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Wolbachia in Parasitoids Attacking Native European and Introduced Eastern Cherry Fruit Flies in Europe

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Abstract

The eastern cherry fruit fly, *Rhagoletis cingulata* Loew (Diptera: Tephritidae), is an economically important pest of cherries in North America. In 1983 it was first reported in Europe where it shares its ecological niche with the native European cherry fruit fly, *Rhagoletis cerasi* L. (Diptera: Tephritidae). Their coexistence in Europe led to the recent horizontal transmission of the *Wolbachia* strain *w*Cer1 from *R. cerasi* to *R. cingulata*. Horizontal *Wolbachia* transmission is mediated by either sharing of ecological niches or by interacting species such as parasitoids. Here we describe for the first time that two braconid wasps, *Psyttalia rhagoleticola* Sachtleben (Hymenoptera: Braconidae) and *Utetes magnus* Fischer (Hymenoptera: Braconidae), naturally parasitizing *R. cerasi*, use the invasive *R. cingulata* in Europe as a new host. In contrast, no parasitoids that parasitize *R. cingulata* in its native American range were detected in the introduced European range. Diagnostic *Wolbachia* PCR screening and sequence analyses demonstrated that all *P. rhagoleticola* individuals were infected with the newly described *Wolbachia* strain *w*Rha while all *U. magnus* individuals were uninfected. *w*Rha is different from *w*Cer1 but had an *Wolbachia surface protein* (*wsp*) gene sequence that was identical to *w*Cer2 of *R. cerasi* and *w*Cin2 of *R. cingulata*. However, multi locus sequence typing revealed differences in all loci between *w*Rha and the tephritid's strains. The horizontal transmission of *w*Cer1 between the two tephritid species did not result in fixed heritable infections in the parasitoids. However, the parasitoids may have acted as a transient *w*Cer1 vector.

Key words: invasive species, *Wolbachia*, parasitoid, *Rhagoletis*, horizontal transmission

The introduction of exotic insects into new environments poses a major threat to global biodiversity (Traveset and Richardson 2006). Although just a small proportion of introduced species may be established in any new environment and become invasive (Williamson 1996), human activity and globalization lead to an ever-growing number of translocated species (Mack et al. 2000, Levine and D'Antonio 2003). Once in new environments, alien species can influence native species and their ecosystems mainly by competition over resources (Dorcas et al. 2012), hybridization with native species (Fitzpatrick et al. 2010), and introduction of parasites and pathogens that can impact native species (Stout and Morales 2009, Meeus et al. 2011). Natural enemies are hypothesized to play a major role in biological invasions (Dunn et al. 2012). The release from natural enemies during the invasion process can allow fast adaptation and rapid spread of alien species within new environments (Keane and Crawley 2002). The lack of enemies in new habitats can facilitate alien species establishment (Torchin et al. 2003) and may enhance its ability to impact native species via competition (Tilman 1999). Conversely, invasive species can provide new food sources for natural enemies (Strauss et al. 2012). For example, invasive insect species may be a new resource for native parasitoids; however, only few studies have investigated this (Klug et al. 2008).

The European cherry fruit fly *Rhagoletis cerasi* L. (Diptera, Tephritidae) is the major pest of sweet and sour cherries (*Prunus* spp.) in Europe (Boller et al. 1970, Daniel and Grunder 2012). In addition to cherries, *R. cerasi* also infests berries of honeysuckle, *Lonicera* spp. (Boller and Bush 1974, Schwarz et al. 2003). It occurs in all cherry-growing countries in Europe and is also present in Russia and western Asia (Fimiani 1989, Namin and Rasoulian 2009). It has a univoltine life cycle with an obligatory diapause (Boller and Prokopy 1976). Females oviposit usually one egg per cherry, and emerging larvae feed in the pulp of the growing fruit for 4–10 wk and overwinter as pupae in the soil (Boller and Prokopy 1976). After oviposition, *R. cerasi* larvae and pupae are exposed to up to 12 parasitoid species (Hoffmeister 1990, 1992). Parasitization rates fluctuate significantly between different life stages of *R. cerasi*. Larvae can be attacked only by well-synchronized parasitoids, and pupae by more generalist parasitoids. Considering the short time of larval occurrence in the field, generalist pupal parasitoids do usually not require to be synchronized since pupae are available for most of the year (Hoffmeister 1990). Parasitization rates also differ between the two host plant genera and geographically (Hoffmeister 1990).

