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ORIGINAL ARTICLE

The internal bacterial diversity of stored product pests

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Abstract Symbiotic bacteria associated with insects play important roles in different physiological processes such as digestion, insect behavior, defense and providing essential nutrition in insect gut. In addition, these bacteria can be used in biocontrol of insect pests using genetic engineering techniques. The first step is to isolate and identify symbiotic bacteria from insects to elucidate their roles, and to use in the development of transgenic strains. For this purpose, we isolated and characterized the bacterial isolates from stored product pests using a combination of conventional tests and 16S rRNA sequence analysis. The bacterial flora of *Callosobruchus maculatus* included *Bacillus pumilus*, *Staphylococcus* sp. and *Pantoea* sp. *Acanthoscelides obtectus* flora included *Staphylococcus kloosii*, *Staphylococcus* sp., *S. saprophyticus* and *Enterococcus faecalis*. The internal flora of *Sitotroga cerealella* included *Staphylococcus succinus*, *Enterococcus* sp. and *Staphylococcus* sp. Finally, *Phthorimaea operculella* flora included *Bacillus* sp., *Staphylococcus sciuri*, *Enterococcus mundtii*, *E. casseliflavus*, *Alcaligenes faecalis*, *Enterobacter* sp., *Pantoea agglomerans* and *Pseudomonas fluorescens*.

Keywords Stored product pests · Symbiosis · Bacteria · Microbial control

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Introduction

Many insect species infest food and non-food products which originate from plant and animal. Collectively, this group of insects is referred to as stored product pests (Suiter et al. 2014). Most are small beetles or moths such as *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae), *Callosobruchus maculatus* (F) (Coleoptera: Bruchidae), *Sitotroga cerealella* (Oliv) (Lepidoptera: Gelechiidae) and, *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) (Özdem 1997; Coll et al. 2000; Cope and Fox 2003; Akter et al. 2013; Suiter et al. 2014). These pests cause significant quantitative and qualitative losses to the multibillion dollar grain, food, and retail industries each year through their feeding, product adulteration, customer complaints, product rejection at the time of sale, and costs associated with their management (Hagstrum and Subramanyam 2009). Besides their direct damage to food, the presence of these insects has the potential to vector disease organisms. Many stored grain insects possess hairs and indentations on their exoskeletons that can act as mechanical vectors of pathogens. Maize weevils have been shown to carry numerous fungi species, including *Aspergillus niger* Van Tieghem, *A. glaucus* (L.), *A. candidus* (L.), *Penicillium islandicum* Sopp, *P. citrinum* Thom, *Paecilomyces, Acremonium, Epicoccum, Fusarium semitectum* Berkeley & Ravenel and yeasts (Dix 1984; Mason and McDonough 2012).

Control of stored product pests is necessary to prevent the damage of these pests, such as boring, feeding and mold germination (Mason and McDonough 2012). Up to now, many control strategies such as physical control, inert dust, ionizing irradiation, light and sound, thermal control, ozonation, fumigation, semiochemicals and some kinds of repellents have been applied to prevent the damage to food (El-Aziz 2011). Many available chemical insecticides are not advised for

controlling stored product pests since the residues of the chemicals cause health problems when eaten by human beings or livestock (De Groot 2004; Matthias 2009). This has led to an increased interest in alternative control options, including biological control by mass-reared natural enemies such as parasitoids and predators of insect pests and microbial control agents (also called microbial control), such as bacteria, fungi, viruses, nematodes and protozoa (Khetan 2001; Prozell and Schöller 2002; Matthias 2009).

Microbial control agents for insect pests have gained a great importance in recent years since they have many advantages with respect to environmental benefits, including safety for humans and other non-target organisms, reduction of pesticide residues in food, increased activity of most other natural enemies, and increased biodiversity in managed ecosystems (Khetan 2001; Lacey et al. 2001). Up to now, there have been some basic studies on microbial control of stored product pests. Sabbour (2003) tested two microbial entomopathogens [*Bacillus thuringiensis* and *Beauveria bassiana* (Balsamo) Vuillemin] and three botanical extracts against *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), *Ephestia cautella* (Walk.) (Lepidoptera: Phycitidae) and, *E. kuehniella* (Zeller) (Lepidoptera: Pyralidae). Botanical extracts combined with *B. thuringiensis* caused a significant mortality in almost all cases. Buda and Peciulyte (2008) showed that four entomopathogenic fungal species [*B. bassiana* (Balsamo) Vuillemin, *Verticillium lecanii* (Zimmerman) Viegas, *Metarhizium anisopliae* var. *anisopliae* (Metchnikoff) Sorokin and *Isaria farinosa* (Holm) Brown & Smith (formerly *Paecilomyces farinosus*)] were pathogenic to *Plodia interpunctella*. Sabbour and Abd El-Aziz (2010) mentioned that the efficacy of oil-formulated *Isaria fumosorosea* Wize (formerly *Paecilomyces fumosoroseus*) and *Nomuraea rileyi* (Farlow) Samson against *Bruchidius incarnatus* (Boh.) (Coleoptera: Bruchiade) resulted in reducing ovoposition and adult emergence.

Interactions between insects and bacteria may be symbiotic or pathogenic (Sanchez-Contreras and Vlisidou 2008). Until now, numerous bacteria have been isolated, classified and demonstrated in the laboratory to be pathogenic for various insects. Most of these bacteria are classified in the families of Pseudomonadaceae, Enterobacteriaceae, Lactobacillaceae, Micrococcaceae and Bacillaceae (Bulla et al. 1975). Among the known bacterial pathogens, *Bacillus thuringiensis* (*Bt*) is the most successful and holds considerable potential for further development. *Bt* has been used to control many insect pests worldwide (Burges 1982; Sevim et al. 2012a). Apart from bacterial pathogens, the bacterial symbionts of insects could be used in microbial control of insect pests and they are possible contenders for future pest control (Dillon and Dillon 2004; Demirci et al. 2013). The gut bacteria of insects can be modified using genetic engineering techniques, and different characteristics could be imparted to these bacteria (Sevim et al. 2012a, b). Kuzina et al. (2002) showed that *Enterobacter gergoviae* isolated from the gut microbiota of the pink bollworm was transformed to express *Cyt*1A. Medina et al. (2009) also showed that fire ant midgut bacteria could be transformed using pZeoDsRed shuttle vector, which can be served for the expression of insect toxic proteins. Additionally, it has been demonstrated that *Rhodococcus rhodnii*, which is a bacterial symbiont of the Chagas' disease vector, was transformed to express an anti-trypanosomal agent in the gut (Beard et al. 1992). Moreover, Watanabe et al. (2000) demonstrated that the transgenic strain of *Enterobacter cloacae*, which expresses the ice-nucleation gene (*inaA*), caused the increased mortality of colonized insects. Another approach for using symbiotic bacteria in the control of insect pests is based on the dynamics between bacterial species in the insect gut (Ishikawa 2003). Harada and Ishikawa (1997) used bacterial species which were isolated from the gut of *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae) (the pea aphid) and grown on plates, and pea aphids were fed on these bacteria by mixing them in a synthetic diet. As a result, *Erwinia aphidicola* and *E. herbicola* could grow in the aphid gut to kill the hosts, although they are the predominant species of the aphid gut flora. This suggests that the vigorous growth of *E. aphidicola* is suppressed by the other enterobacteria in the gut (Harada and Ishikawa 1997).

Up to now, studies of the internal bacterial flora of insects have been performed using different kinds of insects belonging to the order of Lepidoptera, Coleoptera, etc. (Mrazek et al. 2008; Yaman et al. 2010; Sevim et al. 2010, 2012a, b; Demirci et al. 2013). However, there has been no study to determine the internal bacterial flora of stored product pests. Therefore, in this study, we aimed to determine the culturable bacterial flora of stored product pests for use in future biocontrol programs of these pests.

