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The bacterial community associated to an Italian population of *Psacothea hilaris*: a preliminary study

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Abstract

The yellow-spotted longicorn beetle, *Psacothea hilaris* (Pascoe) (Coleoptera Cerambycidae), native to eastern Asia, is an invasive species for Europe, where it is present since 2005 as a pest of *Morus* and *Ficus* spp. Up to date, no study on the bacterial community associated with *P. hilaris* has been carried out. The aim of the present work is to characterize the bacterial community associated to an Italian population of *P. hilaris* collected on *F. carica* L. through a culture-independent method (i.e., 454 pyrosequencing) targeting the 16S rRNA gene. The DNA used for bacterial characterization has been extracted from the whole abdomen of 15 adults (seven males and eight females) sampled on the host plant immediately after their emergence in Alserio (Como, Italy) between August and September 2012. The sequencing strategy led to a total of ≈ 2,350 bacterial 16S rRNA gene sequences that have been clustered into 141 bacterial operational taxonomic units. Results shown that the bacterial community was dominated by Proteobacteria (86%) belonging to Oxalobacteraceae and Enterobacteriaceae (respectively 36.4% and 34.8%). *Pantoea* resulted the most abundant genus (28.4%), and the other relevant bacterial genera associated with *P. hilaris* are *Ralstonia* (18.6%), *Methylobacterium* (3%), *Lactococcus* (2%) and *Propionibacterium* (1.4%).

Key words: yellow-spotted longicorn beetle, culture-independent method, 454 pyrotag, Enterobacteriaceae, Italy.

Introduction

The yellow-spotted longicorn beetle, *Psacothea hilaris* (Pascoe) (Coleoptera Cerambycidae), is one of the most common longicorn beetles in Japan, but it is also widely distributed in eastern Asia with several subspecies (Kusama and Takakuwa, 1984). This polyphagous species infests Moraceae trees, in particular figs and mulberries, causing many problems for the sericulture in the native area (Iba, 1993). In Europe, the yellow-spotted longicorn beetle was firstly spotted in England in 1997, and since 2005 it was reported in Italy (Jucker *et al*., 2006), where it is a major pest of *Morus* and *Ficus* spp. Even if these plants are mainly ornamental, in the Mediterranean area *Ficus carica* L. is important also for fig production; for this reason, the presence of *P. hilaris* should be carefully monitored. The damages on the trees are caused by larvae tunneling inside the trunk (xylem), while adults feed on the leaves (Iba, 1993). This invasive species can complete the life cycle in one or two years, with two generations per years, depending on the time of eggs deposition (Watari *et al*., 2002).

Like other long-horned beetles, *P. hilaris* is able to digest polysaccharides, particularly cellulose, through the endogenous enzymes secreted in the gut of larvae and adults (Scrivener *et al*., 1997). Cellulose is not easily digestible for the majority of the eukaryotes, but groups of mutualistic bacteria help their host in lignocellulolytic activity and in the supplement of essential substances missing in the diet (i.e. aminoacids, vitamins and cofactors) (Moran, 2006; Douglas, 2009; McCutcheon *et al*., 2009; Montagna *et al*., 2011).

The aim of the present study is to characterize the bacterial community associated to an Italian population of *P. hilaris* in order to acquire the basic knowledge useful for the development of symbiont-based biocontrol strategies of the host.

Materials and methods

Collection of samples

Fifteen adult specimens of the longhorn beetle *Psacothea hilaris* were sampled on a single tree of *Ficus carica* in Alserio - Como (North Italy) between August 28th and September 15th 2012. The geographic coordinates of the sampling site are: 45°46'40.38"N 9°12'32.62"E and 267 m a.s.l.. Samples were daily collected from the plant and stored in acetone. In order to preserve both the host and the bacterial DNA, injections of acetone were performed with a sterile syringe on each sample.

Sample preparation and DNA extraction

DNA was extracted from all the collected specimens (seven males and eight females). Before the dissection, elytra and wings were cut and the exoskeleton sterilized following the procedures described by Montagna *et al*. (2014). The abdomen was removed under a stereomicroscope using sterilized forceps. The whole gut and the abdomen content were processed for the DNA extraction. DNA was individually extracted, using the DNeasy Blood and Tissue Kit (Qiagen, Heidelberg), according to manufacturer's instructions, with a final elution in 100 µl of AE buffer. Total DNA was quantified by spectrophotometry (Nanodrop 1000, Thermo Scientific, Wilmington, DE, USA).

