Diversification, selective sweep, and body size in the invasive Palearctic alfalfa weevil infected with Wolbachia

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The alfalfa weevil Hypera postica, native to the Western Palearctic, is an invasive legume pest with two divergent mitochondrial clades in its invading regions, the Western clade and the Eastern/Egyptian clade. However, knowledge regarding the native populations is limited. The Western clade is infected with the endosymbiotic bacteria Wolbachia that cause cytoplasmic incompatibility in host weevils. Our aim was to elucidate the spatial genetic structure of this insect and the effect of Wolbachia on its population diversity. We analyzed two mitochondrial and two nuclear genes of the weevil from its native ranges. The Western clade was distributed in western/central Europe, whereas the Eastern/Egyptian clade was distributed from the Mediterranean basin to central Asia. Intermediate mitotypes were found from the Balkans to central Asia. Most Western clade individuals in western Europe were infected with an identical Wolbachia strain. Mitochondrial genetic diversity of the infected individuals was minimal. The infected clades demonstrated a higher nonsynonymous/synonymous substitution rate ratio than the uninfected clades, suggesting a higher fixation of nonsynonymous mutations due to a selective sweep by Wolbachia. Trans-Mediterranean and within-European dispersal routes were supported. We suggest that the ancestral populations diversified by geographic isolation due to glaciations and that the diversity was reduced in the west by a recent Wolbachia-driven sweep(s). The intermediate clade exhibited a body size and host plant that differed from the other clades. Pros and cons of the possible use of infected-clade males to control uninfected populations are discussed.

Recent invasion events and routes of alien agricultural pests are of particular importance for the management and control of pests1–6. The knowledge of historical diversification and dispersal of agricultural pests in their native range provides insights to understand their natural and biological selective environments, including the role played by endosymbionts in pest emergence7–9.

The alfalfa weevil Hypera postica (Gyllenhal) (Coleoptera: Curculionidae: Hyperini), native to the Western Palearctic region, is a serious pest of alfalfa and other beneficial legumes in its invading territories, such as Medicago, Vicia, Trifolium, and Astragalus10–12 (Nearctic, Japan, Southeast Asia, and Oceania13–15). Invading populations in the USA comprise the Western (North American) type that invaded Utah in 190416, the Egyptian type that invaded Arizona in 193917, and the Eastern (North American) type that invaded Maryland in 195118.
These types are different in their ecological, behavioral, and defensive traits (pupation site and aggregation during aestivation\textsuperscript{15}, defensive behavior\textsuperscript{16,17}, and encapsulation of immature endoparasitoid\textsuperscript{18–22}) but are morphologically indistinguishable\textsuperscript{23}. Allozyme and mitochondrial DNA markers distinguish the Western type from the Egyptian and Eastern types\textsuperscript{22–25}, whereas the Egyptian and Eastern types are distinguishable only by a slight difference (1–2 SNPs in tRNA\textsuperscript{Ser}) in a mitochondrial gene sequence\textsuperscript{24,25}. The nuclear DNA polymorphism indicates that the three types share a gene pool, namely, a single species\textsuperscript{25,26}. They also mate with each other to reproduce (but see later for incompatibility\textsuperscript{27,28}).

Wolbachia (Alphaproteobacteria: Rickettsiales: Rickettsiaceae) are maternally (vertically) transmitted intracellular bacteria that infect approximately 40% of insects and other arthropods alongside nematodes\textsuperscript{29}. These endosymbiotic bacteria can manipulate host reproduction via reproductive cells and the genetic mechanism of this manipulation has recently been uncovered\textsuperscript{10}. Cytoplasmic incompatibility (CI), or postzygotic incompatibility between infected and uninfected gametes, is the most commonly observed phenotype of Wolbachia. Theoretically, bidirectional CI most strongly accelerates host speciation. Unidirectional CI, or postzygotic isolation of gametes between infected males and uninfected females, can also promote host speciation. While infection by Wolbachia is favored in females in populations with high Wolbachia prevalence, loss of Wolbachia can also occur through incomplete inheritance from mothers with low Wolbachia density\textsuperscript{29}. The loss (or incomplete transmission) rate of Wolbachia in insect hosts is slightly higher than the gain rate\textsuperscript{31}. Maternal transmission and unidirectional CI eventually reduce host mitochondrial diversity over generations. The selective sweep of mitochondria leads to a close association between the mitochondrial clade and Wolbachia infection\textsuperscript{32–35}. Several studies have discovered that Wolbachia may also accelerate the fixation of nonsynonymous mutations in hosts\textsuperscript{29,36–38}. Various positive fitness effects of endosymbionts on their hosts have been revealed, such as viral suppression and metabolic provisioning\textsuperscript{39,40}. The effect of endosymbionts on maternal mitochondria may also influence coevolution between mitochondria and nuclear genomes\textsuperscript{31,42}.

In H. postica, the Western clade is found to be infected by Wolbachia that induces unidirectional CI\textsuperscript{27,43,44}.

Several invading populations of the Western clade are free of Wolbachia, and a cross between uninfected Western males and Egyptian/Eastern females within these populations does produce viable offspring\textsuperscript{39}. This reconfirms that these clades, while genetically distant, remain conspecific. The CI effect between infected Western males and uninfected Eastern females is almost perfect (only 0.1% of hybrid eggs hatch), while 29.5% of hybrid eggs between infected Eastern males and infected Western females hatch\textsuperscript{27}.