Material and methods

Collection and rearing of insects

Stored product pests (*Acanthoscelides obtectus*, *Callosobruchus maculatus*, *Sitotroga cerealella* and *Phthorimaea operculella*) used in this study were obtained from a variety of homes and warehouses in the vicinity of Rize, Turkey, in 2011 and 2012. The obtained insects and larvae were brought to the laboratory in clean plastic boxes and fed with the proper nutrients such as beans, kidney beans, corn and potatos until the isolation of bacteria was performed.

Isolation of bacteria

The studies of bacterial isolation were carried out on both larvae and adults of *Acanthoscelides obtectus* and

Callosobruchus maculatus. The bacterial isolation was performed on only larvae of *Sitotroga cerealella* and *Phthorimaea operculella*. First of all, ten larvae and adults from *Acanthoscelides obtectus* and *Callosobruchus maculatus*, and ten larvae from *Sitotroga cerealella* and *Phthorimaea operculella* were separately surface-sterilized with 70 % ethanol for 5 min and then washed 2–3 times with sterile distilled water in order to remove any bacteria on the surface of the insects (Lipa and Wiland 1972). After that, larvae and adults which were surface-sterilized were transferred to glass test tubes (10 mL) including 2 mL nutrient broth (Difco, NJ, USA) and then homogenized using a glass tissue grinder, following filtration of homogenates through two layers of sterile muslins to remove insect debris. Each homogenate was diluted from 10^{-1} to 10^{-8}, and 100 μL from these dilutions were spread on nutrient agar plates. Next, the remaining homogenates were heated at 80 °C to eliminate non-spore-forming bacteria, and 100 μL from the heated suspensions were spared on nutrient agar plates. Plates were incubated at 30 °C for 2–3 days, and the growing colonies were selected according to color and colony morphology. Each colony was streaked on the sterile nutrient agar plates, and incubated at 30 °C overnight and for 48 h for slow-growing isolates. Finally, the pure bacterial isolates were stored in 20 % glycerol at −80 °C for further studies. The bacterial isolates were characterized based on their morphological, biochemical and physiological properties using Bergey's *Manual of Systematic Bacteriology*, volumes 1 and 2 (Krieg and Holt 1986; Sneath et al. 1986). Additionally, the isolates were identified by 16S rRNA sequence analysis to confirm the morphological characterization of the isolates. All isolates identified in this study are public-accessible and were deposited at Microbiology Laboratory, Department of Biology, Recep Tayyip Erdoğan University, Rize, Turkey.

Characterization of the bacterial isolates

Phenotypic characterization of the bacterial isolates was performed by direct observations (colony morphology on nutrient agar) and using a various staining techniques such as Gram staining, endospore stains and capsule staining (Claus 1992; Prescott et al. 1996). Following staining, the bacterial perpetrates were inspected by a light microscope at ×1000 magnification. The motility was tested in semisolid medium according to Soutourina et al. (2001).

Tests of KIA, indol, catalase, oxidase, citrate, urease, nitrate and gelatinase were performed based on the Bergey's *Manual of Systematic Bacteriology*, volumes 1 and 2 (Krieg and Holt 1986; Sneath et al. 1986). Additionally, the activity of chitinase, lipase, proteinase, amylase and cellulose were determined (Teather and Wood 1982; Kouker and Jaeger 1987; Sandallı et al. 2008; Yu et al. 2009). Temperature, pH, and NaCl tolerance tests were also performed in LB broth to determine physiological properties of the isolates.

Antimicrobial susceptibility testing

The susceptibilities of the bacterial isolates to the various antibiotics were determined by the standard disk diffusion method based on the guidelines of National Committee for Clinical Laboratory Standards (NCCL Standards 1997). Mueller Hinton Agar (MHA; Merck, Germany) was used as the growth medium. The results were evaluated by using the breakpoints in the same guidelines. The antibiotic disks (Oxoid, UK) of gentamycin (10 μg), amoxicillin (25 μg), tetracycline (30 μg), rifamycin (30 μg), ampicillin (10 μg), sulfamethoxazole (25 μg), kanamycin (30 μg), erythromycin (15 μg), neomycin (30 μg), cephalothin (30 μg), ciprofioxacin (5 μg), chloranphenicol (30 μg) and ceftriaxone (30 μg) were used for both Gram-positive and Gram-negative bacteria. Streptomycin (10 μg), norfloxacin (10 μg), optochin (5 μg), vancomycin (30 μg), methicillin (5 μg), oxacillin (1 μg) and novobiocin (30 μg) were used for only Gram-positive bacteria. Amikasin (30 μg) and ceftazidime (30 μg) were used for only Gram-negative bacteria.

Gene sequencing

Total genomic DNA of the bacterial isolates was extracted according to the standard phenol and chloroform protocol of Sambrook et al. 1989. The extracted DNAs were dissolved in 50 μL Tris-EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and stored at −20 °C until performing PCR.

An approximately 1400 bp of the 16S rRNA gene region of the bacterial isolates was amplified using the primer pairs of 27F (5′-AGAGTTTGATCMTGGCTCAG-3′ as forward) and 1492R (5′-GGYTACCTTGTTACGACTT-3′) as reverse (MACROGEN). PCR reactions were carried out in a 50-μL reaction volume containing 1.5 μL 10 mM dNTP mix, 5 μL 10× *Taq* DNA polymerase reaction buffer, 1 μL 5 U/μL of *Taq* DNA polymerase (Fermentas), 1.5 μL 10 pmol each of the opposing primers, 3 μL MgCl2, 1 μL genomic DNA, and 35.5 μL dH_2O. The PCR reactions was started with 2 min initial denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 1 min, extension at 72 °C for 1.5 min. Finally, the reactions were extended 72 °C for 10 min. The PCR products were analyzed using 1 % agarose gels stained with ethidium bromide, and viewed under UV light. The right PCR products were sequenced using a primer pairs of 518F (5′-CCAGCAGCCGCGGTAATACG-3′) and 800R (5′-TACCAGGGTATCTAATCC-3′) (MACROGEN, The Netherlands). The sequences obtained were used to perform BLAST searches using the NCBI GenBank database (Altschul et al. 1990).

Phylogeny

16S rRNA sequences were initially edited using Bioedit, and then all sequences including the sequences of the related bacterial species from GenBank database were aligned using a multiple-sequence alignment procedure of the Clustal W package in Bioedit (Thomson et al. 1994; Hall 1999). The alignment was refined by eye. Phylogenetic analysis was performed using neighbor-joining phylogenies with p-distance correction, gap omission, and 1.000 bootstrap pseudoreplicates (Felsenstein 1985; Saitou and Nei 1987). All analyses were performed with MEGA 6.0 phylogenetic software (Tamura et al. 2013).

GenBank accession numbers

The 16S rRNA gene sequences for the bacterial isolates isolated during this study have been deposited in GenBank, and have accession numbers from KJ888101 to KJ888143.

Results

A total of 43 bacterial isolates (11 of them from *Callosobruchus maculatus*, 11 of them from *Acanthoscelides obtectus*, 6 of them from *Sitotroga cerealella* and 15 of them from *Phthorimaea operculella*) were isolated from stored product pests, and they were identified using a number of conventional tests and 16S rRNA gene sequencing. Among *C. maculatus* isolates, only one (Be-11) was isolated from larvae and others were isolated from adult individuals. While two isolates (Be-7 and Be-9) caused precipitation in nutrient broth, others caused turbidity. Two isolates (Be-5 and Be-9) were motile while others were not motile. Although only two isolates (Be-5 and Be-9) were Gram-negative, others were Gram-positive. Among *A. obtectus* isolates, three (Fbe-9, Fbe-10 and Fbe-11) were isolated from larvae, others from adults. All isolates lack motility, a capsule and caused turbidity in nutrient broth after growth. All isolates were Gram-positive and their cell shapes were coccus. All isolates from *S. cerealella* were Gram-positive, obtained from larvae and caused turbidity in nutrient broth. Colonies of all isolates were smooth and their cell shapes were coccus. Only one isolate (Ml-1) had a capsule. Other characters varied depending on the isolate. All isolates from *P. operculella* were obtained from larvae. Only two isolates (Pb-1 and Pb-12) formed a capsule. Other morphological characters varied depending on the isolate (Table 1).

