Phylogenetic placement and evidence for horizontal transfer of *Wolbachia* in *Plutella xylostella* (Lepidoptera: Plutellidae) and its parasitoid, *Diadegma insulare* (Hymenoptera: Ichneumonidae)

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Abstract—The diamondback moth, *Plutella xylostella* (L.), is a global pest of cruciferous crops (Brassicaceae). It has developed resistance to virtually all known insecticides, and biological control has become an important management tool. In North America the parasitoid *Diadegma insulare* (Cresson) has been used successfully to reduce diamondback moth populations. We document the presence of the α-proteobacterial endosymbiont *Wolbachia* and its associated bacteriophage WO in *P. xylostella* and *D. insulare* and examine the phylogenetic relationships of *Wolbachia* and WO in both host species. Our results suggest that *Wolbachia* and WO have been horizontally transferred in this insect−parasitoid system in recent evolutionary history. Knowledge of the dynamics of *Wolbachia* in *P. xylostella* and *D. insulare* may be an important factor in future control of this pest in the field.

Résumé—La fausse teigne des crucifères, *Plutella xylostella* (L.), est un ravageur des récoltes de crucifères (Brassicaceae) à l'échelle de la planète. Elle a développé une résistance à presque tous les insecticides connus et la lutte biologique est un outil important pour sa gestion. En Amérique du Nord, le parasitoïde *Diadegma insulare* (Cresson) a servi à réduire avec succès les populations de la fausse teigne des crucifères. Nous démontrons la présence d'un *Wolbachia* endosymbionte α-protéobactérien et de son bactériophage WO chez *P. xylostella* et *D. insulare* et nous examinons les relations phylogénétiques de *Wolbachia* et de WO chez les deux espèces d'hôtes. Nos résultats indiquent que *Wolbachia* et WO ont subi un transfert horizontal dans ce système insecte-parasitoïde durant l'histoire évolutive récente. La connaissance de la dynamique de *Wolbachia chez P. xylostella* et *D. insulare* pourrait être un facteur important dans le contrôle de ce ravageur en nature dans le futur.

[Traduit par la Rédaction]

Introduction

The mustard family, Brassicaceae, comprises approximately 340 genera and over 3350 species, including important agricultural, ornamental, and research plants (Koch *et al.* 2003). The most economically important mustards are species of *Brassica* L., including

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various vegetable and oilseed crops (Koch *et al.* 2003). Numerous insect pests can attack all above- and below-ground portions of *Brassica* crops (Lamb 1989); diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), outbreaks can lead to almost complete crop loss. DBM is common throughout the United States of America, and infestations in Canada are caused by DBM transported by wind from the southern States (Smith and Sears 1982).

Many growers rely on multiple insecticide applications to control infestations of DBM (Sarfraz *et al.* 2006) and this has caused it to become resistant to virtually all known insecticides (Sarfraz and Keddie 2005). Even with newer insecticides such as spinosad and indoxacarb, a few years of extensive application has led to resistance in certain DBM populations (Zhao *et al.* 2006). Because insecticides adversely affect the natural enemies of DBM, infestations often cannot be successfully managed (Sarfraz *et al.* 2005). Pesticide resistance has made the use of parasitoids essential to sustainable management of DBM populations.

Some species of the parasitoid genus *Diadegma* Förster (Hymenoptera: Ichneumonidae) are used for biological control of DBM. One species found in Canada is the larval endoparasitoid *Diadegma insulare* (Cresson) (Sarfraz *et al.* 2005, 2006). This parasitoid apparently does not overwinter in temperate North America but likely disperses northward along with its hosts (Putnam 1978; Dosdall *et al.* 2004).

The α-proteobacteria known as *Wolbachia* are maternally transmitted endosymbionts estimated to infect 20%–76% of all insect species (Jeyaprakash and Hoy 2000; Werren and Windsor 2000; Floate *et al.* 2006; Hilgenboecker *et al.* 2008). In some arthropods *Wolbachia* is capable of inducing a number of reproductive alterations that enhance the vertical transmission of *Wolbachia* in host populations (Stouthamer *et al.* 1999). Reproductive phenotypes induced by *Wolbachia* in arthropod hosts include cytoplasmic incompatibility, parthenogenesis, feminization, and male-killing (Werren 1997). The exact genetic basis of these phenotypes is unknown; however, the presence of genes encoded by extra chromosomal elements, such as a bacteriophage, determines the virulence of many other bacterial pathogens (reviewed in Canchaya *et al.* 2003). To date no plasmids have been identified in *Wolbachia* strains; however, the bacteriophage WO is present in multiple copies (Masui *et al.* 2000). Its role in *Wolbachia*-induced reproductive phenotypes has not been determined (Bordenstein and Wernegreen 2004; Sinkins *et al.* 2005). Based on a survey of 39 lineages of *Wolbachia*, the WO bacteriophage is estimated to infect up to 90% of *Wolbachia* strains. It plays an active role in the evolution of the *Wolbachia* genome and undergoes frequent horizontal transfer between *Wolbachia* in different hosts (Bordenstein and Wernegreen 2004).

