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Isolation and characterization of epiphytic bacteria from the leaves of *Halophila ovalis* (R. Brown) J.D. Hooker, 1858 from South Andaman, India

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Abstract

Seagrasses are one of the most vital components of the world's ocean, nourishing and hosting a wide flux of organisms from an ecological perspective. *Halophila ovalis* or paddle weed is one of the dominant species of seagrasses of the Andaman and Nicobar Islands. The associated microbiome, especially the epiphytic bacteria, plays a critical role in their interaction with the environment. These bacterial associates were isolated from the leaf blades of seagrass and identified using basic microbiological phenotypic identification techniques along with genotypic confirmation by the 16S rRNA gene sequencing. The phylogenetic analysis of the 16S rRNA gene of the bacterial DNA confirmed 18 different species (*Vibrio alginolyticus* (2), *Vibrio parahaemolyticus, Bacillus subterraneus, Photobacterium ganghwense, Vibrio owensii, Bacillus vietnamensis, Pseudomonas pseudoalcaligenes, Pseudomonas mosselii* (3), *Pseudomonas mendocina* (2), *Bacillus haikouensis, Bacillus aquimaris, Pantoea dispersa, Pseudocitrobacter faecalis, Bacillus subtilis, Vibrio neocaledonicus, Citrobacter freundii, Vibrio hyugaensis, and Pseudomonas putida*) of epiphytic bacteria belonging to seven genera. Information on bacterial epiphytes of seagrass provides a better picture of the bacteria-host interaction leading to a healthy ecosystem.

Keywords: Seagrass, *Halophila ovalis*, Epiphytic bacteria, 16S rRNA

1. Introduction

About two-third of our planet, Earth is sheltered by oceanic waters with a luxuriant flourish of an extensive range of biological entities commencing from simple unicellular prokaryotes to complex multicellular eukaryotes, each of its holding its distinction from its terrestrial counterparts. The oceanic realm with a non-terminating recurring supply of water conceals and masks a wide range of organisms, of which significant shares are underexplored or unidentified. Out of these organisms, many species share similar morphological facets irrespective of their variance in genetic makeup. Hence an authentic, dependable and steadfast scheme for proper identification of these species is the need of the hour compared to the traditional taxonomical methods (Clarridge, 2004).

Seagrasses are marine monocotyledon angiosperms dwelling under submerged conditions (Hartog, 1970) and distributed globally along the intertidal region of the tropical sea except for polar regions (Short *et al*. 2007). Due to their wide range of distribution and interaction with other organisms, they are often referred to as an excellent example of a living ecosystem. They are known to nourish a wide range of organisms, from the smallest bacteria to gastropods, fishes, turtles, dugongs, etc. Due to eutrophication, anthropogenic activities, and global warming, there is a marked significant fall in the world's seagrass cover (Costanza *et al*., 1997; Orth *et al*., 2006). This decline in seagrass can easily alter its physical, chemical, and biological interaction with the oceanic environment.

Halophila ovalis (R. Brown) J.D. Hooker, 1858 species (Fig. 1) commonly referred to as paddle weed are one among the 14 reported species of seagrasses from India distributed along the Gulf of Mannar, Palk Bay, Lakshadweep Islands, Gulf of Kachchh, Ashtamudi estuary, Chilka lake, and Andaman and Nicobar Islands (Jagtap *et al*., 2003, Thangaradjou *et al*., 2010, Immanuel *et al*., 2016, Savurirajan *et al*., 2018). These are small herbaceous plants with leaves arranged in pairs with 1- 4 cm long as oval leaf blades with a narrow and thin stem and rhizome submerged beneath the sand and fine roots that originate from the shoot (Lanyon, 1986). Souza *et al*., 2015 studied the importance of bacteria that can enhance the growth of the plant and protecting it from diseases and abiotic stresses. An evident proportion of the productivity of the seagrass meadows are often attributed to the shelter communities of epiphytes (Wetzel and Penhale, 1979), consisting of the populations of bacteria, fungi, and algae playing a pivotal role in the recycling of organic compounds (Hemminga and Duarte, 2000). The interaction between bacteria and its host seagrasses (Kirchman *et al*., 1984; Welsh, 2000; Holmer *et al*., 2001; Kurilenko *et al*., 2007; Garcias-Bonet *et al*., 2012) validates the idea of it creating a holobiont (Thompson *et al*., 2014; Bordenstein and Theis, 2015). The information regarding bacterial epiphytes of seagrass is very scant, with a majority of research focusing on bacterial communities in the sediment cover of the seagrass (Cifuentes *et al*., 2000, Garcia-martinez *et al*., 2009) and endophytic bacteria resident on the seagrass (Kuo, 1993,

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Ivanova *et al*., 2004, Garcias-Bonet *et al*., 2012). Futuristic studies to be focused on the pattern of relationship between epiphytes and seagrasses to understand the ecological status of the epiphytes, in suppression or promotion of the growth of seagrass and surveillance of epiphytes in monitoring the environmental changes in seagrass communities.

