INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19–0360/2019/22–3–497–502
DOI: 10.17957/IJAB/15.1091
http://www.fspublishers.org

Full Length Article

Ochrobactrum pituitosum Causes Kernel Rot and Premature Shedding of Fresh Walnut Fruits

Zhongdong Yu[1*], Yunjing Sui[1], Shaobing Peng[1], Tao Li[2] and Phonepaserd Phanpadith[1]
[1]*College of Forestry, Northwest Agriculture & Forestry University, Yangling 712100, China*
[2]*Sichuan Provence Forest Pest Control and Quarantine Station, Chengdu, 610082, China*
[*]For correspondence: yuzhongdong001@nwsuaf.edu.cn; yu-10083@163.com

Abstract

A new suspected bacterium caused premature shedding of walnut fruit has been observed in commercial orchards in Shaanxi Province, China. Typical symptoms include kernel rot, with the kernel becoming black and liquid at the end, and eventually, the infected fruit falls; however, the exterior of the fruit appears to be healthy, and the fruit exocarp, sarcocarp and endocarp tissues develop normally. Based on the morphological observation, a Biolog GenIII plate, biochemical reactions, phylogenic analysis of 16S rDNA and *gyr*B gene sequences, as well as the fulfilling Koch's postulates on fruits, tobacco leaves and a potato assay, the causal agent was determined as *Ochrobactrum pituitosum*. This is the first report of *O. pituitosum* as a pathogen of walnut in the world, causing fresh walnut premature shedding. © 2019 Friends Science Publishers

Keywords: Walnut kernel rot disease; *Ochrobactrum pituitosum*; Biolog identification; Phylogenic identification

Introduction

Walnut (*Juglans regia* L.), originated in Central Asia, and is a deciduous tree belonging to the genus *Juglans* in the *Juglandaceae*. It plays an important role as a "woody oil plant" and has edible, pharmaceutical and timber value (Akbari *et al*., 2012; Ercisli *et al*., 2012; Pollegioni *et al*., 2015). China produces 1,655,000 t of walnut fruit annually and ranks first in the world for yield (Khadivi-Khub and Ebrahimi, 2015). The walnut-producing industry in China has developed rapidly, and China is also ranked first in the area planted and fresh walnut produced in the world (Khadivi-Khub *et al.,* 2015). Many tree species of *Juglandaceae* in the world have been imported in China and bred for walnut production, including pecan, another genus species, *Carya illinoinensis* from north American. Varieties of *J. regia* called paper walnut with thin wall, early ripening and high yield, were planted in most walnut orchards in China, however, they suffered the heaviest loss from insects and diseases throughout the year.

In recent years, a new bacterial disease has been found in commercial orchard in the Shaanxi Province, China. The young walnut fruits grow slowly with normal color but falls down easily in wind and rain. The kernels of the fallen fruits display a cheese-like rot and are full of liquid, whereas the tissues of the exocarp, sarcocarp and endocarp have developed normally in early stage. This disease is generally found in 10–30 d bearing fruits and can lead to a fruit dropping rate as high as 40% in the field, which seriously affects the yield of the commercial walnut. To clarify the pathogen responsible for kernel rot, methods involving liquid tissue isolation, artificial cultivation, purification of the pathogen, pathogenicity tests, cell wall staining, Biolog microarrays, biochemical analysis and molecular identification were employed for identification in this study and were reported herein.

Materials and Methods

Materials

Walnut fruits of *Juglans regia* were collected from Yangling District (34°16'N, 108°4'E), Qianxian County (34°54'N, 108°25'E) and Shanyang County (33°55'N, 109°91'E) in Shaanxi Province, where kernel rot has frequently occurred. The putative fruits on the trees and the fallen fruits on the ground were both packed in sealed bags, conveyed to the laboratory in an icebox, and then pathogens were isolated and cultured artificially.

Lysogeny broth (LB) agar medium of pH 7.0, with 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g agar per 1000 mL volume, was applied for isolation and purification. Liquid LB medium without agar was applied for fermentation of the purified colony. HL medium, with 2 g tryptone, 5 g NaCl, 0.02 g K_2HPO_4, 3 mL 1% methyl bromide blue water, 3 g agar, and a total volume of 1000 mL, with a final pH of 7.1, was used for physiological and chemical identification.

