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Rhizospheric metagenome of the medicinal fern *Christella dentata* from saline environment of Indian Sundarbans

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Abstract

The soil microbiome plays a key role in controlling carbon and nutrient cycle in terrestrial ecosystems, affecting plant productivity and stress tolerance. Over time, bacterial communities have evolved mechanisms to endure stresses. However, the mechanism governing these strategies is not entirely known. A cloud-based microbial bioinformatics approach was utilized to investigate the diversity and probable ecological roles of uncultured bacterial communities in the active rhizospheric zone of the medicinal fern *Christella dentata* (Forssk (Brownsey) & Jermy. The rhizospheric soil samples were collected from the farthest island village Kumirmari, in the Gosaba block of Indian Sundarbans (22° 13' 11.0" N, 88° 55' 03.3" E). Uncultured bacteria in medicinal ferns were detected using the V3-V4 hypervariable amplicon region of the 16S rRNA gene and mapped using the SILVA 138.1 Small Subunit rRNA Database. A cloud-based microbial bioinformatics study indicated a high richness of operational taxonomic units (673 OTUs), with Proteobacteria (35.46%), Actinobacteriota (25.54%), Firmicutes (20.86), Acidobacteriota (4.13%), and Bacteroidota (3.14%) clustered and over-distributed in the rhizosphere. Gammaproteobacteria (28.27%), Actinobacteria (23.42), and Bacilli (19.78%) displayed substantial phylogenetic redundancy. The most abundant pathways were membrane transporter, ATP-binding cassette transporter, and signal transduction. This plant promoting rhizospheric bacteria trigger osmolyte accumulation, maintain ion homeostasis, boost oxidative stress resilience, biofilm development, and phytohormone secretion whenever they are exposed to an adverse environment like salinity.

Key words : Rrizospheric, metagenomic analysis, Saline environment, *Christella dentata.*

Ferns grow in an incredible array of habitats, ranging from high-altitude tropical and subtropical rain forests and sub-desert regions, as well as the interior, to the rocks of coastal areas and mangrove swamps. The most optimal ecosystem for these spore-bearing

plants, however, is a tropical mesic environment, where 65% of the surviving species of ferns flourish eagerly and develop abundantly[17]. *Christella dentata* (Forssk.) Brownsey & Jermy, often known as the Toothed Cloak Fern, is an herbal remedy listed under the Thelypteridaceae family. It is distinguished by its unusual fronds, that include toothed edges, thus the name "dentata." In conventional medical practices, *Christella dentata* has been used to heal infections of the skin, bruises, and injuries. The extracts from *Christella dentata* have demonstrated promising antibacterial, antifungal, anti-gout, and anti-rheumatism activities in multiple researches[1,9,14,17,18]. The fern under study is endemic to tropical and subtropical countries, may be observed at various sites across the world, including the saline environment of the Indian portion of the Sundarbans. The fern flora of the Indian Sundarbans region was formerly highly rich and widespread. However, various abiotic variables, such as frequent typhoon, storms, flooding, and human influence, present very serious threats. Ganguli *et al*.,[5] argued that ferns spotted in the Indian Sundarbans regions had relocated from their natural habitat to areas where human impact is significantly lesser. The *Christella dentata* is widespread across the Sundarbans. These areas remain humid due to the vicinity of the Matla estuary; the river saline water also contributes salt, resulting in salinity stress, to this pteridophytic plant. Although these pteridophytic plant are non-halophytes and cannot develop viable spores if the soil salinity is excessive. Pangua *et al*.,[12] reported that fern spores are unable to germinate when exposed to 3.26% salinity. Once salinity concentrations reduced, the fern spores were able to recover their germination capacity following a period of saltwater incubation. Earlier, the evolutionary responses of both the spore and gametophyte to salty environments were examined in *Acrostichum aureum* L. and *Asplenium marinum* L[12]. The specific mechanism to resist this abiotic stress is still not established. Plant rhizospheres are dynamic biological systems that act as hotspots for microorganisms that support ecological synergistic linkages while protecting the health of their host plants. Being sessile, plants are susceptible to a broad spectrum of biotic and abiotic stressors that limit their ability to develop and grow. These bacteria manufacture, or stimulate, physiologically active chemicals that assist the host plant adapt to biological stressors. In untapped environments such as salinity, the possibility of finding rhizospheric bacteria with unique metabolic traits is higher[2,6]. The primary goal of the present work was to collect soil samples from the saline environment and analyze the abundance of uncultured bacterial communities and their probable ecological functions around the rhizosphere of *Christella dentata*. In order to estimate the optimum microbial composition of the taxon, the culture-independent amplicon-based 16S metagenome approach was used.

