Diplom- / Masterarbeit

The Endosymbiont *Wolbachia* in *Rhagoletis pomonella* and *R. cingulata* (Diptera, Tephritidae)

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Alles Gescheite ist schon gedacht worden,
man muss nur versuchen, es noch einmal zu denken.
JOHANN WOLFGANG V. GOETHE

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Abstract

Wolbachia is a common intracellular endosymbiontic bacteria found in up to 65% of insects. *Wolbachia* infections have been detected in all major insect orders. *Wolbachia* manipulate the reproduction by inducing male killing, parthenogeneis, feminization and the most frequently effect is cytoplasmic incompatibility (CI). CI is an incompatibility between sperm and egg and occurs when infected males mate with uninfected females or females infected with a different *Wolbachia* strain. The genus *Rhagoletis* belongs to the dipteran family Tephritidae and contains several economically important pest species worldwide. Recently, it was shown that the European Cherry Fruit Fly, *R. cerasi*, harbours five different *Wolbachia* strains containing at least two strains which have the potential to be used in biological control of insect pests.

In this thesis two *Rhagoletis* species were screened by conventional PCR with *wsp* primer for *Wolbachia*. These amplicons were directly sequenced. Further the PCR products have been cloned and about 20 plasmids per species were sequenced. After that the detected strains were characterized by the multilocus strain markers MLST: *hcp*A, *cox*A, *gat*B, *fts*Z and *fbp*A.

The two species screened were the Eastern Cherry Fruit Fly, *Rhagoletis cingulata*, and the Apple Maggot, *Rhagoletis pomonella*. *R. cingulata* is a pest in sweet and sour cherries in North America which was recently introduced in Europe. It has a similar biology as *R. cerasi*. *R. pomonella,* is a serious pest in apple orchards in North America and a model species for sympatric speciation. *R. pomonella* on hawthorn and apple are genetically and ecologically different.

The PCR data revealed that *R. cingulata* and *R. pomonella* are infected with *Wolbachia*. Sequence data proved that *R. cingulata* contains two different *Wolbachia* strains, *w*Cin1 and *w*Cin2. They are ident to the *Wolbachia* strains *w*Cer1 and *w*Cer2 of *R. cerasi* comparing the five different nuclear MLST markers.

R. pomonella collected from apple are infected with one *Wolbachia* strain *w*Pom which was ident to the *w*Cer2 strain based on the *wsp* gene. Analysis with MLST primers showed one single mutation on the *hcp*A locus not being detected before.

Zusammenfassung

Wolbachia ist ein intrazellulär lebendes Bakterium, das in bis zu 65% der Insekten symbiontisch lebt. *Wolbachia* Infektionen wurden in allen größeren Insektenordnungen nachgewiesen. *Wolbachia* manipuliert die Vermehrung der Insekten durch *male-killing*, Parthenogenese, Feminisierung und zytoplasmatische Inkompatibilität (CI). CI bezeichnet eine Inkompatibilität zwischen Spermium und Ei und wird verursacht, wenn sich infizierte Männchen mit nicht infizierten Weibchen kreuzen.

Die Gattung *Rhagoletis* gehört zu der Zweiflügler-Familie der Fruchtfliegen Tephritidae, Arten dieser Gattung gehören weltweit zu den wichtigsten ökonomischen Pflanzenschädlingen.

In dieser Diplomarbeit wurden zwei *Rhagoletis* Arten mit einer konventionellen PCR mit *wsp* Primern auf *Wolbachia* untersucht. Die untersuchten PCR-Produkte wurden direkt sequenziert. Weiters wurden die PCR Produkte kloniert und ca. 20 Plasmide pro Art sequenziert. Die entdeckten Stämme wurden mit den MLST-Markern - *hcp*A, *cox*A, *gat*B, *fts*Z und *fbp*A -charakterisiert.

Es wurden zwei Arten der Gattung *Rhagoletis* untersucht. Einerseits die Amerikanische Kirschfruchtfliege *Rhagoletis cingulata*, einem Schädling bei Süß- und Sauerkirschen in Nordamerika, der kürzlich nach Europa eingeschleppt wurde. Zum anderen handelt es sich um die Apfelfruchtfliege *R. pomonella*, einem Schädling bei Weißdorn und Apfel in Nordamerika. Bei *R. pomonella* wurde zum ersten Mal sympatrische Artbildung beschrieben: Arten an Weißdorn und Apfel unterscheiden sich ökologisch und genetisch.

PCR-Daten bestätigten eine *Wolbachia*-Infektion bei *R. cingulata* und *R. pomonella*. Die Sequenzierung der PCR-Produkte ergab, dass *R. cingulata* mit zwei unterschiedlichen *Wolbachia* Stämmen *w*Cin1 und *w*Cin2 infiziert ist. Eine genaue Analyse mit fünf verschiedenen MLST-Markern bestätigte, dass *Wolbachia* in *R. cingulata* genetisch ident der *Wolbachia* in der europäischen Kirschfruchtfliege *R. cerasi* ist.

Von Apfelbäumen gesammelte *R. pomonella* Individuen sind mit einem *Wolbachia*-Stamm, *w*Pom, genetisch ident dem *w*Cer2 Stamm in *R. cerasi*, infiziert. Genauere Analysen mit den MLST Primern zeigten eine Mutation am *hcp*A locus.

Index of Abbreviations

A	Adenosine
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
C	Cytosine
° C	Degree Celsius
CI	Cytoplasmic incompatibility
cm	Centimetre
*cox*A	Cytochrome c oxidase, subunit I
DNA	Deoxyribonucleic acid
dNTP	2´-deoxyribonucleoside-5´-triphosphate
*fbp*A	Outer surface protein
*fts*Z	Cell division protein
G	Guanosine
g	Gram
*gat*B	Glutamyl-tRNA-(Gln)-amidotransferase
*hcp*A	Conserved hypothetical protein
IIE	Incompatible Insect Technique
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
lacZ	Gene encoding for the enzyme β-galactosidase
LB	Lysogeny broth
Leu	Leucine
M	Molar
m	Milli
$MgCl_2$	Magnesium chloride
min	Minute(s)
MLST	Multilocus sequence typing
n	Nano
NJ	Neighbor Joining Method
NaOH	Sodium hydroxide
PEG	Polyethylenglycole
PCR	Polymerase chain reaction

RLOs	Rickettsia-like organisms
RNase	Ribonuclease
rpm	Rounds per minute
sec	Second(s)
SOC	”Salt optimized + carbon”
spp.	Subspecies
T	Thymidine
TAE	Tris[aminomethyl]aminoethane
Taq	*Thermus aquaticus*
TE-Buffer	Tris-EDTA-Buffer
U	Unit
UV	Ultraviolet (light)
*w*Au	*Wolbachia* variant from *Drosophila simulans* (Australia)
*w*Aso	*Wolbachia* variant from *Asobara tabida*
*w*Bo	*Wolbachia* variant from *Drosophila borealis*
*w*Cal	*Wolbachia* variant from *Calyptratae sp*.
*w*Cer	*Wolbachia* variant from *Rhagoletis cerasi*
*w*Chl	*Wolbachia* variant from *Chloropidae sp.*
*w*Cin	*Wolbachia* variant from *Rhagoletis cingulata*
*w*Dana	*Wolbachia* variant from *Drosophila anassae*
*w*Dia	*Wolbachia* variant from *Diabrotica barberi*
*w*Dmun	*Wolbachia* variant from *Drosophila munda*
*w*Mel	*Wolbachia* variant from *Drosophila melanogaster*
*w*Mono	*Wolbachia* variant from *Monomorum chinense*
*w*Muni	*Wolbachia* variant from *Muscidifurax uniraptor*
*w*Na	*Wolbachia* variant from *Nasonia vitripennens*
*w*Ngi	*Wolbachia* variant from *Nasonia giraulti*
*w*Pom	*Wolbachia* variant from *Rhagoletis pomonella*
*w*Sol	*Wolbachia* variant from *Solenopsis spp*.
wsp	*Wolbachia* surface protein
*wsp*81F	Primer for amplifying *wsp*
*wsp*691R	Primer for amplifying *wsp*
X-Gal	5-bromo-4-chloro-3-indolyl- β-D-Galactopyranoside

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1. Introduction

1.1 *The genus* Wolbachia

Wolbachia is a gramnegativ endosymbiontic bacterium. Hertig and Wolbach described this endosymbiont in the year 1924 (HERTIG & WOLBACH 1924). They were the first to find this Ricketsia Like Organism (RLOs) in *Culex pipiens* (Diptera). HERTIG (1936) named it endosymbiont, *Wolbachia pipiens*.

Results from different studies show an infection rate from 15 to 75% of all insect species (JEYAPRAKASH & HOY 2000, WEST ET AL. 1998, WERREN ET AL. 1995, WERREN & WINDSOR 2000). Recently, HILGERBOECKER ET AL. (2008) concluded from a metapopulation study that 65% of all insects are infected with this endosymbiont. Low titre infections and low frequency infections mostly lead to an underestimation, e.g. ARTHOFER ET AL. (2009A) characterized two *Wolbachia* strains in *Pityogenes chalcographus* (Col., Curculionidae) and described that *Wolbachia* can persist in host species at low densities and low frequencies. Based on a study with wild populations of *Drosophila innubila,* UNCKLESS ET AL. (2009) showed that there is substantial (20.000-fold) variation in *Wolbachia* density and that within host *Wolbachia,* density is positively correlated with both the efficacy of male killing and maternal transmission fidelity.

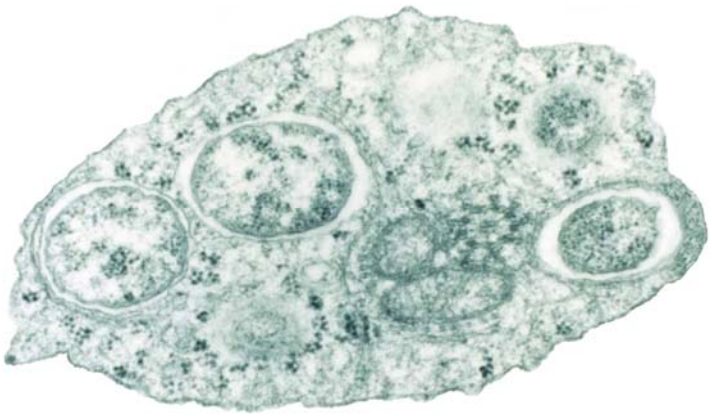

Figure 1.1 Transmission electron micrograph of *Wolbachia* in a developing spermatid of the moth *Ephesia cautella* (taken from O'NEILL & RIEGLER 2006)

Infections have been detected in all major orders of insects and some other arthropod classes like the arachnids or the isopods. Further *Wolbachia* was detected in nematodes (SIRONI ET AL. 1995, BANDI ET AL. 1998). *Wolbachia* is supposed to switch frequently between different species (BRAIG ET AL. 1994). In

most cases *Wolbachia* changes the reproduction abilities of the hosts to enhance its vertical transmission (WERREN 1997). In some cases *Wolbachia* can have positive effects on fertility, sperm competition and can be essential for oogenesis (DEDEINE ET AL. 2001). The transmission occurs transovarial through the cytoplasm of host eggs like the mitochondrial DNA and that is the reason why *Wolbachia* always infects female germlines (SERBUS & SULLIVAN 2007).

On the basis of nuclear 16S ribosomal sequences, *Wolbachia* has been grouped into 7 supergroups. In the supergroup A and B are the major *Wolbachia* strains of the arthropods and supergroup C and D include the most *Wolbachia* strains of filarial nematodes. Supergroup E includes *Wolbachia* that infects wing-less insects like the Collembola and *Wolbachia* strains of the supergroup F are mostly detected in termites. Supergroup G includes *Wolbachia* strains from Australian spiders (for review see WERREN ET AL. 2008).

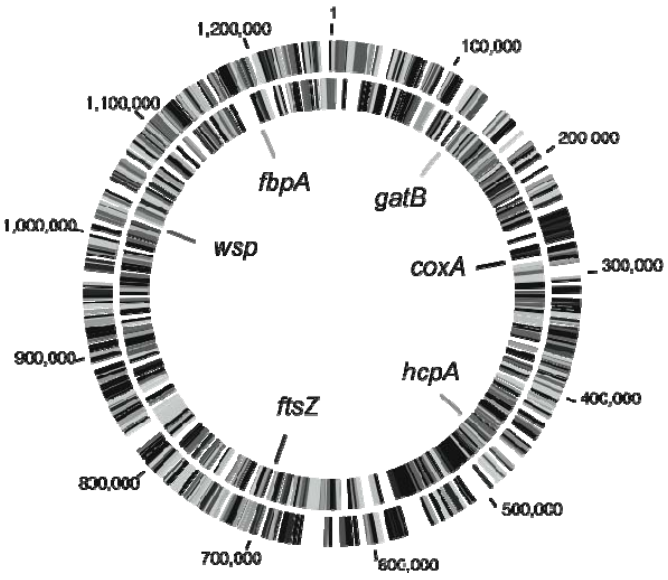

Figure 1.2 Map of the *w*Mel genome (*Wolbachia* in *Drosophila melanogaster*) with the location of the five MLST loci and the *wsp* locus (taken from BALDO ET AL. 2006); see also Table 3.1.

Three *Wolbachia* surface proteins *wsp* have commonly for screening: *wsp, wsp*A and *wsp*B (BRAIG ET AL. 1998, ZHOU ET AL. 1998). *wsp* is divided into four hypervariable regions (HVRs) (WERREN ET AL. 2008) and is quite polymorphic (BALDO ET AL. 2006) and prone to recombination (WERREN & BARTOS 2001). Thus this locus is not ideal for the phylogenetic characterization of *Wolbachia* (BALDO ET AL. 2006). Still, it is a good marker to be used for detection (ARTHOFER ET AL. 2009B).

In consequence, a supplemental typing system to the *wsp* genes was developed by BALDO ET AL. (2006): The Multilocus Sequence Typing System MLST. MLST uses five housekeeping genes *gat*B, *cox*A, *hcp*A *fts*Z and *fbp*A (for details see table 3.1) that are broadly distributed across the *w*Mel genome of *Drosophila melanogaster* (Figure 1.2). Strains with similar *wsp* sequences often have different MLST allelic profiles (BALDO ET AL. 2006). The MLST alleles can be submitted to the *Wolbachia* MLST database (http://pubmlst.org/*wolbachia*/) a site hosted at "The Peter Medawar Building for Pathogen Research" at the University of Oxford, UK.

Wolbachia can precipitate parthenogenetic induction, feminization of males, male killing or cytoplasmic incompatibility (CI). Multipotent *Wolbachia* strains can induce different effects on different hosts (for review see WERREN ET AL. 2008). In the following I will concentrate on CI as it the most frequent effect detected in insects and plays a major role in that thesis.

CI can be detected when a cross between *Wolbachia* infected males and uninfected females results in none or in limited offspring (Fig 1.3). This incompatibility is unidirectional as the reciprocal cross i.e. between an uninfected male crossed with a *Wolbachia* infected female results in normal *Wolbachia* infected offspring. O'NEILL & KARR (1990) differentiate unidirectional mechanism by a bifactorial mechanism for the first time in *Drosophila simulants*: *Wolbachia* modifies the sperm and the sperm can only be rescued if the female has the rescue mechanism. If the female is not infected by the same *Wolbachia* strain the eggs will remain unfertilized.

Wolbachia can be involved in speciation (BORDENSTEIN ET AL. 2001). The authors reported about reproductive isolation between the sibling species *Nasonia vitripennis* and *Nasonia girauli*. A single *Wolbachia* infection of *N. vitripennis* is bidirectional incompatible with a single *Wolbachia* infection of *N. girauli*. These different strains have independent origins. They are required as example for horizontal *Wolbachia* transfer between different species.

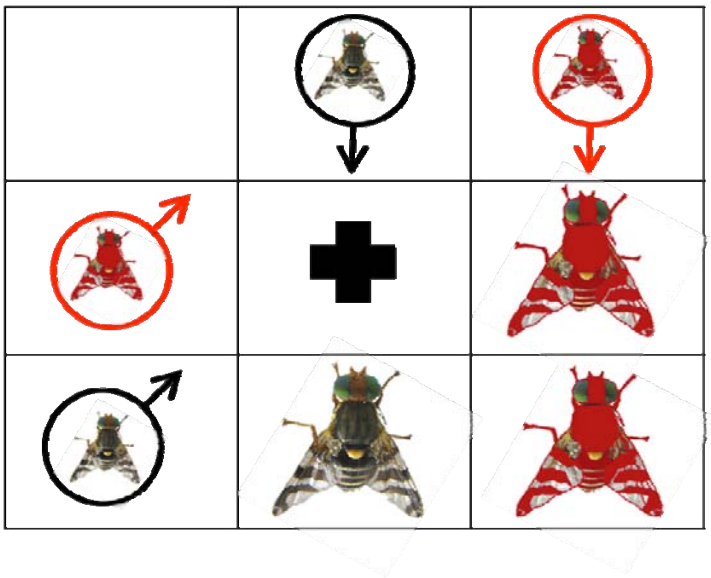

Figure 1.3 The phenotype of *Wolbachia* inducing cytoplasmic incompatibility CI. *Wolbachia* infected males (red) crossed with not infected females result in no offspring (indicated by black cross) in contrast to the reciprocal crossing which results in a vital *Wolbachia* infected offspring.

Wolbachia has been suggested as a potential tool for environment-friendly strategies for the control of arthropod species, the major agricultural pests or disease vectors (BEARD ET AL. 1993, BOURTZIS & O'NEILL 1998, BOURTZIS & BRAIG 1999). A similar way is using *Wolbachia*-induced CI for pest control. Crosses between uninfected females and infected males result in an egg mortality for nearly 100%. Incompatible Insect Technique (IIT) (BOLLER & BUSH 1974, BOLLER ET AL. 1976) is the use of the mechanism of *Wolbachia*-induced CI for the control of populations of pest insects. *Wolbachia* transinfection experiments from infected *R. cerasi* to uninfected medflies *Ceratitis capitata* (ZABALOU ET AL. 2004) showed the potential of *Wolbachia* as a means for insect pest population control. The problem for the practical approach is besides others the difficulty of mass rearing *Wolbachia* infected males only (BOURTZIS 2008).

1.2 Wolbachia *in the dipteran family Tephritidae*

Tephritidae is one of two fly families referred to as "fruit flies". There are almost 5,000 species of tephritids, categorized into almost 500 genera. Tephritid fruit

flies are of major importance in agriculture. Various species of fruit fly cause damage to fruit and other plant crops (WIKIPEDIA 2009).

Wolbachia is described in several tephritid species including the Caribbean fruit fly *Anastrepha suspensa* (WERREN ET AL. 1995), the South American fruit fly *Anastrepha fraterculus* (SELIVON ET AL. 2002), six species of the genus *Anastrepha*: *A. grandis, A. striata, A. pickeli, A. oliqua, A. serpentina, A. amita* (COSCRATO ET AL. Gene bank), *Rhagoletis cerasi* (BLÜMEL & RUSS 1989, RIEGLER & STAUFFER 2002, ARTHOFER ET AL. 2009B), *Ceratitis capitata* (LINCOLN ET AL. 2005), the oriental fruit fly *Bactrocera dorsalis* (SUN ET AL. 2007) and in 12 other species of *Bactocera* in Thailand (SUN ET AL. 2007, JAMNONGLUK ET AL. 2002).

1.2.1 Wolbachia *in the tephritid genus* Rhagoletis

The extensive historic data set on distribution of the geographic complexes (BOLLER ET AL 1976) coupled with field collections over the entire distribution range of the host species for the last decade (RIEGLER & STAUFFER 2002, ARTHOFER ET AL. 2009B) make *R. cerasi* to one of the most attractive field models of *Wolbachia* dynamics. Unidirectional incompatibilities occurred when males from southern and central European populations mated with females from northern European populations (BOLLER ET AL. 1976). BLÜMEL & RUSS (1989) detected Rickettsia Like Organisms (RLOs) in the ovaries of individuals in all populations. RIEGLER & STAUFFER (2002) found an infection with two different *Wolbachia* strains namely *w*Cer1 and *w*Cer2 in the European *R. cerasi* populations. Infections with the *Wolbachia* strain *w*Cer1 were found throughout Europe whereas the southern and central Europe was found to be superinfected with *w*Cer2. A comparison of the two different *Wolbachia* strains with the mating incompatibilities found by BOLLER ET AL. (1976) suggested that most likely *w*Cer2 induces CI. The fact that *w*Cer1 was present in each individual caused the guess, that *w*Cer2 is the cause of CI. A follow up project funded by the Austrian Science Foundation (FWF) revealed that European *R. cerasi* populations are infested by at least five *Wolbachia* strains (*w*Cer1-5) (ARTHOFER ET AL. 2009B) (Fig. 1.5). Recently, K. KÖPPLER (JKI, Dossenheim, personal

communication) confirmed that *w*Cer2 most likely is the CI inducing strain by performing further crossing studies with *w*Cer defined populations.

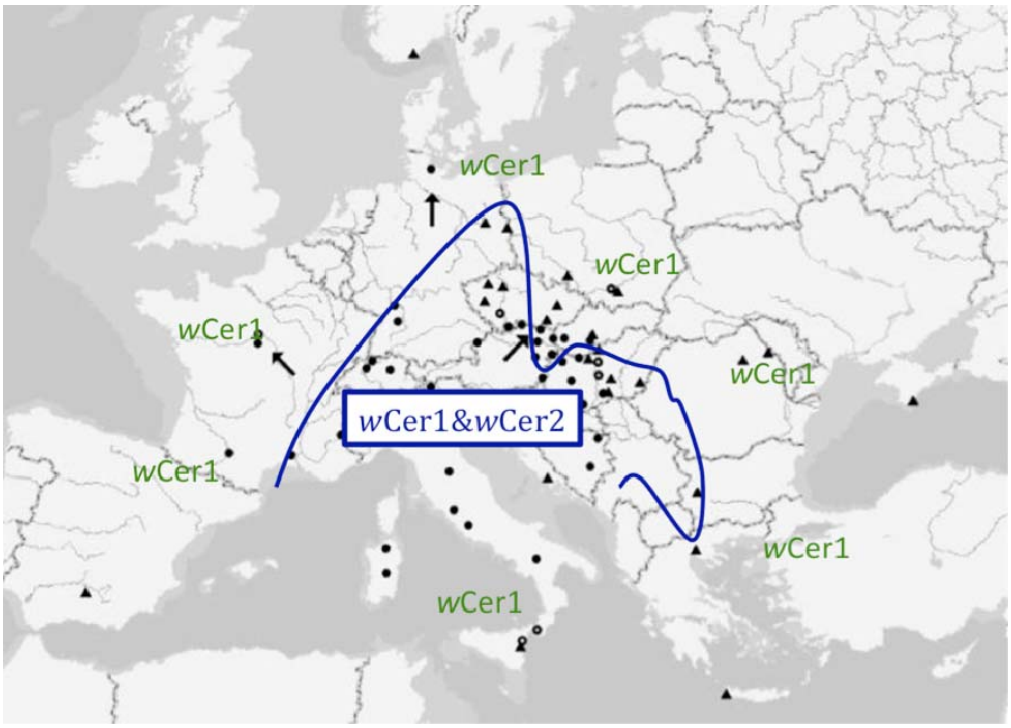

Figure 1.4 Distribution of the unidirectional incompatibility trait between populations of *Rhagoletis cerasi*. Populations from the south (●) and the north and east (▲) exhibited strong cytoplasmic incompatibility CI of 98% in single pair crossings (BOLLER ET AL. 1976). Distribution of *w*Cer1 and *w*Cer2 of *R. cerasi* in Europe according to RIEGLER & STAUFFER (2002). Transition zone is indicated by blue line.

