Diversity and phylogenetic analysis of endosymbiotic bacteria of the date palm root borer *Oryctes agamemnon* (Coleoptera: Scarabaeidae)

Wael S El-Sayed¹ ²* and Reda A Ibrahim¹ ³

**Abstract**

**Background:** The date palm root borer *Oryctes agamemnon* (Coleoptera: Scarabaeidae) is one of the major pests of palms. In Saudi Arabia, both larvae and adults of *Oryctes* are particularly troublesome, especially during the establishment of young date palm orchards. Endosymbiotic bacteria are known to have a key role in food digestion and insecticide resistance mechanisms, and therefore are essential to their host insect. Identification of these bacteria in their insect host can lead to development of new insect pest control strategies.

**Results:** Metagenomic DNA from larval midgut of the date palm root borer, *O. agamemnon*, was analyzed for endosymbiotic bacterial communities using denatured gradient gel electrophoresis (DGGE) utilizing 16S rRNA genes. The DGGE fingerprints with metagenomic DNA showed predominance of eleven major operational taxonomic units (OTUs) identified as members of *Photobacterium*, *Vibrio*, *Alomonas*, *Shewanella*, *Cellulomonas*, and *Citrobacter*, as well as uncultured bacteria, including some uncultured *Vibrio* members. DGGE profiles also showed shifts in the dominant bacterial populations of the original soil compared with those that existed in the larval midguts. The endosymbiotic bacterial community was dominated by members of the family *Vibrionaceae* (54.5%), followed by uncultured bacteria (18.2%), *Enterobacteriaceae* (9.1%), *Shewanellaceae* (9.1%), and *Cellulomonadaceae* (9.1%). Phylogenetic studies confirmed the affiliation of the dominant OTUs into specified families revealed by clustering of each phylotype to its corresponding clade. Relative frequency of each phylotype in larval midguts revealed predominance of *Vibrio furnissii* and *Vibrio navarrensis*, followed by uncultured bacterial spp., then *Cellulomonas hominis*, *Shewanella algae*, and *Citrobacter freundii*.

**Conclusion:** Analysis of metagenomic DNA for endosymbiotic bacterial communities from the midgut of *Oryctes* larvae showed strong selection of specific bacterial populations that may have a key role in digestion, as well as other benefits to the larvae of *O. agamemnon*. Determination of the distinct endosymbiotic community structure and its possible biological functions within the insect could provide us with basic information for future pest control research.

**Keywords:** *Oryctes agamemnon*, Endosymbionts, 16S rRNA gene, DGGE

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Background

Several insect pests attack date palm (Phoenix dactylifera L.) orchards, causing serious damage and economic losses. In many Arabian countries, three species of rhinoceros beetles, Oryctes (Coleoptera: Scarabaeidae), O. elegans, O. agamemnon and O. rhinoceros, are known to infest date palm orchards [1]. The most widespread is O. agamemnon, which is a root borer in its larval stage and a frond borer in the adult stage. The other two species, O. rhinoceros and O. elegans, are fruit stalk borers and can also act as root borers [2]. Oryctes spp. have a wide host range, attacking and causing serious damage and crop loss on many hosts, including date palm, coconut palm, betel nut, sago palm and oil palm [3].

Recently, Oryctes spp. have emerged as major pests of different date palm cultivars. In Saudi Arabia, both larvae and adults of Oryctes are particularly troublesome, especially during the establishment of young date palm orchards. The development time of the larval stage is long and may extend for several years in some species. The larvae feed on roots and rotten wood whereas the adults feed on nectar, plant sap and fruit [4-7].

Certain mutualists may influence host plant range and enable insect pests to modify plant physiology for their own benefit. There is increasing evidence for the role of microbial mutualistic symbioses in insect–plant interactions [8]. The horizontal transmission of mutualists among their host insects can be achieved through a route involving its host plant. Where this transmission occurs, the insect mutualist might either become a plant pathogen and damage the plant or change the way the plant interacts with its natural enemies and host competitors [9].

