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Evidence for a recent horizontal transmission and spatial spread of *Wolbachia* from endemic *Rhagoletis cerasi* (Diptera: Tephritidae) to invasive *Rhagoletis cingulata* in Europe

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Abstract

The widespread occurrence of *Wolbachia* in arthropods and nematodes suggests that this intracellular, maternally inherited endosymbiont has the ability to cross species boundaries. However, direct evidence for such a horizontal transmission of *Wolbachia* in nature is scarce. Here, we compare the well-characterized *Wolbachia* infection of the European cherry fruit fly, *Rhagoletis cerasi*, with that of the North American eastern cherry fruit fly, *Rhagoletis cingulata*, recently introduced to Europe. Molecular genetic analysis of *Wolbachia* based on multilocus sequence typing and the *Wolbachia* surface protein *wsp* showed that all *R. cingulata* individuals are infected with *w*Cin2 identical to *w*Cer2 in *R. cerasi*. In contrast, *w*Cin1, a strain identical to *w*Cer1 in *R. cerasi*, was present in several European populations of *R. cingulata*, but not in any individual from the United States. Surveys of *R. cingulata* from Germany and Hungary indicated that in some populations, the frequency of *w*Cin1 increased significantly in just a few years with at least two independent horizontal transmission events. This is corroborated by the analysis of the mitochondrial cytochrome oxidase II gene that showed association of *w*Cin1 with two distinct haplotypes in Germany, one of which is also infected with *w*Cin1 in Hungary. In summary, our study provides strong evidence for a very recent inter-specific *Wolbachia* transmission with a subsequent spatial spread in field populations.

Keywords: horizontal transmission, invasive species, multilocus sequence typing, *Rhagoletis cerasi*, *Rhagoletis cingulata*, *Wolbachia*

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Introduction

The endosymbiotic α-Proteobacterium *Wolbachia* is probably the most common intracellular symbiont, infecting approximately 40% of all insect species (Zug & Hammerstein 2012). In the majority of cases, maternally

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inherited *Wolbachia* manipulates its host's reproduction to facilitate its own spread (Werren *et al.* 2008). This can lead to a rapid invasion of *Wolbachia* within a new host population (Turelli & Hoffmann 1991). In addition, closely related *Wolbachia* strains have been found in taxonomically unrelated insect hosts, implying the absence of long-term *Wolbachia*-host co-evolution and that the bacteria can spread by horizontal transmission among species (O'Neill *et al.* 1992; Vavre *et al.* 1999; Huigens *et al.* 2004; Baldo *et al.* 2008).

Despite its widespread distribution, direct evidence for the horizontal transmission of *Wolbachia* on an ecological timescale in nature is rare. Heath *et al.* (1999) demonstrated the horizontal transmission of *Wolbachia* from *Drosophila simulans* to one of its parasitoid wasp species, *Leptopilina boulardi*. However, *Wolbachia* failed to establish a long-term association with the novel host and was not inherited efficiently. Huigens *et al.* (2000, 2004) documented inter-specific transmission of *Wolbachia* between *Trichogramma* wasps sharing the same eggs of the moth *Trichoplusia ni*. A recent study on the parasitoid wasp *Leptopilina clavipes* provided evidence that horizontal transmission of a parthenogenesis-inducing *Wolbachia* strain played a role in the expansion of the new infection within a population (Kraaijeveld *et al.* 2011). In summary, the results from previous studies imply extensive horizontal transmission of *Wolbachia* on an evolutionary timescale (O'Neill *et al.* 1992; Baldo *et al.* 2008). On a much shorter ecological timescale, however, it appears that horizontal transmission may be limited by many factors and is therefore uncommon.

Obstacles for horizontal transmission of *Wolbachia* in nature have been shown in laboratory studies. Although *Wolbachia* can be transmitted among species by microinjection into embryos (e.g. Boyle *et al.* 1993; Riegler *et al.* 2004; McMeniman *et al.* 2008; Hughes *et al.* 2011) and adults (Ruang-Areerate & Kittayapong 2006), transinfections among different hosts can fail because of stochastic effects due to *Wolbachia* density, colonization efficiency of the germline (Frydman *et al.* 2006), pathogenic effects to the new host due to maladaptation to its immune system (Le Clec'h *et al.* 2012), low transmission efficiency and negative fitness effects associated with novel host associations (Clancy & Hoffmann 1997; Riegler *et al.* 2004). Not achieving a required threshold of infection prevalence based on these previous parameters can result in rapid loss of *Wolbachia* (Hoffmann & Turelli 1997; Kang *et al.* 2003; Riegler *et al.* 2004). The constraint of maladaptation was recently circumvented by initially adapting *Wolbachia* to a novel host background by transinfection into cell lines of the target species and then subsequently microinjecting the bacteria into individuals of the novel host (McMeniman *et al.* 2008; Hughes *et al.* 2011; Walker *et al.* 2011).