Wolbachia is a diverse genus currently subdivided into 10 supergroups, of which supergroups A and B are found exclusively in arthropods (Ros *et al.* 2009). Comparison of the phylogenies of *Wolbachia* with that of their hosts show little congruence, suggesting either that *Wolbachia* can be horizontally transferred between arthropod species (Werren *et al.* 1995; West *et al.* 1998) or that genetic recombination is occurring (Bordenstein and Wernegreen 2004; Baldo *et al.* 2005, 2006). Possible routes for *Wolbachia* transfer include contact after injury (Rigaud and Juchault 1995) or through plants (Sintupachee *et al.* 2006) or parasitoids (Werren *et al.* 1995; Vavre *et al.* 1999).

Recently *Wolbachia* has been shown to provide resistance to RNA viruses, but not to DNA viruses, in *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Hedges *et al.* 2008; Teixeira *et al.* 2008); this may enhance the occurrence of this symbiont, already widespread in wild populations of *Drosophila* Fallen. No evidence exists for this effect in DBM or *Diadegma*. However, if the same strain of *Wolbachia* exists in the host and the parasitoid as a result of horizontal transmission, and this strain provides resistance to RNA viruses, then DBM and *Diadegma* may gain the same benefit.

In this study we investigated (*i*) the phylogeny of *Wolbachia* found in Alberta populations of DBM and its parasitoid, *D. insulare*, using two *Wolbachia* housekeeping genes,

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groEl (heat-shock protein HSP60) and *ftsZ* (cell-cycle gene), and (*ii*) the phylogeny of the WO bacteriophage, using the minor capsid protein gene *orf7* (Gavotte *et al.* 2007). The selected housekeeping genes are reliable for use in phylogenetic analysis because there is little or no detectable recombination within them (Ros *et al.* 2009).

Materials and methods

DBM and *D. insulare* were collected in commercial canola fields near Lethbridge in southern Alberta (49°38′N, 112°48′W). Specimens were maintained as laboratory colonies on potted *Brassica napus* L. cv. Q2 in cages (base 40.5 cm × 40.5 cm, height 80.5 cm, lined on the sides and top with 500 μm mesh plastic screening) at 20 ± 2 °C and under natural light.

Genomic DNA was extracted from whole insects using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma–Aldrich, Oakville, Ontario). The supergroup-A-specific primers *wsp*136F/*wsp*691r and supergroup-B-specific primers *wsp*81f/*wsp*522r (Braig *et al.* 1998, Zhou *et al.* 1998) were used initially to detect the presence of *Wolbachia* and determine its supergroup. Colonies negative for *Wolbachia* were eliminated from further analysis; positive colonies were maintained for over a year before the phylogenetic analysis was conducted. The presence of the WO phage was tested with the *orf7* gene using the primers WOF and WOR (Masui *et al.* 2000). The *Wolbachia ftsZ* primers (Lo *et al.* 2002) and supergroup-B-specific *groEL* primers (Baldo *et al.* 2006) were used to amplify their respective regions. The polymerase chain reaction (PCR) products *groEL*, *ftsZ*, and *orf7* were cloned into the pCR4-TOPO vector and inserted into One Shot TOP10-competent cells (TOPO TA Cloning Kit for Sequencing, Invitrogen, Carlsbad, California). Three clones of every gene were sequenced using a LI-COR 4300 DNA analyzer (LI-COR, Lincoln, Nebraska) with labeled primers and Thermo Sequenase labeled primer cycle sequencing kit (USB Corporation, Cleveland, Ohio) according to the manufacturer's instructions.