Insect intestinal tracts harbor rich communities of nonpathogenic microorganisms [10]. A single gut can harbor 10^5–10^9 prokaryotic cells [11] that have been affiliated to twenty-six phyla, at least for the insects studied to date. It is increasingly evident that insect microbiota are essential for normal growth and development [12]. It has been shown that about 65% of insects possess symbiotic bacteria. Wolbachia spp. is the most commonly reported genus [13-15]. The symbiotic relationship between bacteria and insects varies from being mutualistic and commensal to pathogenic [16,17]. Based on their role, intracellular symbionts in insects are classified as primary or secondary endosymbionts. Primary (obligate) symbionts are essential for the insect due to their role in nutrient supplementation, whereas secondary symbionts have a useful but not essential role for insect survival [18,19].

Insect endosymbionts are detected in specific organs referred to as bacteriomes or mycetomes, usually resulting in a strict vertical transmission from mother to offspring.

Understanding relationships between endosymbiotic bacteria and their insect hosts is not only relevant from an evolutionary view, but can also lead to the identification of new targets for insect pest control [20]. Since many of the relevant endosymbionts cannot be cultured, their functional characterization and/or identification has been difficult. Certain symbionts have been developed as biological control agents and were found to be effective against Chagas disease vectored by Rhodnius prolixus. In this example, the endosymbiotic organism, Rhodococcus rhodnii was genetically transformed to express an anti-trypanosomal output in the insect gut [21].

The date palm root borers of the genus Oryctes are regarded as devastating and invasive pests in a wide variety of palms worldwide. Little is known about the presence of endosymbionts in the genus Oryctes. Exploring bacteria-insect associations in this regard would be useful for potential insect pest control. For example, if obligate endosymbionts exist in Oryctes, then eliminating them using baits could be a potential control strategy. Investigation of endosymbiosis in this genus may help to understand the host-symbiont interactions and the evolution of different reproductive strategies in these beetles, and ultimately provide a future basis for development of novel pest management strategies. Therefore, the objective of this study was to analyze the diversity of the larval midgut microbiota of the date palm root borer, O. agamemnon.

Results and discussion

Endosymbionts of Oryctes agamemnon larvae

Microbial diversity is defined as the number of elements indicated by species or genes within a system [22]. Most of the microbial world within a system remains unexplored due to the existence of many uncultured bacteria species. Molecular-based approaches are useful for determining diversity of various bacterial populations [23-25]. Several molecular methods based on DNA analyses using polymerase chain reaction (PCR) followed by an analysis of the diversity of PCR products are available [26-28].

Polymerase chain reaction denatured gradient gel electrophoresis (PCR-DGGE) [26] in particular, has been regarded as a powerful genetic fingerprinting technique for evaluation of bacterial community structures in different environmental niches. PCR-DGGE analysis utilizing 16S rRNA genes usually yield patterns that reflect the composition of the dominant microorganisms, including the uncultured members [24]. DGGE has been widely used to investigate several bacterial patterns in soil [29], marine habitats [30], rhizosphere [31], grasslands [32], manure and fertilizers [33], and sites polluted with anthropogenic chemicals [34]. Bacterial diversity and community structure of insect endosymbiotic bacteria have not been investigated by DGGE previously. Therefore, we used DGGE in this study to investigate bacterial populations in the midgut of O.
O. agamemnon larvae. The DGGE patterns obtained with total community DNA from larval midguts showed predominance of eleven major OTUs (Figure 1). DGGE profile of metagenomes belonging to five larval midguts showed the same pattern, confirming a stable and intact endosymbiotic bacterial community structure. DGGE was also used to investigate the distribution pattern of soil bacteria in larva-infested soil. DGGE fingerprinting showed changes in the dominant bacterial populations of the original soil compared with those that existed in the midguts. This shift could be attributed to the strong selection of specific bacterial populations that may have a key role in insect nutrition. The consistency of such midgut endosymbionts suggests the presence of O. agamemnon-specific microbiota. Andert et al. [35] addressed the question whether or not the hindgut of the two closely related scarabs Pachyoda ephipiata and Pachyoda marginata, harbors a specific bacterial microbiota. Terminal restriction fragment length polymorphism (T-RFLP) analysis showed that in both species, the hindgut bacterial community strongly differs from that in the midgut, food soil, and fecal pellets. It was concluded that high intra- and interspecific similarities between the T-RFLP profiles of different larvae indicate the presence of a hindgut-specific microbiota.