The colonization of new habitats due to invasive species can have effects on their endosymbiont community (e.g. Himler *et al.* 2011). *Wolbachia* infections widespread in invasive ant species were lost (Reuter *et al.* 2005) or were present in lower prevalence than in their native range (Ugelvig & Cremer 2012). This is consistent with the assumption that the invasion success of species is often correlated with the release of naturally co-evolved enemies (e.g. predators, parasites and pathogens; Prenter *et al.* 2004). Further, the invasion of species in new areas can create novel interactions with native species that can result in transmission of new pathogens to the native cohabitant (e.g. Kozubíková *et al.* 2009) or an acquisition of new symbionts from an invasive range (Himler *et al.* 2011). The previously reported detection of *Wolbachia* in invasive populations of *Ceratitis capitata* in South America suggests that the fly acquired *Wolbachia* horizontally following its introduction from the Old World, where the bacterium is absent in this species (Rocha *et al.* 2005). These findings illustrate that invasive species provide excellent opportunities to study potential horizontal *Wolbachia* transmissions in the field.

The eastern cherry fruit fly *Rhagoletis cingulata* infests the fruits of several cherry species in the genus *Prunus* in its native range in North America (Bush 1966; Foote 1981). Like other *Rhagoletis* species, *R. cingulata* has a univoltine life cycle, and adults live on average for about one month in nature (Bush 1966; Boller & Prokopy 1976). A female fly can lay up to 300 eggs in her lifetime, depositing eggs singly under the skin of ripening cherry fruits. Eggs hatch within three days and larvae feed in the pulp of the cherry and undergo three larval instars before diapausing as pupae in the soil (Boller & Prokopy 1976). *Rhagoletis* flies are moderately strong fliers and can disperse several kilometres if suitable host trees are not available locally (Boller & Prokopy 1976).

Rhagoletis cingulata was introduced to Europe from eastern North America at the end of the 20th century and has since been found in several European countries, that is, Austria, Belgium, Croatia, France, Germany, Hungary, Italy, the Netherlands and Slovenia (Merz & Niehuis 2001; Szeőke 2006; Bjeliš 2007; EPPO 2007, 2010; Egartner *et al.* 2010). Intensive monitoring programmes in Germany were initiated after *R. cingulata* was first detected in 1993. Population numbers of *R. cingulata* declined dramatically in subsequent years with only a single fly found in Germany in 1999 and just a few individuals in 2002 and 2003 (Lampe *et al.* 2005). In 2004, however, *R. cingulata* numbers increased substantially and the fly spread throughout Germany (Lampe *et al.* 2005; Vogt *et al.* 2010). In other countries, such as Switzerland and Austria, *R. cingulata* appears to have gone locally extinct and/or is currently present in only very small numbers (Daniel & Wyss 2007; Egartner *et al.* 2010).

In Europe, *R. cingulata* infests mainly sour cherries (*Prunus cerasus*) and wild sweet cherries (*Prunus avium*), where it can co-occur with the native European cherry fruit fly *Rhagoletis cerasi*. Although *R. cingulata* and *R. cerasi* attack similar hosts, they are phylogenetically distantly related taxa, belonging into different species groups (Bush 1966; Berlocher & Bush 1982; McPheron & Han 1997; Smith & Bush 1997). Previous studies have shown that *R. cerasi* is infected with at least five different *Wolbachia* strains designated *w*Cer1 to *w*Cer5 (Riegler & Stauffer 2002; Arthofer *et al.* 2009). The strain *w*Cer1 appears to be an obligate infection that is present in all *R. cerasi* flies (Riegler & Stauffer 2002; Arthofer *et al.* 2009), and attempts to cure the host of this strain have all failed (K. Köppler & H. Vogt, personal communication). Strain *w*Cer2 induces cytoplasmic incompatibility and is slowly spreading northward through European *R. cerasi* populations (Riegler & Stauffer 2002). The other three strains present in *R. cerasi* do not show any distinct structure in their distribution and are found in almost all fly populations at varying frequencies (Arthofer *et al.* 2009).

