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Short Communication

16S rRNA Phylogenetic Analysis of the Plant Growth-Promoting Rhizobacteria Associated with Pepper (*Piper nigrum* L.)

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Fourteen indigenous rhizobacterial isolates from pepper (*Piper nigrum* L.) rhizosphere were successfully identified by 16S rRNA sequencing, namely *Acinetobacter radioresistens* (UPMLH1), *Bacillus* spp. (UPMLH8, UPMLH23, UPMLH34 and UPMLH43), *Bacillus cereus* (UPMLH1, UPMLH13, UPMLH24, UPMLH41 and UPMLH42), *Bacillus megaterium* (UPMLH3 and UPMLH22), *Bacillus subtilis* (UPMLH5) and *Leclercia* sp. (UPMLH2). All the identified strains were successfully positioned in the 16S rRNA based phylogenetic tree at the nucleotide and the amino acid sequence levels. Present study found that the relationship structure of translated amino acid phylogenetic tree was simpler than nucleotide based phylogenetic tree. Both phylogenetic trees contained two phyla, Firmicutes and Proteobacteria, with the Firmicutes were the largest proportion of the isolates recovered from this study.

Key words: 16S rRNA, Pepper, *Piper nigrum* L., plant growth-promoting rhizobacteria.

Beneficial traits of rhizobacteria can be exploited to reduce or resolve the negative impact arising from overuse of agrochemicals. Yield-increasing rhizobacteria or commonly known as plant growth-promoting rhizobacteria promote plant growth by several direct and indirect mechanisms such as biological nitrogen fixation, phosphate solubilisation and phytohormone production and deliver benefits to the plant through the root system by colonising the rhizospheric soil or the rhizoplane, or by endophytic colonisation (Qureshi *et al*., 2012; Zakry *et al*., 2012; Aziz *et al*., 2015). PGPR belong to several genera of root associated bacteria, among them *Azospirillum, Bacillus, Pseudomonas* and *Rhizobium* (Bhattacharyya and Jha, 2012; Fatnassi *et al*., 2015). The 16S rRNA sequencing technique has been used widely as a molecular signature for bacterial species identification and analysis of phylogenetic relationship and position among bacterial isolates (Ribeiro and Cardoso, 2012). To exploit further the beneficial properties of PGPR for the establishment of a sustainable ecosystem in pepper production, the identification of new isolates from among native strains from the rhizosphere is essential. In fact, very little is known about the bacteria that reside in the rhizosphere of *Piper nigrum*. The aims of the present study were to identify rhizobacteria associated with the pepper vine and also to determine their 16S rRNA phylogenetic relationships after those strains have been isolated and characterised in the previous studies (Zakry *et al*., 2010; Aziz *et al*., 2012).

Collection of fourteen rhizobacterial strains have been isolated from *Piper nigrum* L. of two varieties namely, Kuching and

Semenggok Emas, and characterised for their plant growth-promoting traits, i.e. nitrogen fixation, phosphate solubilisation and IAA production in the previous studies (Zakry *et al*., 2010; Aziz *et al*., 2012). In the present study, fourteen strains were then subjected to DNA analysis by 16S rRNA (rDNA) sequencing. Extraction and amplification of genomic DNA for 16S rRNA gene was carried out with the bacterial ID PCR kit which amplified the nucleotide region between the highly conserved 16S rRNA (rDNA) primer binding sites. The PCR assay produced an amplicon nearly 1500 bp in size that contained variable regions. All procedures were carried out according to the manufacturer's instructions (Profound Kestrel Laboratories Sdn. Bhd., Shah Alam, Malaysia). The 16S rRNA gene fragment was amplified using two universal primers, known as forward (5'-GAA GGC GAC TTT CTG GTC TG-3') and reverse (5'-CCT TTG AGT TTC AGC CTT GC-3'). The 16S rDNA nucleotide sequence was determined by PCR-direct sequencing using the ABI PRISM 310 Genetic Analyzer and the BigDye terminator cycle system (PE Applied Biosystems).

Newly determined 16S rDNA sequences were aligned with 16S rDNA sequences from the GenBank nucleotide database by using the BLASTn search program and the MEGABLAST algorithm (Searching for high similarity sequences). For the determination of phylogenetic positioning, multiple alignments with sequences of nearest taxa were employed using ClustalW version 1.6 in the MEGA 5 software (Tamura *et al*., 2011). The 16S rRNA sequences of nearest taxa were retrieved from nucleotide collection of the GenBank database. Partial or nearly complete sequences of the 16S rDNA generated from the present study were deposited in the GenBank/EMBL/DDBJ and assigned with accession number.