Recently, the North American eastern cherry fruit fly *R. cingulata* Loew (Diptera: Tephritidae) was introduced from North America into Europe (Lampe et al. 2005, Johannesen et al. 2013). It now co-occurs with *R. cerasi* in cherries, while it is not known to infest honeysuckle. *Rhagoletis cingulata* has a similar univoltine life cycle, with adult emergence from the overwintering pupal stage in the soil 3 wk after its European relative (Vogt et al. 2010). In Europe, *R. cingulata* was reported for the first time in Switzerland in 1983 and thereupon in Austria, Belgium, Croatia, France, Germany, Hungary, Italy, the Netherlands, and Slovenia (EPPO 2007, 2010, 2013, 2014). However, while *R. cingulata* was mostly detected in Germany, Hungary, and the Netherlands, detection in other countries was infrequent (Smit and Dijkstra 2008, Schuler et al. 2013).

Wolbachia is an alphaproteobacterium widespread in different insect taxa, several other arthropods and nematodes (Werren et al. 2008). This endosymbiont is predominantly inherited maternally through the egg cytoplasm. *Wolbachia* strains are usually able to enhance their spread by mechanisms promoting the reproductive success of infected females, like parthenogenesis, male-killing, feminization, or the induction of cytoplasmic incompatibility (Engelstädter and Hurst 2009). However, phylogenetic incongruence between *Wolbachia* and host species demonstrated that this endosymbiont can occasionally move horizontally between species (O'Neill et al. 1992, Vavre et al. 1999, Baldo et al. 2008, Kawasaki et al. 2016), and between individuals of a species (Kraaijeveld et al. 2011, Schuler et al. 2016). Although the mechanisms of horizontal transmission in nature are not fully understood, sharing the same ecological niche and the interaction with natural enemies such as parasitoids and predators are expected to be key factors for horizontal transmission (Raychoudhury et al. 2009, Stahlhut et al. 2010, Gehrer and Vorburger 2012). Therefore, the introduction and establishment of a new species could result in an opportunity for horizontal transmission of endosymbionts between native and introduced species (Schuler et al. 2013).

Although *Wolbachia* is one of the best-studied endosymbionts, knowledge on its influence on invasive species is scarce (Nguyen et al. 2016). For example, prevalence of *Wolbachia* in the European paper wasp, *Polistes dominulus*, is similar in its native and introduced ranges, suggesting that this endosymbiont has been cointroduced (Stahlhut et al. 2006). In contrast, invasive populations of the Argentine ant *Linepithema humile* and the garden ant *Lasius neglectus* showed significantly lower *Wolbachia* prevalences than in their native ranges (Tsutsui et al. 2003, Cremer et al. 2008) or even endosymbiont loss (Reuter et al. 2004). A similar scenario was recently described for the loss of *Wolbachia* but not another insect endosymbiont, *Cardinium*, in the invasive range of a thrips species while native populations have both endosymbionts (Nguyen et al. 2016).

Currently, at least five *Wolbachia* strains (*w*Cer1–*w*Cer5) are described from *R. cerasi* (Riegler and Stauffer 2002, Arthofer et al. 2009, Augustinos et al. 2014, Schuler et al. 2016). American populations of *R. cingulata* are only infected by one *Wolbachia* strain, *w*Cin2, a strain that, based on available sequence information, appears identical to the *w*Cer2 strain of *R. cerasi*. However, European populations of *R. cingulata* also have a second strain, *w*Cin1 (Schuler et al. 2009, 2013). Characterization of the five multi locus sequence typing (MLST) genes and the *Wolbachia surface protein* (*wsp*) gene showed that this strain is identical to *w*Cer1, a strain that is omnipresent in *R. cerasi* (Riegler and Stauffer 2002, Arthofer et al. 2011, Schuler et al. 2013). While these data highlight that the shared ecological niche of the native and introduced *Rhagoletis* species resulted in a horizontal transmission of *w*Cer1 from *R. cerasi* to *R. cingulata* within a short time period (Schuler et al. 2013), the mechanism how this endosymbiont had crossed host species boundaries remained unclear.

Here we studied the *Wolbachia* infection of parasitoids attacking both *Rhagoletis* species. We examined whether the introduced fruit fly species *R. cingulata* in Europe is attacked by any parasitoids, either from North America or from its European congeneric *R. cerasi*. Furthermore, we tested whether these parasitoids are also infected by *w*Cer1, and therefore constitute intermediate *Wolbachia* hosts with a potential role in horizontal *w*Cer1 transmission between the native *R. cerasi* and the introduced *R. cingulata* in Europe.