Initial studies of European *R. cingulata* indicated that European populations harbour at least two different *Wolbachia* strains (Schuler *et al.* 2009; Drosopoulou *et al.* 2011). A study based on *wsp* described two alleles identical to those of the *R. cerasi* strains *w*Cer1 and *w*Cer2 (Schuler *et al.* 2009). However, due to recombination events, identical *wsp* genes can be present in divergent *Wolbachia* strains (Baldo *et al.* 2006a) without reflecting their evolutionary history (Baldo *et al.* 2008) and therefore sequence analysis of more than just a single gene is essential to exclude misinterpretation (Baldo *et al.* 2008). A recent study using multilocus sequence typing (MLST) of *Wolbachia* infected *R. cingulata* individuals collected in Europe revealed up to three different alleles closely related to *w*Cer2 (Drosopoulou *et al.* 2011). The infection status of Native American *R. cingulata* populations is not known.

The recent introduction of *R. cingulata* to Europe, its ecological overlap with *R. cerasi*, the well-characterized nature of the *Wolbachia* infections in *R. cerasi* and the presence of similar *Wolbachia* strains in the invasive species provide a unique opportunity to study interactions between *Wolbachia* communities in a natural environment in real time. Here, we genetically survey *Wolbachia* based on MLST (Baldo *et al.* 2006b) and the surface protein gene *wsp* (Braig *et al.* 1998) to assess the infection status of four *R. cingulata* populations distributed across their native range in the United States and compare it with six recently established European populations. We demonstrate recent multiple horizontal transmissions of one *Wolbachia* strain (*w*Cin1) from *R. cerasi* to *R. cingulata* in Europe and the spatial spread of this strain in host populations within a few generations. Our results provide new insights into horizontal transmission events and the invasion rates of a new strain in new host populations in nature.

Material and methods

Sample collection

North American samples of *R. cingulata* were collected from four different sites in 2007 and 2010 encompassing a representative portion of the distribution of the fly in the United States (Fig. 1; see Table 1 for site descriptions). European populations of *R. cingulata* were collected between 2006 and 2012 at four locations in Germany and two locations in Hungary (Table 1). Two different life stages of flies were collected for analysis. In the United States, flies were collected as larvae in infested fruit, reared in the laboratory, and genetically scored in the overwintering pupal stage. For the European samples as well as for one population in the United States (USA1), *R. cingulata* and co-occurring *R. cerasi* adult flies were captured on yellow sticky traps hung in host trees in the field and, following capture, individuals were submerged in absolute ethanol and stored at −20 °C (Table 1). DNA was extracted using the Mammalian DNA Mini-Prep Kit (Sigma) following the protocol of the manufacturer. DNA was eluted in

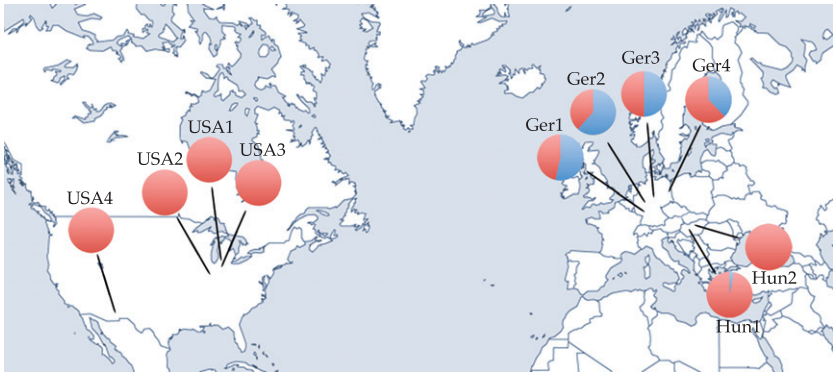

Fig. 1 *Wolbachia* infection frequencies in *Rhagoletis cingulata* across collection sites between 2009 (Ger2, Hun1) and 2010 (all other populations from USA, Ger and Hun; see Table 1 for site designations and information); red = portion of single-infected (*w*Cin2) individuals at site, blue = portion of double-infected (*w*Cin1&2) individuals at site.

Table 1 Locality information (latitude, longitude and years collected), site abbreviations (code) and ontogenetic stage of *Rhagoletis cingulata* analysed in the study

Locality	Code	Latitude/Longitude	Year	Stage
Fennville, Michigan, USA	USA1	42°36′06″N 86°09′32″W	2007, 2010	Adult, pupae
Urbana, Illinois, USA	USA2	40°13′21″N 88°22′14″W	2010	Pupae
Granger, Indiana, USA	USA3	41°45′30″N 86°11′54″W	2010	Pupae
Portal, Arizona, USA	USA4	31°54′51″N 109°08′24″W	2010	Pupae
Mannheim, Baden-Württemberg, Germany	Ger1	49°29′54″N, 8°27′51″E	2010	Adult
Heidesheim, Rhineland-Palatinate, Germany	Ger2	49°59′25″N, 8°06′43″E	2006–2009	Adult
Ammern, Thuringia, Germany	Ger3	51°13′18″N, 10°27′20″E	2010	Adult
Frankfurt/Oder, Brandenburg, Germany	Ger4	52°20′46″N, 14°32′00″E	2010	Adult
Veszprém,Veszprém, Hungary	Hun1	47°04′01″N, 17°55′28″E	2009, 2012	Adult
Sóskút, Pest, Hungary	Hun2	47°24′46″N, 18°49′15″E	2010	Adult

100 μL elution solution (10 mM Tris, 1 mM EDTA) and stored at 4 °C.

