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Presence of *Wolbachia* in Three Hymenopteran Species: *Diprion pini* (Hymenoptera: Diprionidae), *Neodiprion sertifer* (Hymenoptera: Diprionidae), and *Dahlbominus fuscipennis* (Hymenoptera: Eulophidae)

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ABSTRACT. Sawflies are important pests of various plant species. *Diprion pini* (L.) and *Neodiprion sertifer* (Geoffroy) (Hymenoptera: Diprionidae) are two of the most important sawfly pests in Italy, and both species are parasitized by the hymenopteran parasitoid *Dahlbominus fuscipennis* (Zetterstedt). Bacterial endosymbionts are currently studied for their high potential in strategies of biocontrol in a number of insect species. In this study, we investigated the presence of symbiotic bacteria (*Wolbachia* and *Cardinium*) in the three species of hymenoptera mentioned earlier, both in wild and laboratory populations. Although all samples were negative for the presence of *Cardinium*, 100% prevalence for *Wolbachia* was detected, as all examined individuals resulted to be PCR positive. Furthermore, 16S rDNA and *ftsZ* gene sequencing indicated that all individuals from the three hymenopteran species are infected by a single *Wolbachia* strain. Additionally, we report the presence of gynandromorphic individuals in *D. pini*, both in wild and laboratory-reared populations. Heat treatments on *D. pini* colonies removed the *Wolbachia* symbionts, but they also prevented the development of adults.

Key Words: endosymbiont, gynandromorphism, molecular screening, diprionid wasp

Phytophagous insects represent a major threat for worldwide forests by causing direct damage, consuming leaves or other parts of the host, and also by vectoring micro-organisms that can trigger diseases in plants (Ayres and Lombardero 2000, Hulcr and Dunn 2011). Adults of *Diprion pini* (L.) (Hymenoptera: Diprionidae) feed preferentially on leaves of plants of the genus *Pinus*. The areal of distribution of this species covers the majority of continental Europe, in all areas where the host plants are present. In Italy, *D. pini* is frequently encountered in alpine environments (Pornbacher 1992), but it has also been reported in the Etna area in Sicily (Turrisi and Bella 1999). The species *Neodiprion sertifer* (Geoffroy) (Hymenoptera: Diprionidae) also occurs in a variety of habitats, on pine host plants from northern and central Europe, as well as in Asia and North America (Olofsson 1987). The larvae of both sawfly species are massive defoliators that can cause important damage to the plants they feed on, also affecting the esthetic value in ornamental tree plantations. In both species, the adult females lay eggs inside the needles of host plants, and the damage is determined by trophic activity of the larva consuming the leaves (Augustaitis 2007). Besides, as the larvae mature, their feeding becomes more extensive and needles are stripped. Complete defoliation of the trees may occur, affecting the growth of the parasitized plants. The larvae, however, do not feed on new grown leaves, so the trees usually survive (Kurkela et al. 2005). Periodically, sawfly populations may increase to outbreak proportion, and reduction in plant increment and tree mortality can be registered over widespread areas (Coppel and Benjamin 1965). *Dahlbominus fuscipennis* (Zetterstedt) (Hymenoptera: Eulophidae) is a parasitoid of many sawfly species, including *D. pini* and *N. sertifer*, and it is largely reared and released for pest control purposes. Females of *Da. fuscipennis* lay eggs in the larvae of diprionid cocoons. Several eggs are laid with a single insertion of the ovipositor, and up to 40 parasitoid larvae can develop in one cocoon. The winter is spent as a full-grown larva or pupa within the host cocoon.