2. Materials and Methods

Samples of seagrass were collected with sterile scissors and forceps from the intertidal region of Chidiyatapu, Haddo, and Mazar Pahar (Fig. 2) South Andaman, in sterile polythene bags and were transported to the laboratory. The leaf blades of the collected seagrasses were rinsed thrice with autoclaved sterile seawater to remove the loosely bound sand particles and other settlements. After washing, gentle swiping was done on the leaf blades using sterile cotton swabs and inoculated in alkaline peptone water at 25 °C for 12 hours. After incubation, about 100 µl was then spread on the pre-prepared Zobell Marine Agar plate and incubated at 28 °C for 24 hours. After the incubation, the plates were checked for growth and based on morphology, colonies were selected and re-streaked. The re-streaked pure colonies were maintained on slants, stabs, and 10% glycerol cultures at -20 °C.

The DNA from the bacterial isolates was extracted using the method described by Mohandass *et al.* (2012) with slight modification. Bacterial isolates were grown overnight in Zobell Marine Broth (HiMedia, India) and were subjected to centrifugation at 8000 rpm at 4 ºC for 10 minutes. The pellet obtained post centrifugation was re-suspended in 1.5 ml of preheated genomic DNA extraction buffer (100 mM Na_2EDTA, 100 mM Tris HCl, 1.5 M NaCl, and 1% CTAB). After the addition of extraction buffer, the samples were well mixed with a sterile loop and were kept for incubation for 60 minutes at 65 ºC. The samples were again centrifuged at 14000 rpm at 20 ºC for 10 minutes. The supernatant was collected in a sterile tube containing 500 µl of phenol: chloroform: iso-amyl alcohol mixture (25:24:1) and was mixed properly and centrifuged at 14000 rpm at 20 ºC for 10 minutes. This step was repeated, and the supernatant was collected in a fresh sterile tube. 500 µl of chloroform was

