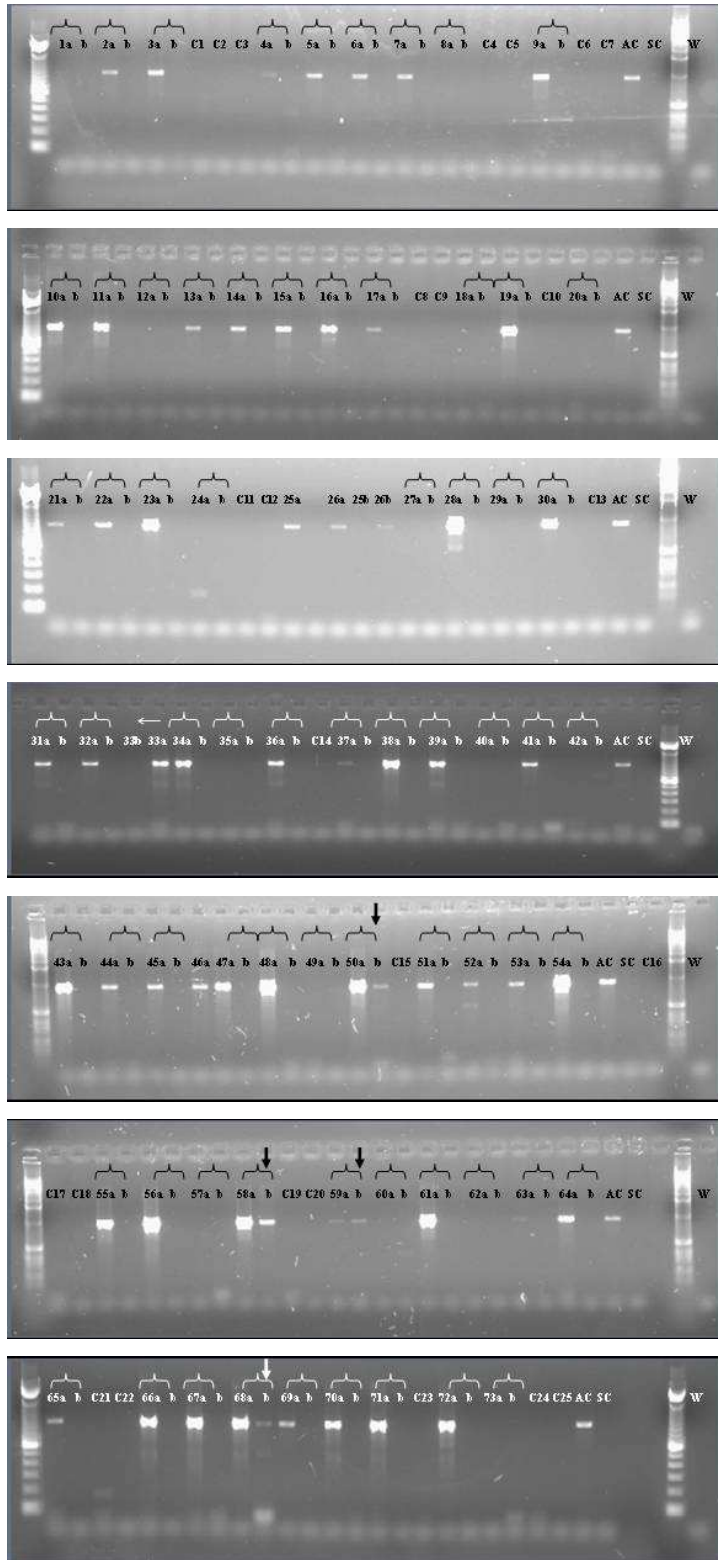
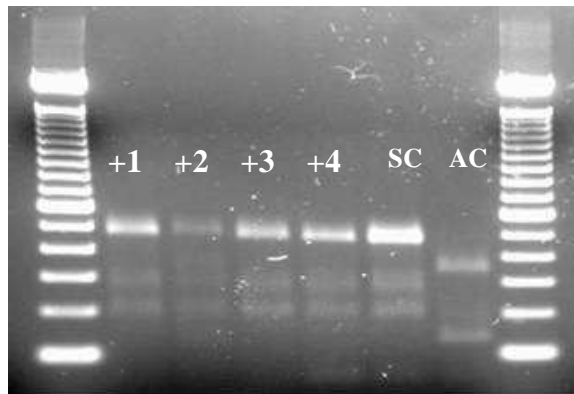