In terms of biochemical tests, all isolates of *C. maculatus* were catalase-positive, and indol- and coagulase-negative. No isolates formed H_2S or gas in KIA medium. Only one isolate (Be-9) was citrate-positive and produced chitinase. Among *A. obtectus* isolates, none of them formed H_2S or gas in KIA medium, and were methyl red-positive, and Voges–Proskauer-, citrate-, gelatinase-, indol-, coagulase-, and chitinase-negative. Only one isolate (Fbe-8) was oxidase-positive. Among *S. cerealella* isolates, no isolates formed H_2S or gas in KIA, and all of them were citrate-, nitrate reduction-, indol-, coagulase-, cellulose-, amylase- and chitinase-negative. Only one isolate (Ml-6) was Voges–Proskauer-positive. While Ml-1 was oxidase-positive, Ml-3 was gelatinase-positive. Only one isolate (Ml-5) produced lipase. As for *P. operculella* isolates, all of them were indol-negative. While Pb-13 was Voges–Proskauer-positive, Pb-1 was amylase-positive. Other biochemical properties of isolates were variable (Table 2).

All bacterial strains isolated from four stored product pests can grow in nutrient broth including 3 and 5 % NaCl, except for Pb-11 from *P. operculella*. While it could grow in 3 % NaCl, it could not grow in 5 % NaCl. All isolates of *C. maculatus* could tolerate 7, 10 and 12 % NaCl concentrations in nutrient broth, except for Be-7 which could not grow in 12 % NaCl. All *A. obtectus* and *S. cerealella* isolates (Fbe isolates) can tolerate from 3 to 15 % NaCl concentrations. The ability to grow in various NaCl concentrations of *P. operculella* isolates was variable. None of the bacterial isolates used in this study could grow in nutrient broth with pH 3.0 and 4.0, while all of them can showed growth in pH 6.0, 7.0 and 8.0, except for Pb-11 which could not grow in pH 8.0. All *C. maculatus* isolates can also grow in pH 9.0, while all *A. obtectus* and *S. cerealella* isolates can grow from pH 5.0 to 12.0. The ability to tolerate pH of other isolates was variable. While the ability of *C. maculatus* and *P. operculella* isolates to grow at 10 °C was variable, all isolates of *A. obtectus and S. cerealella* can grow at 10 °C. All isolates of *A. obtectus*, *S. cerealella* and *P. operculella* can grow at 15 °C. Also, all isolates used in this study can grow at 30 and 37 °C. Only Be-3, Be-4, Be-8, Be-9, Be-10 and Be-11 isolates from *C. maculatus* could grow at 55 °C (Table 3). Antibiotic resistance profiles of the bacterial isolates were variable depending on the isolate and are given in Table 4.

We also performed the partial sequencing of 16S rRNA gene for all bacterial isolates. We used these sequences to carry out phylogenetic analysis using all isolates from this study and their closely related bacterial strains from GenBank to confirm conventional identification of isolates. All accession numbers are shown in Table 5. Phylogenetic analyses also supported conventional tests (Figs. 1, 2, 3, 4, 5). Based on all identification studies, the bacterial isolates were identified as shown in Table 5.

Table 1 The cultural and cell properties of the bacterial isolates

Isolate code	Colony color	Colony shape	Shape of bacteria	Gram stain	Spore stain	Capsule	Motility	Turbidity in NB[a]	Source
C. maculatus									
Be-1	Cream	Rough	Bacil	+	+	+	−	Turbid	Adult
Be-2	Cream	Smooth	Bacil	+	+	−	−	Turbid	Adult
Be-3	Cream	Smooth	Coccobacil	+	+	−	−	Turbid	Adult
Be-4	Orange	Smooth	Coccus	+	−	−	−	Turbid	Adult
Be-5	Yellow	Smooth	Bacil	−	−	−	+	Turbid	Adult
Be-6	Cream	Smooth	Coccus	+	−	−	−	Turbid	Adult
Be-7	Cream	Smooth	Bacil	+	+	+	−	Precipitation	Adult
Be-8	Light orange	Rough	Coccus	+	−	−	−	Turbid	Adult
Be-9	Yellow	Rough	Bacil	−	−	−	+	Precipitation	Adult
Be-10	Cream	Smooth	Bacil	+	+	+	−	Turbid	Adult
Be-11	Cream	Smooth	Bacil	+	+	−	−	Turbid	Larvae
A. obtectus									
Fbe-1	Cream	Mucoid	Coccus	+	ND[b]	−	−	Turbid	Adult
Fbe-2	Cream	Mucoid	Coccus	+	ND	−	−	Turbid	Adult
Fbe-3	Cream	Rough	Coccus	+	ND	−	−	Turbid	Adult
Fbe-4	Cream	Mucoid	Coccus	+	ND	−	−	Turbid	Adult
Fbe-5	Orange	Mucoid	Coccus	+	ND	−	−	Turbid	Adult
Fbe-6	Cream	Mucoid	Coccus	+	ND	−	−	Turbid	Adult
Fbe-7	Clear	Mucoid	Coccus	+	ND	−	−	Turbid	Adult
Fbe-8	Cream	Smooth	Coccus	+	ND	−	−	Turbid	Adult
Fbe-9	Cream	Rough	Coccus	+	ND	−	−	Turbid	Larvae
Fbe-10	Cream	Smooth	Coccus	+	ND	−	−	Turbid	Larvae
Fbe-11	Cream	Mucoid	Coccus	+	ND	−	−	Turbid	Larvae
S. cerealella									
Ml-1	Yellow	Smooth	Coccus	+	ND	+	−	Turbid	Larvae
Ml-2	Light orange	Smooth	Coccus	+	ND	−	−	Turbid	Larvae
Ml-3	Orange	Smooth	Coccus	+	ND	−	−	Turbid	Larvae
Ml-4	Clear cream	Smooth	Coccus	+	ND	−	+	Turbid	Larvae
Ml-5	Cream	Smooth	Coccus	+	ND	−	−	Turbid	Larvae
Ml-6	Cream	Smooth	Coccus	+	ND	−	+	Turbid	Larvae
P. operculella									
Pb-1	White	Smooth	Bacil	+	+	+	+	Precipitation	Larvae
Pb-2	Orange	Smooth	Coccus	+	−	−	+	Turbid	Larvae
Pb-3	Cream	Smooth	Coccus	+	−	−	+	Turbid	Larvae
Pb-4	Cream	Smooth	Coccus	−	−	−	−	Turbid	Larvae
Pb-5	White	Smooth	Coccus	+	−	−	+	Turbid	Larvae
Pb-6	White	Smooth	Coccus	+	−	−	+	Turbid	Larvae
Pb-7	Cream	Smooth	Coccus	−	−	−	−	Turbid	Larvae
Pb-8	Cream	Smooth	Coccus	+	−	−	+	Turbid	Larvae
Pb-9	Yellow	Smooth	Coccus	+	−	−	+	Turbid	Larvae
Pb-10	Cream	Smooth	Coccobacil	−	+	−	−	Turbid	Larvae
Pb-11	Yellow	Rough	Bacil	−	+	−	−	Turbid	Larvae
Pb-12	Cream	Smooth	Bacil	+	+	+	+	Precipitation	Larvae
Pb-13	Cream	Smooth	Coccus	+	−	−	+	Turbid	Larvae
Pb-14	Cream	Rough	Bacil	−	+	−	−	Precipitation	Larvae
Pb-15	Cream	Rough	Coccobacil	−	+	−	−	Precipitation	Larvae

[a] Nutrient Broth

[b] No data

Table 2 The biochemical and enzymatic characterization of the bacterial isolates based on conventional tests

