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Diversity of Endophtic Bacteria in *Aerides crispa*, an Epiphytic Orchid

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ABSTRACT

Aerides crispa is an epiphytic flowering orchid belongs to the family Orchidaceae.we have identified five different endophytic bacteria from this orchid. Genotypic characterization of the bacterial culture was done using 16S rDNA sequencing after PCR amplification. These sequences were compared with the known similar bacterial sequences from the NCBI GenBank database. Homological relationship of the bacteria was also compared by Phylogenetic tree generation. Bacteria obtained were *Bacillus pumilus MT463728, Bacillus megaterium MT540506, Lysinibacillus fusiformis MT540507, Bacillus cereus MT540510, Aneurinibacillus migulanus*. From the study, we have molecularly characterized and documented five different endophytic bacteria from the epiphytic orchid, *A crispa*. This shows the diversity of endophytes in orchids which speaks a lot about the urgent necessity of bioconservation without words.

Keywords : *Aerides crispa,* endophytic bacteria, 16S rDNA, similarity matrix, phylogenetic tree, bioconservation

I. INTRODUCTION

Kodagu district in Karnataka is located in the central part of the Western Ghats, situated in south India comprises of 50% forest and agro-forestry area. Trees of Western Ghats harbor a variety of epiphytic orchid [10].The forests of Western Ghats are known to be a varietal storehouse of economically important plants. The tropical climate, heavy rainfall from southwest monsoon and favorable soil factors made the area ideal for the rich biodiversity. The central Western Ghats area of Karnataka covers places viz., Kodagu, Hassan, Chikmagalur, Shivamogga, and Uttara kannada [1]. Orchids are one among the most threatened of all flowering plants. The Orchidaceae is one of the largest plant families with more than 25,000 species globally. These plants are known for its beauty and medicinal property. *Aerides crispa*, epiphytic flowering plants that habitat on tree trunks

in broad leaved evergreen forests and semi-evergreen forest which belongs to Orchidaceae family.

Endophyte, by definition, is one which resides in the tissues beneath the epidermal cell layers and causes no apparent harm to the host [16]. They form inconspicuous infections within tissues of healthy plants for all or nearly all their life cycle [12] .Endophytic bacteria can be defined as those that can be isolated from healthy, superficially disinfected plant tissues and do not cause any damage to the host plant [2][3]. Orchid species are critically dependent on mycorrhizal fungi for completion of their life cycle, particularly during the early stages of their development when nutritional resources are scarce. Mycorrhizal specificity was low, but significant variation in mycorrhizal community composition was observed between species inhabiting different ecological habitats. Molecular identification of endophytic bacteria from the epiphytic plant *Vanda testacea* were discussed in our previous study [4]. The aim of the present study is to identify endophytic bacteria from the epiphytic orchid plant, *Aerides crispa* to its genotypic level.

II. MATERIALS AND METHODS

The plant specimens were collected from the Somwarpet region (located at 10.42°N 74.73°E latitude) agro-forestry area of Kodagu district, Karnataka which is the central part of Western Ghats, hottest hot spot of biological diversity in the world. The parts of the plant such can be randomly cut off with a disinfected sickle and placed separately in sterile polythene bags to avoid moisture loss. The materials were transported to the laboratory within 24 hours and stored at 4°C until the isolation procedures were completed.

The plant brought into the laboratory were processed within 6hours.The collected plant parts were thoroughly washed in running tap water to remove dirt and debries. Fresh healthy leaves and root were selected for endophyte isolation. Epiphytes were removed from the surface by disinfecting the specimen by 70%ethanol for 1minute, 4% sodium hypochlorite solution for 3minutes; 70% ethanol for 30s and two rinses in sterilized distilled water. After removing the excess water, the leaf and root were excised in to the size of 0.5X0.5cm with the help of a sterile blade. A total of 50 segments were screened from each and these segments were placed in petri-plates nutrient agar with chloramphenicol (150 mg/L).These plates were incubated at 28°Cfor 24-48 hours and after getting visible bacterial colony. Each different colonies were cultured separately by streak plate method for obtaining pure culture. These pure cultures were maintained for further experiments [9] [14].