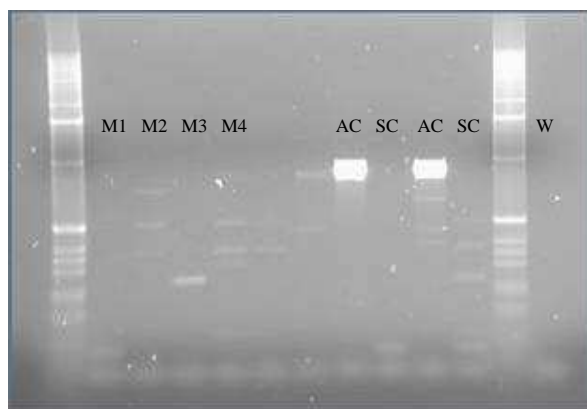
Figure 10. PCR amplification of a *16S* gene fragment from the 145 larvae involved in the 73 experiments plus 25 arrhenotokous larvae used as controls (C). Note: ↓ = Couples with 2 infected individuals (possible horizontal transmission), AC=Australian control, SC=Spanish control, W= Water control.

The NruI digestion on ITS2 PCR products regarding the *Wolbachia* infected samples, highlighted the presence of 4 infected Spanish larvae (see again Figure 10, samples with vertical arrows mark *Wolbachia* infection). In Figure 11 is possible to observe clear bands at the same level of the Spanish control for the mentioned four *Wolbachia* infected samples. These results confirm that the four *Wolbachia* positive infected larvae belong to the Spanish population of *E. mundus*.



**Figure 11.** PCR amplification of the digested *ITS2* product obtained from the four positive *Wolbachia* infected larvae to identify their origins. Samples one to four show the same band that the Spanish control has at 450 bp. SC= Spanish control, AC= Australian control. 1 kb ladder (Gibco).

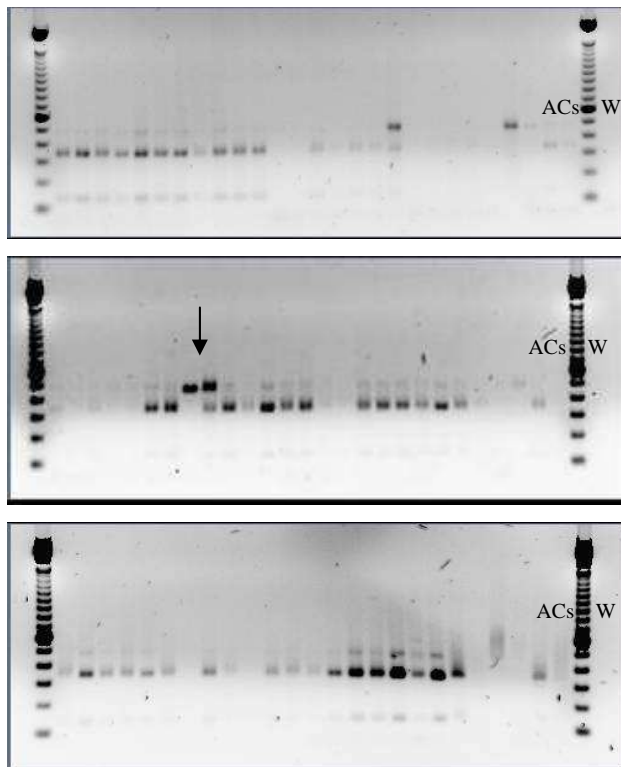
PCR amplification of a *16S* gene fragment was also done to detect the presence of *Wolbachia* in the mothers of the four infected larvae. The four mothers did not present bands (Figure 12) compared to the Australian control that showed strong bands. These analyses discarded the possibility of vertical transmission of *Wolbachia* and may confirm that PI *Wolbachia* bacteria were successfully horizontally transmitted in four cases in this research.



**Figure 12.** PCR amplification of a *16S* gene fragment to detect the presence of *Wolbachia* for mothers of the four larvae infected in the experiments (M1 to M4), AC=Australian control, SC=Spanish control, W=Water control.