Haplodiploid sex determination, in which females develop from fertilized diploid eggs and males develop from unfertilized haploid eggs, is the most frequent and presumably ancestral mechanism of sex determination in the insect order Hymenoptera (Heimpel and de Boer 2008). Such is the case of Diprionidae, including *D. pini* and *N. sertifer*, where sexual chromosomes are absent and sex is determined by ploidy of the newborn (Rousselet et al. 1998). Males are aploid (e.g., $n = 14$ in *D. pini*) while females are diploid (e.g., $2n = 28$ in *D. pini*). Gynandromorphism is an anomaly affecting sexual and somatic characters in different tissues of an individual. An abnormal chimeric organism presenting a mixture of male and female physical features is defined a gynandromorph (Narita et al. 2010). The occurrence of gynandromorphic individuals was previously reported in *D. pini* (Beaudoin et al. 1994).

Micro-organisms of the genus *Wolbachia* are gram-negative α-proteobacteria belonging to the order Rickettsiales, family Anaplasmataceae. *Wolbachia* is an obligated intracellular bacterium infecting a wide range of hosts, from arthropods to filarial nematodes (Bandi et al. 2001). Vertical maternal inheritance is considered the primary route of *Wolbachia* transmission from one host generation to another. The role of *Wolbachia* in the host is also variable; in fact, these bacteria can behave as mutualists, pathogens, or even reproductive parasites. *Wolbachia* bacteria can exploit all known mechanisms of reproductive parasitism, manipulating the host reproduction with different strategies, including parthenogenesis, male killing, cytoplasmic incompatibility, and feminization of genetic males (Werren et al. 2008). In general, *Wolbachia* bacteria are strictly associated with the gonads but might also be present in different somatic tissues (Dobson et al. 1999). *Cardinium*, a recently described bacterial genus of maternally acquired

Table 1. Number of male, female, and gynandromorph *D. pini* individuals for each of the three laboratory colonies for each generation

Colony	Laboratory line 1 (Dpini1)			Laboratory line 2 (Dipini2)			Laboratory line 3 (1×2) (Dpini3)		
Sex	M	F	Gyn	M	F	Gyn	M	F	Gyn
Generation 0	168 (2)	111 (4)	21 (2)	19	20	3	4 (2)	11 (2)	1
Generation I	143	10	14	68 (2)	35 (4)	12 (2)	1	1	1
Generation II	13	15	3	20	9	1	1	1	4
Generation III	11	9	1				7	10	10
Generation IV	44 (4)	52 (2)	7 (2)				19 (2)	84 (2)	8
Generation V	18	45	5				31	36	51
Generation VI	14	4	2				1	2	0
Generation VII	52 (4)	10 (4)	7 (2)				83 (4)	33 (2)	26 (2)
Total Gyn/total individual			60/779			16/187			101/426

Gyn, gynandromorph; F, female; M, male.

endosymbionts, widespread in spiders, arachnids, and other arthropod groups (Martin and Goodacre 2009), is associated with reproductive manipulations such as cytoplasmic incompatibility, parthenogenesis, and feminization in different arthropod species (Wu and Hoy 2012).

Investigations on the presence of endosymbionts in pests represent the first step in developing a potential strategy for biocontrol purposes. For example, a study focused on the agent of Pierce disease in grapevines, the glassy-winged sharpshooter, showed that the spread of the disease could be controlled by an antagonistic bacterium that resides in the same insect vector (Bextine et al. 2004, Newman et al. 2004). On the other hand, the discovery of *Wolbachia* in filarial nematodes led to the development of antisymbiotic therapy to cure filarial diseases (Taylor et al. 2000). In general, considering that the wide interactions between the host and the associated microbiota are a fundamental aspect of insect biology (Weiss et al. 2012), investigations on the role of bacterial symbionts are allowing the development of novel strategies to control pest and parasitic arthropods and to reduce their vector competence.

In this study, we collected and reared individuals of two sawfly species (*D. pini* and *N. sertifer*) and of their parasitoid *Da. fuscipennis* (Hymenoptera: Eulophide). We then investigated the presence and the potential role of two important reproductive manipulator bacteria (*Wolbachia* and *Cardinium*) in these three hymenopteran species. Besides, in accordance with a potential implication of endosymbionts in the occurrence of gynandromorphism (Stouthamer et al. 1997), we also investigated the occurrence of this phenotype in *D. pini* in the new light of the presence of endosymbiotic bacteria.