DNA sequences were added to data sets used by Holden *et al.* (1993), Werren *et al.* (1995), Masui *et al.* (1997), Casiraghi *et al.* (2005), and Baldo et al. (2006) for *groEL* and *ftsZ* and Masui *et al.* (2000, Bordenstein and Wernegreen (2004), and Gavotte *et al.* (2007) for *orf7*. The *groEL* and *ftsZ* sequences were concatenated for each species prior to alignment. Sequences were aligned with ClustalW and modified with BioEdit version 7.0.9 (Hall 1999). The resulting alignment contained the largest overlap with *Wolbachia* from *D. insulare* and *P. xylostella*. For all genes, evolutionary history was inferred and validated using the maximum-parsimony and maximum-likelihood methods, respectively. Phylogenetic trees were generated using PAUP version 4.0*b* 10 (Swofford 2000). Modeltest version 3.7 and Akaike's information criterion were used to select a DNA substitution model for each data set prior to maximum-likelihood analysis: *orf7* (TVM+G) and concatenated *ftsZ* and *groEL* (GTR+G). (Posada and Crandall 1998). Maximum-likelihood heuristic searches were conducted with 100 random additions and tree bisection and reconnection branch swapping. Bootstrap values were calculated from 100 replicates using maximum-parsimony and maximum-likelihood methods with 10 random taxon-addition replicates. Both trees generated are midpoint-rooted.

Results

PCR using *wsp*, *ftsZ*, *groEL*, and *orf7* primers showed that *D. insulare* and *P. xylostella* were each infected with a single *Wolbachia* strain and its WO phage. Phylogenetic analysis was performed to determine the evolutionary relationship of *Wolbachia* between DBM and *D. insulare*. Using maximum-parsimony and maximum-likelihood phylogenetic trees based on the concatenated alignment of the housekeeping genes *ftsZ* and *groEL* from *Wolbachia* in DBM and *D. insulare*, a single strain was detected in supergroup B (Fig. 1).

The bacteriophage WO detected in this study is closely related to that found in *Wolbachia* in the parasitoid *Trichogramma kaykai* Pinto and Stouthamer (Hymenoptera:

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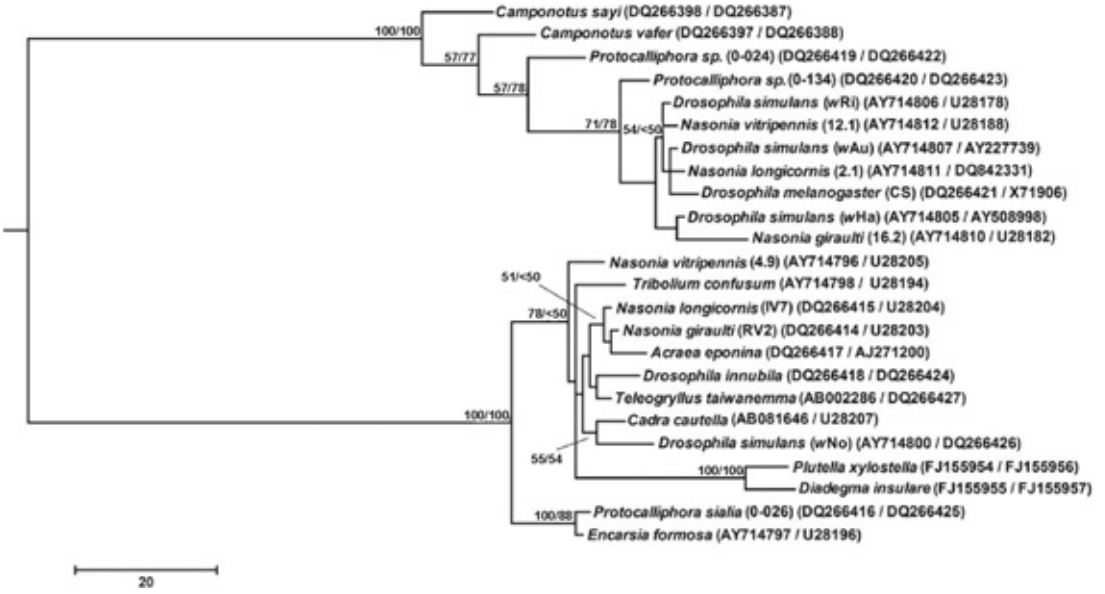
Fig. 1. Phylogenetic tree of *Wolbachia* based on the concatenated alignment of *groEL* and *ftsZ*. The name of the host arthropod species is followed in order by the accession numbers of *groEL* and *ftsZ*. The consensus tree was generated by means of maximum parsimony heuristic searches; bootstrap values for nodes are listed for maximum-parsimony and maximum-likelihood analyses.



Trichogrammatidae) and its host *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) (Fig. 2). Our results show that the WO phage strains in DBM and *D. insulare* are 98% identical, with only three amino acid differences between the WO *orf7* translated product at positions 27 (Asp in DBM, Glu in *D. insulare*), 98 (Tyr in DBM, His in *D. insulare*), and 130 (Gly in DBM, Ser in *D. insulare*), suggesting that the presence of this phage may have been due to horizontal bacterial transfer between DBM and *D. insulare*.

