Masterarbeit

Infection dynamics of the *Wolbachia* strain *w*Cer2 in a German transect of *Rhagoletis cerasi*

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Abstract

Wolbachia is the most common and widespread intracellular bacterium found in arthropods and nematodes. This endosymbiont attracted considerable interest in the past decade for its fascinating manipulating effects on hosts. To ensure its own survival, it successfully alters host reproduction in many ways, including cytoplasmatic incompatibility. This mechanism results in high egg mortality if infected males mate with females that lack the same *Wolbachia* type.

The European cherry fruit fly *Rhagoletis cerasi* (Diptera, Tephritidae) is a serious pest of sweet and sour cherry cultures in Europe. Crossing experiments of flies between southern and central European populations with populations from northern territories resulted in strong unidirectional incompatibility, caused by the *Wolbachia* strain *w*Cer2. In central Germany, geographical distribution of *w*Cer2 in populations of *R. cerasi* shows clear transition zones in which *w*Cer2 infected fruit flies introgress into populations not infected by this strain. These areas offer a unique opportunity to study the distribution and spread of the endosymbiont in action.

In this thesis, I study the infection dynamics of *w*Cer2 and its association with two different mitochondrial haplotypes in *R. cerasi* in Hesse, Germany. Screening of 295 individuals from 19 different locations showed fixation of *w*Cer2 in three populations, 13 transitional populations with infection rates ranging from 6.2% to 75%, and three populations not infected by *w*Cer2. *w*Cer2 uninfected cherry fruit flies were almost perfectly associated with haplotype 1 (with one single individual that was associated with haplotype 2), 87.8% of the *w*Cer2 infected individuals were associated with haplotype 2, suggesting frequent intraspecific horizontal transmission of *w*Cer2. Comparison of new data from 2016 with previous data from the last 15 years showed a general increase in infection rates of *w*Cer2 in Hesse.

Zusammenfassung

Wolbachia ist ein in Arthropoden und Nematoden weit verbreitetes intrazelluläres Bakterium. Um seinen Fortbestand und seine Verbreitung zu sichern, manipuliert dieser Endosymbiont die Reproduktion seiner Wirte. Die Induktion einer zytoplasmatischen Incompatibilität führt zu einer hohen Sterblichkeitsrate der Nachkommen, wenn sich *Wolbachia*-infizierte Männchen mit nicht infizierten Weibchen paaren.

Die Europäische Kirschfruchtfliege *Rhagoletis cerasi* (Diptera, Tephritidae) ist ein in Europa weit verbreiteter Schädling an der Kulturkirsche. Kreuzungsversuche zwischen Fliegen aus Süd- und Zentraleuropa mit Fliegen aus nordeuropäischen Populationen zeigten starke unidirektionale Inkompatibilitäten verursacht durch den *Wolbachia*-Stamm *w*Cer2. In den letzten 15 Jahren hat sich dieser Bakterienstamm in Deutschland ausgebreitet. In Zentraldeutschland sind zahlreiche Populationen noch uninfiziert. Zwischen den *w*Cer2 infizierten Populationen im Norden und den uninfizierten Populationen in Zentraldeutschland liegen Populationen in denen sich derzeit *w*Cer2 infizierte Fliegen ausbreiten. Der Bundesstaat Hessen ist umrundet von solchen Transekten und bietet daher eine einmalige Möglichkeit um die Verbreitung von *Wolbachia* zu studieren.

Diese Arbeit befasst sich mit der Verbreitung der *Wolbachia*-Stamms *w*Cer2 in 19 Populationen in Hessen. Darüber hinaus wird die Assoziation von *w*Cer2 infizierten und uninfizierten Fliegen mit deren mitochondrialen Haplotypen HT1 und HT2 untersucht. Die Charakterisierung von 295 Individuen zeigen eine Fixierung von *w*Cer2 in drei Populationen, Infektionsraten von 6,2% bis 75% in 13 Populationen der Übergangszonen, sowie keine *w*Cer2 Infektion in drei weiteren Populationen. Individuen die nicht mit *w*Cer2 infiziert sind zeigen eine Assoziationsrate mit HT1 von fast 100%, während nur 87,7% der *w*Cer2-infizierten Kirschfruchtfliegen mit HT2 assoziiert sind. Dies deutet auf häufige horizontale Transmission innerhalb dieser Art hin.

Danksagung

Ich möchte mich an dieser Stelle bei den vielen Menschen bedanken die es mir ermöglicht haben, diese Arbeit zu vollenden.

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1. Introduction

1.1 The European cherry fruit fly *Rhagoletis cerasi*

1.1.1 Distribution and origins

Rhagoletis cerasi, the European cherry fruit fly, is a serious pest of sweet and sour cherries in Europe, that belongs to the family of Tephritidae (Diptera) (Boller and Prokopy, 1976; Daniel and Grunder, 2012; Stamenković et al., 2012). The genus *Rhagoletis* includes about 65 known species that are important pest species all over the world, attacking apple (*Rhagoletis pomonella)*, blueberry (*Rhagoletis mendax)*, cherry (*Rhagoletis cerasi*, *Rhagoletis cingulata*, *Rhagoletis indifferens* and *Rhagoletis fausta*) and walnut (*Rhagoletis suavis*, *Rhagoletis completa*, *Rhagoletis juglandis*, *Rhagoletis zoqui*, *Rhagoletis boycei* and *Rhagoletis ramosae*) (White and Elson-Harris, 1992; Boller and Prokopy, 1976; Daniel and Grunder, 2012).

R. cerasi is widely distributed throughout Europe, Russia and to some extent in the more temperate parts of Asia (Moraiti et al., 2012). It mainly infests fruits of various *Prunus* sp. (Rosaceae; *Prunus cerasus, Prunus avium, Prunus mahaleb*) and *Lonicera* sp. (Caprifoliaceae; *Lonicera xylosteum, Lonicera tatarica*) (Boller and Bush, 1974; White and Elson-Harris, 1992; Daniel and Grunder, 2012; Stamenković et al., 2012).

1.1.2 Biology

Adult *R. cerasi* show sizes ranging from 4-5 mm, females are typically slightly larger than males. A bright black thorax, striped wing patterns and a yellow scutellum are typical characteristics that can be used to distinguish the species from other congeners (Figure 1.1). Like most *Rhagoletis* species, cherry fruit flies are univoltine and oligophagous, nutrients include honeydew, floral nectar and bird feces (Boller and Prokopy, 1976; Daniel and Grunder., 2012).

To detect the host-plant, several different factors have been identified: foliage color, tree shape and tree size, the odor, shape and color of the host-fruit seem to play a major role in host detection (Boller and Prokopy, 1976).

Figure 1.1 *Adult cherry fruit fly R. cerasi: typical black body with yellow markings on thorax and characteristic wing patterns (image courtesy of Ben Hamers, www.diptera.info/photogallery.php?photo_id=3213).*

Once a suitable spot has been established, male *R. cerasi* defend their territories on the fruit and wait for females, while females use pheromones to attract males and therefore increase the probability for mating which is also initiated near or on the host fruit (Boller and Prokopy, 1976).

Under optimal environmental conditions, female cherry fruit flies oviposit about 300-400 eggs into suitable growing fruits, usually not more than one egg per clutch is laid. To prevent repeated oviposition into the same fruit, a marking pheromone is deposited on the surface of the fruit (Boller and Prokopy, 1976; Daniel and Grunder, 2012; Stamenković et al., 2012).

Embryonic development lasts between two to ten days and largely depends on temperature and abiotic factors (Daniel and Grunder, 2012). To protect against predators, emerged larvae immediately move towards the cherry pit until their larval development is completed. High sugar content and low acidity in ripe cherry fruits and high temperature is preferred for faster development which can last between 17 to 30 days (Boller and Prokopy, 1976; Daniel and Grunder, 2012).

When fruit abscise from the tree, mature larvae bore exit holes through the fruit skin and burrow into the soil. They pupate within a few days, during this time they are especially vulnerable to predators such as ants as well as desiccation (Boller and Prokopy, 1976; Papanastasiou and Papadopoulos, 2014).

Being a univoltine species, pupae of *R. cerasi* remain in the soil and enter diapause (Boller and Prokopy, 1976; Stamenković et al., 2012). To proceed with their development, a period of low temperature is necessary (Boller and Prokopy, 1976; Daniel and Grunder, 2012; Moraiti et al., 2014). Pupae emerge in the following year in spring, synchronized to the phenology of their host, to ensure that maladaptation to fruiting time or temperature fluctuation does not eliminate the entire population they often remain for another two to five years in the soil before evolving to adults (Boller and Prokopy, 1976; Daniel and Grunder, 2012).

Figure 1.2 *Infested cherry by R. cerasi. The white larvae develop inside the fruits and can reach a size of up to 6 mm. After their larval development is complete they leave the fruit and fall to the ground (https://upload.wikimedia.org/wikipedia/commons/thumb/6/67/Kirschmade.jpg/266px-Kirschmade.jpg).*

Before oviposition, adults need to go through a maturation process which lasts from six to 13 days and is influenced by temperature and fruit maturity phase. Lifespan of *R. cerasi* is estimated to last between four and seven weeks, flight periods overall range from seven to eleven weeks (Daniel and Grunder, 2012; Stamenković et al., 2012).

1.1.3 Management and pest control

Because of the low tolerance for infested cherries, a number of different pest control approaches have been suggested and tested in the field (Boller and Prokopy, 1976; Daniel and Grunder, 2012; Stamenković et al., 2012). Chemical, biological and biotechnological control, cultural practices and precautionary measures as well as the use of attractants and repellents have been used to more or less success. The Sterile insect technique (SIT), a species-specific method for insect control where large numbers of sterile males that exceed a certain threshold are released in order to decrease the female reproductive potential and suppress or even eliminate pest populations, has been tested to reduce infestation levels. Due to difficulty in rearing of *R. cerasi* to produce a critical mass of sterile insects, the method never got commercially introduced (Boller and Prokopy, 1976; Daniel and Grunder, 2012; Zhang et al., 2015).

1.2 The intracellular bacterium *Wolbachia*

1.2.1 Distribution, origin and genomics

Wolbachia are intracellular, maternally transmitted α-proteobacteria that are found in numerous insects (Werren, 1997; Werren and Windsor, 2000; Jiggins et al., 2001; Lo et al., 2007). These gram-negative microbes are typically located within reproductive organs (ovaries and testes), depending on different *Wolbachia*-host associations they can also be found in somatic cells distributed throughout most other tissues, i.a. brain, muscles, midgut, wings and haemolymph (Werren, 1997; Dobson et al., 1999; Sicard et al, 2014).

Wolbachia are common and highly widespread bacteria, infecting a broad range of different insect species, isopods, mites and filarial nematodes (Werren, 1997; Zhou et al., 1998). In terrestrial arthropods, they are the predominant bacterial endosymbiont, estimated to infect 52% of arthropod insect species (Weinert et al., 2015).

In a survey on Rickettsia-like microorganisms in 1924, it was first discovered as an inhabitant of the mosquito *Culex pipiens* (Hertig and Wolbach, 1924). In 1936 it was formally described and the name *Wolbachia pipientis* was proposed (Hertig, 1936).

In recent decades, advances in molecular methods – most notably the use of polymerase chain reaction (PCR) – as well as modern sequencing techniques have revealed that *Wolbachia* are members of the order *Rickettsiales* and are related to the genera *Anaplasma*, *Ehrlichia* and *Rickettsia* (Figure 1.3; Werren, 1997; Lo et al., 2007; Werren et al., 2008).

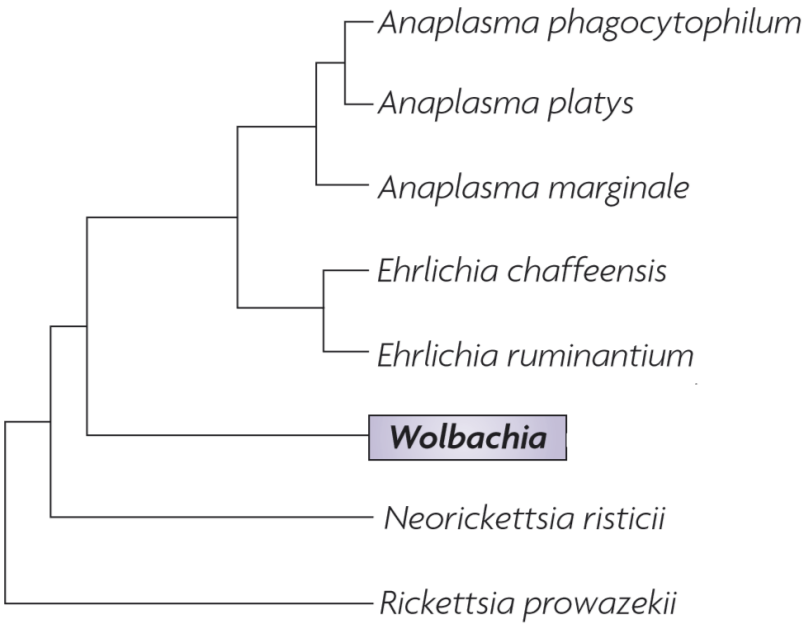

Figure 1.3 *Phylogeny of Wolbachia: phylogenetic tree showing genetic relationships to the genera Anaplasma, Ehrlichia and Rickettsia (Werren et al., 2008).*

Similar to other obligate endosymbionts, *Wolbachia* have relatively small genomes ranging from 1.08 to 1.7 Mb, that show large segments of repetitive and mobile elements (Baldo et al., 2005; Werren et al., 2008; Correa and Ballard, 2016). Molecular data on the 16S rRNA gene, five multilocus sequence typing loci (MLST) as well as the *Wolbachia* surface protein gene (*wsp*) show genetical similarities that are used to divide *Wolbachia* into so called “supergroups”, a term that refers to main evolutionary lineages (Zhou et al., 1998; Lo et al., 2002; Werren et al., 2008; Glowska et al., 2015). Given the high genetic diversity of the bacteria and the improvement in detection accuracy, 16 of these groups have already been described so far (Ros et al., 2009; Glowska et al., 2015).