To cite this paper: Yu, Z., Y. Sui, S. Peng, T. Li and P. Phanpadith, 2019. *Ochrobactrum pituitosum* causes kernel rot and premature shedding of fresh walnut fruits. *Intl. J. Agric. Biol*., 22: 497–502

Isolation of the Putative Pathogen

The diseased fruits were surface sterilized using 70% ethanol for 2 min, rinsed 3 times with sterile distilled water (SDW), and dried on sterilized filter paper in a laminar flow cabinet. The fruits were then cut into two halves with a knife, an inoculating loop was then immersed into the rot kernel and scaled lines on the surface of the LB medium. When colonies developed in the medium at 28°C for 72 h, three single colonies were picked and transferred to a new LB medium Petri dish for mono-colonization, respectively. The single colony was cultured in the LB liquid medium at 28°C and 180 rpm for 48 h, and then an equal volume of glycerinum at a 40% concentration was dropped into the LB liquid for extended storage at 20°C below zero.

Pathogenicity Tests

The putative causing isolates were inoculated into the tobacco vivo seedlings by slight injection 1×10^7 cfu/mL LB suspending into the abaxial leaf, respectively. A potato assay was also employed for checking secondary pathogen by needle puncturing 1 mL LB suspending with 1×10^7 cfu/mL in each inoculation hole. Each group with 10 repeats and a same repeat of control by inoculation SDW instead of causing isolate suspending under the room temperature of 25–28°C. The causing isolate with a typical hypersensitive response (HR) or a tissues broken was believed a potential pathogen and forwarded to inoculate walnut fruits. Healthy fruits about 12 days old and with a diameter of 3–4 cm were selected for artificial inoculation in the field by injecting 0.1 mL bacteria LB suspended with 10^5 cfu/mL into the endocarp from the pistillar point of the walnut after surface sterilization. Each treatment was repeated 10 times, and the control was injected with SDW and 10 repetitions. Fruits for the test were enclosed within a translucent plastic bag to prevent them from contacting with rain and exotic pathogens. The symptoms of the tested fruits were checked every day until the fruit fall, and the pathogen was re-isolated from the fallen fruit according to method above.

Pathogen Morphology and Biochemical Analyses

The putative isolate identified in the method above was further observed under a scanning electron microscope (SEM) and grown in different media to check aerobic and anaerobic requirements, catalase activities, starch dissolution, indole production, H_2S production, lipolysis digestion, gelatin decomposition and Gram staining, according to the methods of Schaad *et al*. (2001). Samples were prepared and coated with gold using a carbon evaporation coater (E-1045, HITACHI, Japan) as described by Zhang *et al*. (2016), and then observed and photographed with a SEM (S-4800, HITACHI, Japan) in the electron microscopy facility at 5 KV of accelerating voltage.

The OmniLog® system, Gen III MicroPlate and the Biolog database from the Biolog Company were employed for identification of the putative bacteria. The putative isolate was cultured on BUG (growth medium for agricultural bacteria, Biolog #1506) agar plats at 28°C for 24 h. Then, 150 μL of the bacterial suspension (OD_{260} = 2.5) was added to each well of a MicroPlate and incubated at 28°C for 24 h. The MicroPlate was read using a MicroStation 2 Reader, and the results were compared with analyzed using the database (Wragg *et al*., 2014). All Biolog procedures were completed at the Qingdao Technology standard R & D center, China.

Molecular Identification of the Putative Bacteria

For DNA extraction, the putative bacterium was cultivated in LB broth at 28°C and at 160 rpm for 48 h before being centrifuged at 8000 rpm for 1 min. The genomic DNA of the bacteria was extracted using the Bacterial Genomic DNA Rapid Extraction Kit (Sangon Biotech Ltd., #B518225-0050) according to the manual, and the dissolved DNA was stored at -20°C for future using.