Isolate	Test[a]																
	KIA	MR	VP	C	O	U	CI	NR	GH	I	CO	P	CE	A	L	CHI	LE
C. maculatus																	
Be-1	Basic/Basic/−/−	−	+	+	−	−	−	−	+	−	−	+	+	−	+	−	+
Be-2	Basic/Basic/−/−	−	+	+	−	−	−	−	+	−	−	+	+	−	+	−	+
Be-3	Basic/Basic/−/−	−	+	+	+	−	−	−	+	−	−	+	+	−	+	−	+
Be-4	Acid/Acid/−/−	+	−	+	−	+	−	−	+	−	−	+	−	−	+	−	−
Be-5	Basic/Acid/−/−	+	+	+	−	−	−	+	−	−	−	−	−	+	−	−	−
Be-6	Acid/Acid/−/−	+	−	+	−	−	−	+	−	−	−	−	−	+	−	−	−
Be-7	Basic/Basic/−/−	−	+	+	+	+	−	−	+	−	−	+	−	+	+	−	+
Be-8	Acid/Acid/−/−	+	−	+	−	+	−	−	−	−	−	+	−	−	+	−	−
Be-9	Acid/Acid/−/−	+	+	+	−	−	+	+	−	−	−	+	+	−	−	+	−
Be-10	Basic/Basic/−/−	−	+	+	+	−	−	−	+	−	−	+	−	−	+	−	+
Be-11	Basic/Basic/−/−	−	+	+	−	−	−	−	+	−	−	+	−	−	+	−	+
A. obtectus																	
Fbe-1	Basic/Basic/−/−	+	−	+	−	+	−	−	−	−	−	−	+	−	+	−	ND[b]
Fbe-2	Acid/Acid/−/−	+	−	+	−	+	−	+	−	−	−	+	+	−	+	−	ND
Fbe-3	Acid/Acid/−/−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	ND
Fbe-4	Basic/Basic/−/−	+	−	+	−	+	−	−	−	−	−	−	+	+	+	−	ND
Fbe-5	Acid/Acid/−/−	+	−	+	−	+	−	+	−	−	−	+	+	−	+	−	ND
Fbe-6	Acid/Acid/−/−	+	−	+	−	+	−	+	−	−	−	−	−	−	+	−	ND
Fbe-7	Basic/Basic/−/−	+	−	−	−	+	−	−	−	−	−	−	−	−	+	−	ND
Fbe-8	Acid/Acid/−/−	+	−	−	+	−	−	−	−	−	−	+	−	−	−	−	ND
Fbe-9	Acid/Acid/−/−	+	−	+	−	+	−	+	−	−	−	+	+	−	−	−	ND
Fbe-10	Basic/Basic/−/−	+	−	+	−	+	−	−	−	−	−	−	+	−	+	−	ND
Fbe-11	Acid/Acid/−/−	+	−	+	−	+	−	+	−	−	−	+	−	−	+	−	ND
S. cerealella																	
Ml-1	Acid/Basic/−/−	+	−	+	+	−	−	−	−	−	−	+	−	−	−	−	ND
Ml-2	Acid/Acid/−/−	+	−	+	−	+	−	−	−	−	−	+	−	−	−	−	ND
Ml-3	Acid/Basic/−/−	+	−	+	−	−	−	−	+	−	−	+	−	−	−	−	ND
Ml-4	Acid/Acid/−/−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	ND
Ml-5	Basic/Acid/−/−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	ND
Ml-6	Acid/Acid/−/−	+	+	−	−	−	−	−	−	−	−	+	−	−	−	−	ND
P. operculella																	
Pb-1	Basic/Basic/−/−	+	−	+	+	−	−	−	−	−	−	+	+	+	+	−	+
Pb-2	Acid/Acid/−/−	+	−	+	−	−	−	+	−	−	−	+	−	−	−	−	−
Pb-3	Acid/Acid/−/−	+	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−
Pb-4	Basic/Basic/−/−	−	−	+	+	−	+	−	−	−	−	−	−	−	−	−	−
Pb-5	Basic/Basic/+/−	−	−	−	+	+	+	+	−	−	+	−	−	−	−	+	−
Pb-6	Acid/Acid/−/−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Pb-7	Acid/Acid/−/−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Pb-8	Acid/Acid/−/−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Pb-9	Acid/Acid/−/−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Pb-10	Basic/Basic/−/−	−	−	+	+	+	+	+	−	−	+	−	−	−	−	+	+
Pb-11	Basic/Basic/−/+	−	−	−	+	+	+	−	+	−	−	+	−	−	−	+	+
Pb-12	Basic/Basic/−/−	+	−	+	−	−	−	−	−	−	−	+	+	−	+	−	+
Pb-13	Acid/Acid/−/−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Pb-14	Basic/Basic/−/+	−	−	+	+	+	+	−	+	−	−	+	−	−	−	+	+
Pb-15	Basic/Basic/−/+	−	−	+	+	+	+	−	+	−	−	+	−	−	−	+	+

[a] *KIA* (Deep/surface/H_2S/gas); *MR* methyl red test; *VP* Voges–Proskauer; *C* catalase; *O* oxidase; *U* hydrolysis of urea; *CI* citrate; *NR* nitrate reduction; *GH* gelatin hydrolysis; *I* indol; *CO* coagulase; *P* protease, *CE* cellulose, *A* amylase, *L* lipase, *K* chitinase, *LE* lesitinase

[b] *ND* No data

Table 3 The physiological characterization of the bacterial isolates; Luria–Bertani broth was used as growth medium

Isolate	Growth																					
	NaCl (%)						pH									Temperature (°C)						
	3	5	7	10	12	15	3	4	5	6	7	8	9	10	12	10	15	30	37	45	50	55
C. maculatus																						
Be-1	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Be-2	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	−	−	+	+	+	+	−
Be-3	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Be-4	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Be-5	+	+	+	+	+	+	−	−	+	+	+	+	+	−	−	+	+	+	+	+	+	−
Be-6	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Be-7	+	+	+	+	−	−	−	−	+	+	+	+	+	−	−	−	−	+	+	+	+	−
Be-8	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Be-9	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Be-10	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+
Be-11	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A. obtectus																						
Fbe-1	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Fbe-2	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Fbe-3	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Fbe-4	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Fbe-5	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Fbe-6	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Fbe-7	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Fbe-8	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Fbe-9	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Fbe-10	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Fbe-11	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
S. cerealella																						
Ml-1	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Ml-2	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Ml-3	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Ml-4	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Ml-5	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Ml-6	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
P. operculella																						
Pb-1	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−
Pb-2	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−
Pb-3	+	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Pb-4	+	+	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−
Pb-5	+	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−
Pb-6	+	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Pb-7	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−
Pb-8	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+	−	+	+	+	+	−	−
Pb-9	+	+	+	−	−	−	−	−	+	+	+	+	+	+	+	−	+	+	+	+	−	−
Pb-10	+	+	+	−	−	−	−	−	+	+	+	+	+	+	+	−	+	+	+	−	−	−
Pb-11	+	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−	+	+	+	−	−	−
Pb-12	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−
Pb-13	+	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−
Pb-14	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−	+	+	+	+	−	−	−
Pb-15	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−	+	+	+	+	−	−	−