The presence of diverged clades and Wolbachia infection history in Palearctic H. postica in its primary range is not known to date. Here, this study aims to explore the process of selection and diversification in H. postica in its native range by revealing and testing mitochondrial and nuclear genetic variation geographically and phylogenetically. We also aim to test if the endosymbiont Wolbachia affected evolution in host weevils. The benefits and risks of the Incompatible Insect Technique\textsuperscript{45} using infected clade males to control the uninfected clade populations are discussed.

**Results**

**Haplotype networks and diversity.** Sequenced segments were 2001 bp; 527 bp for COI-tRNA\textsuperscript{Leu}-COII, 281 bp for Cyt b-tRNA\textsuperscript{Ser}-ND1 (n = 149), 801 bp for 28S (n = 122) and 392 bp for EF-1\textalpha{} (n = 62) (Table 1). Despite our sizable effort, PCR failed for nuclear gene segments (especially EF-1\textalpha{}) for a part of the specimens. The mitotype network revealed two main clades alongside intermediate variants (Fig. 1). The first group corresponded to the Eastern/Egyptian clade (Fig. 1) and consisted of diverse mitotypes with multiple connections, which contributed to a high mitochondrial genetic diversity (Table 2). This clade was widely distributed from central Asia to the Mediterranean region (Fig. 2). Within this clade, populations from the Balkan peninsula displayed high mitochondrial and nuclear genetic diversity (Table 3). The second clade corresponded to the Western clade and exhibited substantially fewer mitotypes, one dominant mitotype and rarer, closely related mitotypes in a star-shape topology, which corresponds to low genetic diversity in both gene fragments (Fig. 1, Table 2). This clade was distributed in western and central Europe, north of the Alps and Pyrenees (Fig. 2). Within this clade, 50.0% of individuals and 50.0% of populations were infected with Wolbachia (Fig. 2). The Wolbachia-infected populations demonstrated lower mitochondrial and nuclear genetic diversity than the uninfected populations (Table 2). Compared with the uninfected Eastern/Egyptian clade, the infected Western clade displayed 22 (Cyt b-tRNA\textsuperscript{Ser}-ND1) to 82 (COI-tRNA\textsuperscript{Leu}-COII) times lower mitochondrial genetic diversity (Table 2). The intermediate clade was distributed from the Balkans to central Asia (Fig. 2). The network for the nuclear fragments, 28S and EF-1\textalpha{}, appeared incongruent with the mitochondrial network (Figs. 1, 3), but as in mitotype variation, there was a significant difference in nuclear haplotype variation between individuals belonging to the different mitochondrial clades (EF-1\textalpha{}, Table 2).

**Wolbachia infection.** The Western clade individuals in northern France, the Netherlands, the Czech Republic, and Poland were infected with Wolbachia (Fig. 2), and all of them had identical ftsZ (699 bp), coxA (432 bp), and hcpA (463 bp) sequences as reported previously\textsuperscript{47}, which corresponds to the prevailing strain wHypera\textsuperscript{1}. In contrast, Western clade individuals found in coastal southern France, Spain, Latvia, Hungary, and Croatia were uninfected (Fig. 2). Additionally, we confirmed that Wolbachia was absent from all native populations studied in the Eastern/Egyptian clade. A reconstructed phylogenetic relationship confirms that wHypera belongs to Supergroup B (Fig. 4). The Wolbachia strain closest to wHypera to date is the one that infects the mite Bryobia pratitosa (Acari: Tetranychidae)\textsuperscript{46}.