Two genes were employed for molecular identification in this study using universal primers. Pair of primers 27F/1492R (27F: 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R: 5'-GGTTACCTTG TTACGACTT-3') (Maestre *et al*., 2010) was used for amplifying the 16S rDNA of the putative bacteria. The PCR reaction consisted of an initial denaturation at 94°C for 5 min and 35 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 2 min, followed by a final extension at 72°C for 10 min. Primer pair F1/R1 (F1: 5'-GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNA ART TYGA-3'; R1: 5'-AGCAG GGTACGGATGTGCGAGCCRTCNACRTCNGCRTCN GTCAT-3') (Kakinuma *et al*., 2003) was used for amplifying the *gyr*B gene. The PCR reaction consisted of an initial denaturation at 94°C for 5 min and 30 cycles of 94°C for 1 min, 65°C for 30 s, and 72°C for 2 min, followed by a final extension at 72°C for 10 min. Both primer pairs were synthesized by the Sangon Biotech (Shanghai) Company Limited, China. Each amplification was performed in a total volume of 25 μL containing 12.5 μL 2 × Es Taq MasterMix (Beijing ComWin Biotech Co., Ltd., China), 1 μL each of forward and reverse primer (10 μM), 1 μL DNA (80 ng/μL) and 9.5 μL of sterilized distilled water. Three microliters of each PCR product were checked by electrophoresis on a 1% agarose gel.

DNA fragments were purified and sequenced by the Aoke Biotech (Shanghai) Company, China. After revising and joining, the sequence was used as the query in a BLAST search performed *via* the NCBI (http://www.ncbi.nlm.nih.gov.), and the homological sequences of type materials with high coverage were downloaded for constructing phylogeny trees. All sequences were aligned first with Clustal X 1.81 (Thompson *et al*., 1997) and then adjusted based on the algorithm of Nei and Kumar (2000). All positions containing gaps and missing data were completely eliminated from the dataset. Data

were analyzed by neighbor-joining (NJ) using the close-neighbor-interchange algorithm (Nei and Kumar, 2000) as implemented in MEGA 5.0 (Tamura *et al*., 2007). Support for the branches based on parsimony criteria was estimated by bootstrap analysis using 1000 replicates.

Results

Symptom Description and Pathogenesis Test

Kernel rot occurs during April and May every year. The fruit appears healthy but easily falls. The rot kernel becomes cheese-like initially, and the endocarp gradually fills with liquid. Finally, the whole endocarp becomes dark and the young fruit falls after bearing approximately 10 days. A dominant colony isolated from the endocarp liquid in this study was named XF and preserved in China Forestry Culture Collection (XF; accession no. cfcc 15139). Isolate XF showed a typical hypersensitive response (HR) *in vivo* tobacco leaves (Fig. 1), but without tissues broken in the potato assay inoculations (Fig. 2). It can also cause young bearing fruit to fall 7 days after inoculation by artificial injection inside the endocarp (Fig. 3). All inoculated young bearing fruits fell within 20 dpi. However, no fruits fell in the control group until the field test ended. The symptoms of inoculated fruits developed similarly as the field-diseased fruit, and re-isolation from inoculated fruits resulted in the same pathogenic bacteria, which caused kernel rot isolated from the field samples. This finding proved Koch's postulates that the putative isolate XF is a pathogen of walnut kernel rot rather than a secondary agent.

Morphologic, Physiologic and Chemical Identification of the Pathogenic Isolate

Isolate XF grows well on the LB medium; its colony has a round shape and is embossed, sticky, white and semitransparent with a surface that is wet, smooth and glossy, and with a regular boundary. Under SEM, the bacteria are rod shaped with sizes of (0.4～0.6) μm × (0.6～1.6) μm, holomastigotes and binary fission (Fig. 4). Under the microscope, the isolate XF bacilliform was considered Gram negative. The XF colony is aerobic, with good catalase activities, indole production, and lipolysis activities. However, the isolate XF cannot dissolve starch and gelatin, and no H_2S was produced during culture (Table 1).

A microarray with a 96-well GEN test revealed that isolate XF has high similarity with *Ochrobactrum intermedium* after 24 h of incubation reaction, with a similarity coefficient of 0.617 and a distance of 5.529 between XF and *O. intermedium*.