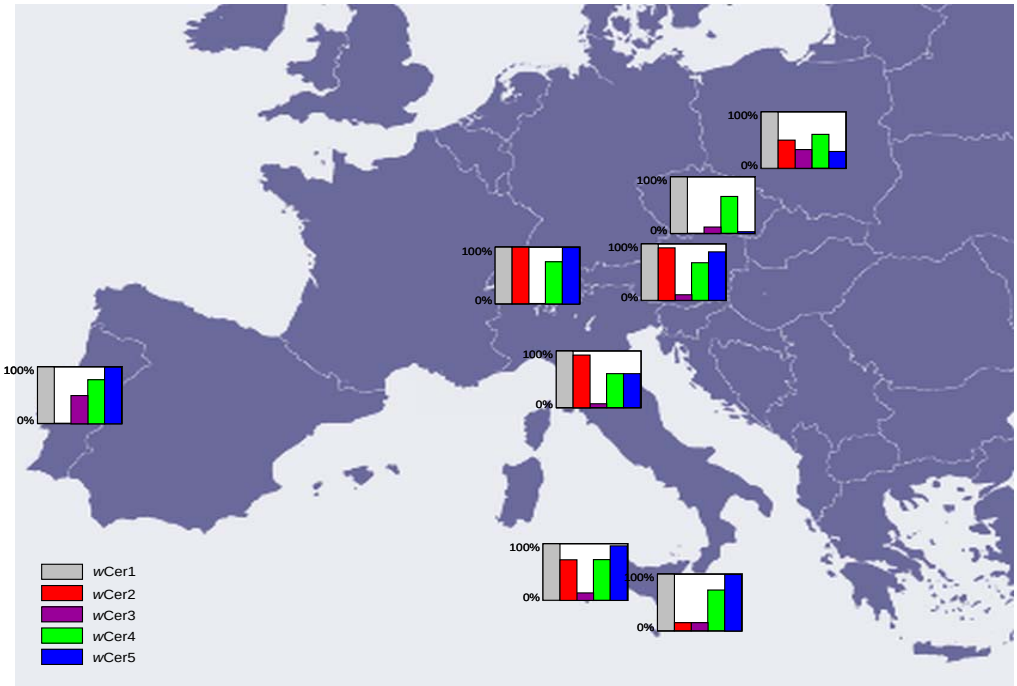

Figure 1.5 The distribution of *w*Cer1-5 of *Rhagoletis cerasi* in eight European populations, 2007. Only *w*Cer1 was detected in all individuals analysed. Figure taken from ARTHOFER ET. AL (2009B).

1.3 Biology of Rhagoletis *spp.*

The genus *Rhagoletis* belongs to the family tephritids and contains important pest species like *R. cerasi*, the Apple Maggot, *R. pomonella*, and the Eastern Cherry Fruit Fly, *R. cingulata*. The biology of these different *Rhagoletis* species is quite similar (Fig. 1.6).

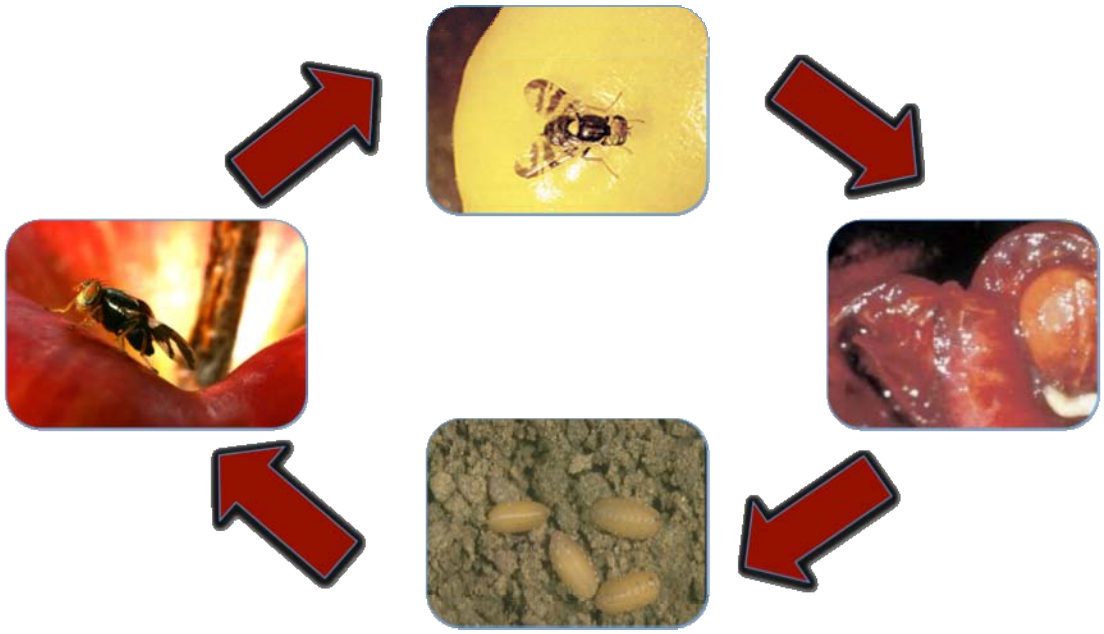

Figure 1.6 Biology of *Rhagoletis* species: Adult females lay eggs singly in host fruit and after hatching the larva feeds on the pulp. Mature larvae drop to the ground where they pupate and hibernate in the soil (BOLLER & PROKOPY 1976)

Adult females move to larval host plants for mating and oviposition. Various reasons for host detection play the foliage colour, tree shape and tree size. Further, the odour of the host fruits act as kairomone attracting flies (MOERICKE ET AL. 1975). Males are waiting on the host plants for females and defend the territories for concurrent males (ALINIAZEE 1974, BOYCE 1934). In order to attract virgin females they emit a highly concentrated pheromone (PROKOPY & BUSH 1972). In order to copulate, males jump or fly on the female's abdomen and prepare for the fertilization (BROOKS 1921, BUSH 1969). After fertilization females fly to the maturing fruits to deposit mostly one egg per fruit e.g. *R. cerasi* or more eggs per fruit e.g. *R. completa* who is laying 15 or more eggs at each fruit (BOYCE 1934, DEAN 1969). Under ideal conditions *Rhagoletis* spp. lay up to 300-400 eggs per female.

A few days after the oviposition the larvae hatch and start feeding in the pulp of the fruits. The larval development is completed in about two to three weeks. Sugar contents and acidity play an important role for the development (BOLLER 1966, DEAN & CHAPMAN 1973). At last larval instars bore through the fruit skin and drop to the ground. They burrow into the soil and pupate (FRICK ET AL. 1954, LATHROP ET AL. 1932). Most *Rhagoletis* species undergo an obligate diapause in the pupal stage and remain in the soil till the next spring (NEILSON 1962). Under unfavourable conditions they can stay in the litter of the soil for several years (BALDUF 1959).

In *R. pomonella* diapause is regulated mainly by photoperiod (PROKOPY 1968). *R. pomonella* has a flexible diapause strategy. After a few weeks pupae can develop to adults. The second generation is often unable to perform a complete development and will be wiped out by low temperatures during winter (CHAPMAN 1941).

1.3.1 The Eastern Cherry Fruit Fly R. cingulata

The Eastern Cherry Fruit Fly *R. cingulata* infests cherry orchards, particularly sour cherries, *Prunus cerasus,* but also wild cherrys *P. avium* in North America. *R. cingulata* is a close relative to the Western Cherry Fruit Fly *R. indifferens*, which infests also *P. avium* and Chinese plum, *P. salicina* (EPPO, CABI 1996). Morphologically these two species are hardly distinguishable (BUSH 1966). Genetically, however, they could be clearly distinguished (MCPHERON & HAN 1997, SMITH & BUSH 1997).

R. cingulata has a similar biology as *R. cerasi*. *R. cingulata* pupae requires a higher temperature to reach maturity and emergence of adults from the soil starts later than the one of *R. cerasi.* Adults are quite sessile as they fly only short distances (LAMPE ET AL. 2005).

Figure 1.7 Adult fly of *R. cingulata.*

R. cingulata is a serious pest on cherries, plums and even olives mainly in North American regions (WEEMS JR., 2001). In 1983, *R. cingulata* was discovered on cherry trees in Europe, namely in Switzerland for the first time (MERZ 1994). Between 1991 and 1993 *R. cingulata* was found many times in the south of Switzerland. Adults were collected by yellow sticky traps. Attacked cherries were collected and hatched in the laboratory (BOLLER & MANI 1994). Later, *R. cingulata* was detected in Germany (LAMPE ET. AL 2005), in northern Italy (EPPO 1996) and in the Netherlands (EPPO 2004). In Germany, *R. cingulata* was detected in Rhineland in the area of Kassel (Hessen) (LAMPE ET. AL 2005). In the latter case, it is reported that 18% of collected maggots were *R. cingulata*. Recently individuals of *R. cingulata* were detected in Slovenia (EPPO 2007) and two individuals in Austria (EGARTNER ET AL. 2008).

R. cingulata infests cherries later than *R. cerasi* (BOLLER 1966, JUBB & COX 1974), which indicates, that *R. cingulata* has the potential to fill an ecological niece namely infesting later ripening cherries (LAMPE ET. AL 2005).

1.3.2 The Apple Maggot R. pomonella

The Apple Maggot, *R. pomonella*, is an important pest species on several orchard species in North America (EPPO 1996). Adults lay their eggs on the fruits and larvae begin to feed on the pulp of ripening fruits. The natural host plant of this species is hawthorn, *Crataegus* spp. In 1860, WALSH described a shift from hawthorn to apple, *Malus domestica*. Since then, *R. pomonella* was recorded on several *Prunus* species like apricot, *Prunus armeniaca*, peach, *P. persica*, and cherry, *P. avium* and *P. cerasus* (ALLDRED & JORGENSEN 1993). Larvae (but no adults) have been found in pears (*P. communis*), medlars (*Amelanchier*), chokeberries (Aronia), cranberry (*Vaccinium macrocarpum)* cotoneasters (*Cotoneaster atropurpureus)* and roses (Rosa) (EPPO 1996). Apple orchards appear to have the highest density rate of *R. pomonella* in the geographically range of North America. So far this pest is absent in the EU. Due to it is potential aggressivity on apple species, *R. pomonella* has been classified as quarantine pest in the EU (EPPO 1996).

Figure 1.8 Adult fly of *R. pomonella*

Besides it's economically importance, *R. pomonella* is a model organism for sympatric speciation. Sympatric speciation is the genetic

differentiation of populations without geographic isolation. In contrast, allopatric speciation is the genetic differentiation caused by geographic isolation (MAYR 1963 or for recent review see BERLOCHER & FEDER 2002). BUSH (1969) described sympatric speciation for the first time in *R. pomonella* as genetically differentiated host races exist on apples and hawthorns. The author hypothesized that this differentiation was caused by the shift from hawthorn to apple (BUSH 1969). Host fidelity prevents gene flow between populations from apple and hawthorn. Host associated fitness trade offs cause post zygotic barriers to gene flow (FEDER 1998) (Figure 1.9). Species collected of hawthorn and samples from apples show genetic differences on three different regions of the genome (BERLOCHER & SMITH 1983, FEDER ET AL. 1989). Studies on the oviposition of the different races reveal that there are differences on the host preference. Females of both host races prefer to oviposit on hawthorns. Females originating from hawthorn are indisposed to lay eggs on apples in contrast to apple originating females, which can lay eggs on both hosts (PROKOPY ET AL., 1972, 1988).

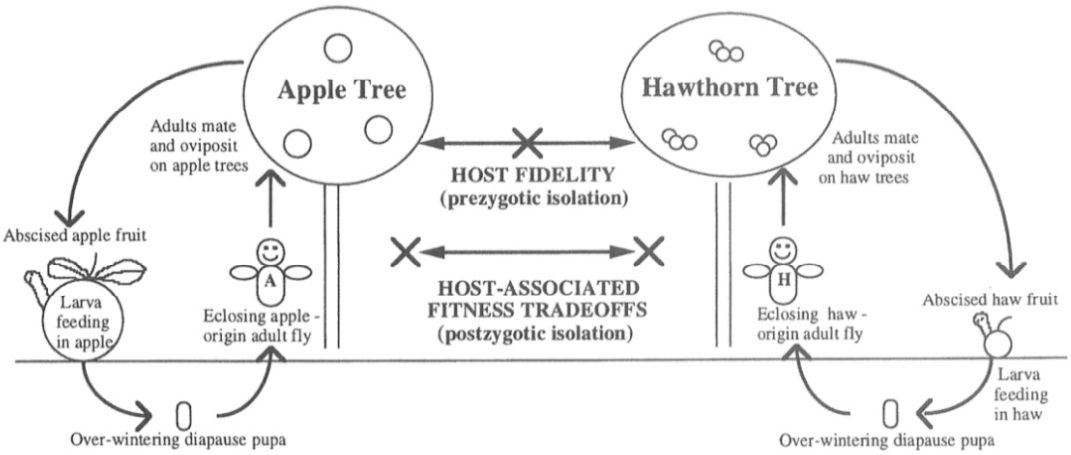

Figure 1.9 The life cycle of *R. pomonella* emphasing the roles of host fidelity and fitness trade-offs play in isolating apple- and hawthorn-infesting races of the fly (taken from from FEDER 1998)

2. Aims

In this thesis the aim was to search for the presence of *Wolbachia* in *R. pomonella* and *R. cingulata*. *R. cingulata* was analysed as this species was introduced in Europe recently and co-infests cherries together with the European Cherry Fruit Fly, *R. cerasi*. *R. cerasi* is known to host up to five *Wolbachia* strains (ARTHOFER ET AL. 2009B) and the question was if these strains were transferred to the introduced species.

R. pomonella was taken, as this prominent species for sympatric speciation was never analysed for the existence of *Wolbachia* to my knowledge. As BORDENSTEIN ET AL. (2001) showed, *Wolbachia* can cause speciation and thus the presence of *Wolbachia* raises a new aspect in this story (for review see HOWARD & BERLOCHER 1998).

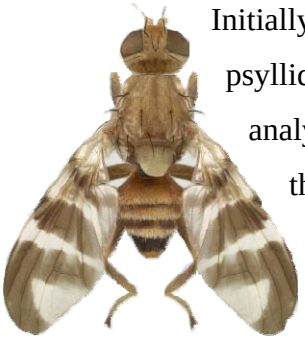

Figure 2.1 Adult fly of *R. completa*

Initially also *Rhagoletis mendax, Rhagoletis completa* and two different psyllid species, *Cacopsylla picta* and *Cacopsylla melanoneura* were analyzed. Latter two psyllid species are important orchard pests as they are vectors for phytoplasmas. *R. mendax* proved to be infected with *Wolbachia* but will be elaborated in a later stage.

R. completa and the two *Cacopsylla* species revealed no amplicon by conventional *wsp* PCR. Thus they were neglected for further studies.

The *Wolbachia* detection was done with general *wsp* primers described by BRAIG ET AL. (1998). These amplicons from *R. cingulata* and *R. pomonella* were sequenced directly. Further, *wsp* amplicons were cloned in order to detect if more than one *Wolbachia* strain was present.

All *Wolbachia* strains detected were characterized by five additional MLST markers (BALDO ET AL. 2006). For all loci a phylogenetic analysis was done.

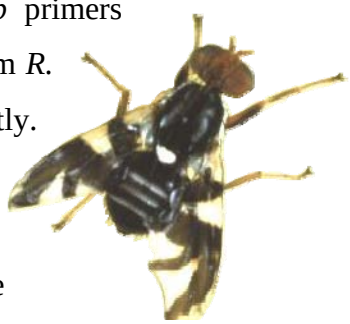

Figure 2.2 Adult fly of *R. mendax*

3. Methods

3.1 Samples

Rhagoletis pomonella adults were collected by Prof. Dr. Luís AF Teixeira (Michigan State University, Department of Entomology, East Lansing, USA) on apple trees (*Malus domestica*) from a population collected in Michigan in 2006. *Rhagoletis cingulata* pupae were collected by Dr. Kirsten Köppler and Dr. Heidrun Vogt (Julius Kühn Institute JKI, Dossenheim, Germany) in Heidesheim in Germany in 2008. All samples were stored in absolute ethanol, sent to the Institute of Forest Entomology, Forest Pathology & Forest Protection, Vienna, Austria and stored at -20°C.

Further blueberry pupae of *R. mendax* collected by Prof. Dr. Luís AF Teixeira (Michigan State University, Department of Entomology, East Lansing, USA) were sent from populations collected in Michigan in 2006. Further walnut husk pupae, *Rhagoletis completa*, collected from immature walnuts were collected at the garden of the Institute of Forest Entomology, Forest Pathology & Forest Protection, Boku Vienna in autumn 2008. Two psyllid species, *Cacopsylla picta* and *Cacopsylla melanoneura* were collected by Mag. Manfred Wolf from the Land- und Forstwirtschaftliches Versuchszentrum Laimburg in Italy, stored in absolute ethanol and brought to Vienna and stored there at -20°C.

3.2 Extraction of the DNA

From each of the species the DNA of two individuals was extracted using the DNA Mini-Prep SIGMA Kit (Appendix I). The insects were put in an Eppendorf tube and overlayed with 180 µl lysis solution, homogenized thoroughly, 20µl SIGMA proteinase K were added and put in the heating block at 55°C. After adding 20 µl of RNAse, the tubes were incubated for 2 min and 200 µl of lysis solution were added. The solution was incubated for 10 min at 70°C. 200 µl absolute ethanol were added to the samples with and transferred to the prepared binding columns. After a few washing steps with a washing solution, the DNA was eluted in 50µl elution buffer and stored at 4°C.

3.3 PCR with general *wsp* primer

To analyse the samples for a *Wolbachia* infection, the *wsp* primers 81F and 691R described by BRAIG ET AL. (1998) were used. The reactions were set up in 20 µl volumes containing, 1x NH_4 buffer (Fermentas), 4mM $MgCl_2$, 200 µM dNTPs, 0,4 µM of each primer, 0,5 U Taq polymerase (Fermentas) and 2 µl of the template DNA. PCR was started for 2 min at 95°C and followed by 32 cycles at 94°C for 30 sec, 60°C for 45 sec, 72°C for 1 min and a final extension at 68°C for 15 min.

For detection 10 µl of DNA fragments were used a submarine horizontal gel system using a 1x TAE running buffer. Gels with a 1 to 2% agarose concentration supplemented with 0,5 µg/ml ethidium bromide were used. DNA was visualized on a UV transilluminator

3.4 PCR with MLST

For a supplemental analysis the five housekeeping genes *gat*B, *cox*A, *hcp*A *fts*Z and *fbp*A from the MLST described by BALDO ET AL. 2006 were used.

Cluster category[a]	Locus code (wMel)	Gene	Product	Primer		Gene length (bp)[b]	Amplified nucleotide range (bp)[b]	MLST fragment size (bp)
				Designation	Sequence (5'–3')			
α-Proteobacteria	WD_0146	*gatB*	Glutamyl-tRNA(Gln) amidotransferase, subunit B	gatB_F1	GAKTTAAAYCGYGCAGGBGTT	1,425	421–891	369
				gatB_R1	TGGYAAYTCRGGYAAAGATGA			
Escherichia coli	WD_0301	*coxA*	Cytochrome *c* oxidase, subunit I	coxA_F1	TTGGRGCRATYAACTTTATAG	1,551	491–977	402
				coxA_R1	CTAAAGACTTTKACRCCAGT			
Escherichia coli	WD_0484	*hcpA*	Conserved hypothetical protein	hcpA_F1	GAAATARCAGTTGCTGCAAA	741	91–605	444
				hcpA_R1	GAAAGTYRAGCAAGYTCTG			
Escherichia coli	WD_0723	*ftsZ*	Cell division protein	ftsZ_F1	ATYATGGARCATATAAARGATAG	1,197	274–798	435
				ftsZ_R1	TCRAGYAATGGATTRGATAT			
Rickettsiales	WD_1238	*fbpA*	Fructose-bisphosphate aldolase	fbpA_F1	GCTGCTCCRCTTGGYWTGAT	900	241–749	429
				fbpA_R1	CCRCCAGARAAAAYYACTATTC			
Wolbachia	WD_1063	*wsp*	Outer surface protein	wsp_F1	GTCCAATARSTGATGARGAAAC	714	85–688	546
				wsp_R1	CYGCACCAAYAGYRCTRTAAA			

Table 3.1 MLST primer names and length described by BALDO ET AL. (2006). Table taken from BALDO ET AL. (2006).

The reactions were set up in 10 µl volume containing, 1x NH_4 buffer (Fermentas), 2mM Mg_2Cl, 100 µM dNTPs, 0,2 µM of each primer. 0,25 U Taq polymerase (Fermentas) and 1 µl of the template DNA. PCR was started for 2 min at 95°C and followed by 32 cycles at 94°C for 30 sec, 60°C for 45 sec, 72°C

for 1 min and a final extension at 68°C for 15 min. With the primer *fts*Z who did not work at a TM of 60°C PCR was successful only with a TM of 50°C.

3.5 Purification of DNA

Plasmid purification was achieved by an alkaline lysis procedure (SAMBROOK ET AL. 1989), with a peqGOLD Cycle-Pure Kit. DNA Product were mixed with the same volume XP1 buffer, vortexed and pipetted in columns and centrifuge for 1 min at 10.000 rpm. Afterwards columns were washed two times with 650 µl SPW-washing buffer and centrifuged for one min on 10.000 rpm. For drying the empty column were centrifuged for 1 min on 10.000 rpm. Purified DNA was resolved in 20 µl elution buffer and centrifuged for 1 min at 10.000 rpm.

3.6 Cloning

The working protocol of this procedure is also listed in appendix I. For cloning an 0,8 µl aliquot of the PCR product was mixed with 0,2 µl of the vector pTZ57R (InstarClone PCR, Fermentas), 0,3 µl polyethylenglycol (PEG3350), 0,2 µl T4 buffer and 0,2 U T4 ligase and constantly held at 15°C over night.

For the transformation competent JM109 *E. coli* cells were used. Preserved in a freezer at -80°C these cells were placed on ice. On each sample 50 µl of *E. coli*, were pipetted, vortexed carefully and placed on ice for 20 min. The samples were heated for 50 sec at 42°C. 950 µl of a SOC-media were added placed at 37°C in the oven for 60 min. The samples were centrifuged for 5 min at 4°C at 2.500 rpm. Most of the supernatant was taken off and the bacteria were discarded in a Petri dish. To select the positive bacteria, the agar contained 100 µg/ml ampicilin, 160 µg/ml xGal and 48 µg/ml IPTG. Plates were stored on 37 degrees over night. The next day plates were controlled for white bacteria colonies. They were marked and transformed with a toothpick on another Petri dish containing agar. After another day, these bacteria were inoculated in LB broth (SAMBROOK ET AL. 1989) (Appendix II) A PCR was done with M13 primers. An agarose gel electrophoresis showed which bacteria contained plasmids with PCR products and which ones without. The plasmid purification was undertaken by an alkaline lysis mini prep procedure (Appendix I). For sequencing the purified DNA was

sent to the Cancer Research Centre DNA Sequencing & Genotyping Facility in Chicago (IL, USA).

