Deciphering microbiota associated to *Rhynchophorus ferrugineus* in Italian samples: a preliminary study

M. Valzano, G. Achille, F. Burzacca, I. Ricci, C. Damiani, P. Scuppa, G. Favia

Scuola di Bioscienze e Biotecnologie, Università degli Studi di Camerino, Italy

Abstract

The Red Palm Weevil, *Rhynchophorus ferrugineus* (Olivier, 1790) is the most dangerous and deadly pest of date, coconut, oil, sago and other palms. Recently introduced in the Mediterranean basin, it became the most relevant insect pest for ornamental palms in the urban environment. Given the development of an innovative control method based on symbiotic control, we have performed a pilot project to decrypt the microbiota associated to both adults and larval stages of the insect to identify potential tools for biocontrol agents against the Palm Weevil. A number of bacterial species were found associated with the insect. In particular, species of the genera *Lactococcus*, *Proteus*, and others were detected.

Introduction

Rhynchophorus ferrugineus (Olivier, 1790) also known as the invasive Red Palm Weevil (RPW) (Wattanapongsiri, 1966) is one of the most destructive pests of date palms, the primary fruit tree of arid, tropical and sub-tropical areas including many Arab Gulf and North Africa countries (Esser & Meredith, 1987; Bokhari & Abuzuhari, 1992; Barranco *et al.*, 1996).

Symptoms of infestation are the presence of tunnels in the trunk, oozing of thick sap from the tree, presence of fermented odor from the

fluid inside infested tunnels, breaking of the trunk and/or the toppling of the crown (Kaakeh, 2006).

An attack from *R. ferrugineus* might inflict death of the palm in 6-8 months (Murphy & Briscoe, 1999) and infestation is often not visible until the tree has fallen down.

As a consequence of the heavy introduction of ornamental date palms in the Mediterranean Basin, *R. ferrugineus* has recently colonized different European regions (*i.e.,* France, Greece, Spain, Albania, Cyprus and Italy) posing serious economical and environmental concerns (Llacer *et al.*, 2010).

In Italy, the first report dates back to 2004 when the insect was detected on some plants of *Phoenix canariensis* in a nursery of the Pistoia province in Tuscany (Sacchetti *et al.*, 2006). Later, *R. ferrugineus* infestations were detected in Sicily and Campania (since 2005), Latium and Apulia (since 2006), Abruzzo, Calabria, Liguria, Marche, Molise Sardinia (since 2007) and Basilicata (since 2010) (Longo & Tamburini, 2005; EPPO, 2008; Massa *et al.*, 2010).

For years, several control methods have been applied against *R. ferrugineus* and other weevil species within the Integrated Pest Management (IPM) strategy (Hallett *et al.*, 1993; Rajamanickam *et al.*, 1995; Abraham *et al.*, 1999). This integrated approach mainly includes phyto-sanitation, which involves cutting down and burning of the infested palms, and the use of insecticides and pheromone traps for adult monitoring and mass trapping (Murphy & Briscoe, 1999; Faleiro, 2006).

The use of a broad-spectrum of insecticides implicates a heavy environmental impact, especially for *non-targeting* invertebrate species. Therefore, the setting and applicability of alternative control strategies is of the greatest importance. In this context, the development of biocontrol methods has attracted much interest in the few last years. This mostly regards the employment of self-pathogens such as *Pseudomonas aeruginosa*, *Beauveria bassiana* (El-Sufty *et al.*, 2007; Güerri-Agulló *et al.*, 2010), different species of *Bacillus* (Banerjee & Dangar, 1995; Salama *et al.*, 2004; Longo & Colazza, 2009) and yeasts (Dangar, 1997), while several studies have been conducted on the natural enemies of *R. ferrugineus* that might be successfully used as tools for its biological control (Murphy & Briscoe, 1999; Hanounik *et al.*, 2000; Salama *et al.*, 2004).

Recently, much effort has been made to identify more effective and safer biological control agents against several insect pests (Sezen *et al.*, 2005). In this framework, characterization of the microbiota associated with insect pest may provide additional tools for insect control.

