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Potentially entomopathogenic nematode isolated from *Popillia japonica*: bioassay, molecular characterization and the associated microbiota

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Abstract

The Japanese beetle, *Popillia japonica* Newman (Coleoptera Scarabaeidae), is a highly invasive pest recently introduced in Europe. In the current study a nematode is isolated from the third larvae instar of *P. japonica* collected in northern Italy. Both BLAST search and the phylogenetic maximum likelihood tree inferred from 18S rRNA sequences confirm the attribution of the isolated nematode to the genus *Oscheius* (Nematoda Rhabditidae). The entomopathogenicity of the isolated nematode was tested on larvae of the model organism *Galleria mellonella* L. (Lepidoptera Pyralidae). The mortality of the host after five days varied from 54% to 60%, depending on nematodes concentration. Furthermore, the microbiota associated with the isolated nematode was characterized using a metabarcoding approach. Our results suggest that the bacterial community of the isolated nematode is dominated by bacteria belonging to the genus *Ochrobactrum*, that includes entomopathogenic species. Further studies are needed to test the possibility of using this nematode as a biocontrol agent of *P. japonica* in Europe.

Key words: *Oscheius myriophilus*, *Ochrobactrum*, biological control.

Introduction

The Japanese beetle, *Popillia japonica* Newman (Coleoptera Scarabaeidae), native to Japan, northern China and the far eastern Russian island of Kuril, was first reported outside its native range in the United States in 1916. Later, it has become an established pest in North America, the Azores and more recently in Europe (EPPO, https://gd.eppo.int/taxon/POPIJA/distribution). In 2014, *P. japonica* was recorded for the first time on the European mainland when an outbreak was reported within the Ticino Valley Natural Park, Italy (Pavesi, 2014; EPPO, https://gd.eppo.int/reporting/article-3272). The species is polyphagous and attacks different plants such as *Acer* spp. L., *Malus* spp. (Mill) (ornamental species), *Prunus* spp. L., *Rosa* spp. L., *Ulmus* spp. L., and *Vitis vinifera* L. (Bragard *et al*., 2018). The three larval instars attack roots causing plant mortality while adults feed on leaves, causing their skeletonization, and on the early ripening fruit (e.g., apples, peaches, nectarines), causing severe damages and affecting the quality of fruit (Bragard *et al*., 2018). In addition, *P. japonica* has an indirect impact on agriculture since in the USA it is reported as a vector of Southern bean mosaic virus and Bean pod mottle virus (Wickizer and Gergerich, 2007).

Several methods have been developed to control *P. japonica*. The main strategies involve the use of chemicals to target larval stages and adults (Morris and Grewal, 2011). More environmentally friendly strategies rely on the use of organisms like the parasitoid wasps *Tiphia vernalis* Rohwer and *Tiphia popilliavora* Rohwer, which attack overwintering larvae and newly emerged adults, or predators such as staphylinids and carabids, which attack young larvae and eggs (Potter and Held, 2002). Nematodes represent interesting alternative biocontrol agents since they have a wide host range and can quickly kill the host. In association with other biocontrol agents, nematodes are important parts of integrated pest management strategies (Grewal *et al*., 2005). Entomopathogenic nematodes (EPNs) are a group of parasitic nematodes, which have evolved an association with insect pathogenic bacteria, able to cause the death of the insect host (Kaya and Gaugler, 1993; Laznik *et al*., 2010; Dillman *et al*., 2012). The infective juvenile of the nematodes is the only free-living stage able to attack and colonize the host. Once the colonization occurred, the symbiotic bacteria [e.g. *Photorhabdus* spp. (Boemare *et al*.) and *Xenorhabdus* spp. (Thomas et Poinar)] harboured in the nematode's intestine are released into the host's haemolymph where they propagate and kill the host by septicaemia within 48 hours (Kaya and Gaugler, 1993). A variety of nematodes such as *Steinernema* spp. Travassos and *Heterorhabditis* spp. Poinar have been observed within the body cavity of *P. japonica* larvae and used as a biocontrol agent against this pest (Cappaert and Smitley, 2002). The commercial species *Heterorhabditis bacteriophora* Poinar resulted an efficient biological control agent of *P. japonica* larvae in Italy (Marianelli *et al*., 2018). Due to the economic impact of the pest, several studies searching for potential natural enemies have been carried out and are still ongoing, especially in the recently colonized regions. In the USA, species in the genus *Psammomermis* Polozhentsev has been reported as the first nematode parasitizing *P. japonica* (Klein *et al*., 1976) and recently a new species *Hexamermis popilliae* Poinar was described from *P. japonica* individuals collected in Italy (Mazza *et al*., 2017).