Genomic DNA of bacterial culture were isolated by phenol-chloroform method according to Mora [13]. 16S rDNA obtained were quantified and amplified using the primers: Forward BSF: 5'GAGTTTGATCCTGGCTCAGG 3'; Reverse BSR: 5' TCATCT GTCCC ACCTTCGGC 3'. The process of PCR was done using the set up; 10X PCR buffer: 2.5 µl, $MgCl_2$:2 µl, dNTP's mix (1mM each):5 µl, Primer (10µM): F-0.5µl, R: 0.5µl, *Taq* polymerase (3U/µl): 0.3 µl DNA template (50ng/µl): 4 µl. The PCR programme employed was as follows: primary denaturation for 5 minutes at 94ºC; 35 cycles of denaturation at 94ºC for 30s; annealing for 30 s, and extension at 72ºC for 1 min; and a final extension for 10 minutes at 72ºC. The sequence similarity matrix was generated by comparing the sequences of known bacterial culture from NCBI data base and submitted for the accession number. Phylogenetic tree is constructed by comparing with strains from GenBank with highest similarities. Tree is constructed using kimura 2 model with bootstrap method. The neighbor joining tree and subtree were generated using MEGA5.2 software.

III. RESULTS

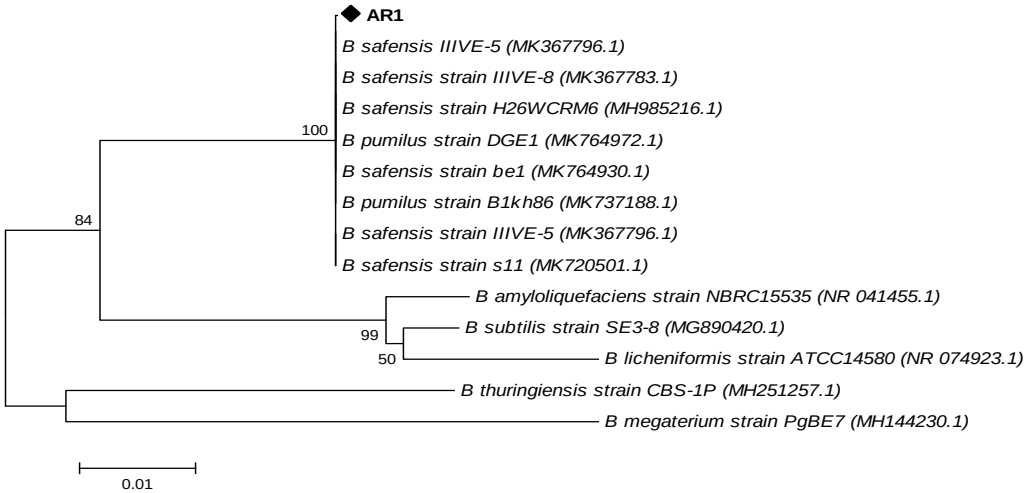
From the epiphytic orchid plant *Aerides crispa*, different plants were used for the isolation of endophytic bacteria. A number of endophytes were obtained. We have selected a few for molecular identification. Those selected ones were AR1, AR2, AR3, AR4 and AL2 (names for temporary convenience). The sequences obtained were submitted to Genbank for Accession numbers; submission code and the accession number of each bacteria were given in the Table 1.