Materials and Methods

Larvae of *D. pini* and *N. sertifer* were collected in northern Italy. In detail, a total of 300 larvae of *D. pini* were collected on *Pinus sylvestris* plants in 2007, in two sampling sites in the municipalities of San Martino in Monte (Bolzano County) and San Dorligo (Trieste county) della Valle (Trento County). These individuals were used to establish a colony that was named Dpini1. A further sampling was performed in 2007 in the municipality of Velturno (Bolzano County). In this occasion, 42 larvae of *D. pini* were sampled and used to establish a colony called Dpini2. A third colony of individuals, named Dpini1×2, was created by the mating of two females of the Dpini2 line, one male of Dpini1, and one male of Dpini2; these four individuals derived from the first generation of each population. Additionally, 35 *N. sertifer* cocoons were collected in Ozzano dell'Emilia (Bologna County) in 2007, and four of them resulted to be parasitized by *Da. fuscipennis.*

The rearing was performed as follows: *D. pini* larvae from the third to the last instar (fourth instar for males and fifth for females) and *N. sertifer* cocoons were placed in an artificial cell at a constant temperature of 20°C, with a relative humidity of 80%, and a photoperiod of 17 h on white neon light (Philips TL-D master 58W/84, Philips, Amsterdam, Netherlands). After measuring the development time of the insects at 20°C, we studied the effects of higher temperatures on development. Two heat treatment experiments were performed each on 15 *D. pini* by increasing the rearing temperature. The first experiment consisted of a stable increase in the rearing temperature to 25°C, while the second consisted of an increase to 30°C for three intervals of 24 h separated by 48 h at 20°C. The hymenopteran parasitoid *Da. fuscipennis* was reared only on *N. sertifer*, at the conditions described earlier.

In total, 30 individuals of *N. sertifer* (17 males and 13 females), 30 of *Da. fuscipennis* (11 males and 19 females), and 50 individuals of *D. pini* (20 females, 20 males, and 10 gynandromorphs) were subjected to molecular screening for the presence of symbionts (see Table 1 for details). All individuals were stored in absolute ethanol, then washed in sterile water, homogenized with a sterile pestle, and processed for DNA extraction using a commercial kit (DNeasy Blood & Tissue kit, Qiagen, Hilden, Germany) following manufacturer's instructions. A molecular screening was performed on the extracted DNAs to investigate the presence of endosymbiotic bacteria belonging to the genera *Wolbachia* and *Cardinium*. Three specific Polymerase Chain Reaction (PCR) protocols, targeting two different *Wolbachia* genes (16s rDNA and *ftsZ*) and one *Cardinium* gene (16s rDNA), respectively, were applied, using previously published assays (Casiraghi et al. 2001, 2005; Marzorati et al. 2006). A subset of the *Wolbachia*-related PCR products (1,393 and 735 bp, respectively) was sequenced using Applied Biosystems (ABI) technology. Phylogenetic analysis was performed on the obtained sequences, using an in-house pipeline comprising MUSCLE (Edgar 2004), Gblocks (Castresana 2000), and PhyML (Guindon et al. 2010).

In *D. pini*, the sexual dimorphism is strong, and the marked differences between males and females allow the identification of gynandromorphic individuals in the population and a detailed description of the distribution of male and female tissues on the soma of abnormal specimens. In the laboratory-reared *D. pini* colonies, gynandromorphic individuals were described and the distribution of male and female tissues in these individuals was reported. In each individual, the male and female areas can be distributed in a strikingly bilateral way, patchily or uniformly mixed. In particular, we annotated the frequency of somatic alterations in each body segment (head, thorax, abdomen, and external genitalia) in these individuals. The distribution of the three tissue types is described with different colors: male (black), female (white), and mosaic (gray; see Fig. 1 for an example pattern).