In fact, there is much evidence to show that insect microbial flora could play an important role in the host biology, such as the production of indispensable compounds and essential nutrients (amino acids and vitamins), to provide protection towards natural enemies, and to influence some significant factors such as insect development, fertility, longevity, immunological competences and vector capability. The knowledge of microbiota composition associated to the target insect, especially those living in the gut, where they often accomplish fundamental functions for their hosts, is a necessary prerequisite for the development of *symbiont-based* approaches (Ricci *et al.*, 2012; Kikuchi *et al.*, 2012).

In the last few years, *a new strategy*, called symbiotic control (SC) has been proposed to control insect pest (Douglas *et al.*, 2007). The SC measure mainly refers to two main approaches: i) interference with microbial symbionts required by insect pests; and ii) the genetic manipulation of symbionts to deliver specific molecules against the insect and/or the pathogens they transmit.

There is still little information about RPW microbiota. Here we report the bacterial communities associated with larvae and adults of *R. ferrugineus* sampled in Central Italy and the identification of microorganisms that might be exploited for its biological control.

Materials and methods

Samples collection and gut dissections

Larvae and adults of RPW were captured on infected palms on the Cupra Marittima seafront (Ascoli Piceno, Marche Region, Italy; latitude 43°1'51''24 N; longitude 13°51'31''68 E).

These were stored in plastic holders and carried to the Parasitology Laboratory of the University of Camerino. Insect surface was washed several times with 70% ethanol and alcohol was allowed to evaporate before further processing of the samples. Insects were fixed on a *foam* table and dissected with sterile needles and sterile fine-tip forceps. Intestinal tracts were placed in 50 μL of Grind Buffer (1% SDS, 25 mM EDTA pH 8, 25 mM NaCl, 50 mM Tris-HCl pH 8) and utilized for DNA extractions.

DNA extraction

Samples were homogenized in 50 μL of grind buffer using sterile polypropylene pestles and placed 30 min at 65°C. After addition of 14 μL of 8 M Potassium Acetate (pH 7.2), the homogenates were kept on ice for 30 min and then centrifuged for 10 min at 13,000 rpm. DNA was precipitated in 100% ethanol. DNA pellets were dried and then rehydrated in 50 μL of ultra-pure water for polymerase chain reaction (PCR) applications and the nucleic acid concentration estimated by spectrophotometer analysis.

R. ferrugineus specific semi-nested polymerase chain reaction assay

Three oligos were designed against 18S rRNA gene of our weevil species:

R18S-for (5′-ACGGGTAACGGGGAATCAGGGT-3′);
Rhyncf-rev (5′-GTCGTAACCACGACGCGCAC-3′) and
R18S-rev (5′-TAATCATTACCTCGGGGGTTCCGA-3′).

Each PCR mixture was prepared, under sterile conditions, in a final volume of 25 μL containing: 1×PCR buffer, 0.25 mM dNTPs, 10 μM of each primers, 1 U Dream Taq DNA Polymerase (Thermo Scientific Inc., Waltham, MA, USA) and 50 ng of DNA. Reactions were run for 2 min at 95°C and cycled 30 times through 30 s at 95°C, 30 s at 60°C and 30 s at 72°C. Finally, reactions were kept at 72°C for 8 min. PCR products were visualized by electrophoresis on ethidium bromide agarose gel (1%) in 0.5×TBE buffer under ultraviolet (UV) light.

Bacterial DNA amplification by polymerase chain reaction

Extracted DNAs were targeted for PCR reactions using the universal 16S rRNA bacterial primers 27F (5′-TCGACATCGTTTACGGCGTG-3′) and 805R (5′-AGAGTTTGATCCTGGCTCAG-3′). Reaction mixtures were carried out in a final volume of 30 μL, using 1×PCR buffer, 0.25 mM dNTPs, 10 μM of each primers, 1 U Dream Taq DNA Polymerase (Thermo Scientific Inc.) and 50 ng of DNA. Reactions were run for 2

min at 95°C and cycled 20 times through 30 s at 95°C, 30 s at 56°C and 30 s at 72°C. Finally, reactions were kept at 72°C for 10 min.

Bacterial library constructions and colony screening

To characterize the bacteria inhabiting *R. ferrugineus* intestinal tract, the 16S rRNA gene amplicons were purified using the DNA Extraction kit (Fermentas, Thermo Scientific Inc.) and were cloned by the pGEM®-T Vector System (Promega, Fitchburg, WI, USA). The recombinant plasmids were transformed into *Escherichia coli* JM109 competent cells (Promega, USA).