Phylogenic Analysis of the Pathogenic Isolate

Polymerase chain reaction (PCR) of the 16S small subunit of ribosomal DNA produced a fragment of approximately 1.5 kb DNA (GenBank Accession No. MF140273). After purification, sequencing and verification, this 1.5 kb DNA was used as the query sequence in a BLAST search performed *via* NCBI (https://www.ncbi.nlm.nih.gov/) and showed a 99% sequence identity and 100% coverage with sequences of the genus *Ochrobactrum*, most of which were reportedly isolated from the *Zea mays* root system (Niu *et al*., 2016). Sequences with high identity with XF were selected for constructing a neighbor-joining phylogeny tree (Fig. 5). The isolate XF was grouped within the clad of most *O. pituitosum* and differentiated from the other species of the genus *Ochrobactrum* and another important pathogen, *Xanthomonas campestris*, which causes walnut black spot

Table 1: Main bacteriological characteristics of isolate XF

Item	Result
gram stain	-
aerobic or anaerobic	aerobic strictly
catalase test	+
starch	-
indole production	+
H_2S production	-
lipolysis	+
gelatin liquefaction	-

"+", Positive reaction. "-", Negative reaction

Fig. 1: A hypersensitive response (HR) on tobacco leaves
In the tobacco vivo assay, the vein left inoculated isolate XF showed a hypersensitive response after 24 h (purple circles), the vein right without HR are the controls inoculated with SDW (red circles)

Fig. 2: Secondary pathogenesis checking of XF (7dip)
A potato assay followed one treatment by one control inoculation, each puncture inoculated line show no tissues broken down, the left column is the control inoculated SDW instead

Fig. 3: The symptoms of bacterial kernel-rot of walnut caused by isolate XF (7dip)
A, The symptoms of dropping walnut; **B**, Artificial inoculation test. **C**, Results of artificial inoculation resulted in kernel getting dark. **D**, The control with SDW resulted in kernel retain white

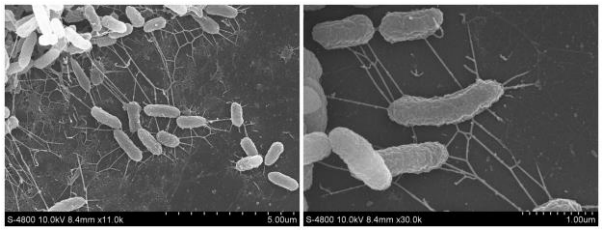

Fig. 4: Rod-shape bacteria with holomastigote (left & right) and a new born cell by binary fission (left picture) under SEM

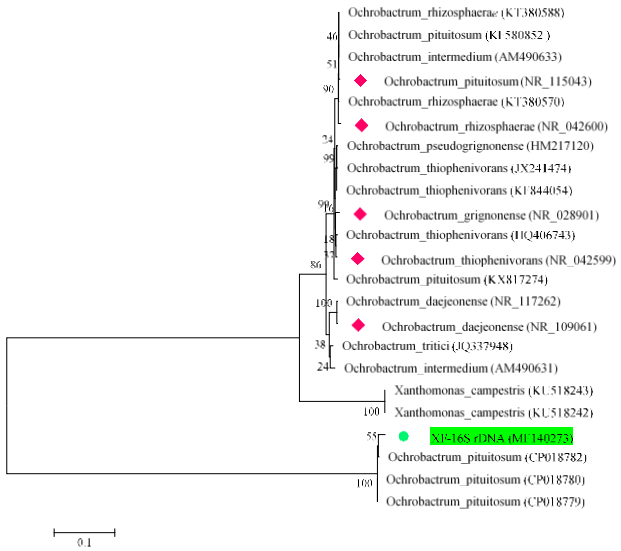

Fig. 5: Phylogenetic tree of 16S rDNA gene sequences by the neighbor joining (Bootstrap values based on 1000 replications are indicated above the branches. The scale bar represents 0.1 nucleotide substitutions per site. ●The tested isolate, ◆Type strain)

