Wolbachia-driven selective sweep in a range expanding insect species

Junchen Deng

Lunds Universitet

Giacomo Assandri

Istituto Superiore per la Protezione e la Ricerca Ambientale

Pallavi Chauhan

Lunds Universitet

Ryo Futahashi

Trukuba

Andrea Galimberti

University of Milano−Bicocca: Universita degli Studi di Milano-Bicocca

Bengt Hansson

Lunds Universitet

Lesley Lancaster

University of Aberdeen

Yuma Takahashi

Chiba University graduate school of science

Erik I Svensson

Lund University: Lunds Universitet

Anne Duplouy (✉ anne.duplouy@helsinki.fi)

University of Helsinki: Helsingin Yliopisto   https://orcid.org/0000-0002-7147-5199

Abstract

Background Evolutionary processes can cause strong spatial genetic signatures, such as local loss of genetic diversity, or conflicting histories from mitochondrial versus nuclear markers. Investigating these genetic patterns is important, as they may reveal obscured processes and players. The maternally inherited bacterium *Wolbachia* is among the most widespread symbionts in insects. *Wolbachia* typically spreads within host species by conferring direct fitness benefits, or by manipulating its host reproduction to favour infected over uninfected females. Under sufficient selective advantage, the mitochondrial haplotype associated with the favoured symbiotic strains will spread (*i.e.* hitchhike), resulting in low mitochondrial genetic variation across the host species range. The common bluetail damselfly (*Ischnura elegans:* van der Linden, 1820) has recently emerged as a model organism of the genetics and genomic signatures of range expansion during climate change. Although there is accumulating data on the consequences of such expansion on the genetic of *I. elegans*, no study has screened for *Wolbachia* in the damselfly genus *Ischnura*. Here, we present the biogeographic variation in *Wolbachia* prevalence and penetrance in 17 *I. elegans* populations across Europe and Japan, and from close relatives in the Mediterranean area (i.e. *I. genei*: Rambur, 1842; and *I. saharensis*: Aguesse, 1958).

Results Our data reveal (a) multiple *Wolbachia*-strains, (b) potential transfer of the symbiont through hybridization, (c) higher infection rates at higher latitudes, and (d) reduced mitochondrial diversity in the north-west populations, indicative of hitchhiking associated with the selective sweep of the most common strain. We found low mitochondrial haplotype diversity in the *Wolbachia*-infected north-western European populations (Sweden, Scotland, the Netherlands, Belgium, France and Italy) of *I. elegans*, and, conversely, higher mitochondrial diversity in populations with low penetrance of *Wolbachia* (Ukraine, Greece, Montenegro and Cyprus). The timing of the selective sweep associated with infected lineages was estimated between 20 000 to 44 000 years before present, which is consistent with the end of the last glacial period about 20 000 ya.

Conclusions Our findings provide an example of how endosymbiont infections ca shape spatial variation in their host evolutionary genetics during postglacial expansion. These results also challenge population genetic studies that do not consider the prevalence of symbionts in many insects, which can impact geographic patterns of mitochondrial genetic diversity.

Background

Range expansion studies have uncovered waves of demographic expansion in many species by comparing the genetic diversity of the initial source to that of the edge populations. Often, but not always (eg. [1]), range expansions lead to reduced genetic diversity and stronger genetic differentiation at a species range limits compared to the source populations, due to the action of drift during rapid demographic spatial expansion and colonization [2, 3]. Under these conditions, certain alleles and genotypes have been shown to spread in the newly colonized regions due to allele surfing [4], or due to selection for local adaptation in novel environments at the range limits [1, 5-8]. Hidden processes and players may however confound these patterns, and challenge our full understanding of the evolutionary histories and genetic diversity of source and edge populations. Infections with maternally inherited symbionts, for example, can cause loss of genetic diversity across entire populations because of differential selection pressures on the infected versus uninfected host lineages. In these conditions, the selective sweep of maternally inherited symbionts can lead to the hitchhiking of certain host haplotypes, which may not themselves be targets of selection [9-12].

The maternally inherited symbiotic bacteria *Wolbachia* can be found in up to half of all arthropod species [13, 14]. These bacteria are selfish passengers known for manipulating their host reproductive system via, for example, inducing the killing or feminization of the male progeny [15, 16], and the overproduction of daughters from unfertilized

eggs (thelytoky [17]), or causing incompatibility between males and females of different infection status [18]. In addition, some *Wolbachia* strains have been shown to provide nutrients essential to the survival of their hosts [19], protection against natural enemies [20-22], or can influence their host behaviours in ways that enhance fitness (e.g. mating rate [23], lekking [24], host choice [25], and more - see review [26]). These phenotypes evolved to improve the fitness of infected hosts over their uninfected counterparts, and therefore provide efficient means for the spread of the symbiont through generations, which can affect the inheritance pattern of the host DNA [27]. Rapid spread of maternally transmitted *Wolbachia* across populations within species can lead to hitchhiking of the co-inherited mitochondrial haplotypes, increasing their frequencies in the host population. As a result, the overall mitochondrial diversity has been shown to decrease within infected populations [28]. If such symbionts were to remain hidden or not screened for, this could mislead, or challenge, inferences based on the mitochondrion in population genetic and phylogeographic studies.

The insect order Odonata (dragonflies and damselflies) includes approximately 6,400 species belonging to 32 families [29]. Within these, the damselfly genus *Ischnura* (bluetails/forktails; Zygoptera: Coenagrionidae) is broadly ditributed in both the Old and the New Worlds. Members of this damselfly genus and other odonates have shown to readily undergo range shifts and expansion in response to climate change [30]. In the clade containing the common bluetail damselfly (*Ischnura elegans*), hybridization and introgression have been reported between *I. elegans* and the island bluetail (*I. genei*), the Sahara bluetail (*I. saharensis*) [31], and the Iberian bluetail (*I. graellsii*) [32], under secondary sympatry following recent range shifts [31-33]. Other studies have shown significant adaptive allele frequency changes along the northward range expansion gradient in *I. elegans*, consistent with selection caused by diverse novel environmental conditions, such as temperature, precipitation and wind speed [1]. Several *Ischnura* species, including *I. elegans*, often have heritable, female-limited colour polymorphisms that include a heritable male-mimic (androchrome; blue females) and one to two other morphs (red or green) [34-37]. Recent studies have shown that these morphs vary in their resistance and tolerance to parasitism by water mites [38], and in various aspects of thermal performance at the northward edge of their range [39, 40].