Wolbachia genotyping and strain identification

Diagnostic PCR was performed using the *wsp* primers 81F and 691R (Braig *et al.* 1998). All PCRs were performed on a 2720 thermal cycler (Applied Biosystems) in a total volume of 10 μL containing 1 × NH_4 Buffer (Fermentas), 2 mM $MgCl_2$, 100 μM dNTPs, 0.2 μM of each primer, 0.2 U Taq polymerase (Fermentas) and 0.8 μL template DNA. Cycling conditions were 95 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min, followed by a final extension at 72 °C for 15 min.

In addition, we also surveyed populations for the five housekeeping genes *coxA, gatB, ftsZ, fbpA, hcpA* that constitute the *Wolbachia* MLST system (Baldo *et al.* 2006b) for strain characterization. PCR conditions were as described above, except that the annealing temperature was changed to 50 °C for *ftsZ*. For in vitro strain segregation, we cloned and sequenced a portion of the PCRs. To accomplish this, 0.8 μL PCR products were ligated into the pTZ57R/T vector (Fermentas) and transformed into competent JM109 *Eschericha coli* cells according to the protocol of the manufacturer. Plasmid DNA was extracted by alkaline lysis (Sambrook *et al.* 1989), insert size was determined by PCR with M13 primers and plasmids with full-size inserts were Sanger sequenced by Eurofins MWG Operon (Ebersberg, Germany). Sequences were edited manually and aligned with CodonCode Aligner (CodonCode Corporation). To assess the infection status of single individuals, strain-specific primers were used as described in Arthofer *et al.* (2009).

Mitochondrial genotyping

To rule out introgression as a source of horizontal *Wolbachia* transmission (e.g. Raychoudhury *et al.* 2009) and to screen for potential genotypic differences among invasive and native *R. cingulata* populations, we amplified a 588-bp fragment of the cytochrome oxidase II (COII) gene from 209 *R. cingulata* individuals – 49 from four United States populations and 160 from five European populations (Table 2) – and from 21 European *R. cerasi* individuals from the same locations where *R. cingulata* was collected: Ger1 (n = 4), Ger2 (n = 3), Ger3 (n = 6) and Hun1 (n = 8) using the primers C2-J-3138 and TK-N-3782 (Simon *et al.* 1994). To exclude PCR artefacts, haplotypes were confirmed by replicate, independent PCR runs. To safeguard against NUMT contamination (e.g. Bertheau *et al.* 2011), five to six cloned plasmids from a PCR product of each haplotype (eight individuals in total) were sequenced, aligned and checked for aberrant bases and compositional abnormalities. Uncorrected *p*-distances among *R. cingulata* and *R. cerasi* were calculated using Mega 5 (Tamura *et al.* 2011).

Results

Wolbachia genotyping

Sequencing results of a total of 109 *wsp* clones derived from 24 different *R. cingulata* flies (seven from North America, 12 from Germany and five from Hungary) revealed the presence of two different bacterial strains designated *w*Cin1 (GenBank JX073680) and *w*Cin2 (JX073681). The *wsp* sequences of *w*Cin1 and *w*Cin2 were identical to those of *w*Cer1 and *w*Cer2, respectively, previously characterized from *R. cerasi*. All 24 *R. cingulata* flies analysed from North America and Europe were infected with *w*Cin2, suggesting that this strain may be widely distributed in the genus *Rhagoletis* in general. In contrast, *w*Cin1 was absent from the seven *R. cingulata* individuals genotyped from North America, but present at varying frequencies in European populations (four of 12 flies from Germany and one of five flies from Hungary).