**Endosymbiotic bacterial community structure**

Endosymbiotic bacterial community structure for insects in general based on culture dependent methods will remain uncertain. Culture-independent methods allow a deeper understanding of the composition of microbial communities in different ecosystems [23]. In this study we examined the endosymbiotic bacterial community structure of date palm root borer larvae with DGGE for rapid comparison of data from many communities and specific phylogenetic information derived from excised bands [27]. Sequence analysis of 16S rRNA gene fragments obtained from DGGE revealed the identity of the endosymbionts in the larval midgut of O. agamemnon. Based on BLAST best similarity matches, phylotypes frequently detected in fingerprinting analysis have been affiliated to Photobacterium sp., Vibrio sp., Allomonas sp., Shewanella sp., Cellulomonas sp., and Citrobacter sp., as well as some uncultured bacteria, including uncultured Vibrio sp. (Table 1). These bacteria might be responsible for palm tissue fermentation in the tunnels where O. agamemnon larvae thrive and might have a key role in the insect’s nutrition. Many of the Enterobacteriaceae produce digestive enzymes and therefore have a role in insect nutrition [36].

Gut bacteria have been reported to exert many useful functions, such as preventing disease, degrading insecticides, and directly or indirectly contributing to food digestion [15]. Food materials may be important in regulating the dynamics of the bacterial community within the insect gut. For example, S. marcescens is a facultative anaerobe that aids in consuming oxygen at the periphery of the Formosan termite’s stomach, thereby maintaining a habitable gut for the strict anaerobes that digest cellulose [37]. In addition to aiding digestion, Citrobacter detected in our study is believed to have the same role in establishing anaerobic conditions for the succession of Shewanella spp. involved in anaerobic fermentation of ingested materials.

Analysis of larval midgut bacterial populations in O. agamemnon revealed a predominance of members belonging to the genus Vibrio. Dominance of certain bacterial taxa as endosymbionts in some insects has been reported. Using sequence-based bacterial typing,
Hirsch et al. [17] identified bacterial endosymbionts in four species of *Otiorhynchus*. More than 90% of all sequence reads belonged to the genus *Rickettsia*. Tagliavia et al. [38] analyzed the gut microbiota of larvae of the red palm weevil. High abundance of Enterobacteriaceae was detected. Fujiwara et al. [39] surveyed symbiotic bacteria from *Bemisia tabaci* species and reported the dominance of *Rickettsia* in all examined whitefly species.

In contrast to our results with larvae, in a study of gut microbiota of adult *Oryctes monoceros* by Desai and Bhamre [40], a completely different microbial population, except for *Citrobacter*, was reported, and included *Dienococcus proteolyticus*, *Micrococcus varians*, *Micrococcus kristinae*, *Micrococcus roseus*, *Micrococcus lylae*, *Citrobacter amalonacticus*, *Corynebacterium xerosis* and *Bacillus fermentas*.

Cellulolytic bacteria are important for digestion of cellulosic materials. In our study, *Cellulomonas* sp. has been detected as a member of the *O. agamemnon* midgut bacterial population indicating its involvement in the digestion process. Huang et al. [41] isolated strains of aerobic and facultatively anaerobic cellulolytic bacteria