Supergroups A and B are most commonly found in arthropods, other supergroups are primarily found in filarial nematodes (C and D), springtails (E), termites (H), aphids (M and N) and mites (F) (Werren et al., 2008; Ros et al., 2009; Glowska et al., 2015). Depending on the relationship with their host, *Wolbachia* shows a broad range of interactions. The endosymbiont can have negative (parasitism), beneficial (mutualism) or neutral (commensialism) effects on its host. In general, interactions can represent a continuum from mutualism to parasitism (Figure 1.4; Werren et al., 2008).

There has been an ongoing debate on accurate taxonomy and a uniform nomenclature system in the *Wolbachia* field, by convention the bacteria are currently commonly referred to simply as *Wolbachia* with additional strain designation based on their host and supergroup identification (Lo et al. 2007; Werren et al., 2008). Lindsey et al. (2016a) recommend to continue the current classification system until further evidence for species designations within this clade occurs (Lo et al. 2007; Werren et al., 2008).

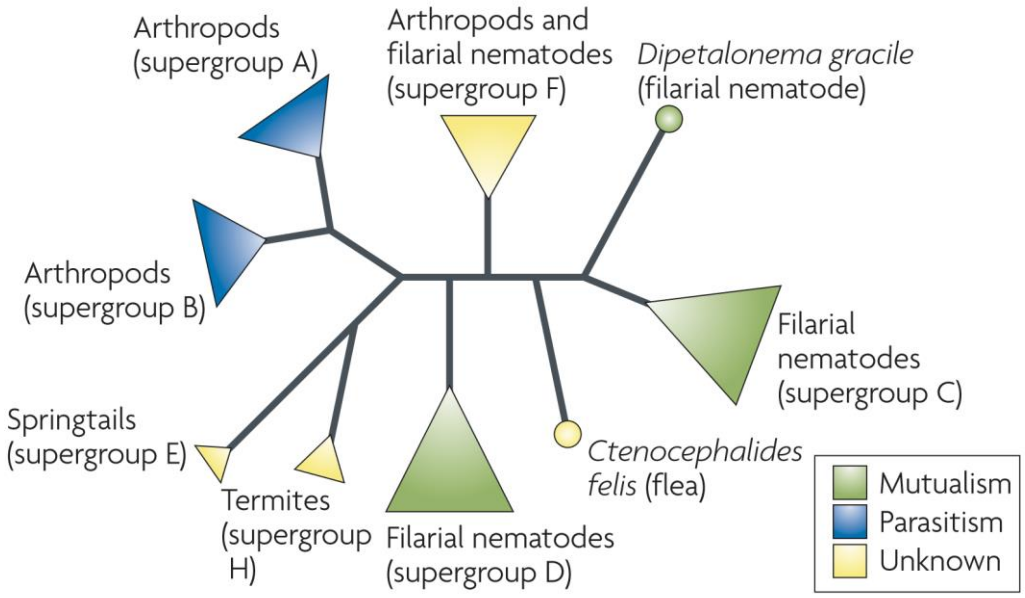

Figure 1.4 *Main supergroups of Wolbachia including dominant patterns of parasitism and mutualism in the host (Werren et al., 2008).*

1.2.2 Biology and transmission

Early observations of *Wolbachia* describe it as a small, spherical or elongate pleomorphic bacterium with sizes ranging from 0.5 to 1.5µm. They have two cell membranes and commonly appear enclosed within a host vacuole (Figure 1.5; Dobson, 2003; Riegler and O'Neill, 2006).

Wolbachia has a very high infection frequency among insects and is probably the most widespread intracellular bacteria known so far (Baldo et al., 2006). This is mainly achieved through maternal inheritance, strains are typically vertically transmitted through the cytoplasm of the host eggs (Werren, 1997; Hong et al., 2002; Baldo et al., 2006; Werren et al., 2008; Zug and Hammerstein, 2012; Correa and Ballard, 2016). Paternal transmission of *Wolbachia* has been described, but seem to occur only rarely if at all (Hoffmann et al., 1990; Turelli and Hoffmann, 1995; Werren, 1997; Arthofer et al., 2009b).

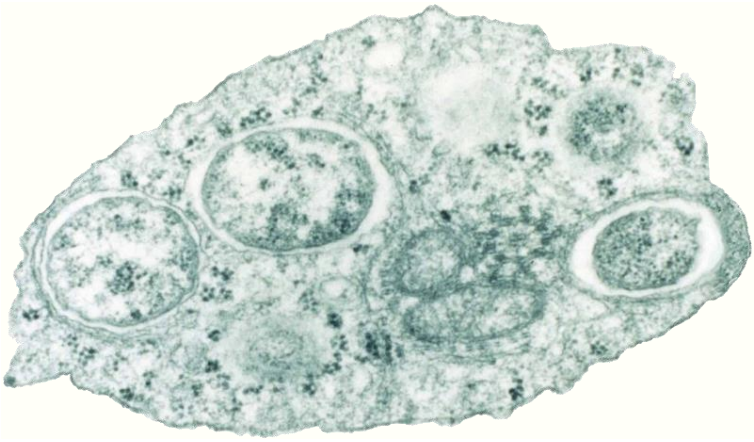

Figure 1.5 *Transmission electron micrograph of Wolbachia inside an insect cell. Commonly the endosymbiont is observed within a host vacuole membrane (Riegler and O'Neill, 2006).*

1.2.3 *Wolbachia* induced phenotypes

Wolbachia evolved various mechanisms that promote its distribution into new populations and species. By altering and manipulating cellular and reproductive processes, they successfully increase the fitness of infected females and thus ensure their own spread (Figure 1.6; Werren et al., 2008; Engelstädter and Hurst, 2009; Correa and Ballard, 2016). The occurring phenotypic alterations on the hosts generally promote the reproduction of infected females and/or discriminate uninfected ones, leading to the transmission and spread of the endosymbiont (Correa and Ballard, 2016).

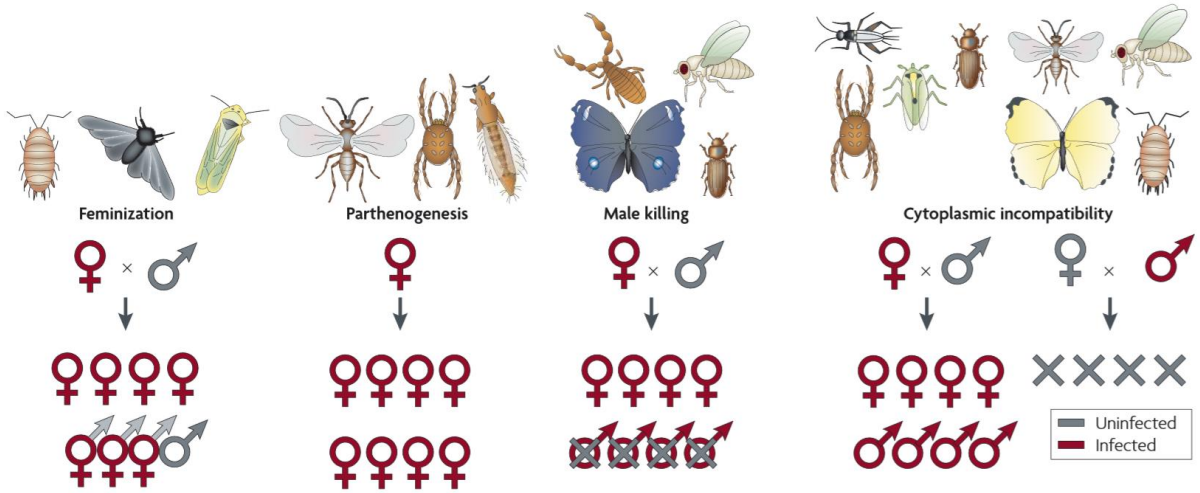

Figure 1.6 *Wolbachia mainly induces four distinct reproductive phenotypes: Feminization, Parthenogenesis, Male-killing and cytoplasmatic incompatibility. Depending on different strains and interaction with the host, these altering mechanisms ensure reproduction and survival of the endosymbionts (Werren et al., 2008).*

1.2.3.1 Feminization

Wolbachia-induced feminization was first described as a phenomenon in Isopoda but has been also observed in Hemiptera and Lepidoptera orders (Vandekerckhove et al., 2003; Werren et al., 2008; Kern et al., 2015). The manipulating abilities of the endosymbiont alter the hosts reproductive system and result in genetic males that develop as females. This sex ratio distorting mechanism leads to an increase in the number of females relative to males, resulting in infection spread of *Wolbachia* and female sex bias of the host population (Correa and Ballard., 2016). While the exact mechanism of feminization is still unclear, research on different infected species have shown interference and complex interactions with their sex-determination systems that

occur at an early embryonic event (Vandekerckhove et al., 2003; Negri et al., 2006; Narita et al., 2007; Werren et al., 2008).

1.2.3.2 Parthenogenesis

Parthenogenesis has been described only in species with arrhenotokous development (where haploid males develop from unfertilized eggs). *Wolbachia* is able to manipulate the ploidy of its host that leads to the development of females from unfertilized eggs. This mechanism has been described in mites, thrips and hymenopterans such as wasps (Stouthamer and Kazmer, 1994; Werren et al., 2008). Stouthamer et al. (1990) showed that parthenogenetic *Trichogramma* wasps can be rendered permanently bisexual by treating the insects with antibiotics or high temperatures, removing their *Wolbachia*. Because of these findings, the authors concluded that maternally inherited microorganisms cause parthenogenesis in these wasps. The altering mechanisms caused by parthenogenesis-inducing *Wolbachia* strains are manifold and include the merging of nuclei, a failed anaphase during the first embryonic cell division and functional apomixis (Lindsey et al., 2016b). After successful manipulation, infected females do not produce sons from unfertilized eggs, but daughters that can maternally transmit the bacteria to the next generation (Werren et al., 2008).

1.2.3.3 Male killing

Another reproductive manipulation process caused by different *Wolbachia*-strains, called male-killing, has been observed in many different arthropod orders (Hurst et al., 1999; Fialho and Stevens, 2000; Werren et al., 2008). If a female is infected with a male-killing-inducing *Wolbachia* strain, it produces mostly daughters while the sons are being killed during the embryonic development. Numerous benefits are assumed to arise from this manipulation mechanism, including reduced intensity of antagonistic interactions between siblings (resource allocation, cannibalism avoidance) and decreased probability of inbreeding among siblings (Charlat et al., 2003; Elnagdy et al., 2011). Further, female offspring can consume the eggs of their dead brothers (Elnagdy et al., 2011) and therefore daughters produced by infected females have a higher probability of survival than daughters of uninfected ones, leading to a fitness benefit of

Wolbachia infected females (Charlat et al., 2003; Unckless and Jaenike, 2011). The symbiont-host interactions have been investigated intensively, findings show that male-killing can lead to male death during embryogenesis (early male-killing) or mortality of late larval instars (late male-killing) (Zeh et al., 2005; Charlat et al., 2007; Werren et al., 2008).

1.2.3.4 Cytoplasmatic incompatibility

Cytoplasmatic incompatibility is widespread in insects and the most common *Wolbachia*-induced phenotype that has been described in arachnids, isopods and insects (Hoffmann and Turelli, 1997; Poinsot et al., 2003; Werren et al., 2008). This reproductive incompatibility leads to embryonic mortality when *Wolbachia*-uninfected females mate with infected males. Infected females on the other hand remain fully fertile regardless if the male is infected by the endosymbiont or not (Poinsot et al., 2003). Considerable amount of work and research has been done on this phenomenon and while precise molecular mechanisms of CI are still unknown, evidence suggests that *Wolbachia* modifies the sperm of infected males during spermatogenesis so that a subsequent fusion with uninfected (or differentially infected) eggs are not viable (Poinsot et al., 2003; Duron et al., 2007; Werren et al., 2008; Correa and Ballard, 2016). In a recent study, LePage et al. (2017) discovered two factor genes (*cif*A and *cif*B) in the *Wolbachia* strain *w*Mel that mediate CI.

The manipulations ultimately lead to an increase in female fitness, but reduce the fitness on infected males and therefore benefit the maternally inherited bacteria (Poinsot et al., 2003; Correa and Ballard, 2016).