Collection and Sample preparation :

Rhizospheric soil samples were taken in January 2019 from a nearby pond at Paschim Kumirmari near, Abytanik Prathamik Vidyalay in Kumirmari, Gosaba Block, West Bengal (22° 13' 11.0" N, 88° 55' 03.3" E), India. The soil is alluvial consisting of clay, silt, fine and sand with a low to moderate salinity[7]. Soil samples are obtained in triplets (10 × 10 × 10 cm) at depths of 5-10 cm. Soil samples were collected into sterile falcon tubes and transported to the laboratory, where they were stored at 4 °C for hours before being processed.

DNA extraction, 16S rRNA gene Amplification, Sequencing and cloud-based metagenomic analysis :

Less than 1 g of rhizospheric soil sample were taken for DNA isolation. The environmental genomic DNA was extracted by following Ganguli *et al*.,[5] protocol. DNA concentration was measured using Qubit Fluorometer 3.0 and DNA was stored at -20 °C. V3-V4 region of 16S rRNA gene was amplified. Metagenomic 16S amplicon sequencing was performed by taking 1µg of DNA using Illumina HiSeq 2000 sequencing platform with 300 × 2 PE chemistry. The quality control of raw fastq reads were carried out using FASTQC toolkit (http:/ /www.bioinformatics. babraham.ac.uk/ projects/fastqc), the processed reads were than assembled into contigs. The metagenomic analysis was conducted using a cloud-based approach, following the guidelines provided by Vishal *et al*.,[19] for taxonomic assignment and associated hypothetical ecological inference. The data is available at the NCBI- Sequence Read Archive with accession number: SRX6860832.

A study on *Christella dentata* active rhizospheric zone recovered approximately 0.43 million quality control reads, using the 16S rDNA amplicon gene to identify uncultured bacterial communities. Bacterial taxonomic profiling was performed using the QIIME 2 pipeline of the NCBI-NIH Nephele web server. The control reads were clustered into operational taxonomic units (OTUs) and classified using >97% sequence homology. The results showed 673 microbial OTUs, divided into 44 bacterial phyla, 116 groups, 276 orders, and 440 families, alongside 03 archaeal phyla, 05 classes, 05 orders, and 08 families (Fig. 1). The Shannon and Simpson diversity indices of alpha diversity were 5.7 and 0.93, reflecting a higher richness and more equal distribution of rhizospheric microbial communities (Table-1). The active rhizospheric zones of the medicinal fern *Christella dentata* was dominated by Gram-negative Proteobacteria (35.46%), followed by Actinobacteriota (25.54%), Firmicutes (20.86%), Acidobacteriota (4.13%), and Bacteroidota (3.14%). Planctomycetota, Desulfobacterota, and Gemmatomonadota were less abundant (Fig. 1A). The most abundant classes in the soil were Gammaproteobacteria (27.27%), Actinobacteria (23.42%), and Bacilli (19.78%); however, Bacillaceae (18.22%), Micrococcaceae (17.19%), and Pesudoalteromonadaceae (14.48%) were the most enriched families (Fig. 1B). Additional microbial bioinformatic investigations revealed that 67.7% of total bacteria were classified, with *Bacillus* (18.08% of those classified) being the most enriched genus (Fig. 1C) found in the active zone of *Christella* rhizospheric soil, a rod-shaped Gram-positive endospore forming firmicutes.

Table-1. Overall statistics

Total reads	Quality Control				OTUs	Alpha Diversity Indices	
	Sequences	Mean Seq. Length	GC (%)	Remove chimera/ adapter		Simpson	Shannon
Pre-QC	488826	301	57	58,826	673	0.93	5.7
Post-QC	430000	301	57				