We applied a Pearson's chi-square test to evaluate any significant differences between the frequencies of gynandromorphic individuals in the three different *D. pini* populations. The test was applied between the frequency of gynandromorphs and normal individuals, respectively, in Dpini1 and Dpini2 and between each of the two populations and the inbreed line Dpini1×2 (2 × 2 contingency table, 1 df).

Results

We investigated the presence of two insect symbionts, *Wolbachia* and *Cardinium*, in the three different *D. pini* populations. *Cardinium* was never detected in any of the sampled individuals, whereas all *D. pini* samples investigated resulted positive to *Wolbachia*. DNA

samples from a population of *N. sertifer* and *Da. fuscipennis* were also tested for the presence of two endosymbionts, and both resulted positive to *Wolbachia* with 100% prevalence and negative to *Cardinium*. See Table 1 for a detailed description of the examined individuals, sexes, and rearing generations.

Partial 16s rDNA and *ftsZ Wolbachia* gene sequences were generated from individuals belonging to the three species investigated in this study (*D. pini*, *N. sertifer*, and *Da. fuscipennis*). For each gene, all the obtained sequences showed 100% identity between them, excluding artifacts, indicating that we detected a single *Wolbachia* strain that presents a high capacity of colonizing the three examined hymenopteran species. Gene sequences were submitted to the National Center for Biotechnology Information (NCBI) database under the accession numbers HE814622 (978 bp) and HE814623 (690 bp), respectively, for 16S rDNA and *ftsZ*. Phylogenetic analyses were performed aligning the obtained *ftsZ* gene sequence with the homologous sequences of 21 *Wolbachia* strains retrieved from the database. The resulting tree (Fig. 2) shows that the novel strain belongs to the A *Wolbachia* supergroup (Casiraghi et al. 2005) and that it is most closely related with two other Wolbachia strains, one infecting a member of Diptera (*Drosophila borealis*) and one infecting a member of Hymenoptera (*Technomyrex albipes*).

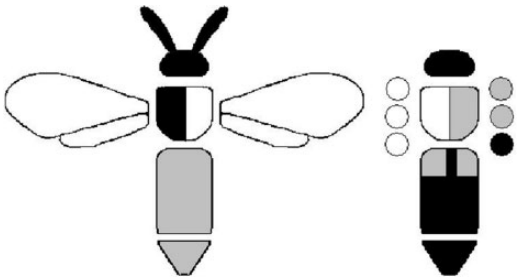

Fig. 1. Dorsal and ventral vision representing the tissue distribution of a gynandromorphic individual: male (black), female (white), and gynandromorph (gray). This specific pattern is an example of an individual belonging to the Dpini1×2.

We performed two heat treatments on the colony of *D. pini* to investigate the effect of temperature increase on the presence of *Wolbachia*. In fact, it has been shown in the literature that an increase in temperature can be an effective way to "cure" *Wolbachia* infection (van Opijnen and Breeuwer 1999, Sakamoto et al. 2008). Additionally, it must be considered that to complete the *D. pini* biological cycle under standard laboratory experimental conditions (Bombosch and Ramakers 1976, Eichhorn et al. 1976, Köpke et al. 2010), an average time of 60 d is required (16-d embryonic, 28-d larval, and 16-d pupal developments). We hypothesized that a temperature increase could reduce this period. For the first treatment, the temperature was set up at 25°C, resulting in a complete biological cycle of 48 d instead of 60 d, but with a great reduction of success in egg hatching. The second heat treatment (30°C) led to interruption of development of the *D. pini* larvae. In fact, none of the larvae subjected to the treatment were able to molt into adults. Molecular screening on these larvae revealed that *Wolbachia* had been eliminated (all 11 tested larvae were negative to both *Wolbachia*-specific PCRs). Further studies should be carried out to establish whether these effects are due to the absence of *Wolbachia* symbionts or simply to the temperature increase.