Individuals that are infected by one CI-inducing strain commonly express unidirectional CI (Telschow et al., 2005). This CI-type occurs, if infected males mate with females uninfected by *Wolbachia*, while all other crosses are fully compatible (Dobson, 2003; Telschow et al., 2005). However, if a male and a female harbor different CI-inducing strains of *Wolbachia*, bidirectional CI can develop, resulting in mutual incompatibility of individuals with different *Wolbachia* strains (Figure 1.7; Werren, 1997; Bordenstein and Werren, 2007).

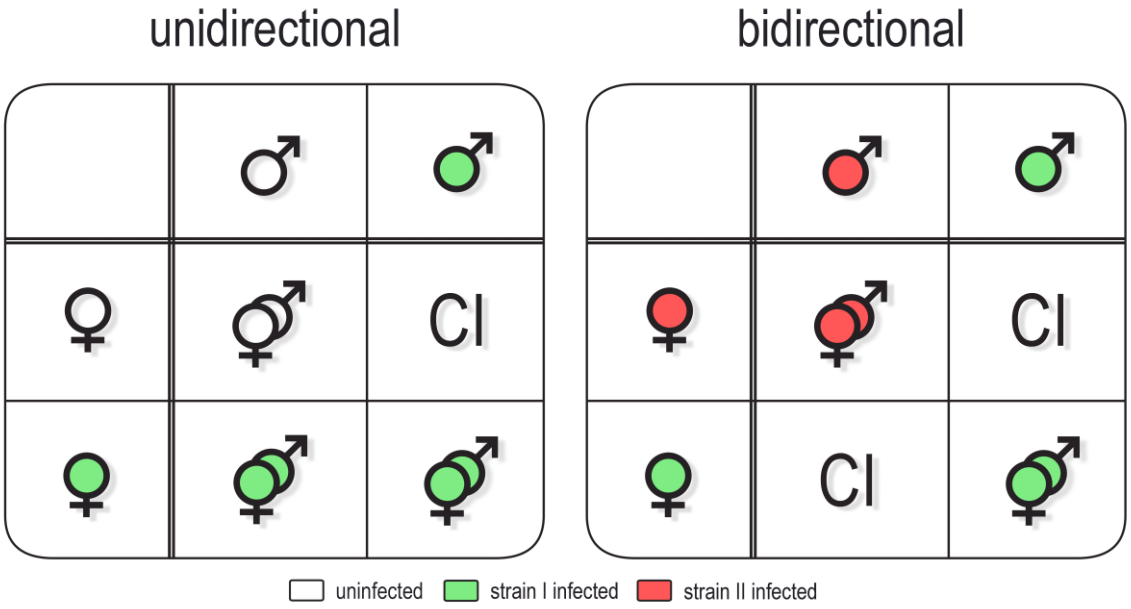

Figure 1.7 *Unidirectional cytoplasmatic incompatibility is expressed when infected males are crossed with uninfected females. All other combinations are fully compatible (Dobson, 2003). This variation of CI also occurs in Wolbachia-infected cherry fruit flies. Bidirectional CI occurs if the mating partners are infected by two different Wolbachia strains (Telschow et al., 2005).*

1.2.4 Horizontal transmission

The wide range of different hosts that are infected by *Wolbachia* cannot be explained by vertical transmission through the maternal lineage alone (Vavre et al., 1999; Baldo et al., 2006). Furthermore, molecular phylogeny of the endosymbiont is incongruent with that of its hosts (O'Neill et al., 1992). This strongly suggests that the bacterium also spreads into new species by horizontal transmission (Heath et al., 1999; Vavre et al., 1999; Werren and Windsor, 2000; Baldo et al., 2006). Different hypotheses have been stated on this matter, the exact mechanisms on how *Wolbachia* invades new host populations through horizontal transmission are still unclear (Vavre et al., 1999; Duron and Hurst, 2013; Correa and Ballard, 2016; Schuler et al., 2016b). Observations show that *Wolbachia* are able to survive briefly in extracellular environments while still maintaining the ability to invade and infect new hosts. The bacteria seem to be able to colonize new female germlines through surrounding somatic cells. Moreover, different ecological routes for lateral transfer of *Wolbachia* such as haemolymph contact between infected and uninfected individuals and horizontal transmission through predation and cannibalism have been described (Le Clec'h et al., 2013). Ahmed et al. (2015) discovered that ovipositors and mouthparts of parasitoids can get contaminated

with *Wolbachia* by feeding or ovipositing on infected whitefly nymphs. Thus, they can transmit *Wolbachia* horizontally to uninfected individuals. Schuler et al. (2013) states several factors that are necessary for horizontal transmission to be successful, including close physiological contact between the individuals, the ability to quickly adapt to the cellular environment of the host and colonization of the female germline that results in a reproductive advantage.

1.2.5 Infection dynamics of *Wolbachia*

The broad distribution of *Wolbachia* across different species can be explained by spread through horizontal transmission, resulting in the colonization of new mitochondrial lineages (Schuler et al., 2016a). Different studies of the infection dynamics of *Wolbachia* and the co-evolution with their hosts have been conducted to gain a better understanding of the bacteria and their potential usefulness in manipulating insect populations and disease suppression.

In 1991 Turelli and Hoffmann presented a study on the infection dynamics and the rate of spread of a CI-inducing *Wolbachia*-like parasite in *Drosophila simulans* in California. Monitoring of populations over different years showed that the infection was spreading at a fast rate of more than 100 km per year and populations with low infection rates became almost completely infected within 3 years. Possible explanations for the fast spread were occasional long-distance dispersal, commercial fruit transport and recolonization after local extinction in winter through immigrant flies (Turelli and Hoffmann, 1991).

Kriesner et al. (2013) conducted a similar study in eastern Australia where they described the sequential spread of two different *Wolbachia* strains (*w*Ri and *w*Au) over 20 years in natural populations of *D. simulans*. While *w*Au does not induce CI, its spread in multiple populations implies that it leads to fitness benefits. The *Wolbachia* variant *w*Ri does cause CI and rapidly displaces *w*Au. This found data has also been used to model and interpret the spread of *Wolbachia* in *Aedes aegypti* mosquitos in Australia, where field released mosquitos, that have been artificially infected with the *w*Mel strain, show a small but significant reduction in fitness that can reduce or prevent further spread of *Wolbachia* outside the released areas (Hoffmann et al., 2014; Schuler et al., 2016a).

In 2013, Schuler et al. showed that frequent horizontal transmission leads to spread of *Wolbachia* from *R. cerasi* to the closely related invasive fly *Rhagoletis cingulata,* proving that *Wolbachia* can adapt quickly to a new host environment and spread rapidly within a new host species.

In 2016, Schuler et al. described the infection dynamics of an ongoing *Wolbachia* invasion in *R. cerasi* in Europe. This work laid the basis of this thesis and will be discussed more in detail in chapter 1.3.

1.2.6 *Wolbachia* and mitochondrial DNA

Mitochondria are extra-nuclear parts of the genome present in the cytoplasm of most eukaryotic cells. Mitochondrial DNA (mtDNA) is commonly and widely used as a marker of choice in demographic, taxonomic, biogeographic and phylogenetic studies. It can easily be amplified from a variety of different taxa, has a mutation rate much higher than in nuclear DNA and shows low recombination. However, using mtDNA as a sole marker in studies is not without complications, as mitochondria are only transmitted through the maternal line. This leads to results that only reflect the history of the female portion of a species (Hurst and Jiggins, 2005).

Wolbachia share many similarities with mitochondria and are vertically co-transmitted through the egg cytoplasm of the mother (Charlat et al., 2003; Correa and Ballard, 2016). The genetic variety of mitochondria can be influenced by the presence of *Wolbachia*, as the effects of the endosymbiont can lead to fitness or reproductive advantages for its host. The mitochondrial genomes of *Wolbachia*-infected individuals will then hitchhike with the spreading endosymbiont, resulting in haplotype replacement and a reduction of haplotype diversity (Charlat et al., 2009; Schuler et al., 2016a). Populations that have recently been infected with *Wolbachia* can therefore show fewer mitochondrial lineages than uninfected populations (Schuler et al., 2016a).

1.2.7 Practical applications

Given their manipulating abilities and drastic effects on host biology, *Wolbachia* have been a major research focus in recent years for possible applications on human disease prevention and biological pest control (Zabalou et al., 2004; Werren et al., 2008; Brelsfoard and Dobson, 2009; Correa and Ballard, 2016). Similar to the Sterile Insect Technique (SIT), a *Wolbachia*-based method called Incompatible Insect Technique (IIT) for vector control has been developed (Zabalou et al., 2004). SIT is a species-specific method for insect control where large numbers of sterile males are released to decrease the female reproductive potential and suppress pest populations. Analogous to this procedure, IIT aims to control field populations by using the specific capabilities of CI-inducing *Wolbachia* strains to artificially sustain female sterility. Repeated releases of males infected by a CI-inducing *Wolbachia* strain lead to high egg mortality if the naturally uninfected females mate with the released infected males. In this case *Wolbachia*-induced CI can then be used to suppress natural populations (Bourtzis, 2008; Alphey et al., 2010; Zhang et al., 2015). The accidental release of *Wolbachia* infected females may result in the establishment of *Wolbachia* infected endosymbionts in the field that can lead to population replacement rather than population suppression (Zhang et al., 2015). Therefore, a practical approach is necessary to avoid the release of infected females and mass rearing techniques have to be established to rear *Wolbachia* infected males only (Bourtzis, 2008).

Other applications aim to reduce the lifespan and decrease the ability of vector carrying populations to transmit diseases through *Wolbachia*. Successful introduction of the life-shortening *w*MelPOP strain from *Drosophila melanogaster* in the mosquito *Aedes aegypti* led to a 50% reduction in the lifespan of infected females that blocks the virus replication and limits the transmission of human diseases. However, detrimental effects on the host fitness hinder a successful spread of transinfected lines in natural host populations. Transinfection of another *Wolbachia* strain from *D. melanogaster*, *w*Mel, showed that *Wolbachia* can interfere with the replication of certain pathogens in insects (Moreira et al., 2009; Walker et al., 2011; Hoffmann et al., 2011; Hoffmann et al., 2015). Other promising approaches aim to decrease vector-borne diseases like Dengue fever (Bull and Turelli, 2013), Malaria (Hughes et al., 2011), Chikungunya virus (Raquin et al., 2015) and the recent outbreak of the Zika virus (Caragata et al., 2016).

1.3 *Wolbachia* in *Rhagoletis cerasi*

1.3.1 Incompatibility in *R. cerasi*

In the 1970s, Boller et al. (1976) conducted extensive crossing experiments with European populations of cherry fruit flies. The authors found strong unidirectional incompatibility between males from southern and central European populations with females from northern and Eastern European populations, resulting in high egg mortality rates of more than 98%, while reciprocal crosses produced normal fertility rates and viable offspring (Figure 1.8; Boller et al., 1976).

Several different hypotheses on the reasons of this incompatibility including genetic, cytoplasmatic and symbiont-induced explanations have been stated by Boller et al. (1976) but could not be verified at that time. In 1989, Blümel and Russ detected *Rickettsia*-like-organisms (RLOs) in the ovaries of individuals in all populations, but did not uncover the identity of these bacteria (Blümel and Russ, 1989).

Figure 1.8 *Map showing the distribution of R. cerasi populations in Europe. Findings show unidirectional incompatibility between southern (red) and northern (grey) populations. Orange dots symbolize transitional populations in coinciding areas. The data is based on research of Boller and Bush (1974) and has been modified by Schuler et al. (2016a).*

1.3.2 *Wolbachia* in *R. cerasi*

In 2002, Riegler and Stauffer conducted further research and found that almost all *R. cerasi* individuals are infected by *Wolbachia*. Direct sequencing of the *wsp* gene showed the presence of the same *Wolbachia* strain, *w*Cer1, in all *R. cerasi* populations. However, ambiguous patterns and double peaks in southern populations showed that those individuals are infected by an additional *Wolbachia* strain, *w*Cer2. They then confirmed the two strains in *R. cerasi* and compared their geographical distribution with the data on mating incompatibilities by Boller and Bush (1974). After screening over 1200 individuals across Europe, the results showed that all collected flies were either single infected by *w*Cer1 or superinfected by both strains, *w*Cer1 and *w*Cer2. The distribution of *w*Cer2 was congruent with the unidirectional incompatible populations found by Boller et al. (Figure 1.9; Boller et al., 1976; Riegler and Stauffer, 2002). This led to the conclusion, that the endosymbiont *Wolbachia* is the cause for the unidirectional incompatibility by causing CI between males infected by *w*Cer2 and females that are uninfected by this *Wolbachia* strain (Riegler and Stauffer, 2002).