Although patterns and consequences of *Wolbachia* host interactions have been studied extensively in other insect groups (e.g. Hymenoptera [41]; Lepidoptera [10]; Diptera [42]), the recent studies by Thipaksorn et al. [43], Salunkhe et al. [44] and Lorenzo-Carballa et al. [45] possibly represent the only three systematic studies on *Wolbachia* infection in Odonata, and no previously published study have focused on *Wolbachia* in the genus *Ischnura.* Here, we investigated the *Wolbachia* strain diversity in three *Ishnura* species (*I. elegans*, *I. genei*, and *I. saharensis*) and provide a first low-coverage assembly of the most-commonly found strain infecting *I. elegans* in Europe. By sequencing four mitochondrial markers (2375bp) and one nuclear marker (512bp), we quantified host genetic diversity, and (I) tested whether any *Wolbachia*-induced selective sweep that might have reduced genetic diversity across the *I. elegans* species range, and (II) looked for evidence of horizontal transfer of the symbiont between the three *Ischnura* species. Finally, by comparing the infection status of the three female colour morphs and the males of *I. elegans*, we tested whether there was any indication that the morphs differ in their *Wolbachia* infection status, just as they do in terms of ectoparasitic water mite infection [38]. This study thus presents novel data on previously hidden players in the ecology and evolution of the range expanding species *I. elegans*, and three of its relatives*.*

Results

Wolbachia penetrance and prevalence in *I. elegans* and closely related species

There were geographical variations in *Wolbachia* penetrance across the *I. elegans* populations (Table 1; Figure 1). In Europe, our data revealed that *Wolbachia* infection was more prevalent in the north-western than in the south-eastern

regions (Figure 1). The infection frequency was over 50% in ten populations, including Sweden [SW], Scotland [AB], the Netherlands and Belgium [VW], France [FR], Finland mainland [FL], Italy (NI, MI, SI), Ukraine [VC] and Northern Japan [IK]. In Sweden, where the majority of our specimens came from, the infection was nearly fixed, with a frequency of 97.7% (85 individuals infected out of 87 samples tested). In contrast, all specimens from Montenegro [BJ], Greece [GR], Cyprus [CY], Finland Åland [ÅL], Ukraine (DR, PC) and Central Japan [RK] were *Wolbachia*-free. Additionally, all 13 *I. pumilio* specimens from Sweden were infected. The *I. genei* specimen from Corsica Island [CO] was infected, while the other two from Sicily [SI] and Sardinia [SD] were not. The single *I. saharensis* specimen from Morocco was also uninfected.

Wolbachia strain diversity in *Ischnura elegans*, *I. genei* and *I. saharensis*

In total, three *Wolbachia* strains, denoted *w*Ele1, *w*Ele2, and *w*Ele3, were identified in *I. elegans* (Table 1). The strain *w*Ele1 was widespread and found in Sweden [SW], Scotland [AB], Belgium and the Netherlands [VW], France [FR], Italy (MI, SI) and Ukraine [VC] (Figure 1). In contrast, the strain *w*Ele2 was restricted to Italy (NI, MI), and *w*Ele3 was found in mainland Finland [FL] and Japan [IK]. The *I. genei* specimen from Corsica was also infected with the strain *w*Ele1; while *I. pumilio* was found to carry two divergent strains, denoted *w*Pum1 and *w*Pum2, with eight specimens (out of 13) potentially carrying the two strains simultaneously. Raw reads from the *I. elegans* whole genome sequencing project [46] were assigned to *Wolbachia*, and were used to build a *de novo Wolbachia* assembly of strain *w*Ele, consisting of under 900 scaffolds. This fragmented *Wolbachia* assembly is 1.4Mb long, suggesting it represents the full genome of a *Wolbachia* strain [47]. Although the circular genome assembly could not be built, we are confident these scaffolds are from a single bacterial infection that are not inserted in the genome of the host, as each scaffold did not contain any host genomic material.

The *Wolbachia* phylogeny based on the *ftsZ* and *wsp* genes (Figure 2a) showed that all strains characterized in this study were from the B-supergroups (genetic distance between *w*Pip and all five strains ranged from 0.077 to 0.113). The three strains from *I. elegans* formed a monophyletic group, divergent from the two strains from *I. pumilio*, *w*Pum1 and *w*Pum2. The pairwise genetic distance ranged from $1.06e^{-3}$ to $2.04e^{-2}$ among the three *I. elegans* strains, and from $3.24e^{-2}$ to $7.49e^{-2}$ between *w*Ele and *w*Pum strains (Table S1). When compared to the previous strain records from the *Wolbachia* PubMLST database [48], almost all strains carried the *ftsZ* allele #7, except the strain *w*Pum2, which carried the *ftsZ* allele #73 (only one nucleotide difference from allele #7). The *wsp* allele #61 was characterized from *w*Pum2, while the four other *wsp* alleles were new to the *Wolbachia* PubMLST database, for a total of 75 polymorphic sites.

Genetic diversity and *Wolbachia*-induced selective sweep in *I. elegans*

We identified 25 polymorphic sites over the analysed 400bp of the nuclear locus *PMRT* sequenced from five *I. elegans* populations. We found one to ten genotype(s) per population showing no less than 98.1% similarity. The haplotype diversity (*Hd*) at *PMRT* was 0.88 for the *Wolbachia*-infected specimens versus 0.91 for the uninfected specimens (Table 2). Additionally, observed heterozygosity levels at two informative polymorphic sites within the *PMRT* gene (position n=47 and 228) were similar between infected and uninfected specimens: 25% and 50% for infected vs 23.5% and 43% for uninfected specimens (two-tailed *P*=0.86; and *P*=0.61, respectively).

Based on the analysis of the *COI* locus only, we detected a total of 24 mitochondrial haplotypes (Haplo1 to 24) from 169 specimens (Figure 3a & 3c). The analysis of all four mitochondrial markers revealed additional mitotypes (17 mitotypes, Haplo_A to Q), despite being based on 47 *I. elegans* specimens only (Figure 3b & 3d). Both mitochondrial haplotype network analyses (*COI* and *COI+COIb+COII+NDI*), however, showed similar patterns, with three main distinct clades emerging (Clade 1: Japan; Clade 2: Cyprus; and Clade 3: all other populations dominated by one common mitotype, the Haplo1 or Haplo_A; Figure 3). Note that three haplotypes, (Haplo23 from Italy, Haplo24 from Japan and

Haplo19 from Cyprus, characterized with the *COI* gene only, fell outside the major clades of the tree. A BLAST search for these three haplotypes in the Barcode of Life Data system [BOLD v.4, 49] showed that Haplo19 grouped with an unidentified *Ischnura* species from Iraq (99.5% similarity to an 'early-released' BOLD sample - Data not provided), while Haplo24 and Haplo23 grouped with *I. elegans* specimens from Pakistan (99.85% & 99.63% similarity to BOLD accession #MAODO254-11 & #MAODO255-11, respectively). The divergence may indicate that these three mitotypes represent either polymorphism in the *COI* gene, or species misidentification. By precaution, we removed the specimens from the rest of the analyses.

Most *w*Ele1-infected specimens carried one mitochondrial haplotype (Haplo1 or Haplo_A), and few specimens carried three closely related mitotypes only characterized with the four genes analysis: Haplo_B, Haplo_C and Haplo_F (Figure 3b & 3d). The low mitochondrial haplotype diversity in *w*Ele1-infected individuals (Hd=0.13) contrasts with the high haplotype diversity found in uninfected individuals (Hd=0.70; when only *COI* is considered, Table 2). The *Wolbachia*-free Cyprus [CY] population maintained the largest mitochondrial haplotype diversity in our samples, with 11 haplotypes for 20 samples sequenced (Haplo1, Haplo13 to 22, Ho=0.55). Altogether, these results are indicative of a *w*Ele1-induced selective sweep in *I. elegans,* which is also supported by significantly negative results from the neutrality tests (Tajima's D, Fu and Li's F; Table 2). The other two strains characterized from *I. elegans* were associated with divergent mitochondrial haplotypes. The strain *w*Ele2 was associated with Haplo7 (Haplo_D and E) in Italy, while *w*Ele3 was associated to Haplo24 (Haplo_P and Q) in Japan and Haplo9 in mainland Finland (Figure 3). Their relationships with mitochondrial haplotype diversity remain unclear due to our small population sample size. The Haplo1 commonly found in *I. elegans*, was also characterized in the *w*Elel1-infected *I. genei* from Corsica, and the uninfected *I. saharensis* sample from Morocco, which could suggest hybridization between these species or, alternatively, shared infection pre-speciation of these taxa. In contrast, the *Wolbachia*-infected Swedish *I. pumilio* specimens carried Haplo4, and the three uninfected *I. genei* specimens from Italy carried either Haplo9 or Haplo10 (Figure 3).