Table 2 Frequencies of the eight different COII haplotypes (HT1–8) found in four *R. cingulata* populations surveyed from the United States (USA1–4), four from Germany (Ger1–4) and one from Hungary (Hun1)

	n	HT1	HT2	HT3	HT4	HT5	HT6	HT7	HT8
USA1 - *w*Cin2	16	62.5		6.3	12.5		6.3	12.5	
USA2 - *w*Cin2	12	66.7		16.7		8.3	8.3		
USA3 - *w*Cin2	12	58.3	8.3		8.3	16.7			8.3
USA4 - *w*Cin2	9	88.9	11.1						
Ger1 - *w*Cin2	8	87.5	12.5						
Ger1 - *w*Cin1&2	11	81.8	18.2						
Ger2 - *w*Cin2	42	85.7	14.3						
Ger2 - *w*Cin1&2	20	70.0	30.0						
Ger3 - *w*Cin2	7	71.4	28.6						
Ger3 - *w*Cin1&2	32	90.6	9.4						
Ger4 - *w*Cin2	7	100.0							
Ger4 - *w*Cin1&2	9	77.8	22.2						
Hun1 - *w*Cin2	16	93.8	6.3						
Hun1 - *w*Cin1&2	8	100.0							

Infection status of flies is given – single-infected (*w*Cin2) or double-infected (*w*Cin1&2) – along with the total sample sizes of flies sequenced (*n*).

To confirm the identity of the shared strains of *Wolbachia* from *R. cingulata* and *R. cerasi*, we sequenced a total of 291 plasmid clones for the loci *fbpA* ($n = 81$), *ftsZ* ($n = 73$), *coxA* ($n = 54$), *hcpA* ($n = 48$) and *gatB* ($n = 35$) from five *R. cingulata* individuals from Germany (two singly and three doubly infected individuals according to *wsp* genotyping) and five singly infected individuals from the United States. Single-infected individuals were used to indentify the alleles of strain *w*Cin1, while additional alleles in double-infected individuals were assigned to *w*Cin2 (Arthofer *et al.* 2011). The results for the five MLST loci confirmed that *w*Cin1 and *w*Cin2 are identical to *w*Cer1 and *w*Cer2, respectively, by means of the *wsp* gene and all MLST loci.

In addition to *w*Cin1 and *w*Cin2, we found in both European and American populations a few sequences highly similar to *w*Cin2, but with SNPs on the *fbpA*, *ftsZ*, *gatB* and *wsp* genes. To rule out sequence and cloning errors, only SNPs present in more than one individual or from at least two different PCRs were considered. The results suggested that besides *w*Cin2, different subtypes of this strain are present in low frequency in natural populations. The different low-frequency subtypes of *w*Cin2 were found in both single- and double-infected individuals, with no significant difference in frequency between the two infection types (data not shown). Of particular note, 20 of 109 plasmids of the *wsp* gene sequenced displayed a frame-shift deletion on hypervariable region 4. This sequence was found to be identical with a low-frequency strain in the apple host race of *R. pomonella* in the United States (Schuler *et al.* 2011). The low frequency and the overall low level of sequence divergence among variant *w*Cin2 subtypes prevented the development of specific primers for rapid large-scale screening.

To assess strain distribution and frequency on a larger scale, we performed diagnostic PCR-based genotyping of 111 *R. cingulata* flies collected from four different populations distributed across the United States and 336 individuals from six European populations (four in Germany and two in Hungary) using *w*Cin1- and *w*Cin2-specific *wsp* primers. As was the case for the cloned samples, all 447 *R. cingulata* flies genotyped from the United States and Europe were infected with *w*Cin2 (Fig. 1, Table S1, Supporting information). However, *w*Cin1 was found in some individuals of European *R. cingulata* populations. The frequency of *w*Cin1 infections varied widely among European *R. cingulata* sites (Fig. 1). Individuals collected in 2009 and 2010 harboured *w*Cin1 at a mean frequency of 1.7% in Hungary: one of 32 individuals in Veszprém (Hun1) was infected with *w*Cin1 (3.1%), while *w*Cin1 was still absent in Sóskút (Hun2; Fig. 1). In contrast, 85 of 168 German individuals (50.6%) were infected with *w*Cin1. Infection rates of *w*Cin1 were heterogeneous within Germany, being highest in Rhineland-Palatinate (Ger2; 61.5%; $n = 26$) and lowest in Brandenburg (Ger4; 37.9%; $n = 29$; Fig. 1, Table S1, Supporting information).

For Rhineland-Palatinate (Ger2) and Veszprém (Hun1), collections were made across years to test for temporal differences in *w*Cin1 infection frequencies. In Rhineland-Palatinate (Ger2), 8% of the analysed individuals in 2006 were *w*Cin1 infected, with the rate increasing to 20.6% ($\chi^2 = 1.77$, df = 1 and $P = 0.17$) in 2008 and to 61.5% in 2009 ($\chi^2 = 10.45$, df = 1 and $P < 0.001$; Fig. 2, Table S1, Supporting information). The Hungarian