Sl. No.	Bacteria	Submission code	Accession number	Temporary Names
1	Bacillus pumilus	SUB7444699	MT463728	AR1
2	Bacillus megaterium	SUB7526079	MT540506	AR2
3	Lysinibacillus fusiformis	SUB7526079	MT540507	AR3
4	Bacillus cereus	SUB7526079	MT463728	AL2

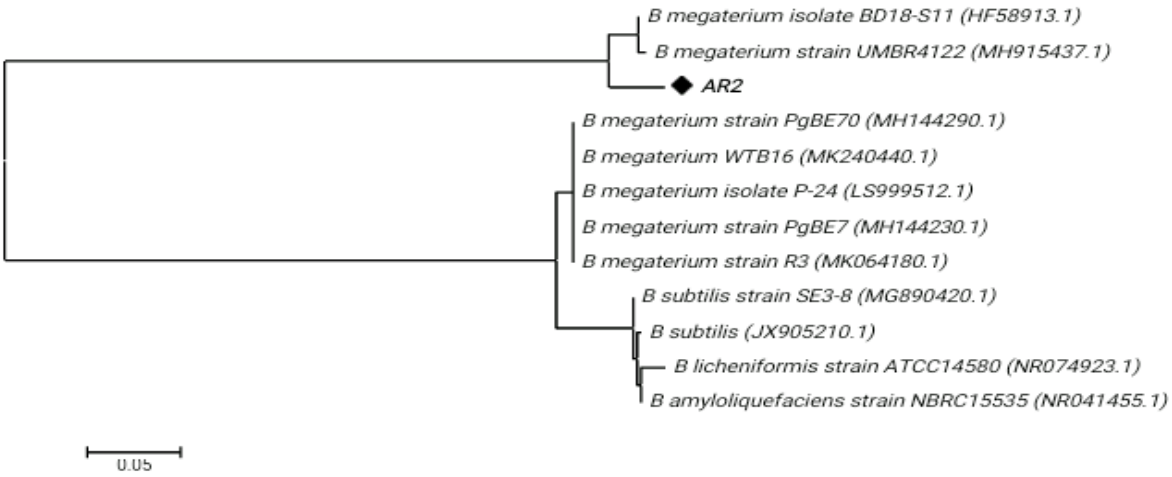
Similarity matrix of each bacteria were given in the tables named Table 2, Table 3, Table 4, Table 5 and Table 6 respectively. AR1: The culture 16S rDNA sequence has 100% similarity with *Bacillus* sp. with maximum homology with *Bacillus pumilus.* AR2: The culture 16S rDNA sequence has 95.0% similarity with *Bacillus* sp. with maximum homology with *Bacillus megaterium.* AR3: The culture 16S rDNA sequence has 99.67% similarity with *Lysinibacillus* sp. with maximum homology with *Lysinibacillus fusiformis.* AR4: The culture 16S rDNA sequence has 98.89% similarity with *Aneurinibacillus* sp. with maximum homology with *A. migulanus.* AL2: The culture 16S rDNA sequence has 100% similarity with *Bacillus* sp. with maximum homology with *Bacillus cereus.*

Phylogenetic tree is constructed by comparing with strains from GenBank with highest similarities. Tree is constructed using kimura 2 model with bootstrap method. The neighbor-joining tree and subtree were generated using MEGA5.2 software. Numbers show the level of bootstrap support from 1,000 repetitions. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. From the experiments, identified bacteria were *Bacillus pumilus, Bacillus megaterium, Lysinibacillus fusiformis, Bacillus cereus, Aneurinibacillus migulanus.* Constructed phylogenetic tree were mentioned in figures (Figure 1 for AR1, Figure 2 for AR2, Figure 3 for AR3, Figure 4 for AR4, Figure 5 for AL2).