**Body size.** Elytral length was significantly different among different clades (χ\textsuperscript{2} = 10.96, p = 0.004) and between sexes (χ\textsuperscript{2} = 7.52, p = 0.006) and marginally different between infected and uninfected individuals.
| Number on the map | Code | Collection site | n  | Year | Latitude, longitude | GenBank accession | GenBank accession Nuclear genes |
|------------------|------|-----------------|----|------|---------------------|------------------|-------------------------------|
| Western Europe   |      |                 |    |      |                     |                  |                               |
| 1                | Cz   | Prague, Czech Republic | 5  | 2012 | 50°05'16"N,14°17'54"E | KX372573 (CO), KX372620 (CB) | KX372667 (28S), MW392102 (EF1a) |
| 2                | Ne   | Amsterdam, the Netherlands | 2  | 2014 | 52°21'35"N,4°57'00"E | MW393903 (CO), MW393922 (CB) | MW383444 (28S), MW389094 (EF1a) |
| 3                | Po   | Nida Basin, Poland | 2  | 2012 | 50°24'N,20°38'E | MW393912 (CO), MW393931 (CB) | MW383460 (28S), MW389110 (EF1a) |
| 4                | Lt   | Daugavpils, Latvia | 2  | 2007 | 55°52"N,26°27"E | MW393914 (CO), MW393933 (CB) |                               |
| Central Europe   |      |                 |    |      |                     |                  |                               |
| 5                | BuH  | Budapest, Hungary | 4  | 2012 | 47°23'41"N,19°01'00"E | MW393904 (CO), MW393923 (CB) | MW383445 (28S), MW389095 (EF1a) |
| 6                | AdH  | Adlyigit, Budapest, Hungary | 9  | 2014 | 47°33'40"N,18°55'58"E | KX372574–75–76, (CO), KX372621–22 (CB) | MW383446 (28S), MW389096 (EF1a) |
| France           |      |                 |    |      |                     |                  |                               |
| 7                | ChF  | Chaussy, France | 4  | 2013 | 49°07'12"N,1°42'02"E | MW393905 (CO), MW393924 (CB) | MW383448 (28S), MW389098 (EF1a) |
| 8                | OrF  | Orleàns, France | 12 | 2013 | 47°53'59"N,1°56'24"E | MW393906,16 (CO), MW393925,35 (CB) | MW383449,62 (28S), MW389099,112 (EF1a) |
| 9                | AuF  | Aurade, France | 5  | 2014 | 43°33'36"N,1°03'01"E | KX372576 (CO), KX372623 (CB) | MW383447 (28S), MW389097 (EF1a) |
| 10               | ComF | Combaillaux, France | 9  | 2016 | 43°40'12"N,3°46'47"E | MW393915 (CO), MW393934 (CB) |                               |
| 11               | AlpF | Saint-Paul-sur-Ubaye, France | 2  | 2010 | 44°31'12"N,6°45'02"E | KX372579 (CO), KX372626 (CB) | MW383450 (28S), MW389100 (EF1a) |
| 12               | CoF  | Casamozza, Corse Island, France | 5  | 2016 | 42°30'35"N,9°26'23"E | MW393910 (CO), MW393929 (CB) | MW383458 (28S), MW389108 (EF1a) |
| Spain            |      |                 |    |      |                     |                  |                               |
| 13               | Sp   | La Cañada, Spain | 6  | 2014 | 40°36'00"N,4°30'35"W | KX372577,82 (CO), KX372624,29 (CB) | MW383452,61 (28S), MW389102,11 (EF1a) |
| Balkans and Italy|      |                 |    |      |                     |                  |                               |
| 14               | Malt | Maremma, Toscana, Italy | 3  | 2015 | 42°38'17"N,11°07'29"E | MW393913 (CO), MW393932 (CB) |                               |
| 15               | Pult | Puglia, Italy | 2  | 2002 | 41°04'N,16°26"E | KX372599–600 (CO), KX372646,47 (CB) | MW383455 (28S), MW389105 (EF1a) |
| 16               | Gr   | Sparti, Peloponnisos, Greece | 5  | 2005 | 36°51'N,22°39"E | KX372586–87,614–16 (CO), KX372633–34,61–63 (CB) |                               |
| 17               | Ro   | Crucea, Romania | 5  | 2009 | 44°31'12"N,28°11'59"E | KX372588,96–98,617 (CO), KX372635,43–45,64 (CB) | MW383450 (28S), MW389100 (EF1a) |
| 18               | KnBu | Knezha, Bulgaria | 10 | 2016 | 43°28'48"N,24°03'36"E | MW393917 (CO), MW393936 (CB) |                               |
| 19               | LoBu | Lozitsa, Bulgaria | 8  | 2016 | 43°34'48"N,25°00'01"E | MW393918 (CO), MW393937 (CB) |                               |
| 20               | Cr   | Zagreb, Croatia | 2  | 2006 | 45°50'35"N,15°44'55"E | KX372578 (CO), KX372625 (CB) |                               |
| Africa           |      |                 |    |      |                     |                  |                               |
| 21               | faMo | Ouzoud falls, Morocco | 10 | 2016 | 32°00'54"N,6°43'24"W | MW393909 (CO), MW393928 (CB) | MW383457 (28S), MW389107 (EF1a) |
| 22               | OuMo | Ouarzazate, Morocco | 2  | 1994 | 30°56'N,6°56'W | KX372589,618 (CO), KX372636,65 (CB) |                               |
| 23               | Li   | Benghazi, Libya | 5  | 1980 | 32°03'N,20°09"E | KX372603–04,13 (CO), KX372650–51,60 (CB) |                               |
| 24               | Eg   | Sakha, Kafr El-Sheikh Government, Egypt | 3  | 2013 | 31°05'13"N,30°56'56"E | KX372580, MW393907 (CO), KX372627, MW393926 (CB) | MW383451 (28S), MW389101 (EF1a) |
| Middle East and central Asia|      |                 |    |      |                     |                  |                               |
| 25               | Isr  | Gal'ash, Israel | 2  | 2014 | 32°13'48"N,34°49'12"E | KX372590 (CO), KX372637 (CB) | MW383453 (28S), MW389103 (EF1a) |
| 26               | Tur  | Catalan vill., Adana, Turkey | 4  | 2002 | 37°15'01"N,35°18'10"E | KX372581,601–02, MW393908 (CO), KX372628,48–49, MW393927 (CB) | MW383456 (28S), MW389106 (EF1a) |
| 27               | Ar   | Metsamor, Armenia | 2  | 2013 | 40°09'19"N,44°07'30"E | KX372606 (CO), KX372653 (CB) |                               |
| 28               | ArIr | Azerbaijan, Iran | 2  | 1999 | 37°56′N,47°23′E | KX372584–85 (CO), KX372631–32 (CB) |                               |
| 29               | Talr | Taleghan, Iran | 8  | 2015 | 36°12′12″N,50°51′47″E | MW393919 (CO), MW393938 (CB) |                               |
| Continued        |      |                 |    |      |                     |                  |                               |
Table 1. Sample collection information for *Hypera postica*. COI: t-RNA*14u*-COII. CB: Cyt b-t-RNASer-ND1.