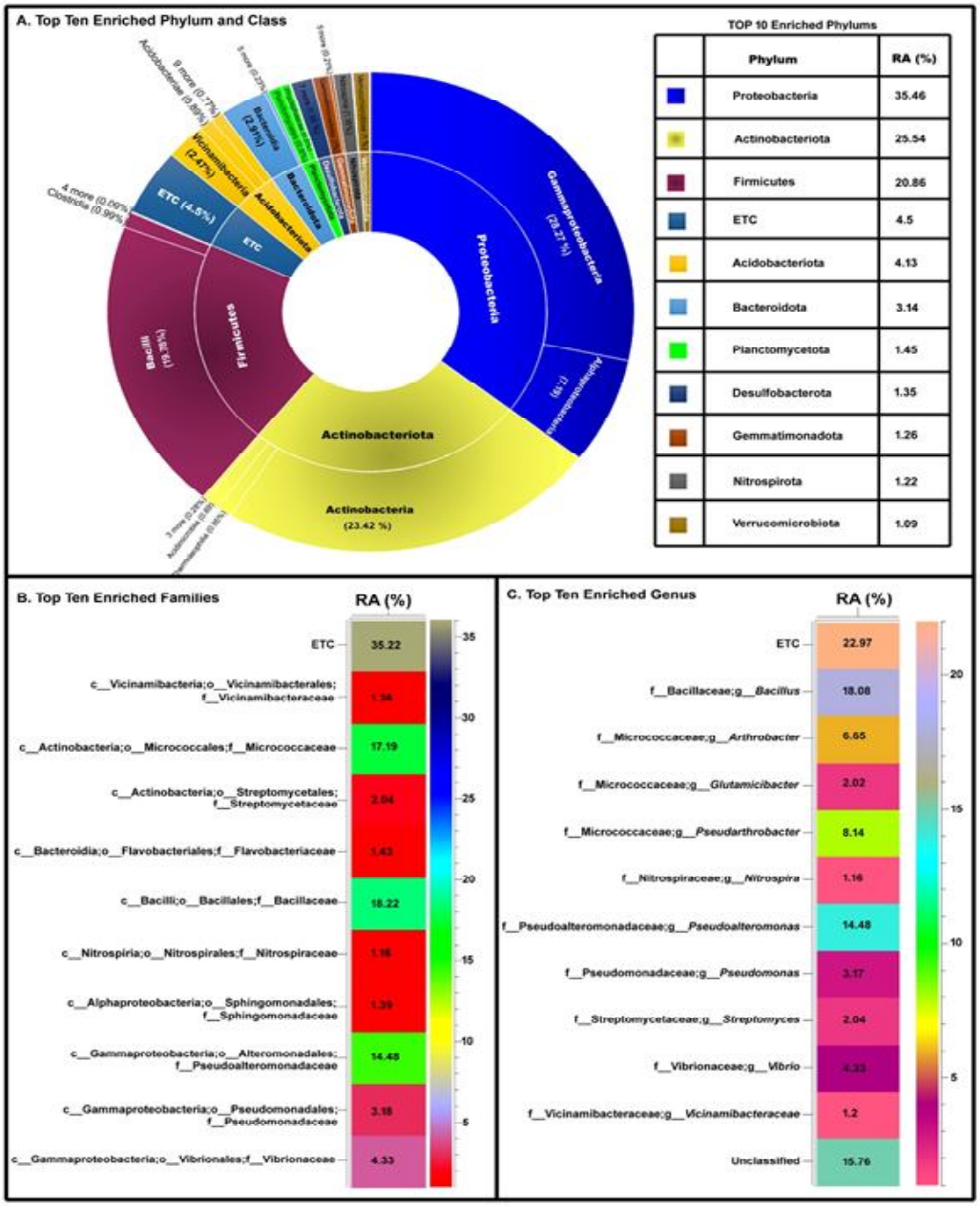

Figure 1. Taxonomic profiling of *Christella dentata* rhizosphere. **A.** Sunburst pie chart showing taxonomic coverage of rhizospheric bacterial communities. Inner circle represents the phylum, while outer circle represent class with their relative abundance in percentage; **B. s**tacked bar showing the distribution of relative abundant family rhizospheric bacteria; **C. s**tacked bar showing of distribution of most relative abundant rhizospheric bacterial genera.