Table 4 Antibiotic resistance profiles of the bacterial isolates

Isolate	Antibiotic																				
	GN[a]	AX	TE	RF	A	SXT	K	E	N	KF	CPR	C	CRO	S	NOR	OP	VA	ME	OX	NV	AK	CAZ
C. maculatus																						
Be-1	S[b]	S	S	S	S	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S	–	–
Be-2	S	R	S	S	S	S	S	S	S	S	R	S	R	S	S	R	S	R	S	S	–	–
Be-3	S	R	S	S	S	S	S	S	S	R	R	S	R	S	S	R	S	R	S	S	–	–
Be-4	S	S	S	S	S	S	S	R	S	S	R	S	R	S	S	R	S	R	S	S	–	–
Be-5	S	R	S	R	S	S	S	S	S	S	R	S	R	S	S	R	S	R	R	S	–	–
Be-6	S	R	S	S	S	S	S	R	S	R	R	S	S	–	–	–	–	–	–	–	S	R
Be-7	S	R	S	S	S	S	S	S	S	R	R	S	R	S	S	R	S	R	R	S	–	–
Be-8	S	R	S	S	S	S	S	S	S	S	R	S	R	S	S	R	S	R	S	S	–	–
Be-9	S	R	S	S	S	S	R	S	S	R	R	S	R	S	S	R	S	R	R	S	–	–
Be-10	S	R	S	R	S	S	S	R	S	R	S	S	S	–	–	–	–	–	–	–	S	R
Be-11	S	R	S	S	S	S	S	S	S	S	R	S	R	S	S	R	S	R	S	S	–	–
A. obtectus																						
Fbe-1	S	R	S	S	S	S	S	S	S	R	S	S	R	S	S	R	S	R	R	S	–	–
Fbe-2	S	R	S	S	R	S	S	R	S	R	S	S	R	S	S	R	R	R	R	S	–	–
Fbe-3	S	R	S	S	S	S	S	R	S	R	S	S	R	S	S	R	S	R	R	S	–	–
Fbe-4	S	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S	R	R	S	–	–
Fbe-5	S	R	S	S	R	S	S	S	S	R	S	S	R	S	S	R	S	R	R	S	–	–
Fbe-6	S	R	S	S	R	S	S	R	S	R	S	S	R	S	S	R	R	R	R	S	–	–
Fbe-7	S	S	S	S	S	S	S	S	S	R	S	S	R	S	S	R	S	R	R	S	–	–
Fbe-8	R	R	S	S	S	S	R	R	S	R	R	S	R	S	R	R	S	R	R	S	–	–
Fbe-9	S	R	S	S	R	S	S	S	S	R	S	S	R	S	S	R	R	R	R	S	–	–
Fbe-10	S	R	S	S	S	S	S	S	S	R	S	S	R	S	S	R	S	R	R	R	–	–
Fbe-11	S	R	S	S	S	S	S	S	S	R	S	S	R	S	S	R	S	R	R	S	–	–
S. cerealella																						
Ml-1	S	S	S	S	S	S	S	S	S	S	R	S	R	S	S	R	S	R	R	S	–	–
Ml-2	S	R	S	S	S	S	S	S	S	R	R	S	R	S	S	R	S	R	R	S	–	–
Ml-3	S	R	S	S	S	S	S	S	S	S	R	S	R	S	S	R	S	R	R	S	–	–
Ml-4	R	R	S	S	S	S	R	S	R	R	R	S	R	R	S	R	S	R	R	S	–	–
Ml-5	S	S	S	S	S	S	S	S	S	R	S	S	R	S	S	S	S	S	S	S	–	–
Ml-6	R	R	S	S	S	S	R	R	R	R	R	S	R	R	R	R	S	R	R	S	–	–
P. operculella																						
Pb-1	S	R	S	ND[c]	S	S	ND	S	ND	S	S	R	R	ND	S	R	S	ND	S	S	R	–
Pb-2	S	R	S	ND	S	S	ND	S	ND	S	S	S	R	ND	S	R	S	ND	S	S	R	–
Pb-3	R	R	S	ND	S	S	ND	R	ND	R	S	S	R	–	R	R	S	–	R	S	R	ND
Pb-4	S	R	S	ND	R	S	ND	R	ND	R	S	S	S	–	R	R	R	–	R	R	S	ND
Pb-5	S	R	S	ND	R	S	ND	R	ND	R	S	S	R	–	S	R	R	–	R	R	R	ND
Pb-6	R	R	S	ND	S	S	ND	R	ND	R	S	S	S	–	R	R	S	–	R	S	S	ND
Pb-7	R	R	S	ND	S	S	ND	R	ND	R	R	S	R	–	R	R	R	–	R	R	R	ND
Pb-8	R	R	S	ND	S	S	ND	R	ND	R	R	S	R	–	R	R	S	–	R	S	R	ND
Pb-9	R	R	R	ND	S	S	ND	R	ND	R	S	S	R	–	R	R	S	–	S	S	R	ND
Pb-10	S	R	S	ND	R	S	ND	R	ND	R	S	S	S	–	S	R	R	–	R	R	S	ND
Pb-11	S	R	R	ND	R	R	ND	R	ND	R	S	R	R	–	R	R	R	–	R	R	S	ND
Pb-12	S	R	S	ND	S	S	ND	R	ND	S	S	S	R	ND	S	R	S	ND	S	S	R	–
Pb-13	R	R	S	ND	S	S	ND	R	ND	R	S	S	R	–	R	R	S	–	S	S	R	ND
Pb-14	S	R	R	ND	R	R	ND	R	ND	R	R	R	R	–	S	R	R	–	R	R	S	ND
Pb-15	S	R	R	ND	R	S	ND	R	ND	R	R	R	S	–	R	R	R	–	R	R	S	ND

[a] *GN* Gentamycin; *AX* Amoxicillin; *TE* Tetracycline; *RF* Rifamycin; *A* Ampicillin; *SXT* Sulfamethoxazole; *K* Kanamycin; *E* Erytromycin; *N* Neomycin; *KF* Cephalothin; *CPR* Ciprofioxacin; *C* Chloranphenicol; *CRO* Ceftriaxone; *S* Streptomycin; *NOR* Norfloxacin; *OP* Optochin; *VA* Vancomycin; *ME* Methicillin; *OX* Oxacillin; *NV* Novobiocin; *AK* Amikacin; *CAZ* Ceftazidime

[b] *R* Resistance, *S* Sensitive

[c] No data

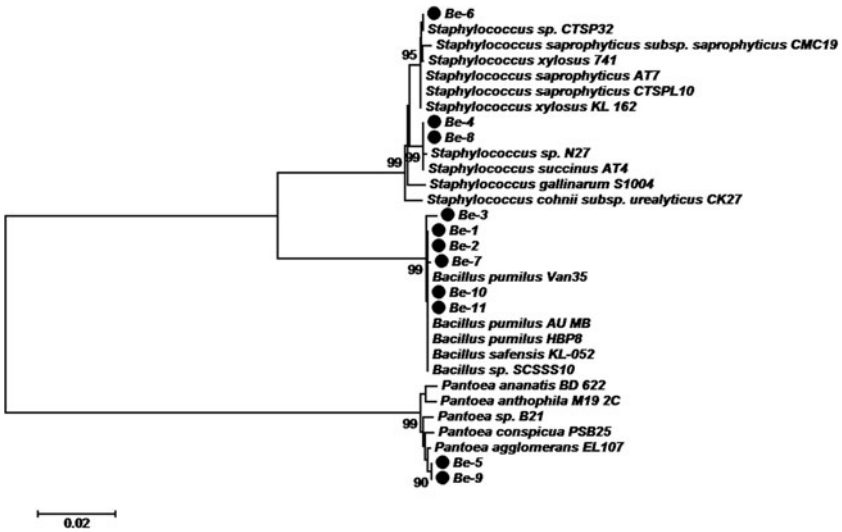

Fig. 1 The neighbor-joining phylogeny with p-distance correction inferred by 16S rRNA sequences of the bacteria isolates of *C. maculatus* and their closely related bacterial species from GenBank. Bootstrap values which are ≥70 are indicated at each node and were based on 1.000 replicates. *C. maculatus* isolates are indicated by black circles. The scale on the bottom of the dendrogram shows the degree of dissimilarity

Discussion

Up to now, studies on bacteria which are associated with insects have been focused on insect–microbial pathogen relationships and finally produce microbial insecticides to control many pest insects worldwide. However, recently, the study of microbial communities of the insect gastrointestinal tract has gained speed for their use in the microbial control of insect pests in various ways, such as genetic engineering of bacterial symbionts and because of the development of molecular techniques for studying complex microbial communities. In both approaches, the first step is to define bacterial species which are associated with the target insects. Therefore, in this study, we aimed to determine the internal bacterial species which are associated with stored product pests such as *Acanthoscelides obtectus*, *Callosobruchus maculatus*, *Sitotroga cerealella* and *Phthorimaea operculella*.