| Number on the map | Code | Collection site                  | n  | Year | Latitude, longitude | GenBank accession Mitochondrial genes | GenBank accession Nuclear genes |
|-------------------|------|----------------------------------|----|------|---------------------|--------------------------------------|--------------------------------|
| 30                | Halr| Hamedan, Iran                     | 3  | 2014 | 34°31′16″N, 48°18′43″E | MW393911 (CO), MW393930 (CB)          | MW383459 (28S), MW389109 (EF1a) |
| 31                | Falr| Dash-A-Abshen, Zagros Mts., Fars Prov., Iran | 2  | 2000 | 29°34′ N, 51°56′ E    | KX372656-09 (CO), KX372655-56 (CB)   |                                 |
| 32                | Tm  | Anau, Turkmenistan                | 2  | 1988 | 37°54′ N, 58°30′ E    | MW393920 (CO), MW393939 (CB)         |                                 |
| 33                | Ky  | Jangy-Talap, Kyrgyzstan           | 4  | 2015 | 41°27′01″N, 75°01′12″E | KX372592 (CO), KX372639 (CB)         | MW383454 (28S), MW389104 (EF1a) |

($χ^2_1 = 3.77, p = 0.052$). The elytron was longer in the intermediate variants ($3.83 ± 0.110$ mm, mean ± SE, $n = 21$) than in the Egyptian/Eastern ($3.48 ± 0.025$ mm, $n = 64$, $p = 0.003$) and Western ($3.50 ± 0.057$ mm, $n = 29$, $p = 0.041$) clades (no difference between the Egyptian/Eastern and Western clades, $p = 0.934$), longer in females ($3.62 ± 0.042$ mm, $n = 60$) than in males ($3.47 ± 0.044$ mm, $n = 52$), and marginally longer in uninfected individuals ($3.57 ± 0.032$ mm, $n = 102$) than in infected individuals ($3.36 ± 0.111$ mm, $n = 11$).

Selective neutrality test and positive selection test. For the Western clade, selective neutrality for mitochondrial segments was rejected by all indices with minus values ($D, D^*, F^*$), while for nuclear genes, selective neutrality was rejected by none of the indices (Table 4), suggesting recent sudden population growth after bottleneck event(s) in the mitochondrial lineage. For the Eastern/Egyptian clade, selective neutrality for both mitochondrial and nuclear segments was rejected by all indices (Table 4), suggesting recent sudden population growth after bottleneck event(s) in the mitochondrial lineage and nuclear variants. For the intermediate clade, selective neutrality for mitochondrial segments was rejected by none of the indices (Table 4). Selective neutrality for nuclear segments was untested because of the insufficient sample size.

A model with different $\omega$ (dN/dS) assigned for infected and uninfected clades improved the model fit most, compared to models with different $\omega$ for two or three different clades, although the improvement was nonsignificant (Table 5). The $\omega$ for infected clade was three times higher than the $\omega$ for uninfected clade even though both were $< 1$ (Table 5).

Geographic history. Isolation by distance. Isolation by distance (IBD) was supported for all populations ($p = 0.003$, number of pairwise comparisons $n = 528$) and for the Eastern/Egyptian clade excluding populations with intermediate mitotypes ($p = 0.050$, $n = 153$), but not for the Western clade ($p = 0.102$, $n = 55$). When the populations with intermediate mitotypes were included, the IBD within the Eastern/Egyptian clade was not supported ($p = 0.215$, $n = 231$).

Phylogeography. Based on available samples, the Balkan/Italian peninsula and the Middle East are the most likely area of the origin of *H. postica*, from which the Western clade diversified via France (Fig. 5a). France is the likely area where the ancestral population was first infected with *Wolbachia* (Fig. 5a, right). We found two connections between regions that were highly supported with BF > 3: France and western Europe (BF = 7.98) and Balkan/Italy and North Africa (BF = 7.90) (BSSVS analysis, Fig. 5b).

Discussion

This study revealed the large-scale geographic distribution and genetic diversity of *H. postica* in its native range. Intermediate mitotypes with larger body sizes were found from the Balkans to central Asia. The observed reduced diversity within the Western clade is likely due to a high percentage of *Wolbachia* infection within this clade, which is known in other species. We also identified a higher substitution rate of nonsynonymous mutations, suggesting promoted (fixation of) nonsynonymous mutations in the infected Western clade. In the Western clade, recent sudden population growth after a bottleneck was suggested only for mitochondrial genes and not for nuclear genes, supporting a recent selective sweep on mitochondria by *Wolbachia* infection.

Our results demonstrated a clear pattern of geographic distribution of the two divergent mitochondrial clades across the area of study, the Eastern/Egyptian and Western clades. The low genetic variation and star-like haplotype network within the Western clade is a signature of a recent demographic expansion from a few founders. If the populations experienced ancient demographic bottlenecks, mitochondrial and nuclear genes are expected to have a concordant population structure. There was a weak concordance (Table 2), suggesting that these genes may have shared similar evolutionary trajectories. Two bottleneck events are likely; postglacial recolonization (see next section) and a recent mitochondrial sweep by *Wolbachia*. The former may also serve as a major driver of IBD that was supported for overall geographic populations (two clades distributed separately in the north and south with intermediate mitotypes in between). The latter likely accelerates the fixation of nonsynonymous mutations in the Western clade.