Two types of phylogenetic trees were constructed i.e. one based on nucleotide sequences and the other based on translated amino acid sequences. The construction was performed using the Neighbor-joining method (Saitou and Nei, 1987). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic relationships. For the phylogenetic tree based on nucleotide sequences, the evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei, 1993) and were expressed in the units of the number of base substitutions per site. Gaps and missing data in all positions were eliminated. For the phylogenetic tree based on amino acid sequences, the evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and were in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. All evolutionary analyses were conducted using MEGA 5 software (Tamura *et al*., 2011).

The 16S rRNA sequence analysis conducted in the present study showed that the 16S rDNA (rRNA) sequences of the 14 strains showed highest levels of identity (100-99 %) when compared with sequences from the nucleotide collection in the GenBank database (Table 1).

Of 14 strains, 9 strains were successfully identified up to species level, viz. *Acinetobacter radioresistens* strain (UPMLH19), *Bacillus cereus* strains (UPMLH1, UPMLH13, UPMLH24, UPMLH41 and UPMLH42), *Bacillus megaterium* strains (UPMLH3 and UPMLH22), *Bacillus subtilis* strain (UPMLH5). Five strains were identified at genus level, namely *Leclercia* sp. strain (UPMLH2) and *Bacillus* sp. strains (UPMLH8, UPMLH23, UPMLH34 and UPMLH43).

Table 1: BLASTn comparison between partial 16S ribosomal RNA gene of locally isolated rhizobacterial strains from pepper (*Piper nigrum* L.) and nucleotide collection of GenBank databases

Isolate	Description	E-value	% Identity	Accession no.[a]
UPMLH1 (HQ876003)[a]	*Bacillus cereus* S74	0.0	100	JX293338
(1397)[b]	*Bacillus cereus* Ps-5	0.0	100	JQ248587
	Bacillus cereus RJ1	0.0	100	JN159662
	Bacillus cereus Wu2	0.0	100	JF267369
UPMLH2 (JX891467)	Uncultured bacterium clone 2.1	0.0	99	EF179821
(1387)	Uncultured bacterium clone 0-172	0.0	99	GU444081
	Leclercia sp. 1185/07	0.0	99	GQ856079
	Leclercia adecarboxylata EPCC5	0.0	99	JQ313580
UPMLH3 (JN012241)	*Bacillus megaterium* SMPG13	0.0	100	JX415549
(390)	*Bacillus* sp. WS13	0.0	100	JN688166
	Bacillus megaterium A8-1	0.0	100	HE981752
	Bacillus megaterium TDB-2	0.0	100	JX393073
UPMLH5 (JX195183)	*Bacillus subtilis* 30C1-2	0.0	99	JN366786
(1302)	*Bacillus* sp. M103	0.0	99	GQ340508
	Bacillus subtilis YY1	0.0	99	JX482116
	Bacillus subtilis SP11	0.0	99	JX499253
UPMLH8 (JX195184)	*Bacillus aryabhattai* YNA12	0.0	100	JN700193
(377)	*Bacillus megaterium* EN2	0.0	100	JN642548
	Bacillus megaterium CBG_LBI30	0.0	100	JF909575
	Bacillus aryabhattai Hc15	0.0	100	JF899293
UPMLH13 (JX195185)	*Bacillus cereus* P14	0.0	100	JN700160
(1404)	*Bacillus cereus* Se02	0.0	100	JN700119
	Bacillus cereus Se07	0.0	100	JN700112
	Bacillus sp. TAW	0.0	100	JX155396
UPMLH19 (JX195186)	*Acinetobacter radioresistens* 7m36-1	0.0	99	JQ661248
(1397)	*Acinetobacter radioresistens* 7m34	0.0	99	JQ661246
	Acinetobacter radioresistens 7m12-1	0.0	99	JQ661242
	Acinetobacter radioresistens 4m124	0.0	99	JQ661237
UPMLH22 (JX891468)	*Bacillus* sp. SG21	0.0	100	JX402436
(445)	*Bacillus megaterium* GMB5003-b	0.0	100	AB739003
	Bacillus megaterium MHT6	0.0	100	JX402906
	Bacillus megaterium GMA479	0.0	100	AB738793
UPMLH23 (JX891469)	*Bacillus cereus* TJBDU2	6e-141	100	JX503931
(275)	*Bacillus thuringiensis*	6e-141	100	JX088537
	Bacillus cereus BC1-PS	6e-141	100	JX294518
	Bacillus sp. G41	6e-141	100	JX293302
UPMLH24 (HQ876004)	*Bacillus cereus* ANctcri1	0.0	100	HQ286640
(1418)	*Bacillus cereus* 68-3	0.0	100	HM104657
	Bacillus sp. DB-6	0.0	100	JF734332
	Bacillus sp. A-BT-nw	0.0	100	JF901711
UPMLH34 (JX891470)	*Bacillus cereus* TJBDU2	0.0	100	JX503931
(373)	*Bacillus thuringiensis* VKK-1.9	0.0	100	JX307077
	Bacillus sp. TAW	0.0	100	JX155396
	Bacillus sp. C44	0.0	100	JX010998
UPMLH41 (JX891471)	*Bacillus cereus* Y-S	0.0	99	EU240976
(1416)	*Bacillus* sp. TAW	0.0	99	JX155396
	Bacillus sp. KV1	0.0	99	JQ433949
	Bacillus cereus HVR22	0.0	99	JQ739719
UPMLH42 (JX891472)	*Bacillus* sp. TAW	0.0	100	JX155396
(1413)	*Bacillus* sp. C44	0.0	100	JX010998
	Bacillus cereus P14	0.0	100	JN700160
	Bacillus cereus Se07	0.0	100	JN700112
UPMLH43 (JX891473)	*Bacillus* sp. SBK-8	0.0	99	AB366349
(357)	*Bacillus cereus* MCM B-1045	0.0	99	DQ983946
	Bacillus thuringiensis MC28	0.0	99	CP003687
	Bacillus cereus 2	0.0	99	JX439638

