16S rRNA gene taxonomic profiling of endophytic bacteria associated with *Phalaenopsis* roots

D. Girija¹, P.K. Rajeevan, Swathi Balakrishnan¹, P.S. Panchami¹ and Mahesh Mohan¹
Department of Agricultural Microbiology¹, Department of Floriculture and Landscaping²
Kerala Agricultural University, Vellanikkara, 680656
*Email: devakovita@gmail.com

ABSTRACT

Orchids are one of the main groups of ornamental plants commercially exploited. In the present study, we analyzed the diversity of bacterial community in *Phalaenopsis* root using metagenomic approach. The diversity of bacterial taxonomic category was assessed at different Operational Taxonomic Unit (OTU) levels using Ribosomal Database Project (RDP) pipeline and MG-RAST. At phylum level, Proteobacteria (61.34%) was the most dominant group followed by unclassified derived from bacteria (24.74%) and Actinobacteria (12.52%). Genus level analysis revealed the abundance of *Rubrobacter*, *Pseudomonas* and *Acinetobacter*. The study revealed that of the total species detected 50.83 per cent were unclassified, stressing the importance of metagenomics to assess the diversity of endophytes associated with orchid roots.

Keywords: Endophyte, orchid, diversity

INTRODUCTION

Orchidaceae is one of the largest plant families, including almost 10% of all flowering plant species. Among these, the monopodial epiphytic *Phalaenopsis* or ‘Moth Orchid is one of the most popular orchids due to its ease of production and blooming year-round. The orchid roots are associated with various fungi and endotrophic bacteria (Teixeira et al., 2015). Apart from mycorrhizal fungi, previous reports revealed the abundance of endophytic bacteria on the roots of the cultivated tropical orchids of genera *Calanthe*, *Acampe* and *Dendrobium* (Tsavkelova et al., 2003). Orchids are characterized by low survival rate in the green house due to the germination under asymbiotic conditions in vitro. Generally, endophytes play an important role in promoting plant growth and yield, suppress pathogens, aid in removing heavy metal contaminants, solubilize phosphate or contribute to nitrogen assimilation for plants (Hallmann et al., 2006). Over the past decade, our understanding of microbial diversity and function in complex environments has increased significantly, primarily because of the introduction of next generation sequencing (NGS) (Lozupone and Knight, 2007). The culture-independent, high-throughput sequencing-based community analysis allows us to observe the microbiome associated with the plants. Since the endophytes have a strong impact on orchids growth, it is very important to study their relationships with plant for developing new strategies for orchid conservation and better exploitation of their medicinal principles. Therefore, in the present study, we employed NGS technology to unveil the culturable and unculturable endophytic bacteria in *Phalaenopsis* root to elucidate the microbial plant colonisation pattern and evaluate its microbial diversity.

The *Phalaenopsis* plants grown in Sphagnum moss under green house conditions were collected from the Department of Pomology and Floriculture, College of Horticulture, Vellanikkara. Samples were immediately transferred and processed for further studies. The roots were detached with sterile knife and washed with sterile distilled water plus a few drops of Tween-20 and left for 10–15 min to drain. These were then cut into 4–5 pieces (2–3 cm in size). Surface sterilization was performed by immersing separately in 90% ethanol (5 min), followed by sodium hypochlorite (3%) solution (2 min), and 75% ethanol (3 min). The disinfected roots were rinsed three times in sterile distilled water. Total genomic DNA was extracted from the surface sterilized root tissues using QIAGEN DNeasy plant kit following the manufacturer’s protocol. Extracted DNA was
suspended in QIAGN elution buffer and stored at 
-20°C. PCR amplification was carried out to amplify V3 conserved region of 16S rRNA gene sequences using the 16S rRNA gene primers (forward primer 5¹-
CCTACGGGNGGCWGCAG-3¹ and reverse 5¹-
GACTACHVGGGTATCTA-3¹). The amplicons were 
purified and sequenced on the Illumina Miseq platform 
at Scigenom Pvt. Ltd. Cochin. The FASTQ sequences 
were filtered to remove chimeric sequences and 
singletons to obtain preprocessed reads, which was 
than clustered to obtain OTUs. The chloroplast 
sequences that comprised of almost 97.5 per cent 
of the total reads were removed using QIIME analysis. 
Further taxonomic annotation of the 301 OTUs 
obtained were done using QIIME and MG-RAST tools.

Total DNA was isolated from the roots of Phalaenopsis plants and the presence of 16S rRNA 
gene was confirmed by amplification with universal 
primers. Total raw sequencing reads (paired end) of 
1,96,595 with average sequence length of 151 bp each 
was obtained from Illumina MiSeq™ sequencer.