Discussion

This study provides evidence that a single *Wolbachia* strain in DBM and *D. insulare* resulted from horizontal transfer between host and parasitoid in recent evolutionary history. Previous phylogenetic studies have shown that there is often a lack of congruence between the phylogenies of *Wolbachia* and its hosts (Werren *et al.* 1995). Based on gene sequences, related strains of *Wolbachia* are found among unrelated insects, which suggests frequent horizontal transmission (Van Meer *et al.* 1999; Vavre *et al.* 1999; Werren *et al.* 1995). One mechanism for this transmission may be host–parasitoid associations. Van Meer *et al.* (1999), Vavre *et al.* (1999), and Gavotte *et al.* (2007) provide strong arguments for horizontal transfer, based on the phylogenetic relationships of *Wolbachia* strains in hymenopteran parasitoids with their hosts. Van Meer *et al.* (1999) found evidence for horizontal transmission between the egg parasitoids *T. kaykai* and *Trichogramma bourarachae* Pintureau and Babault and their host, *E. kuehniella.* Five instances of possible horizontal transmission were found by Vavre *et al.* (1999), including transfer between the larval parasitoids *Leptopilina heterotoma* (Thomson) (Hymenoptera: Eucoilidae) and *Asobara tabida* (Nees) (Hymenoptera: Braconidae) and their respective hosts, *Drosophila simulans* Sturtevant and *D. melanogaster*. Vavre *et al.* (1999) also provided evidence for horizontal transfer between two different hymenopteran parasitoids, possibly through hyper- or multiparasitism, such as between two variants

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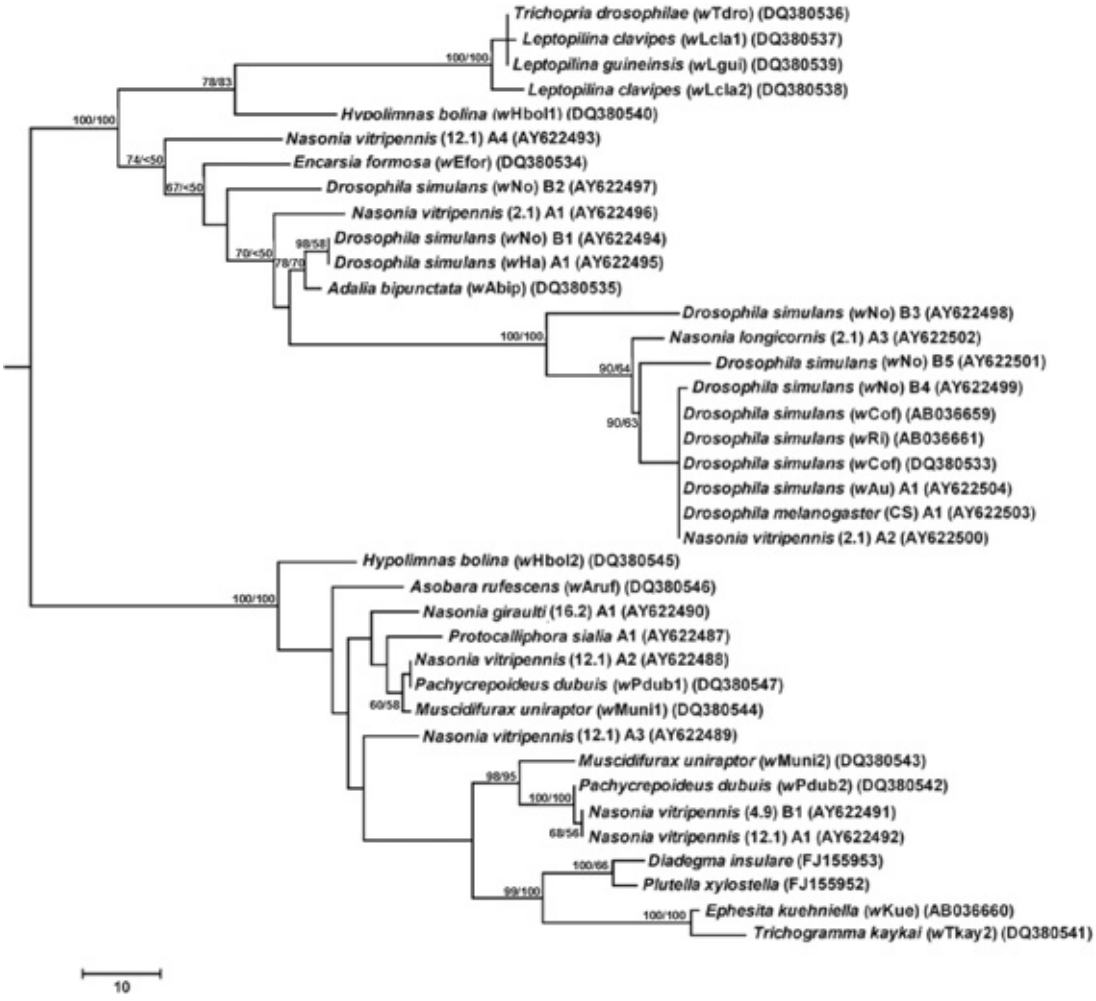
Fig. 2. Phylogenetic tree of the bacteriophage WO based on *orf7*. The name of the host arthropod species is followed by the accession number. The consensus tree was generated by means of maximum parsimony heuristic searches; bootstrap values for nodes are listed for maximum-parsimony and maximum-likelihood analyses.