PCR amplification and pyrosequencing

In order to obtain a good representation of the bacterial community associated with the insect population, the DNAs extracted by the 15 specimens were pooled using 100 ng of DNA from each specimen.

A fragment of approximately 400 bp in the hypervariable V1-V3 region of the bacterial 16S rRNA gene was amplified using bacterial universal primers 27Fmod 5'–AGR GTT TGA TCM TGG CTC AG–3' and 519Rmod bio 5'–GTN TTA CNG CGG CKG CTG–3', both modified by the addition of a GS FLX Titanium Key-Primer 5'–AGR GTT TGA TCM TGG CTC AG–3', and multiplex identifiers. PCR reactions were performed accordingly to previous published protocols (Montagna *et al*., 2014). Amplicons obtained after PCR were sequenced using Roche FLX 454 titanium. PCR reaction and pyrosequencing were commercially performed (MR DNA, Shallowater, TX, USA).

The obtained raw 16S rRNA gene sequences were trimmed to remove pyrosequencing adaptors and low-quality base calls using the python script split_libraries.py implemented in QIIME (Caporaso *et al*., 2010). Analyses on the 16S rRNA sequences were performed using QIIME pipeline scripts (Caporaso *et al*., 2010) according to Montagna *et al.* (2014). The 16S rRNA gene sequences were deposited in European Nucleotide Archive with accession number PRJEB6583.

Bacterial taxonomic assignment and diversity analyses

The obtained high-quality 454 sequences were clustered into operational taxonomic units (OTUs) using UCLUST (Edgar, 2010) with a 97% sequence-identity threshold. One sequence for each identified OTU was selected as representative and aligned to 'greengenes' (http://greengenes.lbl.gov/) using PyNast (Caporaso *et al*., 2010). The OTUs were taxonomically classified using blastn-based searches on the 'greengenes' and SILVA databases. Non-bacterial OTUs were removed from the dataset and the remaining data were used for diversity analyses. The obtained 16S rRNA sequences, belonging to unclassified OTUs, were subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/blast) and compared with sequences available in GeneBank (http://www.ncbi.nlm.nih.gov/genbank/, 30 May 2014).

The diversity indices were estimated on the obtained bacterial OTU table using the R package *vegan* (Oksanen *et al*., 2007), as described in Montagna *et al*. (2014).

Results and discussion

Alpha-diversity

After the removal of low quality base calls, chimeras and chloroplast sequences, a total of 2,349 bacterial 16S rRNA gene sequences were obtained (2,580 raw sequences). These sequences were clustered into 141 bacterial OTUs adopting a similarity threshold of 97%. The coverage of bacterial α-diversity was inspected by the rarefaction curves (figure 1), which indicated that the libraries provided a good representation of total microbial α-diversity associated with the analyzed population of *P. hilaris*. Based on the estimated value of Chao-1 index (corresponding to 211 OTUs), ≈ 67% of the estimated α-diversity has been recovered by the present analysis. The microbiota associated to the *P. hilaris* population had Shannon and Pielou's evenness indices of 2.92 and of 0.41, respectively. The three most abundant OTUs, representing ≈ 60% of the total obtained bacterial 16S rRNA gene sequences, confirm the unbalanced structure of the bacterial community. Compared to the bacterial communities associated with different specimens of *Rhynchophorus ferrugineus* (Olivier) and *Rhynchophorus vulneratus* (Panzer) (unpublished data), the analyzed population of *P. hilaris* shows a lower value of identified OTUs and estimated Shannon index. However, comparing our results to a study carried out on the metagenomic profiles of the microbial community associated with a close relative species from the subfamily Lamiinae, *Anoplophora glabripennis* (Motschulsky), we found similar values of diversity, 166 bacterial OTU (Scully *et al*., 2013).

Taxonomic classification of OTUs

A summary of the taxonomic assignments for the 141 OTUs is reported in figure 2 and table 1. At the phylum level (figure 2A), the microbiota of the yellow-spotted longicorn beetle is dominated by taxa belonging to Proteobacteria (86%). Figure 2B reports the relative abundance of the isolated bacterial families that are represented by more than 1%. Oxalobacteraceae and Enterobacteriaceae turned out to be the dominant bacterial families (represented by 36.4% and 34.8%, respectively). Unclassified Enterobacteriaceae were the most abundant members in the bacterial communities associated with *P. hilaris* (28.4%). Among the unclassified Enterobacteriaceae, the most abundant OTU (6.5%) was identified using BLAST and showed 100% identity with *Pantoea agglomerans* (Ewing and Fife, 1972). The most abundant OTU (16.6%) within unclassified Oxalobacteraceae matched with 100% identity to an uncultured bacteria of the genus *Massilia* (La Scola *et al.,* 2000). *Ralstonia* was the dominant classified bacterium (18.6%) and other bacteria characterized by abundance higher than 1% were *Methylobacterium* (3%), *Lactococcus* (2%) and *Propionibacterium* (1.4%) (table 1).