[a]GenBank accession numbers of the sequences, [b]Sequence length in base.

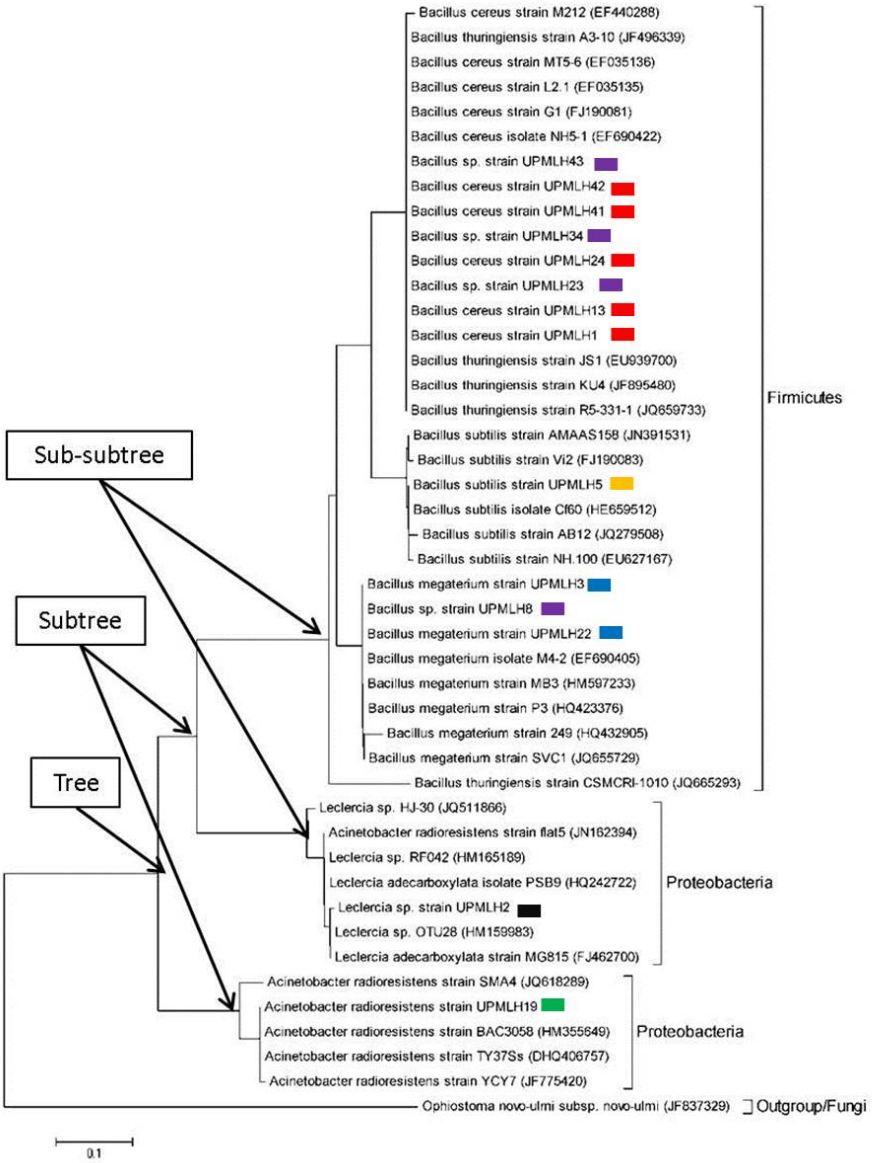

Figure 1: Neighbour joining tree based on alignment of nucleotide sequences of the 16S rRNA gene from 14 newly isolated rhizobacterial strains (UPMLH) and reference strains (Genbank accession number). Bar denotes 0.1 substitutions per nucleotide position. *Ophiostoma novo-ulmi* subsp. *novo-ulmi* was used to form a tree. Different coloured bars indicate position and different species of newly isolated rhizobacteria from pepper (*Piper nigrum* L.).

MEGABLAST analysis of 16S rRNA sequences of strain UPMLH2 was unable to discriminate with reasonable certainty between *Leclercia* sp. and *Leclercia adecarboxylata* sequences. Similarly, UPMLH8 16S rRNA sequence analysis was unable to discriminate between the corresponding *Bacillus aryabhattai* and *Bacillus megaterium* sequences. Moreover, 16S rRNA MEGA BLAST analysis of UPMLH23, UPMLH34 and UPMLH43 sequences failed to discriminate between *Bacillus cereus* and *Bacillus thuringiensis* sequences. The bacterial species isolated from soil were more diverse when compared with those from the rhizoplane or endophyte.

Neighbour-joining tree placed the 14 identified strains with their nearest related isolates in a nucleotide-based tree and translated amino acid-based phylogenetic tree. In the nucleotide-based phylogenetic tree (Figure 1), newly isolated strains were classified into two phyla, Firmicutes (containing most of the newly isolated strains) and Proteobacteria. The nucleotide-based tree formed two distinct subtrees with components from both phyla, Firmicutes and Proteobacteria.