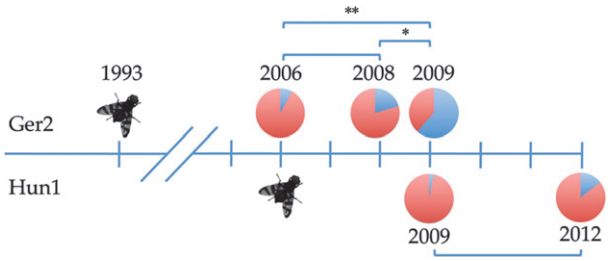

Fig. 2 Infection rates of *Wolbachia* in different *Rhagoletis cingulata* populations in different years in Germany (Ger2, Rhineland-Palatinate) and Hungary (Hun1, Veszprém); red = portion of single-infected (*w*Cin2) flies, blue = portion of double-infected (*w*Cin1&2) flies. Asterisks indicate statistically significant differences (chi-square test; $*P < 0.01$, $**P < 0.001$); the position of the fly along the timeline indicates the first description of *R. cingulata* in the countries.

Veszprém (Hun1) population showed an increase in the *w*Cin1 infection from 3.1% in 2009 to 12.2% in 2012 ($\chi^2 = 2.04$, df = 1 and $P = 0.15$; Fig. 2, Table S1, Supporting information).

Genotyping of different R. cingulata and R. cerasi populations

Sequencing a part of the COII gene of different *R. cingulata* individuals from the United States and Europe revealed eight different haplotypes (Table 2). Haplotype 1 was the most common, being present in all European and American *R. cingulata* populations; at lower frequency, also haplotype 2 was present on both continents. Haplotypes 3 to 8 were exclusively found at lower frequencies in various American populations. Haplotypes 2 to 7 each differed by one nucleotide substitution from haplotype 1, and haplotype 8 also differed by a single nucleotide substitution from haplotype 2. All SNPs were synonymous except for the substitution in haplotype 7 which resulted in a nonsynonymous change from alanine to threonine. In Germany, the strain *w*Cin1 was found in flies possessing COII haplotypes 1 and 2, while in Hungary, *w*Cin1 was associated only with haplotype 1 (Table 2). In contrast, the 21 sequenced *R. cerasi* individuals from four different populations in Europe showed no genetic variation on the COII gene. Sequence divergence between *R. cingulata* and *R. cerasi* ranged from 13.3% to 13.6% confirming previous results (Smith & Bush 1997). No mtDNA haplotypes from *R. cerasi* were found in individuals from *R. cingulata* and vice versa.

Discussion

Here we present the first direct and in-depth comparison of the *Wolbachia* infections of native American and invasive European *R. cingulata* populations. Our results provide strong evidence for multiple horizontal transmission events of a *Wolbachia* strain (*w*Cer1/*w*Cin1) from a European native congener, *R. cerasi*, to the recently introduced *R. cingulata* in Europe, and its successful spatial spread in the novel host within a few years. Our study documents one of the few examples of a successful horizontal transmission of *Wolbachia* in nature within a short ecological timescale.

The apparent ease with which *w*Cin1 has crossed species barriers in *Rhagoletis* raises the issue of why similar cases of horizontal transfer are not more often detected in other systems. Several factors may contribute to restricting the horizontal transmission of *Wolbachia* in nature (Combes 2001; Vavre *et al.* 2003; Riegler *et al.* 2004). First, horizontal transmission requires close physical contact between donor and recipient. Second, the transmitted *Wolbachia* has to adapt quickly to the new host's cellular environment in order to multiply. Third, the bacteria must colonize the female germline for efficient vertical transmission (Frydman *et al.* 2006; Fast *et al.* 2011). Fourth, colonization of the host's germline must go hand in hand with some reproductive advantage (e.g. cytoplasmic incompatibility, Hoffmann & Turelli 1997) to drive the novel infection through a population.

The opportunity for physical contact of donor and recipient species could be constituted by the close interactions between insect parasitoid and host taxa (Huigens *et al.* 2000) or by phytophagous species sharing the same plant (Stahlhut *et al.* 2010). This should provide an avenue for the horizontal spread of *Wolbachia*. Further, the adaptation to the cellular environment of a novel host may be difficult to overcome. In this regard, artificial *Wolbachia* transmission by microinjection usually results in stably inherited infections using bacterial strains from closely related donor species (Zabalou *et al.* 2004), whereas transmission to phylogenetically distant hosts is less likely to succeed (McMeniman *et al.* 2008; Hughes *et al.* 2011; Walker *et al.* 2011). Artificial transfer experiments of *Wolbachia* between two isopod species of two different families have shown that within a new genetic host background *Wolbachia* can be highly virulent, resulting in the death of the host (Le Clec'h *et al.* 2012). Also with respect to population dynamics, successful transmission does not guarantee establishment, as *Wolbachia* may fail to become fixed within a population due to inefficient vertical transmission (Heath *et al.* 1999) or significant negative fitness effects on the host (Hoffmann *et al.* 1990). Thus, despite evidence for frequent horizontal transmission events on an evolutionary timescale, actual incidences of horizontal transmission of *Wolbachia* among species may be relatively hard to detect.