Materials and Methods

Insect Sampling, DNA Extraction, DNA Barcoding, and Sequence Analysis

Infested cherries were collected in four localities in Austria (AT: Prinzersdorf), the Czech Republic (CZ: Brno), and Germany (Ger1: Ingelheim; Ger2: Dresden). Infested fruits were transported to the laboratory where *Rhagoletis* larvae were allowed to emerge and pupate. In this way we obtained pupae of *R. cerasi* from Austria and the Czech Republic where *R. cingulata* is very rare (Egartner et al. 2010, EPPO 2014). From the two localities in Germany we obtained pupae of *R. cingulata*. Pupae were kept at diapause conditions of 4 °C and 65–70% relative humidity for 5 mo (Vallo et al. 1976) followed by incubation at 24 °C. Emerging parasitoids were collected directly after eclosion and stored in ethanol. They were then morphologically grouped into two morphospecies.

All emerged parasitoid individuals were DNA extracted using the GenElute Mammalian DNA Mini-Prep Kit (Sigma) according the manufacturer's protocol. The DNA was dissolved in 50 µl elution buffer and stored at 4 °C. To confirm the morphological identification, we amplified and sequenced a 611 bp fragment of the mitochondrial *cytochrome oxidase I (COI)* gene, a region used for DNA barcoding using the primers LCO1490 and HCO2198 (Folmer et al. 1994). PCR was performed in 10 µl reactions containing 1× NH_4 buffer, 2 mM $MgCl_2$, 100 µM dNTPs, 0.2 µM of each primer, 0.2 U *Taq* polymerase (Thermo Scientific), and 1 µl

Table 1. Information about the occurrence of haplotypes of two parasitoid species (Psy—*Psyttalia rhagoletticola*, Ut—*Utetes magnus*) in different cherry fruit fly species and populations

Species	Locality	Host	*n*	Psy1	Psy2	Psy3	Psy4	Psy5	Psy6	Ut1
P. rhagoletticola	AT—Prinzersdorf	*R. cerasi*	7	4	2			1		
P. rhagoletticola	CZ—Brno	*R. cerasi*	4	2	1			1		
P. rhagoletticola	GER—Ingelheim	*R. cingulata*	6	2		2	1		1	
U. magnus	AT—Prinzersdorf	*R. cerasi*	1							1
U. magnus	GER—Ingelheim	*R. cingulata*	5							5
U. magnus	GER—Dresden	*R. cingulata*	5							5

of template DNA. PCR amplifications were performed in a 2720 thermal cycler (Applied Biosystems) with the following cycling conditions: 2 min at 94 °C followed by 5 cycles at 94 °C for 30 s, 45 °C for 90 s, and 72 °C for 1 min and 35 cycles at 94 °C for 30 s, 51 °C for 1 min, and 72 °C for 1 min with a final extension at 72 °C for 5 min. PCR products were then Sanger sequenced by Eurofins MWG Operon (Ebersberg, Germany). *COI* sequences were analyzed and a phylogenetic tree constructed with the inclusion of other parasitoid sequences from Rugman-Jones et al. (2009). Maximum Likelihood analyses was performed in Mega 5.2 (Tamura et al. 2011) using the Hasegawa–Kishino–Yano (HKY) model.

Wolbachia Characterization Using *wsp* and MLST

All parasitoids were screened for the presence of *Wolbachia* using the *wsp* primers 81F and 691R (Braig et al. 1998). Positive samples were further characterized for the five MLST genes *gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA* (Baldo et al. 2006). PCR was set up using the same conditions as described above. Cycling conditions were 2 min denaturation at 95 °C, followed by 35 cycles at 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min, and a final extension at 72 °C for 15 min. PCR conditions of all primer sets were identical except for *ftsZ* with an annealing temperature of 50 °C. PCR products were then Sanger sequenced by Eurofins MWG Operon (Ebersberg, Germany).

Phylogenetic Analyses of *Wolbachia*

Sequence chromatograms were carefully screened for ambiguous sites to exclude the presence of multiple infections, edited manually, and assembled by CodonCode Aligner vers. 3.7 (Codon Code Corporation). To exclude PCR artifacts, all genotypes were confirmed by independent PCRs. DNA sequences were determined by BLAST analysis and *Wolbachia* sequences were compared with the MLST database (http://pubmlst.org/wolbachia). Obtained sequences were deposited in GenBank and the *Wolbachia* MLST database. The pairwise genetic distances between the different *Wolbachia* strains were calculated using the Kimura 2 parameter model in Mega 5.2 (Tamura et al. 2011).