The study of the bacterial diversity of stored product pests is very limited. Prabha Kumari et al. (2011) studied the microflora of the red flour beetle [*Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae] and isolated different bacteria including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Escherichia coli* and *Enterobacter* sp., which are similar to the species in our study. Behar et al. (2010) isolated *Rickettsia felis* infecting *Liposcelis bostrychophila* (Badonnel) (Psocoptera: Liposcelidae). Yusuf and Turner (2004) defined a *Wolbachia*-like bacterium, which can manipulate the reproduction of their arthropods hosts, thus ensuring maternal transmission from *L. bostrychophila*. Lakshmikantha et al. (2006) isolated several *Enterococcus* species (*E. faecalis*, *E. faecium*, *E. gallinarum* and, *E. casseliflavus*) from stored product insects. In this study, we obtained a total of 43 bacterial strains from four stored product pests including *A. obtectus*, *C. maculatus*, *S. cerealella* and, *P. operculella*. Some of the strains such as *Bacillus pumilus*, *Pantoea* sp., *Staphylococcus kloosii*, *S. saprophyticus*, *S. succinus*, *S. sciuri*, *Enterococcus mundtii*, *Alcaligenes faecalis*, *Pantoea agglomerans* and *Pseudomonas fluorescens* were isolated for the first time from any stored product pest.

Bacillus is a genus of Gram-positive, rod-shaped bacteria including both free-living (non-pathogenic) and pathogenic species (Madigan and Martinko 2005). Within this genus, there are many bacterial species which are related to insects. The best-known examples are *B. thuringiensis*, *B. popilliae* and, *B. sphaericus* (Stahly et al. 2006). In this study, we isolated eight *Bacillus* strains (six of them were *B. pumilus* from *C. maculatus* and two of them were *Bacillus* sp. from *P. operculella*). *Bacillus pumilus* is a naturally occurring bacterium that is especially common in soil and on dead plant tissue (Priest 1993). Molina et al. (2010) also showed that this bacterium is toxic to *Ceratitis capitata* larvae (Weidemann) (Diptera: Tephritidae). With this study, we also showed that the genus of *Bacillus* might be associated with stored product pests in different ways, pathogenic or symbiotic. In both relationships, these strains have a remarkable potential for use in the control of these pests, considering easy mass production of this genus for pathogenic ones, and easy to cultivate, genetically modify, and reintroduce back into host insect for symbiotics ones.

The members of *Staphylococcus* genus is a Gram-positive bacteria appearing round, and forming grape-like clusters (Ryan and Ray 2004). Most members of this genus are harmless and normally present on the skin and mucous membranes of humans and other organisms (Madigan and Martinko 2005). Recently, there has been some evidence showing the relationship of some members of *Staphylococcus* genus with insects. Up to now, many *Staphylococcus* species such as

Fig. 2 The neighbor-joining phylogeny with p-distance correction inferred by 16S rRNA sequences of the bacteria isolates of *A. obtectus* and their closely related bacterial species from GenBank. Bootstrap values which are ≥70 are indicated at each node and were based on 1.000 replicates. *A. obtectus* isolates were indicated by black circles. The scale on the bottom of the dendrogram shows the degree of dissimilarity

Staphylococcus sp., *S. aureus*, *S. carnosus*, *S. xylosus*, *S. warneri*, *S. gallinarum*, *S. scirui* and, *S. kloosii* have been isolated from different insects belonging to the order of Homoptera, Diptera and Lepidoptera (Kuzina et al. 2001; Osborn et al. 2002; Yu et al. 2008; Sevim et al. 2012b; Demirci et al. 2013). In this study, we also isolated 17 *Staphylococcus* species (three from *C. maculatus*, nine from *A. obtectus*, four from *S. cerealella* and one from *P. operculella*) from stored product pests. All these studies suggest that *Staphylococcus* species might have close relationships with insects in different ways, from pathogenic to obligate mutualism. There has been one study showing the pathogenic properties of this genus. Danismazoglu et al. (2012) found that *Staphylococcus pasteuri* had an important mortality against *Agriotes lineatus* (Coleoptera: Elateridae) under laboratory conditions. Latterly, the discovery of recent developments in restriction–modification systems has greatly improved the ability to genetically manipulate some members of *Staphylococcus* genus (Monk and Foster 2012). We showed that *Staphylococcus* species seems to be related to stored product pests and some strains isolated during this study could be genetically modified and used as host organism for expression of insect-toxic proteins.

We isolated two *Pantoea* sp., one *P. agglomerans* and one *Enterobacter* sp., which are members of the family of Enterobacteriaceae. The Enterobacteriaceae is a large family

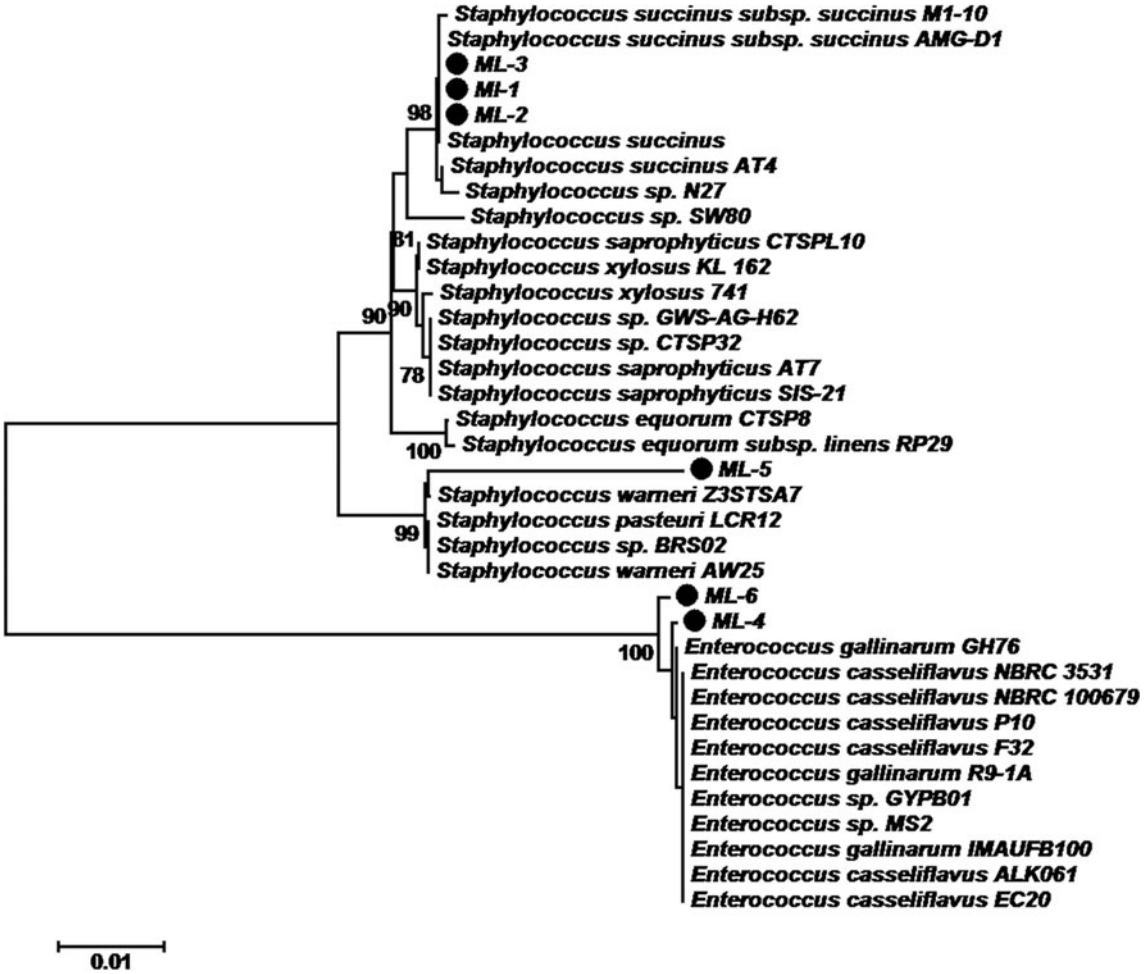

Fig. 3 The neighbor-joining phylogeny with p-distance correction inferred by 16S rRNA sequences of the bacteria isolates of *S. cerealella* and their closely related bacterial species from GenBank. Bootstrap values which are ≥70 are indicated at each node and were based on 1.000 replicates. S. *cerealella* isolates are indicated by black circles. The scale on the bottom of the dendrogram shows the degree of dissimilarity