The dynamics of Wolbachia infection in R. cingulata

The population dynamics of both the host fly *R. cingulata* and its novel *Wolbachia* strain *w*Cin1 appear to vary across Europe and may reflect differences in the history of horizontal transmission in the system. At the present time, *R. cingulata* is fairly common and widely dispersed in Germany and Hungary (Vogt *et al.* 2010; F. Lakatos & K. Tuba, unpublished). In contrast, the fly is rare or absent in most other European countries (e.g. Daniel & Wyss 2007; Egartner *et al.* 2010). With respect to Germany, *R. cingulata* began to spread extensively in 2004 and has now been detected in all cherry-producing areas of the country (Vogt *et al.* 2010). In Hungary, *R. cingulata* was first recorded in 2006 (Szeőke 2006) and may have been introduced separately than German populations. This hypothesis is supported by a recent microsatellite analysis of North American and European *R. cingulata* populations implying separate introductions of *R. cingulata* from North America into Germany and Hungary (Johannesen *et al.* 2013). If true, a later introduction of *R. cingulata* into Hungary could have resulted in a more recent independent transfer of *w*Cin1 into the fly, providing less time for host adaptation and the spread of the strain. Consistent with this scenario, the portion of *R. cingulata* infected with *w*Cin1 is much higher in Germany than in Hungary, where the strain was still absent in one population in 2010. However, because no spatial segregation between German and Hungarian populations could be found, we cannot exclude migration of some *w*Cin1 infected flies from Germany to Hungary (e.g. due to trading activities) as source for the double infection in Hungary.

The absence of an association between the maternally transmitted mitochondrial genome and *Wolbachia* at higher taxonomic levels can be seen as in support of horizontal transmission on an evolutionary timescale (Hurst & Jiggins 2005; Baldo *et al.* 2008). Partial sequencing of the COII gene of *R. cingulata* provided in-depth information about the genetic diversity of *R. cingulata* and the horizontal transmission event of *w*Cin1. Just two of the eight haplotypes present in the native population in the United States were found in Europe. The reduced genetic diversity in Europe may be interpreted as reflecting a founder effect following *R. cingulata*'s introduction. However, the spread of the horizontally acquired *w*Cin1 is associated with the haplotype of the initially infected female and would therefore be expected to deplete the uninfected haplotypes, also in the absence of a founder effect (Turelli *et al.* 1992; Hurst & Jiggins 2005). The fact that individuals of both haplotypes present in Europe are infected with *w*Cin1 provides evidence that the presence of *w*Cin1 in European *R. cingulata* populations is not the result of a single horizontal transmission event. However, we cannot rule out occasional leakage of paternal mtDNA (e.g. Nunes *et al.* 2013) as possible alternative hypotheses to account for the pattern in addition to multiple horizontal transmissions.

How *w*Cin1 has accomplished its rapid rise in *R. cingulata* remains to be resolved. Once established in a new host species, *Wolbachia* has the potential to spread quickly through the new population (Turelli & Hoffmann 1991). Although novel infections are initially expected to have low transmission rates and cause reduced fecundity in infected females (Hoffmann & Turelli 1988), these detrimental consequences of *Wolbachia* can be offset by positive fitness effects of the bacteria for its host (Hedges *et al.* 2008; Teixeira *et al.* 2008; Hosokawa *et al.* 2010) and the induction of cytoplasmic incompatibility (Hoffmann & Turelli 1997). As no results of crossing experiments between *w*Cin1 infected and noninfected *R. cingulata* are currently available – rearing of *R. cingulata* on an artificial diet failed (K. Köppler, unpublished) – we can only speculate about the fitness effects and the reproductive phenotype of *w*Cin1 in the new host. However, the high percentage of doubly infected individuals and the rapid spread of *w*Cin1 over the last few years suggest that it may have minimal, if any, negative fitness effects on *R. cingulata* and/or is able to induce cytoplasmic incompatibility. It is also possible that multiple horizontal transmissions of *w*Cin1, as previously described in parasitoid wasps (Kraaijeveld *et al.* 2011), have contributed to the spread of the strain, as well.

