**Isolation and Identification of taxonomically diverse bacterial
endophytes from citrus in Punjab Pakistan**

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Abstract

Citrus is an economically important fruit crop grown in all provinces of Pakistan, while Punjab accounting for 95 percent of total production due to its favorable climate for citrus production. Commercially grown varieties in Pakistan include sweet oranges, grapefruits, Mandarine, Lime, and lemon. The goal of this research was to see how diverse the cultivable bacterial populations are found in citrus cultivars. Out of 90 isolated cultures, 37 endophytic bacterial species and 15 different genera of bacteria were characterized based on morphological, biochemical, and molecular methods from citrus leaves. All the isolated bacteria were subjected to PCR amplification through 16S rDNA followed by sequencing. RDP base classification revealed that class Bacilli has the largest percentage of isolates, whereas class Alpha, Beta, and Gamma Proteobacteria have the lowest percentage among all genotypes used. According to the findings, the phylum Firmicutes contains a common genus (*Brevibacterium*, 1%; *Enterococcus*, 6%; *Staphylococcus*, 7% and *Bacillus*, 60%). Alpha (*Rhizobium*) beta (*Burkholderia cepacia*; *Comamonas terigena*) gamma Proteobacteria (*Enterobacter hermachei* (1%), *Klebsiella pneumoniae* (1%), *Proteus mirabilis* (8%), *Pseudomonas aeruginosa* (5%), *Psychrobacter pulmonis* and *Yersinia molalretti* (1%) respectively. These results revealed that cultivars of the plants might contribute to the structure and endophytic bacterial communities associated with citrus. Endophytes extracted from leaf samples of different citrus cultivars in Pakistan are reported for the first time. The idea of employing endophytes bacteria to produce enzymes stimulate plant growth, and its purpose as a biological control agent will be investigated in the future.

Keywords: Diversity, morphological, biochemical, Molecular characterization, endophytes, Citrus, Classification, 16S rRNA

Introduction

Citrus has traditionally been recognized as one of the most popular fruit, as it is enriched with minerals, vitamins A, B, and C, ascorbic acid, and also possesses strong antioxidant potential [1, 2]. Pakistan is the world's number one producer of Kinnow mandarin [3]. A widespread kind of microorganisms exists in the phyllosphere (Surface of the plant). Bacteria are the most common microbes found in the phyllosphere. Endophytes (bacteria that live inside plant tissues) and epiphytes (bacteria that live on the surface of plants) are both examples of phyllosphere bacteria [4, 5]. The research on plant-based endophytes is critical for understanding the diverse bacterial interactions that occur in certain environments, which help to improve their biotechnological applications [6, 7]. The endophytes that reside within the surface of the leaf form complex population dynamics that are important for agriculture and the environment. Plant health can be improved or arrested by the bacterial diversity associated with the leaf, which can also drive the colonization and infection of tissues by phytopathogens [8, 9].

Microorganisms that live in a mutualistic connection with a plant are known as endophytes [10, 11]. Plant processes such as growth promotion, nutrient uptake, abiotic stress tolerance, and pathogen infection inhibition are thought to be supported by them [6]. Bacterial endophytes (BE) are found in different parts of plants such as roots, stems, and leaves [12, 13]. The population density of BE is affected by multiple factors, including the plant's developmental phase [7, 14], cultivar (genotype) [14, 15], the portion under examination [16], comprising the type of plant being studied, and the

interaction between microbes and ecological factors [17]. The population density of BE could be 10^2 to 10^9 people per square kilometer [18]. Commonly, BE present in lower numbers as compared to the rhizospheric bacteria [19]. They are not limited to a single species in a single plant host, but they could be observed in multiple genera and species. Endophytic bacteria have been extracted from different segments of *M. micrantha*, including the roots, lamina, and petiole [20]. In previous studies, endophytes have been isolated from cottonwood (*Populus deltoids*) [21], grapevine (*Viti vinifera*) [22], poplar (*Populus alba*) [23], sweet potato (*Ipomoea batatas*) [24], potato [25, 26], soybean (*Glycine max*) [27] and tomato (*Solanum lycopersicum*) [28].