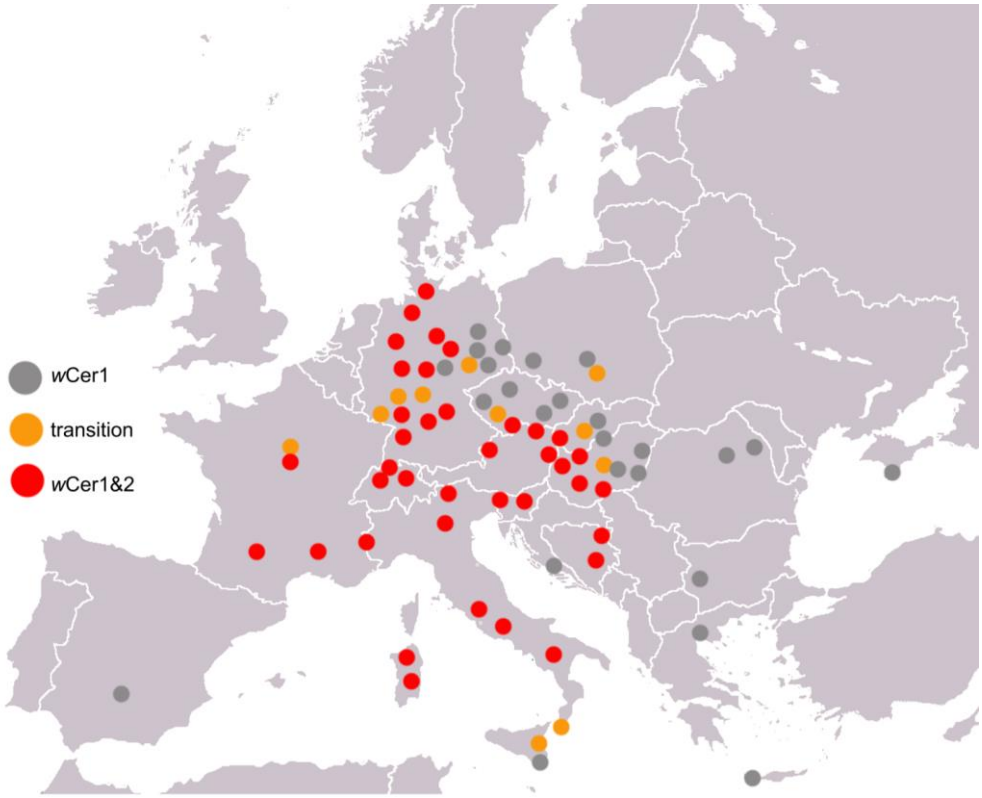

Figure 1.9 *Distribution of Wolbachia strain wCer2 infected and uninfected as well as transitional populations of R. cerasi. Data was taken from Riegler and Stauffer 2002 and has been modified by Schuler et al. (2016a).*

Arthofer et al. (2009a) discovered three additional *Wolbachia* strains, *w*Cer3, *w*Cer4, and *w*Cer5, distributed at different frequencies in most European populations of *R. cerasi*. However, the infection patterns of these strains did not show accordance to the observed distribution of CI (Schuler et al., 2016a). Thus, screening of the additional strains was not conducted in this work.

1.3.3 Further research and different haplotypes

Based on the works by Boller et al. in the 1970s, Riegler and Stauffer in 2002 as well as additional groundwork by Arthofer et al. (2009a), Schuler et al. (2016a) studied the infection dynamics of *Wolbachia* in the cherry fruit fly in Europe. Following a 15-year-long survey (1999-2014), the authors focused on the spread and expansion history of the endosymbiont in different transects in central Germany. Additionally, they characterized the mtDNA of the collected *R. cerasi* and compared the infection status with the mitochondrial haplotypes of the fly. The study revealed two closely related haplotypes, HT1 and HT2, that are differentiated only by a single nucleotide polymorphism (SNP). Further, the authors identified a clear correlation between the two mainly occurring *Wolbachia* strains with the two different mitochondrial haplotypes and explained the low mitochondrial diversity in all populations by two consecutive sweeps of *w*Cer1 and *w*Cer2. (Figure 1.10). This association suggests CI-driven selective sweep and frequent intraspecific horizontal transmission, provides new insight into the invasion dynamics of *Wolbachia* and further confirms maternal inheritance and the induction of CI (Schuler et al., 2016a).

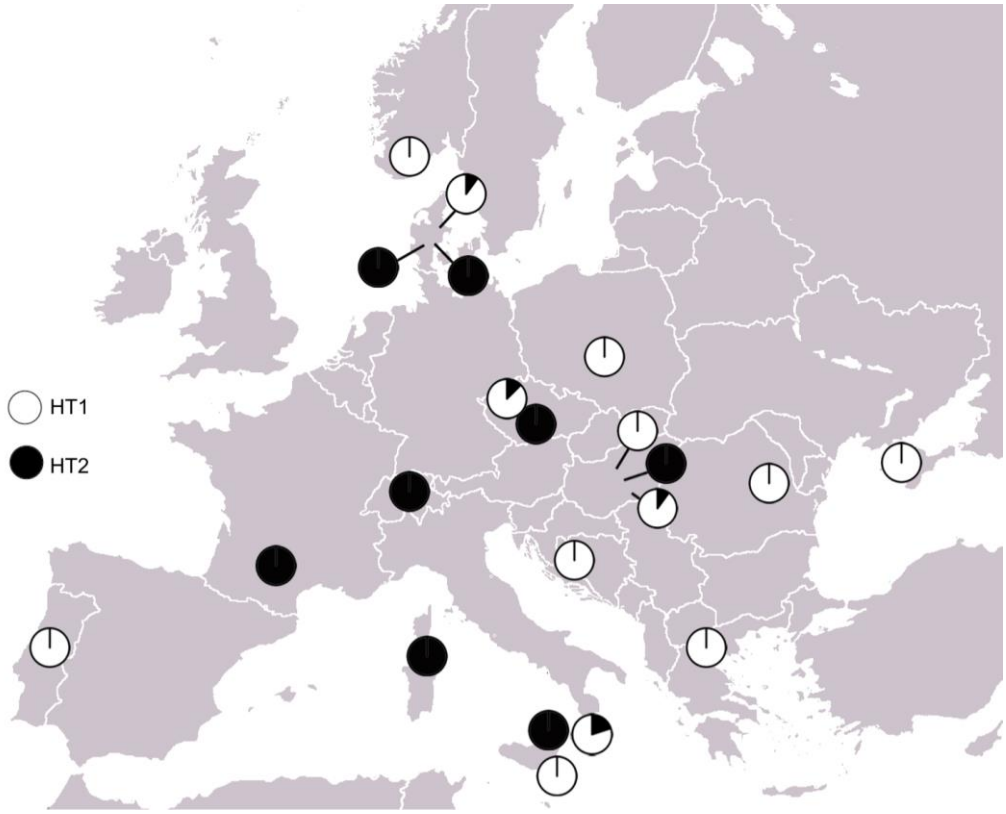

Figure 1.10 *Map showing the distribution of two different mitochondrial haplotypes HT1 and HT2 in populations of R. cerasi in Europe (Schuler et al., 2016a).*

2. Aims

Schuler et al. (2016a) focused on the *Wolbachia* strain *w*Cer2 and described the distribution and the mitochondrial haplotypes of its host in Germany. The results of the survey of *Wolbachia* in *R. cerasi* are visualized in Figure 2.1. In this figure, samples from northern and southern Germany show fixation of *w*Cer2, while areas in central Germany mostly consist of uninfected or transitional populations.

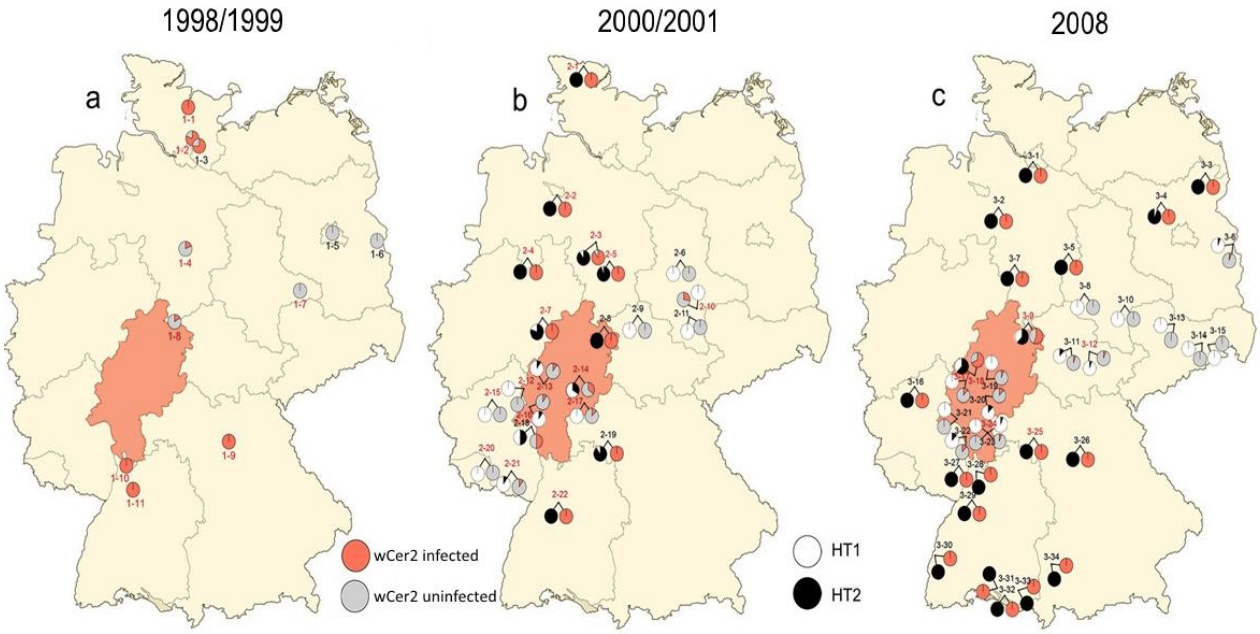

Figure 2.1 *Geographic distribution of Wolbachia strain wCer2 and associated haplotypes of R.cerasi from 1998-2008 in Germany with the highlighted state of Hesse. Graphics were taken from Schuler et al. (2016) and were slightly modified.*

The state of Hesse in Central Germany is especially interesting because it contains populations uninfected by *w*Cer2 and is surrounded by transitional populations with both *w*Cer2 infected and *w*Cer2 uninfected individuals. The aim of this work is to continue the observations on field population and interactions between *Wolbachia* and *R. cerasi* on a smaller scale by focusing exclusively on this transect area. Fine scale sampling in these transition zones and comparison of new results with existing data will give new insights on the temporal and spatial spread of *Wolbachia*.

3. Materials and Methods

3.1 Collection of *R. cerasi*

Between 04.07 - 09.07.2016 cherry fruit flies were collected by Hannes Schuler and Christian Stauffer at 18 locations on infested *Prunus* and *Lonicera* plants in Hesse, Germany (Figure 3.1). One additional population was provided by Heidrun Vogt (Julius Kühn Institute, Dossenheim). Part of the larvae were extracted on site directly out of infested cherry fruits and stored in absolute ethanol, in two populations adult flies were also caught with an exhaustor in the field. Additionally, infested cherries were collected and transported to the laboratory. Emerging larvae were collected after pupation and stored in absolute ethanol. DNA extraction was performed with larvae, pupae and adult flies. All samples were stored in absolute ethanol at -20° C.

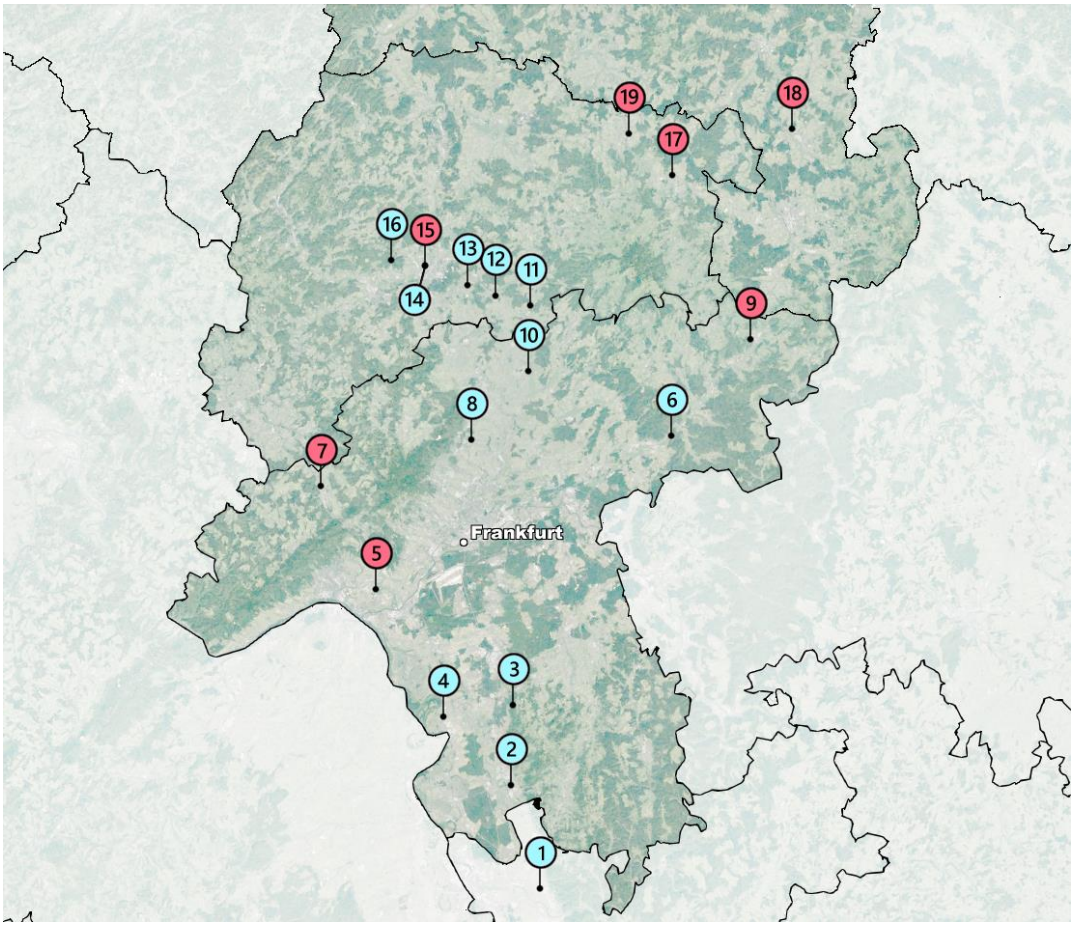

Figure 3.1 *Map showing collected locations across Hesse (1-19, see Tab. 5.1 for specific site information). Blue dots represent Prunus, red dots Lonicera plants.*

3.2 DNA Extraction

From every site, 10-16 individuals were chosen for DNA extraction using the SIGMA GenElute™ Mammalian Genomic DNA Miniprep Kit. For more detailed information on the extraction protocol please refer to Appendix 8.5.