of *Trichopria* Ashmead (Diapriidae) and *L. heterotoma*, between the pupal parasitoid *Trichopria drosophilae* (Perkins) and *A. tabida*, and between *Muscidifurax uniraptor* (Kogan and Legner) and *Pachycrepoideus vindemmiae* (Rondani) (Pteromalidae). Although the bacteriophage WO may be horizontally transferred without *Wolbachia*, there are cases (*e.g.*, between *T. kaykai* and *E. kuehniella* and between *M. uniraptor* and *Pachycrepoideus dubius*), where *Wolbachia* and its associated phage are transferred together (Gavotte *et al.* 2007). The close phylogenetic relationship between *Wolbachia* and its phage infection in DBM and *D. insulare* suggests that they were transferred together between the two insect hosts.

Wolbachia has previously been detected in DBM in the United States of America using the sensitive long PCR technique. Phylogenetic analysis based on *wsp* sequence data demonstrated that DBM had two strains of *Wolbachia*, *w*Sus-A1 and *w*Xyl-B1, belonging to the *w*Mel and *w*Con group in supergroups

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A and B, respectively (Jeyaprakash and Hoy 2000). More recently, Delgado *et al.* (2009) found three *Wolbachia* strains in DBM outside North America. Sequences from the dominant strain *plutWB1*, found in Africa and Asia, were about 17% divergent from sequences from the *w*Xyl-B1 strain. In our study, the only one to report on a Canadian DBM population, a single *Wolbachia* infection belonging to supergroup B was found. The *wsp* sequence data from our DBM population are the same as the *wsp* sequence in *plutWB1* (<1% sequence divergence) (unpublished data). The difference in our population's *wsp* sequence and those previously described from the United States of America may indicate multiple colonization events by DBM in North America. Delgado and Cook (2009) described sex-ratio distortion resulting from the presence of *plutWB1*. This was not investigated in our study.

Wolbachia inhibits RNA virus infection but not DNA virus infection in *D. melanogaster* (Hedges *et al.* 2008; Teixeira *et al.* 2008). *Wolbachia* has also been shown to modify the host immune response. In the alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae), rates of encapsulation of eggs of the larval parasitoid *Bathyplectes curculionis* (Thomson) (Hymenoptera: Ichneumonidae) are higher when *Wolbachia* is present than when it is absent. In the same host, *Wolbachia* also influences the success rate of the adult parasitoid *Microctonus aethiopoides* Loan (Hymenoptera: Braconidae). Parasitoids develop normally in weevils without *Wolbachia* but are less successful in weevils with *Wolbachia* (Hsiao 1996). In a study where the host *D. simulans* and the parasitoid *L. heterotoma* were both infected with *Wolbachia*, both showed adverse, immunity-related effects (Fytrou *et al.* 2006). Encapsulation of parasitoid eggs in the infected host is reduced, and the presence of *Wolbachia* in the parasitoid alone also reduced its success, but the mechanisms of these effects have not been determined (Fytrou *et al.* 2006). Because virus replication in an insect host is suppressed by *Wolbachia*, it is tempting to suggest that *Wolbachia* is interfering with virus replication in the parasitoid. Reduction of the parasitoid virus (polydnavirus) may make eggs in an insect host more susceptible to encapsulation. Further study is required to elucidate the role of the *Wolbachia* WO virus in parasitization of DBM.

We have identified a single strain of *Wolbachia* and the bacteriophage WO in *P. xylostella* and *D. insulare*. This complex relationship among viruses, bacteria, parasitoids, and hosts requires further study. Knowledge about this interaction may permit the development of effective pest-management strategies. Future investigation of *Wolbachia* dynamics may be an important factor in the control of DBM in the field and the use of *Wolbachia* as a vector for genetic transformation.

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