To study the effects of inbreeding on production of gynandromorphs, three different populations of *D. pini* were followed under controlled laboratory conditions for up to seven generations. We succeeded in maintaining the two field-derived colonies (Dpini1 and Dpini2), respectively, for seven and three generations, whereas the third colony (Dpini1×2), obtained from a cross between individuals of the two colonies, was more successful and was interrupted after seven generations, while still actively reproducing. In all experimental populations, we observed the appearance of gynandromorphic individuals. The total number of gynandromorphs was 177 of 1,392 individuals (mean frequency, 0.12): 60/719 in Dpini1 (mean frequency, 0.08), 16/187 in Dpini2 (mean frequency, 0.08), and 101/426 in Dpini1×2 (mean frequency, 0.23). In the laboratory-interbred population of *D. pini* (Dpini1×2), we report an increase in the frequency of gynandromorphism. Indeed, we

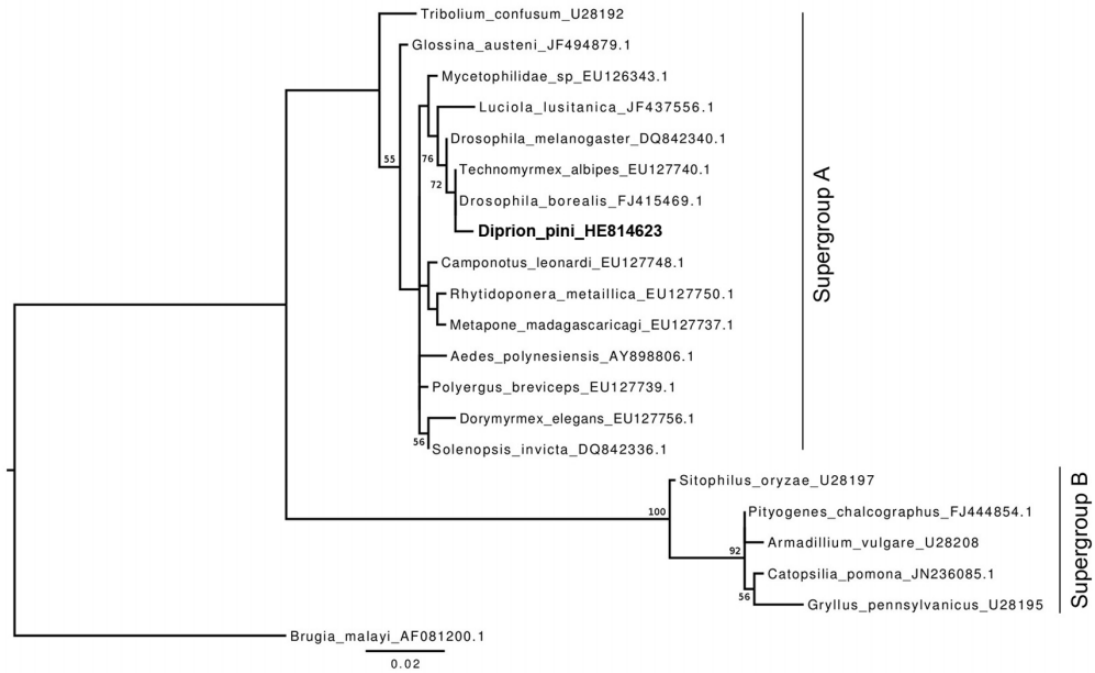

Fig. 2. Phylogenetic analysis based on a 437-bp portion of the *ftsZ Wolbachia* gene. The alignment was analyzed with PhyML. Bootstrap support values above 50 are indicated above each node. The name of the host species and the sequence accession number are indicated on each branch.