Depending on the collection of the different populations, larvae, pupae or adult flies were put in a 1.5 ml Eppendorf tube prefilled with 180 µl lysis solution. Each individual was mashed thoroughly inside the tube before adding 20 µl of SIGMA proteinase K, an enzyme used to digest proteins and remove contaminations. Samples were incubated on a heating block at 55°C for 2 hours. Subsequently, 20 µl of RNAse was added to the sample. Following the provided protocol, 200 µl of lysis solution was added and the solution was again incubated for 10 minutes at 70°C. To prevent the DNA from dissolving in the water, 200 µl of absolute ethanol was added to the samples before continuing with several washing steps using the provided wash solution. Finally, the DNA was eluted in 100 µl elution solution and stored at 4°C.

3.3 Polymerase chain reaction (PCR)

All individuals were screened for *Wolbachia* infection by PCR, using *w*Cer1 and *w*Cer2 specific primers targeting specific fragments of the *Wolbachia* surface protein (*wsp*) (Table 3.1; Braig et al., 1998; Riegler and Stauffer, 2002; Arthofer et al., 2009). Each reaction was set up in 10 µl volumes containing 1 mg/ml BSA (Bovine Serum Albumin), 2 mM y-Buffer (PeqLab), 800 µM dNTPs, 0,2 µM of each primer, 0.5 U *Taq* polymerase (PeqLab) and 1 µl of the template DNA. PCR conditions were 2 min at 94°C and continued with 35 cycles at 94°C for 30 sec, 55°C for 45 sec, 72°C for 1 min followed by a final extension at 72°C for 10 min. PCR amplification was verified on 2% agarose gels stained with GelRed Nucleic Acid Gel Stain (Biotium). Gel-electrophoresis was accomplished with 1.6 µl of DNA mixed with 6 µl loading buffer. The amplified fragments were visualized on a UV transilluminator (PeqLab) after 15 to 20 minutes.

Name	Sequence	Ori.	Specifity	Size
***w*sp226F**	5‘-GTGGTGGTGCATTTGGTGATAAAATGG-3‘	F	*w*Cer1	428bp
spec1R	5’-GCCTTTATCAGCAACCTTTTTGTTTGC-3’	R		
spec2F	5’-CTATAAGAAAGACAAGAGTGATTAC-3’	F	*w*Cer2	479bp
spec2R	5’-CTGCATCAGTAACCTGTATGGTTGAATC-3’	R		

Table 3.1 *Primer sets used for screening all individuals for Wolbachia infection by amplification of fragments of the Wolbachia surface protein (wsp) (Arthofer et al., 2009a).*

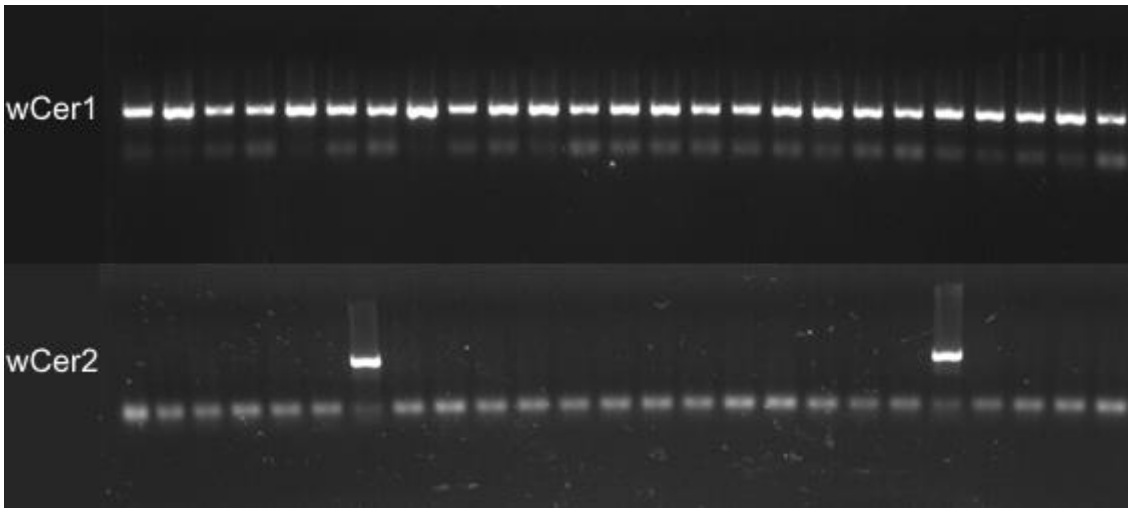

Figure 3.2: *Agarose gels after electrophoresis showing amplified fragments of the wsp gene. In this example, all individuals of R. cerasi show infection with Wolbachia strain wCer1 (first row) and two individuals also show infection with strain wCer2 (second row).*

3.4 Restriction Fragment Length Polymorphism (RFLP)

The determination of mitochondrial haplotypes was performed by restriction fragment length polymorphism (RFLP). Following the protocols of Schuler et al. (2016a), a specific fragment of the mitochondrial cytochrome c oxidase subunit I was amplified using the mitochondrial primers Pat and Dick (Table 3.2; Simon et al., 1994). PCR was performed using the same conditions as described in 3.3. Additionally, 10 µl of the PCR product was incubated with 2 U of *HaeIII* (Thermo Scientific™) at 37° for 3 hours. While haplotype 1 (HT1) remains undigested, haplotype 2 is cut into two fragments (342bp and 204bp). After gel-electrophoresis, the two haplotypes can be distinguished under UV-light on the agarose gel (Schuler et al., 2016a).

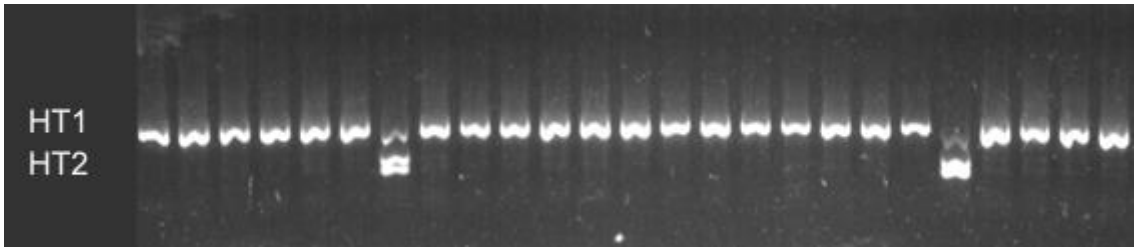

Figure 3.3: *Identification of different haplotypes HT1 and HT2 using the same samples shown in Figure 3.2. In two individuals, the restriction enzyme cut the amplicon into two separate fragments (342bp and 204bp).*

3.5 Sequencing

The mtDNA of individuals that did not show an infection with *Wolbachia* was additionally sequenced. DNA purification of the samples was performed using the peqGOLD Cycle-Pure Kit (PeqLab). The PCR product was mixed with the same volume of XP1 buffer before pipetting the mixture into new columns. Samples were centrifuged for 1 min at 10.000 rpm and washed with 650 µl SPW-washing buffer before adding 20 µl of elution buffer to resolve the purified PCR product. After incubating for 2 min and centrifuging for 1 min at 10.000 rpm the procedure was completed. Samples were sent to the Cancer Research Centre DNA Sequencing & Genotyping Facility in Chicago (IL, USA) for sequencing.

Sequences were analyzed with Chromas 2.5.1, edited in Gene Runner 6.0.2 and aligned with ClustalX 2.1 (Thompson et al., 1997; Larkin et al., 2007). Comparison of the nucleotide sequences with gene databases was achieved using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI).

4. Results

The goal of this thesis was to study the spatial distribution of *w*Cer2 in *R. cerasi* in a transect in Germany. The state of Hesse is comprised by a transition zone in which *w*Cer2 infected fruit flies introgress into *w*Cer2 uninfected populations. This chapter presents the results of this survey on 19 populations of *R. cerasi* collected in Hesse.

4.1 Fixation of *w*Cer1

Approximately 16 individuals from each of the 19 population of *R. cerasi* were screened for infection with *Wolbachia* strain *w*Cer1 using specific primers wsp226F and spec1R (Table. 3.1) that target the *Wolbachia* surface protein (*wsp*) (Arthofer et al., 2009a). As described by Riegler and Stauffer (2002), Arthofer et al. (2009a) and Schuler et al. (2016a), infection rates of *w*Cer1 should be 100%, thus screening for this strain was mainly performed to ensure the DNA quality of the extracts. PCR amplification with *w*Cer1 specific primers showed positive results on the agarose gel on 294 out of 295 individuals (99.6%).

4.2 Distribution of *w*Cer2 and association with mitochondrial haplotypes

Population	Location	Host	Individuals	% wCer2 infected	HT1	HT2
1	Dossenheim	*Prunus*	16	100%	0	16
2	Bensheim	*Prunus*	16	6.2%	15	1
3	Ober-Ramstadt	*Prunus*	16	6.2%	15	1
4	Stockstadt	*Prunus*	16	18,7%	13	3
5	Erbenheim	*Lonicera*	15	6.6%	14	1
6	Hailer	*Prunus*	16	100%	0	16
7	Idstein	*Lonicera*	10	10%	9	1
8	Rosbach	*Prunus*	16	0%	16	0
9	Schlüchtern	*Lonicera*	16	37.5%	10	6
10	Weckesheim	*Prunus*	16	25%	12	4
11	Utphe	*Prunus*	16	12.5%	14	2
12	Langsdorf	*Prunus*	16	18.7%	13	3
13	Lich	*Prunus*	16	0%	16	0
14	Gießen 1	*Prunus*	16	100%	5	11
15	Gießen 2	*Lonicera*	16	75%	6	10
16	Lahnau	*Prunus*	16	18.7%	16	0
17	Wallenrod	*Lonicera*	16	0%	16	0
18	Grossenmoor	*Lonicera*	14	14.2%	12	2
19	Alsfeld	*Lonicera*	16	12.5%	13	3

Table 4.1 *Screening results of the 19 populations that have been analyzed for wCer2 infection and haplotypes HT1 and HT2. One individual from Alsfeld [19] did not show an infection with Wolbachia. For a more detailed version see Appendix 8.4.*