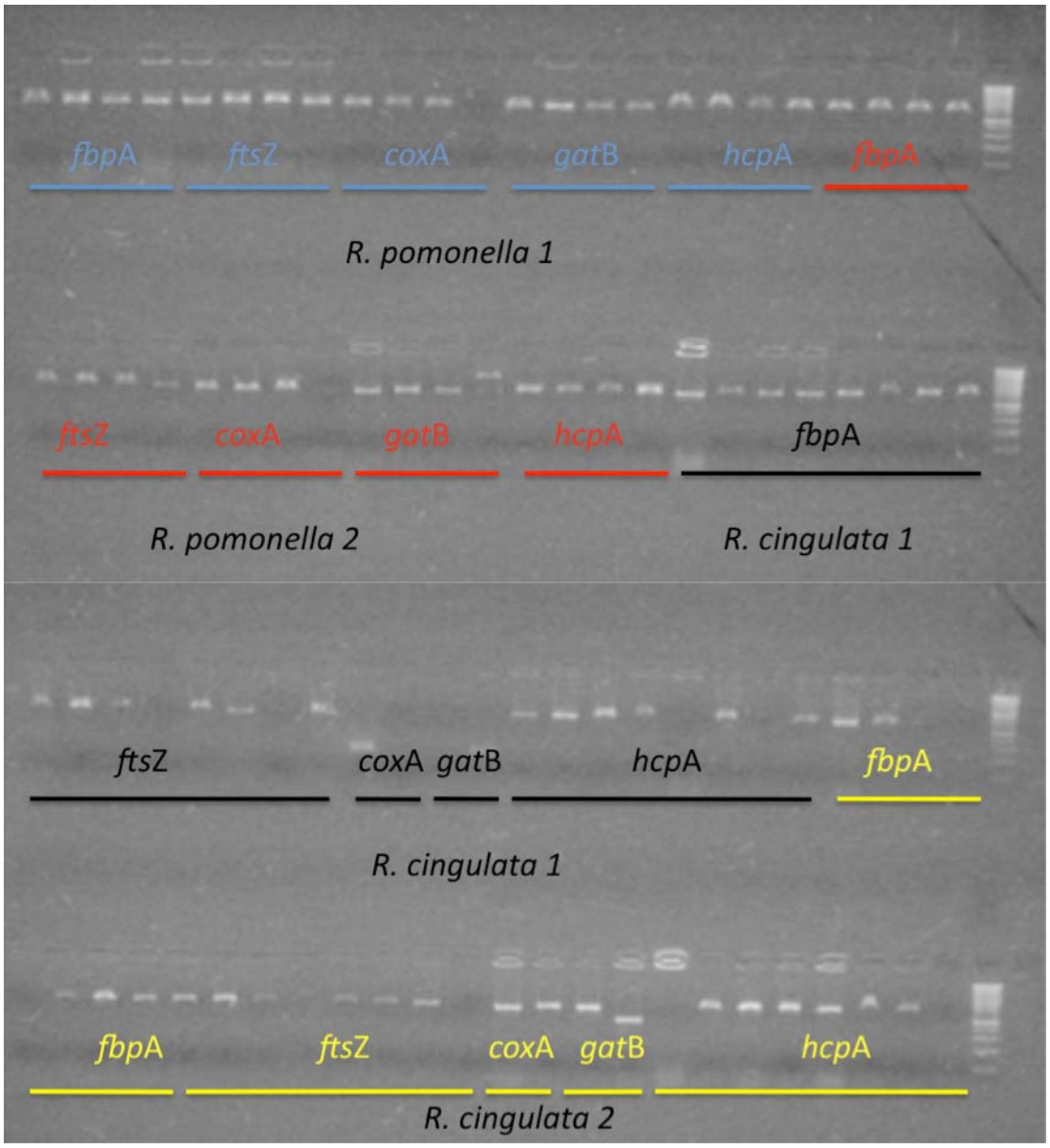

Figure 3.1 Agarose gel showing the MLST products of *R. cingulata* and *R. pomonella*. Samples of the positive amplicons were purified and sequenced.

3.7 Phylogenetic analyses of the sequence data

Retrieved sequences were edited and aligned with Codon Code Aligner (CodonCode Corporation, USA). Further ClustalX (THOMPSON ET AL. 1997) was used for tree construction using the neighbor-joining (NJ) algorithm with Kimura-2-parameter distances as implemented in MEGA4 (TAMURA ET AL. 2007).

4. Results & Discussion

The aim of my master thesis was the detection of *Wolbachia* strains in species of the genus *Rhagoletis* and in the two psyllid species *Cacopsylla picta* and *Cacopsylla melanoneura*. *Rhagoletis cerasi* proved to carry several high and low titre *w*Cer strains (ARTHOFER ET AL. 2009B). Some of these strains expressed high CI after transinfection into the economically important medfly species, *Ceratitis capitata*, making *Wolbachia* a potential means in biological insect control (ZABALOU ET AL. 2004). Other *Rhagoletis* species have been not analysed for *Wolbachia* and thus I aimed to detect *Wolbachia* by applying general *wsp* primers (BRAIG ET AL. 1998) and to characterize these strains with five nuclear loci described by BALDO (2006).

4.1 Detection of *Wolbachia* by PCR using *wsp* primers

The PCR analyses with the *wsp*81F and *wsp*691R primers developed by BRAIG ET AL. (1998) showed positive amplicons with *Rhagoletis mendax* (1, 2), *Rhagoletis cingulata* (3, 4) and *Rhagoletis pomonella*. (5, 6) The amplicon + was the positive sample *Rhagoletis cerasi* and is about 500bp long. *Rhagoletis completa* (7, 8, 9, 10), *Cacopsylla picta* (11, 12, 13, 14) and *Cacopsylla melanoneura* (15, 16) did not result in visible amplicons (Figure 4.1).

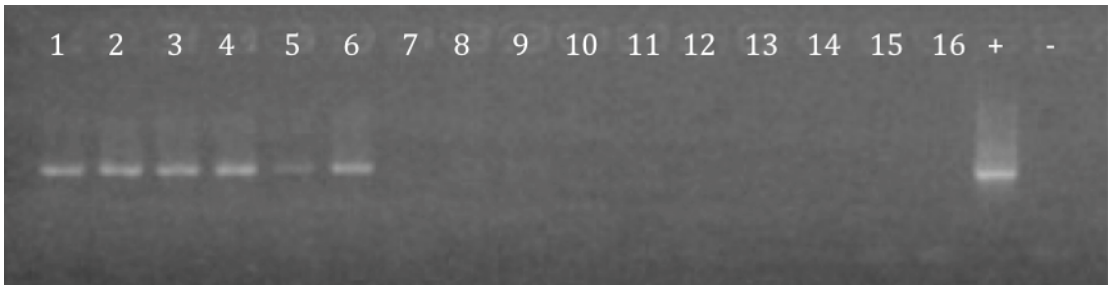

Figure 4.1 Agarose gel showing the *wsp* products of *R. mendax* (1, 2) *R. cingulata* (3, 4) and *R. pomonella* (5, 6). The amplicons were at the same size as the positive control, *R. cerasi*. The negative probe was a Mastermix sample. *R. completa* (7, 8, 9, 10), *Cacopsylla picta* (11, 12, 13, 14) and *C. melanoneura* (15, 16) did not result in an amplicon.

ARTHOFER ET AL. (2009B) report low densities of the *w*Cer3 and *w*Cer4 strains in *R. cerasi* populations. In that species, hybridisation of the amplicon with a *wsp* probe was used as this method is more sensitive than conventional PCR. The bark beetle *Pityogenes chalcographus* was analysed with nested PCR lowering the detection limit also some potencies and also here two low density strains were detected – *w*Cha1, *w*Cha2 (ARTHOFER ET AL. 2009A). Thus negative results by conventional PCR like here in *R. completa* and the two psyllids *C. picta* and *C. melanoneura* do only show that there are not high titre *Wolbachia* strains. Sensitive detection methods like Southern blot or nested PCR should be applied and also more individuals should be analysed as the density might also vary widely between individuals as suggested by HILGERBOECKER ET AL. (2008) or UNCKLESS ET AL. (2009).

4.2. *Wolbachia* in *Rhagoletis cingulata*

4.2.1 Phylogenetic characterization of *w*Cin strain by *wsp*

Purifying and sequencing the *wsp* products of the two individuals of *R. cingulata,* the sequences were edited and aligned. Further, the *wsp* products were cloned with a TA vector and about 20 plasmids were sequenced. These sequences were 100% ident to the ones from direct sequencing (Appendix III). The alignment of the sequences revealed two types of sequences - *w*Cin1 and *w*Cin2 differing by 0,25% (for distances see Appendix IX).

The most similar sequences were retrieved from the Genbank by the BLAST search option. These were included in the alignment and a phylogenetic tree was constructed with both distance and parsimony method. Here the Neighbour-Joining NJ tree applying Kimura-2-parameter distances and bootstrapping is shown (Fig. 4.2). The phylogenetic analyses revealed that *w*Cin1 and *w*Cin2 were ident to *w*Cer1 (AF418556) and *w*Cer2 (AF418557) of *R. cerasi,* respectively (Appendix IX).

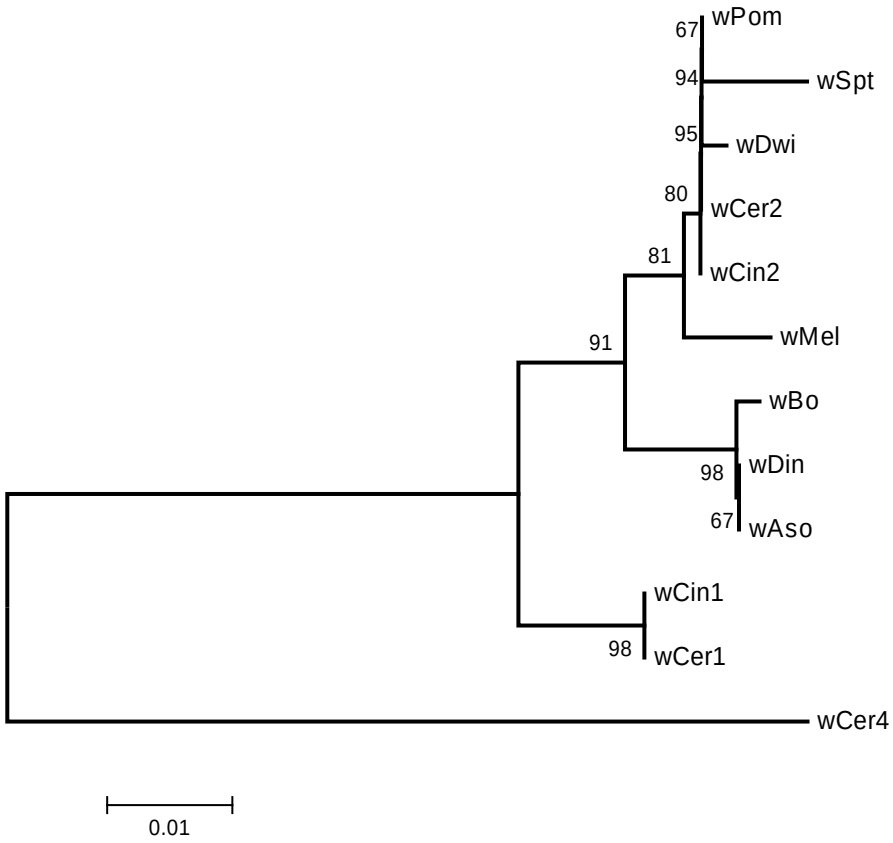

Figure 4.2 Phylogenetic analysis of *w*Cin1 and *w*Cin2 of *R. cingulata*, *w*Pom of *R. pomonella* and other *Wolbachia* strains retrieved from the Genbank. Analysis was done with Neighbour Joining method using the *wsp* sequences. Bootstrap analysis was done with 100 replicates and are the numbers above the node. *w*Cer= *R. cerasi*, *w*Dwi= *Drosophila willistoni* (AY620229), *w*Spt= *D. septentrionalis* (AY620214), *w*Aso= *Asobara tabida* (AY581186), *w*Dbo= *D. borealis* (FJ415468), *w*Mel= *D. innubila* (AY552553)

*w*Cin2 is ident to the *w*Cer2 strain and the *w*Au strain of *Drosophila simulans* (DQ235407) (Fig 4.2). *w*Cin1 is ident to *w*Cer1, a *Wolbachia* strain detected in all European *R. cerasi* populations analysed (ARTHOFER ET AL. 2009B, RIEGLER & STAUFFER 2002). Until now, eight supergroups A to H have been designated based on clustering patterns of 16S-DNA and the genes *fts*Z and *wsp* (WERREN ET AL. 1995, BANDI ET AL. 1998, ZHOU ET AL. 1998, LO ET AL. 2002, WERREN ET AL. 2008). *w*Cin1 and *w*Cin2 belong to the supergroup A according.

4.2.2 Characterization of the *w*Cin strains by MLST

In the following, the *w*Cin 1 and *w*Cin2 were characterized by the five MLST loci (BALDO ET AL. 2006). These five loci are listed in Table 1.1. Figure 4.3

shows the agarose gel electrophoresis and reveals the successful amplification of all 5 loci: *gat*B, *cox*A, *hcp*A, and *fbp*A. *fts*Z did not amplify with a T_M temperature of 60°C but with 50°C (data not shown).

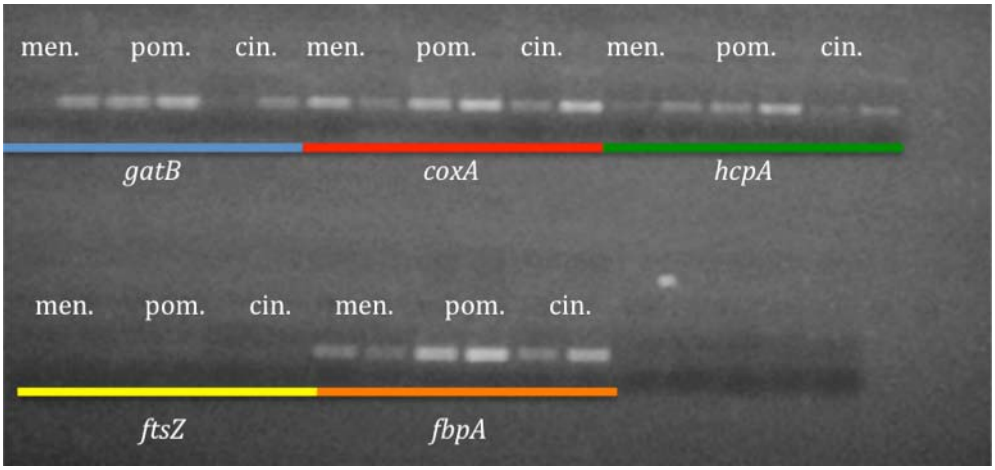

Figure 4.3: Agarose gel showing the products amplified with the primers of the five MLST loci (Table 1.1). Four loci's worked well whereas the locus *fts*Z did not result in an amplicon. This locus was successfully amplified with a TM of 50°C**.**

The amplicons of the five gene products from two individuals of *R. cingulata* were directly sequenced after purification. Further, they were cloned with a TA vector and after transformation into bacterial cells about 8 positive plasmids from each of the loci were picked and sequenced. These sequences revealed 100% identity to the sequences from the direct product (Appendix III). The MLST loci revealed also two different sequences confirming the existence of *w*Cin1 and *w*Cin2. Only *cox*A and *gat*B revealed the *w*Cin1. In these loci *w*Cin2 might be ident with *w*Cin1 or have mismatches in primer sites or might be present in too low titre for detection. In latter case more plasmids should be taken in order to characterize that strain by all MLST loci.

Most similar sequences were retrieved from the Genbank by the BLAST search option. These were included in that alignment and phylogenetic trees from each loci were constructed with distance method using Neighbor-Joining NJ tree applying Kimura-2-parameter distances and bootstrap analysis (Fig. 4.4-4.8).

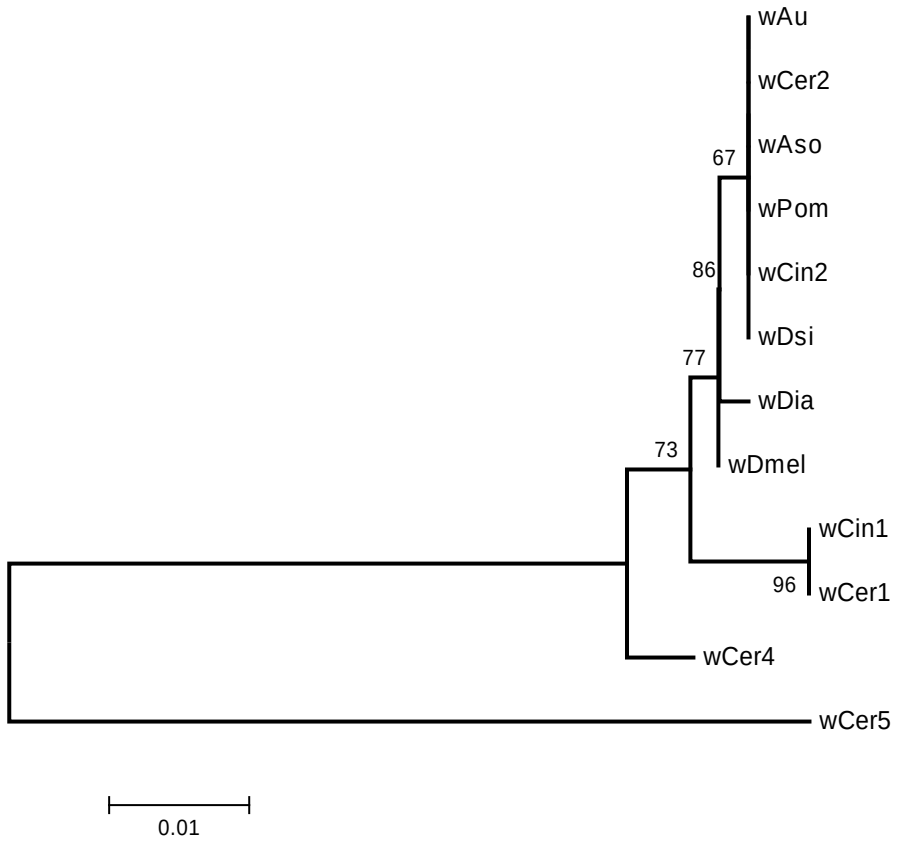

Figure 4.4 Phylogenetic analysis of *w*Cin1 and *w*Cin2 of *R. cingulata*, *w*Pom of *R. pomonella* and other *Wolbachia* strains. Analysis was done with Neighbour Joining method using the *fts*Z sequences. Bootstrap analyses were done with 100 replicates and are the numbers above the node. *w*Cer = *R. cerasi*, *w*Dsi= *Drosophila simulants* (EF423735), *w*Dia= *Diabrotica barberi* (AY136554), *w*Aso= *Asobara tabida* (AY567704), *w*Au= *D. simulants* (AY227739), *w*Mel= *Drosophila melanogaster* (AE017196)

The alignment and distance matrix of the *fts*Z gene is shown in appendix IV and IX, respectively. The NJ tree based on the *fts*Z gene showed that *w*Cin1 is genetically ident to the *w*Cer1 (AY227737) strain and *w*Cin2 (AY227738) to *w*Cer2 of *R. cerasi* and to *Wolbachia* detected in *D. simulans* (EF423735) and *Asobara tabida* (AY567704) (Fig. 4.4).

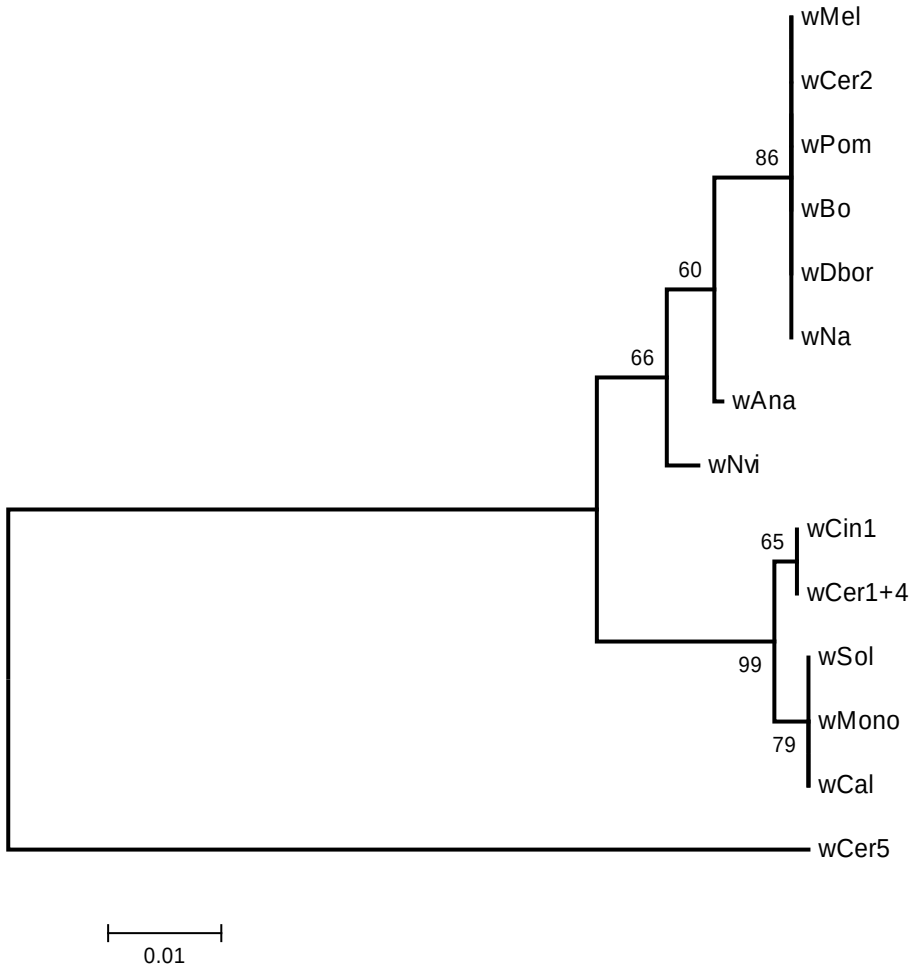

Figure 4.5 Phylogenetic analysis of *w*Cin1 and *w*Cin2 of *R. cingulata*, *w*Pom of *R. pomonella* and other *Wolbachia* strains. Analysis was done with Neighbour Joining method using the *cox*A sequences. Bootstrap analyses were done with 100 replicates and are the numbers above the node. *w*Cer= *R. cerasi*, *w*Mel= *Drosophila melanogaster*, *w*Bo= *Drosophila borealis* (FJ415470), *w*Na= *Nasonia longicornis* (FJ390239), *w*Ana= *Drosophila ananassae* (EF611963), *w*Nvi= *Nasonia vitripennens* FJ390240), *w*Sol= *Solenopsis* spp.(EU127565), *w*Mono= Monomorium chinense (EU127553), *w*Cal= *Calyptratae* sp. (EU126210)

The alignment and distance matrix of the *cox*A gene is shown in appendix IV, and IX, respectively. Here only *w*Cin1 was detected and the NJ analysis showed that *w*Cin1 is ident to *w*Cer1. With a genetic distance of 0,05% *w*Cin1 is related to a *Wolbachia* strain detected in the fire ant, *Solenopsis* spp. (EU127565) (Fig. 4.5).