Based on the analysis of the sequences of the four mitochondrial loci, there were nine amino acid differences between the mitochondrial sequences of *I. pumilio* and *I. elegans*. Among the unchanged amino acid sites, we found 97 and 81 synonymous nucleotide differences among the 245 fourfold and 332 twofold degenerate codons, respectively. To correct for multiple substitutions and other mechanisms that cause higher number of substitutions more than the observed, we used the Jukes-Cantor correction. Given that we found four observed polymorphic sites in *w*Ele1-infected individuals, we estimated the selective sweep of wEle1 to have happened between 20 860 and 43 543 years before present. When the timing was evaluated based on the mitochondrial *COI* locus only (598bp), which was sequenced in more samples, the sweep was estimated between 10 159 and 21 174 years before present.

Ischnura elegans colour polymorphism, sex differences, and *Wolbachia* infection in Sweden

Within Sweden only, the area where most of our samples come from, we found that the three female colour morphs show similar *Wolbachia* infection frequencies (Fisher's exact test, p=0.63, Table 3) (Infection frequency: (A: blue) 1=27/27; (I: green) 1=22/22; (O: red) 0.96=23/24). Similarly, females and males were equally likely to be infected (Fisher's exact test, p=0.31) (Females: 0.99% or 72/73; Males: 0.93% or 14/15).

Discussion

The spread of maternally inherited symbionts would mostly affect the mitochondrial haplotype diversity of its host [12, 50], while external ecological and demographic factors like population range expansions and bottlenecks would be expected to reduce genetic diversity at both mitochondrial and nuclear levels. We found five B-supergroup *Wolbachia* strains in the three damselfly species of the genus *Ischnura* that we investigated. The common strain *w*Ele1 was

characterized in *I. elegans* and *I. genei,* while two additional strains (*w*Ele2 and 3) were found in *I. elegans* only, and two divergent strains were found in the sympatric species *I. pumilio* (*w*Pum1 and 2), which is sympatric with *I. elegans* in Sweden. In accord with a selective sweep driven by *w*Ele1 in *I. elegans* across Western Europe, we would particularly like to highlight (I) the reduced mitochondrial haplotype diversity and non-neutral evolution of the mitochondrial haplotypes associated to *w*Ele1-infected specimens, (II) the conserved nuclear haplotype diversity and levels of heterozygosity between infected and uninfected specimens. We estimated this selective sweep of *Wolbachia* to have occurred between 10 159 to 43 543 years ago, which is recent in the history of the host species (*I. elegans* and *I. graellsii* diverged 0.14 Mya [51]), and largely concordant with the timing of the last glacial maximum (20 000ya) in Europe [52]. Like other species of insects, *I. elegans* is currently shifting its geographic range northward in response to climate change [1, 30, 53, 54]. The timing of the selective sweep could suggest that *I. elegans* acquired the *w*Ele1 during its ongoing northward range expansion since the last glacial period. In such situation, one would expect that the mitochondrial diversity would be affected by both the sweep and bottlenecks due to range shift, while the nuclear diversity would be only affected by bottlenecks due to range shift. Although our Swedish population show the lowest mitochondrial diversity of all populations, the nuclear diversity is high. Our sample size is however small, and more comprehensive investigations of this may inform about the consequences of the spread of *Wolbachia* for the genetic diversity, the long-term success and the dynamics of this range expanding damselfly species.

Interactions between hosts and facultative symbionts are driven by complex sets of conflicts of interests, between the hosts and the symbionts, which outcomes also depend on the environment [55, 56]. These dynamic systems will then either result in the fixation of the symbiont in the populations [57, 58], its decline [59, 60], or stability at intermediate frequencies [61, 62]. The striking success of *w*Ele1 across the North-Western European range of *I. elegans* might suggest some benefits to the infected over the uninfected damselflies. *Ischnura elegans* is affected by parasitic water mites in nature, and previous studies have shown that tolerance and/or resistance levels to this parasite differ between sexes and female colour morphs [38]. As *Wolbachia* is known to affect its host fitness in the presence of parasites [21, 22, 63], we tested whether the variation in response to parasites between female colour morphs and sexes was linked to their infection status with the *Wolbachia* strain *w*Ele1. We found no such sex or morph differences associated with the strain *w*Ele1 in *I. elegans*, but this result does of course not rule out the possibility that the symbiont may still protect against parasitism in *I. elegans*. *Wolbachia* are also known to manipulate their hosts in various other ways that may similarly support its success and spread in the host populations [9, 17, 64, 65]. The strain *w*Ele1 is unlikely to manipulate its host reproductive system via feminization or male killing, as both females and males were found infected and the population sex-ratios were not systematically female biased [35, 38]. The strain could however induce cytoplasmic incompatibility, a type of sperm-egg incompatibility between males and females of different infection status [66], which would not affect population sex-ratio. Future mesocosm studies and mating experiments in semi-natural conditions [37] would allow the investigation of these hypotheses, to reveal the costs and/or benefits of *Wolbachia* in the damselfly species.

Although we found *w*Ele1 at almost fixed frequencies in the Western European populations of *I. elegans*, the strain was rare in Eastern Europe (*i.e.* Greece [GR], Ukraine (DR, PC), and Montenegro [BJ]), and absent from a few other populations (*i.e.* Cyprus [CY], Italy (NI, MI), Åland [ÅL]). We showed that the few uninfected individuals found in the Western European populations carry the same mitochondrial haplotypes as their *w*Ele1-infected conspecifics (Haplo1 and Haplo3), contrasting with the uninfected specimens from Eastern Europe that show a wide diversity of divergent mitochondrial haplotypes. This may suggest that the maternal transmission of the strain is not perfect and that few offspring from infected mothers can hatch uninfected in these populations. Additionally, two hypotheses could explain differences between populations: (I) *w*Ele1 has spread across the western populations but did not yet invade the remaining populations, or (II) *w*Ele1 has spread across the whole Europe in the past, but the infection was consequently lost. For the first hypothesis, *Wolbachia*-infected population would show reduced mitochondrial

haplotype diversity, while uninfected populations would have high mitochondrial diversity [10, 67]. In contrast, the second hypothesis suggests reduced haplotype diversity at the mitochondrial level and shared mitochondrial haplotypes, or closely related haplotypes, in both infected and uninfected populations [12, 64]. There is some support for each of these hypotheses, as we discuss more below.

