

# Diversity of endophytic bacteria in medicinally important *Nepenthes* species

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## Abstract

**Background:** *Nepenthes* species are used in traditional medicines to treat various health ailments. However, we do not know which types of endophytic bacteria (EB) are associated with *Nepenthes* spp. **Objective:** The objective of this study was to isolate and to identify EB associated with *Nepenthes* spp. **Materials and Methods:** Surface-sterilized leaf and stem tissues from nine *Nepenthes* spp. collected from Peninsular Malaysia were used to isolate EB. Isolates were identified using the polymerase chain reaction-amplified 16S ribosomal DNA (rDNA) sequence similarity based method. **Results:** Cultivable, 96 isolates were analyzed; and the 16S rDNA sequences analysis suggest that diverse bacterial species are associated with *Nepenthes* spp. Majority (55.2%) of the isolates were from *Bacillus* genus, and *Bacillus cereus* was the most dominant (14.6%) among isolates. **Conclusion:** *Nepenthes* spp. do harbor a wide array of cultivable endophytic bacteria.

**Key words:** 16S ribosomal DNA (rDNA), bacteria, diversity, endemic, endophytes, Malaysia, *Nepenthes*

## INTRODUCTION

Malaysia (Borneo) and Indonesia hosts the largest number of endemic *Nepenthes* spp.<sup>[1,2]</sup> The fluid from young unopened pitchers is used in cleaning wounds or treating incontinence, distress and pain.<sup>[3]</sup> The decoction of *Nepenthes* spp. aerial parts are used in the treatment of kidney stones, hypertension, fever and cough (<http://www.forestry.gov.my/>).

The earlier studies have shown that endophytic microorganisms isolated from medicinal plants produce the same metabolites as their hosts.<sup>[4]</sup> Therefore, there is a great potential in exploring endophytes as a source of

therapeutic natural products. However, despite several traditional medicinal applications of *Nepenthes* spp., what types of endophytes are associated with them is not known. The objective of this study was to isolate and to identify the endophytic bacteria (EB) from *Nepenthes* spp.

## MATERIALS AND METHODS

*Nepenthes* spp., namely, *Nepenthes ampullaria*, *N. gracilis*, *N. macfarlanei*, *N. mirabilis*, *N. rafflesiana* and *N. sanguinea* were collected from FRIM, Selangor, Malaysia. However, leaves and twigs from one to three individual plants of *Nepenthes* spp., namely, *N. alba*, *N. albomarginata*, *N. gracillima* and *N. sanguinea* were collected from the Gunung Jerai (GJ), Gurun, Kedah, Malaysia.

Leaves with petioles were thoroughly washed under running tap-water and the surface-sterilization of plant material samples was carried out as reported elsewhere.<sup>[5]</sup> The stem pieces were soaked in 70% ethanol and flamed to make their surface sterile. Aseptically, the leaf and stem tissue pieces were inoculated in the Petri plates containing Luria-Bertani (LB) agar medium. The plates were incubated

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in an incubator at 37°C (±3°C) for 18-20 h in the dark. The isolation, cultivation of endophytes, amplification of 16S rDNA, sequencing of 16S rDNA, identification of endophytes and rooted phylogenetic tree construction was carried out as reported by Bhore *et al.*<sup>[5]</sup>

## RESULTS

Incubation of the inoculated leaf discs and stems pieces on LB agar medium enabled cultivable EB to grow, and the colonies of grown EB were visible on the margins of the leaf and stem tissues. Ninety-six (96) isolates from nine *Nepenthes* spp. were analyzed.

All 96 isolates were identified based on 16S rDNA sequence BLAST (megablast) hits analysis. The annotated 16S rRNA gene fragment (16S rDNA) nucleotide sequences of all isolates have been submitted to the international DNA database (GenBank/DDBJ/EMBL) under accession numbers: JF819686-JF819713 and JF938974-JF939041. Analysis of the identified isolates showed that majority of the isolates from *Nepenthes* spp. were from the Bacilli (59.4%) class, followed by Gammaproteobacteria (35.4%) and Betaproteobacteria (5.2%) [Table 1]. The data analysis also suggests that *Bacillus* spp exist in all nine *Nepenthes* spp.

The 16S rDNA multiple sequence alignment output from CLUSTALW was used in the construction of a rooted dendrogram. The constructed dendrogram is depicted in Figure 1.