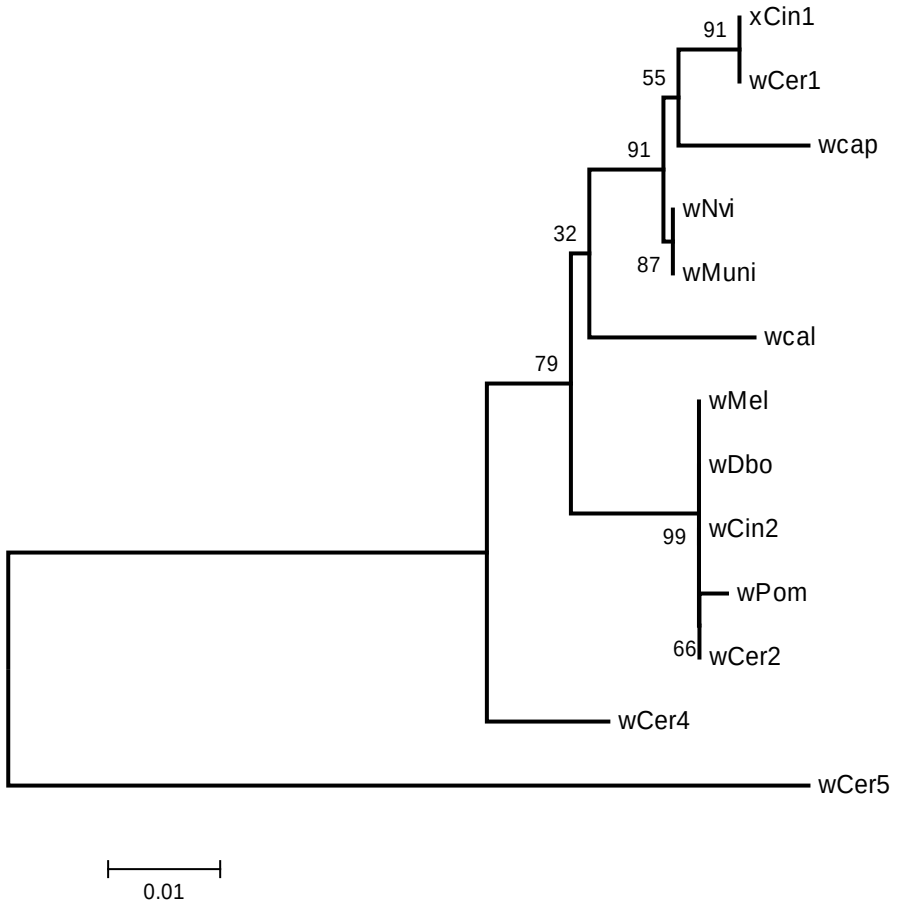

Figure 4.6 Phylogenetic analysis of *w*Cin1 and *w*Cin2 of *R. cingulata*, *w*Pom of *R. pomonella* and other *Wolbachia* strains. Analysis was done with Neighbour Joining method using the *hcp*A sequences. Bootstrap analyses were done with 100 replicates and are the numbers above the node. *w*Cin= *Wolbachia* in *Rhagoletis cingulata*, *w*Pom= R. pomonella. *w*Cer= *R. cerasi*, *w*Mel= *Drosophila melanogaster* (AE017196), *w*Cap= *Camponontus leonardi* (EU127639), *w*Nvi= *Nasonia vitripennis* (DQ842407), *w*Muni= *Muscidifurax uniraptor* (DQ842404), *w*Cal= *Calyptrata muscoid* fly (EU126321), *w*Dbo= *Drosophila borealis* (FJ415472),

The alignment and distance matrix of the *hcp*A gene is shown in appendix XI and IX, respectively. The NJ tree based on the *hcp*A gene confirmed that *w*Cin1 and *w*Cin2 is ident with *w*Cer1 and *w*Cer2. The *w*Cin1 strain differs by 0,17% compared to the *Wolbachia* detected in the ant *Camponontus leonardi* (EU127639). The *w*Cin2 strain is genetically ident to *Wolbachia* in *Drosophila melanogaster* (AE017196) and *D. borealis* (FJ415472).

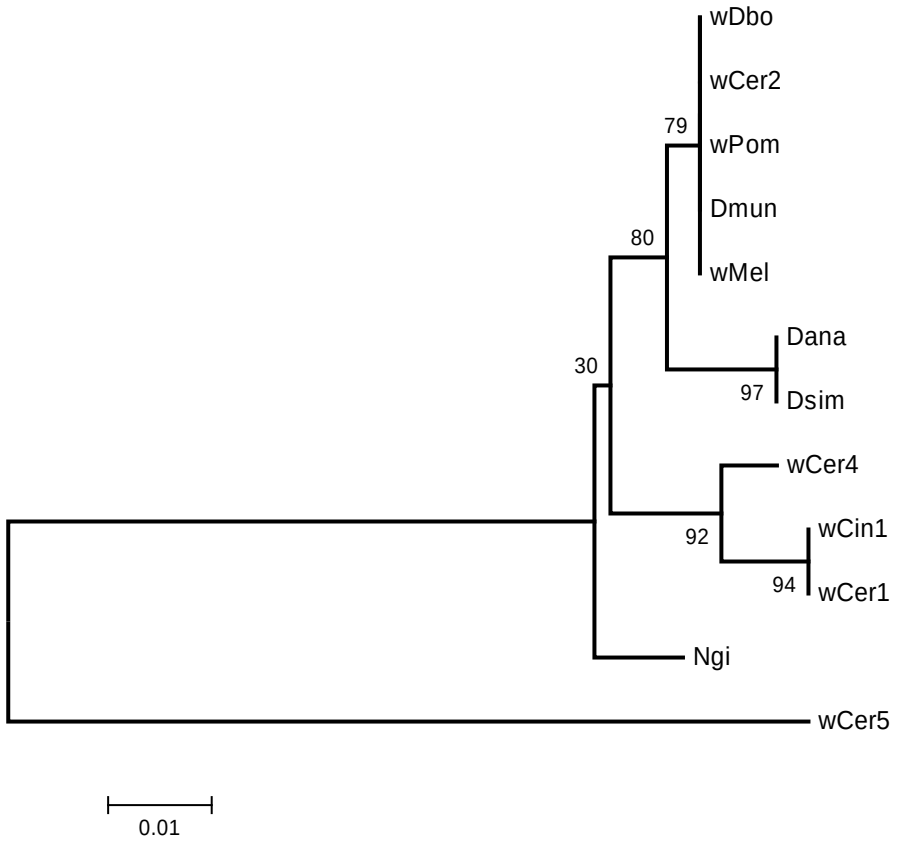

Figure 4.7 Phylogenetic analysis of *w*Cin1 and *w*Cin2 of *R. cingulata*, *w*Pom of *R. pomonella* and other *Wolbachia* strains. Analysis was done with Neighbour Joining method using the *gat*B sequences. Bootstrap analyses were done with 100 replicates and are the numbers above the node. *w*Cer= *R. cerasi*, *w*Mel= *Drosophila melanogaster* (DQ842452), *w*Dmun= *D. munda* (EU126167), *w*Dbo= *D. borealis* (FJ415471), *w*Dana= *D. anassae* (EF611906), *w*Dsim= *D. simulans* (DQ842433), *w*Ngi= *Nasonia giraulti* (DQ842442)

The alignment and distance matrix of the *gat*B gene is shown in appendix VII and IX, respectively. The sequences analysis revealed only *w*Cin1. The phylogenetic analysis by NJ showed *w*Cin1 ident *w*Cer1 of *R. cerasi*. With a genetic difference of 0,14% *w*Cin1 is related to *w*Cer4 of *R. cerasi* on that locus.

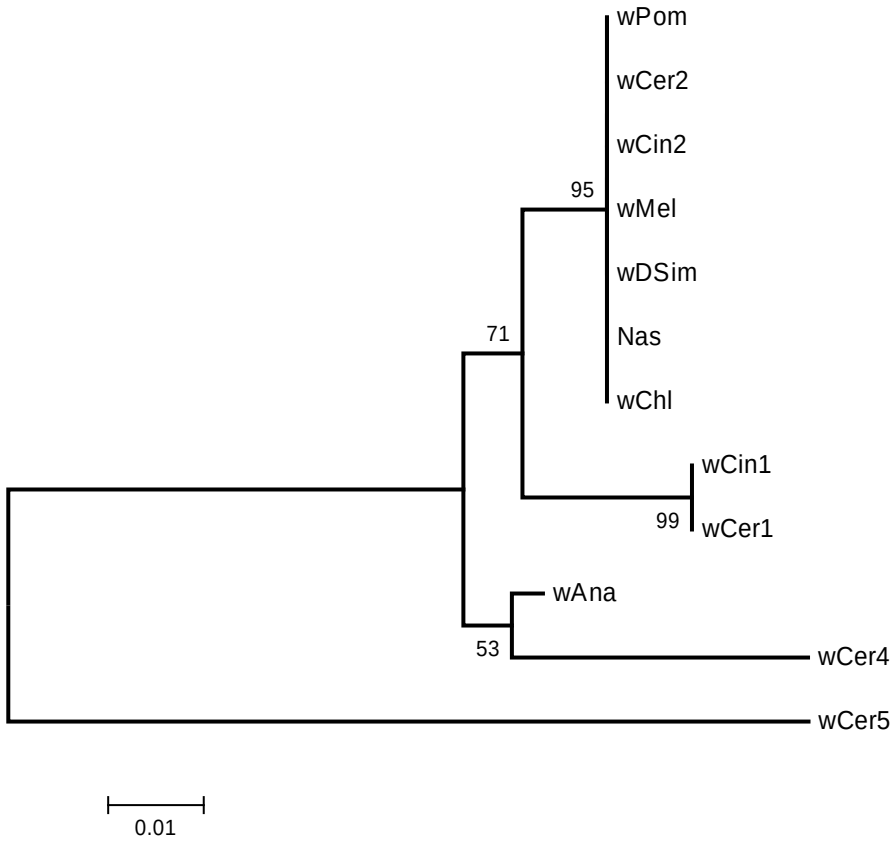

Figure 4.8 Phylogenetic analysis of *w*Cin1 and *w*Cin2 of *R. cingulata*, *w*Pom of *R. pomonella* and other *Wolbachia* strains. Analysis was done with Neighbour Joining method using the *fbp*A sequences. Bootstrap analyses were done with 100 replicates and are the numbers above the node. *w*Cer= *R. cerasi*, *w*Mel= *Drosophila melanogaster* (AE017196), *w*DSim= *D. simulans* (AE017196) *w*Nas= *Nasonia vitripennis* (DQ842370), *w*Chl= *Chloropidae* sp. (EU126395), *w*Ana= *Drosophila anassae* (EF611894)

The alignment and distance matrix of the *fbp*A gene is shown in appendix VIII and IX, respectively. The NJ tree based on the *fbp*A gene revealed *w*Cin1 and *w*Cin2 ident to *w*Cer1 and *w*Cer2 of *R. cerasi*, respectively. As shown before *w*Cin2 is closely related to the *Wolbachia* detected in *D. melanogaster*.

4.2.3 *Wolbachia* in *Rhagoletis cingulata* and consequences

R. cingulata harbours at least two *Wolbachia* strains named *w*Cin1 and *w*Cin2. They seem to be ident to the *w*Cer1 and *w*Cer2 detected in the European Cherry Fruit Fly, *R. cerasi* (RIEGLER & STAUFFER 2002). In latter species ARTHOFER ET AL. (2009B) detected five strains, recently. *w*Cer3 and *w*Cer4 could be only detected with blotting methods, a method which is more sensitive compared to conventional PCR - detection limit for single copy *wsp* genes of 10^{-2} ng plasmid DNA could be lowered to 10^{-7} ng. However, in this thesis only conventional PCR was applied and thus it cannot be excluded that other *Wolbachia* strains are in *R. cingulata*. Low titre strains are frequently detected in insects (HILGERBOECKER ET AL. 2008, UNCKLESS ET AL. 2009).

In this thesis two individuals were analysed and one of those was infected only with *w*Cin2, the other one with both strains. More individuals and more geographic different populations have to be analyzed to detect the distribution pattern of infection in Europe. The *R. cingulata* individuals analyzed were collected in Germany. We did not have access to an American population. The question arises if also the American species contain *w*Cin1 or *w*Cin2. The infection would be originating from Europe if the American populations do not harbour these two strains and the infection would be quite young as *R. cingulata* was introduced to Europe quite recently. The possibility of a horizontal transfer was shown by ZABALOU ET AL. (2004) by infecting artificially uninfected med flies with *w*Cer2 and *w*Cer4 and by RIEGLER ET AL. (2004) by transferring *w*Cer2 into *D. simulans* and establishing 5 *Drosophila* lines expressing different CI. As RAYCHOUDHURY ET AL. (2009) OR RIEGLER (2002) proposed the most likely transfer in field might be done by parasitism. Another possibility might be that during competition in the cherry *R. cingulata* larvae feed and kill the competing *R. cerasi* larvae. Migration of *Wolbachia* from sexual to autosomal cells has been reported by FRYDMAN ET AL. (2006).

If American populations of *R. cingulata* are infected with *w*Cin1 and *w*Cin2 this would support the hypotheses that *w*Cin1 and *w*Cin2 are common strains being quite omnipresent in dipteran species. *w*Cin2 belongs to the *w*Mel group, who is present in different Diptera, Homoptera and Lepidoptera species (JEYAPRAKASH & HOY 2000).

4.3. *Wolbachia* in *Rhagoletis pomonella*

4.3.1 Phylogenetic characterization of the *w*Pom strain by *wsp*

The amplicons of *R. pomonella* were sequenced after purification of the PCR products. The sequences were edited and aligned with Codon Code Aligner. For that purpose also the most similar sequences obtained after a BLAST search in the Genbank were compared.

R. pomonella was infected with one *Wolbachia* strain in the following named *w*Pom. The *wsp* sequence was 100% ident to *w*Cer2 of *R. cerasi*. The *w*Pom strain belongs to the supergroup A and with a genetic divergence of 0,08% (see Appendix IX) is closely related to the *w*Mel-group (Fig. 4.2).

The following trees were constructed using the Neighbour Joining (NJ) algorithm applying Kimura-2-parameter distances and bootstrapping as implemented in MEGA4 (TAMURA ET AL. 2007) Further the *wsp* PCR products were cloned with a TA vector and after transformation into bacterial cells 20 plasmids were picked and sent for sequencing. These sequences revealed 100% homologous sequences as shown in the alignment (Appendix IX).

Two individuals from *R. pomonella* were sequenced plus 20 plasmids from one individual. Alignment of the sequences revealed one *Wolbachia* strain ident to the *w*Cer2 (AF418557) strain of *R. cerasi.* (Appendix III).

4.3.2 Characterization of the *w*Pom strains by MLST

*w*Pom was also characterized by the five MLST loci (BALDO ET AL. 2006). The amplicons of the five gene products from two individuals of *R. pomonella* were directly sequenced after purification. Further, they were cloned with a TA vector and after transformation into bacterial cells about eight positive plasmids from each of the loci were taken and sequenced.

The analyses of *R. pomonella* with the five MLST loci confirmed the results on the *wsp* gene. Each locus detected the *w*Pom strain, ident to the *w*Cer2 of *R. cerasi* (Fig 4.4, 4.5, 4.7, 4.8). Only the *hcp*A locus revealed one single mutation on the codon position 2 indicating that the amino acid is changed. All eight sequences of two different individuals had the same mutation compared to *w*Cer2 (Fig. 4.6 and Fig 4.9).

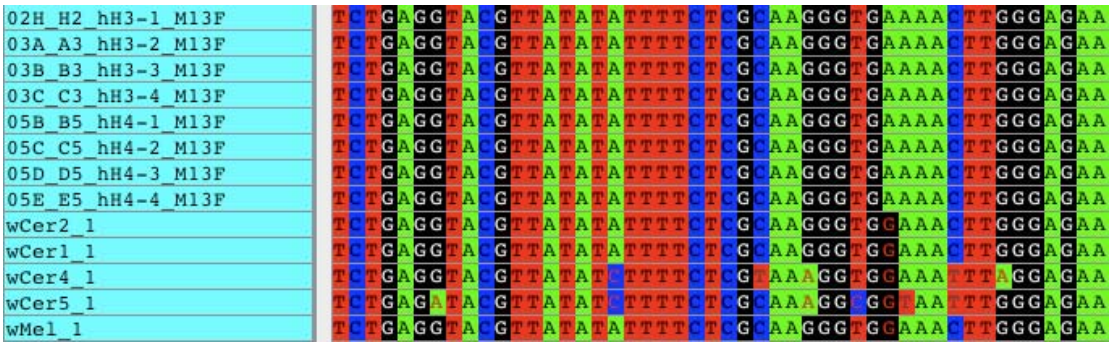

Figure 4.9 Alignment of the *hcp*A sequences from two individuals of *R. pomonella* (02H_H2 to 05E_E5) with four *w*Cer strains of *R. cerasi* and the *Wolbachia* strain of *D. melanogaster*.

4.3.3 *Wolbachia* in *R. pomonella* and consequences

Here I analyzed only one single population collected from apple trees. *R. pomonella* harbours at least one *Wolbachia* strains named *w*Pom which seem to be closely related to the *w*Cer2 detected in the European Cherry Fruit Fly, *R. cerasi* (RIEGLER & STAUFFER 2002). However, in this thesis only conventional PCR was applied and thus it cannot be excluded that other *Wolbachia* strains might be harboured by *R. pomonella*. Low titre strains are frequently detected in insects. ARTHOFER ET AL (2009B) reported a detection limit for single copy *wsp* genes of 10^{-2} ng plasmid DNA and the same author reported in ARTHOFER ET AL (2009A) that by nested PCR the detection can be lowered from 10^{-4} to 10^{-5} ng. So it might be that *R. pomonella* is infected with other *Wolbachia* strains.

The individuals of *R. pomonella* analyzed were collected in apple trees, only. A population from hawthorn should be analyzed in a follow up work in order to detect if also in that population *w*Pom can be detected. Unless, *w*Pom will add a new aspect on the process involved in sympatric speciation. *Wolbachia* can be involved in processes of speciation (LAVEN 1959, 1967). BORDENSTEIN ET AL. (2001) described that microbes act in producing reproductive isolation between *Nasonia* wasps. In that case, different *Wolbachia* strains in a defined population or species can cause CI. As a consequence the gene flow of the two species was reduced. Sympatric speciation is described as splitting one evolutionary lineage into two without the occurrence of geographic isolation (BERLOCHER & FEDER 2002). *Wolbachia* is described to be restrictive for sympatric speciation (WERREN 2002).

5. References:

ALINIAZEE MT (1974) The Western cherry fruit fly, *Rhagoletis indifferens*. 2. Aggressive behaviour. *Canadian Entomology* **106**, 1021-1024.

ALLDRED DB, JORGENSEN CD (1993) Hosts, adult emergence, and distribution of the apple maggot (Diptera: Tephritidae) in Utah. *Pan-Pacific-Entomologist* **69**, 236-246.

ARTHOFER W, RIEGLER M, AVTZIS DN, STAUFFER C (2009A) Evidence for low titre infections in insect symbiosis: *Wolbachia* in the bark beetle *Pityogenes chalcographus* (Coleoptera, Scolytinae). *Environmental Microbiology*, Published Online: Apr 6 2009 2:32AM, DOI: 10.1111/j.1462-2920.2009.01914.x

ARTHOFER W, SCHNEIDER D, KRAMMER M, RIEGLER M, MILLER WJ, STAUFFER C (2009B) Hidden *Wolbachia* diversity in field populations of the European cherry fruit fly, *Rhagoletis cerasi* (Diptera, Tephritidae). *Molecular Ecology*, in submission.

BALDO L, LO N, WERREN JH (2005) Mosaic nature of the *Wolbachia* surface protein. *Journal of Bacteriology* **187**, 5406-5418.

BALDO L, DUNNING HOTOPP JC, JOLLEY KA, BORDENSTEIN SA, BIBER SA, CHOUDHURY RR, HAYASHI C, MAIDEN MCJ, TETTELIN H, WERREN JH (2006) Multilocus Sequence Typing System for the endosymbiont *Wolbachia pipientis*. *Applied and Environmental Microbiology* **72**, 7098-7110.

BALDUF WV (1959) Obligatory and facultative insects in rose-hips, their recognition and bionomics. *3. Biological Monographs* **26,** 194.

BANDI C, ANDERSON TJ, GENCHI C, BLAXTER ML (1998) Phylogeny of *Wolbachia* in filarial nematodes. *Proceedings of Biological Sciences* **265**, 2407-13.

BEARD CB, O'NEILL SL, TESH RB, RICHARDS FF, AKSOY S (1993) Modification of Arthropod vector competence via symbiotic bacteria. *Parasitology Today* **9**, 179-183.

BERLOCHER SH, FEDER JL (2002) Sympatric speciation in phytophagous insects: moving beyond controversy. *Annual Review of Entomology* **47**, 773-815.

BERLOCHER SH, SMITH DC (1983) Segregation and mapping of allozymes of the apple maggot fly *Journal of Heredity* **74**, 337-340.

BLÜMEL S, RUSS K (1989) Manipulation of races. In: ROBINSON AS, HOOPER G (*eds*.) Fruit Flies: Their Biology, Natural Enemies and Control, Vol. 3B. 387-389 *Elsevier, Amsterdam.*

BOLLER EF (1974) Progress Report of the IOBC Working Group on Genetic conrtrol of *Rhagoletis cerasi*. presented at General Assembly of IOBC/WPRS Madrid.

BOLLER EF (1966) Beitrag zur Kenntnis der Eiablage und Fertilität der Kirschenfliege *Rhagoletis cerasi* L. Vorläufige Mitteilung. *Bulletin de la Societe Entomologique Suisse* **38**, 193-202.

BOLLER E, MANI E, (1994) Two North American *Rhagoletis* spp. in Europe. *IOBC/WPRS Bulletin* **17**, 83-83.

BOLLER EF, PROKOPY RJ (1976) Bionomics and management of *Rhagoletis*. *Annual Review of Entomology* **21**, 223-246.

BOLLER EF, RUSS K, VALLO V, BUSH GL (1976) Incompatible races of European cherry fruit fly, *Rhagoletis cerasi* (Diptera: Tephritidae), their origin and potential use in biological control. *Entomologia Experimetalis & Applicata* **20**, 237-247.

BORDENSTEIN R, O'HARA FP, WERREN JH (2001) *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. *Nature* **409**, 707-710.

BOURTZIS K, O´NEILL SL (1998) *Wolbachia* infections and arthropod reproduction. *Bioscience* **48**, 287-293.

BOURTZIS K**,** BRAIG HR (1999) The many faces of *Wolbachi*a. In: RAOULT D, BROUQUI P (*eds*.) Rickettsiae and Rickettsial Diseases at the Turn of the Third Millelium. *Elsevier, Amsterdam*, 199-219.

BOURTZIS K (2008) *Wolbachia*-Induced Cytoplasmic Incompatibility to Control Insect Pests. In: VREYSEN MJB, ROBINSOPN AS, HENDRICHS J (*eds.*), Area Wide Control of Insect Pests. *Springer Netherland*, 125-135

BOYCE AM (1934) Bionomics of the walnut husk fly, *Rhagoletis completa. Hilgardia* **8**, 363-579.

BRAIG HR, GUZMAN H, TESH RB, O'NEILL SL (1994) Replacement of the natural *Wolbachia* symbiont of *Drosophila simulans* with a mosquito counterpart. *Nature* **367**, 354-355.

BRAIG HR, ZHOU W, DOBSON SL, O'NEILL SL (1998) Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. *The Journal of Bacteriology* **180**, 2373-2378.

BROOKS FE (1921) Walnut husk maggot. *US Departement of Agricultural Bulletin* **992**, 1-8.

BUSH GL (1969) Mating behavior, host specificity, and the ecological significance of sibling species in frugivorous flies of the genus *Rhagoletis* (Diptera, Tephritidae). *American Naturalist* **103**, 669-672.

CHAPMAN PJ, HESS AD (1941) Mortality of the apple maggot in fruit held in cold storage. *Circular US Departement of Agriculture* **600**, 1-9.

DEAN RW (1969) Infestation of peaches by *Rhagoletis suavis*. *Journal of Economical Entomology* **31**, 940-941.

DEAN RW, CHAPMAN PJ (1973) Bionomics of the Apple Maggot in Eastern New York. *Search Agricultural Entomology, Geneva* **3**, 62.

DEDEINE F, VAVRE F, FLEURY F, LOPPIN B, HOCHBERG ME, BOULETREAU M (2001) Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proceedings of the Natural Academy of Sciences* **98**, 6247-6252.

EGARTNER A, ZEISNER N, HAUSDORF H, LETHMAYER C & BLÜMEL S (2008) Investigations on the occurrence of the quarantine fruit fly species *Rhagoletis cingulata* and *Rhagoletis indifferens* on *Prunus avium* and *Prunus cerasus* in Austria. In: International Conference on Integrated Fruit Production, *IOBC/WPRS* Avignon, France, Abstracts, 82.

EPPO (2004) *Rhagoletis cingulata* occurs in the Netherlands, but not *R. indifferens EPPO Reporting Service* **06**, 87.

EPPO (2007) First outbreak of *Rhagoletis cingulata* in Slovenia EPPO Reporting Service **08**, 148.

EPPO/CABI (1996): *Rhagoletis cingulata* and *Rhagoletis indifferens* In: SMITH IM, MCNAMARA DG, SCOTT PR, HOLDERNESS M: Quarantine Pests for Europe. Second Edition. Data sheets on quarantine pests for the European Union and for the European and Mediterranean Plant Protection Organization. *CAB International*, Wallingford, 470-474.