The Italian peninsula has represented a refugium for many species during the last glacial maximum [52, 68]. In this region, *I. elegans* damselflies carry two closely related mitochondria (Haplo1 and Haplo6), found in association with two closely related *Wolbachia* strains (*w*Ele1 and *w*Ele2, respectively). Both mitotypes and *Wolbachia* strains may have diverged after isolation in geographically separated refugia on the Italian peninsula. In contrast, *w*Ele3, the third strain characterized in all *I. elegans* specimens collected from Japan, and from mainland Finland, is highly divergent from both *w*Ele1 and *w*Ele2. The strain *w*Ele3 is found in association with two mitochondrial haplotypes (Haplo9 and Haplo24), suggesting either haplotype diversity across the large range of this particular infection, or yet undetected bacterial diversity. These results may suggest a history of short-time separation between the Italian populations, and of longer-time separation between the Japanese and European populations. Additionally, although four other *Wolbachia* strains have been described from the genus *Ischnura* [in I. senegalensis: 44, in I. taitensis: 69], the true strain diversity in *Ischnura* is likely to be higher than the current data suggest, as only few genetic markers were genotyped, plus the sampling efforts in each study were not representative of the entire ranges of the targeted host species.

The two populations originating from the Cyprus island in the Mediterranean Sea and from the Åland islands in the Baltic Sea, were both *Wolbachia*-uninfected. The Cyprus population is located at the southern range limit of *I. elegans* in Europe. There, the strong divergence of the mitochondrial haplotypes to those associated to the infection in Western Europe suggests that the population has remained uninfected potentially due to its geographic isolation. The population differ phenotypically in several aspects from other continental populations, particularly in terms of smaller average body size and deviant colour morph frequencies [70]. Although also geographically isolated, the island population on the Åland archipelago carries one unique haplotype that only differs by one nucleotide from the most common haplotype associated with *w*Ele1 in Sweden, and is identical to the haplotype associated with *w*Ele3 in mainland Finland (although based on one unique mitochondrial gene). These results are better supporting the second hypothesis described above, in which the Åland population may have lost its infection recently. However, more mitochondrial genetic data will be needed to infer which of Sweden or Finland is the original source population of Åland.

The uninfected individuals from Greece and Ukraine carry similar to identical mitochondrial haplotypes than *w*Elel1-infected samples, which is also consistent with recent infection loss and divergence from an original population. As a facultative symbiont, *Wolbachia* is frequently lost [59, 69], either due to drift following the colonisation of new habitats [71] or selective pressures on the symbiont. High temperatures can also negatively affect *Wolbachia* titers in some *Drosophila* species [72], and lead to the loss of the infection in mosquitoes [73] and mites [74] reared under laboratory conditions. Similarly, studies of the species *Hyposoter horticola* (Hymenoptera) have revealed strong variations in *Wolbachia* penetrance across local populations in Åland [from 0 to 100% infection rate, 62]. Such local population variations were partly explained by imperfect transmission of the symbiont through generations, combined with locally variable negative selective pressures on the infected wasps [63]. The *I. elegans* specimens collected in Åland all came from a single collection site in the southern part of the main island (*i.e.* Nåtö). Thus, these specimens might not be representative of the true average infection status of the entire Åland populations. To resolve (I) whether *I. elegans* colonized the Åland population after the sweep of *w*Ele1 in Sweden or that of *w*Ele3 in Finland, and subsequently lost the infection and diverged at the mitochondrial level, and/or whether (II) these patterns reflect a dynamic population history with local variations in infection status and penetrance, further collection across the whole Åland archipelago would be required.

Although a maternally inherited symbiont, *Wolbachia* has been shown to also transfer horizontally between species. Examples include, but are not restricted to, the horizontal transfer of *Wolbachia* between damselfly species of the genera *Nesobasis* and *Melanesobasis* in Fiji Islands in the Pacific Ocean [45]. *Wolbachia* can be horizontally transmitted via different means, including hybridization between host species [45], shared resources (e.g. shared hostplant [75]), or shared parasitism (e.g. shared mites [76], or shared parasitoids [77]). Damselflies are well-known for carrying and sharing ectoparasitic water mites [38, 78], however the role of such parasites as vectors of *Wolbachia* between *Ischnura* species remains unknown. Additionally, the presence of *w*Ele1 in both *I. elegans* and *I. genei* may rather suggest that horizontal transfer of the strain between the two species occurred through hybridization, as both species carry the same mitochondrial haplotype. Evidences of frequent hybridization and introgression have been shown in some Odonate species due to latitudinal range expansion and the increasing sympatric interactions between closely related species [79], including in the genus *Ischnura* [31-33, 80].

Conclusion

The present biogeographic study of *Wolbachia* in the damselfly genus *Ischnura* revealed a wide diversity of previously hidden inherited symbiotic *Wolbachia* strains in the three species investigated. Furthermore, we detected a recent selective sweep of the *Wolbachia* strain *w*Ele1 across the Western European populations of *I. elegans*, and we discuss the potential horizontal transfer of the strain through hybridization. The biogeographical pattern of the infection and the estimated timing of the sweep suggested that *w*Ele1 might have spread across *I. elegans* populations during its host's northern expansion after the last glacial maximum. Consequently, the mitochondrial haplotype diversity in this range expanding species has been highly reduced but started to recover from the successful spread of the symbiont. We found the symbiont in specimens of all colour morphs and both sexes, and thus the costs and benefits from the infections remain to be investigated. We hope that the data presented here will further stimulate research on the consequences of symbiont infection, the associated loss of genetic diversity and consequences for host species in terms of their ongoing range expansions in response to climate change.

Materials And Methods

Samples collection

Ischnura damselflies were collected during the summer of 2015, 2016, 2019 or 2020 depending on their geographical origins. Individuals were caught in the field and stored in 95% ethanol in a -20°C freezer until further analysis. Specimens include 87 individuals from seven local populations in South Sweden (15 males and 72 females of all three morphs), and 105 other individuals from twelve other geographic regions, including Finland (Åland islands and mainland), Scotland, France, Cyprus, Greece, Ukraine, Belgium, the Netherlands, Italy, Montenegro and Japan (Table 1). The seven Swedish local populations are all located within a few kilometres from each other; thus, samples were grouped under a unique 'Southwest' population for the rest of the study. Similarly, samples from Belgium and the Netherlands were grouped as one unique population, denoted as 'Vinne-Walem'. In contrast, the samples from isolated parts of Italy were separated in three populations based on their relative geographical locations, and denoted either 'Northern', 'Central', or 'Southern' (Figure 1, Table 1).

We also included 20 specimens belonging to three other *Ischnura* species in this study:

- Thirteen specimens of *pumilio,* collected in 2019 from four Swedish populations. *Ischnura pumilio* co-occurs sympatrically with *I. elegans* in this region but falls in another major phylogenetic clade of the *Ischnura* tree, and is less closely related to *I. elegans* than are the following two species [81].

- Three specimens of *genei,* an allopatric species to *I. elegans* endemic of the western Mediterranean region. Two samples of *I. genei* were collected from the two insular populations of Sardinia and Sicily (Italy), and the third sample is from Corsica (France) [82]
- One unique specimen of *saharensis* collected from Morocco. The species is also an allopatric species to *I. elegans* and is distributed across North Africa.

Molecular work

Specimens were dissected in sterile conditions to avoid cross specimen contamination. We extracted the DNA from the abdomen of each damselfly, except for the Japanese samples, for which DNA was extracted from one leg, following the protocol of a Qiagen DNeasy Blood & Tissue Extraction Kit (Qiagen, USA). The quality of all DNA extracts was tested by PCR, through the amplification of the 5'-end region (~654bp) of the *cytochrome oxidase I* (*COI*) mitochondrial gene using the primers LCO-1490/HCO-2198 designed by Folmer et al. [83]. Only samples that were positive for the *COI* amplification were included in the following analyses.