registered a statistically significant difference in the frequency of gynandromorphs within the population of the line Dpini1×2 compared with both Dpini1 ($\chi^2 = 60.956$; 2 × 2 contingency table; 1 df; two-tailed $P < 0.0001$) and Dpini2 ($\chi^2 = 19.321$; 2 × 2 contingency table; 1 df; two-tailed $P < 0.0001$), whereas there is no significant difference in the frequencies of gynandromorphs between the lines Dpini1 and Dpini2 ($\chi^2 = 0.152$, 1 df, two-tailed $P = 0.6969$). In the majority of gynandromorphic individuals (90.3%), we reported a complex pattern of external male, female, or gynandromorphic tissues distributed in more than one body district (see Fig. 1 for an example pattern). Worthy of note, external genitalia were frequently affected by gynandromorphism (61/177 = 34.5%).

Discussion

We investigated the presence of *Wolbachia* and *Cardinium* symbionts in *D. pini*, *N. sertifer*, and *Da. fuscipennis* by molecular methods. Our results indicate the presence of *Wolbachia* in all examined individuals of all three hymenopteran species, whereas *Cardinium* was not found in any of the examined samples. Although 100% prevalence of *Wolbachia* in individuals collected in the wild suggests a complete pervasiveness of the symbiont in the examined species, additional sampling from different geographical sites would be required to allow a definitive conclusion on this issue. The second part of the study was focused on *D. pini* to investigate the occurrence of gynandromorphism in wild and laboratory populations. Finally, heat treatments were performed in *D. pini* to try to evaluate whether removal of the *Wolbachia* symbionts was possible and whether this would have an effect on the host, particularly in terms of frequency of occurrence of gynandromorph individuals. We were not able to link the presence of gynandromorphic individuals in *D. pini* to the presence of *Wolbachia* because these bacteria are always present in all investigated individuals (i.e., males, females, and gynandromorphs).

The lack of genetic variability between bacteria of the genus *Wolbachia* recorded in *D. pini*, *N. sertifer*, and *Da. fuscipennis* suggests a horizontal transfer event between these three species. Because *Da. fuscipennis* were reared on *N. sertifer* in our laboratory, the acquisition of *Wolbachia* in the parasitoid could also be explained through an event of symbiont transfer between these two species. We highlight that both *N. sertifer* and *Da. fuscipennis* were reared separately from *D. pini* populations, so we can exclude the occurrence of horizontal transfer between the laboratory populations. Nevertheless, horizontal *Wolbachia* transmission from infected to uninfected individuals within (intraspecific) and between (interspecific) host species has been previously observed both in the laboratory and over evolutionary timescales (Cordaux et al. 2001, 2012; Huigens et al. 2004; Viljakainen et al. 2008). Horizontal transmission allows *Wolbachia* to infect several host species and contributes to its vast host range. Interspecific horizontal transmission has been reported, e.g., in wasps of the genus *Trichogramma* (Huigens et al. 2004) and in the parasitoid wasp *Nasonia* (Raychoudhury et al. 2009). We can hypothesize that the *Wolbachia* strain detected in this study was horizontally transferred between the three analyzed hymenopteran species in the wild. It seems reasonable to hypothesize that the parasitoid behavior is involved in this pattern of transmission. The parasitoid wasp *Da. fuscipennis* could have acquired the bacteria while feeding on the caterpillar of diprionid wasps, and it could also have transmitted it to additional hosts. For this model to be acceptable, the efficiency of infestation of the parasitoid must be <100%; otherwise, all infected host individuals would be killed by the infestation. A study on 16 caterpillar species highlighted the existence of immune response mechanisms that allow the host to survive parasitoid infestations (Smilanich et al. 2009). We currently cannot conclude which was the original host of the detected *Wolbachia* strain or whether the symbiont exerts specific effects on the three hosts. *Wolbachia* infection in a parasitoid was previously reported in the uzifly, *Exorista sorbillans* (Wiedemann) (Diptera: Tachinidae), which parasitizes the silkworm, *Bombyx mori* (L.) (Lepidoptera: Bombycidae). Administration of tetracycline to the adult uziflies removed *Wolbachia* endosymbionts and resulted in several reproductive disorders. However, tetracycline treatment did not show marked effects on the life expectancy of the uzifly (Puttaraju and Prakash 2005).