The abundance of major bacterial groups in each 
taxonomic category is given in Table 1. Altogether, 
10 bacterial phyla were detected and among these, 
Proteobacteria (61.34%) was the most dominant group 
followed by unclassified derived from bacteria 
(24.74%) and Actinobacteria (12.52%). Reads 
belonging to Acidobacteria, Bacteriodetes, Chloroflexi, 
Cyanobacteria, Spirochaetes, Tenericutes, Firmicutes 
and Bacteriodetes were found to be the other phyla 
with less than 1 per cent (Fig 1). The higher abundance 
of Proteobacteria in the roots of orchids suggests that 
members of this phylum are particularly well adapted 
to colonize inner plant tissues and establish as root 
endophytes. The phylum Proteobacteria comprises 
several species that promote plant growth and act as 
biological control agents of different diseases (Bulgarelli et al., 2013). Actinobacteria play specific roles, for 
instance, protecting the host plants against insects and 
diseases especially by the production of bioactive 
compounds. Firmicutes were found to be metabolically 
the most versatile group with production of multiple 
enzyme activities. Cyanobacteria are photosynthetic; 
some are capable of fixing nitrogen and others improve 
soil-aggregation stability (Issa et al., 2007), a key 
aspect of soil conservation. Results of the present 
investigation are in agreement with the earlier reports 
on Proteobacteria, Actinobacteria, Firmicutes and 
Bacteriodetes being the prominent phyla in the roots of 
tree peony (Yang et al., 2017).

A total of 7 bacterial classes were identified 
and among them Gammaproteobacteria was the 
most dominant group (41.90%) followed by 
unclassified (derived from bacteria) (31.35%) 
Actinobacteria (25.11%) and Bacilli (1.37%) 
(Fig 2). A total of 17 bacterial orders were detected. 
The most dominant group was unclassified derived 
from bacteria (35.99%) followed by 
Pseudomonadales (30.69%) and Rubrobacterales

---

**Fig. 1.** Abundance of endophytic bacteria at phylum level constructed in MG-RAST with illumina sequencing data set

**Fig. 2.** Abundance of endophytic bacteria at genus level constructed in MG-RAST with illumina sequencing data set

**J. Hortl. Sci.**
**Vol. 13(1) : 103-107, 2018**
Table 1. Abundance of major taxonomic category from phyla to species level of endophytic bacteria in *Phalaenopsis* root

| Sl No | Phylum                | Class                  | Order           | Family               | Genus             |
|-------|-----------------------|------------------------|-----------------|----------------------|-------------------|
| 1     | Proteobacteria        | Gammaproteobacteria    | Pseudomonadales | Pseudomonadaceae     | Pseudomonas       |
|       | (61.34 %)             | (41.90 %)              | (30.68 %)       | (21.67 %)            | (21.67 %)         |
|       | Betaproteobacteria    | (19.60 %)              | Enterocheckterales | (9.00 %)          | Acinetobacter     |
|       | (0.04 %)              |                        | Xanthomonadales | (1.96 %)            | (9.00 %)          |
|       |                       |                        | Burkholderiales | (0.71 %)            | Entebactera        |
|       |                       |                        |                 |                      | (1.78 %)          |
|       |                       |                        |                 |                      | Achromobacter     |
|       |                       |                        |                 |                      | (0.03 %)          |
| 2     | unclassified          | unclassified (derived from Bacteria) | unclassified (derived from Bacteria) | unclassified (derived from Bacteria) | unclassified (derived from Bacteria) |
|       | (derived from Bacteria) | (31.35 %)               | (35.99 %)       | (35.99 %)            | (35.99 %)         |
| 3     | Actinobacteria        | Actinobacteria         | Actinomycetesales | Rubrobacteraceae     | Rubrobacter       |
|       | (12.52 %)             | (25.11 %)              | (0.5 %)         | (27.47 %)            | (27.47 %)         |
|       |                       |                        | Rubrobacterales | (0.69 %)            | Pseudonocardia     |
|       |                       |                        |                 |                      | (0.66 %)          |
|       |                       |                        |                 |                      | Kribbella         |
|       |                       |                        |                 |                      | (0.44 %)          |
| 4     | Firmicutes            | Bacilli                | Bacillales      | Paenibacillaceae     | Aneurinobacillus  |
|       | (0.83 %)              | (1.57 %)               | (1.57 %)        | (1.27 %)             | (1.27 %)          |
|       |                       | Clostridia             |                 |                      | Bacillus          |
|       |                       | (0.002 %)              |                 |                      | (0.14 %)          |
| 5     | Cyanobacteria         | Unclassified (derived from cyanobacteria) |              |                      |                   |
|       | (0.38 %)              | (0.001 %)              |                 |                      |                   |
| 6     | Bacteroidetes         | Bacteroidetes          | Bacteroidiales  | Bacteroidiales       | Rikenellaceae     |
|       | (0.17 %)              | (0.14 %)               | (0.14 %)        | (0.09 %)             | (0.09 %)          |
|       |                       | Flavobacteria          |                 |                      |                   |
|       |                       | (0.03 %)               |                 |                      |                   |
|       |                       | Cytophagia             |                 |                      |                   |
|       |                       | (0.002 %)              |                 |                      |                   |
| 7     | Acidobacteria         |                        |                 |                      |                   |
|       | (0.02 %)              |                        |                 |                      |                   |
| 8     | Chelomobile            |                        |                 |                      |                   |
|       | (0.002 %)             |                        |                 |                      |                   |
| 9     | Spirochaetes          |                        |                 |                      |                   |
|       | (0.002 %)             |                        |                 |                      |                   |
| 10    | Tenericutes            |                        |                 |                      |                   |
|       | (0.002 %)             |                        |                 |                      |                   |