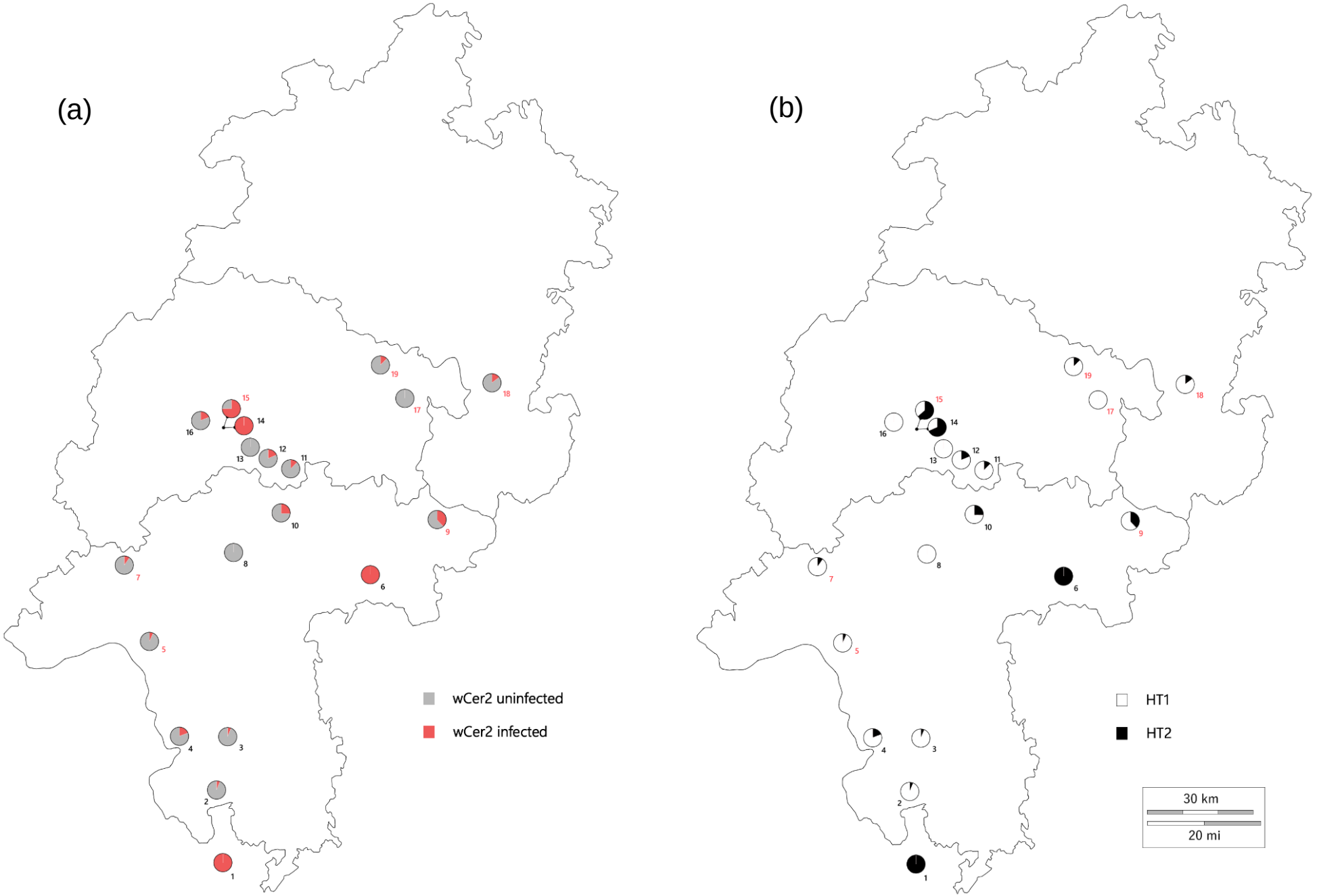

Figure 4.1 *Visual display of the screening results of 295 individuals of R. cerasi in Hesse, Germany. Pie charts on map (a) show the distribution of wCer2 infected (red) and uninfected (grey) cherry fruit flies. Map (b) shows the allocation of mitochondrial haplotypes HT1 (white) and HT2 (black). Locations are numbered 1-19 (Table 4.1), black numbers indicate flies collected on Prunus plants, red numbers flies collected on Loniceras plants. Direct comparison of the pie charts show the association of wCer2 infected flies with HT2 and wCer2 uninfected flies with HT1.*

4.2.1 Distribution of *w*Cer2

*w*Cer2 was present in a total of 89 (out of 295) individuals from 19 populations (30.1%). While three populations showed fixation of *w*Cer2, 16 were transitional with three populations that were uninfected by this strain. The southernmost population of Dossenheim [1] as well as central population from Hailer [6] and Gießen 1 [14] showed 100% infection rate. Samples from 3 locations (Rosbach [8], Lich [13], Wallenrod [17]) were completely *w*Cer2 uninfected. Transitional populations with *w*Cer2 infected and *w*Cer2 uninfected individuals showed different infection rates ranging from 6.2% in Ober-Ramstadt [3] to 75% in Gießen 2 [15]. Locations in the center of Hesse indicate strong variety with a fully infected population at Gießen 1 [14] and high infection rate in Gießen 2 [15], that are in close distance (approximately 11km) to low infected populations in Lahnau [16] and completely uninfected flies collected from Lich [13]. Screening of flies collected in the north-eastern part of Hesse resulted in low infestation values between 0% to 14.2%, southern populations – apart from Dossenheim [1] – followed a similar trend with values ranging from 6.2 to 18.7%.

4.2.2 Distribution of haplotypes HT1 and HT2

Haplotype distribution of HT1 associated strongly with *w*Cer2 uninfected individuals in all populations (205 out of 206, 99.5%) and HT2 showed strict association with *Wolbachia* strain *w*Cer2 in most areas (80 out of 89, 89.9%). Apart from four populations, results show 100% congruence levels in infection and haplotype distribution. Exceptions to this pattern have been identified in geographically close localities (~11km) Gießen 1 [14], Gießen 2 [15] and Lahnau [16], with nine individuals that were *w*Cer2 infected but associated with HT1. Another misassociation was found in Alsfeld [19] with one *w*Cer2 infected individual associated with HT2.

4.3 Comparison of the data with previous studies

To draw conclusions on the infection dynamics of *w*Cer2, my data were compared to the previous data from Schuler et al. (2016a). This study characterized nine populations from Hesse from 1999-2016 with six populations collected multiple times that were used for this comparative approach (Table 4.2; Figure 4.2).

Location	#Population	Host	Year	Individuals	%*w*Cer2 infected	HT1	HT2
Dossenheim	1-10	*Lonicera*	1999	10	100%	n.a.	n.a.
	3-28	*Prunus*	2008	16	100%	0	16
	1	*Prunus*	2016	16	100%	0	16
Ober-Ramstadt	3-24	*Lonicera*	2008	15	0%	15	0
	3-23	*Prunus*	2008	15	6.6%	14	1
	3	*Prunus*	2016	16	6.2%	15	1
Stockstadt	3-22	*Prunus*	2008	16	12.5%	14	2
	4	*Prunus*	2016	16	18.7%	13	3
Lich	3-19	*Prunus*	2008	16	6.2%	16	0
	4-1	*Prunus*	2014	10	0%	10	0
	13	*Prunus*	2016	16	0%	16	0
Gießen 1 2	2-13	*Lonicera*	2001	10	10%	9	1
	3-18	*Lonicera*	2008	16	68.7%	6	10
	15	*Lonicera*	2016	16	75%	6	10
	14	*Prunus*	2016	16	100%	5	11
Lahnau	3-17	*Lonicera*	2008	16	12.5%	16	0
	16	*Prunus*	2016	16	18.7%	16	0
Alsfeld	2-14	*Lonicera*	2000	10	50%	7	3
	19	*Lonicera*	2016	16	12.5%	14	3
Reichelsheim* **Weckesheim**	3-20	*Prunus*	2008	16	12.5%	14	2
	10	*Prunus*	2016	16	25%	12	4
Mechtildshausen** **Erbenheim**	3-21	*Prunus*	2008	16	0%	16	0
	5	*Lonicera*	2016	15	6.6%	14	1

Table 4.2 *Direct comparison of the screening results in seven locations conducted in this thesis (2016) with latest available data from years 2000-2014 (Schuler et al., 2016a). Nine populations located in Hesse have been analyzed by Schuler et al., six of these at same locations as in this work. *Distance between Reichelsheim and Weckesheim is approximately 1.7 km. **Distance between Mechtildshausen and Erbenheim is approximately 2.5 km.*

4.3.1 Comparison of *w*Cer2 infections across different years

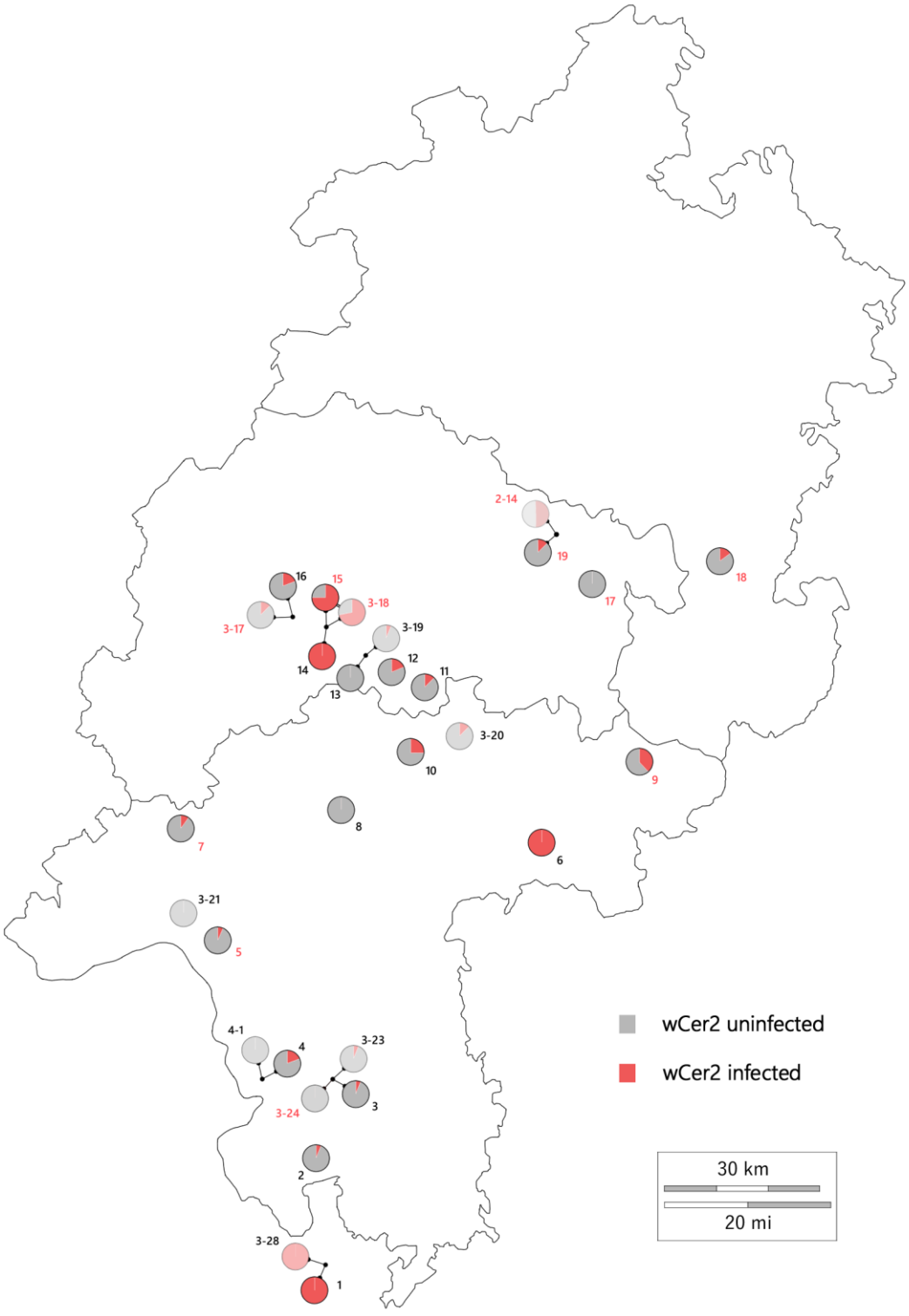

Figure 4.2 *Geographic distribution of wCer2 across Hesse from 1999-2016. Results from previous years (Schuler et al., 2016a) are depicted as transparent pie charts. Populations that have been collected at the same locations are connected by a black line. Black numbers indicate flies collected from Prunus plants, red numbers flies collected from Lonicera.*

Fixation of *w*Cer2 remained in Dossenheim [1], a population that has been fully infected by this strain in previous years as well. Direct comparison of screening results in Stockstadt [4] and Lahnau [16] between the years 2008 and 2016 showed a slight increase in infection rates in both populations from 12.5% to 18.7%. In Ober-Ramstadt [3] the number of infected individuals remained constant. While in 2008 one out of 15 flies, in 2016 one out of 16 flies was infected by *w*Cer2. The population in Lich [13] had an infection rate of 6.2% in 2008 but was not infected in 2014 and 2016. A high loss of *w*Cer2 occurred in Alsfeld [19] where 50% of the individuals were infected by *w*Cer2 in 2000 (with just 12.5% in 2016). Population from Mechtildshausen and Reichelsheim from 2008 could not be compared directly due to lack of samples in that exact area from 2016. Samples from Mechtildshausen were completely uninfected by *w*Cer2, the geographically closest population collected in 2016 at Erbenheim [5] shows a low infection rate of 6.6% (Table 4.1). With an infection value of 12.5%, Reichelsheim fits into the pattern seen in geographically close populations Weckesheim [10] (25%) and Utphe [11] (12.5%).

A possible influence of the host was found in Gießen: Flies collected on *Lonicera* showed an infection rate of 10% in 2001 and 68.7% in 2014. Direct comparison with the same locality (Gießen 2 [15]) in 2016 showed an infection increase to 75%. However, flies collected on *Prunus* plants in Gießen 1 [14] were already completely infected by *w*Cer2.

4.3.2 Comparison of *Wolbachia*-haplotype association across different years

A comparison of the association of *w*Cer2 uninfected and infected individuals with the two occurring haplotypes HT1 and HT2 in transitional populations has been conducted. Results showed that *w*Cer2 uninfected cherry fruit flies were strongly associated with HT1. Schuler et al. (2016a) reported one single individual in 2016 from Alsfeld [19] not infected by *w*Cer2 but associated with HT2. Congruence rate between *w*Cer2 infected individuals with HT2 fluctuated across different years ranging from 69% in 2000 to 75% in 2014 and 87.8% in 2016.