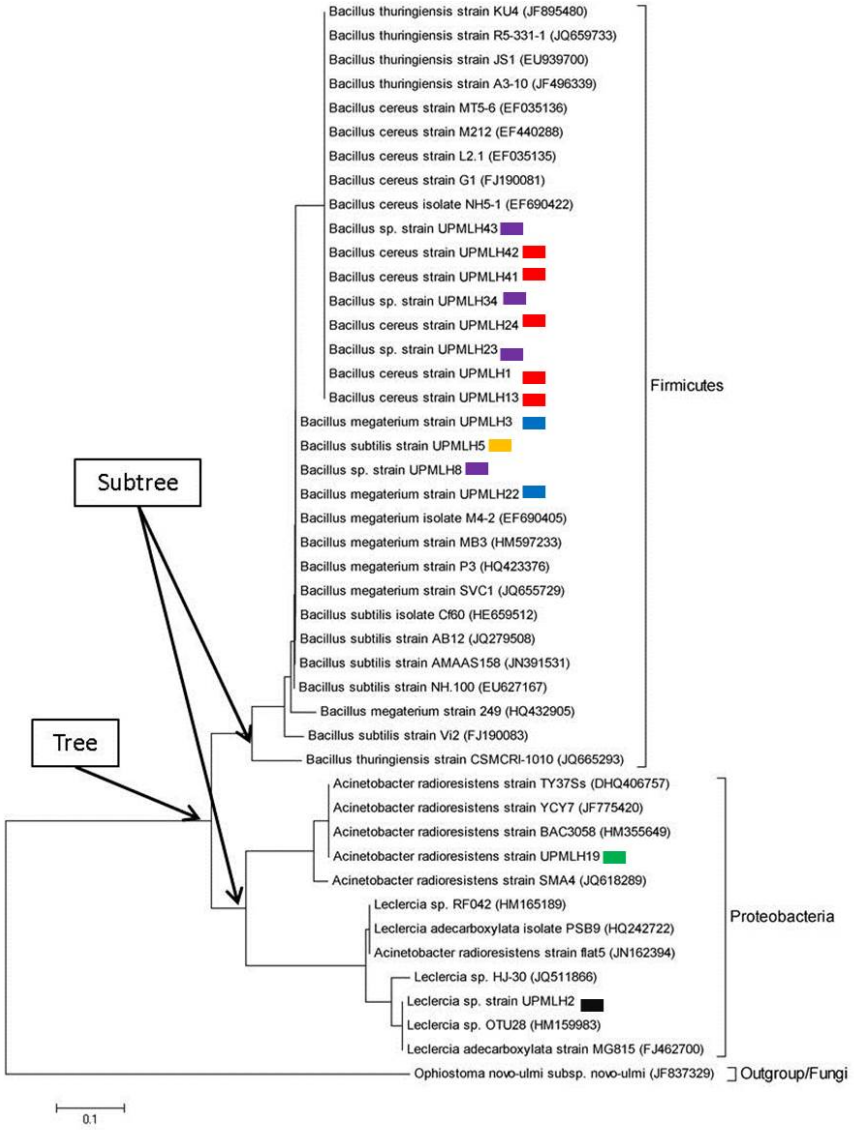

Figure 2: Neighbour joining tree based on alignment of translated amino acid sequences of the 16S rRNA gene from 14 newly isolated rhizobacterial strains (UPMLH) and reference strains (Genbank accession number). Bar 0.1 substitutions per translated amino acid position. *Ophiostoma novo-ulmi* subsp. *novo-ulmi* was used to form a tree. Different coloured bars indicate position and different species of newly isolated rhizobacteria from pepper (*Piper nigrum* L.).

The phylum Proteobacteria was made up mostly of *Leclercia* sp. while Firmicutes made up another branch of the subtree. The Proteobacteria-Leclercia

subtree was, hence, separated from the phylum Firmicutes at the sub-subtree level. The Firmicutes sub-subtree showed that *Bacillus cereus* strains were closely related to the *Bacillus subtilis* strains. At the same time, *Bacillus cereus* strains shared high identity with *Bacillus thuringiensis*.

In the translated amino acid-based phylogenetic tree (Figure 2), all novel strains formed two distinct subtrees of two phyla and were closely related with each other, especially in the case of *Bacillus subtilis* and *Bacillus megaterium*. The phylogenetic tree based on translated amino acid sequences was less complex as compared with the nucleotide-based phylogenetic tree.

The present study used PCR amplification and nucleotide sequencing of the ribosomal 16S rRNA to identify rhizobacterial strains from the *P. nigrum* rhizosphere. Almost all the isolates were successfully identified at least to genus level, although there are five rhizobacterial strains where it had not been possible to identify to the species level. This is may be because of lack of sequence information in the database. 16S rDNA sequence of strain UPMLH2 was identified as a species of *Leclercia* with 99 % homology with *Leclercia adecarboxylata* strain EPCC5.