All sequences were deposited into the GenBank databases (Accession #MW509059-66). In total, we amplified and sequenced four mitochondrial regions (*COI*, *COIb*, *COIIa* and *NDI*) to test for a selective sweep, one nuclear marker (*PRMT*) to test for population bottlenecks, and two *Wolbachia* genes (*ftsZ* and *wsp*) to characterize strain diversity. Note: an extra *Wolbachia* gene (*fbpa*) was sequenced for few *w*Ele1-infected specimens from Sweden (primers details in Table S2). Purified PCR products were sent to Macrogen (Macrogen Europe, Inc.) for single strand direct forward Sanger sequencing. All sequences were manually curated and aligned using Geneious Prime 2020.2.4 (https://www.geneious.com) and AliView [84]. Double peaks in the chromatograms were treated as either evidence of contamination (for mtDNA), multiple infections (for *Wolbachia* DNA), polymorphism (for mtDNA and nuclear DNA), or sequencing noises (all). *Wolbachia* and mtDNA sequences showing such patterns were not included in the following analyses, while the analysis of those double peaks in the nuclear locus sequences allowed us to identify heterozygotic and homozygotic specimens at polymorphic sites (see below).

Ischnura elegans nuclear haplotype diversity

To identify diversity at the nuclear level, we isolated 400bp of the successfully sequenced nuclear gene *PRMT* from 48 specimens (27 uninfected and 21 *Wolbachia*-infected) from 10 populations ([AB], [RK], [IK], [SW], [VC], [MI], [NI], [SI], [CY] and [GR]), with 2 to 12 specimens per population. However, Italy ([MI], [NI] & [SI]) and Japan ([RK] & [IK]) carry *Wolbachia* strains that are divergent from *w*Ele1, and which may have altered the genetic of those populations in ways that are impossible to fully test with the current dataset. Therefore, we did not include these sequences in further analyses. The final sample size was 31 sequences, including 23 *Wolbachia*-uninfected specimens and 8 *Wolbachia*-infected specimens from five populations (Table 1). To test whether the nuclear gene from *Wolbachia*-infected and uninfected damselflies has evolved under neutrality, we performed neutrality tests in DnaSP v6.0 [85] by calculating Tajima's *D* (Tajima, 1989) and Fu and Li's *F* [86] metrics, and by estimating nucleotide diversity (π) and haplotype diversity (*Hd*) (Table 2). We also estimated the observed heterozygosity levels at two nuclear polymorphic sites for both the *Wolbachia*-infected and uninfected specimens.

Wolbachia and mitochondrial haplotype diversity, phylogenies and haplotype networks

All *Wolbachia* Sanger-sequences were BLAST-ed against the *Wolbachia* PubMLST database (https://pubmlst.org/wolbachia/) [48] to find the corresponding or closest alleles at each locus. Additionally, all five MLST genes (*ftsz*, *gatB*, *hcpA*, *coxA*, and *fbpA*) and the *wsp* gene of the strain *w*Ele1 were also extracted from the whole genome project of a Swedish *I. elegans* [46]. All *Wolbachia* reads were identified and isolated from the raw read

data of the I. elegans genome project [46]. The wEle1 assembly was built by mapping reads to two previously sequenced *Wolbachia* genomes (wPip [87] and wMel [88]) using bwa mem version 0.7.8 [89]. The properly mapped pairs were extracted using samtools 1.8. The isolated *Wolbachia* paired reads were assembled into a draft genome using spades version 3.9.0 at kmers 21,33,55,77, and 99. The wEle1 draft genome assembly ($N_{scaffold}$= 893; N50= 5 523bp; longest scaffold=53 331bp, genome size= 1.4MB) is available as supplementary fasta file.

We concatenated sequences of the *ftsZ* and *wsp* genes from each *Wolbachia* strain characterized in this study, and from five additional strains (wMel, wRi, wClec, wBm, wPip; Isolate id number: 1, 11, 36, 37, 1808 in *Wolbachia* pubMLST database, respectively) previously assigned to the A-, A-, F-, D-, and B-*Wolbachia* supergroups, respectively. The phylogenetic analyses were conducted in IQ-Tree on XSEDE [90] implemented in CIPRES [91], using the genes separately (Figure S1) or concatenated (Figure 2). 'Model Selection' [92] was selected to allow for the search of the best model in CIPRES. The partition type was set to allow the two partitions (one for each gene) to have different speeds [93]. The best fit substitution models were decided by running '-m TESTNEW' in IQ-Tree. Bootstrapping was conducted using 'Ultrafast' and 'SH-aLRT' bootstrap methods (Hoang et al. 2018) in IQ-Tree with 1000 replicates. The 'TN+F+I' and 'TPM3+F+G4' models were applied to *ftsZ* and *wsp* genes, respectively, as the best fit models with highest BIC (Bayesian information criterion) scores. All other setting options were left as default. The pairwise genetic distances between *Wolbachia* strains were calculated in MEGA-X [94]. The best phylogenetic trees were visualized in FigTree (http://tree.bio.ed.ac.uk/software/figtree/), and rooted using the wBm-D and wClec-F strains as outgroups (Figure 2).

Additionally, we built two types of mitochondrial haplotype networks: (A) one based on the *COI* 5'-end region only (598bp), and (B) a second based on all four mitochondrial regions (2375bp). The networks were built using POPART [95] with the median joining method [96] (Figure 3). To the mitochondrial sequences produced by the present study, we added mitochondrial sequences from the same markers from any species of the *I. elegans* clade (*i.e. I. elegans, I. genei, I. saharensis, I. graellsii* and *I. fountaineae*), publicly available in GenBank before July 2020 (Table S3). Note: only sequences with a length equal or longer to 600bp were included to ensure the performance of the analyses.

Wolbachia selective sweep

The estimation of the timing of the *Wolbachia* sweep in *I. elegans* was carried out following the method described by Rich et al. [97]. We first estimated the neutral mutation rates at the third position of fourfold and twofold synonymous codons of the four mitochondrial genes separately. The open reading frames of mitochondrial genes were found by blasting the nucleotide sequence against the mitochondrial proteome of *I. elegans* [98]. The mitochondrial sequences of *I. elegans* and *I. pumilio* were aligned in MEGA X [94] in order to calculate the number of nucleotide differences and the number of fourfold and twofold synonymous sites between the two species. Jukes-Cantor correction [99] was applied to correct for multiple substitutions. Lastly, the neutral mutation rates were calculated based on the divergence time between *I. elegans* and *I. pumilio*, estimated between 10.4 to 21.7 My before present [51] (calculations were repeated twice, using each extreme of that divergence time range). Consequently, the age of the infection can be estimated using the following equation:

$$t = \frac{S}{\mu_a \Sigma n_i l_i + \mu_b \Sigma n_i m_i} \qquad (1)$$

Where t is the estimated time since the infection; *S* is the number of observed neutral polymorphisms in a set of mitochondrial haplotypes; u_a and u_b are the neutral mutation rates at the third position of fourfold and twofold synonymous codons, respectively; n_i is the number of sampled sequences at the ith locus; l_i and m_i are the number of fourfold and twofold synonymous sites at the ith locus. Our estimation was only based on mitochondrial haplotypes that were carried by the infected individuals. With this method, the corrected number of substitutions was estimated as

137.91 = 245(-3/4)ln[1 − (4/3) x (97/245)] and 111.11 = 332(-1/2)ln[1 − 2 x (81/332)] among fourfold and twofold degenerate codons, respectively. Assuming the maximum estimate divergence time at 21.7 Mya [51], we estimated that neutral mutation rates on fourfold and twofold synonymous sites would be expected to be 1.30% and 0.77% per site per million years, respectively. If the minimum estimate of 10.4 Mya was assumed, the neutral mutation rates are estimated to be 2.71% and 1.61%, respectively. These estimations are biologically reasonable if we assume the general divergence rate of mtDNA in arthropods at 1.1-1.2% per site per million years (Brower 1994).