## DISCUSSION

In this short and snappy study, we isolated and identified 96 isolates from nine *Nepenthes* spp. EB have been

reported from several medicinal plants, for instance *Glycyrrhiza* spp.,<sup>[6]</sup> *Artemisia annua*<sup>[7]</sup> and *Gynura procumbens*.<sup>[5]</sup> However, to the best of our knowledge, our study is the first to illustrate diversity and types of EB in nine stated *Nepenthes* spp.

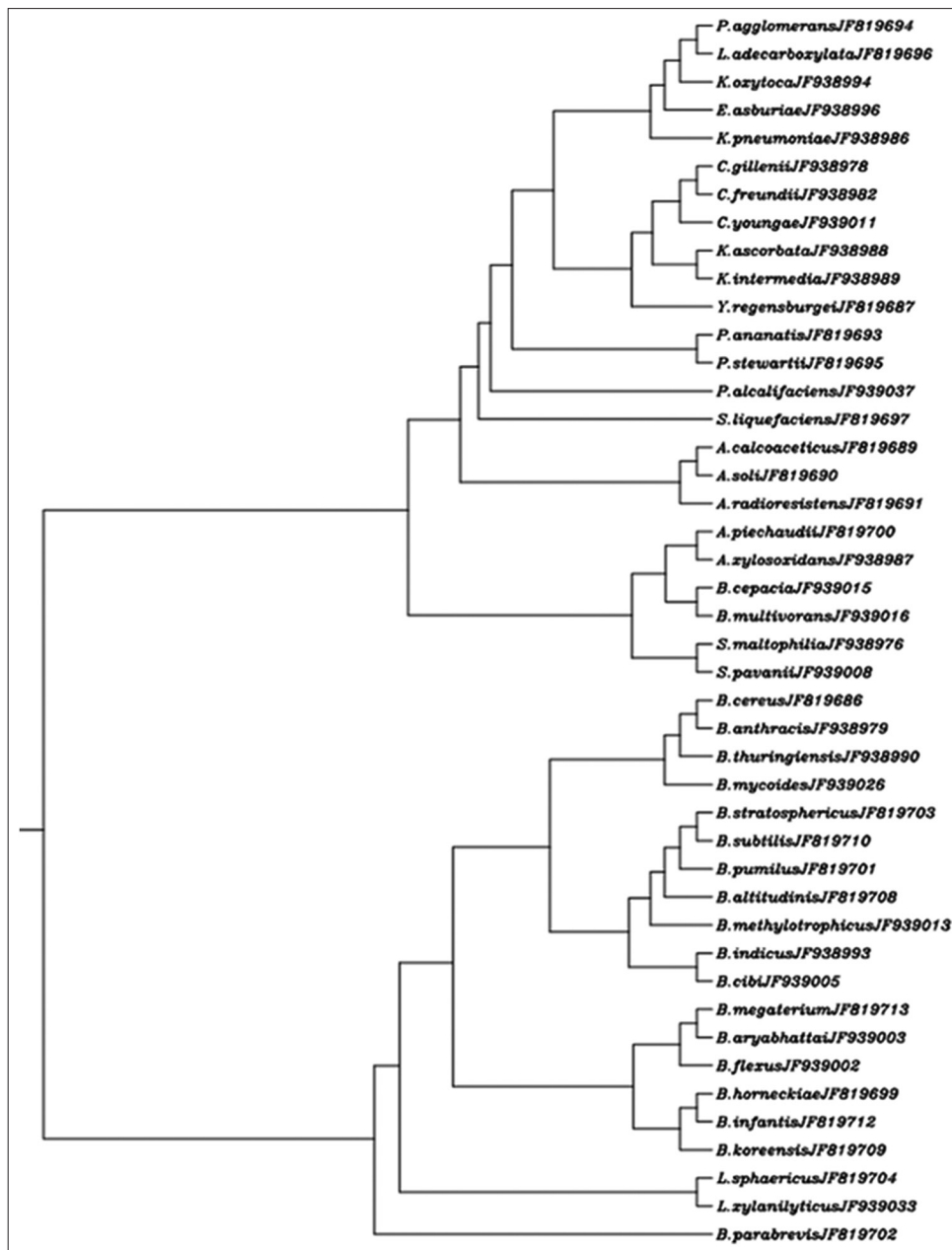
Comparison between the annotated 16S rDNA fragment sequences from isolates and the sequences from GenBank/DDBJ/EMBL database using the BLASTN program revealed the identity of the respective isolates. It appears that the diversity of EB varies from species to species.<sup>[8]</sup> The rooted dendrogram clearly showed the clustering of the species from the Bacilli, Betaproteobacteria and Gammaproteobacteria groups.