The *ITS2* products of the *Wolbachia* infected samples were used for their digestion with *Nru I* enzyme to confirm the origin of the larvae (population from Australia) (Figure 13). Therefore, it is confirmed that all the other *Wolbachia* positive samples tested belong to the Australian population exempt one (marked with a vertical arrow). This result has to be rechecked.

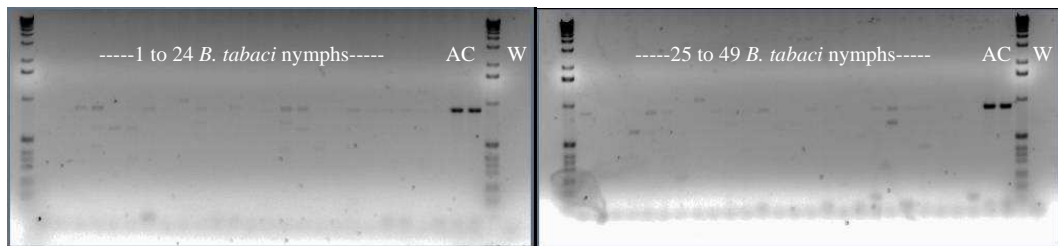


**Figure 13.** *Wolbachia* infected samples (except the mentioned four) digested with *Nru I* enzyme. All the samples tested were from the Australian population except one which is marked with a vertical arrow. ACs=Australian controls (2), W=Water control.

Horizontal transmission of parthenogenesis-inducing *Wolbachia* was successfully done in this research; a rate of 5.47% (4/73) could be presented. This relative low rate of horizontal transmission was expected since previous studies in *Trichogramma* (Grenier et al. 1998; Cook & Butcher 1999; Huigens et al. 2000) mention *Wolbachia* is not easily transmitted horizontally. Nevertheless, this rate can be increased if we take into account only the samples that were positive for *Wolbachia* infection and not the total of experiments performed. On the other hand, Cook & Butcher in 1999 summarize experimental evidence for horizontal transmission of *Wolbachia* mentioning frequencies “as high as” 0.6%-3.0% for horizontal transmission between hosts and parasitoids, parasitoids and hosts, and parasitoids via multiparasitism or conspecific superparasitism of hosts in different solitary parasitoid-host systems. The same authors mention “A major

concern here is that a rate of horizontal transmission as low as 0.001% may be important over evolutionary time yet below a realistically detectable threshold in laboratory experiments”. According to Grenier et al 1998, it is also important to take in account for a successful horizontal transfer of PI *Wolbachia* the bacterial density.

One question remains open: is there any chance *Wolbachia* can come from *B. tabaci*? A number of 49 samples of *B. tabaci* nymphs were analyzed for *16S*. Slight a-specific bands were present in PCR pictures (Figure 14).



**Figure 14. Samples of *B. tabaci* nymphs analyzed for *16S*. Slight a-specific bands were present in PCR pictures (AC=Two Australian controls, W=Water control).**

Sequencing *16S-Wolbachia* from the experimented larvae of *E. mundus* Spanish strain to compare it with the *16S-Wolbachia* from the Australian strain is suggested. Then, the next step will be to complete the development of the infected larvae to have a new population and then to study inheritance and fixation of *Wolbachia* in the new infected population.

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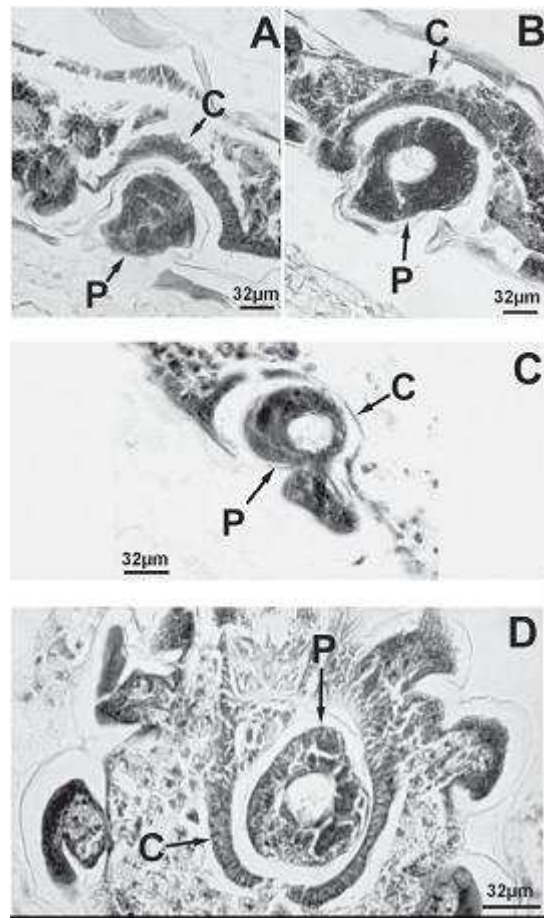
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## Appendix I