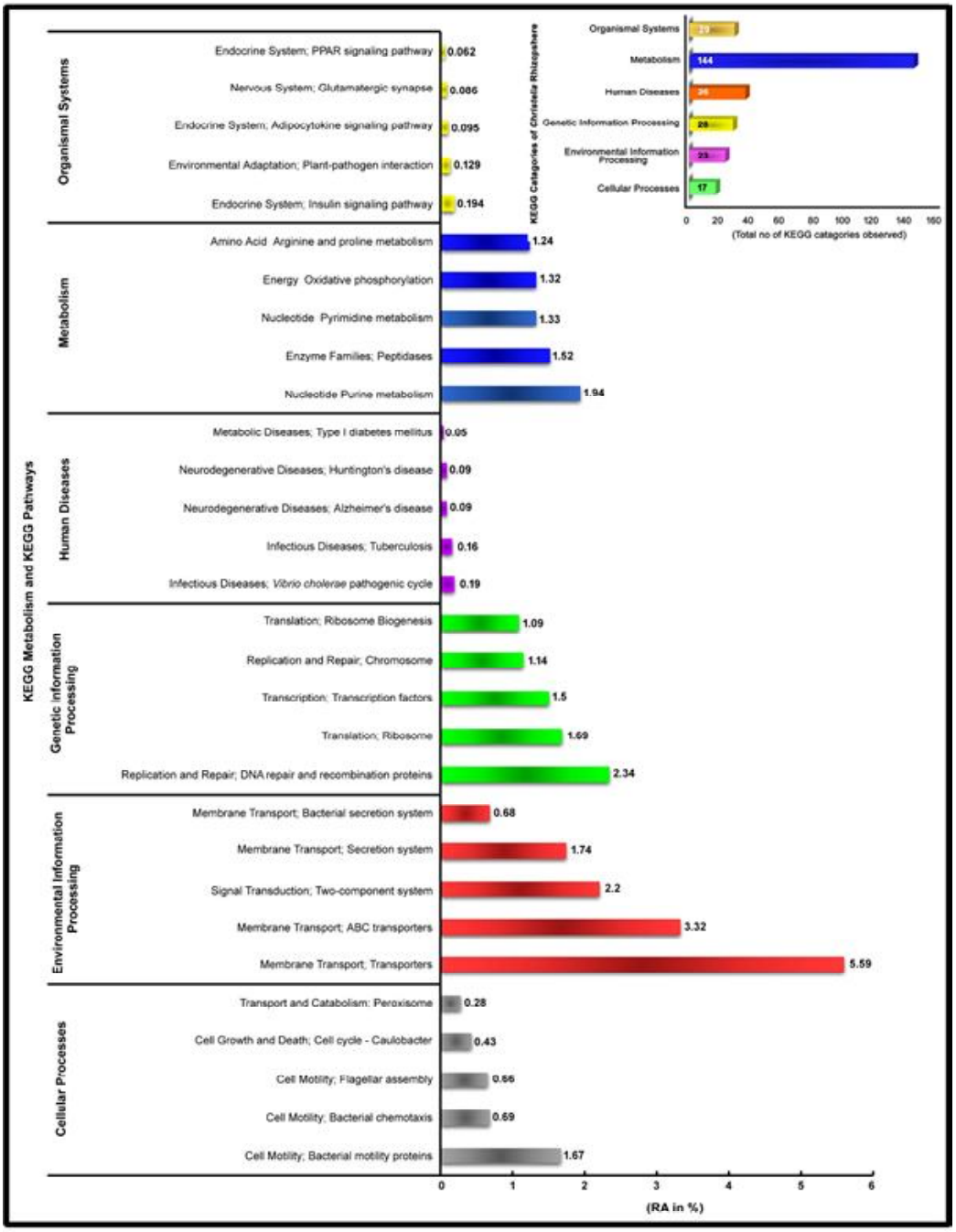

Figure 2. Top enriched KEGG categories with their pathways detected in the *Christella dentata* rhizosphere.

The PICRUSt investigation has discovered hypothetical functional roles for these soil microorganisms (Fig. 2). This study discovered six separate KEGG categories, namely cellular processes, environmental information processing, genetic information processing, human diseases, metabolism, and organ systems. Metabolism and human diseases were the two most enriched KEGG categories. Further investigation revealed that membrane transporter (KO2000), ATP-binding cassette transporter pathway (KO2010), signal transduction pathway (KO02020), DNA repair and recombination proteins pathway (KO03032), and nucleotide purine metabolism pathway (KO00230) were the most enriched pathways associated with the active zone of rhizospheric soil.

This study investigates the structure, dynamics, and ecological roles of bacterial communities in the rhizosphere soils of *Christella dentata*. The soil samples were collected from a low to moderately alluvial saline environment in the Indian Sundarbans. *Christella* species are non-halophytic, and low to moderate salinity can disrupt sporulation process, potentially inhibiting or altering spore formation in ferns due to the species sensitivity to salt concentrations[12]. However, specific studies on fern species are limited[16]. This study uses 16S metagenomic sequencing to identify uncultured plant growth promoting rhizospheric (PGPR) bacteria in *Christella dentata* from a salty environment, offering insights into saline rhizosphere microbial communities and suggesting strategies for soil ecology restoration. The rhizosphere is a microbial hub where plant roots release organic carbon, boosting the population of microbial species[5,13,19]. This study discovered 44 bacterial phyla, 116 groups, 276 orders, 440 families, and 673 genera, accounting for 67.7% of the total reads of the cellular organism (Fig. 1). The remaining 32.3% is unclassified at the genus level. Generally, increasing salinity minimizes or alters the microbial population[2]. However, our analysis indicates the highest Shannon index (5.7), suggesting the highest diversity level. The inverse Simpson indices were 0.93, with 1 being highest evenness, indicating an uneven distribution of rhizospheric bacterial populations (Table-1). The high alpha diversity indices maybe suggest that the abundance of bacterial diversity in our samples is reliant on both the fern genotype and the environment where it thrives[8].