The transformed cells were plated onto LB agar supplemented with 100 g/mL ampicillin, 0.5 mM IPTG, 50 mg/mL X-Gal and cultured overnight at 37°C. White colonies were picked up and used in PCR reactions with the primer T7 (5′-AATACGACTCACTATAGGG-3′) and Sp6 (5′-ATTTAGGTGACACTATAGAAT-3′). The PCR reactions (10 μL) contained 1×PCR buffer, 0.25 mM dNTPs, μM of each primer, 1 U Dream Taq DNA Polymerase (Thermo Scientific Inc). Reactions were run for 2 min at 95°C and cycled 20 times through 30 s at 95°C, 30 s at 52°C and 30 s at 72°C. Finally, reactions were kept at 72°C for 8 min. PCR products were then resolved in an 1% agarose gel stained with ethidium bromide. To avoid the sequencing redundancy of the same clones, restriction analysis of the bacterial amplified inserts was performed. Each reaction was carried out in a final volume of 15 μL containing: 1× of buffer, 10×Y(Yellow)/Tango™, 2 U Hin6I, 2 U of Hpa II, 2 U of Bsu15I and 5 μL of amplicon. The samples were incubated at 37°C for 1 h and 30 min, and were subsequently loaded on ethidium bromide agarose gel (2%) in 0.5×TBE buffer. All the clones exhibiting an exclusive restriction pattern were amplified in 25 μL and purified through DNA Extraction Kit (Fermentas, Thermo Scientific Inc.) for sequencing.

Sequencing

Bacterial purified fragments were sequenced at the Eurofins MWG Operon Company (Ebersberg, Germany). Sequences were compared with the database at the National Centre for Biotechnology Information (NCBI) by using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

R. ferrugineus identification by specific semi-nested polymerase chain reaction assay

A morphological analysis had initially been performed (EPPO, 2007) (Figure 1A-B). To confirm insect identity as RPW, a molecular screening was performed using two generic primers for Coleoptera/Curculionidae and an internal primer specifically designed on the 18S rRNA locus gene of *R. ferrugineus*.

A nested PCR assay was then applied to DNA of larvae, adult females and adult males (Figure 1C) with the aim to obtain a species-specific amplification product to precisely identify *R. ferrugineus* samples.

Indeed, two PCR products were detected: a 611 bp fragment and a 428 bp fragment. The smaller band represented the specific fragment for *R. ferrugineus*. To confirm their specificity, the DNA fragments of 428 bp were purified by gel excision and sequenced. Sequences showed 100% similarity with *R. ferrugineus* sequence in GeneBank (Accession n. EF125057.1) confirming the morphological identification of the samples used in the subsequent molecular studies.