The strain UPMLH8 16S rDNA sequence had 100 % similarity both to *Bacillus aryabhattai* strain YNA12 and *Bacillus megaterium* strain EN2. Hence, the strain UPMLH8 is, for the time being, identified as *Bacillus* sp. strain UPMLH8, pending further analysis to determine its species. Strains UPMLH23, UPMLH34, UPMLH43 were identified as *Bacillus* sp., and according to 16S rDNA sequences, they have the highest similarity (100-99 %) between *Bacillus cereus* and *Bacillus thuringiensis*. The *Bacillus cereus* group includes *B. cereus*, *B. thuringiensis*, *B. anthracis*, all having similar biochemical, morphological and genetic characteristics (Jensen *et al*., 2003; Bavykin *et al*., 2004). *B. cereus* can be distinguished from *B. thuringiensis* by screening for the presence of the *cry* gene or its crystal protein that is associated with the latter (Chen and Tsen, 2002).

Phylogenetic analysis conducted in the present study indicated that all rhizobacterial strains isolated from *P. nigrum* rhizosphere were successfully positioned and clustered according to their genetic background. However, translated amino acid phylogenetic tree showed a simpler structure of relationships. Since cell-cell communication always occurs in the root zone with the exchanging and sharing for food source and genetic materials (Federle and Bassler, 2003), the isolated rhizobacteria are closely related at amino acid level, especially for the bacteria under the phylum Firmicutes.

In conclusion, the present study showed that fourteen rhizobacterial isolates that have been identified by 16S rRNA sequencing and were shown to belong to several species, namely *Acinetobacter radioresistens* (UPMLH1), *Bacillus* spp. (UPMLH8, UPMLH23, UPMLH34 and UPMLH43), *Bacillus cereus* (UPMLH1, UPMLH13, UPMLH24, UPMLH41 and UPMLH42), *Bacillus megaterium* (UPMLH3 and UPMLH22), *Bacillus subtilis* (UPMLH5) and *Leclercia* sp. (UPMLH2). All the identified strains were successfully positioned in the phylogenetic tree at the nucleotide and the amino acid sequence levels.

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