The *Christella* rhizosphere's metagenomic study indicates a diverse microbial community, with Proteobacteria being the leading phylum, followed by Actinobacteriota and Firmicutes (Fig. 1A). This corresponds with earlier studies characterizing the microbial community composition of *Azolla* from the paddy soil[20]. The phylogenetically diversified groups of prokaryotes are enriched due to increased stressors (osmotic pressure or organic resources) in moderately to hypersaline soil in coastal wetlands, as highlighted by Zhang *et al.*,[21]. This study reveals that Gammaproteobacteria, facultatively anaerobic mesophilic bacteria, were the most abundant classes in saline environments, with some members such as Pseudoalteromonadaceae, Pseudomonadaceae, and Vibrionaceae (Fig. 1B) being salt-tolerant. This emphasizes their importance in adapting to high salinity conditions, as well as their potential in metagenomic studies on osmotolerance mechanisms. Actinobacteriota the Gram-positive bacteria represent second-

largest phylum in the *Christella* rhizosphere. They play a crucial role in facilitating plant growth through allowing plants to manage salt stress through the synthesis of osmolytes, plant hormones, and enzymes. They additionally assist in maintain osmotic equilibrium and enhance nutrient availability[11,12,15]. Micrococcaceae, Streptomycetaceae, Nocardioidaceae, PeM15, and Nocardiaceae are Actinobacteriota families (Fig. 1B) found to be prevalent in the rhizosphere of ferns. Firmicute, the third-largest phylum, and *Bacillus* (Fig. 1C), one of the most dominant genera in the rhizosphere of ferns, are gram-positive, rod-shaped, facultative anaerobes. These endospore-forming bacteria perform a key role during salinity stress. Additionally, when *Bacillus* is put in an adverse environment, it produces stress-tolerant spores that can withstand high salt levels by inhibiting entry into the sporulation pathway at those levels. *Bacillus* strains can promote *Christella* growth under saline conditions by stimulating ion homeostasis-related genes. Chen *et al*.,[3] discovered that several *Bacillus* stains helped *Arabidopsis thaliana* grow better in salty environments by upregulating the expression NHX1, NHX7, gs and gr genes. In contrast with this, our metagenomic analysis discovered certain PGPR phylotypes, viz., *Pseudoalteromonas, Pseudarthrobacter, Arthrobacter, Vibrio, Pseudomonas, Streptomyces, Glutamicibacter*, *Vicinamibacteraceae*, and *Nitrospira*. These bacteria can trigger osmolyte accumulation, maintain ion homeostasis, boost oxidative stress resilience, biofilm development, and phytohormone secretion whenever they are exposed to an adverse environment like salinity[10]. The present study hypothetically predicts the ecological roles for these soil microorganisms obtained via PICRUSt analysis (Fig. 2). Metabolism and human diseases were the two most abundant KEGG categories in this low- to moderately saline environment of the fern. However, the membrane transporter pathway (KO2000), the ATP-binding cassette transporter pathway (KO2010), and the signal transduction pathway (KO02020) were the most abundant pathways. In this low- to moderate-saline environment, majority of the bacterium genes use these pathways to skip or circumvent the salinity stress. This environmental information processing category of KEGG encompasses a diversity of biological activities such as maintaining plant homeostasis under salt stressors, intake of nutrients, stomatal closure, and transport of phytohormones[4]. Thus, a huge number of microorganisms engage in membrane transport channels in the fern rhizosphere.

Additionally, comparative metagenomic investigation of the soil rhizospheres of *Acrostichum aureum* (Tiger fern)[5] and *Marsilea minuta* L[13] previously examined in the Indian Sundarbans indicates a comparable bacterial community composition. But the *Christella dentata* soil metagenome displayed quite distinct results, which highlighted the largest abundance of Gammaproteobacteria, followed by Actinobacteria. This may have happened due to the variation of bacterial diversity in our samples, which depends on both the fern genotype and the rhizospheric environment.

Soil metagenomic analyses of *Christella dentata* revealed a unique assemblage of *Gammaproteobacteria* and *Bacillus* in the rhizosphere. These bacteria maintain ion homeostasis, boost oxidative stress resilience, and protect host plants. However, the presence of unassigned taxa in other prokaryotic associates suggests a lack of information about

the richness of rhizospheric communities.

Declaration on conflict of interest

The author declares no conflict of interest.

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