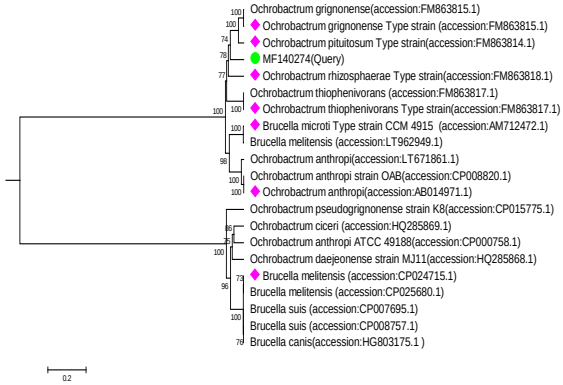

Fig. 6: Phylogenetic tree of gyr-B gene sequences by the neighbor joining (Bootstrap values based on 1000 replications are indicated above the branches. ●The tested isolate, ◆Type strain)

disease in the worldwide (Pollegioni *et al*., 2015).

Another gene of *gyr*B was amplified with a product of 1.2 kb DNA fragment (GenBank Accession No. MF140274). After sequencing and BLAST searching, this sequence of XF also showed high identity with sequences of the genus *Ochrobactrum*. Among them, XF showed sequence identities of 86%, 86%, and 88% with *O. pituitosum* (CP018780.1), *O. pseudogrignonense* (FM863816.1), and *O. grignonense* (FM863815.1), respectively. Type strains of sequences with high similarity were then selected for construction of a phylogeny tree, in which XF was also grouped within the clad of a type strain *O. pituitosum* but failed to differentiate from the other type strain *O. grignonense* (Fig. 6).

Discussion

O. pituitosum belongs to class α-proteobacteria, order Rhizobiales, family Brucellaceae, and genus *Ochrobactrum*. There are 17 effective named species in total (Lebuhn *et al*.,

2006; Scholz *et al*., 2008; Huber *et al*., 2010), and the model species, *O. anthropi*, is an opportunistic pathogen of human beings (Bezza *et al*., 2016). In recent years, the number of human infections caused by opportunistic pathogens belonging to the *Ochrobactrum* genus has increased dramatically (Chain *et al*., 2011). *Ochrobactrum* contains root-associated strains that can be involved in bivalent interactions with both plant and human hosts (Berg *et al*., 2005; Woo *et al*., 2011; Aguirre-Garrido *et al*., 2012; Gohil *et al*., 2016). *O. intermedium* CN3 produces a lipopeptidal bio-surfactant that showed up to 70% degradation of the most hydrophobic components of petroleum sludge, presenting potential application in petroleum sludge treatment and environmental remediation (Bezza *et al*., 2016). *O. intermedium* strain 2745-2 can degrade crude oil and was isolated from the formation water of the Changqing oilfield in Shaanxi, China (Chai *et al*., 2015). Species of *Ochrobactrum* have the ability to digest hydrocarbons, proteins and fats. Physiological and biochemical tests showed that isolate XF is an aerobic bacterium that can decompose kernel fats and transfer tyrosine protein into indoles, a form of auxin that probably accelerates the process of young bearing fruits falling from the trees.

Gene *gyr*B, a subunit of the gyrase, is a typical exon DNA, *gyr*B involved in most bacteria genomes with different substitution ratios at a certain protein and a certain protein locus, and few horizontal transfers occur, and *gyr*B is widely used for cryptic species identification, new species identification and diversity studies of bacterial communities (Kumar *et al*., 2006). 16S rDNA, a relatively high conservative intermediate repeat gene, is universally used for bacteria identification; however, the small fragment of its PCR produced and the limited information involved made it difficult to differentiate the cryptic species (Kakinuma *et al*., 2003; Peat *et al*., 2010). From this point of view, the *gyr*B gene showed more advantages than 16S rDNA for distinguishing cryptic phylogenic species. However, the *gyr*B in this study showed a high diversity in *Ochrobactrum* bacteria, and revealed that isolate XF only 86% homogenesis with *O. pituitosum*, and as there are only 17 effective named species found in the genus *Ochrobactrum*, both *gyr*B and 16S rDNA can provide limited phylogenic information of this cryptic species.