FEDER JL (1998) The apple maggot fly, *Rhagoletis pomonella*: flies in the face of conventional wisdom about speciation. in: HOWARD D, BERLOCHER SH (*eds*) Endless Forms: Species and Speciation, 130-144 *Oxford University Press.*

FEDER JL, BUSH GL (1989) Gene frequency clines in host races of *Rhagoletis pomonella* (Diptera: Tephritidae) in the midwestern United States. *Heredity*, **63**, 245-266.

FRICK KE, SIMKOVER HG UND TELFORD HS (1954) Bionomics of the Cherry Fruit Flies in Eastern Washington. *Washington Agricultural Experiment Station Bulletin* **13,** 1-66.

FRYDMAN HM, LI JM, ROBSON DN, WIESCHAUS E (2006) Somatic stem cell niche tropism in *Wolbachia*. *Nature* **441**, 509-12.

HERTIG M (1936) The Rickettsia, *Wolbachia pipientis* and associated inclusions of the mosquito, *Culex pipiens*. *Parasitology* **28**, 453-490.

HERTIG M, WOLBACH SB (1924) Studies on *Rickettsia*-like microorganisms in insects. *Journal of Medical Research* **44**, 329-74.

HILGENBOECKER K, HAMMERSTEIN P, SCHLATTMANN P, TELSCHOW A, WERREN JH (2008) How many species are infected with *Wolbachia*? – a statistical analysis of current data. *FEMS Microbiology Letters* **281**, 215-220.

HOFFMANN AA, CLANCY D, DUNCAN J (1996) Naturally occurring *Wolbachia* infection causing cytoplasmic incompatibility in *Drosophila melanogaster*. *Genetics* **148**, 221-231.

JAMNONGLUK W, KITTAYAPONG P, VISUT B, O'NEILL SL (2002) *Wolbachia* infections of Tephritid Fruit Flies: molecular evidence for five distinct strains in a single host species. *Current Microbiology* **45**, 255-260.

JEYAPRAKASH A, HOY MA (2000) Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. *Insect Molecular Biology* **9**, 393-405.

KIMURA M (1980) A simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111-116.

LAMPE I, BURGHAUSE F, KRAUTHAUSEN HJ (2005) Introduction and Distribution of the American Eastern Cherry Fruit Fly, *Rhagoletis cingulata*, in the Rhine Valley, Germany. In: Proceedings of Plant Protection and Plant Health in Europe: 1st Symposium 2005: Introduction and Spread of Invasive Species, 09.06.-11.06.2005, Berlin.

LAMPE I, DAHLBENDER W, HARZER U, HENSEL G, KRAUTHAUSEN HJ (2006) Die Amerikanische Kirschfruchtfliege (*Rhagoletis cingulata*) - Untersuchungen zum Auftreten in Rheinland-Pfalz. *Obstbau* 414-416.

LATHROP FH, NICKELS CB (1932) The biology and control of the blueberry maggot in Washington County Maine. *Technical Bulletin 275 United States Dept. of Agriculture* 77.

LAVEN H (1959) Speciation by cytoplasmic isolation in the *Culex pipiens* complex. *Cold Spring Harbor Symposia on Quantitative Biology* **24**, 166-173.

LAVEN H (1967) Speciation and evolution in Culex pipiens. In WRIGHT JW, PAL R (*eds.*) Gentics of insect vectors of disease. *Amsterdam: Elsevier.*

LINCOLN SR, RODRIGO OM, ANDRÈ LP, PERONDINI I, DENISE S (2005) Occurrence of *Wolbachia* in Brazilian samples of Ceratitis capitata (Wiedemann) (Diptera: Tephritidae). *Neotropical Entomology* **34**, 1013-1015.

JUBB GL, COX JA (1974) Seasonal emergence of two cherry fruit fly species in Erie County, Pennsylvania: 25-year summary. *Journal of Economic Entomology* **67**, 613-615.

LO N, CASIRAGHI M, SALATI E, BAZZOCCHI C, BANDI C (2002) How many *Wolbachia* supergroups exist. Molecular Biology and Evolution **19**, 341-346.

MATOLIN S (1976) Mechansim causing incompatibility between different strains of *Rhagoletis cerasi* (Diptera, Tephritidae). *Acta Entomologia Bohemoslovakia* **73**, 289-292.

MAYR E (1963) Animal Species and Evolution. *Harvard University Press.*

MCPHERON BA, HAN HY (1997) Phylogenetic analysis of North American *Rhagoletis* (Diptera: Tephritidae) and related genera using mitochondrial DNA sequence data. *Molecular Phylogenetics and Evolution* **7**, 1-16.

MERZ B (1994) Diptera, Tephritidae. Insecta Helvetica, Fauna. *10. Schweizerische Entomologische Gesellschaft, Genève*.

MOERICKE V, PROKOPY RJ, BERLOCHER S, BUSH GL (1975) Visual stimuli eliciting attraction of *Rhagoletis pomonella* flies to trees. *Entomologia Experimentalis et Applicata* **18**, 497-507.

MCGRAW, O'NEILL (2004) *Wolbachia* pipientis: intracellular infection and pathogenesis in Drosophila. *Current opinion in Microbiology* **7**, 67-70.

MCPHERON BA, HAN HY (1997) Phylogenetic analysis of North American *Rhagoletis* and related genera using mitochondrial DNA sequence data. *Molecular Phylogenetics and Evolution* **7**, 1-16.

NEILSON TWA (1962) Effects of temperature on development of overwintering pupae of the apple maggot, *Rhagoletis pomonella* (Walsh). *Canadian Entomology* **94**, 924-928.

O'NEILL SL, KARR TL (1990) Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. Nature **348**, 178-180.

PROKOPY RJ (1968) Influence of photoperiod, temperature, and foot on initiation of diapause in the apple maggot. *Canadian Entomology* **100**, 403-422.

PROKOPY RJ**,** BUSH GL (1972) Mating behavior in *Rhagoletis pomonella* (Diptera: Tephritidae). 3. Male aggregation in response to an arrestant. *Canadian Entomology* **104**, 275-283.

PROKOPY RJ & BUSH GL (1973) Ovipositional responses to different sizes of artificial fruit by flies of *Rhagoletis pomonella* species group. *Annals of the Entomological Society of America* **66**, 927-929.

PROKOPY RJ, DIEHL SR, COOLEY SS (1988) Behavioral evidence for host races in *Rhagoletis pomonella* flies. *Oecologia* **76**, 138-147.

RAYCHOUDHURY R, BALDO L, OLIVEIRA DCSG, WERREN JH (2009) Modes of acquisition of *Wolbachia*: horizontal transfer, hybrid introgression, and codivergence in the *Nasonia* species complex. *Evolution* **63**, 165-183.

RIEGLER M, STAUFFER C (2002) *Wolbachia* infections and superinfections in cytoplasmically incompatible populations of the European cherry fruit fly *Rhagoletis cerasi* (Diptera, Tephritidae). *Molecular Ecology* **11**, 2425-2434.

RIEGLER M, CHARLAT S, STAUFFER C, MERÇOT H (2004) *Wolbachia* transfer from a true fly into the real fruit fly: Investigating the outcomes of host/symbiont co-evolution *Applied and Environmental Microbiology* **70**, 273-279.

SAMBROOK J, FRITSCH EF, MANIATIS T (1989) Molecular Cloning a Laboratory Manual *2nd edn. Cold Spring Harbour Laboratory Press*,New York.

SERBUS LR, SULLIVAN W (2007) A cellular basis for *Wolbachia* recruitment to the host germline. *Plos Pathogens* **3**, 1930-1937.

SELIVON D, PERONDINI ALP, RIBEIRO AF, MARINO CL, LIMA MMA, COSCRATO VE (2002) *Wolbachia* endosymbiont in a species of the *Anastrepha fraterculus* complex (Diptera, Tephritidae). *Invertebrate Reproduction and Development* **42**, 121-127.

SIRONI M, BANDI C, SACCHI L, DI SACCO B, DAMIANI B, GENCHI C (1995) Molecular evidence for a close relative of the arthropod endosymbiont *Wolbachia* in a filarial worm. *Molecular and Biochemical Parasitology* **74**, 223-227.

SMITH JJ, BUSH GL (1997) Phylogeny of the genus *Rhagoletis* (Diptera: Tephritidae) inferred from DNA sequences of mitochondrial cytochrome oxidase II. *Molecular Phylogenetic Evolution* **7**, 33-43.

SUN X, CUI L, LI Z (2007) Diversity and Phylogeny of *Wolbachia* Infecting *Bactrocera dorsalis* (Diptera: Tephritidae) Populations from China. *Environmental Entomology* **36**, 1283-1289.

TAMURA K, DUDLEY J, NEI M, KUMAR S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596-1599.

THOMPSON JD, GIBSONTJ, PLEWNIAK F, JEANMOUGIN F, HIGGINS DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**, 4876-4882.

UNCKLESS RL, BOELIO LM, HERREN JK, JAENIKE J (2009) *Wolbachia* as populations within individual insects: causes and consequences of density variation in natural populations. *Proceedings of the royal Society B Series* (online publication).

WADE MJ (2001) Infectious Speciation. *Nature* **409**, 675-677.

WALSH BD (1867) The apple-worm and the apple maggot. *Journal of Horticulture* **2**, 338-343.

WEEMS JR HV (2001) Eastern Cherry Fruit Fly, *Rhagoletis cingulata* (Loew) (Insecta: Diptera: Tephritidae). *DPI Entomology Circular* **116** in Featured Creatures from the Entomology and Nematology Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida.

WERREN JH, ZHANG W, GUO LR (1995) Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. *Proceedings of the Royal Society of London B Series* **261**, 55-63.

WERREN JH (1997) Biology of *Wolbachia*. *Annual Review of Entomology* **42**, 587-609.

WERREN JH (2002) *Wolbachia* and Speciation. In: HOWARD D, BERLOCHER SH (*eds*) Endless Forms: Species and Speciation, 130-144. *Oxford University Press*, Oxford.

WERREN JH, WINDSOR DM (2000) *Wolbachia* infection frequencies in insects: evidence of a global equilibrium. *Proceedings of the Royal Society of London B Series* **267**, 1277-1285.

WERREN JH, BARTOS JD (2001) Recombination in *Wolbachia*. *Current Biology* **11**, 431-435.

WERREN JH, BALDO L, CLARK ME (2008) *Wolbachia*: master manipulators of invertrebrate biology. *Nature Reviews Microbiology* **6**, 741-751.

WEST SA, COOK JM, WERREN JH, GODFRAY HCJ (1998) *Wolbachia* in two insect host-parasitoid communities. *Molecular Ecology* **11**, 1457-1465.

WIKIPEDIA.ORG (2009) retrieved on 15.05.2009.

ZABALOU S, RIEGLER M, THEDORAKOPOULOU M, STAUFFER C, SAVAKIS C, BOURTZIS K (2004) *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. *Proceedings of the Natural Academy of Sciences USA* **101**, 15042-15045.

ZABALOU S, LIVADARAS I, FRANZ G, ROBINSON A, SAVAKIS C, BOURTZIS K (2009) Incompatible Insect Technique: Incompatible Males from a Ceratitis capitata (Order: Tephritidae) Genetic Sexing Strain. *Annual Review of Entomology* (in submission).

ZHOU W, ROUSSET F, O´NEILL SL (1998) Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. *Proceedings of the Royal Society of London B Series* **265**, 509-515.

6. Appendix

Appendix I Working Protocols

- Extraction after SIGMA kit

- pipette 180 µl of lysis solution T (B-6678) in a 1,5 ml Eppendorf tube
- add the insect specimen and mince with the drill – put the samples on ice
- add 20 µl of proteinase K
- vortex (ca. 15 sec.) and put on the heating block at 55°C/450 rpm for 2 hrs
- add 20 µl RNAse and let tubes stand for 2 min at room temperature
- add 200 µl of lysis solution C (B-8803)
- vortex carefully and incubate at 70° for 10 min
- during incubation prepare the tubes and columns - add 500 µl column preperation Solution to the column and spin at 13.000 rpm for 1 minute
- discard flow- through and put column back into the same tube
- add 200 µl absolute ethanol to the sample
- vortex for 15 sec
- transfer the samples to the binding columns (approx. 650 µl)
- spin at 8.000 rpm for 1 min
- discard tube with flow-trough and put column in a fresh tube
- add 500 µl of wash solution
- spin at 8.000 rpm for one minute
- discard flow-trough and put column back into the same tube

- add 500 µl wash solution
- spin at 13.000 rpm for 3 minutes
- discard flow-through and put column back into the same tube
- spin again for 1 min at 13.000 rpm to get rid of any remaining alcohol
- put column in a fresh tube
- add 50 µl of elution solution and let column stand for 5 minutes
- spin at 8.000 rpm for 1 min
- store DNA in the fridge

- DNA Purification

- mix DNA product with the same volume XP1 buffer
- vortex carefully
- pipette the mixture in the columns
- centrifuge for 1 min at 10.000 rpm
- wash columns with 650 µl SPW-Wash buffer
- centrifuge for 1 min 10.000 rpm
- wash columns with 650 µl SPW-Wash buffer
- centrifuge for 1 min 10.000 rpm
- centrifuge the empty column for 1 min at 10.000 rpm
- add 20 µl elution buffer
- incubate for 2 min
- centrifuge 1 min at 10.000 rpm

Appendix II Cloning

Day 1

- mix PCR product with 1,4 µl H_2O, 0,1 µl ptZ57R, 0,3 µl PEG3350, 0,3µl T4 Buffer, 0,1 µl T4 ligase
- add 0,8 µl DNA
- incubate over night

Day 2

- thaw 35 µl competent cells per reaction on ice
- pre-cool ligation reaction mixtures on ice in 0.5 ml reaction tubes
- add competent cells to the ligations
- incubate on ice for 20 min
- heat shock bacterial suspensions in a 42 °C hot water bath for 50 sec
- put reactions back on ice for 1-2 min immediately
- add 300 µl of SOC medium were added to each tube
- incubate at 37 °C for 1-2 hrs
- prepare LB-Amp plates in the meantime: plate 40 µl X-Gal (20 mg/ml) and 40 µl IPTG (24 mg/ml) on each plate with a a Drigalski spatula
- plate transformation reactions on the plates
- incubate upside down over night at 37 °C

Day 3

- transfer 0.5 ml up to 2 ml of overnight *E. coli* cultures into 1.5 ml reaction tubes.
- tip with a sterile toothpick

- transfer into Eppendorf tubes (containing Mastermix for PCR) and a cap-o-test vial containing 3 ml LB broth containing 50 µg/ml ampicillin.
- vials were incubated at 37 °C overnight under vigorous shaking (180 – 200 rpm).

Day 4

- transfer 0.5 ml up to 2 ml of overnight *E. coli* cultures into 1.5 ml reaction tubes.
- pellet cells by centrifugation: 10.000 rpm, 4 min
- discard supernatant and re-suspend pellets in 100 µl resuspension solution
- add 1µl RNAse
- incubate for 2-5 min
- add 200 µl NaOH-SDS
- vortex at 1.400 rpm
- add 150 µl ice cold Kac-solution, vortex 10 sec
- put samples 5 min on ice
- centrifuge 5 min on 4°C at 15.000 rpm
- pipette supernatant in a new tube
- add 900 µl EtOH and vortex carefully
- incubate for 2 min and centrifuge for 5 min at 4°C on 15.000 rpm
- discard flow-through and dry the tube
- add cold 70% EtOH vortex carefully and centrifuge for 5 min at 4°C on 15.000 rpm
- discard ethanol and air-dry pellets for approximately 2 hours
- re-suspend pellet in 10 mM Tris

LB broth (SAMBROOK ET AL. 1989)

Per Liter

Bacto-tryptone	10.0 g
Bacto-yeast Extract	5.0 g
NaCl	10.0 g
Agar	15.0 g

Adjust the pH to 7,0 with 5N NaOH, adjust the volume of the solution to 1 liter with deionized H2O and sterile by autoclaving

Appendix III Alignment of the *wsp* sequences of *R. cingulata* (H5, H6) and *R. pomonella* (H3, H4)

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H3-1
ACAAAAGTTGATGGTATTACCTATAAGAAAGACAAGAGTGATTACAGTCCATTAAAACCA
H4-1
ACAAAAGTTGATGGTATTACCTATAAGAAAGACAAGAGTGATTACAGTCCATTAAAACCA
H5-1
ACAAAAGTTGATGGTATTACCTATAAGAAAGACAAGAGTGATTACAGTCCATTAAAACCA
H6-1
ACAAAAGTTGATGGTATTACCTATAAGAAAGACAAGAGTGATTACAGTCCATTAAAACCA

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H3-1
TCTTTTATAGCTGGTGGTGGTGCATTTGGTTACAAAATGGACGACATCAGGGTTGATGTT
H4-1
TCTTTTATAGCTGGTGGTGGTGCATTTGGTTACAAAATGGACGACATCAGGGTTGATGTT
H5-1
TCTTTTATAGCTGGTGGTGGTGCATTTGGTTACAAAATGGACGACATCAGGGTTGATGTT
H6-1
TCTTTTATAGCTGGTGGTGGTGCATTTGGTTACAAAATGGACGACATCAGGGTTGATGTT

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H3-1
GAAGGAGTTTATTCATACCTAAACAAAAATGATGTTAAAGATGTAACATTTGACCCAGCA
H4-1
GAAGGAGTTTATTCATACCTAAACAAAAATGATGTTAAAGATGTAACATTTGACCCAGCA
H5-1
GAAGGAGTTTATTCATACCTAAACAAAAATGATGTTAAAGATGTAACATTTGACCCAGCA
H6-1
GAAGGAGTTTATTCATACCTAAACAAAAATGATGTTAAAGATGTAACATTTGACCCAGCA

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H3-1
AATACTATTGCAGACAGTGTAACAGCAATTTCAGGATTAGTGAACGTGTATTACGATATA
H4-1
AATACTATTGCAGACAGTGTAACAGCAATTTCAGGATTAGTGAACGTGTATTACGATATA
H5-1
AATACTATTGCAGACAGTGTAACAGCAATTTCAGGATTAGTGAACGTGTATTACGATATA
H6-1
AATACTATTGCAGACAGTGTAACAGCAATTTCAGGATTAGTGAACGTGTATTACGATATA

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H3-1
GCAATTGAAGATATGCCTATCACTCCATACATTGGTGTTGGTGTTGGTGCAGCGTATATT
H4-1
GCAATTGAAGATATGCCTATCACTCCATACATTGGTGTTGGTGTTGGTGCAGCGTATATT
H5-1
GCAATTGAAGATATGCCTATCACTCCATACATTGGTGTTGGTGTTGGTGCAGCGTATATT
H6-1
GCAATTGAAGATATGCCTATCACTCCATACATTGGTGTTGGTGTTGGTGCAGCGTATATT

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H3-1
AGCACTCCTTTGGAACCCGCTGTGAATGATCAAAAAAGTAAATTTGGTTTTGCTGGTCAA
H4-1
AGCACTCCTTTGGAACCCGCTGTGAATGATCAAAAAAGTAAATTTGGTTTTGCTGGTCAA
H5-1
AGCACTCCTTTGGAACCCGCTGTGAATGATCAAAAAAGTAAATTTGGTTTTGCTGGTCAA
H6-1
AGCACTCCTTTGGAACCCGCTGTGAATGATCAAAAAAGTAAATTTGGTTTTGCTGGTCAA

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H3-1
GTAAAAGCTGGTGTTAGTTATGATGTAACTCCAGAAGTCAAACTTTATGCTGGAGCTCGT
H4-1
GTAAAAGCTGGTGTTAGTTATGATGTAACTCCAGAAGTCAAACTTTATGCTGGAGCTCGT
H5-1
GTAAAAGCTGGTGTTAGTTATGATGTAACTCCAGAAGTCAAACTTTATGCTGGAGCTCGT
H6-1
GTAAAAGCTGGTGTTAGTTATGATGTAACTCCAGAAGTCAAACTTTATGCTGGAGCTCGT