Possible routes of horizontal transmission

The exact route of *w*Cin1 acquisition by *R. cingulata* remains to be determined. It is probable that the shared habitats of *R. cerasi* and *R. cingulata* in feeding within cherries as larvae played an important role in the horizontal transmission of *w*Cin1. For example, due to different flight periods of the species, it is possible that *w*Cin1 was transmitted from the earlier active *R. cerasi* to *R. cingulata*, which emerges 3–4 weeks later. Parasitoids naturally infesting *R. cerasi* have been found to attack and develop in *R. cingulata* as well (H. Vogt, personal communication). Thus, a parasitoid first attacking *R. cerasi* could transfer *Wolbachia* with its ovipositor – a 'dirty needle' – to *R. cingulata* (Houck *et al.* 1991; Hughes *et al.* 2004).

Another putative mechanism for *Wolbachia* transmission is cannibalism (Le Clec'h *et al.* 2013). The developmental delay of *R. cingulata* makes larval cannibalism unlikely, but it is possible that feeding larvae or larvae that die in fruit may disperse *Wolbachia* into the environment, facilitating its transmission between flies. A recent

investigation on horizontal transmission of *Rickettsia* from the whitefly *Bemisia tabaci* to other whiteflies (Caspi-Fluger *et al.* 2012) showed that bacteria can be transmitted via the host plant: *Rickettsia*, and perhaps other Proteobacteria, are able to survive inside the phloem from which they were acquired by other whiteflies. A similar transmission path was suggested for closely related *Wolbachia* strains of different insect taxa infesting the same pumpkin hosts (Sintupachee *et al.* 2006).

Finally, inter-specific hybridization of infected and uninfected species can lead to introgression of inherited bacteria such as *Wolbachia* and be interpreted as horizontal transmission. However, this could be identified by the cointrogression of mtDNA from the originally infected host insect (e.g. Raychoudhury *et al.* 2009). We consider hybridization as unlikely because mtDNA sequence data for 160 European specimens of *R. cingulata* – 80 singly and 80 doubly infected flies – revealed various *R. cingulata*-specific haplotypes which were not associated with *R. cerasi*.

Conclusion

In this study, we present evidence for the recent horizontal transmission of a *Wolbachia* strain from the fruit fly *R. cerasi* to the closely related invasive species *R. cingulata* in Europe. The high frequency of double infections across German populations showed that the newly introduced *w*Cin1 strain was able to adapt to its new host and to invade populations so that more than half of the individuals were infected within a few host generations. The fact that various mitochondrial haplotypes of *R. cingulata* from Germany are infected with *w*Cin1 suggests that multiple horizontal transmission events enforced the spread of *w*Cin1 in the new host population. The geographic isolation and perhaps different invasion history of the Hungarian population of *R. cingulata* and the different increase in *w*Cin1 prevalence over time suggest that it may represent an independent horizontal transmission event. However, this presumption and the course of the parallel *w*Cin1 spread have to be investigated in future studies. The *R. cingulata* system therefore provides a promising opportunity to gain novel insights into the dynamics of the early stages of natural horizontal *Wolbachia* transmission.

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This study was part of H.S.'s PhD thesis on the biology and evolution of *Wolbachia* in *Rhagoletis* species. C.B.'s postdoctoral research focuses on ecology and evolution of plant-insect relationships. S.P.E. and J.L.F. are evolutionary biologists interested in the role that ecological adaptation plays in speciation. M.R. studies the biology and role of microbial insect symbiosis in insect-plant interactions. The research of B.C.S. and F.M.S. deals with a broad range of molecular ecological topics, among others focusing on terrestrial arthropods and their symbionts. J.J. is a molecular ecologist interested in speciation processes in tephritids. P.K. is PhD student with M.R. working on *Wolbachia* and sex determination in Australian butterflies. K.T. and F.L. are entomologists working on invasive species and their biology. K.K. is researcher and consultant in plant protection of horticultural pests. W.A. is interested in arthropod phylogeography and population genetics, biotic factors influencing species dispersal and the ecology and evolution of symbiosis. C.S.'s research focuses on scolytids and tephritids and addresses questions on host-parasite co-evolution and its link to population genetics.

Data accessibility

DNA sequences: GenBank accessions JX073680 – JX073691 (*Wolbachia*) and KC480164 – KC480171 (COII *R. cingulata*) and KC812339 (COII *R. cerasi*).

Wolbachia MLST database information (http://pubmlst.org/wolbachia) *w*Cin1 id = 509, *w*Cin2 id = 510.

mtDNA and *Wolbachia* alignments, and *Wolbachia* infection and mitochondrial haplotype of each individual: Dryad doi:10.5061/dryad.36077.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 *Wolbachia* infection status of *Rhagoletis cingulata* at each collection site in different years. Table contains sample size (*n*), the number of singly infected (*w*Cin2) and doubly infected (*w*Cin&2) flies, and the percentage of doubly infected flies at sites.