Fig. 1. *Halophila ovalis*

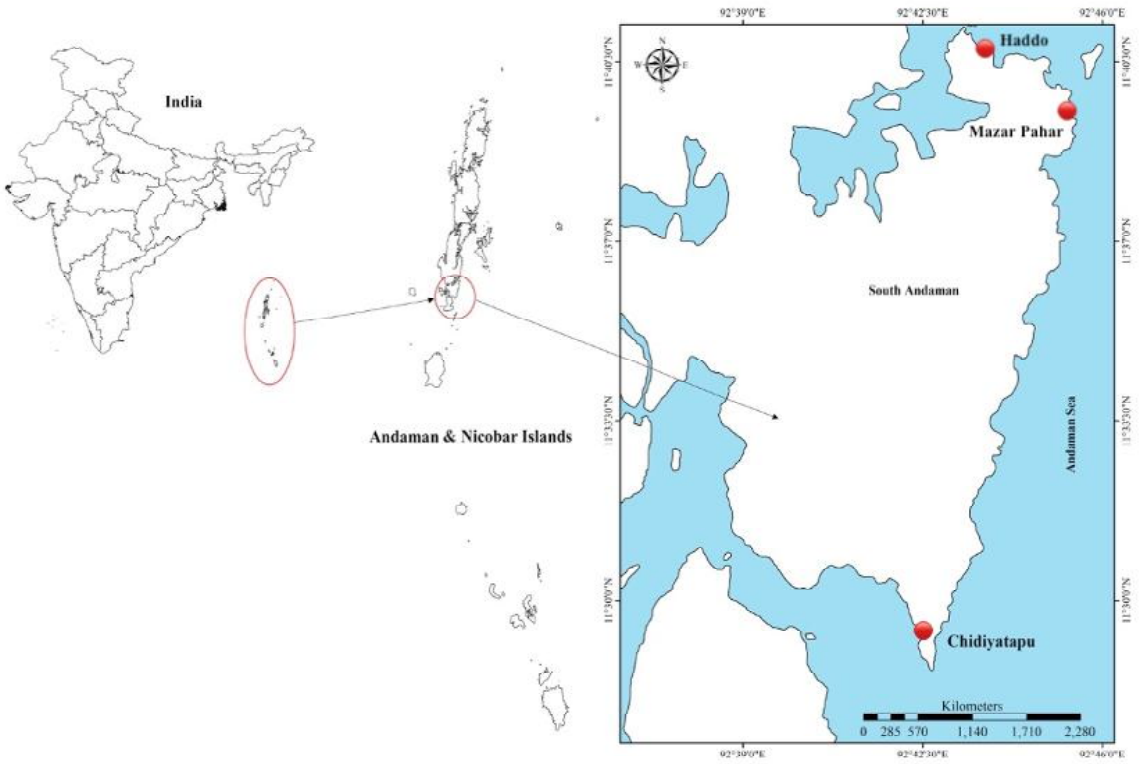

Fig. 2. Map showing the location of Sampling stations

added to the supernatant and centrifuged at 14000 rpm at 20 ºC for 10 minutes, followed by the addition of ice-cold ethyl alcohol to the supernatant for the precipitation of nucleic acid in the supernatant fraction. The sample was then centrifuged and purified with 70% ethyl alcohol, and the final pellets were dried and were re-suspended in molecular biology grade water. The DNA was diluted to 100 ng/µl and was stored at -20 ºC for further study.

After the genomic DNA extraction, the PCR amplification of the 16S rRNA gene was carried out by using universal forward and reverse primer 27F [5'-AGAGTTTGATCCTGGCTCAG-3'] and 1492R [5'-GGTTACCTTGTTACGACTT3'], respectively (Lane, 1991). The PCR was carried out in a final reaction volume of 25 µl. The composition of reaction mixture: 10x PCR buffer, 10 µM dNTPs, 25 mM $MgCl_2$, 1 U of Taq DNA polymerase, 10 pmol of each forward primer and reverse primer, molecular grade water, and approximately 20 ng of bacterial genomic DNA. The amplification program: starting with an initial denaturation at 94 ºC for 3 minutes, following 35 cycles at 94 ºC for 1 minute, 55 ºC for 1 minute, and 72 ºC for 1 minute, ending by a final extension step of 72 ºC for 5 minutes. The final product was run on 1% agarose gel stained with ethidium bromide, and the clear bands were visualized on the gel documentation unit. The amplified PCR products were sequenced using ABI 3500 DNA sequencer (Applied Biosystems).

3. Results and Discussion

A total of 380 colonies were observed from 18 plates, out of which 22 isolates were selected based on the morphological characters. These 22 isolates were subjected to various phenotypic and genotypic characterization. All 22 strains were subjected to routine physiological and biochemical examinations. All the strains were Gram-negative, except GRb4, GRb9, GRb14, GRB15, and GRb18 and growth was not observed in seven strains GRb2, GRb3, GRb5, GRb6, GRb7, GRb28, and GRb30 in Nutrient agar in the absence of NaCl. All the isolates were rod-shaped. Except for GRb4, GRb9, GRb14, GRB15, and GRb18, all strains were motile. Based on these phenotypic examinations, all strains were identified up to the genus level as *Vibrio* (4), *Bacillus* (2), *Photobacterium* (1), and *Pseudomonas* (4) from *Halophila ovalis* of Chidiyatapu (Table 1), *Bacillus* (3), *Pantoea* (1), and *Pseudocitrobacter* (1) from Mazar Pahar (Table. 2), and *Pseudomonas* (3), *Vibrio* (2), and *Citrobacter* (1) from Haddo (Table 2).