Analysis of bacterial libraries

Three 16S rRNA bacterial gene libraries were established from total DNA isolated from midguts of adult female, adult male and larva previously characterized by both morphology and molecular analysis.
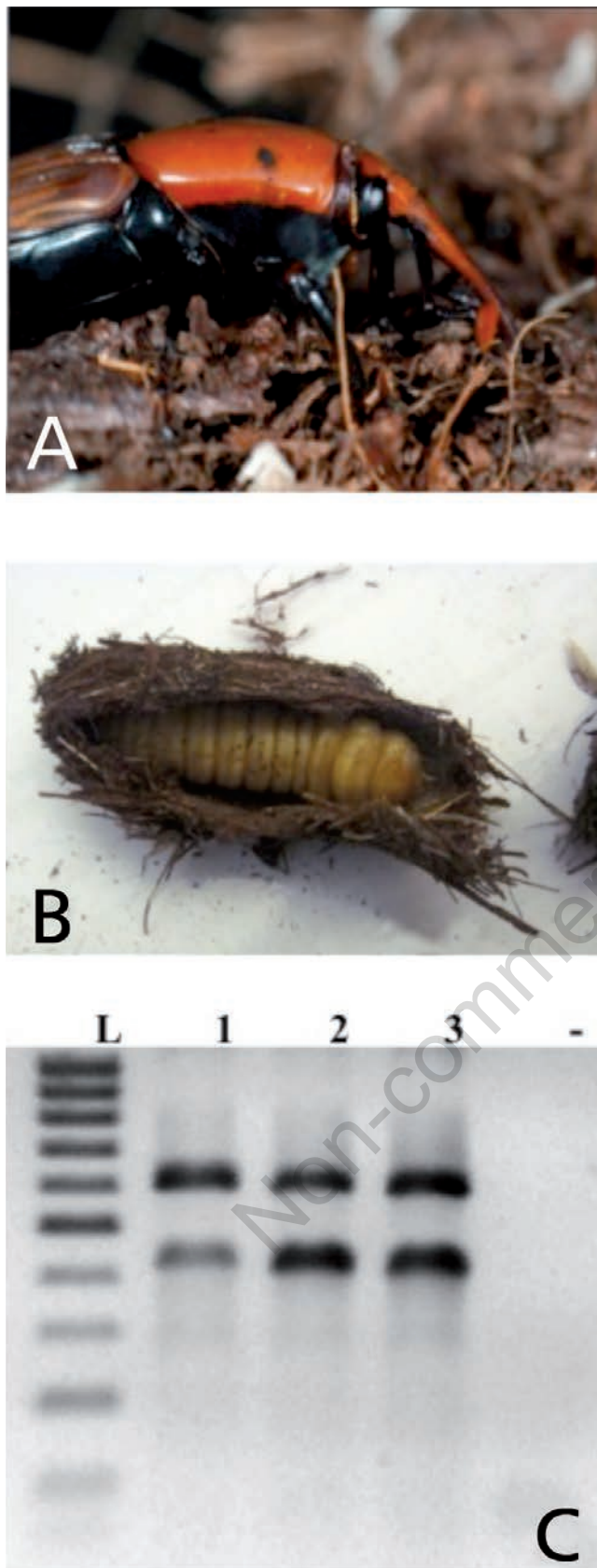
Figure 1. An adult (A) and a larva (B) of *R. ferrugineus*, sampled in Cupra Marittima. (C) Semi-nested polymerase chain reaction for the identification of *R. ferrugineus*. L, ladder 100 bp; 1, larva; 2, adult female; 3, adult male; - , no DNA control.

The majority of the clones in the library obtained by adult female were closely related to *Lactococcus lactis* (30%) a Gram-positive lactic acid bacterium that is a common member of the microbial communities of termite gut and the major folate secretor together with *Serratia grimesii* into termites (Graber & Breznak, 2005). Several clones instead gave high sequence similarity with *Cedecea davisae* (20%) and with an uncultured *Enterobacter* sp. (20%). Five clones were affiliated with an uncultured gamma-proteobacterium (25%) while only one clone was related to an uncultured bacterium (5%).

The analysis of the library obtained by adult male showed that the prevailing bacterium was once again *L. lactis* (66.7%). Few further clones showed high sequence similarity with *Proteus vulgaris* (6.7%). Interestingly, this Gram-negative bacterium has been reported to be associated to the digestive tract of *Bombyx mori* L. (Lepidoptera: Bombycidae) (Anand *et al.*, 2010). Other clones are associated to *Pseudomonas* sp. (6.7%) and uncultured bacteria (6.7%).

Clones from the library obtained by larvae were identified as an uncultured *Staphylococcus* sp. (21%), *Pseudomonas* sp. (15.8%), *Pseudomonas aeruginosa* (10.5%), an uncultured *Acinetobacter* sp. (15.8%) and three uncultured bacteria (21%; 10.5%; 5.2%) (Table 1).

Discussion and conclusions

We have performed a pilot project aimed at characterizing the microbial communities associated with the midgut of both larvae and adults of *R. ferrugineus* specimens to identify possible candidates for the development of symbiotic control approaches of this insect pest. In this framework, some operational taxonomic units (OTU) have been identified offering interesting future perspectives in the elaboration of applied research-projects.

Different and specific bacterial species have been found in association with the insect at both developmental stages.

Some of the characterized species may perform important biological functions inside the RPW and could be exploited for future applications into the biological control of this pest. Two bacteria look particularly interesting: *L. lactis*, a cultivable Gram-positive lactic acid bacterium, which has been shown to be the prevalent bacterium in adults of both sexes, and *P. vulgaris,* a cultivable Gram-negative bacterium.