Finally, to test whether the mitochondrial genes from *Wolbachia* infected and uninfected damselflies have evolved under neutrality, we performed neutrality tests in DnaSP v6.0 [85] by calculating two population genetic statistics: Tajima's *D* (Tajima, 1989) and Fu and Li's *F* [86], and by estimating nucleotide diversity (π) and haplotype diversity (*Hd*).

Ischnura elegans colour polymorphism, sex differences, and *Wolbachia* infection in Sweden

All statistical analyses were performed in R version 3.6.1 [100]. We used *Chi*-square test to investigate the association between *Wolbachia* infection and sex, and between infection and colour morph in female *I. elegans*. As most populations had a limited and incomplete sampling per sex and colour morph (e.g. only one female from Åland and from Finland mainland; only two morphs present in the Japan, Cyprus and Scotland populations, see Zenodo open data submission doi: 10.5281/zenodo.4445061), this test was only performed using the Swedish specimens. Fisher's exact test was applied as an improvement of *Chi*-square test when the expected value of any cells of the contingency table is below five (Table 3).

Declarations

Ethics approval and consent to participate

No consent to participate was needed for this study. The Italian Ministry of the environment, Land and Sea released a national permit for the collection of species included in European and Italian conservation directives, or to collect samples in regional or national protected areas (Prot. #0031783.20-11-2019).

Consent for publication

Not applicable

Availability of data and materials

The dataset supporting the conclusions of this article is available in the Zenodo repository doi: 10.5281/zenodo.4445061; and in GenBank database (Accession #MW509059-66) as stated in the text.

Competing interests

The authors declare no conflict of interests

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Author's contributions

AD & ES conceived the study. JD, ES, GA, RF, AG, BH, LTL, YT & AD collected samples. JD, PC & AD produced the data. JD & AD analyzed the data and wrote the manuscript. All authors reviewed and agreed on the manuscript.

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Diversity, Equality and Inclusion statement (https://www.nature.com/articles/d41586-020-02429-8)

The authors highly value equity, diversity and inclusion in science. We would like to acknowledge the international character of our team, which significantly contributed to the completion and quality of the present study. It includes researchers from different countries, backgrounds and career stages. The first author is from China, the last author is from France, other authors are from England, India, Italy, Japan, and Sweden. There are three female and seven male authors. We cite a large body of studies from many of our peers without checking whether the citations are equally distributed across groups. We thus acknowledge some shortcomings in our study and strive to address these issues in future work.

ORCIDs

Anne DUPLOUY - 0000-0002-7147-5199

Erik SVENSSON - 0000-0001-9006-016X

Andrea GALIMBERTI - 0000-0003-3140-3024

Giacomo ASSANDRI - 0000-0001-5161-5353

Bengt HANSSON – 0000-0001-6694-8169

Pallavi CHAUHAN – 0000-0002-5160-6673

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Tables

Table 1: Sample size, *Wolbachia* strains penetrance, and mitochondrial haplotypes (mitotypes based on *COI* 5'end only, or four mitochondrial regions: *COI, COIb, COIIa, & NDI*) across all populations of four *Ischnura* species. Shaded cells for data on *I. genei*, *I. pumilio*, and *I. saharensis*. N: number of samples, No: Number of haplotypes, *Ho*: Observed haplotype diversity (as N_H/N_t), Na: failed sequencing.

Country	Population [abbreviation]	Species	Strain	Infection rate	Ho (N_H/N_t)		
					Mitochondrial		Nuclear
					COI 5'end	4 regions	$PMRT$
Cyprus	Germasogetia Reservoir [CY]	*I. elegans*	-	0% (0/10)	0.85 (11/13)	0.88 (7/8)	0.7 (7/10)
Finland	Åland [ÅL]	*I. elegans*	-	0% (0/8)	0.2 (1/5)	Na	Na
	Mainland [FL]		*w*Ele3	100% (4/4)	0.25 (1/4)	0.25 (1/4)	Na
France	West [FR]	*I. elegans*	*w*Ele1	100% (1/1)	1 (1/1)	1 (1/1)	1 (1/1)
	Corsica [CO]	*I. genei*	*w*Ele1	100% (1/1)	1 (1/1)	1 (1/1)	1 (1/1)
Greece	Limni Dhistos [GR]	*I. elegans*	-	0% (0/10)	0.25 (2/8)	0.5 (2/4)	1 (10/10)
Japan	Ikeda [IK]	*I. elegans*	*w*Ele3	100% (10/10)	0.11 (1/9)	0.25 (1/4)	0.33 (3/9)
	Rokkasho [RK]	*I. elegans*	-	0% (0/3)	0.33 (1/3)	1 (1/1)	0.67 (2/3)
Italy	North [NI]	*I. elegans*	*w*Ele2	66.7% (2/3)	0.67 (2/3)	0.75 (3/4)	1 (2/2)
	Middle [MI]	*I. elegans*	*w*Ele2	100% (4/4)	0.25 (1/4)	1 (1/1)	1 (2/2)
		I. elegans	*w*Ele1	100% (1/1)	1 (1/1)	Na	Na
	South [SI]	*I. elegans*	*w*Ele1	50% (1/2)	1 (2/2)	1 (2/2)	1 (2/2)
	Sicily [SC]	*I. genei*	-	0% (0/1)	1 (1/1)	1 (1/1)	1 (1/1)
	Sardinia [SD]	*I. genei*	-	0% (0/1)	1 (1/1)	-	1 (1/1)
Montenegro	Bojana [BJ]	*I. elegans*	-	0% (0/1)	1 (1/1)	1 (1/1)	1 (1/1)
Morocco	Ain Isker [AI]	*I. saharensis*	-	0% (0/1)	1 (1/1)	1 (1/1)	1 (1/1)
Netherlands & Belgium	Vinne-Walem [VW]	*I. elegans*	*w*Ele1	52.9% (9/17)	0.06 (1/16)	Na	Na
Scotland	Aberdeen [AB]	*I. elegans*	*w*Ele1	100% (20/20)	0.05 (1/20)	0.5 (2/4)	0.5 (1/2)
Sweden	Southwest [SW]	*I. pumilio*	*w*Pum1	100% (4/4)	Na	Na	0.33 (1/3)
			*w*Pum2	100% (9/9)	Na	Na	
		I. elegans	*w*Ele1	97.7% (85/87)	0.09 (4/42)	0.3 (3/10)	1 (4/4)
Ukraine	Vostochne[VC]	*I. elegans*	*w*Ele1	50%	0.33	Na	1 (4/4)

			(2/4)	(1/3)		
Dniestr river [DR]	*I. elegans*	-	0% (0/2)	1 (1/1)	1 (1/1)	1 (1/1)
Pelican city [PC]	*I. elegans*	-	0% (0/3)	0.5 (1/2)	Na	1 (1/1)

Table 2: Mitochondrial and nuclear nucleotide diversity estimates, and neutrality tests of *Wolbachia* infected and uninfected *I. elegans* specimens based on the mitochondrial or nuclear loci. N_t- number of samples, N_H- Number of haplotypes, *Ho*- Observed haplotype diversity (as N_H/N_t), *Hd*- Haplotype diversity (as the probability of two haplotypes to be different), *S*- Number of polymorphic sites, *π*- nucleotide diversity; * $P<0.05$, ** $p<0.02$, *** $P<0.001$. In bold the significant data, and in grey the data for *w*Ele1 infected samples, for visualisation.