Bacterial DNA of all the 22 isolates was amplified with the universal ribosomal primers and have shown a band pattern of approximately 1500 base pairs, which matched with the 16S rRNA genes from the universal primer pair used for the bacterial DNA amplification. Sequences obtained were aligned in ClustalW (Thompson *et al.*, 1994), with a set of referral sequences corresponding to our sequences obtained from the NCBI databank. The phylogenetic analysis of the 16S rRNA gene of the bacterial DNA confirmed 18 species (Table. 3) (*Vibrio alginolyticus* (2), *Vibrio parahaemolyticus*, *Bacillus subterraneus*, *Photobacterium ganghwense*, *Vibrio owensii*, *Bacillus vietnamensis*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas mosselii* (3), *Pseudomonas mendocina* (2), *Bacillus haikouensis*, *Bacillus aquimaris*, *Pantoea dispersa*, *Pseudocitrobacter faecalis*, *Bacillus subtilis*, *Vibrio neocaledonicus*, *Citrobacter freundii*, *Vibrio hyugaensis*, and *Pseudomonas putida*) belonging to phyla Proteobacteria (*Vibrio*, *Photobacterium*, *Pseudomonas*, *Pantoea*, *Pseudocitrobacter*, and *Citrobacter*) and Firmicutes (*Bacillus*). Results obtained were found to match with the findings of Weidner *et al.* (2000), in which the bacterial associates of seagrass *Halophila stipulacea* was studied. Murthy *et al.* (2013) and Mishra and Mohanraju, (2018) also reported similar kinds of bacteria from the intertidal regions of the Andaman and Nicobar Islands. The Phylogenetic tree was constructed using the neighbour-joining tree method (Fig. 3, and 4). The genotypic characterization based on the 16S rRNA gene sequencing was matching the phenotypic characterization based on physiological and biochemical examinations.

Bacterial diversity was high in Chidiyatapu compared to Mazar Pahar and Haddo. The Haddo region was dominated by bacteria belonging to the genus *Bacillus*. Nine different species belonging to four genera were isolated from *Halophila ovalis* of Chidiyatapu. Five different species belonging to three genera were isolated from *Halophila ovalis* of Mazar Pahar six different species belonging to three genera were isolated from *Halophila ovalis* of Haddo. *Pantoea and Pseudocitrobacter* were seen only in Mazar Pahar, and *Photobacterium* and *Citrobacter* were seen exclusively in Chidiyatapu and Haddo. Even though *Vibrio* acts as pathogens for plants and animals, they play a vital role in the nitrogen fixation of seagrass (Criminger *et al.*, 2007). The dominance of the bacteria such as *Vibrio* and *Pseudomonas* in Chidiyatapu and Haddo might be due to the discharge of hydrocarbons in these areas from ships and small motorized boats whereas they were absent in Mazar Pahar, which is an isolated inter-tidal region, and free from these water transportation. The presence of oil-degrading bacteria such as *Pseudomonas putida* (Ward *et al.*, 2006) from these study areas supports our observation. The focus of the present study was to understand the epiphytic bacterial community associated with the leaf blades of the seagrass *Halophila ovalis* with the help of both conventional taxonomic identification and molecular identification methods. Eighteen different species of bacterial epiphytes belonging to seven genera were identified. This study helps in understanding the host-bacteria interaction in the seagrass ecosystem.

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Table 1. Results of Biochemical tests of epiphytic bacteria of *Halophila ovalis* of Chidiyatapu

	Bacterial epiphytes of *Halophila ovalis* of Chidiyatapu										
Strain ID	GRb2	GRb3	GRb4	GRb5	GRb6	GRb7	GRb9	GRb10	GRb11	GRb12	GRb13
Morphology											
Gram stain	-	-	+	-	-	-	+	-	-	-	-
Motility	+	+	-	+	+	+	-	+	+	+	+
Shape	R	R	R	R	R	R	R	R	R	R	R
Biochemical Tests											
Catalase	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	-	+	+	+	+	+	+	+	+
Nitrate	+	+	+	+	+	+	-	-	-	-	+
Citrate	-	-	-	+	-	-	-	+	+	+	+
Urease	-	-	-	-	-	-	-	-	-	-	-
Indole	-	+	-	+	-	+	-	-	-	+	-
Hydrogen Sulphide	-	-	-	-	-	+	-	-	-	-	-
Methyl Red	-	+	-	-	-	+	-	-	-	-	-
Voges-Proskauer	+	-	-	-	+	-	-	-	-	-	-
Gelatinase	+	+	+	+	+	+	+	+	+	+	-
Aesculin Hydrolysis	-	-	+	-	-	-	+	-	-	-	-
Growth at 0% NaCl	-	-	+	-	-	-	+	+	+	+	+
Growth at 3% NaCl	+	+	+	+	+	+	+	+	+	+	+
Growth at 6% NaCl	+	+	+	+	+	+	+	+	+	+	+
Growth at 8% NaCl	+	+	+	+	+	+	+	-	-	-	-
Growth at 10% NaCl	-	-	-	+	-	-	+	-	-	-	-
Growth at 4 °C	-	-	-	-	-	-	-	-	-	-	-
Growth at 20 °C	+	-	+	+	+	+	+	-	+	+	-
Growth at 35 °C	+	+	+	+	+	+	+	+	+	+	+
Growth at 40 °C	+	+	+	+	+	-	+	+	-	-	+
Growth at 50 °C	-	-	-	-	-	-	+	-	-	-	-
Acids from											
Sucrose	+	-	-	-	+	+	+	-	+	+	+
Dextrose	+	+	+	+	+	+	+	-	+	+	+
Lactose	-	-	+	-	-	-	-	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	-	+	-	-	-
Mannitol	+	+	-	+	+	+	+	-	+	+	-
Inositol	-	-	-	+	-	-	-	-	-	-	-
Mannose	+	+	-	-	+	+	+	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	-
Arabinose	-	+	-	-	-	-	-	-	-	-	-
Species Identified	*Vibrio alginolyticus*	*Vibrio parahaemolyticus*	*Bacillus subterraneus*	*Photobacterium ganghwense*	*Vibrio alginolyticus*	*Vibrio owensii*	*Bacillus vietnamensis*	*Pseudomonas pseudoalcaligenes*	*Pseudomonas mosselii*	*Pseudomonas mosselii*	*Pseudomonas mendocina*