| DGGE band | Accession no. | Closest matches | Accession no. | Similarity (%) | Phyligenetic affiliation |
|-----------|---------------|----------------|---------------|----------------|-------------------------|
| 1         | LC009469      | *Photobacterium ganghwaense* FR1311 | NR043295      | 100            | Gammaproteobacteria/Vibrionaceae |
|           |               | *Photobacterium sp.* | AB583193      | 100            | Gammaproteobacteria/Vibrionaceae |
|           |               | *Photobacterium sp. RSBAUOCA50005B* | HM641040      | 100            | Gammaproteobacteria/Vibrionaceae |
| 2         | LC009470      | *Vibrio fluvialis* MBTD-CMFRI-Vf05 | KF317830      | 100            | Gammaproteobacteria/Vibrionaceae |
|           |               | *Vibrio sp. BTOK10* | JQ923505      | 100            | Gammaproteobacteria/Vibrionaceae |
|           |               | *Vibrio vulnificus* MP-4 | AY911393      | 100            | Gammaproteobacteria/Vibrionaceae |
| 3         | LC009471      | *Photobacterium ganghwaense* FR1311 | NR043295      | 100            | Gammaproteobacteria/Vibrionaceae |
|           |               | *Vibrio foris* HO83 | KJ577078      | 100            | Gammaproteobacteria/Vibrionaceae |
|           |               | Uncultured bacterium clone SWH04_PR | JQ480712      | 98            | Gammaproteobacteria/Vibrionaceae |
| 4         | LC009472      | Uncultured bacterium clone BT12G08 | KC208438      | 99            | Bacteria/Environmental sample |
|           |               | Uncultured bacterium clone SWG11_M5 | JQ480736      | 99            | Bacteria/Environmental sample |
|           |               | Uncultured bacterium clone nbw223h08c1 | KFO64992      | 99            | Bacteria/Environmental sample |
| 5         | LC009473      | *Allomonas enterica* JC102, D09-37 | FR837603      | 98            | Gammaproteobacteria/Vibrionaceae |
|           |               | Uncultured *Vibrio* sp. clone D004025FO4 | GU179548      | 98            | Gammaproteobacteria/Vibrionaceae |
|           |               | Uncultured bacterium clone LGH02-B-135 | HQ916550      | 98            | Gammaproteobacteria/Vibrionaceae |
| 6         | LC009474      | *Vibrio navarrensis* AM37820 | KJ807107      | 100            | Gammaproteobacteria/Vibrionaceae |
|           |               | *Vibrio navarrensis* 2544-86 | KJ807099      | 100            | Gammaproteobacteria/Vibrionaceae |
|           |               | *Vibrio navarrensis* 1397-6T | KJ807092      | 100            | Gammaproteobacteria/Vibrionaceae |
| 7         | LC009475      | *Vibrio sp. U15* | HF968434      | 100            | Gammaproteobacteria/Vibrionaceae |
|           |               | *Vibrio furnisi* (ATCC 35016T) | X74704        | 100            | Gammaproteobacteria/Vibrionaceae |
|           |               | Uncultured *Vibrio* sp. clone KR-SUC-9-A10 | AM183773      | 99            | Gammaproteobacteria/Vibrionaceae |
| 8         | LC009476      | *Shewanella algae* HS | KM007068      | 99            | Gammaproteobacteria/Shewanelleaceae |
|           |               | *Shewanella haloidis* NIOT-CS16 | KJ371072      | 99            | Gammaproteobacteria/Shewanelleaceae |
|           |               | *Shewanella sp. MPTDBS* | KJ796480      | 99            | Gammaproteobacteria/Shewanelleaceae |
| 9         | LC009477      | *Cellulomonas hominis* PuiC5.18 | LM0994741     | 99            | Actinobacteria/Cellulomonadaceae |
|           |               | *Cellulosimicrobium cellulans* 517 | KJ947163      | 99            | Actinobacteria/ Promicromonosporaceae |
|           |               | *Cellulomonas aerilata* JCM 16376 | AB910521      | 99            | Actinobacteria/Cellulomonadaceae |
| 10        | LC009478      | *Citrobacter freundii* C09 | KM222617      | 99            | Gammaproteobacteria/Enterobacteriaceae |
|           |               | *Citrobacter youngae* GTC 01314 | AB741661      | 99            | Gammaproteobacteria/Enterobacteriaceae |
|           |               | *Citrobacter muriniae* M-T-MRS_22 | JQ979823      | 99            | Gammaproteobacteria/Enterobacteriaceae |
| 11        | LC009479      | Uncultured bacterium clone SWH04_PR | JQ480712      | 98            | Bacteria/Environmental sample |
|           |               | Uncultured bacterium clone BT12G08 | KC208438      | 99            | Bacteria/Environmental sample |
|           |               | Uncultured bacterium clone SWG11_M5 | JQ480736      | 99            | Bacteria/Environmental sample |
from the gut of *Holotrichia parallela* (Coleoptera: Scarabaeidae) larvae. The cellulolytic bacterial community was dominated by Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes (1.45%). However, *Cellulomonas* sp. in particular, was not detected among this community.

**Diversity of *Oryctes agamemnon* endosymbionts**

The versatility and diversity of insect-bacteria interactions leads to an enormous potential regarding the mechanisms for the modulation and control of insect pests with both medical and agricultural implications [42]. Through TFLP analyses of bacterial rRNA extracted from the guts of *Harpalus pensylvanicus* and *Anisodactylus sanctaeclausi* (Coleoptera: Carabidae), Lundgren et al. [8] revealed that gut-associated bacterial communities were of low diversity. The bacterial community in these beetles comprised *Serratia* sp., *Burkholderia fungorum*, *H. alvei*, *Phenylbacterium* sp., *Caedibacter* sp., *Spiroplasma* sp., *Enterobacter* strain B-14, and *Weissella viridescens*. Some of these organisms, but not all have been previously associated with insects. However, none of them has been detected in *O. agamemnon*, suggesting that their larvae have a unique bacterial community. In comparison to previously reported insect microbiota, our study revealed low diversity and a highly unique pattern for *O. agamemnon* microbiota.