During a sampling campaign aiming to collect *P. japonica* individuals to study changes in the composition of the associated microbiota throughout the host developmental stages (Chouaia *et al*., 2019), an individual was found colonized by nematodes. This study aims to identify the isolated nematodes using molecular taxonomy and to evaluate its efficiency in suppressing individuals of an insect model organism. Furthermore, we characterized the microbiota associated with the nematode in order to look for potential bacterial symbionts with entomopathogenic activity.

Materials and methods

Collection of *P. japonica* individuals, nematode isolation and manipulation

Nematodes were obtained from the body cavity of a third- instar larvae specimen of *P. japonica* collected in Oleggio (45°36'N 08°38'E, 230 m a.s.l. - Novara, Piedmont, Italy) within a project that aims to characterize the microbiota associated to this invasive pest (Chouaia *et al*., 2019). The nematodes were isolated from the infected *P. japonica* larva and then reared using the last instar of the model organism *Galleria mellonella* L. (Lepidoptera Pyralidae) as host, following the method described by Kaya and Stock (1997). The parasitized *G. mellonella* cadavers were rinsed in distilled water and placed in modified White traps (White, 1927) at 24 °C ± 0.5 °C for two weeks. During that time, the emerging infective juveniles (IJs) were collected for the following experiments. A suspension of the isolated IJs in sterilized distilled water was stored at 10 °C in order to obtain individuals for the in vivo test of "mortality". A sample of the isolated nematodes was stored in absolute ethanol for DNA extraction, molecular identification and characterization of the associated microbiota.

DNA extraction and molecular identification of the nematode

After surface sterilization of about 100 nematodes following previously published protocol (Montagna *et al*., 2015; Mereghetti *et al*., 2019), total DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen). A fragment of 890 base pairs of the 18S rRNA gene was amplified using the primers 18SF2/18SR2 (Pernin *et al*., 2015), PCR amplification was performed in 25 µL reaction mix containing: 1X Taq reaction Buffer (10 mM Tris-HCl at pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$), 0.2 mM of each deoxynucleotide triphosphate, 0.5 pmol of each primer, 0.6 U of GoTaq DNA Polymerase and 10 ng of template DNA. PCR thermal profile was as following: an initial denaturation of 3 minutes at 95 °C followed by 35 cycles of 30 seconds denaturation at 95 °C, 30 seconds annealing at 52 °C and 1 minute 20 seconds extension at 72 °C, with a final single extra extension step of 10 minutes at 72 °C. The obtained amplicon was Sanger sequenced, electropherograms were manually checked and then forward and reverse were assembled in a consensus sequence using Geneious R10 (Biomatters Ltd). The consensus sequence (accession number: MN263255) was subject to BLAST search and compared with sequences available in GenBank. Homologous sequences of close relatives taxa, according to Liu *et al*. (2012), were aligned using MAFFT with Q-INS-i algorithm that consider RNA secondary structure (Katoh *et al*., 2017). The obtained aligned sequences were tested using jModelTest2 (Darriba *et al*., 2012) to select the best model of nucleotide substitutions, which resulted to be the Generalised time-reversible model (GTR) (Tavaré, 1986). The single locus phylogeny was inferred under maximum likelihood using PhyML 3.0 (Guindon *et al*., 2010) and the previously selected nucleotide substitution model, with 100 bootstrap replicates.

Entomopathogenic activity on *G. mellonella*

We tested the virulence of the nematode using the last instar larvae of greater wax moth (*G. mellonella*), following the procedure described by Torrini *et al*. (2015). Briefly, each moth larva was placed in a Petri dish (3.5 cm diameter) with two layers of filter paper (Whatman No. 1) and inoculated with nematodes at two concentrations: C_1 = 300 nematodes / 250 µl H_2O (Treatment 1: T1) and C_2 = 400 nematodes / 250 µl H_2O (Treatment 2: T2). The control consisted of moth larvae with 250 µl of distilled water. For each assay three replicates were performed, each replicate containing five individuals. Petri dishes were stored at 24 °C ± 0.5 °C in darkness. The mortality of larvae for each trial was evaluated over a period of five days by counting dead individuals at a 24 hours interval. The dead larvae were placed in modified White traps (White, 1927), for 72 hours after death, in order to recover the nematode infective juvenile stages (IJs) from the host cadaver. In order to test for differences between the treatments and the control, data collected on the number of individuals survived after five days were analysed using Kruskal-Wallis test in R (version 3.5.1).