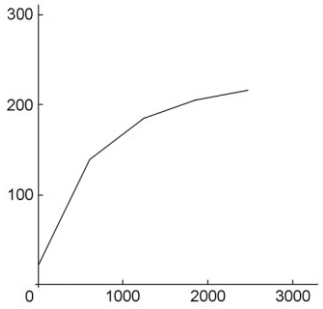

Figure 1. Rarefaction curve for total bacterial community (3% identity cut-off) associated with the analysed population of *P. hilaris*. On the x-axis is reported the sequencing effort expressed as number of obtained 16S rRNA sequences, while on the y-axis is reported the cumulative number of observed OTUs.

Table 1. Families and genera of bacteria identified in the microbiota associated with *P. hilaris* with their relative abundance expressed as percentage.

Phylum/Family	Relative abundance	Phylum/Genus	Relative abundance
Unclassified	0.5	Thermi	0.34
Other bacteria	0.7	*Deinococcus*	0.34
Acidobacteria		Proteobacteria	46.86
Solibacteraceae	0.3	*Pseudomonas*	0.09
Other Acidobacteria	0.3	*Yersinia*	0.04
Actinobacteria		*Erwinia*	0.04
Geodermatophilaceae	0.2	*Ralstonia*	18.56
Kineosporiaceae	0.2	*Massilia*	16.60
Microbacteriaceae	3.0	*Janthinobacterium*	0.09
Micrococcaceae	0.4	*Hydrogenophaga*	0.51
Nocardioidaceae	0.1	*Lautropia*	0.34
Propionibacteriaceae	1.4	*Achromobacter*	0.09
Pseudonocardiaceae	0.3	*Sphingomonas*	0.51
Rubrobacteraceae	0.2	*Roseomonas*	0.21
Other Actinobacteria	0.5	*Gluconobacter*	0.09
Bacteroidetes		*Paracoccus*	0.04
Chitinophagaceae	0.6	*Agrobacterium*	0.04
Flexibacteraceae	0.8	*Methylobacterium*	3.02
Sphingobacteriaceae	0.2	*Devosia*	0.09
Firmicutes		*Pantoea*	6.50
Staphylococcaceae	0.1	Fusobacteria	0.47
Streptococcaceae	2.8	*Leptotrichia*	0.47
Fusobacteria		Firmicutes	2.13
Leptotrichiaceae	0.5	*Lactococcus*	2.00
Planctomycetes		*Staphylococcus*	0.13
Isosphaeraceae	0.2	Bacteroidetes	0.63
Proteobacteria		*Pedobacter*	0.21
Caulobacteraceae	0.3	*Spirosoma*	0.21
Aurantimonadaceae	0.2	*Hymenobacter*	0.17
Bradyrhizobiaceae	0.1	*Flavobacterium*	0.04
Hyphomicrobiaceae	0.1	Armatimonadetes	0.17
Methylobacteriaceae	3.6	*Fimbriimonas*	0.17
Rhizobiaceae	0.6	Actinobacteria	4.08
Acetobacteraceae	1.3	*Rubrobacter*	0.17
Sphingomonadaceae	0.5	*Actinomycetospora*	0.26
Alcaligenaceae	0.1	*Propionibacterium*	1.36
Burkholderiaceae	0.3	*Propionicimonas*	0.13
Comamonadaceae	0.6	*Kocuria*	0.38
Oxalobacteraceae	36.4	*Microbacterium*	0.81
Enterobacteriaceae	34.8	*Frigoribacterium*	0.34
Pseudomonadaceae	0.1	*Curtobacterium*	0.21
Other Proteobacteria	7.0	*Brevibacterium*	0.04
Thermi		*Parascardovia*	0.04
Deinococcaceae	0.3	"*Ca.* Solibacter"	0.34
		Unclassified	45.32