The midgut bacterial populations of *O. agamemnon* larvae were taxonomically restricted to two major groups, with 80% of the natural bacterial microbiota composed of only three bacterial families within Gammaproteobacteria. The dominant bacterial taxa are members of Vibrionaceae (54.5%), Enterobacteriaceae (9.1%), and Shewanellaceae (9.1%). In addition to Gammaproteobacteria, one family belonging to Actinobacteria was detected (Cellulomonadaceae (9.1%)). Host diet plays a major role in shaping the insect bacterial microbiota. Chandler et al. [43] found that Drosophilid flies have taxonomically restricted bacterial communities, with 85% of the natural bacterial microbiome composed of only a few bacterial families (Enterobacteriaceae, Lactobacillales and Acetobacteraceae). 18.2% from the total bacterial population was detected as uncultured bacterial members. Several indices, including species richness and evenness, are used to describe the structural diversity of a community [44]. (Figure 2A) shows the diversity and richness of bacterial species of *O. agamemnon* larvae compared with those of the soil where the larvae live. The reduction in diversity and richness of bacterial species of larvae compared with soil was attributed to the selection of specific bacterial populations that may have a key role in food digestion for the benefit to the larvae. (Figure 2B) shows the relative frequency of each bacterial species and the predominance of *Vibrio* spp. among the endosymbiotic bacterial population.

**Phylogenetic analysis**

The 16S rRNA genes are used for phylogenetic affiliation of Eubacteria and Archaea. Partial sequences of 16S rRNA gene of bacterial microbiota from the larval midgut of *O. agamemnon* have been analyzed. Sequences were compared with their closest matches with BLAST search tool to obtain the nearest phylogenetic neighbors. About 72.7% of the bacterial community was assigned to Gammaproteobacteria. The remainder of the bacterial community was assigned to Actinobacteria (9.1%) and uncultured bacterial members (18.2%). Bacteria belonging to Gammaproteobacteria were classified as members of three families: Vibrionaceae, Enterobacteriaceae, and Shewanellaceae, with predominance of the former. Actinobacteria comprised only one family, Cellulomonadaceae enclosing *Cellulomonas* sp.

Tagliavia et al. [38] analyzed the gut microbiota of larvae of the red palm weevil. They assigned 98% of the total population to only three phyla: Proteobacteria, Bacteroidetes, and Firmicutes, and three main families (Enterobacteriaceae, Porphyromonadaceae and Streptococaceae). Bacterial members have been identified as *Dysgonomonas*, *Lactococcus*, *Salmonella*, *Enterobacter*, *Budvicia*, *Entomoplasma*, *Bacteroides* and *Comamonas*. The major phylogenetic microbiota of the hindgut of *P. ephippiata* were identified through a 16S rRNA gene clone library and revealed that Clostridia, Betaproteobacteria, and Bacteroidetes, followed by Bacillales and Deltaproteobacteria, were dominant.