Species	Subsets	N_t	N_H	*Ho* (N_H/N_t)	*Hd*	*S*	*π (Pi)*	*Tajima's D*	*Fu & Li's F*
I. elegans Mitochondrial locus: *COI* Size: 598bp	All	135	16	0.12	0.533	44	0.009	-1.24	**-2.84***
	*w*Ele1	73	5	0.07	0.133	23	0.001	**-2.65*****	**-5.98****
	*w*Ele2	6	1	0.17	0	0	0	NA	NA
	*w*Ele3	13	2	0.15	0.46	20	0.015	1.85	**1.86****
	Uninfected	43	13	0.30	0.703	43	0.011	-1.32	-1.84
I. elegans Mitochondrial loci: *COI &COIb, COIIa, & NDI* Size: 2375bp	All	41	16	0.39	0.796	87	0.008	-0.183	0.60
	*w*Ele1	15	4	0.27	0.371	5	0.0003	**-1.91***	**-2.67***
	*w*Ele2	4	2	0.50	0.500	1	0.0002	-0.612	-0.48
	*w*Ele3	5	2	0.40	0.400	1	0.0002	-0.817	-0.77
	Uninfected	17	11	0.65	0.882	43	0.006	0.423	-0.09
I. elegans Nuclear locus: *PMRT* Size: 400bp	All	31	25	0.81	0.897 (N=26, No=18)!	18	0.008	-0.909	-1.19
	*w*Ele1	8	7	0.86	0.879 (N=7, No=6)!	9	0.007	1.026	1.43
	Uninfected	23	20	0.87	0.908 (N=19, No=17)!	18	0.008	-1.076	-1.64

!: Sample size and haplotype numbers for the calculations of *Hd*, S, *π, Tajima's D* and *Fu & Li's* indexes were slightly different for the nuclear gene, due to an indel in some sequences.

Table 3: Sexual and colour polymorphism in our *Wolbachia* infected and uninfected specimens from Sweden. A: androchrome, I: infuscans, O: obsolete.

	Female	Male	A (blue)	I (green)	O (red)
Infected (N=)	72	14	27	22	23
Uninfected (N=)	1	1	0	0	1

1. Infection ~ sex, $p=0.31$, not significant
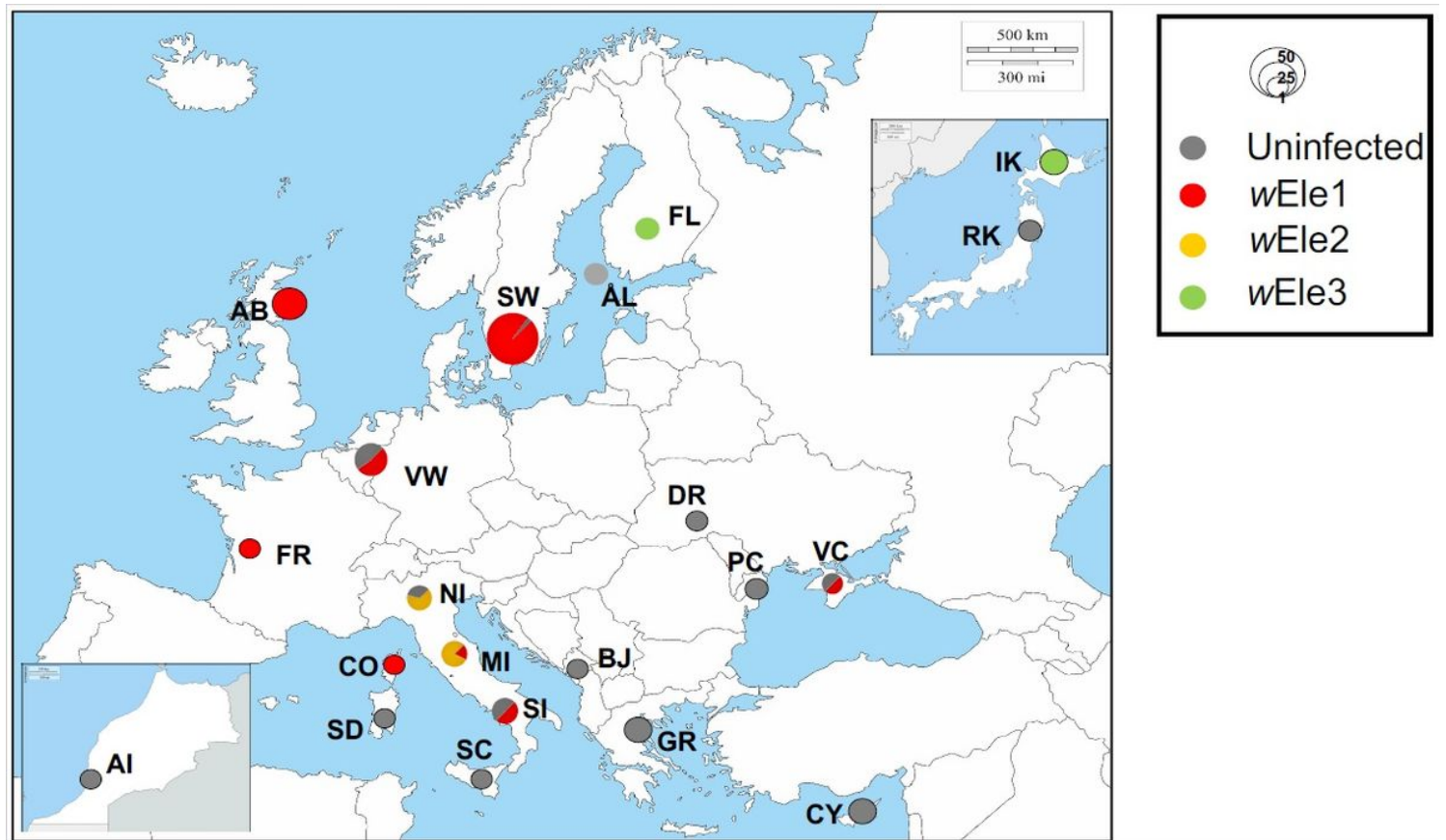2. Infection ~ three morphs (A, I, O), $p=0.63$, not significant