The sequences of the five MLST genes were concatenated and aligned with the MLST sequences of *w*Cer1, *w*Cer2, *w*Cer4, and *w*Cer5 in *R. cerasi* (Arthofer et al. 2011), and *w*Cin1 and *w*Cin2 in *R. cingulata* (Schuler et al. 2013). *w*Cer3, a strain that arose from recombination between *w*Cer2 and *w*Cer5, is present in low titers and at low prevalence in *R. cerasi* (Arthofer et al. 2009). Therefore, it was not included in this study. The optimal substitution model was determined by jModeltest (Posada and Crandall 1998), and maximum likelihood analyses of *wsp* and MLST alignments were performed using the Hasegawa–Kishino–Yano (HKY+G+I) substitution model in MEGA 5.2 (Tamura et al. 2011).

Results

Identification of Parasitoids in *R. cerasi* and *R. cingulata*

In total, we investigated 28 parasitoid wasps that emerged from *R. cerasi* and *R. cingulata* from four different localities of three countries (Table 1). The wasps were grouped into two morphospecies that were then identified as *Psyttalia rhagoletticola* Sachtleben (1934) and *Utetes magnus* Fischer (1958), both of the braconid subfamily of Opiinae. A total of 17 individuals were identified as *P. rhagoletticola*—seven in Austria, four in Czech Republic, and six in Ingelheim (Ger1). Eleven individuals were identified as *U. magnus*—five in Ingelheim (Ger1), five in Dresden (Ger2), and one in Austria (Table 1). *Psyttalia rhagoletticola* was collected exclusively from *R. cerasi* pupae in Austria and Czech Republic, and exclusively from *R. cingulata* pupae in both German populations. *Utetes magnus* was less common and found in one *R. cerasi* pupa in Austria and in ten *R. cingulata* pupae from both German populations.

The *COI* sequences of the 17 *P. rhagoletticola* individuals contained between one to six SNPs (over a length of about 600 bp) and were assigned to six haplotypes (GenBank KX503389–KX503394). These six haplotypes were monophyletic when compared with *COI* sequences of other *Psyttalia* species (Rugman-Jones et al. 2009), thereby confirming the species status based on morphological identification (Fig. 1). According to the phylogenetic analysis the closest relative was *Psyttalia ponerophaga* from Pakistan. The abundance of the *P. rhagoletticola* haplotypes varied, as they occurred as single individual (Psy4 and Psy8) or up to eight individuals (Psy1) (Table 1). The most common haplotype, Psy1, was found in all three localities where *P. rhagoletticola* occurred. German *P. rhagoletticola* was most polymorphic and had four haplotypes found across six individuals (Psy1, Psy3, Psy4, Psy6). Three haplotypes (Psy3, Psy4, Psy6) were found exclusively in Germany. Austrian *P. rhagoletticola* had three different haplotypes (Psy1, Psy2, Psy5) across seven individuals. All three haplotypes were also found in the Czech Republic, while only Psy1 was shared with German populations. In contrast to the diverse *P. rhagoletticola*, all 11 *U. magnus* specimens shared a single haplotype (GenBank KX503395).

Characterization of *Wolbachia* in Parasitoids of *Rhagoletis*

Irrespective of host fruit fly species, all individuals of *P. rhagoletticola* were positive for *Wolbachia*, while *Wolbachia* was not detected in any specimens of *U. magnus*. Characterization of the *wsp* gene and the MLST loci revealed that all *P. rhagoletticola* individuals were infected by the same *Wolbachia* strain. This strain was named *w*Rha according to Zhou et al. (1998). BLAST search of the *wsp* fragment revealed identical sequences for *w*Rha and *w*Cer2 in *R. cerasi* (Riegler and Stauffer 2002) and *w*Cin2 in *R. cingulata* (Schuler et al. 2009; Fig. 2a). However, all *w*Rha MLST alleles were different to