of Gram-negative bacteria including many harmless symbionts and some pathogens such as *Salmonella*, *Escherichia coli*, *Serratia* and *Enterobacter* (Don et al. 2005). Based on molecular phylogenetic analyses, this family is closely associated with insects as symbionts (Moran 2001). This family also includes important insect pathogens such as *Serratia marcescens* and *Enterobacter aerogenes* (Bulla et al. 1975). There are also many studies showing the isolation of *Pantoea agglomerans* and *Enterobacter* sp. from many insect species (Sevim et al. 2012b; Demirci et al. 2013; Çakıcı et al. 2014). Based on these studies, it is possible to say that members of the family of Enterobacteriacae are common members of insect gut flora and that they are suitable candidates for use in genetic engineering to produce transgenic strains in the control of many pest species. Watanabe et al. (2000) cloned and transformed the ice nucleation (IN) gene into *Enterobacter cloacae* and tested cold-hardiness of *Glyphodes duplicalis* (Inoue, Munroe and Mutuura 1981) (Lepidoptera: Crambidae) larvae fed on mulberry leaves including transgenic strain. They found that these larvae died at −5 °C while the control group was alive. In another study, *Bacillus thuringiensis* toxin (Cyt1A which is a cytolytic protein toxin lethal to mosquito and black fly larvae) was cloned into *Enterobacter gergoviae*, and it was suggested that the transgenic strain might be used to spread Cyt1A gene to populations of agricultural insects (Kuzina et al. 2002). Bacterial species belonging to Enterobacteriaceae isolated during this study could be used for candidate organisms for the expression of foreign genes which can be suitable for insect control.

The most common isolated bacterial species during this study belong to the genus of *Enterococcus*. This genus is Gram-positive cocci include lactic acid bacteria which often occur in pairs or short chains. Two species of this genus (*E. faecalis* and *E. faecium*) are commonly commensal in human intestines. Some species of this genus such as *E. casseliflavus*, *E. gallinarum* and, *E. raffinosus* cause

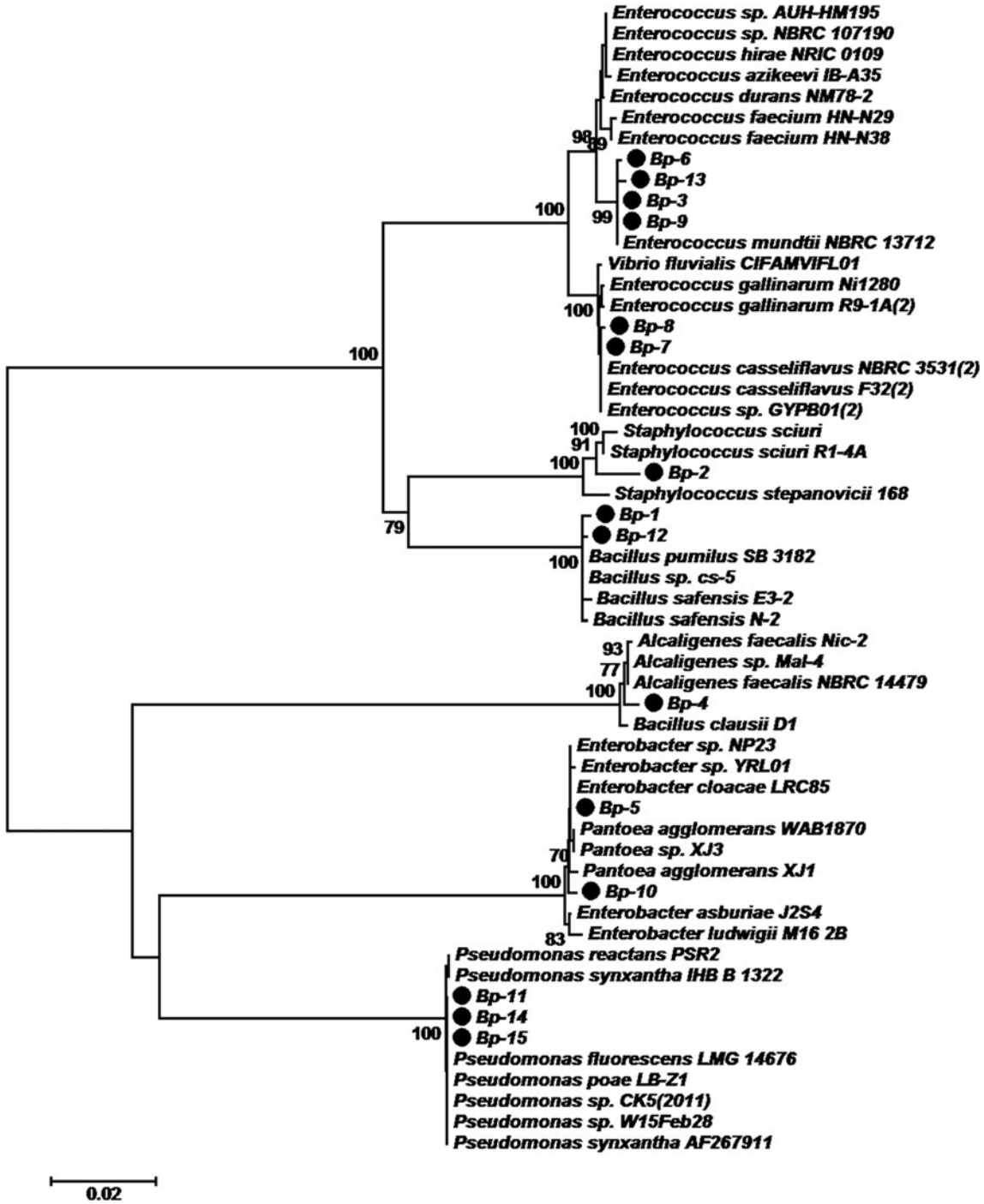

Fig. 5 The neighbor-joining phylogeny with p-distance correction inferred by 16S rRNA sequences of all bacterial isolates used in this study and their closely related bacterial species from GenBank. Bootstrap values which are ≥70 are indicated at each node and were based on 1.000 replicates. *C. maculatus* isolates were indicated wbyith black circles, *A. obtectus* isolates by black squares, *S. cerealella* isolates by black diamonds and *P. operculella* isolates by black triangles. The scale on the bottom of the dendrogram shows the degree of dissimilarity

infections in human (Gilmore et al. 2002). The members of this genus are also closely related to insects, and they occur randomly among insect species (Martin and Mundt 1972: Sevim et al. 2010: Danismazoglu et al. 2012). Lakshmikantha et al. (2006) isolated many *Enterococcus* species (*E. gallinarum*, *E. faecalis*, *E. faecium* and, *E. casseliflavus*) from stored product pests. Yezerski et al. (2005) also found that the presence of some stored product pests in flour affected the microflora of a flour community. They mostly isolated *Bacillus* and *Enterococcus* species (*E. mundtii* and *E. faecium*) from flours which were contaminated by some stored product pests. We also mostly isolated

similar species of *Enterococcus* from stored product pests with these studies, showing that Enterococci are common bacterial species within stored product pests.

The genus of *Alcaligenes* includes bacteria which are Gram-negative and rod-shaped species. The most common species of this genus is *A. faecalis* which is commonly found in the intestinal canal of vertebrates, decaying materials, dairy products, water and soil (Busse and Stolz 2006). *Alcaligenes faecalis* has also been isolated from many insect species such as *Ips sexdentatus* (Boern) (Coleoptera: Curculionidae), *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) and *Tapinoma melanocephalum* (Fabricius) (Hymenoptera: Formicidae) (Sevim et al. 2012a: Secil et al. 2012: Pesquero et al. 2012). We also isolated one strain of *A. faecalis* from *P. operculella*, showing a first isolation of this species from any stored product pest.