Developmental defects, which occur at low frequencies under natural conditions, can lead to morphologically anomalous individuals with both male and female traits. Even though gynandromorphic individuals are described in many insect taxa (Sassaman and Fugate 1997, Narita et al. 2010), the specific mechanisms and driving forces causing this peculiar developmental abnormality are not known. In the examined populations of *D. pini*, the phenomenon of gynandromorphism occurred with a high frequency in the three populations (8% in Dpini1 and Dpini2 and 23% in Dpini1×2, respectively) with marked variability throughout different generations. Hence, random events, such as the occurrence of binucleate eggs, dispermy/polyspermy, reduction/duplication of the chromosome number, and loss of part of the genetic material, implicated in cases of gynandromorphism in natural and laboratory populations of other arthropods (Kamping et al. 2007, Narita et al. 2010, Ford 2012), unlikely explain the recurrence of this condition in *D. pini*. Environmental temperature is considered another key factor involved in gynandromorphism occurrence in arthropods (Narita et al. 2010). In fact, increase in temperature has been demonstrated as an important factor for the production of gynandromorphic individuals in *Ooencyrtus submetallicus* (Wilson 1962). In the parasitic wasp *Nasonia vitripennis*, the proportion of gynandromorphic individuals can also be increased by exposing the mother or early-stage embryos to high temperature (Kamping et al. 2007). In our work, the three different *D. pini* populations were kept for the whole duration of the main experiment at a constant temperature (20°C) inside the rearing cell. Under these persistent conditions, we could appreciate a high variability in the number of gynandromorphic individuals between the different generations. Unfortunately, our studies on the experimental increase in rearing temperature of *D. pini* were not sufficient to elucidate the role of this factor in *D. pini* because these experiments highly decreased the hatching success of the eggs and increased the larval mortality rate (25°C), and they also blocked the development of adults (30°C).

The association between the presence of *Wolbachia* and the occurrence of gynandromorphism and other developmental abnormalities has been proposed in diverse arthropod groups (Pereira et al. 2003). Intriguingly, a partial *Wolbachia* reduction using antibiotics led to the appearance of *Ostrinia scapulalis* (Walker) intersexes having exclusively male genotype (Kageyama and Traut 2004). In *Zyginidia pullula* (Hemiptera: Cicadellidae), the infection by *Wolbachia* induces feminization of genetic males. These feminized males are characterized by intersexual phenotypes, females presenting upper pygofer appendages, a typical male secondary sexual feature (Negri et al. 2008). Another example can be found in the isopod *Armadillidium vulgare*, where a conflict between feminizing sex ratio distorters and autosomal masculinizing genes can be traced to the origin of intersexes (Rigaud and Juchault 1993). In the case of *D. pini*, a possible link between the presence of *Wolbachia* and the occurrence of gynandromorphism remains an issue to be clarified with specific experiments, such as the use of antibiotics. Besides, the possible discovery of *Wolbachia*-free populations of *D. pini* could also shed light on the mechanisms leading to gynandromorphism in this species. The findings presented here spring a number of intriguing questions to be answered before a full picture of the many genetically ecological and evolutionary aspects of gynandromorphism in *D. pini* will be clarified.

The transinfection of different host species with *Wolbachia* also suggests a promising biocontrol approach. Fruit fly *Wolbachia* strains were demonstrated to be able to invade and prosper in mosquito populations, also reducing adult host lifespan, affecting host reproduction, and interfering with pathogen replication (Iturbe-Ormaetxe et al. 2011). Our work on the presence of *Wolbachia* endosymbionts in the three hymenopteran species represents an initial step for potential biocontrol applications.

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