Characterization of the nematode microbiota using 16S rRNA metabarcoding

V1-V2 and V4 regions of 16S rRNA gene were sequenced using Ion Torrent platform (Life Technologies). The total DNA of the nematodes was used as template to amplify the V1-V2 region using the primers 27FYM (Frank *et al*., 2008) and 338R (Amann *et al*., 1990), and to amplify the V4 region using the primers 515F (Caporaso *et al*., 2011) and 802R (Claesson *et al*., 2009) and 806R (Caporaso *et al*., 2011). The PCR primers were tailed with two different GC rich sequences enabling barcoding in a second amplification. 16S rRNA V1-V2 PCR was performed in 20 µL volume reaction containing 8 µL of HotMasterMix 5 Prime 2.5X (Quanta Bio), 0.4 µL of BSA (20 µg/µL) (Sigma-Aldrich), 1 µL of EvaGreen™ 20X (Biotium), 0.8 µL of 27FYM (10 µM) (5' modified with unitail 1 5'- GTGAGAGTTTGATYMTGGCTCAG -3'), 0.8 µL of 338R (10 µM) (5' modified with unitail 2 5'- GCTGCCTCCCGTAGGAGT -3'), and 1 µL (corresponding to 50 ng) of DNA template. Library were prepared accordingly to Chouaia *et al*. (2019) and sequencing was performed at Life Sciences Department of Trieste University, Italy. The obtained reads were analysed using QIIME 2 version 2018.11 (Bolyen *et al*., 2019). After trimming, 16S rRNA sequences were clustered into Operational Taxonomic Units (OTUs) with a similarity

cut-off of 97% using the de novo clustering method implemented in the q2-vsearch plugin (Rognes *et al*., 2016). Chimeras were identified and filtered using the uchime-denovo method implemented in q2-vsearch (Edgar *et al*., 2011). Taxonomic assignment of OTU representative sequences was performed using q2-feature-classifier (Bokulich *et al.*, 2018) and adopting Greengenes 13.8 (McDonald *et al*., 2012) as reference database. The 16S rRNA reads were deposited in the Sequence Read Archive (SRA) of NCBI with accession number SRS5367118.

Results

Molecular characterization of the nematode

The 897 bp long 18S rRNA fragment amplified from the nematode DNA was assigned to the Rhabditidae family based on a BLAST identity of 100%. The sequence showed an identity of 100% with sequences from *Oscheius myriophilus* (Poinar) and *Rhabditis myriophila* (Poinar), synonymized with *O. myriophilus* by Sudhaus (2011) (see also Abolafia and Peña-Santiago, 2019). Interestingly, the sequence identity was high (99.5%) even with *Oscheius microvilli* (Zhou *et al*.) (accession number: KT825913). The phylogenetic maximum likelihood tree inferred on the 18S rRNA sequence dataset confirmed the assignment of the isolated nematode to *Oscheius myriophilus* (bootstrap value of 95%) (figure 1).

Virulence tests of the nematode

In order to investigate the possibility to use the isolated nematode as a biocontrol agent, its virulence activity has been evaluated using the model organism *G. mellonella*. After inoculation of the insects, their survival was monitored for five days. At the end, six and seven larvae (corresponding to 40% and 46% of the individuals involved in T1 and T2, respectively) survived for the two tested nematode concentrations. In contrast, 14 individuals (corresponding to 93%) survived in the control experiment (table 1). IJs were recovered from the insect host cadaver using White traps. The results of the Kruskal-Wallis test indicate a significant difference in term of survival among the groups (χ^2 = 6.79, df = 2, p-value = 0.0336; table 2). Post-hoc test (Tukey's HSD) showed a significant difference between control and both treatments while no difference has been detected between the two treatments (table 2).