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H3-1
TATTTCGGTTCTTATGGTGCTAATTTTGATGGAAAAAAAACAGATCCTAAAGATTCAACC
H4-1
TATTTCGGTTCTTATGGTGCTAATTTTGATGGAAAAAAAACAGATCCTAAAGATTCAACC
H5-1
TATTTCGGTTCTTATGGTGCTAATTTTGATGGAAAAAAAACAGATCCTAAAGATTCAACC
H6-1
TATTTCGGTTCTTATGGTGCTCATTTTGATGGAGAAAAAGTAGATCCTAGAGATGCAAAC
                 ********************** ************ *****  ******** ****
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H3-1             AGACAGGTTACTGATGCAGGCGCATACAAAGTT
H4-1             AGACAGGTTACTGATGCAGGCGCATACAAAGTT
H5-1             AGACAGGTTACTGATGCAGGCGCATACAAAGTG
H6-1             AAAAAGGTTGCTGATAAAGGCGCATATAAAGTC
                 * * ***** *****  ********* *****
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Appendix IV Alignment of the *fts*Z sequences of *R. cingulata* (Rcf) and *R. pomonella* (Rpf)

```
Rcf3-3
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rpf5-1
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rpf5-3
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rcf3-4
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rcf4-1
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rcf4-4
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rpf5-2
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rpf6-7
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rpf5-5
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rpf5-8
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rcf3-2
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rcf4-3
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rpf5-6
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rcf4-2
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rcf3-1
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rpf6-1
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rpf6-2
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rpf6-3
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rpf6-4
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC
Rpf6-5
GGTGGTACTGGAACCGGTGCAGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCC

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Rcf3-3
GCAGTTAAGGATAGAGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rpf5-1
GCAGTTAAGGATAGAGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rpf5-3
GCAGTTAAGGATAGAGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rcf3-4
GCAGTTAAGGATAGAGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rcf4-1
GCAGTTAAGGATAGAGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rcf4-4
GCAGTTAAGGATAGAGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
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Rpf5-2
GCAGTTAAGGATAGAGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rpf6-7
GTAGTTAAGGATAGAGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rpf5-5
GCAGTTAAGGATAGAGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rpf5-8
GCAGTTAAGGATAGAGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rcf3-2
GCAGTTAAGGATAGAGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rcf4-3
GCAGTTAAGGATAGAGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rpf5-6
GCAGTTAAGGATAGAGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rcf4-2
GCAGTTAAGGATAGAGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rcf3-1
GCAGTTAAGGATAGAGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rpf6-1
GCAGTTAAGGATAGGGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rpf6-2
GCAGTTAAGGATAGGGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rpf6-3
GCAGTTAAGGATAGGGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rpf6-4
GCAGTTAAGGATAGGGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
Rpf6-5
GCAGTTAAGGATAGGGCGCCAAAAGAAAAAAAGATATTGACTGTTGGAGTTGTAACTAAA
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Rcf3-3
CCGTTCGGTTTTGAAGGTGTGCGCCGTATGCGCATTGCAGAGCTTGGACTTGAAGAACTG
Rpf5-1
CCGTTCGGTTTTGAAGGTGTGCGCCGTATGCGCATTGCAGAGCTTGGACTTGAAGAACTG
Rpf5-3
CCGTTCGGTTTTGAAGGTGTGCGCCGTATGCGCATTGCAGAGCTTGGACTTGAAGAACTG
Rcf3-4
CCGTTCGGTTTTGAAGGTGTGCGCCGTATGCTCATTGCAGAGCTTGGACTTGAAGAATTG
Rcf4-1
CCGTTCGGTTTTGAAGGTGTGCGCCGTATGCGCATTGCAGAGCTTGGACTTGAAGAACTG
Rcf4-4
CCGTTCGGTTTTGAAGGTGTGCGCCGTATGCGCATTGCAGAGCTTGGACTTGAAGAACTG
Rpf5-2
CCGTTCGGTTTTGAAGGTGTGCGCCGTATGCGCATTGCAGAGCTTGGACTTGAAGAACTG
Rpf6-7
CCGTTCGGTTTTGAAGGTGTGCGCCGTATGCGCATTGCAGAGCTTGGACTTGAAGAACTG
Rpf5-5
CCGTTCGGTTTTGAAGGTGTGCGCCGTATGCGCATTGCAGAGCTTGGACTTGAAGAACTG
Rpf5-8
CCGTTCGGTTTTGAAGGTGTGCGCCGTATGCGCATTGCAGAGCTTGGACTTGAAGAACTG
Rcf3-2
CCGTTCGGTTTTGAAGGTGTGCGCCGTATGCGCATTGCAGAGCTTGGACTTGAAGAACTG
Rcf4-3
CCGTTCGGTTTTGAAGGTGTGCGTCGTATGCGCATTGCAGAGCTTGGACTTGAAGAACTG
Rpf5-6
CCGTTCGGTTTTGAAGGTGTGCGCCGTATGCGCATTGCAGAGCTTGGACTTGAAGAACTG
Rcf4-2
CCGTTCGGTTTTGAAGGTGTGCGCCGTATGCGCATTGCAGAGCTTGGACTTGAAGAACTG
Rcf3-1
CCGTTCGGTTTTGAAGGTGTGCGCCGTATGCGCATTGCAGAGCTTGGACTTGAAGAACTG
Rpf6-1
CCGTTCGGCTTTGAAGGTGTGCGCCGTATGCGTATTGCAGAGCTTGGACTTGAAGAACTG
Rpf6-2
CCGTTCGGCTTTGAAGGTGTGCGCCGTATGCGTATTGCAGAGCTTGGACTTGAAGAACTG
Rpf6-3
CCGTTCGGCTTTGAAGGTGTGCGCCGTATGCGTATTGCAGAGCTTGGACTTGAAGAACTG
Rpf6-4
CCGTTCGGCTTTGAAGGTGTGCGCCGTATGCGTATTGCAGAGCTTGGACTTGAAGAACTG
Rpf6-5
CCGTTCGGCTTTGAAGGTGTGCGCCGTATGCGTATTGCAGAGCTTGGACTTGAAGAACTG
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Rcf3-3
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rpf5-1
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rpf5-3
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rcf3-4
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rcf4-1
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rcf4-4
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rpf5-2
CAAAAATACGTGGATACACTTATTGTCATCCCAAATCAGAATTTATTTAGAATTGCAAAT
Rpf6-7
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rpf5-5
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
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Rpf5-8
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rcf3-2
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rcf4-3
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rpf5-6
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rcf4-2
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rcf3-1
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rpf6-1
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rpf6-2
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rpf6-3
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rpf6-4
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
Rpf6-5
CAAAAATACGTGGATACACTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAAT
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Rcf3-3
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rpf5-1
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATCGGCATC
Rpf5-3
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rcf3-4
GAAAAAACTACNTTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rcf4-1
GAAAAAACTACGTTCTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rcf4-4
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rpf5-2
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rpf6-7
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rpf5-5
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rpf5-8
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rcf3-2
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rcf4-3
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rpf5-6
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rcf4-2
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rcf3-1
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rpf6-1
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rpf6-2
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rpf6-3
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
Rpf6-4
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATN
Rpf6-5
GAAAAAACTACATTTTCTGATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATC
                 *********** ** ************************************
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Rcf3-3
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATCAATCTTGACTTCGCTGATATA
Rpf5-1
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATCAATCTTGACTTCGCTGATATA
Rpf5-3
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATCAATCTTGACTTCGCTGATATA
Rcf3-4
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATCAATCTTGACTTCGCTGATATA
Rcf4-1
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATCAATCTTGACTTCGCTGATATA
Rcf4-4
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATCAATCTTGACTTCGCTGATATA
Rpf5-2
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATCAATCTTGACTTCGCTGATATA
Rpf6-7
AGAGGAGTACCTGACTTGATGGTCATGCCAGGGCTTATCAATCTTGACTTCGCTGATATA
Rpf5-5
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATCAATCTTGACTTCGCTGATATA
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Rpf5-8
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATCAATCTTGACTTCGCTGATATA
Rcf3-2
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATCAATCTTGACTTCGCTGATATA
Rcf4-3
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATCAATCTTGACTTCGCTGATATA
Rpf5-6
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATCAATCTTGACTTCGCTGATATA
Rcf4-2
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATCAATCTTGACTTCGCTGATATA
Rcf3-1
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATCAATCTTGACTTCGCTGATATA
Rpf6-1
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATTAATCTTGACTTCGCTGATATA
Rpf6-2
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATTAATCTTGACTTCGCTGATATA
Rpf6-3
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATTAATCTTGACTTCGCTGATATA
Rpf6-4
NGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATTAATCTTGACTTCGCTGATATA
Rpf6-5
AGAGGAGTAACTGACTTGATGGTCATGCCAGGGCTTATTAATCTTGACTTCGCTGATATA
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Rcf3-3
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACCGGAGAGGCAGAAGGAGAA
Rpf5-1
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACCGGAGAGGCAGAAGGAGAA
Rpf5-3
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACCGGAGAGGCAGAAGGAGAA
Rcf3-4
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACCGGAGAGGCAGAAGGAGAA
Rcf4-1
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACCGGAGAGGCAGAAGGAGAA
Rcf4-4
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACCGGAGAGGCAGAAGGAGAA
Rpf5-2
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACCGGAGAGGCAGAAGGAGAA
Rpf6-7
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACCGGAGAGGCAGAAGGAGAA
Rpf5-5
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACCGGAGAGGCAGAAGGAGAA
Rpf5-8
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACCGGAGAGGCAGAAGGAGAA
Rcf3-2
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACCGGAGAGGCAGAAGGAGAA
Rcf4-3
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACCGGAGAGGCAGAAGGAGAA
Rpf5-6
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACCGGAGAGGCAGAAGGAGAA
Rcf4-2
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Rcf3-1
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Rpf6-1
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Rpf6-2
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACTGGAGAGGCAGAAGGAGAA
Rpf6-3
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACTGGAGAGGCAGAAGGAGAA
Rpf6-4
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACTGGAGAGGCAGAAGGAGAA
Rpf6-5
GAAACAGTAATGAGCGAGATGGGCAAAGCGATGATCGGCACTGGAGAGGCAGAAGGAGAA
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Rcf3-3          GATAGAGCAATTAGT
Rpf5-1          GATAGAGCAATTAGT
Rpf5-3          GATAGAGCAATTAGT
Rcf3-4          GATAGAGCAATTAGT
Rcf4-1          GATAGAGCAATTAGT
Rcf4-4          GATAGAGCAATTAGT
Rpf5-2          GATAGAGCAATTAGT
Rpf6-7          GATAGAGCAATTAGT
Rpf5-5          GATAGAGCAATTAGT
Rpf5-8          GATAGAGCAATTAGT
Rcf3-2          GATAGAGCAATTAGT
Rcf4-3          GATAGAGCAATTAGT
Rpf5-6          GATAGAGCAATTAGT
Rcf4-2          GATAGAGCAATTAGT
Rcf3-1          GATAGAGCAATTAGT
Rpf6-1          GATAGAGCAATTAGT
Rpf6-2          GATAGAGCAATTAGT
Rpf6-3          GATAGAGCAATTAGT
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Rpf6-4          GATAGAGCAATTAGT
Rpf6-5          GATAGAGCAATTAGT
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Appendix V Alignment of the *cox*A sequences of *R. cingulata* (Rcx) and *R. pomonella* (Rpx)

```
Rpx3-2
ATGCGCACAAAAGGAATGTCATTAACTAAGATGCCACTGTTTGTTTGGTCTGTCTTGCTA
Rpx3-3
ATGCGCACAAAAGGAATGTCATTAACTAAGATGCCACTGTTTGTTTGGTCTGTCTTGCTA
Rpx4-1
ATGCGCACAAAAGGAATGTCATTAACTAAGATGCCACTGTTTGTTTGGTCTGTCTTGCTA
Rpx4-3
ATGCGCACAAAAGGAATGTCATTAACTAAGATGCCACTGTTTGTTTGGTCTGTCTTGCTA
Rpx4-2
ATGCGCACAAAAGGAATGTCATTAACTAAGATGCCACTGTTTGTTTGGTCTGTCTTGCTA
Rpx3-1
ATGCGCACAAAAGGAATGTCATTAACTAAGATGCCACTGTTTGTTTGGTCTGTCTTGCTA
Rcx6-1
ATGCGTGCGAAAGGCATGTCGTTGACTAAGATGCCACTATTTGTTTGGTCTGTCTTGCTA
Rcx6-2
ATGCGTGCGAAAGGCATGTCGTTGACTAAGATGCCACTATTTGTTTGGTCTGTCTTGCTA
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Rpx3-2
ACAGCATTTATGTTGATTGTTGCCTTACCAGTGCTTGCCGGTGCTATAACTATGCTTCTT
Rpx3-3
ACAGCATTTATGTTGATTGTTGCCTTACCAGTGCTTGCCGGTGCTATAACTATGCTTCTT
Rpx4-1
ACAGCATTTATGTTGATTGTTGCCTTACCAGTGCTTGCCGGTGCTATAACTATGCTTCTT
Rpx4-3
ACAGCATTTATGTTGATTGTTGCCTTATCAGTGCTTGCCGGTGCTATAACTATGCTTCTT
Rpx4-2
ACAGCATTTATGTTGATTGTTGCCTTACCAGTGCTTGCCGGTGCTATAACTATGCTTCTT
Rpx3-1
ACAGCATTTATGTTGATTGTTGCCTTACCAGTGCTTGCCGGTGCTATAACTATGCTTCTT
Rcx6-1
ACAGCATTTATGTTGATTGTTGCCTTACCGGTGCTTGCCGGTGCTATAACTATGCTTCTT
Rcx6-2
ACAGCATTTATGTTGATTGTTGCCTTACCGGTGCTTGCCGGTGCTATAACTATGCTTCTT
                 *************************** *
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Rpx3-2
ACTGATCGCAATATTGGTACTTCCTTTTTTGATCCTGCCGGTGGCGGCGATCCTGTGTTA
Rpx3-3
ACTGATCGCAATATTGGTACTTCCTTTTTTGATCCTGCCGGTGGCGGCGATCCTGTGTTA
Rpx4-1
ACTGATCGCAATATTGGTACTTCCTTTTTTGATCCTGCCGGTGGCGGCGATCCTGTGTTA
Rpx4-3
ACTGATCGCAATATTGGTACTTCCTTTTTTGATCCTGCCGGTGGCGGCGATCCTGTGTTA
Rpx4-2
ACTGATCGCAATATTGGTACTTCCTTTTTTGATCCTGCCGGTGGCGGCGATCCTGTGTTA
Rpx3-1
ACTGATCGCAATATTGGTACTTCCTTTTTTGATCCTGCCGGTGGCGGCGATCCTGTGTTA
Rcx6-1
ACTGATCGCAATATTGGTACTTCCTTTTTTGATCCTGCAGGTGGTGGTGACCCTGTGTTA
Rcx6-2
ACTGATCGCAATATTGGTACTTCCTTTTTTGATCCTGCAGGTGGTGGTGACCCTGTGTTA
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Rpx3-2
TTTCAACATCTATTTTGGTTTTTTGGTCATCCAGAAGTTTACGTAATTATTTTTCCTGCA
Rpx3-3
TTTCAACATCTATTTTGGTTTTTTGGTCATCCAGAAGTTTACGTAATTATTTTTCCTGCA
Rpx4-1
TTTCAACATCTATTTTGGTTTTTTGGTCATCCAGAAGTTTACGTAATTATTTTTCCTGCA
Rpx4-3
TTTCAACATCTATTTTGGTTTTTTGGTCATCCAGAAGTTTACGTAATTATTTTTCCTGCA
Rpx4-2
TTTCAACATCTATTTTGGTTTTTTGGTCATCCAGAAGTTTACGTAATTATTTTTCCTGCA
Rpx3-1
TTTCAACATCTATTTTGGTTTTTTGGTCATCCAGAAGTTTACGTAATTATTTTTCCTGCA
Rcx6-1
TTTCAACATTTATTTTGGTTTTTTGGTCATCCAGAAGTTTACGTAATTATTTTTCCTGCA
Rcx6-2
TTTCAACATTTATTTTGGTTTTTTGGTCATCCAGAAGTTTACGTAATTATTTTTCCTGCA
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Rpx3-2
TTTGGCATCATAAGTCAGGTTGTATCAACTTTTTCTCACAGACCTGTATTTGGTTACATA
Rpx3-3
TTTGGCATCATAAGTCAGGTTGTATCAACTTTTTCTCACAGACCTGTATTTGGTTACATA
Rpx4-1
TTTGGCATCATAAGTCAGGTTGTATCAACTTTTTCTCACAGACCTGTATTTGGTTACATA
Rpx4-3
TTTGGCATCATAAGTCAGGTTGTATCAACTTTTTCTCACAGACCTGTATTTGGTTACATA
Rpx4-2
TTTGGCATCATAAGTCAGGTTGTATCAACTTTTTCTCACAGACCTGTATTTGGTTACATA
Rpx3-1
TTTGGCATCATAAGTCAGGTTGTATCAACTTTTTCTCACAGACCTGTATTTGGTTACATA
Rcx6-1
TTTGGCATCATAAGTCAGGTTGTATCAACTTTTTCTCACAGACCTGTATTTGGTTACATA
Rcx6-2
TTTGGCATCATAAGTCAGGTTGTATCAACTTTTTCTCACAGACCTGTATTTGGTTACATA

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Rpx3-2
GGGATGGTTTATGCAATGATAGGTATAGCAGTATTTGGCTTTATGGTTTGGGCTCACCAT
Rpx3-3
GGGATGGTTTATGCAATGATAGGTATAGCAGTATTTGGCTTTATGGTTTGGGCTCACCAT
Rpx4-1
GGGATGGTTTATGCAATGATAGGTATAGCAGTATTTGGCTTTATGGTTTGGGCTCACCAT
Rpx4-3
GGGATGGTTTATGCAATGATAGGTATAGCAGTATTTGGCTTTATGGTTTGGGCTCACCAT
Rpx4-2
GGGATGGTTTATGCAATGATAGGTATAGCAGTATTTGGCTTTATGGTTTGGGCTCACCAT
Rpx3-1
GGGATGGTTTATGCAATGATAGGTATAGCAGTATTTGGCTTTATGGTTTGGGCTCACCAT
Rcx6-1
GGGATGGTTTATGCAATGATAGGTATAGCAGTATTTGGCTTTATGGTTTGGGCTCATCAT
Rcx6-2
GGGATGGTTTATGCAATGATAGGTATAGCAGTATTTGGCTTTATGGTTTGGGCTCATCAT

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Rpx3-2          ATGTTCACTGTTGGGCTTAGTGCTGACGCTGCTGCATTTTTT
Rpx3-3          ATGTTCACTGTTGGGCTTAGTGCTGACGCTGCTGCATTTTTT
Rpx4-1          ATGTTCACTGTTGGGCTTAGTGCTGACGCTGCTGCATTTTTT
Rpx4-3          ATGTTCACTGTTGGGCTTAGTGCTGACGCTGCTGCATTTTTT
Rpx4-2          ATGTTCACTGTTGGGCTTAGTGCTGACGCTGCTGCATTTTTT
Rpx3-1          ATGTTCACTGTTGGGCTTAGTGCTGACGCTGCTGCATTTTTT
Rcx6-1          ATGTTCACTGTTGGGCTTAGTGCTGACGCTGCTGCATTTTTT
Rcx6-2          ATGTTCACTGTTGGGCTTAGTGCTGACGCTGCTGCATTTTTT
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Appendix VI Alignment of the *hcp*A sequences of *R. cingulata* (Rch) and *R. pomonella* (Rph)

```
Rph3-1
GATCCCGAACTCAACCCGCGCCTTCGCTCTGCTATATTTGCTGCACGCAAGGAAAATCTA
Rph4-1
GATCCCGAACTCAACCCGCGCCTTCGCTCTGCTATATTTGCTGCACGCAAGGAAAATCTA
Rph3-2
GATCCCGAACTCAACCCGCGCCTTCGCTCTGCTATATTTGCTGCACGCAAGGAAAATCTA
Rph3-3
GATCCCGAACTCAACCCGCGCCTTCGCTCTGCTATATTTGCTGCACGCAAGGAAAATCTA
Rph3-4
GATCCCGAACTCAACCCGCGCCTTCGCTCTGCTATATTTGCTGCACGCAAGGAAAATCTA
Rph4-2
GATCCCGAACTCAACCCGCGCCTTCGCTCTGCTATATTTGCTGCACGCAAGGAAAATCTA
Rph4-3
GATCCCGAACTCAACCCGCGCCTTCGCTCTGCTATATTTGCTGCACGCAAGGAAAATCTA
Rph4-4
GATCCCGAACTCAACCCGCGCCTTCGCTCTGCTATATTTGCTGCACGCAAGGAAAATCTA
Rch6-5
CTGCCCGAACTCAACCCGCGCCTTCGCTCTGCTATATTTGCTGCACGCAAGGAAAATCTA
Rch6-1
CTGCCCGAACTCAACCCGCGCCTTCGCTCTGCTATATTTGCTGCACGCAAGGAAAATCTA
Rch6-3
CTGCCCGAACTCAACCCGCGCCTTCGCTCTGCTATATTTGCTGCACGCAAGGAAAATCTA
Rch6-2
CTGCCCGAACTCAACCCGCGCCTTCGCTCTGCTATATTTGCTGCACGCAAGGAAAATCTA

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Rph3-1
CCAAAAGATAAAATAGAAACAGCAATAAAAAATGCAACTGGTAACGTTGCTGGAGAAAAT
Rph4-1
CCAAAAGCTAAAATAGAAACAGCAATAAAAAATGCAACTGGTAACGTTGCTGGAGAAAAT
Rph3-2
CCAAAAGATAAAATAGAAACAGCAATAAAAAATGCAACTGGTAACGTTGCTGGAGAAAAT
Rph3-3
CCAAAAGATAAAATAGAAACAGCAATAAAAAATGCAACTGGTAACGTTGCTGGAGAAAAT
Rph3-4
CCAAAAGATAAAATAGAAACAGCAATAAAAAATGCAACTGGTAACGTTGCTGGAGAAAAT
Rph4-2
CCAAAAGATAAAATAGAAACAGCAATAAAAAATGCAACTGGTAACGTTGCTGGAGAAAAT
Rph4-3
CCAAAAGATAAAATAGAAACAGCAATAAAAAATGCAACTGGTAACGTTGCTGGAGAAAAT
Rph4-4
CCAAAAGATAAAATAGAAACAGCAATAAAAAATGCAACTGGTAACGTTGCTGGAGAAAAT
Rch6-5
CCAAAAGATAAAATAGAAACAGCAATAAAAAATGCAACTGGTAACGTTGCTGGAGAAAAT
Rch6-1
CCAAAAGATAAAATAGAAACAGCAATAAAAAATGCAACTGGTAACATTGCTGGAGAAAAT
Rch6-3
CCAAAAGATAAAATAGAAACAGCAATAAAAAATGCAACTGGTAACATTGCTGGAGAAAAT
Rch6-2
CCAAAAGATAAAATAGAAACAGCAATAAAAAATGCAACTGGTAACATTGCTGGAGAAAAT
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Rph3-1
TACGAGGAAATCCAATATGAAGGTCATGGGCCTTCTGGCACTGCACTCATTGTCCATGTT
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Rph4-1
TACGAGGAAATCCAATATGAAGGTCATGGGCCTTCTGGCACTGCACTCATTGTCCATGTT
Rph3-2
TACGAGGAAATCCAATATGAAGGTCATGGGCCTTCTGGCACTGCACTCATTGTCCATGTT
Rph3-3
TACGAGGAAATCCAATATGAAGGTCATGGGCCTTCTGGCACTGCACTCATTGTCCATGTT
Rph3-4
TACGAGGAAATCCAATATGAAGGTCATGGGCCTTCTGGCACTGCACTCATTGTCCATGTT
Rph4-2
TACGAGGAAATCCAATATGAAGGTCATGGGCCTTCTGGCACTGCACTCATTGTCCATGTT
Rph4-3
TACGAGGAAATCCAATATGAAGGTCATGGGCCTTCTGGCACTGCACTCATTGTCCATGTT
Rph4-4
TACGAGGAAATCCAATATGAAGGTCATGGGCCTTCTGGCACTGCACTCATTGTCCATGTT
Rch6-5
TACGAGGAAATCCAATATGAAGGTCATGGGCCTTCTGGCACTGCACTCATTGTCCATGTT
Rch6-1
TACGAGGAAATACAATATGAAGGTCATGGGCCTTCTGGTACTGCACTCATTGTCCATGCC
Rch6-3
TACGAGGAAATACAATATGAAGGTCATGGGCCTTCTGGTACTGCACTCATTGTCCATGCC
Rch6-2
TACGAGGAAATACAATATGAAGGTCATGGGCCTTCTGGTACTGCACTCATTGTCCATGCC
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Rph3-1
TTGACTAATAATCGCAACCGAACTGCTTCTGAGGTACGTTATATATTTTCTCGCAAGGGT
Rph4-1
TTGACTAATAATCGCAACCGAACTGCTTCTGAGGTACGTTATATATTTTCTCGCAAGGGT
Rph3-2
TTGACTAATAATCGCAACCGAACTGCTTCTGAGGTACGTTATATATTTTCTCGCAAGGGT
Rph3-3
TTGACTAATAATCGCAACCGAACTGCTTCTGAGGTACGTTATATATTTTCTCGCAAGGGT
Rph3-4
TTGACTAATAATCGCAACCGAACTGCTTCTGAGGTACGTTATATATTTTCTCGCAAGGGT
Rph4-2
TTGACTAATAATCGCAACCGAACTGCTTCTGAGGTACGTTATATATTTTCTCGCAAGGGT
Rph4-3
TTGACTAATAATCGCAACCGAACTGCTTCTGAGGTACGTTATATATTTTCTCGCAAGGGT
Rph4-4
TTGACTAATAATCGCAACCGAACTGCTTCTGAGGTACGTTATATATTTTCTCGCAAGGGT
Rch6-5
TTGACTAATAATCGCAACCGAACTGCTTCTGAGGTACGTTATATATTTTCTCGCAAGGGT
Rch6-1
TTGACTAATAACCGCAACCGTACTGCTTCTGAGGTACGTTATATATTTTCTCGCAAGGGT
Rch6-3
TTGACTAATAACCGCAACCGTACTGCTTCTGAGGTACGTTATATATTTTCTCGCAAGGGT
Rch6-2
TTGACTAATAACCGCAACCGTACTGCTTCTGAGGTACGTTATATATTTTCTCGCAAGGGT
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Rph3-1
GAAAACTTGGGAGAAGCAGGAAGTGTTAGTTACCTTTTCGATCATGTAGGCTTAATCGTC
Rph4-1
GAAAACTTGGGAGAAACAGGAAGTGTTAGTTACCTTTTCGATCATGTAGGCTTAATCGTC
Rph3-2
GAAAACTTGGGAGAAACAGGAAGTGTTAGTTACCTTTTCGATCATGTAGGCTTAATCGTC
Rph3-3
GAAAACTTGGGAGAAACAGGAAGTGTTAGTTACCTTTTCGATCATGTAGGCTTAATCGTC
Rph3-4
GAAAACTTGGGAGAAACAGGAAGTGTTAGTTACCTTTTCGATCATGTAGGCTTAATCGTC
Rph4-2
GAAAACTTGGGAGAAACAGGAAGTGTTAGTTACCTTTTCGATCATGTAGGCTTAATCGTC
Rph4-3
GAAAACTTGGGAGAAACAGGAAGTGTTAGTTACCTTTTCGATCATGTAGGCTTAATCGTC
Rph4-4
GAAAACTTGGGAGAAACAGGAAGTGTTAGTTACCTTTTCGATCATGTAGGCTTAATCGTC
Rch6-5
GGAAACTTGGGAGAAACAGGAAGTGTTAGTTACCTTTTCGATCATGTAGGCTTAATCGTC
Rch6-1
GGAAACTTGGGAGAAACAGGAAGTGTTAGTTACCTTTTTGATCATGTAGGTTTAATTGTC
Rch6-3
GGAAACTTGGGAGAAACAGGAAGTGTTAGTTACCTTTTTGATCATGTAGGTTTAATTGTC
Rch6-2
GGAAACTTGGGAGAAACAGGAAGTGTTAGTTACCTTTTTGATCATGTAGGTTTAATTGTC
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Rph3-1
TATAAAGCAGAGGGTGTGAATTTTGATGATTTATTCAGTCATGGAATCGAATTAGAAGTA
Rph4-1
TATAAAGCAGAGGGTGTGAATTTTGATGATTTATTCAGTCATGGAATCGAATTAGAAGTA
Rph3-2
TATAAAGCAGAGGGTGTGAATTTTGATGATTTATTCAGTCATGGAATCGAATTAGAAGTA
Rph3-3
TATAAAGCAGAGGGTGTGAATTTTGATGATTTATTCAGTCATGGAATCGAATTAGAAGTA
Rph3-4
TATAAAGCAGAGGGTGTGAATTTTGATGATTTATTCAGTCATGGAATCGAATTAGAAGTA
Rph4-2
TATAAAGCAGAGGGTGTGAATTTTGATGATTTATTCAGTCATGGAATCGAATTAGAAGTA
Rph4-3
TATAAAGCAGAGGGTGTGAATTTTGATGATTTATTCAGTCATGGAATCGAATTAGAAGTA
Rph4-4
TATAAAGCAGAGGGTGTGAATTTTGATGATTTATTCAGTCATGGAATCGAATTAGAAGTA
Rch6-5
TATAAAGCAGAGGGTGTGAATTTTGATGATTTATTCAGTCATGGAATCGAATTAGAAGTA
Rch6-1
TATAAAGCAGAGGGTGTGAATTTTGATGATTTATTCAGTCATGGAATCGAATTAGAAGTA
Rch6-3
TATAAAGCAGAGGGTGTGAATTTTGATGATTTATTCAGTCATGGAATCGAATTAGAAGTA
Rch6-2
TATAAAGCAGAGGGTGTGAATTTTGATGATTTATTCAGTCATGGAATCGAATTAGAAGTA

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Rph3-1
TTGAATGTTGAGGAAAATGACAAAGAAGGATTACACGTTATAACTTGTGAAATAAAAGAT
Rph4-1
TTGAATGTTGAGGAAAATGACAAAGAAGGATTACACGTTATAACTTGTGAAATAAAAGAT
Rph3-2
TTGAATGTTGAGGAAAATGACAAAGAAGGATTACACGTTATAACTTGTGAAATAAAAGAT
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Rph3-3
TTGAATGTTGAGGAAAATGACAAAGAAGGATTACACGTTATAACTTGTGAAATAAAAGAT
Rph3-4
TTGAATGTTGAGGAAAATGACAAAGAAGGATTACACGTTATAACTTGTGAAATAAAAGAT
Rph4-2
TTGAATGTTGAGGAAAATGACAAAGAAGGATTACACGTTATAACTTGTGAAATAAAAGAT
Rph4-3
TTGAATGTTGAGGAAAATGACAAAGAAGGATTACACGTTATAACTTGTGAAATAAAAGAT
Rph4-4
TTGAATGTTGAGGAAAATGACAAAGAAGGATTACACGTTATAACTTGTGAAATAAAAGAT
Rch6-5
TTGAATGTTGAGGAAAATGACAAAGAAGGATTACACGTTATAACTTGTGAAATAAAAGAT
Rch6-1
TTGAATATTGAGGAAAATGACAAAGAAGGATTACACGTTATAACTTGTGAAATAAAAGAT
Rch6-3
TTGAATATTGAGGAAAATGACAAAGAAGGATTACACGTTATAACTTGTGAAATAAAGGAT
Rch6-2
TTGAATATTGAGGAAAATGACAAAGAAGGATTACACGTTATAACTTGTGAAATAAAAGAT
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Rph3-1          TTTGGTAAAGTACGCGATGCCTTT
Rph4-1          TTTGGTAAAGTACGCGATGCCTTT
Rph3-2          TTTGGTAAAGTACGCGATGCCTTT
Rph3-3          TTTGGTAAAGTACGCGATGCCTTT
Rph3-4          TTTGGTAAAGTACGCGATGCCTTT
Rph4-2          TTTGGTAAAGTACGCGATGCCTTT
Rph4-3          TTTGGTAAAGTACGCGATGCCTTT
Rph4-4          TTTGGTAAAGTACGCGATGCCTTT
Rch6-5          TTTGGTAAAGTACGCGATGCCTTT
Rch6-1          TTTGGTAAAGTACGCGATGCCTTT
Rch6-3          TTTGGTAAAGTACACGATGCCG--
Rch6-2          TTTGGTAAAGTACGCGATGCCTTT
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Appendix VII Alignment of the *gat*B sequences of *R. cingulata* (Rcg) and *R. pomonella* (Rpg)