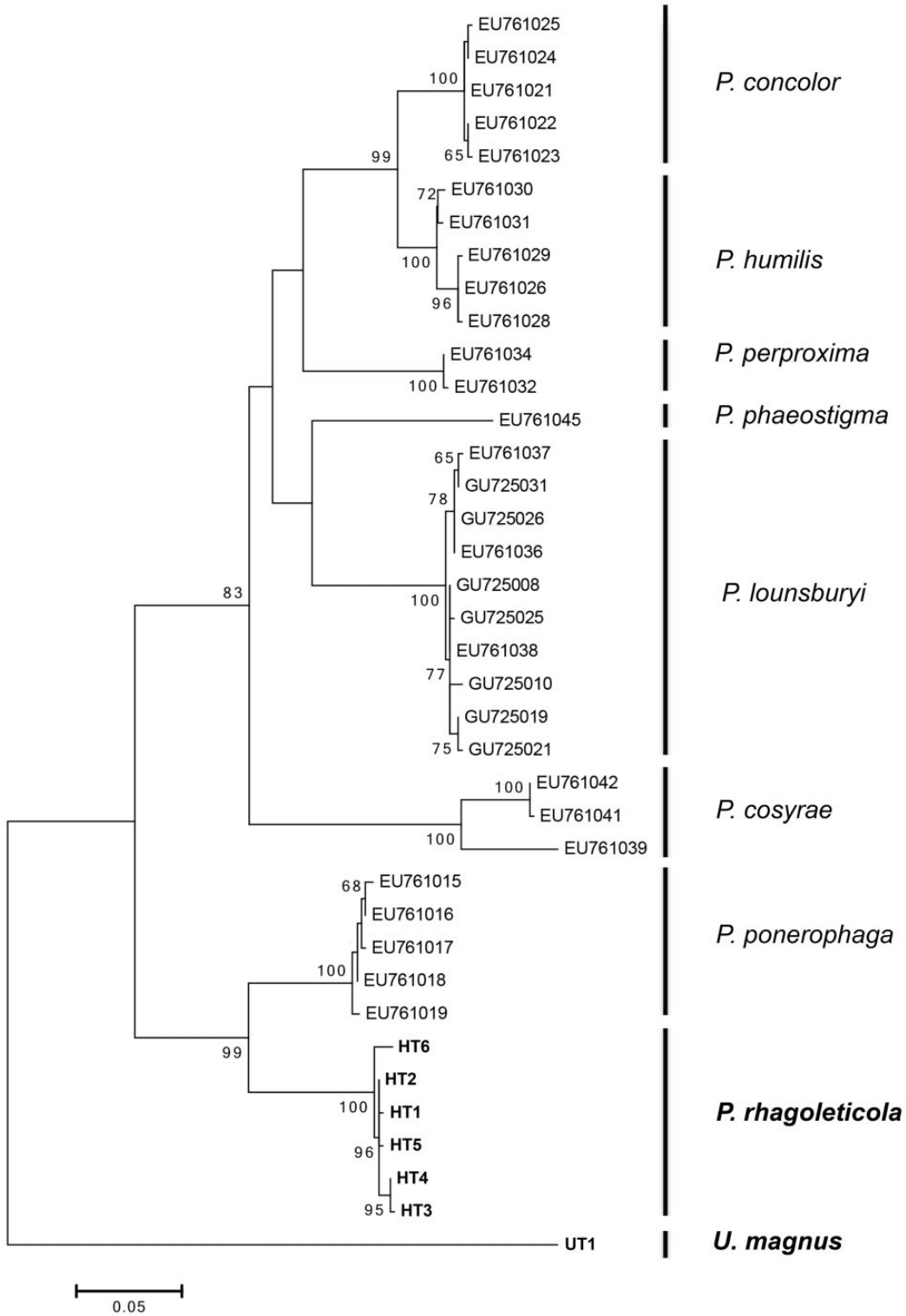

Fig. 1. Phylogenetic tree based on a fragment of the mitochondrial *COI* gene. Consensus sequences from Rugman-Jones et al. (2009) are listed with their GenBank accession numbers, newly sequenced haplotypes of *Psyttalia rhagoleticola* are highlighted in bold, newly sequenced *Utetes magnus* is used as outgroup. Bootstrap values >60% are shown (1,000 replicates). Scale bar represents number of nucleotide substitutions per site.

the MLST alleles of *w*Cer2 from *R. cerasi* (Arthofer et al. 2011) and *w*Cin2 from *R. cingulata* (Schuler et al. 2013), resulting in different phylogenetic placements of the strains (Fig. 2b). Pairwise distances between MLST alleles of *w*Rha, *w*Cer2, and *w*Cin2 ranged from 0.012 on *fbpA* to 0.023 on *hcpA* (Table 2).