Taxonomic composition of microbial community of the nematode

A total of 139,245 bacterial 16S rRNA sequences of the regions V1-V2 and V4 were obtained from the DNA isolated from the nematodes. The microbiota associated with the nematode consisted of 1188 bacterial OTUs, obtained clustering the reads at 97% of sequence similarity. The nematode's bacterial community was characterized by values of Shannon and Pielou indices of 2 and 0.2, respectively indicating low diversity and evenness. The most abundant taxa in the microbiota was represented by the phylum Proteobacteria 99% (1106 OTUs), within this phylum, 46.6% of the 16S rRNA reads were assigned to unclassified family belonging to the order Rhizobiales (400 OTUs) and 50.7% assigned to the family Brucellaceae (259 OTUs), belonging to the same order. At the genus level, the bacterial community is dominated by *Ochrobactrum* (Holmes) (49.7% of the reads, 184 OTUs) followed by an unclassified genus belonging to the order Rhizobiales (43.2% of the reads, 400 OTUs) and the genus *Pseudomonas* (Migula) (4.2% of the reads, 85 OTUs) (figure 2). A single OTU constitutes 95% of the reads as-

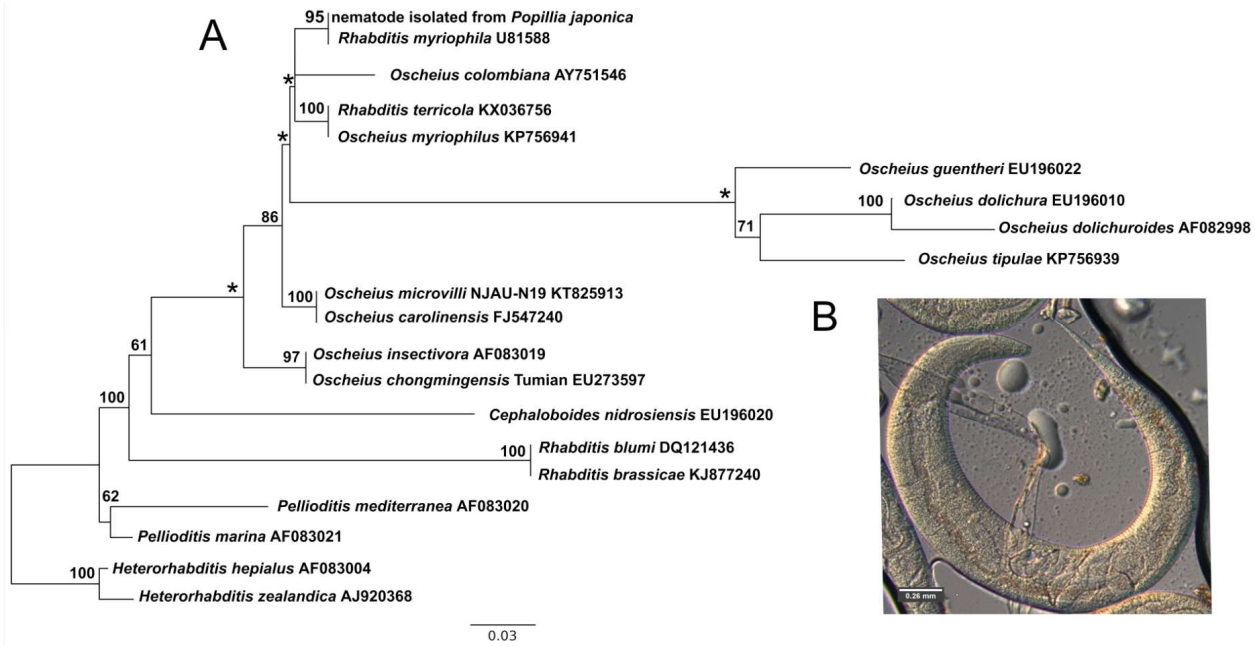

Figure 1. (**A**) Maximum likelihood tree based on 18S rRNA sequences obtained with PhyML 3.0. Bootstrap values above 50 are shown at the branch points, black asterisks indicate bootstrap values lower than 50. (**B**) The isolated nematode captured with scanning light microscope Olympus BX50 optical with BX-Pol simple polarizing.

Table 1. Number of dead *G. mellonella* larvae recovered in each replicate (R1-R3) for each treatment (T1, T2) and control, at a 24 h interval.