Table 2. Results of Biochemical tests of epiphytic bacteria of *Halophila ovalis* of Mazar Pahar and Haddo.

	Bacterial epiphytes of *Halophila ovalis* of Mazar Pahar					**Bacterial epiphytes of *Halophila ovalis* of Haddo**					
Strain ID	GRb14	GRb15	GRb16	GRb17	GRb18	GRb28	GRb29	GRb30	GRb32	GRb33	GRb34
Morphology											
Gram stain	+	+	-	-	+	-	-	-	-	-	-
Motility	-	-	+	+	-	+	+	+	+	+	+
Shape	R	R	R	R	R	R	R	R	R	R	R
Biochemical Tests											
Catalase	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	+	-	+	+	+	+
Nitrate	-	-	-	+	-	+	+	+	+	-	+
Citrate	-	+	-	+	-	-	+	-	+	+	+
Urease	-	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	+	-	+	-	-	-
Hydrogen Sulphide	-	-	-	-	-	+	-	-	-	-	-
Methyl Red	-	-	-	+	-	+	+	-	-	-	-
Voges-Proskauer	-	-	+	-	-	-	-	+	-	-	-
Gelatinase	-	-	-	-	-	+	-	+	-	-	-
Aesculin Hydrolysis	+	+	-	-	+	-	-	-	-	-	-
Growth at 0% NaCl	+	+	+	+	+	-	+	-	+	+	+
Growth at 3% NaCl	+	+	+	+	+	+	+	+	+	+	+
Growth at 6% NaCl	+	-	+	+	+	+	+	+	+	+	+
Growth at 8% NaCl	+	-	-	+	+	+	-	+	-	+	-
Growth at 10% NaCl	+	-	-	-	+	-	-	-	-	-	-
Growth at 4 °C	-	-	-	-	-	-	-	-	-	-	-
Growth at 20 °C	+	+	-	-	+	+	-	+	-	-	-
Growth at 35 °C	+	+	+	+	+	+	+	+	+	+	+
Growth at 40 °C	+	+	+	+	+	-	+	+	+	+	+
Growth at 50 °C	-	-	-	-	-	-	-	-	-	-	-
Acids from											
Sucrose	+	+	-	-	+	+	-	-	+	-	+
Dextrose	+	-	-	+	+	+	+	+	+	+	+
Lactose	-	+	-	-	-	-	-	-	-	-	-
Fructose	+	+	-	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	+	+	-	-	-
Mannitol	+	-	+	+	+	+	+	+	-	-	-
Inositol	-	-	-	-	-	-	-	+	-	-	-
Mannose	+	-	+	-	+	+	+	+	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	-
Arabinose	-	-	+	+	-	-	+	-	-	+	-
Species Identified	*Bacillus haikouensis*	*Bacillus aquimaris*	*Pantoea dispersa*	*Pseudocitrobacter faecalis*	*Bacillus subtilis*	*Vibrio neocaledonicus*	*Citrobacter freundii*	*Vibrio hyugaensis*	*Pseudomonas mendocina*	*Pseudomonas putida*	*Pseudomonas mosselii*