Furthermore, none of the *wsp* and MLST alleles of *w*Rha were identical with *w*Cin1, the strain horizontally acquired by *R. cingulata* from *R. cerasi* in Europe (Schuler et al. 2013). The comparison of the *wsp* sequence of *w*Rha with the other strains in *R. cerasi*, *w*Cer4 and *w*Cer5, showed a sequence divergence of 0.736 and

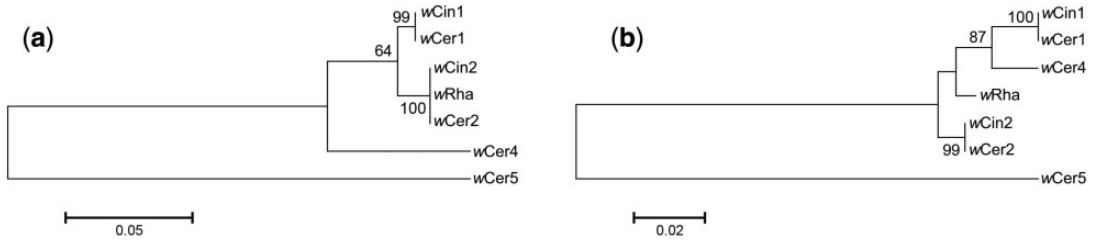

Fig. 2. Maximum likelihood tree of the *wsp* gene (**a**) and the concatenated MLST genes (**b**) of *Wolbachia* in *Psyttalia rhagoletticola* *w*Rha, *Rhagoletis cerasi* *w*Cer1, *w*Cer2, *w*Cer4, and *w*Cer5, and *Rhagoletis cingulata* *w*Cin1 and *w*Cin2. Bootstrap values >60% are shown. Numbers at nodes represent posterior probabilities >50% based on 1,000 replicates. Scale bar represents number of nucleotide substitutions per site.

Table 2. Pairwise genetic distances for *wsp* and the five MLST loci between *w*Rha, *w*Cer, and *w*Cin strains

	wsp	*coxA*	*fbpA*	*ftsZ*	*hcpA*	*gatB*
*w*Rha	0	0	0	0	0	0
*w*Cer1	0.024	0.009	0.019	0.023	0.026	0.048
*w*Cin1	0.024	0.009	0.019	0.023	0.026	0.048
*w*Cer2	0	0.021	0.012	0.016	0.023	0.021
*w*Cin2	0	0.021	0.012	0.016	0.023	0.021
*w*Cer4	0.736	0.009	0.050	0.002	0.007	0.034
*w*Cer5	0.928	0.136	0.281	0.103	0.131	0.135

0.928. However, in three of the five MLST loci *w*Rha and *w*Cer4 were closely related, with sequence divergences ranging from 0.002 in *ftsZ* to 0.05 in *fbpA*. *w*Rha and *w*Cer5 were more divergent, with sequence divergences ranging from 0.103 in *ftsZ* to 0.281 in *fbpA* (Table 2). A comparison with the MLST database showed that the newly described *w*Rha strain is a novel strain with unique *ftsZ*, *coxA*, and *gatB* alleles according to comparisons with alleles deposited in the MLST database. All sequences of *wsp* and the five MLST alleles were assigned to *w*Rha and entered in GenBank (KX503396–KX503401) and the MLST database (ID 1826).

Discussion

The introduction and establishment of alien species in new environments is a significant threat to biodiversity worldwide (Mack et al. 2000). Many studies described the successful invasions of various species by focusing on the genetic (Mooney and Cleland 2001) and ecological dimensions (Davis 2009). Within the latter, many focused on direct effects of alien species on ecosystems, for example via predation or competition over resources, while fewer studies looked at native diversity impacting invasive species in their newly established invasive range. Here we studied parasitoids of the native European cherry fruit fly *R. cerasi* and the introduced North American eastern cherry fruit fly *R. cingulata* in Europe. We examined the parasitoids' *Wolbachia* infection status and potential role as vectors in the horizontal *w*Cer1 transmission recently detected between their native and introduced host fruit fly species (Schuler et al. 2013). While our analysis of four population samples did not detect any American parasitoids of *R. cingulata* in their introduced range in Europe, we found that two European parasitoids of *R. cerasi*, *P. rhagoletticola* and *U. magnus*, were able to attack and develop in the introduced congener. We also surveyed and characterized *Wolbachia* infections across the two trophic levels, and found *Wolbachia* in *P. rhagoletticola* but not in the other parasitoid species. This newly described strain, *w*Rha, has a *wsp* gene sequence that is identical with *wsp* of *w*Cer2 found in both fruit fly species but is different from *w*Cer2 and other strains in all MLST genes. Therefore, the horizontal transmission of the *Wolbachia* strain *w*Cer1 across both fruit fly species has not resulted in the establishment of identical infections in their parasitoids.