Figures

Figure 1

Wolbachia strain diversity and penetrance from 17 populations across the geographical range of the damselfly Ischnura elegans. The top right window shows the data from two populations in Japan. The sample from Morocco [AI] (bottom left window) is from the species I. saharensis, while the samples from [CO], [SC] and [SD] are I. genei specimens. Size of each chart is proportional to the number of individuals included in the study for each population. Maps are freely available here: Europe (https://d-maps.com/carte.php?num_car=2232&lang=en), Japan (https://d-maps.com/carte.php?num_car=354&lang=en), and Morocco (https://d-maps.com/carte.php?num_car=1132&lang=en).
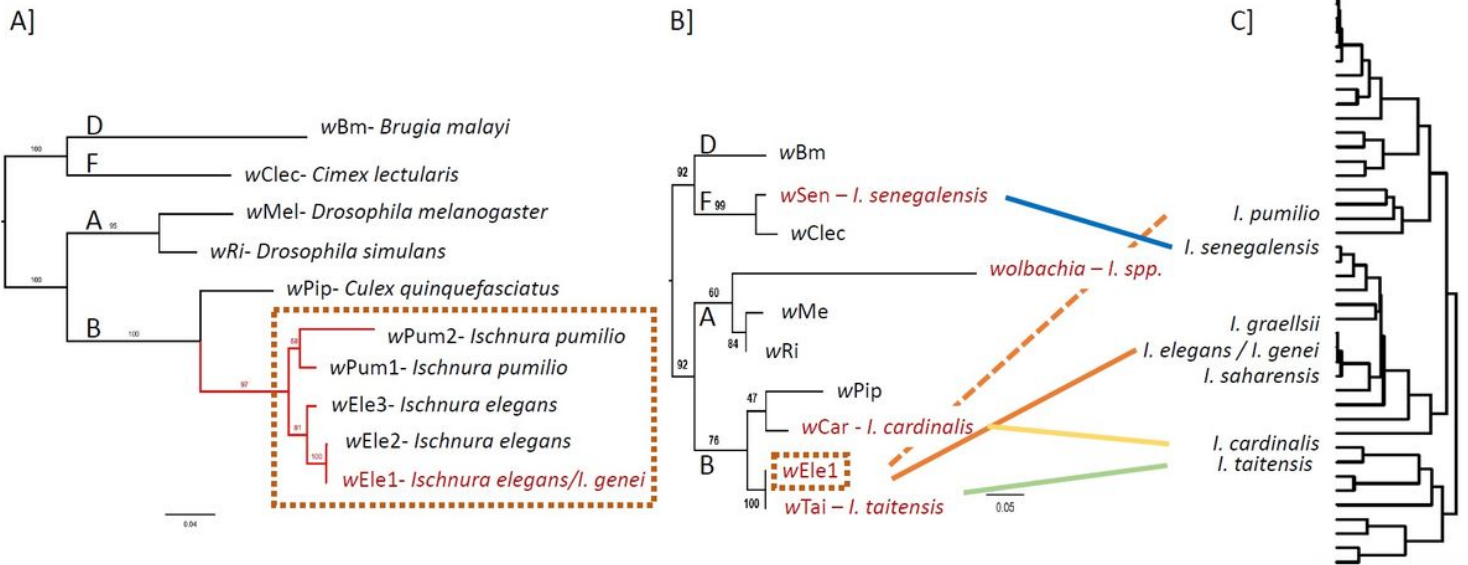
Figure 2

Maximum likelihood tree of (A) the Wolbachia strains characterized in the present study and based on the concatenated sequences of the ftsZ and wsp genes, and of (B) five Wolbachia strains from five Ischnura species, including wEle1, based on the fbpa gene. (C) Phylogenetic tree of the Ischnura genus phylogeny, for comparison, as provided by [51]. In (B): the strains marked with red come from Ischnura species: wSen [44], 'Wolbachia_I. spp.', wCar and wTai [45]. Five additional strains (wBm, wClec, wMel, wRi, wPip) were also included in the trees as references for the different Wolbachia-supergroups A, B, D and F. The two Wolbachia trees were rooted using the D and F-Wolbachia supergroups as outgroups. Bootstrapping was conducted using 'Ultrafast' bootstrap method in IQ-Tree with 1000 replicates. Links between the (B) and (C) trees show the lack of concordance between the symbiont and host trees.
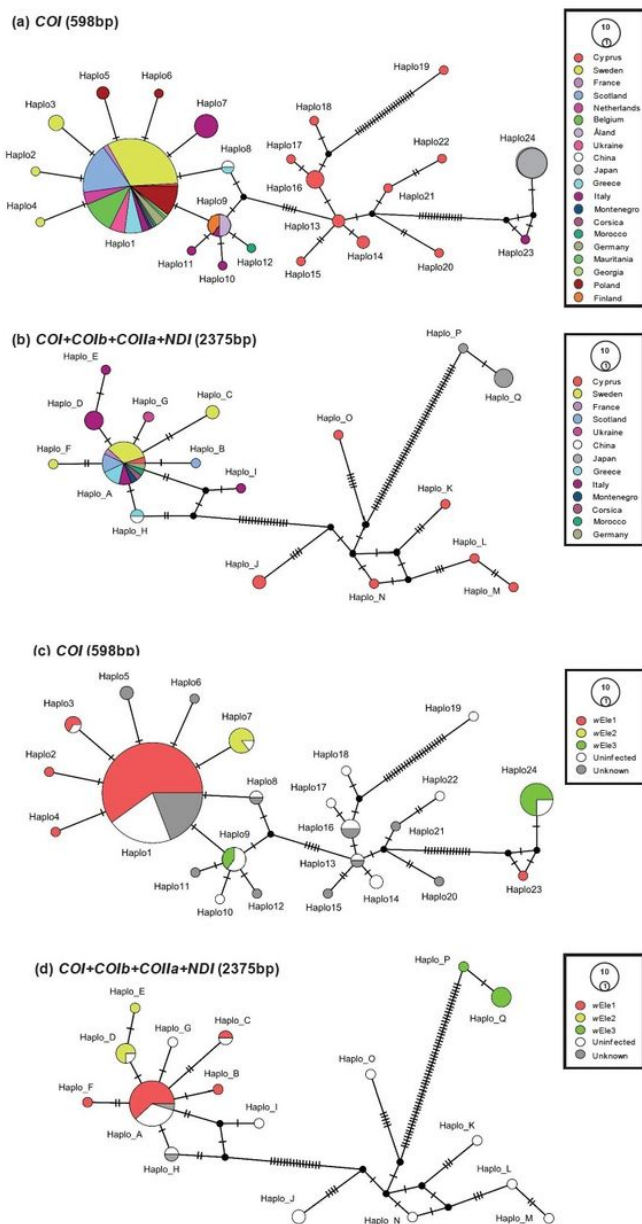
Figure 3

Mitochondrial haplotype networks of I. elegans and two closely related species, I. genei and I. saharensis, based on (a & c) the mitochondrial COI gene only; and (b and d) all four mitochondrial markers, organised per country (a & b) or per infection status (c & d). Each circle represents one unique haplotype. The size of the circle is proportional to the number of specimens carrying the same haplotype. The black nodes indicate unobserved haplotypes. All other nodes were coloured by populations. The number of black bars between two haplotype nodes represent nucleotide differences between haplotypes. The mitotypes of 'Unknown' infection status were collected from Genbank and EMBL (Table S3).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- DengEtalSuppMatDec2020.pdf
- wolbachiaassemblyI.elegans.fasta