**Figure 15.** Capsule formation upon penetration of an *E. mundus* first instar larva into a *B. tabaci* 4th instar nymph. **A:** Host epidermis begins to invaginate as 1st instar parasitoid begins to penetrate. **B:** 1st instar *E. mundus* in a later stage of penetration. **C:** 1st instar *E. mundus* completing penetration. **D:** 2nd instar *E. mundus* completely surrounded by the capsule. P, parasitoid; C, capsule. Scale bars = 32 μm. (Gelman et al. 2005b).

## Appendix II

Table 2. Results of the molecular analyses of 73 pairs (146 *E. mundus* larvae) obtained from the experiments done to get horizontal transmission of *Wolbachia* bacteria. July, 2007. Laboratory of Entomology, WUR.

Sample	ITS 2	16 S	NRU L	Code in samples	Sample	ITS 2	16 S	NRU L	Code in samples	Observations
1 a	1	0	Australian	11.4 D1a	41 a	+	+	Australian	21.4 D5a	
b	+	-		11.4 D1b	b	+	-		21.4 D5b	
2 a	+	+	Australian	11.4 D2a	42 a	1	0	Australian	21.4 D6a	
b	+	-		11.4 D2b	b	+	-		21.4 D6b	
3 a	+	+	Australian	13.4 A1a	43 a	+	+	Australian	21.4 D7a	
b	+	-		13.4 A2a	b	+	-		21.4 D7b	
4 a	+	+	Australian	18.4 B1a	44 a	+	+	Australian	21.4 D8a	
b	+	-		18.4 B1b	b	+	-		21.4 D8b	
5 a	+	+	Australian	18.4 B2a	45 a	+	+	Australian	21.4 D9a	
b	+	-		18.4 B2b	b	+	-		21.4 D9b	
6 a	+	+	Australian	18.4 B3a	46 a	+	+	Australian	21.4 D10a	
b	+	-		18.4 B3b	b	+	-		lost	
7 a	+	+	Australian	18.4 B4a	47 a	+	+	Australian	21.4 D11a	
b	+	-		18.4 B5b	b	+	-		21.4 D11b	
8 a	1	0	Australian	18.4 B4a	48 a	+	+	Australian	21.4 D12a	
b	+	-		18.4 B5b	b	+	-		21.4 D12b	
9 a	+	+	Australian	18.4 C1a	49 a	1	0	Australian	21.4 D13a	
b	+	-		18.4 C1b	b	+	-		21.4 D13b	
10 a	+	+	Australian	18.4 C2a	50 a	+	+	Australian	21.4 D14a	
b	+	-		18.4 C2b	b	+	+	Spanish	21.4 D14b	Infected by HT
11 a	+	+	Australian	18.4 C3a	51 a	+	+	Australian	21.4 E1a	
b	+	-		18.4 C3b	b	+	-		21.4 E1b	
12 a	1	0	Australian	19.4 A1a	52 a	+	+	Australian	21.4 E2a	
b	+	-		19.4 A1b	b	+	-		21.4 E2b	
13 a	0.5	+	Australian	19.4 A2a	53 a	+	+	Australian	21.4 E3a	
b	+	-		19.4 A2b	b	+	-		21.4 E3b	
14 a	+	+	Australian	19.4 A3a	54 a	+	+	Australian	21.4 E4a	
b	+	-		19.4 A3b	b	+	-		21.4 E4b	
15 a	+	+	Australian	19.4 B1a	55 a	+	+	Australian	21.4 A1a	
b	+	-		19.4 B1b	b	+	-		21.4 A1b	
16 a	+	+	Australian	19.4 B2a	56 a	+	+		21.4 A2a	
b	+	-		19.4 B2b	b	+	-		21.4 A2b	
17 a	+	+	Australian	19.4 B3a	57 a	0.2	0	Australian	21.4 A3a	