Biolog GEN III microarray plates are used for rapid bacteria identification, but the identification relies heavily on the data available. Species not in the data will not be identified in practice, and cryptic species cannot be identified because of low similarity. Currently, there are 1327 species stored in the GEN III data, among which Gram-negative and aerobic bacteria account for 568 species (Wragg *et al*., 2014). In general, the Biolog system displays a limited ability for species differentiation, therefore, we obtained different species sensu identification results from those of Biolog and molecular phylogeny tree. However, both of these methods showed isolate XF to be in the same genus *Ochrobactrum*.

Conclusion

O. pituitosum, as a novel pathogen of walnut, caused kernel rot and fruits premature shedding. It is a Gram$^-$ bacteria with flagellum peritrichous, indole produced, and parasite instead of causal pathogen. Combined phylogenetic analysis of 28srDNA and *gyr*B genes and Biolog GEN III microarray method resolve *O. pituitosum* as a novel pathogen, different from another ever reported important walnut pathogen, *X. campestris*, which causes walnut black spot disease.

Acknowledgments

This study was supported by the national key research projects 2017YFD0600103-4-2 in China, Shaanxi Agricultural science and Technology Extension Project (2015NY124).

References

Aguirre-Garrido, J.F., D. Montiel-Lugo, C. Hernandez-Rodriguez, G. Torres-Cortes, V. Millan, N. Toro, F. Martinez-Abarca and H.C. Ramirez-Saad, 2012. Bacterial community structure in the rhizosphere of three cactus species from semi-arid highlands in central Mexico. *Anton. Leeuwen.*, 10: 891–904

Akbari, V., R. Jamei, R. Heidari and A.J. Esfahlan, 2012. Antiradical activity of different parts of Walnut (*Juglans regia* L.) fruit as a function of genotype. *Food Chem.*, 135: 2404–2410

Berg, G., L. Eberl and A. Hartmann, 2005. The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. *Environ. Microbiol.*, 7: 1673–1685

Bezza, F.A., M. Beukes and E.M.N. Chirwa, 2016. Application of biosurfactant produced by *Ochrobactrum intermedium* CN3 for enhancing petroleum sludge bioremediation. *Process Biochem.*, 51: 343–345

Chai, L.J., X.W. Jiang, F. Zhang, B.W. Zheng, F.C. Shu, Z.L. Wang, Q.F. Cui, H.P. Dong, Z.Z. Zhang, D.J. Hou and Y.H. She, 2015. Isolation and characterization of a crude oil degrading bacteria from formation water: comparative genomic analysis of environmental *Ochrobactrum intermedium* isolate versus clinical strains. *J. Zhej. Univ.-Sci. B*, 16: 865–874

Chain, P.S., D.M. Lang, D.J. Comerci, S.A. Malfatti, L.M. Vergez, M. Shin, R.A. Ugalde, E. Garcia and M.E. Tolmasky, 2011. Genome of *Ochrobactrum anthropi* ATCC 49188T, a versatile opportunistic pathogen and symbiont of several eukaryotic hosts. *J. Bacteriol.*, 193: 4274–4275

Ercisli, S., B. Sayinci, M. Kara, C. Yildiz and I. Ozturk, 2012. Determination of size and shape features of walnut (*Juglans regia* L.) cultivars using image processing. *Sci. Hortic.*, 133: 47–55

Gohil, K.N., P.S. Neurgaonkar, A. Paranjpe, S.G. Dastager and M.S. Dharne, 2016. Peeping into genomic architecture by re-sequencing of *Ochrobactrum intermedium* M86 strain during laboratory adapted conditions. *Genomics Data*, 8: 72–76

Huber, B., H.C. Scholz, P. Kampfer, E. Falsen, S. Langer and H.J. Busse, 2010. *Ochrobactrum pituitosum* sp. nov., isolated from an industrial environment. *Intl. J. Syst. Evol. Microbiol.*, 60: 321–326

Kakinuma, K., M. Fukushima and R. Kawaguchi, 2003. Detection and Identification of *Escherichia coli*, Shigella, and Salmonella by Microarrays Using the *gyr*B Gene. *Biotechnol. Bioeng.*, 6: 721–728

Khadivi-Khub, A. and A. Ebrahimi, 2015. The variability in walnut (*Juglans regia* L.) germplasm from different regions in Iran. *Acta Physiol. Plantarum*, 37: 57