The asymmetric inheritance of maternal mitochondrial of an infected host caused by unidirectional CI-inducing *Wolbachia* can potentially lead to a sweep, which likely explains the low mitochondrial genetic variation among infected individuals. The infected clade demonstrated accelerating nonsynonymous mutations or fixation. This result is consistent with a general trend of *Wolbachia* infected insect groups, suggesting fixation of nonsynonymous mutations in mitochondria promoted by its small effective population size under the CI-inducing
Figure 1. Statistical parsimony network of mitochondrial COI-tRNALeu-COII and Cyt b-tRNASer-ND1 of Hypera postica in its native range. Generated using TCS 1.2180.
**Wolbachia** infection. Furthermore, *Wolbachia* infection is advantageous for *H. postica* by enhancing resistance against its adult parasitoid *Microctonus aethiopoides*.22 In the southernmost populations of the Western clade or the geographic contact zone between the two clades, most individuals were uninfected or had lost *Wolbachia*. The imperfect maternal transmission was observed in the interclade crosses of *H. postica*27; fitness costs incurred by cytoplasmic incompatibility and stochasticity during the invasion process45,54 may lower the *Wolbachia* infection rate. Environmental causes (e.g., extreme temperatures) may also accelerate the endosymbiont loss55,56. Resulting uninfected *H. postica* populations (or with lowered *Wolbachia* density) must have regained reproductive compatibility between clades and enabled crosses between the diverged clades.

The intermediate variants exhibited a large body size. Larger genitalia of the males with these mitotypes may inhibit mating with the females of other clades and promote reproductive isolation. These mitotypes also were associated with an ecological niche that differed from the niche of other clades. Bulgaria populations used *Vicia cracca* as a host plant, whereas other populations used *Medicago* and *Trifolium*. *Vicia cracca* has high contents of cyanamide57 and canavanine58 that are toxic to insect herbivores59,60.

The genetic structure of most European biota has been strongly influenced by glacial oscillations of the Holocene61,62, and most temperate species exhibit northward post-glacial recolonization from glacial refugia located in southern Europe through central Asia during the last glacial maxima (southern genetic richness/northern purity63; in beetles64). In *H. postica*, we observed mtDNA differentiation for all clades and within the Eastern/Egyptian clade. Based on the estimated ancestral states in mitochondrial phylogeography and mitochondrial/nuclear genetic diversity, the Balkan and Italian peninsulas are a possible candidate for the origin of

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### Table 2. Mitochondrial and nuclear genetic diversity of the two clades (the Western and the Egyptian/Eastern) and the intermediate clades in *Hypera postica*. n: mean number of pairwise differences; nucleotide diversity (average over loci) (mean ± SD). The genetic distance was calculated based on pairwise differences. Numbers in parentheses are sample sizes (pooled number of individuals, number of populations). The same letters within each column (gene segment) indicate no significant difference between clades (p > 0.01). For population-wise mitochondrial and nuclear diversities and distances, see Supplementary Tables S1 and S2.

| Clade (Wolbachia infection) | Mitochondria | Nuclear |
|-----------------------------|-------------|---------|
|                             | COI-tRNALeu-COI | Cyt b-tRNASer-ND1 | 28S EF-1α |
|                             | n | Nucleotide diversity | n | Nucleotide diversity | n | Nucleotide diversity | n | Nucleotide diversity |
| Western (infected) | 0.074 ± 0.150 (27, 6) | 0.00004 ± 0.00031 | a | 0.148 ± 0.217 (27, 6) | 0.00003 ± 0.00086 | a | 0.262 ± 0.299 (27, 6) | 0.00003 ± 0.00042 | a | 3.134 ± 1.689 (22, 5) | 0.00802 ± 0.00482 | a |
| Western (uninfected) | 3.481 ± 1.832 (27, 6) | 0.00661 ± 0.00387 | a | 0.729 ± 0.560 (27, 6) | 0.00260 ± 0.00222 | a | 1.057 ± 0.724 (25, 6) | 0.00132 ± 0.00101 | a | 6.107 ± 3.251 (8, 3) | 0.01154 ± 0.00942 | a |
| Egyptian/Eastern (uninfected) | 6.027 ± 2.917 (74, 23) | 0.01149 ± 0.00614 | b | 3.295 ± 1.714 (74, 23) | 0.01173 ± 0.00676 | b | 1.052 ± 0.708 (60, 20) | 0.00131 ± 0.00098 | a | 5.335 ± 2.646 (31, 12) | 0.01334 ± 0.00736 | b |
| Intermediate (uninfected) | 10.800 ± 4.897 (21, 6) | 0.01955 ± 0.01037 | c | 0.733 ± 0.567 (21, 4) | 0.00261 ± 0.00225 | c | 2.111 ± 1.282 (10, 3) | 0.00264 ± 0.00181 | a | 0 (1, 1) | 0 | ab |
| Among-clade variation (df=2), p | 72.53%, < 0.00001 | 87.60%, < 0.00001 | 3.34%, 0.0168 | 6.50%, 0.0544 |

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**Figure 2.** Geographic distribution of mitochondrial clades of *Hypera postica* in its native range. Pie chart sizes for clades are proportional to sample sizes. *Wolbachia* infection (dark gray ring: infected; white (partial) ring: uninfected) is only indicated for the Western clade individuals because none of the Egyptian/Eastern clade or the intermediate clade were infected. The background map was obtained from Fotolla, [https://stock.adobe.com/jp/photos/](https://stock.adobe.com/jp/photos/).
the Eastern/Egyptian clade and western Europe (France) for that of the Western clade. The primary center of genetic and species diversity of the main host *Medicago* is the Caucasus (north-western Iran and north-eastern Turkey)\(^6^5\), which may also consist of the area of origin of *H. postica*.

The recent dispersal routes that include the north Mediterranean were highly supported. Anthropogenic factors may allow occasional dispersal of *H. postica* to Europe and North Africa with alfalfa traded for livestock feed (by 2,600 years ago\(^6^6\)). More recent international trade of alfalfa meal and pellets may continue to aid the weevil’s opportunistic long-distance dispersal; France, Spain, and Italy are the major alfalfa exporters among *H. postica*’s native ranges\(^6^7\).