Pseudomonas fluorescens is a common Gram-negative and rod-shaped bacterium which has multiple flagella and an extremely versatile metabolism, and can be found in different environments such as soil and water (Palleroni 1984). This species also has biocontrol properties, protecting the roots of some plant species against pathogenic fungi such as *Fusarium* and *Pythium* (Haas and Keel 2003). Since this bacterium can colonize some plant species, it was found to be a suitable candidate for genetic engineering studies in terms of biological control. Herrera et al. (1994) cloned *cry1A(c)* into *P. fluorescens* strain which can colonize sugarcane, and they found that sugarcane treated with the transgenic *P. fluorescens* strain was more resistant to *Eldana saccharina* (Walker) (Lepidoptera: Pyralidae) than untreated sugarcane. Wenwei et al. (2010) also showed that the transgenic strain of *P. fluorescens* which carried scorpion insect toxin (AaHIT1) showed insecticidal activity and retained the activity against plant pathogens. This bacterium might also have symbiotic relationships with insects because *P. fluorescens* has been isolated from many insect species (Danismazoglu et al. 2012; Sevim et al. 2012a, b). This also makes this bacterium a good candidate for the production of transgenic strains of *P. fluorescens* which can colonize both plants and insect guts.

Symbiotic bacteria which live in insect guts play an important role in many different processes. It is known that these bacteria provide dietary supplements lacking in the diet of insects, such as certain amino acids and essential vitamins. The gut bacteria of insects also provide resistance against their natural enemies and parasitoids. Some gut bacteria have also

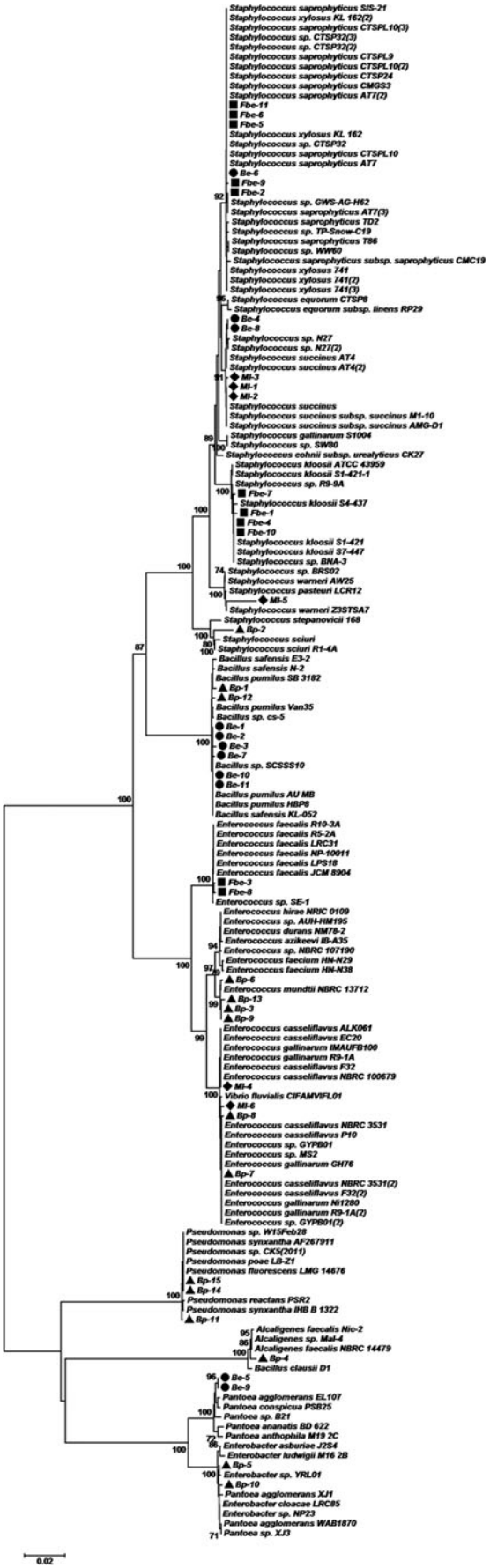

Fig. 5 The neighbor-joining phylogeny with p-distance correction inferred by 16S rRNA sequences of all bacterial isolates used in this study and their closely related bacterial species from GenBank. Bootstrap values which are ≥70 are indicated at each node and were based on 1.000 replicates. *C. maculatus* isolates were indicated wbyith black circles, *A. obtectus* isolates by black squares, *S. cerealella* isolates by black diamonds and *P. operculella* isolates by black triangles. The scale on the bottom of the dendrogram shows the degree of dissimilarity

Table 5 The proposed identification results of the bacterial isolates and their GenBank accession numbers

Isolate	Species	GenBank accession number
C. maculatus		
Be-1	*Bacillus pumilus*	KJ888101
Be-2	*B. pumilus*	KJ888102
Be-3	*B. pumilus*	KJ888103
Be-4	*Staphylococcus* sp.	KJ888104
Be-5	*Pantoea* sp.	KJ888105
Be-6	*Staphylococcus* sp.	KJ888106
Be-7	*B. pumilus*	KJ888107
Be-8	*Staphylococcus* sp.	KJ888108
Be-9	*Pantoea* sp.	KJ888109
Be-10	*B. pumilus*	KJ888110
Be-11	*B. pumilus*	KJ888111
A. obtectus		
Fbe-1	*Staphylococcus kloosii*	KJ888112
Fbe-2	*Staphylococcus* sp.	KJ888113
Fbe-3	*Enterococcus faecalis*	KJ888114
Fbe-4	*S. kloosii*	KJ888115
Fbe-5	*S. saprophyticus*	KJ888116
Fbe-6	*Staphylococcus* sp.	KJ888117
Fbe-7	*S. kloosii*	KJ888118
Fbe-8	*E. faecalis*	KJ888119
Fbe-9	*Staphylococcus* sp.	KJ888120
Fbe-10	*S. kloosii*	KJ888121
Fbe-11	*Staphylococcus* sp.	KJ888122
S. cerealella		
Ml-1	*Staphylococcus succinus*	KJ888123
Ml-2	*S. succinus*	KJ888124
Ml-3	*S. succinus*	KJ888125
Ml-4	*Enterococcus* sp.	KJ888126
Ml-5	*Staphylococcus* sp.	KJ888127
Ml-6	*Enterococcus* sp.	KJ888128
P. operculella		
Pb-1	*Bacillus* sp.	KJ888129
Pb-2	*Staphylococcus sciuri*	KJ888130
Pb-3	*Enterococcus mundtii*	KJ888131
Pb-4	*Alcaligenes faecalis*	KJ888132
Pb-5	*Enterobacter* sp.	KJ888133
Pb-6	*Enterococcus mundtii*	KJ888134
Pb-7	*E. casseliflavus*	KJ888135
Pb-8	*E. casseliflavus*	KJ888136
Pb-9	*E. mundtii*	KJ888137
Pb-10	*Pantoea agglomerans*	KJ888138
Pb-11	*Pseudomonas fluorescens*	KJ888139
Pb-12	*Bacillus* sp.	KJ888140
Pb-13	*E. mundtii*	KJ888141
Pb-14	*P. fluorescens*	KJ888142
Pb-15	*P. fluorescens*	KJ888143

been reported to have impacts on host longevity and influence insect behavior (Sanchez-Contreras and Vlisidou 2008). Also, some gut bacteria produce digestive enzymes which help to digest leaf constituents such as cellulose and pectin (Dillon and Dillon 2004). In this study, we isolated many different bacteria from stored product insects and this is an important step to understand the role of gut bacteria in stored product insects.

The first step for the investigation of the role of bacteria in insects, and developing biocontrol agents in the commercial sense is to isolate and correctly identify bacterial isolates. In this sense, we isolated and characterized different bacterial species from stored product pests using a combination of currently used methods. The isolated bacterial species during this study should be investigated in terms of the expression of insect-active toxins, such as Cry toxins via genetic engineering techniques. In addition, this study would lead to new studies to investigate the role of gut bacteria in stored product insects.

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