```
Rpg3-1
GAAGCTGCAGAATGCATGAAAAAATTGAGGCAGATTTTGCGTTACATTGGTTCGTGTGAT
Rpg3-3
GAAGCTGCAGAATGCATGAAAAAATTGAGGCAGATTTTGCGTTACATTGGTTCGTGTGAT
Rpg4-1
GAAGCTGCAGAATGCATGAAAAAATTGAGGCAGATTTTGCGTTACATTGGTTCGTGTGAT
Rpg3-2
GAAGCTGCAGAATGCATGAAAAAATTGAGGCAGATTTTGCGTTACATTGGTTCGTGTGAT
Rpg3-4
GAAGCTGCAGAATGCATGAAAAAATTGAGGCAGATTTTGCGTTACATTGGTTCGTGTGAT
Rpg4-3
GAAGCTGCAGAATGCATGAAAAAATTGAGGCAGATTTTGCGTTACATTGGTTCGTGTGAT
Rpg4-2
GAAGCTGCAGAATGCATGAAAAAATTGAGGCAGATTTTGCGTTACATTGGTTCGTGTGAT
Rcg5-1
GAAGCTGCAGAATGCATGAAAAAATTGAGGCAGATTTTGCGTTACATTGGTTCGTGTGAT

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Rpg3-1
GGTGATATGGAAAAGGGATCACTTCGTTGTGATGCAAATGTTTCTGTCCGCCTAAAAGGC
Rpg3-3
GGTGATATGGAAAAGGGATCACTTCGTTGTGATGCAAATGTTTCTGTCCGCCTAAAAGGC
Rpg4-1
GGTGATATGGAAAAGGGATCACTTCGTTGTGATGCAAATGTTTCTGTCCGCCTAAAAGGC
Rpg3-2
GGTGATATGGAAAAGGGATCACTTCGTTGTGATGCAAATGTTTCTGTCCGCCTAAAAGGC
Rpg3-4
GGTGATATGGAAAAGGGATCACTTCGTTGTGATGCAAATGTTTCTGTCCGCCTAAAAGGC
Rpg4-3
GGTGATATGGAAAAGGGATCACTTCGTTGTGATGCAAATGTTTCTGTCCGCCTAAAAGGC
Rpg4-2
GGTGATATGGAAAAGGGATCACTTCGTTGTGATGCAAATGTTTCTGTCCGCCTAAAAGGC
Rcg5-1
GGTGATATGGAAAAGGGATCACTTCGTTGTGATGCAAATGTTTCTGTCCGCCTAAAAGGC

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Rpg3-1
AGTAGTACATTTGGCACTCGTTGTGAAATAAAAAATCTGAACTCGATACGTTATATTGTG
Rpg3-3
AGTAGTACATTTGGCACTCGTTGTGAAATAAAAAATCTGAACTCGATACGTTATATTGTG
Rpg4-1
AGTAGTACATTTGGCACTCGTTGTGAAATAAAAAATCTGAACTCGATACGTTATATTGTG
Rpg3-2
AGTAGTACATTTGGCACTCGTTGTGAAATAAAAAATCTGAACTCGATACGTTATATTGTG
Rpg3-4
AGTAGTACATTTGGCACTCGTTGCGAAATAAAAAATCTGAACTCGATACGTTATATTGTG
Rpg4-3
AGTAGTACATTTGGCACTCGTTGTGAAATAAAAAATCTGAACTCGATACGTTATATTGTG
Rpg4-2
AGTAGTACATTTGGCACTCGTTGTGAAATAAAAAATCTGAACTCGATACGTTATATTGTG
Rcg5-1
AGTAGCGCACTTGGCACTCGTTGTGAGATAAAAAATCTGAACTCGATACGTTATATTGTG
                *****  ** ************ **
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Rpg3-1
CAAGCTATAGACTATGAAATACAAAGACAAATTGAAATTTTAGAAAGTGGAGAAGAAATA
Rpg3-3
CAAGCTATAGACTATGAAATACAAAGACAAATTGAAATTTTAGAAAGTGGAGAAGAAATA
Rpg4-1
CAAGCTATAGACTATGAAATACAAAGACAAATTGAAATTTTAGAAAGTGGAGAAGAAATA
Rpg3-2
CAAGCTATAGACTATGAAATACAAAGACAAATTGAAATTTTAGAAAGTGGAGAAGAAATA
Rpg3-4
CAAGCTATAGACTATGAAATACAAAGACAAATTGAAATTTTAGAAAGTGGAGAAGAAATA
Rpg4-3
CAAGCTATAGACTATGAAATACAAAGACAAATTGAAATTTTAGAAAGTGGAGAAGAAATA
Rpg4-2
CAAGCTATAGACTATGAAATACAAAGACAAATTGAAATTTTAGAAAGTGGAGAAGAAATA
Rcg5-1
CAAGCTATAGACTATGAAATACAAAGACAAATTGAAATTTTAGAAAGTGGGGAAGAAATA
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Rpg3-1
AGTCAAGATACCTTATTGTTTGACGTTGCTTCGGGAAAAACAAAAGTGATGAGAAGCAAA
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Rpg3-3
AGTCAAGATACCTTATTGTTTGACGTTGCTTCGGGAAAAACAAAAGTGATGAGAAGCAAA
Rpg4-1
AGTCAAGATACCTTATTGTTTGACGTTGCTTCGGGAAAAACAAAAGTGATGAGAAGCAAA
Rpg3-2
AGTCAAGATACCTTATTGTTTGACGTTGCTTCGGGAAAAACAAAAGTGATGAGAAGCAAA
Rpg3-4
AGTCAAGATACCTTATTGTTTGACGTTGCTTCGGGAAAAACAAAAGTGATGAGAAGCAAA
Rpg4-3
AGTCAAGATACCTTATTGTTTGACGTTGCTTCGGGAAAAACAAAAGTGATGAGAAGCAAA
Rpg4-2
AGTCAAGATACCTTATTGTTTGACGTTGCTTCGGGAAAAACAAAAGTGATGAGAAGCAAA
Rcg5-1
AGTCAAGATACCTTATTGTTTGATGTTGCTTCGGGAAAAACAAAAGTGATGCGAAACAAA
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Rpg3-1
GAGGATGCAAGCGATTATAGATACTTCCCTGAGCCTGATTTATTACCTGTTGAGGTAAGC
Rpg3-3
GAGGATGCAAGCGATTATAGATACTTCCCTGAGCCTGATTTATTACCTGTTGAGGTAAGC
Rpg4-1
GAGGATGCAAGCGATTATAGATACTTCCCTGAGCCTGATTTATTACCTGTTGAGGTAAGC
Rpg3-2
GAGGATGCAAGCGATTATAGATACTTCCCTGAGCCTGATTTATTACCTGTTGAGGTAAGC
Rpg3-4
GAGGATGCAAGCGATTATAGATACTTCCCTGAGCCTGATTTATTACCTGTTGAGGTAAGC
Rpg4-3
GAGGATGCAAGCGATTATAGATACTTCCCTGAGCCTGATTTATTACCTGTTGAGGTAAGC
Rpg4-2
GAGGATGCAAGCGATTATAGATACTTCCCTGAGCCTGATTTATTACCTGTTGAGGTAAGC
Rcg5-1
GAAGATGCAAGCGACTATAGATACTTCCCTGAGCCTGATTTATTACCTGTTGAGGTAAGC
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Rpg3-1            CAGGATAAA
Rpg3-3            CAGGATAAA
Rpg4-1            CAGGATAAA
Rpg3-2            CAGGATAGA
Rpg3-4            CAGGATAAA
Rpg4-3            CAGGATAAA
Rpg4-2            CAGGATAAA
Rcg5-1            CAGGATAAA
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Appendix VIII Alignment of the *fbp*A sequences of *R. cingulata* (Rcp) and *R. pomonella* (Rpp)

```
Rcp6-6
TGAAGCTGGTGCTGCAACTTATGCTGGAATGCTCCCACTTATTTTGAAACTTAATAGTTC
Rcp6-7
TGAAGCTGGTGCTGCAACTTATGCTGGAATGCTCCCACTTATTTTGAAACTTAATAGTTC
Rpp4-2
TGAAGCTGGTGCTGCAACTTATGCTGGAATGCTCCCACTTATTTTGAAACTTAATAGTTC
Rpp3-4
TGAAGCTGGTGCTGCAACTTATGCTGGAATGCTCCCACTTATTTTGAAACTTAATAGTTC
Rpp3-3
TGAAGCTGGTGCTGCAACTTATGCTGGAATGCTCCCACTTATTTTGAAACTTAATAGTTC
Rpp3-2
TGAAGCTGGTGCTGCAACTTATGCTGGAATGCTCCCACTTATTTTGAAACTTAATAGTTC
Rcp6-1
TGAAGCTGGTGCTGCAACTTATGCTGGAATGCTCCCACTTATTTTGAAACTTAATAGTTC
Rcp6-8
TGAAGCTGGCGCTGCAACTTATGCTGGAATGCTCCCACTTATTTTGAAACTTAATAGTTC
Rpp4-3
TGAAGCTGGTGCTGCAACTTATGCTGGAATGCTCCCACTTATTTTGAAACTTAATAGTTC
Rpp4-1
TGAAGCTGGTGCTGCAACTTATGCTGGAATGCTCCCACTTATTTTGAAACTTAATAGTTC
Rpp3-1
TGAAGCTGGTGCTGCAACTTATGCTGGAATGCTCCCACTTATTTTGAAACTTAATAGTTC
Rcp6-2
TGAAGCTGGTGCTTCAACTTATGCTGGAATGCTCCCACTTATTTTGAAACTTAATAGTTC
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Rcp6-6
CAACTCTTTACATTCAAAGGATCTAACCTCTGATCAGGCAATAACCTCTTCTGTGAAAGA
Rcp6-7
CAACTCTTTACATTCAAAGGATCTAACCTCTGATCAGGCAATAACCTCTTCTGTGAAAGA
Rpp4-2
CAACTCTTTACATTCAAAGGATCTAACCTCTGATCAGGCAATAACCTCTTCTGTGAAAGA
Rpp3-4
CAACTCTTTACATTCAAAGGATCTAACCTCTGATCAGGCAATAACCTCTTCTGTGAAAGA
Rpp3-3
CAACTCTTTACATTCAAAGGATCTAACCTCTGATCAGGCAATAACCTCTTCTGTGAAAGA
Rpp3-2
CAACTCTTTACATTCAAAGGATCTAACCTCTGATCAGGCAATAACCTCTTCTGTGAAAGA
Rcp6-1
CAACTCTTTACATTCAAAGGATCTAACCTCTGATCAGGCAATAACCTCTTCTGTGAAAGA
Rcp6-8
CAACTCTTTACATTCAAAGGATCTAACCTCTGATCAGGCAATAACCTCTTCTGTGAAAGA
Rpp4-3
CAACTCTTTACATTCAAAGGATCTAACCTCTGATCAGGCAATAACCTCTTCTGTGAAAGA
Rpp4-1
CAACTCTTTACATTCAAAGGATCTAACCTCTGATCAGGCAATAACCTTTTCTGTGAAAGA
Rpp3-1
CAACTCTTTACATTCAAAGGATCTAACCTCTGATCAGGCAATAACCTCTTCTGTGAAAGA
Rcp6-2
CAACTCTTTACATTCAAAGGATCTAACCTCTGATCAGGCAATAACCTCTTCTGTGAAAGA
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Rcp6-6
TGCGCTGCGTTTGGGATGCTTAGCTGTCGGATTTACTATATATCCTGGTTCTGCTAAGTG
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Rcp6-7
TGCGCTGCGTTTGGGATGCTTAGCTGTCGGATTTACTATATATCCTGGTTCTGCTAAGTG
Rpp4-2
TGCGCTGCGTTTGGGATGCTTAGCTGTCGGATTTACTATATATCCTGGTTCTGCTAAGTG
Rpp3-4
TGCGCTGCGTTTGGGATGCTTAGCTGTCGGATTTACTATATATCCTGGTTCTGCTAAGTG
Rpp3-3
TGCGCTGCGTTTGGGATGCTTAGCTGTCGGATTTACTATATATCCTGGTTCTGCTAAGTG
Rpp3-2
TGCGCTGCGTTTGGGATGCTTAGCTGTCGGATTTACTATATATCCTGGTTCTGCTAAGTG
Rcp6-1
TGCGCTGCGTTTGGGATGCTTAGCTGTCGGATTTACTATATATCCTGGTTCTGCTAAGTG
Rcp6-8
TGCGCTGCGTTTGGGATGCTTAGCTGTCGGATTTACTATATATCCTGGTTCTGCTAAGTG
Rpp4-3
TGCGCTGCGTTTGGGATGCTTAGCTGTCGGATTTACTATATATCCTGGTTCTGCTAAGTG
Rpp4-1
TGCGCTGCGTTTGGGATGCTTAGCTGTCGGATTTACTATATATCCTGGTTCTGCTAAGTG
Rpp3-1
TGCGCTGCGTTTGGGATGCTTAGCTGTCGGATTTACTATATATCCTGGTTCTGCTAAGTG
Rcp6-2
TGCGCTGCGTTTGGGCTGCTTAGCTGTCGGATTTACTATATATCCTGGTTCTGCTAAGTG
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Rcp6-6
TTTCGATATGATGGAGGAAGCCCGTGGAATCATAGCTGAAGCCAAATCTTATGGACTTGC
Rcp6-7
TTTCGATATGATGGAGGAAGCCCGTGGAATCATAGCTGAAGCCAAATCTTATGGACTTGC
Rpp4-2
TTTCGATATGATGGAGGAAGCCCGTGGAATCATAGCTGAAGCCAAATCTTATGGACTTGC
Rpp3-4
TTTCGATATGATGGAGGAAGCCCGTGGAATCATAGCTGAAGCCAAATCTTATGGACTTGC
Rpp3-3
TTTCGATATGATGGAGGAAGCCCGTGGAATCATAGCTGAAGCCAAATCTTATGGACTTGC
Rpp3-2
TTTCGATATGATGGAGGAAGCCCGTGGAATCATAGCTGAAGCCAAATCTTATGGACTTGC
Rcp6-1
TTTCGATATGATGGAGGAAGCCCGTGGAATCATAGCTGAAGCCAAATCTTATGGACTTGC
Rcp6-8
TTTCGATATGATGGAGGAAGCCCGTGGAATCATAGCTGAAGCCAAATCTTATGGACTTGC
Rpp4-3
TTTCGATATGATGGAGGAAGCCCGTGGAATCATAGCTGAAGCCAAATCTTATGGACTTGC
Rpp4-1
TTTCGATATGATGGAGGAAGCCCGTGGAATCATAGCTGAAGCCAAATCTTATGGACTTGC
Rpp3-1
TTTCGATATGATGGAGGAAGCCCGTGGAATCATAGCTGAAGCCAAATCTTATGGACTTGC
Rcp6-2
TTTCGATATGATGGAGGAAGCCCGTGGAATCATAGCTGAAGCCAAATCTTACGGGCTTGC
                ************************************************** **
*****


Rcp6-6
AGTAGTGCTATGGTCTTATCCACGCGGTGAAGGGATTTCCAAAGAAGGTGAAACAGCAGT
Rcp6-7
AGTAGTGCTATGGTCTTATCCACGCGGTGAAGGGATTTCCAAAGAAGGTGAAACAGCAGT
Rpp4-2
AGTAGTGCTATGGTCTTATCCACGCGGTGAAGGGATTTCCAAAGAAGGTGAAACAGCAGT
Rpp3-4
AGTAGTGCTATGGTCTTATCCACGCGGTGAAGGGATTTCCAAAGAAGGTGAAACAGCAGT
```

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Rpp3-3
AGTAGTGCTATGGTCTTATCCACGCGGTGAAGGGATTTCCAAAGAAGGTGAAACAGCAGT
Rpp3-2
AGTAGTGCTATGGTCTTATCCACGCGGTGAAGGGATTTCCAAAGAAGGTGAAACAGCAGT
Rcp6-1
AGTAGTGCTATGATCTTATCCACGCGGTGAAGGGATTTCCAAAGAAGGTGAAACAGCAGT
Rcp6-8
AGTAGTGCTATGGTCTTATCCACGCGGTGAAGGGATTTCCAAAGAAGGTGAAACAGCAGT
Rpp4-3
AGTAGTGCTATGGTCTTATCCACGCGGTGAAGGGATTTCCAAAGAAGGTGAAACAGCAGT
Rpp4-1
AGTAGTGCTATGGTCTTATCCACGCGGTGAAGGGATTTCCAAAGAAGGTGAAACAGCAGT
Rpp3-1
AGTAGTGCTACGGTCTTATCCACGCGGTGAAGGGATTTCCAAAGAAGGTGAAACAGCAGT
Rcp6-2
AGTAGTGCTATGGTCTTATCCACGCGGTGAAGGGATTTCCAAAGAAGGTGAAACAGCAGT
                  ********** *
*********************************************

Rcp6-6
TGATGTTATTGCCTATGCTGCGCACATGGCAGCTTTGCTTGGCGCTAATATAATAAAAGT
Rcp6-7
TGATGTTATTGCCTATGCTGCGCACATGGCAGCTTTGCTTGGCGCTAATATAATAAAAGT
Rpp4-2
TGATGTTATTGCCTATGCTGCGCACATGGCAGCTTTGCTTGGCGCTAATATAATAAAAGT
Rpp3-4
TGATGTTATTGCCTATGCTGCGCACATGGCAGCTTTGCTTGGCGCTAATATAATAAAAGT
Rpp3-3
TGATGTTATTGCCTATGCTGCGCACATGGCAGCTTTGCTTGGCGCTAATATAATAAAAGT
Rpp3-2
TGATGTTATTGCCTATGCTGCGCACATGGCAGCTTTGCTTGGCGCTAATATAATAAAAGT
Rcp6-1
TGATGTTATTGCCTATGCTGCGCACATGGCAGCTTTGCTTGGCGCTAATATAATAAAAGT
Rcp6-8
TGATGTTATTGCCTATGCTGCGCACATGGCAGCTTTGCTTGGCGCTAATATAATAAAAGT
Rpp4-3
TGATGTTATTGCCTATGCTGCGCACATGGCAGCTTTGCTTGGCGCTAATATAATAAAAGT
Rpp4-1
TGATGTTATTGCCTATGCTGCGCACATGGCAGCTTTGCTTGGCGCTAATATAATAAAAGT
Rpp3-1
TGATGTTATTGCCTATGCTGCGCACATGGCAGCTTTGCTTGGCGCTAATATAATAAAAGT
Rcp6-2
TGATGTTATTGCCTATGCTGCACACATGGCAGCTTTGCTTGGCGCTAATATAATCAAAGT
                  ********************* ********************************
*****