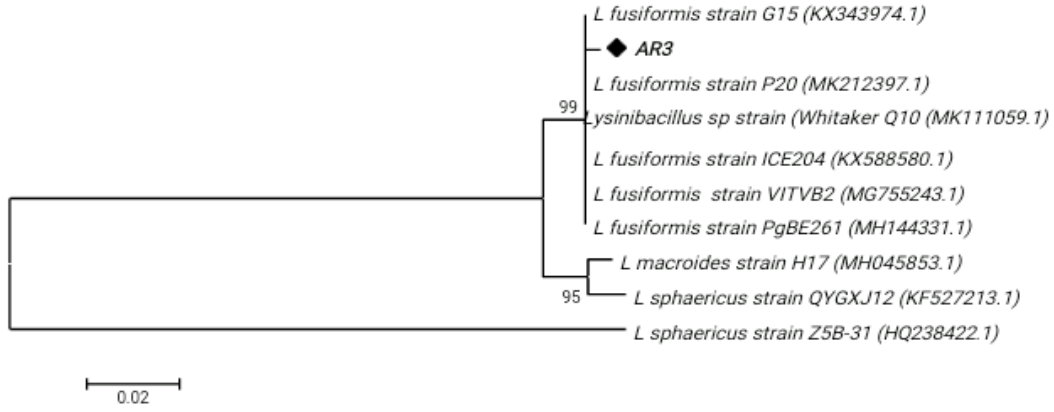
Given below is the **figure 1** indicating phylogenetic tree of bacterial culture AR1



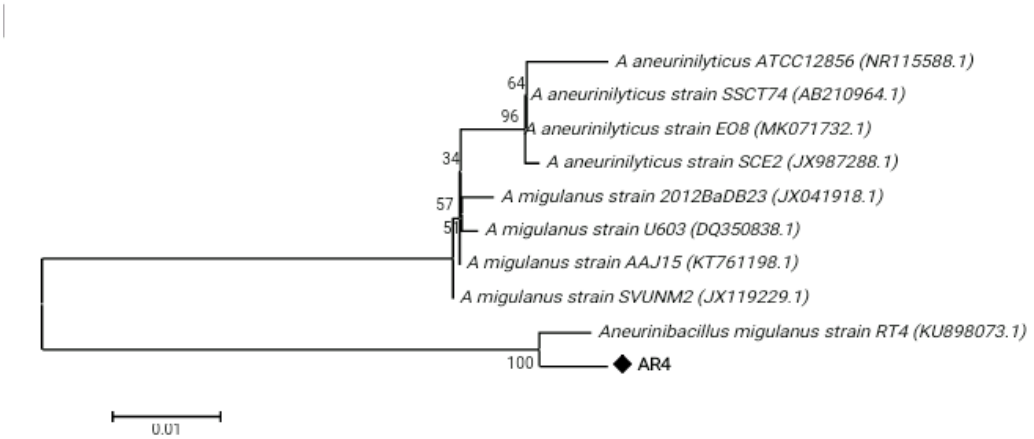
Given below is the **figure 2** indicating phylogenetic tree of bacterial culture AR2



Given below is the **figure 2** indicating phylogenetic tree of bacterial culture AR3



Given below is the **figure 2** indicating phylogenetic tree of bacterial culture AR4



Given below is the **figure 2** indicating phylogenetic tree of bacterial culture AL2