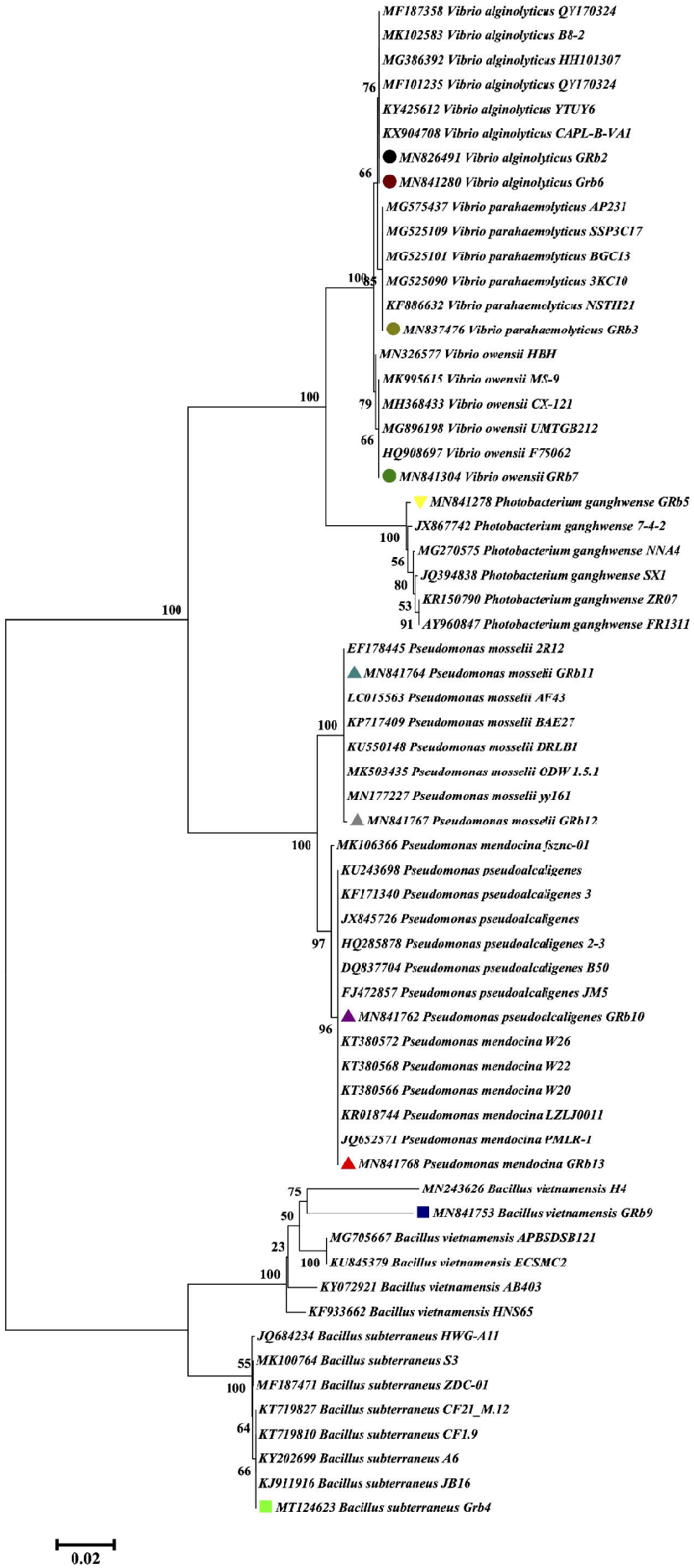

Fig. 3. Neighbour Joining Tree of 16S rRNA gene sequences of bacteria isolated from *Halophila ovalis* of Chidiyatapu