In this research, phylogenetic studies confirmed the affiliation of dominant OTUs from *O. agamemnon* midgut to members of four distinct families, Vibrionaceae, Shewanellaceae, Enterobacteriaceae, and Cellulomonadaceae, revealed by clustering of each individual member to its corresponding group. (Figure 3) represents the phylogenetic tree based on 16S rRNA sequences analysis and showing the relationship between selected dominant phylotypes (OTUs) and representative species, along with other related genera. According to phylogenetic analysis, six phylotypes have been assigned to the family Vibrionaceae including, DGGE-OTU 1, 2, 4, 5, 7, and 11. DGGE-OTU 6 was identified as *Vibrio* member and clustered at a separate phylogenetic branch with *Vibrio parahaemolyticus* indicating its close relation to that species in particular; and finally, DGGE-OTU 3 was specifically clustered with *Vibrio parahaemolyticus* H083 and *Vibrio* sp. S4. Family Shewanellaceae was found to contain only one species, DGGE-OTU 8, with 99% sequence similarity to *Shewanella* sp. and phylogenetically clustered with *Shewanella* spp. branch. The phylotype DGGE-OTU 9 was affiliated to the Actinobacteria and assigned to *Cellulomonas* sp. (99%) or *Cellulosimicrobium cellulans* S17 (99%). Enterobacteriaceae group was only represented by one phylotype, DGGE-OTU 10, that has been affiliated
to *Citrobacter* sp. Phylogenetic analysis confirmed its relation to Enterobacteriaceae members like *Enterobacter*, *Klebsiella*, and *Leclercia*. Members of Enterobacteriaceae have been reported as frequent endosymbionts. Campbell et al. [45] studied the phylogeny of symbiotic bacteria of four weevil species (Coleoptera: Curculionidae) and showed that symbionts from taxonomically divergent weevils are mainly members of the *Enterobacteriaceae*. Uncultured endosymbiotic bacteria were also detected in this study. DGGE OTU 4 and 11 were assigned to uncultured bacterial members.

**Conclusions**

In conclusion, endosymbiotic bacteria are known to be involved in protecting their host insect against natural antagonists, contributing to insecticide resistance mechanisms, and aiding in food digestion and are, therefore, essential for normal growth and development of their host insect. In this regard, endosymbiotic bacteria could be manipulated, potentially offering new approaches for insect control. Therefore, identification of endosymbiotic bacteria of *O. agamemnon* is an important step in this process. Metagenomic DNA from midguts of *Oryctes* larvae was analyzed for endosymbiotic bacterial communities. Except for the Enterobacteriaceae group, *Oryctes* larvae were found to harbor unique endosymbiotic bacteria when compared with previously reported microbiota. Such distinct microbial community structure and its possible biological function within the insect will provide us with basic information for development of pest control strategies utilizing intrinsic endosymbiotic bacteria. Finally, there is an ultimate question

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**Figure 2** Numerical analysis for the diversity of the *O. agamemnon* endosymbionts. (A), DGGE OTUs richness and Shannon diversity index (*H*) determined from DGGE fingerprints of endosymbiotic bacterial communities of larvae midguts (A, B, C, D, and E) compared with soil bacteria (S). (B), Relative frequency of each phylotype in larvae midguts.
Figure 3 (See legend on next page.)
we have to answer, what would be resulted in the absence (either intentional or accidental) of each single symbiont or a specific symbiotic group? If this question is correctly answered, this means a successful control strategy for this insect pest is achieved. Therefore, further studies are now required to clarify the biological function of these endosymbiotic bacteria in Oryctes larvae and their potential as novel targets for beetle control.

Methods

Insects

Oryctes agamemnon larvae were field-collected from a date palm orchard about 80 km north Almadinah Almawarah region, of Saudi Arabia at longitude (39°11'6") and latitude (24°47'6"). The 3rd larval instar was dominant in sampling. Samples of larvae were collected in sterilized plastic containers. The larvae were kept in the laboratory for one week prior to dissection to avoid possible infestations from the field and to reduce any potential insecticide residual effects. All stages were kept in plastic containers half-filled with soil and date palm pieces. The larvae were dissected in dissection trays containing 0.65% saline and the midguts were aseptically removed [46]. The midguts were homogenized in a sterile glass homogenizer containing 0.85% saline. The supernatant suspension was used for bacterial enrichment and DNA extraction. Each sample consisted of the content of three pooled midguts taken from three larvae of the same instar. Metagenomic DNA was extracted from soil infested with larvae for comparative purposes.