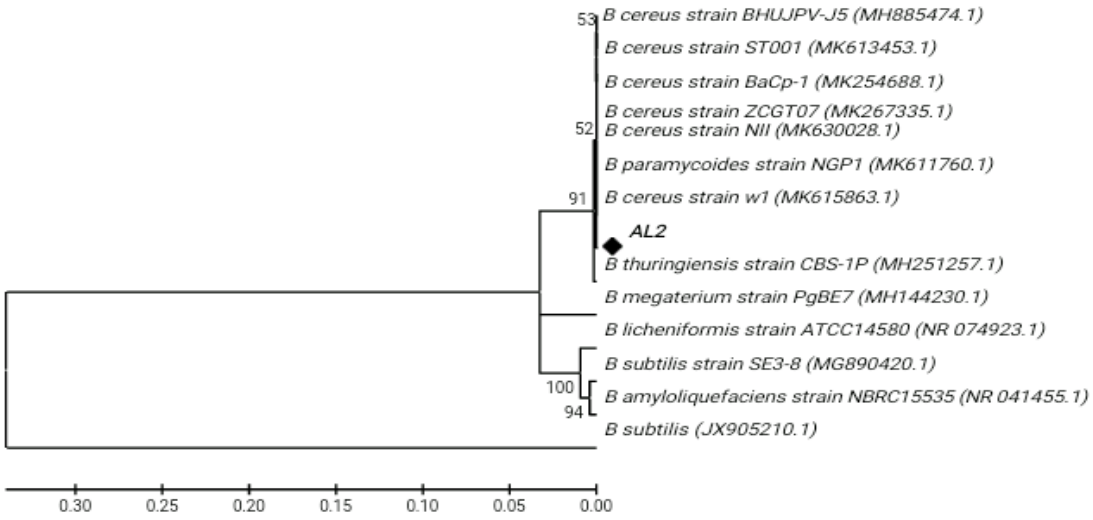


Table 1: Sequence similarity matrix of AR1 16S rRNA partial sequence with the other closely related species

Sequence	1	2	3	4	5	6	7	8	9	10	11	12	13	14
AR1	-													
B_safensis_IIIVE-5 _(MK367796.1)	100	-												
B_safensis_strain_IIIVE-8 _(MK367783.1)	100	100	-											
B_safensis_strain_H26WCRM6 _(MH985216.1)	99.5	99.5	99.5	-										
B_pumilus_strain_DGE1 _(MK764972.1)	100	100	100	99.5	-									
B_safensis_strain_be1 _(MK764930.1)	100	100	100	99.5	100	-								
B_pumilus_strain_B1kh86 _(MK737188.1)	100	100	100	99.5	100	100	-							
B_safensis_strain_IIIVE-5 _(MK367796.1)	100	100	100	99.5	100	100	100	-						
B_safensis_strain_s11 _(MK720501.1)	100	100	100	99.5	100	100	100	100	-					
B_thuringiensis_strain_CBS-1P _(MH251257.1)	92.5	92.5	92.5	92.1	92.5	92.5	92.5	92.5	92.5	-				
B_megaterium_strain_PgBE7 _(MH144230.1)	92.5	92.5	92.5	92.1	92.5	92.5	92.5	92.5	92.5	91.5	-			
B_subtilis_strain_SE3-8 _(MG890420.1)	94	94	94	93.6	94	94	94	94	94	92.5	90.2	-		
B_licheniformis_strain_ ATCC14580	92.6	92.6	92.6	92.2	92.6	92.6	92.6	92.6	92.6	91.9	89.8	97.6	-	

_(NR_074923.1)														
B_amyloliquefaciens_strain _NBRC15535_(NR_041455.1)	94	94	94	93.6	94	94	94	94	94	92.5	89.4	98.7	97.2	-

Table 2: Sequence similarity matrix of AR2 16S rRNA partial sequence with the other closely related species

Sequence	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
B_subtilis (JX905210.1)	-											
B_licheniformis_strain_ATCC14580 (NR074923.1)	98.30	-										
B_amyloliquefaciens_strain_NBRC15535 (NR041455.1)	99.40	98.60	-									
B_subtilis_strain_SE3-8 (MG890 420.1)	99.60	98.30	99.40	-								
B_megaterium_strain_R3 (MK0641 80.1)	94.00	93.40	93.80	94.40	-							
B_megaterium_strain_PgBE7 (MH144230.1)	93.60	93.40	93.60	94.00	99.60	-						
B_megaterium_isolate_P-24 (LS999512.1)	90.50	89.90	90.30	90.80	96.00	96.00	-					
B_megaterium_strain_PgBE70 MH	94.00	93.40	93.80	94.40	100.00	99.60	96.00	-				
B_megaterium_WTB16 (MK240440.1)	92.70	92.10	92.50	93.10	98.60	98.30	97.30	98.60	-			
B_megaterium_strain_UMBR4122 (MH915437.1)	48.80	47.90	48.60	48.80	49.50	49.30	48.20	49.50	48.60	-		
B_megaterium_isolate_BD18-S11_(HF584913.1)	49.00	48.10	48.80	49.00	49.70	49.50	48.40	49.70	48.80	99.20	-	
AR2	47.60	46.90	47.80	47.60	48.70	48.70	48.00	48.70	48.20	92.10	92.70	-