The comparison of the data shows that the association between *w*Cer2 with HT1 generally decreased from 31% in 2000 to 12.2% in 2016 (Figure 4.3).

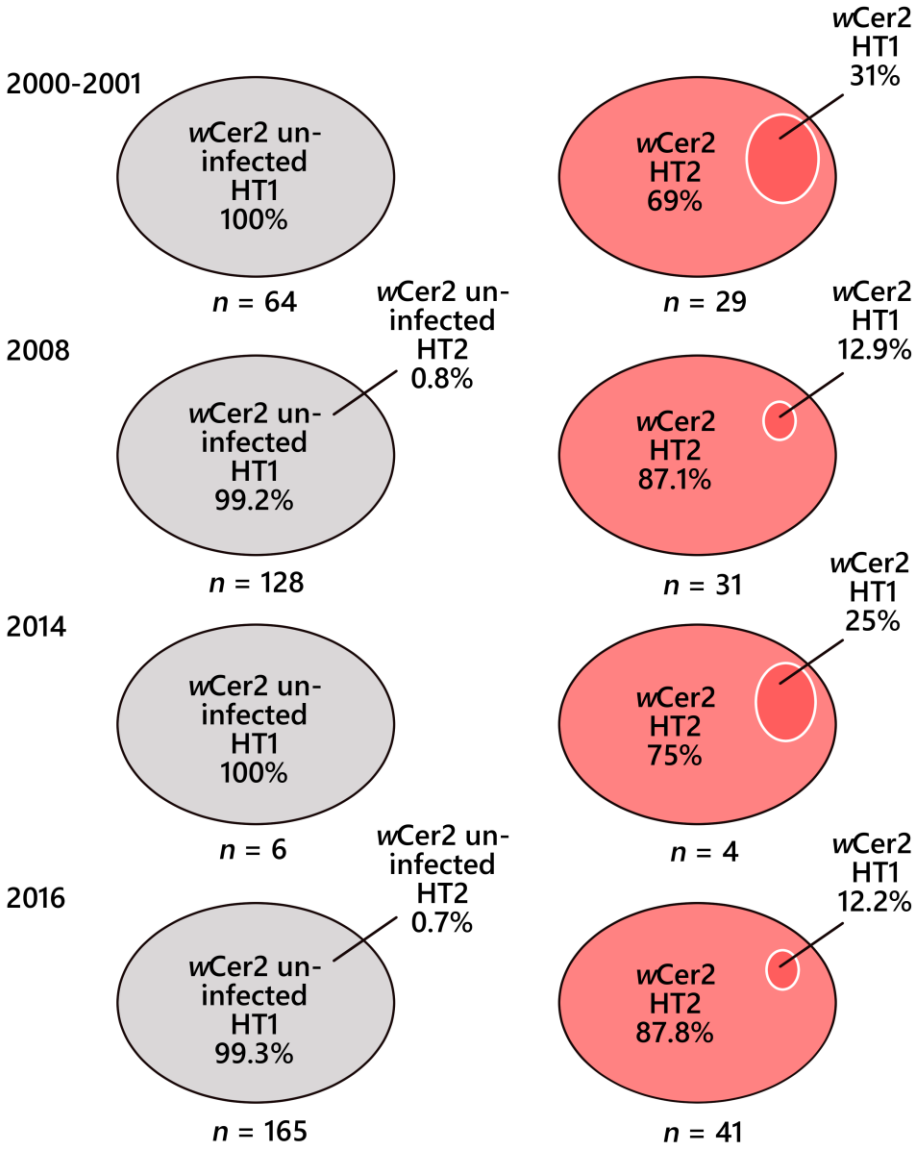

Figure 4.3 *Comparison of the association of wCer2 uninfected (grey) and infected (red) samples with the two occurring haplotypes HT1 and HT2 in transitional populations. Results from 2000-2014 have been taken and adapted from Schuler et al. (2016a). Note that results from Schuler et al. (2016a) do not only represent populations in Hesse, but included also transitional populations that have been found in other regions of Germany.*

4.4 Analysis of *Wolbachia* uninfected individuals

In 18 individuals, PCR revealed no positive signal for *Wolbachia*. All uninfected samples were collected on *Lonicera* plants. While mistakes in the PCR procedure could not be ruled out completely, these individuals might belong to another species. Therefore, all uninfected individuals were Sanger sequenced, analyzed, edited and used for comparison with gene databases using the BLAST tool provided by the NCBI (Figure 4.4).

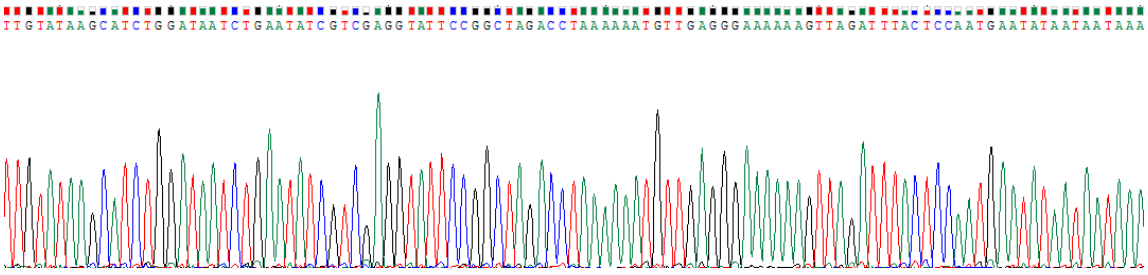

Figure 4.4 *Example of a chromatogram visualized in Chromas 2.5.1. The shown sequence depicts the nucleotides of a fragment of the COI gene of R. cerasi amplified using the primers Pat and Dick (Simon et al., 1994). Unusual high and poorly defined peaks at the beginning and the end of the sequence have been cut before performing a BLAST.*

17 out of the 18 individuals showed congruence values with other *Rhagoletis* species of less than 90% (*Rhagoletis zoqui, Rhagoletis cingulata*) and just 88% with *R. cerasi*. The high divergence rate suggests that the individuals are not *R. cerasi*. However, more detailed determination of the species was not possible since the species was not listed in the GenBank. Furthermore, all analyzed samples that did no show an infection by *Wolbachia* were pupae that hindered a morphological determination of the adults.

One individual showed a congruence value of 100% with a GenBank entry of *Rhagoletis cerasi*. Thus, this individual is either *Wolbachia* uninfected or the result signifies an error in the PCR screening of *Wolbachia*.

5. Discussion

This thesis focused on the spatial distribution of the *Wolbachia* strain *w*Cer2 in *R. cerasi* in Hesse and its association with the mitochondrial haplotype of its host. The comparison with previously published data (Schuler et al., 2016a) allowed a direct comparison of the *w*Cer2 frequency across more than 15 years and highlights a complex infection frequency of *Wolbachia* in Central Germany.

5.1 Fluctuation in infection rates across Hesse

The prevalence of *w*Cer2 across 19 investigated populations showed varying results ranging from complete infection with *w*Cer2 to transitional populations with rates of 6.2% to 75% and locations with no infection by this strain. Schuler et al. (2016a) showed that the state of Hesse was surrounded by populations with fixed *w*Cer2 infection rates in the south, north and west (Figure 2.1). As expected, the southernmost population of Dossenheim [1] showed a fixation of *w*Cer2, as it did in every screening since 1999 (Schuler et al., 2016a). While results in the southern parts of Hesse showed little to no variance and remained relatively stable, unexpected infection rates occurred in central Hesse. While both populations from Gießen [14 and 15] showed high infection by *w*Cer2 (100% and 75%), populations that are in vicinity of approximately 15km (Langsdorf [12], Lich [13], Lahnau [16]) showed low infection rates or were completely uninfected (18.7%, 0% and 18.7%). Populations located in central-eastern areas showed intermediate infection rates in Schlüchtern [9] with 37.5% while Hailer [6] was already completely invaded by *w*Cer2.

The observations by Schuler et al. (2016a) suggest a complete overtake of *w*Cer2 in *R. cerasi* in Hesse over time. While the results of the survey by Schuler et al. (2016a) show that the overall infection rates of *w*Cer2 in transitional populations increased strongly between 2000 to 2008 (69%-87.1%), comparison with the data from 2016 shows only a slight increase between 2008 and 2016 (87.1%-87.8%; Figure 4.3). However, the infection dynamics of *Wolbachia* do not necessarily follow a clear and linear path. Kriesner et al. (2013) for example observed a rapid spread of the strain

*w*Ri in *Drosophila simulans* in Australia, while Atyame et al. (2015) showed that the *w*Pip11 strain in the mosquito *Culex pipiens* in Tunisia stays at a constant level.

5.1.1 Migration of *R. cerasi* vs. accidental release

To invade a new population *Wolbachia* relies on the activity and dispersal of its hosts. A number of studies on the behavior of cherry fruit flies have been conducted, that show estimated flight periods ranging from 7 to 11 weeks starting in May (Daniel and Grunder, 2012). During this period, cherry fruit flies tend to stay relatively close to their established hosts with low migration into new areas (Stamenković et al., 2012; Daniel and Grunder, 2012). Stamenković et al. (2012) describes different surveys that estimate flight distances up to 500m, Boller and Remund (1983) conducted empirical experiments showing that some *R. cerasi* might even disperse as far as 3km if cherry orchards were visible from the release point. On the other hand, they also found that forests surrounding the release area had a very strong inhibitory effect on emigration and immigration of the cherry fruit flies. These findings strongly suggest that topographic conditions like shape and terrain influence the migration process of the flies and therefore also limit the spread of *Wolbachia*.

The spread of *w*Cer2 originated from southern and central European populations of *R. cerasi* and subsequently migrated to the north. However, in 2002 Riegler and Stauffer identified a population in northern Germany (Kiel) migrating to the south that deviated from these observations, showing high infection rates in locations that could not be explained by natural distribution of the flies alone (Riegler and Stauffer, 2002; Schuler et al., 2016a). Evidence suggests that this was caused by accidental release of infected cherry fruit flies due to transport of cherry fruits to the north. This assumption could explain the unusual high infection rates and unusual patterns in some populations. Gießen 1 [14] and Gießen 2 [15] showed unusually high infection rates compared to geographically close areas with low infection rates. This occurrence could be explained by manmade import and export of infected cherries. Further, *Lonicera* shrubs are popular ornamental plants. Tree nurseries might be an additional source on how *w*Cer2 infected pupae can be distributed across different areas.

5.1.2 Loss of *Wolbachia*

On an evolutionary timescale, *Wolbachia* can be lost due to imperfect maternal transmission (Richardson et al., 2012). While infection frequencies in all populations increased or stayed at the same level across different years, the population collected from Lich [13] did not show infection with *w*Cer2 in 2014 and 2016, even if 6.2% of infected flies have been reported in 2008 (Schuler et al., 2016a). With a low initial infection frequency, the spread of *Wolbachia* is assumed to be slow where potential negative fitness effects might result in a loss of the infection.

Wolbachia is expected to establish and spread from a very low starting frequency if there are no fitness costs for the host associated with the infection. Small fitness costs can already hinder the establishment of the bacterium in a population if the initial frequency is low. To overcome negative fitness effects, a certain infection threshold needs to be achieved (Hoffmann et al., 2014; Hoffmann et al., 2015). Thus, low initial infection frequency can lead to a very slow increase or even to a decrease in infection rates as observed in directly comparable populations located in Ober-Ramstadt [3], Stockstadt [4] and Lahnau [16], with little to no change in *Wolbachia* infestation, and in the population of Lich [13], where infection rate dropped from 6.2% in 2008 to 0% in 2016 (Figure 4.2, Table 4.2).

An unexpected high decrease of *w*Cer2 was found in Alsfeld [19] where the infection frequency dropped from 50% in 2000 to 12.5% in 2016. A loss of *Wolbachia* would result in a high amount of individuals from mitochondrial HT2 uninfected with *w*Cer2. Since just a single fly showed this association, a frequent *Wolbachia* loss in this population can be excluded. Migration from uninfected flies from neighboring populations or the accidental release of uninfected flies might be the cause of the high reduction of *w*Cer2 in this population.

5.2 Sporadic misassociation between *Wolbachia* and mitochondrial haplotypes

In 15 out of 19 populations, mitochondrial haplotype distribution HT1 and HT2 showed perfect association with *w*Cer2 uninfected and infected cherry fruit flies. Three populations (Gießen 1 [14], Gießen 2 [15], Lahnau [16], Figure 4.1, Table 4.1) that are in close vicinity deviated from this pattern showing a high percentage (32.2%) of individuals infected with *w*Cer2 but associated with HT1. Considering only transitional populations, Schuler et al. (2016a) also found a high number (21.9%) of individuals showing this misassociation. Reasons for this deviation are interpreted as repeated intraspecific horizontal transmission of *w*Cer2 into singly infected HT1 flies (Schuler et al., 2016a). Another possible explanation could be paternal transmission of *Wolbachia*, an occurrence that has been observed but seem to occur only rarely if at all (Hoffmann et al., 1990; Turelli and Hoffmann, 1995; Arthofer et al., 2009b; Schuler et al., 2016a). However, while the paternal transmission of *w*Cer2 would result in a permanent association of *w*Cer2-infected flies with HT1 also in populations outside the transition zone. Horizontal acquisition of *w*Cer2 that, however, does not get transmitted to their offspring could explain the loss of this misassociation (Schuler et al., 2016a).