Khadivi-Khub, A., A. Ebrahimi, A. Mohammadi and A. Kari, 2015. Characterization and selection of walnut (*Juglans regia* L.) genotypes from seedling origin trees. *Tree Genet. Genomics*, 11: 54

Kumar, H.S., A. Parvathi, I. Karunasagar and I.D. Karunasagar, 2006. A *gyr*B-based PCR for the detection of *Vibrio vulnificus* and its application for direct detection of this pathogen in oyster enrichment broths. *Intl. J. Food Microbiol.*, 111: 216–220

Lebuhn, M., S. Bathe, W. Achouak, A. Hartmann, T. Heulin and M. Schloter, 2006. Comparative sequence analysis of the internal transcribed spacer 1 of *Ochrobactrum* species. *Syst. Appl. Microbiol.*, 29: 265–275

Maestre, J.P., R. Rovira, F.J. Álvarez-Hornos, M. Fortuny, J. Lafuente, X. Gamisans and D. Gabriel, 2010. Bacterial community analysis of a gas-phase biotrickling filter for biogas mimics desulfurization through the rRNA approach. *Chemosphere*, 80: 872–880

Nei, M. and S. Kumar, 2000. *Molecular Evolution and Phylogenetics*, pp: 1–423. Oxford Univ. Press, New York, USA

Niu, B., J.N. Paulson, X. Zheng and R. Kolter, 2016. Simplified and representative bacterial community of maize roots. *Proc. Natl. Acad. Sci. USA*, 114: 2450–2459

Peat, S.M., R.H. ffrench-Constant, N.R. Waterfield, J. Marokhazi, A. Fodor and B.J. Adams, 2010. A robust phylogenetic framework for the bacterial genus *Photorhabdus* and its use in studying the evolution and maintenance of bioluminescence: A case for 16S, *gyr*B, and *gln*A. *Mol. Phylogenet. Evol.*, 57: 728–740

Pollegioni, P., K.E. Woeste, F. Chiocchini, S.D. Lungo, I. Olimpieri, V. Tortolano, J. Clark, G.E. Hemery, S. Mapelli and M.E. Malvolti, 2015. Ancient humans influenced the current spatial genetic structure of common walnut populations in Asia. *PLoS One*, 8: 10–16

Schaad, N.W., J.B. Jones and W. Chun, 2001. *Laboratory Guide for Identification of Plant Pathogenic Bacteria*, pp: 1–265. American Phytopathological Society, Minnesota, USA

Tamura, K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.*, 24: 1596–1599

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins, 1997. The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res.*, 25: 4876–4882

Woo, S.G., L.N. Ten, J. Park and M. Lee, 2011. *Ochrobactrum daejeonense* spp. nov., a nitrate- reducing bacterium isolated from sludge of a leachate treatment plant. *Intl. J. Syst. Evol. Microbiol.*, 61: 2690–2696

Scholz, H.C., A.S. Dahouk, H. Tomaso, H. Neubauer, A. Witte, M. Schloter, P. Kampfer, E. Falsen, M. Pfeffer and M. Engel, 2008. Genetic diversity and phylogenetic relationships of bacteria belonging to the *Ochrobactrum brucella* group by recA and 16S rRNA gene-based comparative sequence analysis. *Syst. Appl. Microbiol.*, 31: 1–16

Wragg, P., L. Randall and A.M. Whatmore, 2014. Comparison of Biolog GEN III MicroStation semi-automated bacterial identification system with matrix-assisted laser desorption ionization-time of flight mass spectrometry and 16S ribosomal RNA gene sequencing for the identification of bacteria of veterinary interest. *J. Microbiol. Meth.*, 105: 16–21

Zhang, M., W. Wei, Y. Sun, X. Jiang, X. Ying, R. Tao and L. Ni, 2016. *Pleurocidin congeners* demonstrate activity against *Streptococcus* and low toxicity on gingival fibroblasts. *Arch. Oral Biol.*, 70: 79–87

[Received 02 Mar 2019; Accepted 27 Mar 2019; Published (online) 12 Jul 2019]