Rcp6-6
AAAACTTCCAACTAAATATTTGGAAAGGGAGAAAATAGAAACAGAAAATATTGAATCATT
Rcp6-7
AAAACTTCCAACTAAATATTTGGAAAGGGAGAAAATAGAAACAGAAAATATTGAATCATT
Rpp4-2
AAAACTTCCAACTAAATATTTGGAAAGGGAGAAAATAGAAACAGAAAATATTGAATCATT
Rpp3-4
AAAACTTCCAACTAAATATTTGGAAAGGGAGAAAATAGAAACAGAAAATATTGAATCATT
Rpp3-3
AAAACTTCCAACTAAATATTTGGAAAGGGAGAAAATAGAAACAGAAAATATTGAATCATT
Rpp3-2
AAAACTTCCAACTAAATATTTGGAAAGGGAGAAAATAGAAACAGAAAATATTGAATCATT
Rcp6-1
AAAACTTCCAACTAAATATTTGGAAAGGGAGAAAATAGAAACAGAAAATATTGAATCATT
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Rcp6-8
AAAACTTCCAACTAAATATTTGGAAAGGGAGAAAATAGAAACAGAAAATATTGAATCATT
Rpp4-3
AAAACTTCCAACTAAATATTTGGAAAGGGAGAAAATAGAAACAGAAAATATTGAATCATT
Rpp4-1
AAAACTTCCAACTAAATATTTGGAAAGGGAGAAAATAGAAACAGAAAATATTGAATCATT
Rpp3-1
AAAACTTCCAACTAAATATTTGGAAAGGGAGAAAATAGAAACAGAAAATATTGAATCATT
Rcp6-2
AAAACTTCCAACTAGATATTTGGAAAAAGAAAAAATAGAAACAGAAAATATTGAATCATT
                ************** ***********  **
******************************

Rcp6-6          ATCTAAAAGAATTGAATATGTTAAAAGGTCTTGTTTTGCAGGGAAAA
Rcp6-7          ATCTAAAAGAATTGAATATGTTAAAAGGTCTTGTTTTGCAGGGAAAA
Rpp4-2          ATCTAAAAGAATTGAATATGTTAAAAGGTCTTGTTTTGCAGGGAAAA
Rpp3-4          ATCTAAAAGAATTGAATATGTTAAAAGGTCTTGTTTTGCAGGGAAAA
Rpp3-3          ATCTAAAAGAATTGAATATGTTAAAAGGTCTTGTTTTGCAGGGAAAA
Rpp3-2          ATCTAAAAGAATTGAATATGTTAAAAGGTCTTGTTTTGCAGGGAAAA
Rcp6-1          ATCTAAAAGAATTGAATATGTTAAAAGGTCTTGTTTTGCAGGGAAAA
Rcp6-8          ATCTAAAAGAATTGAATATGTTAAAAGGTCTTGTTTTGCAGGGAAAA
Rpp4-3          ATCTAAAAGAATTGAATACGTTAAAAGGTCTTGTTTTGCAGGGAAAA
Rpp4-1          ATCTAAAAGAATTGAATATGTTAAAAGGTCTTGTTTTGCAGGGAAAA
Rpp3-1          ATCTAAAAGAATTGAATATGTTAAAAGGTCTTGTTTTGCAGGGAAAA
Rcp6-2          ATCTAAAAGAATTGAATATGTTAAAAAATCTTGTTTTGCAGGAAAAA
                ****************** *******  ************** ****
```

Appendix IX Kimura-2-parameter distances calculated with MEGA4 (Tamura et al. 2007)

- *wsp*

[1] #wCin1	[2] #wCer1	[3] #wPom
[4] #wCer2	[5] #wCin2	[6] #wSpt
[7] #wDwi	[8] #wMel	[9] #wBo
[10] #wDin	[11] #wAso	[12] #wCer4

[1]
[2] 0.000
[3] 0.025 0.025
[4] 0.025 0.025 0.000
[5] 0.025 0.025 0.000 0.000
[6] 0.034 0.034 0.008 0.008 0.008
[7] 0.027 0.027 0.002 0.002 0.002 0.010
[8] 0.029 0.029 0.008 0.008 0.008 0.017 0.010
[9] 0.029 0.029 0.017 0.017 0.017 0.025 0.019 0.025
[10] 0.027 0.027 0.015 0.015 0.015 0.023 0.017 0.023 0.002
[11] 0.027 0.027 0.015 0.015 0.015 0.023 0.017 0.023 0.002 0.000
[12] 0.115 0.115 0.120 0.120 0.120 0.130 0.122 0.120 0.122 0.125 0.125

- *gat*B

[1] #wCin1	[2] #wCer1	[3] #wCer4
[4] #wMel	[5] #Dmun	[6] #wPom
[7] #wDbo	[8] #wCer2	[9] #Dana
[10] #Dsim	[11] #Ngi	[12] #wCer5

[1]
[2] 0.000
[3] 0.014 0.014
[4] 0.028 0.028 0.025
[5] 0.028 0.028 0.025 0.000
[6] 0.028 0.028 0.025 0.000 0.000
[7] 0.028 0.028 0.025 0.000 0.000 0.000
[8] 0.028 0.028 0.025 0.000 0.000 0.000 0.000
[9] 0.036 0.036 0.028 0.014 0.014 0.014 0.014 0.014
[10] 0.036 0.036 0.028 0.014 0.014 0.014 0.014 0.014 0.000
[11] 0.031 0.031 0.028 0.019 0.019 0.019 0.019 0.019 0.022 0.022
[12] 0.149 0.149 0.156 0.143 0.143 0.143 0.143 0.143 0.157 0.157 0.142

- *cox*A

[1] #*w*Cin1	[2] #wCer1+4	[3] #wSol
[4] #wMono	[5] #wCal	[6] #wNa
[7] #wDbor	[8] #wBo	[9] #*w*Pom
[10] #wMel	[11] #wCer2	[12] #wAna
[13] #wNvi	[14] #wCer5	

[1]
[2] 0.000
[3] 0.005 0.005
[4] 0.005 0.005 0.000
[5] 0.005 0.005 0.000 0.000
[6] 0.036 0.036 0.036 0.036 0.036
[7] 0.036 0.036 0.036 0.036 0.036 0.000
[8] 0.036 0.036 0.036 0.036 0.036 0.000 0.000
[9] 0.036 0.036 0.036 0.036 0.036 0.000 0.000 0.000
[10] 0.036 0.036 0.036 0.036 0.036 0.000 0.000 0.000 0.000
[11] 0.036 0.036 0.036 0.036 0.036 0.000 0.000 0.000 0.000 0.000
[12] 0.028 0.028 0.028 0.028 0.028 0.008 0.008 0.008 0.008 0.008 0.008
[13] 0.023 0.023 0.028 0.028 0.028 0.013 0.013 0.013 0.013 0.013 0.013 0.015
[14] 0.139 0.139 0.143 0.143 0.143 0.139 0.139 0.139 0.139 0.139 0.139 0.133 0.139

- *fbp*A

[1] #*w*Cin1	[2] #wCer1	[3] #wChl
[4] #Nas	[5] #wDSim	[6] #wMel
[7] #wCin2	[8] #*w*Pom	[9] #wCer2
[10] #wAna	[11] #wCer4	[12] #wCer5

[1]
[2] 0.000
[3] 0.027 0.027
[4] 0.027 0.027 0.000
[5] 0.027 0.027 0.000 0.000
[6] 0.027 0.027 0.000 0.000 0.000
[7] 0.027 0.027 0.000 0.000 0.000 0.000
[8] 0.027 0.027 0.000 0.000 0.000 0.000 0.000
[9] 0.027 0.027 0.000 0.000 0.000 0.000 0.000 0.000
[10] 0.034 0.034 0.022 0.022 0.022 0.022 0.022 0.022 0.022
[11] 0.060 0.060 0.052 0.052 0.052 0.052 0.052 0.052 0.052 0.034
[12] 0.153 0.153 0.147 0.147 0.147 0.147 0.147 0.147 0.147 0.147 0.160

- *fts*Z

[1] #*w*Cin1	[2] #wCer1	[3] #*w*Cin2
[4] #*w*Pom	[5] #wAso	[6] #wAu
[7] #wCer2	[8] #wDsi	[9] #wDia
[10] #wDmel	[11] #wCer4	[12] #wCer5

```
[ 1]
[ 2]  0.000
[ 3]  0.013 0.013
[ 4]  0.013 0.013 0.000
[ 5]  0.013 0.013 0.000 0.000
[ 6]  0.013 0.013 0.000 0.000 0.000
[ 7]  0.013 0.013 0.000 0.000 0.000 0.000
[ 8]  0.013 0.013 0.000 0.000 0.000 0.000 0.000
[ 9]  0.013 0.013 0.004 0.004 0.004 0.004 0.004 0.004
[10]  0.010 0.010 0.002 0.002 0.002 0.002 0.002 0.002 0.002
[11]  0.021 0.021 0.013 0.013 0.013 0.013 0.013 0.013 0.013 0.010
[12]  0.111 0.111 0.111 0.111 0.111 0.111 0.111 0.111 0.111 0.108 0.106
```

- *hcp*A

[1] #*w*Cin1	[2] #wCer1	[3] #*w*Cin2
[4] #*w*Pom	[5] #wAso	[6] #wAu
[7] #wCer2	[8] #wDsi	[9] #wDia
[10] #wDmel	[11] #wCer4	[12] #wCer5

```
[ 1]
[ 2]  0.000
[ 3]  0.013 0.013
[ 4]  0.013 0.013 0.000
[ 5]  0.013 0.013 0.000 0.000
[ 6]  0.013 0.013 0.000 0.000 0.000
[ 7]  0.013 0.013 0.000 0.000 0.000 0.000
[ 8]  0.013 0.013 0.000 0.000 0.000 0.000 0.000
[ 9]  0.013 0.013 0.004 0.004 0.004 0.004 0.004 0.004
[10]  0.010 0.010 0.002 0.002 0.002 0.002 0.002 0.002 0.002
[11]  0.021 0.021 0.013 0.013 0.013 0.013 0.013 0.013 0.013 0.010
[12]  0.111 0.111 0.111 0.111 0.111 0.111 0.111 0.111 0.111 0.108 0.106
```

Appendix X Sources of Figures and tables

Fig 1.1: http://scilogs.be/starttoknow/gallery/5/Wolbachia.jpg

Fig 1.2: BALDO ET AL. 2006

Fig 1.3: Fly from www.bugguide.net

Fig 1.4: RIEGLER & STAUFFER 2002

Fig 1.5: ARTHOFER ET AL. 2009B

Fig 1.6: (from up to right) www.biohelp.at, www.inra.fr, www.lfl.bayern.de, www.nd.edu

Fig 1.7: www.bugguide.net

Fig 1.8: www.bugguide.net

Fig 1.9: FEDER 1998

Fig 2.1: Forestryimages.org

Fig 2.2: www.virgignafruit.ento.vt.edu

Table 3.1: BALDO ET AL. 2006

7. Curriculum vitae

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Education

2008-2009:	Diploma thesis "The Endosymbiont *Wolbachia* in *Rhagoletis pomonella* and *R. cingulata* (Diptera, Tephritidae)" Institute of Forest Entomology, Forest Pathology & Forest Protection, Department of Forest & Soil Sciences Boku, Vienna
2003 – 2009	student of agriculture and phytomedicine at the University of Natural Resources & Applied Life Sciences Boku in Vienna
1998 – 2003	agricultural high school in Auer, Bozen, Italy
1990 – 1998	primary school

Experiences

	practical work at the parental apple orchards
	tutor for the course *Plant Protection* at the Institute of Plant Protection, Boku
	tutor for the course *Agricultural Diagnostics of Damages* at the Institute of Plant Protection, Boku
	Erasmus Semester in Bologna, Italy
	English language course in Portsmouth, UK

Publications

SCHULER H, ARTHOFER W, KRUMBÖCK S, KÖPPLER K, VOGT H, TEIXEIRA LAF, RIEGLER M, STAUFFER C (2009) The bacterial endosymbiont *Wolbachia* in the invasive cherry pest *Rhagoletis cingulata* (Diptera, Tephritidae). Proceedings of the German Society for General and Applied Entomolgy, submitted.

SCHULER H, ARTHOFER W, KÖPPLER K, VOGT H, TEIXEIRA LAF, RIEGLER M, STAUFFER C (2009) *Wolbachia* in *Rhagoletis* spp. *Entomologentagung DGaaE*, Göttingen – Posterpräsentation

ARTHOFER W, KRUMBOECK S, SCHULER H, RASOOL B, RIEGLER M, KOEPPLER K, STAUFFER C (2009) Thirteen new microsatellite loci in Rhagoletis cerasi (Diptera: Tephritidae), a model host species for Wolbachia symbiosis in field populations. *Molecular Ecology Resources*, submitted

Publications and Poster

The bacterial endosymbiont *Wolbachia* in the invasive cherry pest *Rhagoletis cingulata* (Diptera, Tephritidae)

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Zusammenfassung Wir berichten über zwei *Wolbachia* Isolate in einer europäischen Population der amerikanischen Kirschfruchtfliege *Rhagoletis cingulata*,. Die Isolate *w*Cin1 und *w*Cin2 wurden durch Amplifikation, Klonierung und Sequenzierung des *Wolbachia surface protein* (*wsp*) Gens identifiziert. Eine phlyogenetische Analyse der *wsp* Region ergab, dass *w*Cin1 und *w*Cin2 ident mit *w*Cer1 und *w*Cer2 sind, welche in der Europäischen Kirschfruchtfliege, *R. cerasi*, gefunden wurden. Potentieller horizontaler *Wolbachia* Transfer und mögliche Folgearbeiten werden diskutiert.

Key Words: *Rhagoletis*, *Wolbachia*, invasive species

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Introduction

Wolbachia is a gram negative endosymbiotic bacterium found in up to 65% of insect species (HILGERBOECKER & al. 2008). Infections have been detected in all major orders of insects and some other arthropod taxa (WERREN & al. 2008). Although it's main path

of transmission is transovarial through the cytoplasm of host eggs *Wolbachia* is supposed to occasionally jump horizontally among species (BALDO & al. 2008). *Wolbachia* can change the reproductive traits of its hosts to enhance colonization of the germline and vertical transmission (WERREN & al. 2008). Cytoplasmic incompatibility (CI) is the most common phenotype in insects. It leads to embryonic death of fertilized eggs when infected males mate with uninfected females, while matings between infected males and females are compatible. This results in a reproductive advantage of infected over uninfected females and leads to increased infection rates in host populations over generational cycles (HOFFMANN & TURELLI, 1997).

Based on extensive single pair crossing experiments, BOLLER & al. (1976) concluded that populations of the European cherry fruit fly, *Rhagoletis cerasi*, are divided into two geographic complexes which exhibit unidirectional incompatibility. BLÜMEL & RUSS (1989) detected Rickettsia Like Organisms (RLOs) in the ovaries of individuals in all populations. By applying PCR techniques with *Wolbachia* specific primers, RIEGLER & STAUFFER (2002) detected two different *Wolbachia* strains, *w*Cer1 and *w*Cer2 in cherry fruit fly populations. Transinfection experiments with *w*Cer2 revealed complete CI in the Mediterranean fruit fly, *Ceratitis capitata* and cage experiments demonstrated that *Wolbachia*-induced CI could be used as a tool for population control (ZABALOU & al. 2004).

It has recently been reported that the American cherry fruit fly, *R. cingulata*, is present in Europe. So far, the species has been found in Austria, Germany, Hungary, Slovenia and Switzerland (BOLLER 2000, DANIEL & WYSS 2007, EGARTNER & al. 2008, EPPO 2006, EPPO 2007a, EPPO 2007b, VOGT & al. 2009). *R. cingulata* has a similar biology as *R. cerasi* with the exception of required higher temperatures for *R. cingulata* pupae to reach maturity and delayed emergence of adults from the soil (VOGT & al. 2009). *R. cingulata* is a serious pest in cherries in Northeast American regions (BUSH 1966, ROTHWELL & al. 2006).

Here we investigated *Wolbachia* infections in *R. cingulata* from a German population. We discuss potential horizontal *Wolbachia* transmission between *R. cingulata* and *R. cerasi*, as both species might co-occur in the same cherries. The *Wolbachia* detection was carried out by PCR using *wsp* primers and subsequent cloning and sequencing of the

amplicons.

Materials & Methods

R. cingulata flies were collected from yellow sticky traps in Heidesheim, Germany, in 2008 and stored in absolute ethanol at -20°C. DNA of two individual flies was extracted using the Sigma GenElute Mammalian DNA extraction Kit (Sigma) following the protocol of the manufacturer. DNA was eluted in 50 µl TE (10 mM Tris, 1 mM EDTA, pH=8.0) and stored at -20°C. All PCR reactions were performed on a 2720 thermal cycler (Applied Biosystems) in a total volume of 10 µl containing: 1x Mg-free buffer (Fermentas), 2 mM $MgCl_2$, 100 µM dNTPs, 0.2 µM of each primer, 0.25 U Taq polymerase (Fermentas) and 0.8 µl template DNA. Cycling conditions for universal *wsp* amplification using the primers wsp81F and wsp691R (BRAIG & al. 1998) were 95°C for 2 min followed by 32 cycles at 94°C for 30 sec, 60°C for 45 sec, 72°C for 1 min and a final extension at 68°C for 15 min. For cloning, a 0.8 µl aliquot of PCR product was ligated into the pTZ57R vector of the InstaClone PCR cloning kit (Fermentas) according to the instructions of the manufacturer. The ligated plasmids were used for transformation of competent JM109 *E. coli* cells and after overnight growth white colonies were picked and transferred to liquid LB medium. Insert size was determined by PCR with M13 vector primers and plasmid DNA was extracted by alkaline lysis. Sanger sequencing was performed by a commercial provider. Retrieved sequences were edited manually, aligned using ClustalX (THOMPSON & al. 1997) and compared with *Wolbachia* sequences from GenBank by BLAST analysis.

Results

PCR with the *Wolbachia* specific primers resulted in positive amplicons in the two analysed German individuals. These two amplicons were cloned and from each 21 plasmids were sequenced. Sequence analysis revealed that both individuals are infested by two *Wolbachia* variants which were named *w*Cin1 and *w*Cin2. A BLAST search and subsequent alignment revealed that *wsp* sequnces of *w*Cin1 and *w*Cin2 are identical to those from *w*Cer1 and *w*Cer2 detected in *R. cerasi*.

Discussion

We report about two *Wolbachia* sequence variants in the American cherry fruit fly *R. cingulata*. *wsp* of *w*Cin1 and *w*Cin2 are identical to *w*Cer1 and *w*Cer2 detected in *R.*

cerasi. This suggests a horizontal strain transfer between the two cherry fruit fly species. To further test this hypothesis we will need to characterise more loci of the *w*Cin isolates by Multi Locus Strain Typing (MLST) as described by BALDO & al. (2006) and compare with the MLST loci of *w*Cer. This characterization will give deeper insight into the genomes of the strains and might reveal differences between the Cin and the Cer-strains. In order to interpret the direction of transfer it has to be tested whether American populations of *R. cingulata* are also infected by *w*Cin1 and *w*Cin2.

Acknowledgements

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References

BALDO, L., AYOUB, N.A., HAYASHI, C.Y., RUSSELL, J.A., STAHLHUT, J.K. & WERREN, J.H. (2008): Insight into the routes of *Wolbachia* invasion: high levels of horizontal transfer in the spider genus *Agelenopsis* revealed by *Wolbachia* strain and mitochondrial DNA diversity. -Molecular Ecology 17: 557-569.

BALDO, L., DUNNING HOTOPP, J.C., JOLLEY, K.A., BORDENSTEIN, S.R., BIBER, S.A., CHOUDHURY, R.R., HAYASHI, C., MAIDEN, M.C.J., TETTELIN, H. & WERREN, J.H. (2006): Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. - Applied and Environmental Microbiology 72: 7098-7110.

BLÜMEL, S. & RUSS, K. (1989): Manipulation of races. - In: *Fruit flies: Their Biology, Natural Enemies and Control*. Vol. 3B (eds. ROBINSON, A.S. & HOOPER, G.), pp. 387-389.

BOLLER, E.F., RUSS, K., VALLO, V. & BUSH, G.L. (1976): Incompatible races of European cherry fruit fly, *Rhagoletis cerasi* (Diptera: Tephritidae), their origin and potential use in biological control. - Entomologia Experimetalis & Applicata 20: 237-247.

BOLLER, E., (2000): Situationsbericht über die nordamerikanische Fruchtfliegenarten in der Schweiz (Diptera: Tephritidae). - Eidgenössische Forschungsanstalt für Obst-, Wein- und Gartenbau, Wädenswil, 5pp.

BRAIG, H.R., ZHOU, W., DOBSON, S.L. & O'NEILL, S.L. (1998): Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. - Journal of Bacteriology 180: 2373-2378.

BUSH, G.L. (1966): The taxonomy, cytology and evolution of the genus *Rhagoletis* in North America (Diptera: Tephritidae). - Bulletin of the Museum of Comparative Zoology, 134:431-526.

DANIEL, C. & WYSS, E. (2007): Zum Auftreten der Amerikanischen Kirschfruchtfliege in der Nordwestschweiz und im Tessin. http://orgprints.org/10679.

EGARTNER, A., ZEISNER, N., HAUSDORF, H., LETHMAYER, C. & BLÜMEL, S. (2008): Investigations on the occurrence of the quarantine fruit fly species *Rhagoletis cingulata* and *Rhagoletis indifferens* on *Prunus avium* and *Prunus cerasus* in Austria. - In: *International Conference on Integrated Fruit Production, IOBC wprs*. Avignon, France, 2008, Abstracts, 82.

EPPO (2006): Current situation of *Rhagoletis cingulata* in Germany (2006/003). Reporting Service 2006, 1: p.3. http://www.eppo.org/PUBLICATIONS/reporting/reporting_service.htm

EPPO (2007a): *Rhagoletis cingulata* was reported for the first time in 2006 in Hungary. Reporting Service 2007, 6: p. 2. http://www.eppo.org/PUBLICATIONS/reporting/reporting_service.htm

EPPO (2007b): First outbreak of *Rhagoletis cingulata* in Slovenia (2007/148). Reporting Service 2007, 8, p.3. http://www.eppo.org/PUBLICATIONS/reporting/reporting_service.htm

HILGENBOECKER, K., HAMMERSTEIN, P., SCHLATTMANN, P., TELSCHOW & A., WERREN, J.H. (2008): How many species are infected with *Wolbachia*? – a statistical analysis of current data. - FEMS Microbiology Letters 281: 215-220.

HOFFMANN, A.A., & TURELLI, M. (1997): Cytoplasmatic incompatibility in insects. In: *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction,* Oxford: Oxford University Press, (eds. O'NEILL, S.L., HOFFMANN, A.A., & WERREN, J.H.). pp. 42-80.

RIEGLER, M. & STAUFFER, C. (2002): *Wolbachia* infections and superinfections in cytoplasmically incompatible populations of the European cherry fruit fly *Rhagoletis cerasi* (Diptera, Tephritidae). - Molecular Ecology 11: 2425-2434.

ROTHWELL, N., GUT, L & TEIXEIRA, L. (2006). Cherry fruit fly ecology and management. Michigan State University Fruit CAT Letter. 21 (9).

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D.G. (1997): The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. - Nucleic Acids Research 24: 4876-4882.

VOGT, H.,, KÖPPLER, K., DAHLBENDER, W. & HENSEL, G. (2009): Observations of *Rhagoletis cingulata*, an invasive species from North America, on cherry in Germany 2009. In: *Proceedings of the International Conference on Integrated Fruit Production, IOBC*. woprs, Avignon, France, 2008. Bulletin IOBC wprs *In Press*

WERREN, J.H., BALDO, L. & CLARK, M.E. (2008): *Wolbachia*: master manipulators of invertrebrate biology. - Nature Reviews Microbiology 6: 741-751.

ZABALOU, S., RIEGLER, M., THEODORAKOPOULOU, M., STAUFFER, C., SAVAKIS, C. & BOURTZIS, C. (2004): *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. - Proceedings of the National Academy of Sciences of the USA 101: 15042-15045.