**Conclusion.** While geographic isolation assisted continental diversification of the weevil *H. postica*, recent *Wolbachia* infection reduced diversity in a mitochondrial clade in the host weevil in western Europe. *Wolbachia*-infected males could be used as a control agent for the Incompatible Insect Technique on uninfected populations, however, the risk of heterosis in interclade crosses following accidental cure of *Wolbachia* must be assessed before application.

### Table 3. Mitochondrial and nuclear genetic diversity in *Hypera postica* based on geographic regions.

| Geographic region (country) | Mitochondria | Nuclear |
|----------------------------|--------------|---------|
|                            | COI-TRNA\(^{53}c\)-COII | Cyt b-TRNA\(^{53}c\)-ND1 | 28S | EF-1α |
|                            | n | Nucleotide diversity | n | Nucleotide diversity | n | Nucleotide diversity | n | Nucleotide diversity |
| Western Europe (Cz, Ne, Lit, Po) | 0.51 ± 0.46 (11, 4) | 0.0010 ± 0.0010 | 0.18 ± 0.25 (11, 4) | 0.0006 ± 0.0010 | 0.40 ± 0.40 (10, 4) | 0.0005 ± 0.0006 | 3.07 ± 1.85 (6, 2) | 0.0078 ± 0.0055 |
| France (Fr) | 9.92 ± 4.65 (35, 6) | 0.0188 ± 0.0098 | 7.06 ± 3.40 (35, 6) | 0.0251 ± 0.0134 | 0.58 ± 0.48 (35, 6) | 0.0007 ± 0.0007 | 4.48 ± 2.30 (21, 6) | 0.0112 ± 0.0064 |
| Spain (Sp) | 9.13 ± 4.90 (6, 1) | 0.0173 ± 0.0107 | 8.47 ± 4.57 (6, 1) | 0.0301 ± 0.0188 | 0.93 ± 0.74 (6, 1) | 0.0012 ± 0.0011 | 2.67 ± 1.65 (6, 1) | 0.0068 ± 0.0049 |
| Central Europe (H) | 0.00 ± 0.00 (13, 2) | 0.0000 ± 0.0000 | 0.31 ± 0.34 (13, 2) | 0.0011 ± 0.0014 | 0.73 ± 0.58 (12, 2) | 0.0009 ± 0.0008 | 6.10 ± 3.30 (7, 2) | 0.0155 ± 0.0096 |
| Balkan and Italy (Gr, Bu, Ro, Cz, It) | 15.53 ± 7.10 (35, 7) | 0.0295 ± 0.0150 | 5.08 ± 2.53 (35, 7) | 0.0181 ± 0.0100 | 1.32 ± 0.85 (23, 7) | **0.0016 ± 0.0012** | 6.00 ± 4.58 (2, 2) | **0.0153 ± 0.0166** |
| Africa (Mo, Lib, Eg) | 4.23 ± 2.19 (20, 4) | 0.0080 ± 0.0046 | 2.58 ± 1.44 (20, 4) | 0.0092 ± 0.0057 | 1.00 ± 0.71 (16, 4) | 0.0012 ± 0.0010 | 4.62 ± 2.42 (13, 3) | 0.0117 ± 0.0069 |
| Middle East and central Asia (Ir, Ir, At, Tur, Tm, Ky) | 10.23 ± 4.81 (29, 9) | 0.0194 ± 0.0102 | 3.08 ± 1.65 (29, 9) | 0.0110 ± 0.0065 | 1.08 ± 0.74 (20, 8) | 0.0013 ± 0.0010 | 5.05 ± 2.79 (7, 4) | 0.0129 ± 0.0082 |
| Among geographic region variation, p | 36.6%, <0.00001 | 63.0%, <0.00001 | 13.0%, <0.00001 | 9.2%, 0.00198 |

**Figure 3.** Statistical parsimony network of nuclear 28S and EF-1α. See Fig. 1 for colors for regions. Generated using TCS 1.21**80**.
Figure 4. Bayesian consensus tree of *Wolbachia* strain based on *ftsZ*, *coxA*, and *hcpA*. Strain codes, if available, followed by host species and *Wolbachia* supergroups are shown. The *Wolbachia* strain, wHypera1, infecting *Hypera postica* in its native range, is shown in bold. Bayesian support values (posterior probabilities > 0.7) are shown near nodes. The outgroup is *Anaplasma marginale* (Alphaproteobacteria: Rickettsiales: Anaplasmataceae). Generated using MrBayes 3.2.673. Host strains, *Wolbachia* isolates and GenBank accession numbers are listed in Supplementary Table S3.

| Clade            | Mitochondria | Nuclear          |
|------------------|--------------|------------------|
|                  | *D* (p)      | *D* (p)          | *F* (p) | *n* | *D* (p)      | *D* (p)          | *F* (p) | *n* |
| Western          | -2.389 (0)   | -3.371 (0.0015) | -3.302 (0.0010) | 23  | -0.822 (0.152) | -0.547 (0.267) | -0.666 (0.240) | 15  |
| Egyptian/Eastern | -1.510 (0.039) | -2.872 (0.0175) | -2.673 (0.018) | 39  | -1.491 (0.0255) | -2.007 (0.0125) | -1.959 (0.0165) | 19  |
| Intermediate     | 0.695 (0.763) | 0.566 (0.707)   | 0.596 (0.719)  | 6   |                |                  |         |     |