The success of establishment of introduced pest species is often correlated with the release from parasites and pathogens present in the native range of invasive hosts (Mitchell and Power 2003, Torchin and Mitchell 2004). In its native range in North America, *R. cingulata* is host to five different parasitoid species: *Coptera cingulatae*, *Coptera occidentalis*, *Diachasma ferrugineum*, *Diachasmimorpha mellea*, and *Utetes frequens* (Wharton and Marsh 1978, Muesebeck 1980, Rull et al. 2011, Hood et al. 2015, Hamerlinck et al. 2016). While *Utetes* attack egg or early larval stages, *Diachasma* and *Diachasmimorpha* oviposit into late instar larvae in fruits. *Coptera* species, however, attack their host after pupation in the soil. The introduction of *R. cingulata* to Europe could have been linked with a potential cointroduction of its natural enemies attacking either the egg or larval stages. However, we did not detect any American native parasitoids in *R. cingulata* in Europe, and thus the establishment of *R. cingulata* in Europe may have profited from a release from its natural American enemies prior or during its establishment in Europe.

In contrast to previous studies that described 12 species of parasitoids in *R. cerasi* (Hoffmeister 1990, 1992), in this study we detected just two, *P. rhagoletticola* and *U. magnus*. The low diversity captured in our study may be due to our sampling strategy. In previous studies, *Rhagoletis* pupae were exposed to a wider parasitoid community in the field, including species that specifically target the fly puparium, while in our study we collected fruits with infested larvae that then pupated in a laboratory environment; therefore, we effectively excluded puparium parasitoids. Both *P. rhagoletticola* and *U. magnus* are parasitoids of the last larval instar of *R. cerasi* and complete their life cycle in the pupal stage of their host. Nine of the 12 described parasitoid species attack *R. cerasi* during the pupal stage in the soil (Hoffmeister 1990) and could therefore not be detected in our study. Furthermore, Hoffmeister (1990, 1992) described that some parasitoids are associated exclusively to one of the two different host forms on cherry and honeysuckle: while eleven different parasitoid species were reared from *Lonicera* infesting flies, just six parasitoid species were collected from *Prunus* infesting flies. Similarly, Monaco (1984) found exclusively *U. magnus* in wild cherries (*Prunus mahaleb*), and no parasitoids in cultivated cherries while we found two parasitoid species in cultivated cherries.

The different biology of *R. cerasi* and *R. cingulata* could potentially influence the different occurrence of the two parasitoid species. While *P. rhagoletticola* was reared from both species, all except a single individual of *U. magnus* emerged from *R. cingulata* pupae. It has previously been found that adult *U. magnus* appears in the field ~2 wk later than adult *P. rhagoletticola* (Hoffmeister 1990), and this could result in more parasitization of *R. cingulata*, which

has its peak flight activity 3 wk after *R. cerasi* (Vogt et al. 2010). Furthermore, both parasitoids are generalists that attack larvae of different *Rhagoletis* species. The main host of *P. rhagoleticola* are *R. cerasi* and *Myoleia lucida*, a tephritid fly that also infests *Lonicera* fruits. In contrast, *U. magnus* mainly attacks *Rhagoletis meigenii* and *Rhagoletis alternata* developing in *Berberis* and *Rosa* species that usually fruit at least one month after cherries (Hoffmeister 1990, 1992). The late emergence of *U. magnus* adults may therefore constitute a preadaption to introduced *R. cingulata* that also occurs later in the season. Adults of *P. rhagoleticola*, however, are active for long enough time periods to attack larvae of both *R. cerasi* and *R. cingulata*.

The detection of two shared parasitoid species in native *R. cerasi* and introduced *R. cingulata* may suggest that they are responsible for the horizontal transmission of the *Wolbachia* strain *w*Cer1 from *R. cerasi* to *R. cingulata* (Schuler et al. 2013). Laboratory studies demonstrated that the close interaction of parasitoids with their hosts could result in horizontal *Wolbachia* transmission. Parasitoids can have three different roles in horizontal transmission: 1) They can acquire *Wolbachia* from an infected host and then transmit it vertically to their offspring. For example, parasitic *Leptopilina boulardi* wasps acquired *Wolbachia* by attacking infested *Drosophila* pupae. Subsequently, the bacterium was vertically transmitted within the new host species (Heath et al. 1999). 2) *Wolbachia* can be transmitted from one parasitoid to another by attacking the same host. For example, *Trichogramma* larvae, developing in *Wolbachia*-uninfected moths acquired the bacterium by super- and multiparasitism with infected larvae of the same (Huigens et al. 2000) or closely related parasitoid species (Huigens et al. 2004). Finally, 3) parasitoids can act as vectors by transmitting *Wolbachia* without being infected. For example, parasitic wasps attacking *Wolbachia*-infested *Bemisia tabaci* were able to transmit the bacterium with contaminated mouthparts and ovipositors to uninfected hosts but did not acquire it, as it was not detected in the parasitoid tissues and gonads (Ahmed et al. 2015).