In the population collected from Alsfeld [19] on the other hand, one individual uninfected with *w*Cer2 was associated with HT1. Schuler et al. (2016a) also identified a very low percentage of flies (0.96%) with this association. *Wolbachia* strain *w*Cer2 induces very strong CI and shows high transmission efficiency, an assumption that is also supported by theoretical modeling (Schuler et al., 2016a). Therefore, the evidence suggests that the rare association of *w*Cer2 uninfected flies with HT2 is caused by loss of *Wolbachia* due to incomplete vertical transmission.

5.3 *Wolbachia* uninfected tephritids on *Lonicera*

Seventeen individuals collected from *Lonicera* plants did not show an infection with *Wolbachia*. Barcoding these individuals with sequencing of a partial COI fragment showed low congruence values with COI isolates of *R. cerasi*. Comparison of the sequences with those of the GenBank did not result in a match. Literature research showed that *Lonicera* gets attacked by another tephritid fly *Myoleia lucida* (Hoffmeister, 1992). The alignment of the non-*Rhagoletis* sequences with a previously sequenced COI fragment from a pupa that is assumed to be *M. lucida* (Schuler et al. unpublished data) showed 100% identity (Figure 5.1).

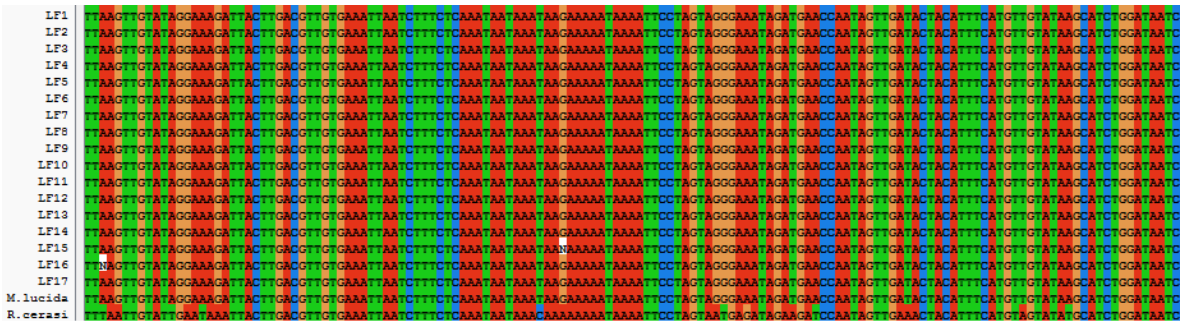

Figure 5.1 *Sector of a complete sequence alignment of a fragment of the mitochondrial COI gene done in ClustalX2.1. Wolbachia uninfected individuals collected from Lonicera plants (LF1-LF17) show high congruence values compared to a sample acquired from previous findings of pupae on Lonicera (supposedly M. lucida), while comparison to a sequence of R. cerasi shows strong differences.*

To further confirm the identity of the *Wolbachia*-uninfected non-*Rhagoletis* individuals, accurate taxonomic identification on adult flies needs to be conducted. If the species is successfully identified, sequences for future comparison can be entered in the GenBank.

6. Conclusion

Screening of 295 individuals from 19 locations showed fixation of *w*Cer2 in three populations, 13 transitional populations with infection rates ranging from 6.2% to 75%, and three populations not infected by *w*Cer2. Comparison of new data from 2016 with observations on the infection dynamics since 1999 showed a general increase in infection rates of *w*Cer2 in Hesse over time.

Reasons for the distribution of the endosymbiont are manifold and difficult to summarize into a clear and linear pattern. The accidental release caused by transport of infected cherry fruits, topographic conditions of the terrain, the natural behavior of the host fly *R. cerasi*, loss of *Wolbachia* caused by imperfect maternal transmission, the inability to establish itself due to negative fitness effects as well as rare paternal transmission influence the results and make it difficult to outline a prediction on migration and infection rates.

Extensive surveys by Riegler and Stauffer (2002) and Schuler et al. (2016a) have outlined clear transitional zones in Europe and Germany that show the spread of the endosymbiont into new areas. However, to accomplish more accurate results on this topic on a smaller scale, it is necessary to collect a high number of samples in the same locations in subsequent years and perform a direct and precise comparison of the observations.

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8 Appendix

8.1 Index of Abbreviations

A	Adenosine
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine Serum Albumin
C	Cytosine
°C	Degree Celsius
CI	Cytoplasmic incompatibility
cm	Centimetre
COI	Cytochrome c oxidase subunit I
DNA	Deoxyribonucleic acid
dNTP	2´-deoxyribonucleoside-5´-triphosphate
G	Guanosine
g	Gram
HT	Haplotype
HaeIII	Restriction enzyme isolated from *Haemophilus aegyptius*
IIT	Incompatible Insect Technique
M	Molar
m	Milli
Mb	Megabyte
min	Minute(s)
MLST	Multilocus sequence typing
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
RLOs	Rickettsia-like organisms
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
sec	Second(s)
SIT	Sterile Insect Technique
SNP	Single Nucleotide Polymorphism

spp.	Subspecies
spec2F	Primer for amplifying *wsp*
spec1R	Primer for amplifying *wsp*
spec2R	Primer for amplifying *wsp*
T	Thymidine
TAE	Tris-acedate-EDTA
Taq	*Thermus aquaticus*
U	Unit
µm	Micrometre
µl	Microlitre
UV	Ultraviolet (light)
*w*Cer	*Wolbachia* variant from *Rhagoletis cerasi*
wsp	*Wolbachia* surface protein
*wsp*81F	Primer for amplifying *wsp*
*w*sp226F	Primer for amplifying *wsp*
*wsp*691R	Primer for amplifying *wsp*

8.2 List of Figures

8.3 List of Tables

8.4 Detailed table of screening results

Population #	Location	Host	Individuals#	*w*Cer2 infected	% *w*Cer2 infection	Haplotype 1	Haplotype 2	*w*Cer2 uninfected HT1	*w*Cer2 uninfected HT2	*w*Cer2 HT1	*w*Cer2 HT2	% *w*Cer2 uninfected HT1	% *w*Cer2 HT2
1	**Dossenheim**	*Prunus*	16	16	100%	0	16	0	0	0	16	100%	100%
2	**Bensheim**	*Prunus*	16	1	6.2%	15	1	15	0	0	1	100%	100%
3	**Ober-Ramstadt**	*Prunus*	16	1	6.2%	15	1	15	0	0	1	100%	100%
4	**Stockstadt**	*Prunus*	16	3	18.7%	13	3	13	0	0	3	100%	100%
5	**Erbenheim**	*Lonicera*	15	1	6.6%	14	1	14	0	0	1	100%	100%
6	**Hailer**	*Prunus*	16	16	100%	0	16	0	0	0	16	100%	100%
7	**Idstein**	*Lonicera*	10	1	10%	9	1	9	0	0	1	100%	100%
8	**Rosbach**	*Prunus*	16	0	0%	16	0	16	0	0	0	100%	100%
9	**Schlüchtern**	*Lonicera*	16	6	37.5%	10	6	10	0	0	6	100%	100%
10	**Weckesheim**	*Prunus*	16	4	25%	12	4	12	0	0	4	100%	100%
11	**Utphe**	*Prunus*	16	2	12.5%	14	2	14	0	0	2	100%	100%
12	**Langsdorf**	*Prunus*	16	3	18.7%	13	3	13	0	0	3	100%	100%
13	**Lich**	*Prunus*	16	0	0%	16	0	16	0	0	0	100%	100%
14	**Gießen 1**	*Prunus*	16	16	100%	5	11	0	0	4	12	0%	75%
15	**Gießen 2**	*Lonicera*	16	12	75%	6	10	4	0	2	10	66.6%	83.3%
16	**Lahnau**	*Prunus*	16	3	18.7%	16	0	13	0	3	0	81.2%	0%
17	**Wallenrod**	*Lonicera*	16	0	0%	16	0	16	0	0	0	100%	100%
18	**Grossenmoor**	*Lonicera*	14	2	14.2%	12	2	12	0	0	2	100%	100%
19	**Alsfeld**	*Lonicera*	16	2	12.5%	13	3	12	1	0	2	92.3%	100%

Supplementary Table 1 *Detailed screening results of 19 populations of R. cerasi from 2016.*

Population #	Location	Host	year	Individuals#	*w*Cer2 infected	% *w*Cer2 infection	Haplotype 1	Haplotype 2	*w*Cer2 uninfected HT1	*w*Cer2 uninfected HT2	*w*Cer2 HT1	*w*Cer2 HT2	% *w*Cer2 uninfected HT1	% *w*Cer2 HT2
1-10	**Dossenheim**	*Lonicera*	1999	10	10	100%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3-28	**Dossenheim**	*Prunus*	2008	16	16	100%	0	16	0	0	0	16	0%	100%
3-23	**Ober-Ramstadt**	*Prunus*	2008	15	1	6.7%	14	1	14	0	0	1	93.3%	0%
3-24	**Ober-Ramstadt**	*Lonicera*	2008	15	0	0%	15	0	15	0	0	0	100%	0%
3-22	**Stockstadt**	*Prunus*	2008	16	2	12.5%	14	2	14	0	0	2	100%	100%
3-19	**Lich**	*Prunus*	2008	16	1	6.3%	16	0	15	0	1	0	93.8%	0%
4-1	**Lich**	*Prunus*	2014	10	0	0%	0	0	10	0	0	0	100%	0%
2-13	**Gießen**	*Lonicera*	2001	10	1	10%	9	1	9	0	0	1	90%	10%
3-18	**Gießen**	*Lonicera*	2008	16	11	68.8%	6	10	5	0	1	10	31.3%	90.9%
3-17	**Lahnau**	*Lonicera*	2008	16	2	12.5%	16	0	14	0	2	0	87.5%	0%
2-14	**Alsfeld**	*Lonicera*	2000	10	5	50%	7	3	5	0	2	3	50%	30%
3-20	**Reichelsheim**	*Prunus*	2008	16	2	12.5%	14	2	14	0	0	2	87.5%	100%
3-21	**Mechtildshausen**	*Prunus*	2008	16	0	0%	16	0	16	0	0	0	100%	0%

Supplementary Table 2 *Detailed screening results of populations of R. cerasi in Hesse between 1999-2014 (Schuler et al., 2016a).*

8.5 Working protocols

8.5.1 DNA Extraction protocol (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 seconds) and put on the heating block at 55°C/450 rpm for 2-3 hours
- 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 preparation
- 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 seconds
- Transfer the samples to the binding columns (approx. 650 µl)
- Spin at 8.000 rpm for 1 min
- Discard tube with flow-through and put column in a fresh tube
- Add 500 µl of wash solution
- Spin at 8.000 rpm for one minute
- Discard flow-through 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

8.5.2 DNA Purification protocol (peqGOLD Cycle-Pure kit)

- Mix PCR-product with the same volume of XP1 buffer
- Vortex carefully
- Pipette the mixture into a HiBind®-column
- Spin at 10,000 rpm for 1 minute
- Wash with 650 µl of SPW-wash buffer and spin at 10,000 rpm for 1 minute
- Discard flow-through and wash again with 650 µl SPW-wash buffer and spin at 10,000 rpm for 1 minute
- Spin empty column at 10,000 rpm for 1 minute
- Add 20 µl elution buffer
- Incubate for 2 minutes
- Spin at 10,000 rpm for 1 minute

8.5.3 PCR protocols

8.5.3.1 PCR with specific *wsp* primers

Mastermix

Primer pairs: *w*sp226F and spec1R (*w*Cer1)

spec2F spec2R (*w*Cer2)

Volume: 10µl, filled up with distilled H_2O

y-Buffer	2mM
BSA	1mg/ml
dNTP's	800µM
Primer	0,2 µM
Taq	0,5 U

Thermocycler program

initial denaturation		94°C	2 min.
35 cycles	Denaturation	94°C	30 sec.
	primer annealing	55°C	45 sec.
	extension	72°C	1 min.
final extension		72°C	10 min.

8.5.3.2 PCR with mitochondrial primers and RFLP

Mastermix

Primer pairs: Pat and Dick

Volume: 10µl, filled up with distilled H_2O

y-Buffer	2mM
BSA	1mg/ml
dNTP's	800µM
Primer	0,2 µM
Taq	0,5 U

Thermocycler program

initial denaturation		94°C	2 min.
35 cycles	Denaturation	94°C	30 sec.
	primer annealing	55°C	45 sec.
	extension	72°C	1 min.
final extension		72°C	10 min.

RFLP

1,6µl of Mastermix (filled up with distilled H_2O) in every PCR product

HaeIII	2U
B-R	1mg/ml