Table 3: Sequence similarity matrix of AR3 16S rRNA partial sequence with the other closely related species

Sequence	1	2	3	4	5	6	7	8	9	10
Lysinibacillus_sp_strain_Whitaker Q10 (MK111059.1)	-									
L_fusiformis_strain_ICE204 (KX 588580.1)	100.00	-								
L_fusiformis_strain_P20 (MK212397.1)	100.00	100.00	-							
L_fusiformis_strain_VITVB2 (MG755243.1)	100.00	100.00	100.00	-						
L_fusiformis_strain_G15 (KX343 974.1)	100.00	100.00	100.00	100.00	-					
L_macroides_strain_H17 MH0458	97.60	97.60	97.60	97.60	97.60	-				
L_sphaericus_strain_QYGXJ12 (KF527213.1)	97.10	97.10	97.10	97.10	97.10	98.50	-			
L_fusiformis_strain PgBE261 (MH144331.1)	100.00	100.00	100.00	100.00	100.00	97.60	97.10	-		
AR3	99.60	99.60	99.60	99.60	99.60	97.30	96.80	99.60	-	
L_sphaericus_strain_Z5B-31 (HQ 238422.1)	75.20	75.20	75.20	75.20	75.20	74.70	74.90	75.20	74.90	-

Table 4:Sequence similarity matrix of AR4 16S rRNA partial sequence with the other closely related species

Sequence	1	2	3	4	5	6	7	8	9	10
A_aneurinilyticus_strain_EO8_(MK071732.1)	-									
A_aneurinilyticus_strain_SCE2_ (JX987288.1)	99.80	-								
A_aneurinilyticus_ATCC12856_(NR115588.1)	99.10	98.90	-							
A_migulanus_strain_2012BaDB23_ (JX041918.1)	98.90	98.80	98.00	-						
A_aneurinilyticus_strain_SSCT74 (AB210964.1)	99.80	99.70	98.90	98.80	-					
A_migulanus_strain_AAJ15_(KT761198.1)	99.20	99.10	98.30	99.40	99.10	-				
A_migulanus_strain_U603_(DQ350 838.1)	99.10	98.90	98.20	99.20	98.90	99.50	-			
A_migulanus_strain_SVUNM2_(JX119229.1)	98.60	98.50	97.70	98.80	98.50	99.10	98.90	-		
Aneurinibacillus_migulanus_strain RT4 (KU898073.1)	83.70	83.70	83.00	83.80	83.50	84.10	84.10	83.70	-	
AR4	83.50	83.50	82.80	83.70	83.40	84.00	84.10	83.50	98.80	-

Table 5: Sequence similarity matrix of AL2 16S rRNA partial sequence with the other closely related species

Sequence	1	2	3	4	5	6	7	8	9	10	11	12	13	14
B_cereus_strain_ZCGT07 (MK267335.1)	-													
B_cereus_strain_NII (MK630028.1)	100.00	-												
B_cereus_strain_BHUJPV-J5 (MH885474.1)	94.30	94.30	-											
B_cereus_strain_BaCp-1 (MK254688.1)	100.00	100.00	94.30	-										
B_paramycoides_strain_NGP1 (MK611760.1)	100.00	100.00	94.30	100.00	-									
B_cereus_strain_w1 (MK615863.1)	100.00	100.00	94.30	100.00	100.00	-								
B_cereus_strain_ST001 (MK613453.1)	99.80	99.80	94.30	99.80	99.80	99.80	-							
AL2	100.00	100.00	94.30	100.00	100.00	100.00	99.80	-						
B_thuringiensis_strain_CBS-1P_(MH251257.1)	99.70	99.70	94.10	99.70	99.70	99.70	99.60	99.70	-					
B_megaterium_strain_PgBE7_(MH144230.1)	93.20	93.20	88.00	93.20	93.20	93.20	93.00	93.20	93.30	-				
B_subtilis_strain_SE3-8_(MG890420.1)	93.40	93.40	88.10	93.40	93.40	93.40	93.30	93.40	93.40	92.40	-			
B_subtilis_(JX905210.1)	46.00	46.00	45.30	46.00	46.00	46.00	46.00	46.00	45.90	44.50	44.90	-		
B_licheniformis_strain_ATCC14580_(NR_074923.1)	93.40	93.40	88.10	93.40	93.40	93.40	93.30	93.40	93.40	92.50	98.10	45.30	-	
B_amyloliquefaciens_strain_NBRC15535_(NR_041455.1)	93.40	93.40	88.10	93.40	93.40	93.40	93.30	93.40	93.40	91.90	99.20	45.00	97.90	-