Table 4. Selective neutrality test results on mitochondrial and nuclear segments. *D*: Tajima’s *D*; *D* and *F*: Fu and Li’s *D* and *F*.
**Table 5.** Test of positive selection on the *Hypera postica* phylogeny. Root: *Brachypera zoilus* and *H. miles*. W: Western. E: Egyptian/Eastern. Inf: infected by *Wolbachia*. Uninf: uninfected by *Wolbachia*. ΔlnL: difference in log likelihood (lnL) of each model from the model with the same single ω for W, intermediate, and E clades (i.e., the ‘Root and W/intermediate/E’ model as a reference model). Δdf: difference of each model in degree of freedom (df) from the model with a same single ω for W, intermediate, and E clades. The – symbol: the same ω value as the one on the left.

| Model (number of ω) | Root | E | Intermediate | W | InW | 2ΔlnL | Δdf | p  |
|---------------------|------|---|-------------|---|-----|-------|-----|----|
| Root and W/intermediate/E (2) | 0.0103 | 0.0578 | – | – | – | 0 | 0 | 1.000 |
| Root, W and intermediate/E (3) | 0.0103 | 0.0575 | – | 0.0590 | – | 0 | 1 | 1.000 |
| Root, W/intermediate and E (3) | 0.0104 | 0.0541 | 0.0640 | – | – | 1.50 | 1 | 0.221 |
| Root, InW and UnInW/intermediate/E (3) | 0.0103 | 0.0571 | – | – | 0.1709 | 1.80 | 1 | 0.180 |
| Root, InW, UnInW and intermediate/E (4) | 0.0103 | 0.0575 | – | 0.0554 | 0.1709 | 1.82 | 2 | 0.403 |
| Root, InW, UnInW/intermediate and E (4) | 0.0104 | 0.0541 | 0.0620 | – | 0.1709 | 0.72 | 2 | 0.698 |

**Methods**

**Sampling.** *Hypera postica* (*n* = 149) were obtained from 33 localities covering most of its native distribution range (Table 1). Adults were collected from cultivated and wild legume vegetation of *Medicago*, *Trifolium* in Egypt, and *Vicia cracca* in Bulgaria, mostly during the latest decade. The samples were then stored in ethanol at 4°C until DNA extraction.

**PCR and sequencing.** DNA was extracted from all specimens using a DNeasy Blood & Tissue kit (Qiagen, Tokyo, Japan). We amplified and sequenced two mitochondrial fragments, *COI*-rRNA*°*-COII and Cyt b-rRNA*°*-ND1 and two nuclear fragments, 28S and EF-1a. The primers used were C1-J-279796 and C2-N-338024 for the *COI*-rRNA*°*-COII fragment, CB-J-11545 and N1-N-118148 for Cyt b-rRNA*°*-ND1, 28S-01 and 28S-R-01 for 28S, and ef415F (5′-AACAGAGAACATGGCTTCTCG-3′) and ef862R (5′-CTCAATTTTCTTTGTTGTCATT-3′) (this study) for EF-1a. PCRs were performed using GoTaq Green Master Mix (Promega, Tokyo, Japan). Cycling conditions for *COI*-rRNA*°*-COII* amplification consisted of preheating at 95°C for 2 min, followed by 38 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and an extension at 60°C for 1 min. Amplification conditions for Cyt b-rRNA*°*-ND1* were identical, except that annealing was performed at 55°C for 1 min. Cycling conditions for 28S* amplification were as follows: preheating as above, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 40 s, and an extension at 70°C for 1 min. Those for EF-1a* consisted of preheating as above, followed by 38 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 40 s, and an extension at 68°C for 1 min. Sequencing was carried out using a BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies/Applied Biosystems, Foster City, CA, USA) on a 3730 DNA Analyzer (Applied Biosystems).

**Wolbachia infection and phylogeny.** We used PCR to screen for possible *Wolbachia* infections. The *Wolbachia* *ftsZ* coding fragment was amplified using the primers fts-Z-f and fts-Z-r. PCRs were performed using preheating as above, followed by 32 cycles at 94°C for 40 s, 55°C for 45 s, and 70°C for 1 min. As a positive control, we used *Wolbachia*-infected *Callisosbruchus chinesis*. Blurred and extremely weak signals compared with the positive control were considered uninfected, which differs from a previous study. *Wolbachia*-positive *H. postica* were further subjected to PCR and sequencing of the genes, coxA and hcpA, in addition to *ftsZ* for multilocus sequence typing of *Wolbachia*.

For the phylogenetic reconstruction of *Wolbachia*, we used sequences from representative supergroups of *Wolbachia* (*nr*/*nt* database, Supplementary Table S1) with sequences of *Anaplasma marginale* as an outgroup. We used the GTR model, which was selected as the best fit model of nucleotide substitution by MRBAYES3.2.6, based on the AICc, using MRAICc.pl 1.3.1. The three gene segments were partitioned. Markov chain Monte Carlo (MCMC) simulations were performed for one million generations, with sampling conducted every 1,000 generations. The convergence of independent parallel runs was checked using TRACER 1.6, and the first 25% of trees were discarded as burn-in.