*J. Hort. Sci.*
*Vol. 13(1): 103-107, 2018*
(27.47%). Orders Actinomy etales, Bacillales and Enterobacteriales were also present more than one per cent. Analyses at family level revealed a total of 22 bacterial families were present in the sample. Major bacterial families present in the sample were unclassified derived from bacteria (36%), followed by Rubrobacteraceae (27.48%), Pseudomonadaceae (21.68%) and Moraxellaceae (9.01%). The Gamma Proteobacteria included Pseudomonas, Pantoea, Acinetobacter, Stenotrophomonas and Xanthomonas making it phylogenetically the most diverse group in the current study. Altogether 31 bacterial genera were present. Unclassified derived from bacteria (35.99%) was the most dominant group in the present study followed by Rubrobacter (27.47%), Pseudomonas (21.67%) and Acinetobacter (9%). Genus Enterobacter and Aneurinibacillus were also present at more than one per cent abundance. Genus Rubrobacter is well known to be a radiation resistant bacterium. Genus Pseudomonas can utilize more than 200 compounds as carbon source, can fix atmospheric N and solubilize P. Several of the genera isolated in the current study, including Pantoea, Pseudomonas, Bacillus and Acinetobacter have been isolated from different plants and shown to possess plant growth promoting activities (Trivedi et al., 2011). It has been previously observed that in many cases, Pseudomonas and members of Enterobacteriaceae are abundant in both the soil environment and the plant interior (Spiers et al., 2000). The prevalence of Pseudomonas and Bacillus endosymbionts was also reported in Australian terrestrial orchids (Wilkinson et al., 1994). The study emphasizes on the importance of metagenomics to assess the diversity and role of endophytic microbes in plants.

This study extends the knowledge on the composition and diversity in the orchid microbial populations. Moreover, most of the endophytes observed in the present study are perhaps good producers of bioactive compounds, which can promote the growth of orchids in seedling stage and also in ex vitro acclimatization.

REFERENCES

Bulgarelli D., Schlaeppi, K., Spaepen, S., Ver Loren and van Themaat E. 2013. Structure and functions of the bacterial microbiota of plants. Annu. Rev. Plant Biol, 64: 807-838.

Hallmann, J., Berg, G and Schulz, B. 2006. Isolation procedures for endophytic microorganisms.in: B.E. Schulz, C.C. Boyle, T. Sieber (Eds.), Microbial Root Endophytes, Vol. 9, Springer, Berlin Heidelberg 2006, p. 299–319.

Issa, O.M., Defarge, C., Le Bissonnais, Y., Marin, B., Duval, O., Bruand, A., D’Acqui, L.P., Nordenberg, S. and Annerman, M., 2007. Effects of the inoculation of cyanobacteria on the microstructure and the structural stability of a tropical soil. Plant Soil, 290:209–219

Lozupone, C.A. and Knight, R. Global patterns in bacterial diversity. 2007. Proc. Natl. Acad. Sci., 104: 11436–11440.

Spiers, A.J., Buckling, A. and Raineythen, P.B. 2000. The causes of Pseudomonas diversity. Microbiology,146:2345–2350.

Teixeira da Silva J.A., Tsavkelova, E.A., Zeng, S., Parthibhan, S. and Rao M.V. 2015. Symbiotic in vitro seed propagation of Dendrobium: fungal and bacterial partners and their influence on plant growth and development. Planta, 24:21–22.

Trivedi, P. Spann, T. and Wang, N. 2011. Isolation and characterization of beneficial bacteria associated with citrus roots in Florida Microb Ecol., 62:324-336
Tsavkelova, E.A., Klimova, Y.S., Cherdyntseva, T.A. and Netrusov, A.I. 2006. Microbial producers of plant growth stimulators and their practical use: a review. Appl. Biochem. Microbiol., 42:133–143.

Yang, R., Liu, P. and Ye, W. 2017. Illumina-based analysis of endophytic bacterial diversity of tree peony (Paeonia Sect. Moutan) roots and leaves. Braz. J. Microbiol., 48: 695-705.

Wilkinson, K., Dixon, K., Sivasithamparam, K. and Ghisalberti, E., 1994. Effect of IAA on symbiotic germination of an Australian orchid and its production by orchid-associated bacteria. Plant Soil, 159: 291–295.

(MS Received 10 August 2017, Revised 03 April 2018, Accepted 30 June 2018)