IV. DISCUSSION

Associative bacteria of terrestrial (*Paphiopedilum appletonianum*) and epiphytic (*Pholidota articulata*) tropical orchids were investigated. Microbial community of epiphytic plant differed from that of the terrestrial one. Observed production of plant growth hormone by the microorganisms and its varying effects were also investigated [18].

Abundant bacteria and diazotrophs were identified in common in different tissues of *D. catenatum* from five planting bases, which might play a great role in the supply of nutrients such as nitrogen. The exact abundance of phylum and genus on the different tissues from different planting bases need deeper sequencing with more samples [11]. Some mycorrhizal fungi themselves have endosymbiotic bacteria *Glomeribacter gigasporarum* [7]. Like mycorrhiza, other endophytic fungi completely depend on the plant and its inside conditions for growth.

Some endophytic fungi have been shown to protect plants from herbivores or to be responsible for the synthesis of novel and useful secondary products [17]. Twelve endophytic bacteria were isolated from the meristem of in vitro *Cymbidium eburneum* orchid, and screened according to indole yield quantified by colorimetric assay, in vitro phosphate solubilization, and potential for plant growth promotion under greenhouse conditions. Suggested that these bacterial effects could be potentially useful to promote plant growth during seedling acclimatization in orchid species other than the species of origin [6] . Fenella and Joshi [8] revealed a definite pattern in the diversity of culturable epiphytic bacteria, host-dependent colonization, microhabitat localization and biofilm formation which play a significant role in plant–microbe interaction. A novel endophytic filamentous bacterium strain was isolated from wild orchid *Grosourdya appendiculata* of Thailand [15].

Associative bacteria of terrestrial (*Paphiopedilum appletonianum*) and epiphytic (*Pholidota articulata*) tropical orchids were investigated. Microbial community of epiphytic plant differed from that of the terrestrial one. Rhizobium and other beneficial microbial diversity of three legumes plants that would help as biofertilizers for the crop from Fabaceae family [5]. Orchid species are critically dependent on mycorrhizal fungi for completion of their life cycle, particularly during the early stages of their development when nutritional resources are scarce. Mycorrhizal specificity was low, but significant variation in mycorrhizal community composition was observed between species inhabiting different ecological habitats. Molecular identification of endophytic fungi from the epiphytic fungi Vanda testacea were discussed in our previos study [4].

Here, we have identified and documented five endophytic bacteria from the epiphytic orchid plant, *Aerides crispa*. These bacteria were *Bacillus pumilus, Bacillus megaterium, Lysinibacillus fusiformis, Bacillus cereus, Aneurinibacillus migulanus*. All are bacillus but different from each other. This shows molecular methods are accurate in identification to avoid misidentification. Presence of variety of endophytic bacteria in one plant shows the importance of conservation of biomes and its habitat. *Aerides crispa* have a habitat favourable for endophytes. Five bacteria were genotypically identified from a single plant. Genotypic characterization is better in comparison with other methods of identifying endophytic microbes from plants. Microbial diversity has also be conserved for future generation along with vulnerable plants such as epiphytic orchids.

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