b	+	-		19.4 B3b	b	+	-		21.4 A3b	
18 a	0.2	0	Australian	19.4 C1a	58 a	+	+	Australian	21.4 A4a	
b	+	-		19.4 C1b	b	+	+	Spanish	21.4 A4b	Infected by HT
19 a	+	+		19.4 C2a	59 a	+	+	Australian	21.4 B1a	
b	+	-		19.4 C2b	b	+	+	Spanish	21.4 B1b	Infected by HT
20 a	0.2	0	Australian	20.4 A1a	60 a	0.2	0	Australian	21.4 B2a	
b	+	-		20.4 A1b	b	+	-		21.4 B2b	
21 a	+	+	Australian	20.4 A2a	61 a	+	+	Australian	21.4 B3a	
b	+	-		20.4 A2b	b	+	-		21.4 B3b	
22 a	+	+	Australian	20.4 A3a	62 a	0	0		21.4 B4a	
b	+	-		20.4 A3b	b	+	-		21.4 B4b	
23 a	+	+	Australian	20.4 A4a	63 a	+	+	Australian	21.4 B5a	
b	+	-		20.4 A4b	b	+	-		21.4 B5b	
24 a	0	0		20.4 A5a	64 a	+	+	Australian	21.4 B6a	
b	+	-		20.4 A5b	b	+	-		21.4 B6b	
25 a	+	+	Australian	20.4 B71	65 a	+	+	Australian	21.4 B7a	
b	+	-		20.4 B74	b	+	-		21.4 B7b	
26 a	+	+	Australian	20.4 B73	66 a	+	+	Australian	21.4 C1a	
b	+	-		20.4 B75	b	+	-		21.4 C1b	
27 a	1	0	Australian	20.4 B3a	67 a	+	+	Australian	21.4 C2a	
b	+	-		20.4 B3b	b	+	-		21.4 C2b	
28 a	+	+	Australian	20.4 B4a	68 a	+	+	Australian	21.4 C3a	
b	0	-		20.4 B4b	b	+	+	Spanish	21.4 C3b	Infected by HT
29 a	0.5	0	Australian	20.4 B5a	69 a	+	+	Australian	21.4 C4a	
b	+	-		20.4 B5b	b	+	-		21.4 C4b	
30 a	+	+	Australian	20.4 B6a	70 a	+	+	Australian	21.4 C5a	
b	+	-		20.4 B6b	b	+	-		21.4 C5b	
31 a	+	+	Australian	20.4 C1a	71 a	+	+	Australian	21.4 C6a	
b	+	-		20.4 C1b	b	+	-		21.4 C6b	
32 a	+	+	Australian	20.4 C2a	72 a	0	+	Australian	22.4 E1a	
b	+	-		20.4 C2b	b	0.2	-		22.4 E1b	
33 a	+	+	Spanish	20.4 C3a	73 a	0.2	0		22.4 E2a	
b	+	-		20.4 C3b	b	-	-		22.4 E2b	
34 a	+	+	Australian	20.4 C4a						
b	+	-		20.4 C4b						
35 a	0.2	0		20.4 C5a						
b	0.2	-		20.4 C5b						
36 a	+	+	Australian	20.4 C6a						
b	+	-		20.4 C6b						
37 a	0.2	+	Australian	21.4 D1a						
b	+	-		21.4 D1b						
38 a	+	+	Australian	21.4 D2a						
b	+	-		21.4 D2b						
39 a	+	+	Australian	21.4 D3a						
b	+	-		21.4 D3b						
40 a	+	0	Australian	21.4 D4a						
b	+	-		21.4 D4b						

1 = clear band  
0.5 = half band  
0.2 = almost imperceptible band  
0 = nonvisible band

All the "a" samples are Australian according to the digestion with NRU L except the sample 33a (20.4 C3a).  
The sample 33a (20.4 C3a) showed a band in the same bp as the Spanish control.  
The samples "b" were not examined with NRU L except the four positive for 16 S (in red).