DNA extraction and PCR amplification of 16S rRNA genes

Total community DNA was extracted with the Ultra Clean Soil DNA purification kit (Mo Bio Laboratories, Solana Beach, Calif.). Harvested cells were transferred to bead beating tubes and vortexed horizontally for 1 min at room temperature. Supernatant was collected and DNA was precipitated and purified according to the instruction manual. Amplification of 16S rRNA genes for DGGE analysis was performed using GC-clamp primers (EUB341F-GC: 5’-CGCCCCGCGCCGCGGC GGGGGGGGGGGGGGGCTACGGAGGAG GCAGCAAGCATG-3’ and EUB517R: 5’-ATTACCGCGGC TGCTGG-3’) that correspond to positions 341 and 517 in Escherichia coli [47]. Amplification were performed in 25 μl reaction vessel containing: 2.5 μl of 10 × Taq buffer (100 mM Tris–HCl, pH 8), 1.25 mM MgCl2, 100 μM dNTPs (Invitrogen, USA), 1.2 μM forward primer and reverse primer set (Invitrogen, USA), 0.5U Taq DNA polymerase (Invitrogen, USA), and about 5 ng of template DNA. PCR was performed in Thermal Cycler (Applied Biosystems 2720, USA). A touchdown PCR program was implemented as follows: initial denaturation step at 95°C for 5 min; 5 cycles of 94°C for 40 sec, annealing at 65°C for 40 sec, and extension at 72°C for 40 sec; 5 cycles of 94°C for 40 sec, annealing at 60°C for 40 sec, and extension at 72°C for 40 sec; 10 cycles of 94°C for 40 sec, annealing at 55°C for 40 sec, and extension at 72°C for 40 sec; 10 cycles of 94°C for 40 sec, annealing at 50°C for 40 sec, and extension at 72°C for 40 sec were performed, followed by a final hold at 72°C for 7 min. Amplicons were analyzed by electrophoresis on 1% agarose gels with the size markers (1 kb DNA ladder, Invitrogen, USA) and visualized using ethidium bromide.

DGGE

DGGE was performed using Dcode Mutation Detection System (Bio-Rad Laboratories Ltd., Hertfordshire, UK). PCR products were electrophoresed with 0.5 × TAE buffer (1 × TAE buffer is 0.04 M Tris base, 0.02 M sodium acetate, and 10 mM EDTA [pH 7.4]) on 8% acrylamide gel containing 25 to 50% denaturing gradient of formamide and urea. DGGE was conducted at 60°C for 5 h at voltage of 200 V. The gel was stained with SYBR Green I Nucleic acid gel stain (Cambrex Bio Science Rockland, USA), photographed and analyzed for DGGE band profile with a UV gel documentation system (Bio-Rad Laboratories Inc., CA, USA).

Numerical analysis of the DGGE fingerprints

The DGGE fingerprints were analyzed using Quantity One 1D software (BioRad). The total number of DGGE bands was used to represent OTUs richness [48]. Bacterial diversity was estimated based on densitometric measurements and Shannon diversity index (H’) [48,49], Equation (1)

\[
H’ = - \sum P_i \ln P_i
\]

\[
P_i = n_i/N_i
\]

where \(P_i\) is a relative intensity of DNA band in the fingerprint, \(n_i\) is densitometrically measured intensity of individual DNA band, and \(N_i\) is the total amount of
DNA in the fingerprint. The relative intensity of each band (Pi) was used to express the relative frequency of each phylotype [50].

Sequencing of DGGE bands
Dominant DGGE bands were cut off with a sterile scalpel and eluted by incubation in 100 μl of TE buffer at 100°C for 5 min. Supernatant was used as template for PCR amplification. Reamplification of 16S rRNA genes from excised DNA fragments was performed using bacterial primers EUB314F without GC clamp and EUB517R. Amplification was verified by electrophoresis on 1% agarose gel. PCR products were directly sequenced using a BigDye terminator cycle sequencing [51] at GenoScreen sequencing facility (Genoscreen, Lille, France).

Sequence analysis
The sequences obtained from the 16S rRNA genes were analyzed by Genetyx-Win MFC application software version 4.0. The reference 16S rRNA gene sequences were retrieved from the GenBank database (National Center for Biotechnology Information, National Library of Medicine, USA) [52]. Sequences were compared with their closest matches in GenBank with nucleotide-nucleotide BLAST to obtain the nearest phylogenetic neighbors (www.ncbi.nlm.nih.gov/BLAST/). Sequence alignments were performed by Clustal W 1.83 XP [53] and phylogenetic trees were constructed using neighbor-joining method [54] using MEGA6 software [55].

Accession numbers and data deposition
The 16S tRNA sequences identified in this study have been deposited in the GenBank database under the accession numbers LC009469 to LC009479. The data of the phylogenetic analysis are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.59h51.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
WE carried out the microbiological and molecular analyses, performed the bioinformatics analyses and constructed the phylogeny trees. RI identified and collected the insects in the field and manipulated them for the gut microbiota analyses. Both authors wrote, read and approved the final manuscript.

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