Treatments	24 h	48 h	72 h	4th day	5th day	Total
T1 (400IJs/250µl)	R1 = 0 R2 = 0 R3 = 0	R1 = 1 R2 = 1 R3 = 1	R1 = 1 R2 = 0 R3 = 1	R1 = 1 R2 = 1 R3 = 2	R1 = 0 R2 = 0 R3 = 0	9
T2 (300IJs/250µl)	R1 = 0 R2 = 0 R3 = 0	R1 = 1 R2 = 0 R3 = 1	R1 = 1 R2 = 1 R3 = 1	R1 = 1 R2 = 0 R3 = 1	R1 = 0 R2 = 1 R3 = 0	8
Control (distilled water)	R1 = 0 R2 = 0 R3 = 0	R1 = 0 R2 = 0 R3 = 0	R1 = 0 R2 = 0 R3 = 0	R1 = 0 R2 = 0 R3 = 0	R1 = 0 R2 = 1 R3 = 0	1

Table 2. Statistical analysis to test the differences in the number of survived individuals of *G. mellonella* larvae among the different treatments (T1 = 400IJs/250µl; T2 = 300IJs/250µl) and the control.

Kruskal-Wallis test	
	Survival score
χ^2	6.79
df	2
p-value	0.0336

Post-hoc Tukey HSD test					
Treats		Mean difference	Std. error	95% confidence interval Lower bound	Upper bound
T1	T2	−0.333	0.385	−1.28	0.61
	C	−2.667*	0.385	−3.61	−1.72
T2	T1	0.333	0.385	−0.61	1.28
	C	−2.333*	0.385	−3.28	−1.39
C	T1	2.667*	0.385	1.72	3.61
	T2	2.333*	0.385	1.39	3.28

* p-value ≤ 0.001.