**Body size.** After collecting specimens, the right elytron lengths of the samples were measured to the precision of 0.01 mm with a microscope (VH-5500, Keyence, Osaka, Japan). The sex of the samples was determined by both external and genital morphology. The effect of sex, clades (Egyptian/Eastern, Western, and intermediate), and *Wolbachia* infection on elytral lengths was tested by nonparametric Wilcoxon/Kruskal-Wallis signed rank tests. Posthoc multiple comparison was performed on the significant factor using the Steel-Dwass test. JMP 14.2.0 was used for statistical analyses.

**Selective neutrality test and positive selection test.** Selective neutrality was tested in each clade with Tajima’s D, Fu and Li’s D* and F*, using DNAsp 6.12.03. P values were derived by coalescent simulations with 2,000 replications. For the coalescent simulations for nuclear segments, an intermediate recombination rate was assumed. We used all mitochondrial (808 bp) or nuclear sequences (1,193 bp) of two individuals sampled per clade from each population to avoid sample size bias between populations.
The equal nonsynonymous/synonymous substitution rate ratio (dN/dS ratio, \( \omega \)) between infected and uninfected clades and between the two major clades was tested with a phylogenetic analysis using the maximum likelihood method (likelihood ratio test) employed by the codeml program in PAML 4.9. The models with three or more different \( \omega \) for each branch were compared with a reference (basal) model with two different \( \omega \) (one for the root and the other for both the Western and Egyptian/Eastern clades). We concatenated all the open reading frames (protein-coding fragments) and removed potential stop codons (leading to 215 codons) of the mitochondrial sequences of two individuals randomly sampled per clade from each population to avoid sample size bias between populations. Codons for invertebrate mitochondria were used.

**Haplotype networks and diversity.** All mitochondrial sequences were assembled using SEQUENCER 5.0 (Gene Codes Corp, Ann Arbor, MI, USA), and we checked for the presence of pseudogenes using commonly employed methods. Statistical parsimony networks were reconstructed based on mitochondrial and nuclear fragments using TCS 1.21 82, in which we allowed connections between haplotypes of 20 steps for mitochondrial sequences of two individuals randomly sampled per clade from each population to avoid sample size bias between populations. Codons for invertebrate mitochondria were used.

**Geographic history.** Isolation by distance. We assessed spatial mitochondrial differentiation by testing for isolation by distance (IBD) 85. With a sweep (e.g., by Wolbachia) followed by rapid spread or frequent anthropogenic long-distance dispersal events, the IBD correlation is predicted to be weak at most. We tested if, as predicted by IBD, pairwise geographic distances and pairwise genetic differences were positively correlated using a one-tailed Mantel test based on 2,000 permutations with the ISOLDE program implemented in Genepop 4.2. 86. For pairwise genetic differences, we employed corrected average pairwise differences between populations \( X \) and \( Y \) [\( \pi_{XY} = (\pi_X + \pi_Y)/2 \)] 88 and their \( p \) values were derived using ARLEQUIN.

**Phylogeography.** We estimated the historical dispersal patterns of Hypera postica, using a Bayesian discrete phylogeographic approach with a Bayesian skyride framework implemented in the software package BEAST 1.10.4 89. We used two mitochondrial segments (808 bp). To avoid sample size bias, we selected only one individual per clade from a given locality but excluded intermediate mitotypes, which reduced the data set to 34 individuals, with \( H. miles \) as an outgroup. We used default settings, applied the same molecular evolution model as presented above, and used an uncorrelated relaxed clock model assuming lognormal rate distribution. We assigned each sequence to one of the seven geographic regions, and the symmetric exchanges between the geographic regions throughout the entire phylogeny were modeled with the Bayesian stochastic search variable selection (BSSVS). MCMC runs were performed for 50 million generations, sampling one tree every 25,000 generations. After confirming the stationarity of parameter estimates using TRACER, the first 40% of trees were discarded as burn-in, and maximum clade credibility (MCC) tree was built using TREEANNOTATOR v1.10.4. As each node in each MCMC sample is annotated with a geographic region and Wolbachia infection state, we assessed the certainty of the geographic reconstruction by looking at the distribution of node states across the MCMC using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Bayes factor (BF) values for exchange rates between each pairwise regions were retrieved from the log file from the BSSVS analysis using SPREAD3 v0.9.7.1rc 82.

**Data availability**

GenBank accessions KX372573–372592, 372596–372619 and MW 393902–393920 for COI-tRNALEU-C0II, KX372620–372639, 372643–372666 and MW393921–393939 for Cyt b-tRNAser-ND1, KX372667 and MW383443–383462 for 28S, MW389094–389112 and 392102 for EF-1a, and MW389113–389118 for ftsZ, coxA and rcpA.

Received: 30 August 2020; Accepted: 15 April 2021

Published online: 06 May 2021

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Acknowledgements
We thank Łukasz Kajtoch for information about the collection site in Poland, Tamás Németh and Zoltán György for guidance at the Hungarian Natural History Museum, Kumiko Kagoshima for assistance with PCR, and Anahi Espíndola for advice on phylogeographic methods.

Author contributions
M.T., S.I., J.H. and J.S. conceived the idea, M.T., S.I. and JH. conducted molecular experiments, J.S., A.P., O.M., N.T., E.S., A.H.E. and K.M. performed morphological identification, M.T. and K.K. analyzed the data and M.T., J.H. and K.K. led the writing.

Funding
Funding was provided by Japan Society for the Promotion of Science (Grant Nos. JP23405008, JP25430194, JP26304016, JP17H04612, JP18H02207 and JP19K06840), Kyushu University (Interdisciplinary Programs in Education and Projects in Research Development (25412)) and Ministerstvo Zemědělství (Grant No. RO0418).

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-88770-y.

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