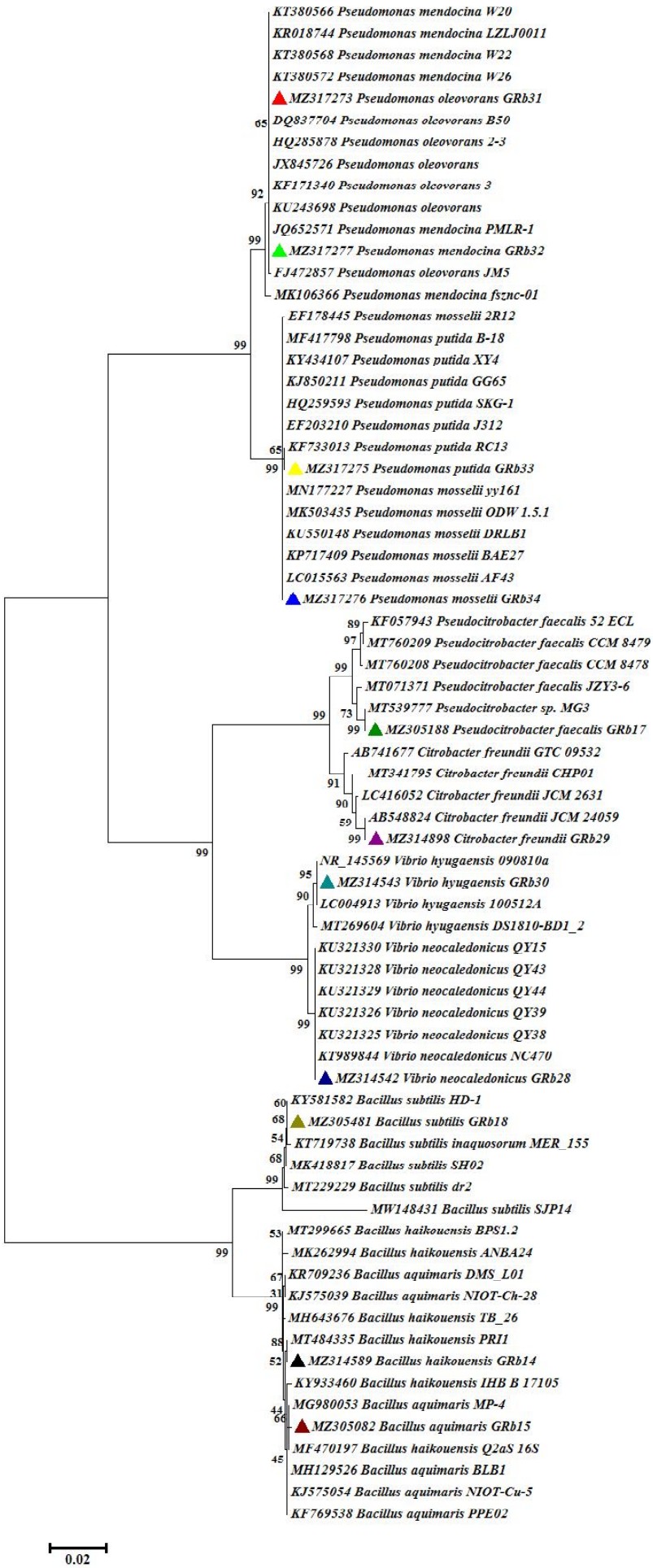

Fig. 4. Neighbour Joining Tree of 16S rRNA gene sequences of bacteria isolated from *Halophila ovalis* of Mazar Pahar and Haddo

Table 3. NCBI accession number of bacterial strains

Sl. No.	Strain code	NCBI Accession No.	Bacteria Identified
1	GRb2	MN826491	*Vibrio alginolyticus*
2	GRb3	MN837476	*Vibrio parahaemolyticus*
3	GRb4	MT124623	*Bacillus subterraneus*
4	GRb5	MN841278	*Photobacterium ganghwense*
5	GRb6	MN841280	*Vibrio alginolyticus*
6	GRb7	MN841304	*Vibrio owensii*
7	GRb9	MN841753	*Bacillus vietnamensis*
8	GRb10	MN841762	*Pseudomonas pseudoalcaligenes*
9	GRb11	MN841764	*Pseudomonas mosselii*
10	GRb12	MN841767	*Pseudomonas mosselii*
11	GRb13	MN841768	*Pseudomonas mendocina*
12	GRb14	MZ314589	*Bacillus haikouensis*
13	GRb15	MZ305082	*Bacillus aquimaris*
14	GRb16	MZ314900	*Pantoea dispersa*
15	GRb17	MZ305188	*Pseudocitrobacter faecalis*
16	GRb18	MZ305481	*Bacillus subtilis*
17	GRb28	MZ314542	*Vibrio neocaledonicus*
18	GRb29	MZ314898	*Citrobacter freundii*
19	GRb30	MZ314543	*Vibrio hyugaensis*
20	GRb32	MZ317277	*Pseudomonas mendocina*
21	GRb33	MZ317275	*Pseudomonas putida*
22	GRb34	MZ317276	*Pseudomonas mosselii*

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