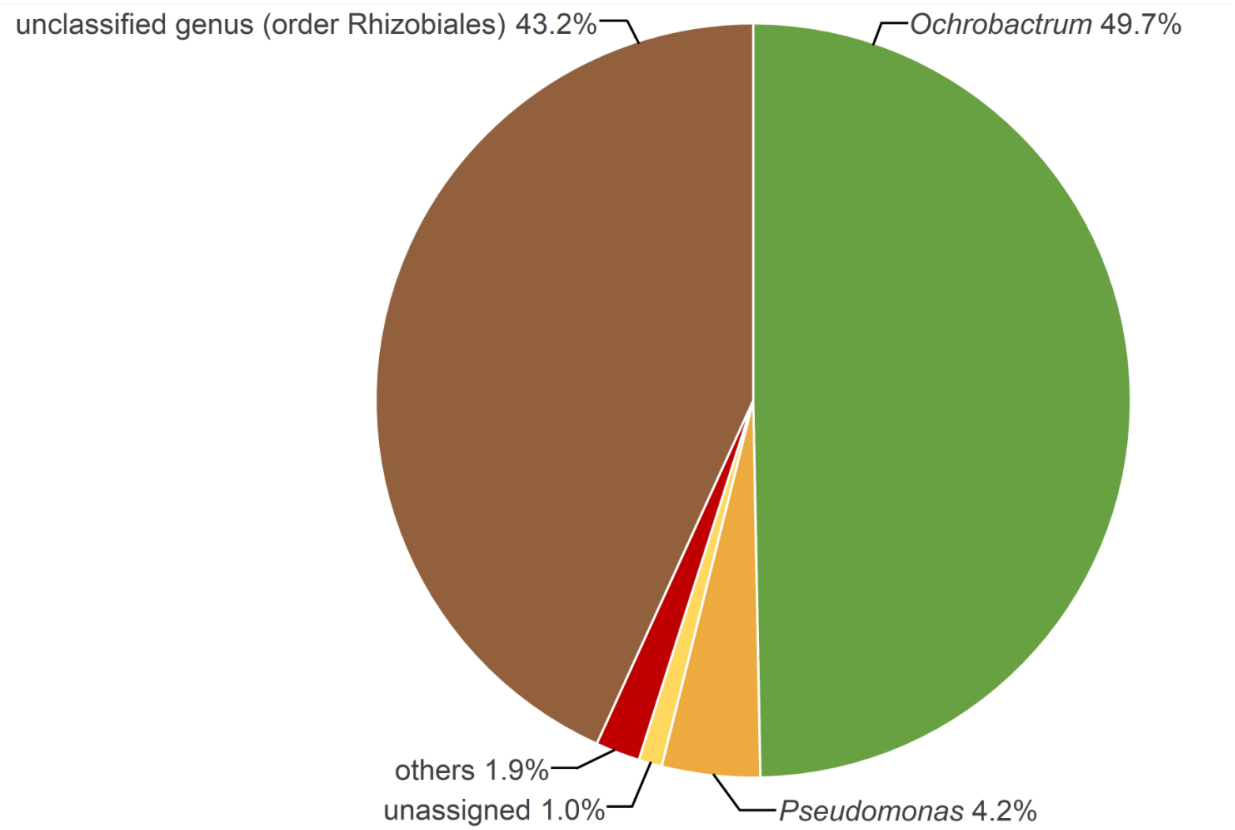

Figure 2. The composition and relative abundances of bacterial genera associated with the nematode isolated from third-instar larvae of *P. japonica*.

signed to the unclassified genus in the order Rhizobiales, so we used the representative sequence of this OTU to perform a search with BLAST on NCBI database and with the classifier tool of RDP (Ribosomal Database Project, https://rdp.cme.msu.edu). BLAST analysis assigns the selected sequence to the genus *Ochrobactrum*, while RDP classifier to unclassified Brucellaceae.

Discussion and conclusion

The present study reports the detection of a nematode associated with the third instar larvae of *P. japonica*, a polyphagous beetle recently reported from north Italy. Our analyses (i.e., BLAST and phylogenetic inference) clearly assigned the nematode to Rhabditidae family. The obtained sequence showed a similarity of 100% with *O. myriophilus*, which was isolated for the first time from the millipede *Oxidis gracilis* (Koch) (Poinar, 1986). Afterwards this nematode was also found in soil samples (Zhang *et al*., 2016; Al-Zaidawi *et al*., 2019) and associated to the European mole cricket (Erbaş *et al*., 2017). The present work represents the first record of *O. myriophilus* in Italy. The taxonomic identification of the nematode is also supported by the phylogenetic tree, where its sequence clusters, even if with low support value, in a group that includes the sequences of *O. myriophilus*. In the same group there are the sequences of *Rhabditis terricola* and *O. colombiana* (Stock *et al*.), the latter previously reported as a necromenic nematode (Stock *et al*., 2005).

Even considering the low sample size of *G. mellonella* larvae, the significant difference between control and trials (T1 and T2) and the amount of individuals killed by nematodes within five days (more than 50%) let us to consider, in agreement with Dillman *et al*. (2012), that the isolated nematode is an entomopathogen. However the virulence is not very high when infecting larvae of this model species (mortality within 48 hours less than 100%).

To further investigate the pathogenicity of the nematode we characterized the associated bacterial community that resulted extremely unbalanced (see Pielou index). This microbiota is dominated by bacterial OTUs assigned to the genus *Ochrobactrum*, Gram-negative bacteria belonging to the family Brucellaceae. Species in this genus have been defined as opportunistic pathogens in human (Holmes *et al*., 1988). In a previous study, *Ochrobactrum anthropi* (Holmes *et al*.) has been reported to be associated with the entomopathogenic nematode *Steinernema scapterisci* Nguyen et Smart (Aguillera and Smart, 1993); in addition, *Ochrobactrum* sp. was isolated from dead larvae of *G. mellonella*, infected by nematodes belonging to the genera *Steinernema* and *Heterorhabditis* (Razia *et al*., 2011). The pathogenic role of bacteria of the genus *Ochrobactrum* was recently demonstrated in the case of *Oscheius chongmingensis* (Zhang *et al*.) on *G. mellonella* larvae (Fu and Liu, 2019). Several biocontrol strategies tend to have a low efficiency when dealing with scarab beetles' larvae, but entomopathogenic nematodes are among the most promising control agent in this context, especially when combined with entomopathogenic fungi (Laznik and Trdan, 2015).

Our results suggest a potential role of the isolated nematode in the biological control of *P. japonica*. Indeed, it harbour high level of bacteria belonging to a genus that has been previously reported as entomopathogen and it causes a quite high mortality of the insect hosts, at least when infecting larvae of the model organism *G. mellonella*. Further studies are needed to test the possibility of using this nematode as a biocontrol agent of *P. japonica* in an integrated pest management framework.

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