Sequence analysis of the *wsp* gene of *w*Rha of *P. rhagoleticola* indicated that it is identical to *wsp* of *w*Cer2, currently spreading in *R. cerasi* in Europe (Riegler and Stauffer 2002, Schuler et al. 2016), and *wsp* of *w*Cin2, omnipresent in *R. cingulata* across North America and Europe (Schuler et al. 2013). The characterization of the MLST genes, however, demonstrated that *w*Rha is genetically distinct from *Wolbachia* strains found in *R. cerasi* and *R. cingulata*. From this perspective, our data do not unequivocally support the hypothesis that parasitoids are responsible for the horizontal transmission of *w*Cer1 from native *R. cerasi* to introduced *R. cingulata*. A previous study that described the horizontal transmission of *Wolbachia* via parasitoids in whiteflies showed that nonendogenous *Wolbachia* can be transmitted by parasitoids within 48 h following the parasitoids contact with host *Wolbachia* via ovipositor and mouthparts (Ahmed et al. 2015). In our study, we collected parasitoids directly after eclosion from their hosts and they did not have access to any new hosts prior to DNA extraction. Therefore, we cannot rule out that under field conditions the parasitoid species can transfer *Wolbachia* via their ovipositor or mouthparts. A follow-up study should therefore test if parasitoids can transmit *Wolbachia* after attacking infected hosts.

The strain *w*Rha is genetically distinct from the *Wolbachia* strains of the fruit fly hosts from which *P. rhagoleticola* emerged. Therefore, it is an inherited infection of *P. rhagoleticola* and we can exclude it as a contamination or not inherited somatic infection present in *P. rhagoleticola*. However, the close genetic relationship of the *Wolbachia* strain found in *P. rhagoleticola* with *Wolbachia* strains in fruit fly hosts, in particular the identical *wsp* sequences, suggest a shared evolutionary history, potentially also involving recombination across MLST loci.

The acquisition of *Wolbachia* can influence the mitochondrial diversity of its host (Hurst and Jiggins 2005). Reproductive advantages of *Wolbachia*-infected individuals result in the spread of the associated mitochondrial genomes replacing the haplotypes of uninfected individuals (Schuler et al. 2016). Therefore, *Wolbachia*-infected species are assumed to display fewer mitochondrial lineages than uninfected ones (Hurst and Jiggins 2005). However, we found that all 11 individuals of *U. magnus* that were not infected by *Wolbachia* shared a single mitochondrial haplotype, while 17 *Wolbachia*-infected *P. rhagoleticola* individuals showed high mitochondrial diversity with six different haplotypes. This could indicate either an ancient *Wolbachia* infection of *P. rhagoleticola* or that the *w*Rha infection is a result of multiple horizontal transmission events into or within this host species. The incidence and prevalence of *Wolbachia* in one out of two tephritid parasitoid species in our study is similar to a previous study that detected *Wolbachia* in one out of two parasitoid species of Australian tephritids (Morrow et al. 2014). In contrast, a study about parasitoids of Malaysian tephritids reported all five tested braconid parasitoid species infected with *Wolbachia* (Muhamad et al. 2015).

In summary, our results showed that the two parasitoids *P. rhagoleticola* and *U. magnus* naturally attacking *R. cerasi* were adapted to introduced *R. cingulata* in Europe. While we could not detect *Wolbachia* in *U. magnus*, all individuals of *P. rhagoleticola* were infected by this endosymbiont. Characterization of the *wsp* and MLST loci showed that the parasitoid harbored a different *Wolbachia* strain than its *Rhagoletis* hosts. Although we cannot exclude a potential role of the parasitoids to have transiently vectored *Wolbachia* between the two *Rhagoletis* species, our data do not support the hypothesis that parasitoids are hosts for a *Wolbachia* strain that has been transferred between the two *Rhagoletis* species.

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