Endophytes are found abundantly in various plant species studied to date, and soil bacteria such as *Bacillus* spp., *Pseudomonas* spp. and *Azospirillum* spp. are commonly associated with plants as endophytes.<sup>[9,10]</sup> However, we did not find any *Pseudomonas* spp. and *Azospirillum* spp. in our isolates. During isolation of EB, the growth medium used might be directly affecting the number and type of endophytic microorganisms that can be isolated from the plant tissues. The tissue samples used in the isolation of EB were from a single or few plants of each *Nepenthes* spp. The location and the conditions in which plant species are grown also determine the types of endophytes in it. We have used plant samples from *Nepenthes* spp. that were collected from their wild habitat in GJ and diverse collection available at FRIM. This could be the reason for the wide diversity of EB in the studied *Nepenthes* spp. It is important to note that from 96 isolates, 22 isolates (representing 15 species) belonged to the Enterobacteriaceae family, which contains human enteric pathogens. A number of species from the Enterobacteriaceae family have been reported as

**Table 1: The genera of endophytic bacterial isolates (EBIs) isolated from 9 *Nepenthes* plant species**

Class	Order	Family	Genus	Species No <sup>1</sup>	Isolates <sup>#</sup>
Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	17	53
		Paenibacillaceae	<i>Lysinibacillus</i>	2	3
			<i>Brevibacillus</i>	1	1
Betaproteobacteria	Burkholderiales	Alcaligenaceae	<i>Achromobacter</i>	2	3
		Burkholderiaceae	<i>Burkholderia</i>	2	2
		Enterobacteriales	Enterobacteriaceae	<i>Citrobacter</i>	3
<i>Enterobacter</i>	1			2	
<i>Klebsiella</i>	2			3	
<i>Kluyvera</i>	2			3	
<i>Leclercia</i>	1			1	
<i>Pantoea</i>	3			4	
<i>Providencia</i>	1			1	
<i>Serratia</i>	1			1	
<i>Yokenella</i>	1			2	
<i>Acinetobacter</i>	3			6	
Xanthomonadales	Xanthomonadaceae	<i>Stenotrophomonas</i>	2	5	
		Total	44	96	

<sup>1</sup>number of species of respective bacterial genus within total isolates obtained from 9 *Nepenthes* spp, <sup>#</sup>total number of isolates representing respective genus of bacteria



**Figure 1:** Rooted dendrogram showing clustering of 44 diverse types of endophytic bacteria (EB) isolated from nine *Nepenthes* spp. The accession number of 16S rDNA (rRNA gene) sequence of the respective isolate is given in front of the species name

endophytes, viz *Enterobacter cloacae* and *Klebsiella pneumoniae* in maize, *Enterobacter asburiae* in cotton, and *Klebsiella* spp. and *Enterobacter cloacae* in banana.<sup>[11,12]</sup>

Of the 44 species of EB isolated from *Nepenthes* spp., 33 species have been reported as endophytes in various plant species. However, we have not found any published record that reported *Acinetobacter soli*, *Bacillus cibi*, *B. horneckiae*, *B. indicus*, *B. koreensis*, *B. stratosphericus*, *Citrobacter gillessii*, *C. youngae*, *Kluyvera ascorbata*, *Providencia alcalifaciens* and *Serratia liquefaciens* as endophytes. Perhaps, this is the first study that reports these bacterial species as endophytes. However, the benefits derived by *Nepenthes* spp. from

these bacterial endophytes and its quantum are not clearly understood yet.

From this study, we concluded that *Nepenthes* spp. contains diverse types of cultivable EB, and that the majority of bacterial endophytes (59.4%) were from the Bacilli class. Nonetheless, these research findings could serve as a foundation in further research on the therapeutic properties of *Nepenthes* spp. in correlation with their bacterial endophytes. We hypothesize that in *Nepenthes* spp., these EB might be involved in producing bioactive compounds of pharmaceutical importance, and further research is required to ascertain the same.

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