Lactic acid bacteria have been identified as common members of the gut microbiota of wood- and soil-feeding termites (Bauer *et al.*, 2000; Eutick *et al.*, 1978; Schultz & Breznak, 1978; Tholen *et al.*, 1997).

Besides the production of lactate and acetate (Schultz & Breznak, 1979; Tholen *et al.*, 1997), these bacteria have also been implicated in the recycling of excretory nitrogen in termite biosynthesis (Potrikus & Breznak, 1980a, 1980b; Potrikus & Breznak, 1981).

Strains of *L. lactis* and *Serratia grimesii* isolated from hindguts of the termite *Zootermopsis angusticollis*, were shown to exert a role in the secretion of 5-formyl-tetrahydrofolate (THF). The THF acts as a C_1 carrier in CO_2-reductive acetogenesis, a microbial mediated process for energy production (Graber & Breznak, 2005).

Furthermore, cross-feeding of 5-formyl-THF by other community members is important for the growth of hindgut symbionts, such as *Treponema primitia*, an essential organism for termite nutrition and survival because of its ability to fix di-nitrogen and produce acetate.

Since *Z. angusticollis* and *R. ferrugineus* are both xylophagous insects, *L. lactis* strains in the weevil species likely execute very similar functions to those found in the termite.

Concerning *P. vulgaris*, it is known that guts of Lepidoptera contain bacteria that produce enzymes for carbohydrates digestion (Dillon & Dillon, 2004). In particular, *P. vulgaris*, *Citrobacter freundii*, *Serratia liquefaciens* and *Klebsiella* sp., were reported to be cellulose-degrading bacteria and xylanolitytic bacteria (Prem Anand & Sripathi, 2004). *Bombix mori* L.

Table 1. Summary of the bacterial library screening.

DNA sources	Bacterial species [Accession number]	Sequence database match		No. clones	Total no. clones
		OTUs (%)	Sequence similarity (%)		
Midgut from adult ♀	*Lactococcus lactis* [CP002365]; [EU869288]	30	99-100	6	
	Cedecea davisae [HQ242718]	20	99	4	
	Uncultured gamma proteobacterium [JF833678]	25	98-99	5	20
	Uncultured enterobacter sp. [JF703600]	20	97	4	
	Uncultured bacterium [GU562599]	5	99	1	
Midgut from adult ♂	*Lactococcus lactis* [CP002365]; [EU869288]	67	99	10	
	Proteus vulgaris [X07652]	6.6	99	1	
	Pseudomonas sp. [DQ118950]	6.6	99	1	
	Uncultured bacterium [HQ602799]	6.6	99	1	15
	Uncultured bacterium [EU459323]	6.6	99	1	
	Uncultured bacterium [EU773759]	6.6	99	1	
Midgut from larva	*Staphylococcus* sp. [EF061904]	21	95	4	
	Pseudomonas sp. [AF521651]	15.8	98	3	
	Pseudomonas aeruginosa [AE004091]	10.5	97	2	
	Uncultured bacterium [GQ476553]	21	99	4	19
	Uncultured bacterium [AB286475]	10.5	99	2	
	Uncultured bacterium [JF276483]	5.3	98	1	
	Uncultured acinetobacter [FJ811887]	15.8	99	3	

OTUs, operational taxonomic units.

(Lepidoptera: Bombycidae) feeds on mulberry leaves that are mainly composed of pectin, xylan, cellulose and starch (Anand *et al.*, 2010) and hosts *P. vulgaris* in its digestive tract. The xylophagous diet of RPW is comparable with that of *Bombyx mori* L. It is, therefore, reasonable to suggest that *R. ferrugineus* digestive tract could contain specific bacteria, such as *P. vulgaris*, able to produce enzymes that digest polysaccharides.

All of these characteristics, coupled with the fact that *L. lactis* and *P. vulgaris* are cultivable, make them potential candidates for the development of SC approaches, particularly those based on paratransgenesis and/or symbiont interference. However, further studies in this direction are required.

In larvae, we have found mostly bacteria of the genera *Pseudomonas* and *Staphylococcus*; interestingly, *Pseudomonas* is the only genus identified both in larvae and adults.

In conclusion, our pilot study, even though performed on a limited number of individuals, showed the potential of deciphering the composition of *R. ferrugineus* microbiota to identify symbionts and provide knowledge of insect-symbionts relationships in order to develop targeted biological control.

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