It is noteworthy that the bacterial community associated with *P. hilaris* is dominated by members of Enterobacteriaceae, a bacterial family in which several symbiotic associations with insect have been described, such as the primary symbiont of Aphids, *Buchnera aphidicola* Munson *et al.* (1991), or *Wigglesworthia glossinidia* (Aksoy, 1995) and *Sodalis glossinidius* (Dale and Maudlin, 1999) the endosymbiont of the tsetse fly (Wamwiri *et al*., 2013), and secondary symbionts such as *Raoultella* with *Rhagoletis completa* Cresson (Martinez-Sañudo *et al*., 2011), or *Klebsiella* with *Ceratitis capitata* Wiedemann (Behar *et al*., 2008). Besides, the acquisition by *P. hilaris* of Enterobacteriaceae from the environment cannot be excluded on the basis of current data. On the other hand, the presence of members of Oxalobacteraceae (i.e., *Ralstonia* and *Massilia*) should be investigated more in detail since the former have been detected in association with different insect hosts, as in leaf beetles, seed-parasitic wasps and glassy-winged sharpshooter (Montagna *et al.*, 2014; Paulson *et al.*, 2014; Rogers *et al.*, 2014); while the latter with cochineal insects of the genus *Dactylopius* (Ramírez-Puebla *et al.*, 2010).

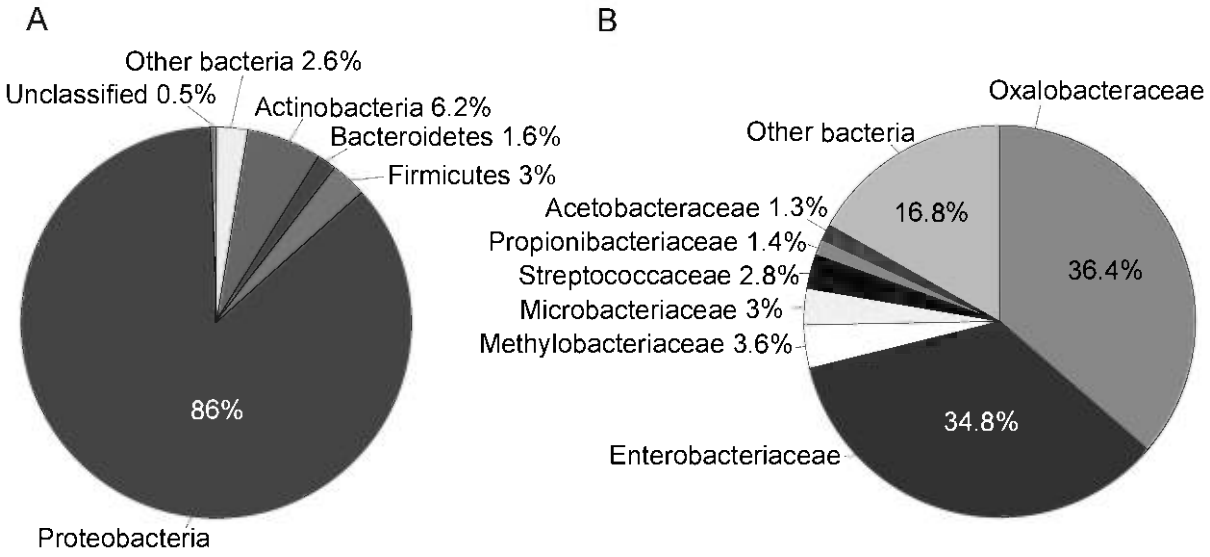

Figure 2. Relative abundance in taxonomic assignment of 16S rRNA gene sequences obtained from the analyzed population of *P. hilaris.* A. taxonomic assignment at phylum level; B. taxonomic assignment at family level.

Moreover, the presence of *P. agglomerans* in the bacterial community associated with *P. hilaris* is intriguing and request further investigations since this bacterium has been discovered in the gut of locusts producing guaiacol and promoting the aggregation of the insect host (Dillon *et al*., 2000). Since bacteria belonging to the genus *Pantoea* sp. have been recovered also in *A. glabripennis* (Geib *et al*., 2009), which shows similar ecology, a possible symbiotic role of this bacterium cannot be excluded.

Conclusion

In conclusion, this preliminary work shows the presence of a relatively simple community of bacteria associated with the analyzed population of *P. hilaris*. Within the identified bacterial community, the presence of *P. agglomerans*, *Ralstonia*, *Massilia* and *Methylobacterium* is noteworthy. In addition, since Enterobacteriaceae resulted the most abundant family and its members are prone to establish symbiotic association with insects, makes these bacteria a possible target for future studies addressed to develop innovative biocontrol strategies based on paratransgenesis. This work will contribute to deepen the knowledge about the ecology of *P. hilaris* and especially its associated microbiota. This step is important for the development of new techniques aimed at controlling populations of invasive species, as suggested by Mazza *et al*. (2011) for *R. ferrugineus*.

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