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 wCin2 identical to wCer2 in R. cerasi. In contrast, wCin1, a strain identical to wCer1 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 wCin1 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 wCin1 with two distinct haplotypes in Germany, one of which is also infected with wCin1 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

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
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), transmissions 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 transfection 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. Kozubiková 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; Bjelis 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 *Ragoletis 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 1 μl double-distilled water.

**Fig. 1** *Wolbachia* infection frequencies in *Ragoletis 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* Cin1) 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

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

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 × NH₄ Buffer (Fermentas), 2 mM MgCl₂, 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 *Escherichia 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 Hun (*n* = 8) using the primers C2-J-3138 and TK-N-3782 (Simón *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 *wCin1* (GenBank JX073680) and *wCin2* (JX073681). The *wsp* sequences of *wCin1* and *wCin2* were identical to those of *wCer1* and *wCer2*, respectively, previously characterized from *R. cerasi*. All 24 *R. cingulata* flies analysed from North America and Europe were infected with *wCin2*, suggesting that this strain may be widely distributed in the genus *Rhagoletis* in general. In contrast, *wCin1* 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).
Infection status of flies is given – single-infected (wCin2) or double-infected (wCin1&wCin2) – along with the total sample sizes of flies sequenced (n).

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).

<table>
<thead>
<tr>
<th>Population</th>
<th>HT1</th>
<th>HT2</th>
<th>HT3</th>
<th>HT4</th>
<th>HT5</th>
<th>HT6</th>
<th>HT7</th>
<th>HT8</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA1 - wCin2</td>
<td>16</td>
<td>62.5</td>
<td>6.3</td>
<td>12.5</td>
<td>6.3</td>
<td>12.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA2 - wCin2</td>
<td>12</td>
<td>66.7</td>
<td>16.7</td>
<td>8.3</td>
<td>8.3</td>
<td>8.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA3 - wCin2</td>
<td>12</td>
<td>58.3</td>
<td>8.3</td>
<td>8.3</td>
<td>16.7</td>
<td>8.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA4 - wCin2</td>
<td>9</td>
<td>88.9</td>
<td>11.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ger1 - wCin2</td>
<td>8</td>
<td>87.5</td>
<td>12.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ger1 - wCin1&amp;wCin2</td>
<td>11</td>
<td>81.8</td>
<td>18.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ger2 - wCin2</td>
<td>42</td>
<td>85.7</td>
<td>14.3</td>
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<td></td>
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<tr>
<td>Ger2 - wCin1&amp;wCin2</td>
<td>20</td>
<td>70.0</td>
<td>30.0</td>
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<tr>
<td>Ger3 - wCin2</td>
<td>7</td>
<td>71.4</td>
<td>28.6</td>
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<tr>
<td>Ger3 - wCin1&amp;wCin2</td>
<td>32</td>
<td>90.6</td>
<td>9.4</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ger4 - wCin2</td>
<td>7</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ger4 - wCin1&amp;wCin2</td>
<td>9</td>
<td>77.8</td>
<td>22.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hun1 - wCin2</td>
<td>16</td>
<td>93.8</td>
<td>6.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hun1 - wCin1&amp;wCin2</td>
<td>8</td>
<td>100.0</td>
<td></td>
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</table>

Infection status of flies is given – single-infected (wCin2) or double-infected (wCin1&wCin2) – 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 wCin1, while additional alleles in double-infected individuals were assigned to wCin2 (Arthofer et al. 2011). The results for the five MLST loci confirmed that wCin1 and wCin2 are identical to wCer1 and wCer2, respectively, by means of the wsp gene and all MLST loci.

In addition to wCin1 and wCin2, we found in both European and American populations a few sequences highly similar to wCin2, 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 PCR s were considered. The results suggested that besides wCin2, different subtypes of this strain are present in low frequency in natural populations. The different low-frequency subtypes of wCin2 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 wCin2 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 wCin1- and wCin2-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 wCin2 (Fig. 1, Table S1, Supporting information). However, wCin1 was found in some individuals of European R. cingulata populations. The frequency of wCin1 infections varied widely among European R. cingulata sites (Fig. 1). Individuals collected in 2009 and 2010 harboured wCin1 at a mean frequency of 1.7% in Hungary: one of 32 individuals in Veszprém (Hun1) was infected with wCin1 (3.1%), while wCin1 was still absent in Sóskút (Hun2; Fig. 1). In contrast, 85 of 168 German individuals (50.6%) were infected with wCin1. Infection rates of wCin1 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 wCin1 infection frequencies. In Rhineland-Palatinate (Ger2), 8% of the analysed individuals in 2006 were wCin1 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 (wCin2) flies, blue = portion of double-infected (wCin1&2) flies. Asterisks indicate statistically significant differences (chi-square test; \( *P < 0.01, \quad **P < 0.001 \)); 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 wCin1 infection from 3.1% in 2009 to 12.2% in 2012 (\( \chi^2 = 2.04, \quad df = 1 \) 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 wCin1 was found in flies possessing COII haplotypes 1 and 2, while in Hungary, wCin1 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 (wCer1/wCin1) 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 wCin1 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 wCin1 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; Egarnter 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 wCin1 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 wCin1 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 wCin1 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 wCin1. 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 wCin1 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. 1991; Hurst & Jiggins 2005). The fact that individuals of both haplotypes present in Europe are infected with wCin1 provides evidence that the presence of wCin1 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 wCin1 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 wCin1 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 wCin1 in the new host. However, the high percentage of doubly infected individuals and the rapid spread of wCin1 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 wCin1, 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 wCin1 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 wCin1. For example, due to different flight periods of the species, it is possible that wCin1 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 ovispositor – 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 co-introgression 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 wCin1 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 wCin1 suggests that multiple horizontal transmission events enforced the spread of wCin1 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 wCin1 prevalence over time suggest that it may represent an independent horizontal transmission event. However, this presumption and the course of the parallel wCin1 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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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) \( w_{\text{Cin1}} \) id = 509; \( w_{\text{Cin2}} \) 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 (\( w_{\text{Cin2}} \)) and doubly infected (\( w_{\text{Cin1} \& 2} \)) flies, and the percentage of doubly infected flies at sites.