Pakistan has an agriculturally dominated economy with large areas of fertile land and a diversified geographical region and climate. There has been limited research on microbial biodiversity, and no such type of research on the diversity of BE from Citrus in Pakistan has ever been reported. It is so believed that bacterial species found in Pakistan carrying a wide variety of endophytic bacteria will be investigated to find the real picture. This study will provide information regarding the different strains of bacteria that are residing in citrus leaves and still need to be identified from different geographical locations of the country.

Methods

Sample collection and isolation of bacteria

Citrus leaf samples were collected from orchards in the Punjab districts of Lahore, Multan, Mian Chanu, Sahiwal, Faisalabad, and Sargodha. For subsequent processing, leaves from thirty-two different citrus varieties were collected and stored at -80°C . To

isolate endophytes, 3-4 cm of citrus leaf midrib part were sanitized with NaOCl solution (0.6%) for three min and then rinsed thrice with sterilized deionized autoclaved water. A homogeneous solution of crushed mid rib portion and deionized water was prepared and streaked on Nutrient agar (NA) medium plates, which were incubated at 28°C for 1-2 days. Purified colonies were culture on NA plates and incubated at 28°C for 1 day until growth appears. Following Bergey's manual of systemic bacteriology, pure cultures of bacterial isolates were identified using morphological and biochemical methods [29].

Identification of bacterial isolates by 16S rDNA:

Genomic DNA of Bacterial endophytes was extracted by the Cetyl trimethyl ammonium bromide (CTAB) method followed by [30]. Single-cell colonies of bacteria were culture in 5mL of NA for 24 hours and centrifuged at 13000 rpm for 2 min. Then the pellet was dissolved in mixture of TAE buffer, 10% SDS, proteinase k (20 mg/ml) and incubated at 37°C for 60 min. 5M NaCl (100µL) and CTAB (80µL) were added and incubated for 10 minutes at 65°C. After that 750µL of Chloroform Isoamyl Alcohol (24:1) were added and centrifuged for 10 min. About 400µL of the upper phase was removed and shifted to another tube. Then (700µl) of Phenol Chloroform was added and centrifuged for 10 min and repeat this step. After that (20µL) of 3M Sodium Acetate and (500µL) of Absolute Ethanol were gently mixed and kept at -20°C for 12 h. After that tubes were spin (centrifuged) again at 13000 rpm for 10 min and the supernatant was wasted. The DNA pellet was washed with 70% of ethanol and dissolved in distilled autoclave water. Extracted DNA was confirmed on 1% agarose gels containing EtBr (ethidium bromide) (0.5 µg/ mL) and visualize under UV light.

Genomic DNA of 90 bacterial isolates was subjected to PCR by 16SrRNA primers 27-F (5' AGAGTTTGATCMTGGCTCAG 3'), 1492-R (5' ACCTTGTTACGACTT 3'), and following PCR conditions reported by (Trivedi et al. 2011). PCR products were Gel purified and sent for Sequencing to Macrogen South Korea. The obtained sequences were aligned with the reported ones in the Gene Bank using BLAST (Basic Local Alignment Search tool) at the NCBI (National Centre for Biotech Information). Apart from NCBI Genbank, the isolated bacterial sequences were classified using the Ribosomal Database Project (RDP Hierarchy Browser). As previously stated, all the obtained sequences of bacterial endophytes after proper identification were submitted to NCBI Gene bank Database and attained their Accession numbers (Table 3).

Evolutionary Study of Bacterial strains

After sequence analysis, MEGA 7.0 software was used to construct a phylogenetic evolutionary tree through multiple sequence alignment of 16S rDNA gene sequences with other reported ones [31]. A bootstrap analysis of 1000 replications was used to calculate a confidence value for the aligned sequence data set. To investigate the evolutionary relationship between bacterial endophytes, a phylogenetic tree was created using the neighbor-joining algorithm. The signature sequence of the endophytes was also identified through MEGA 6.0 (Muscle) software following the alignment of isolated strains with reported strains (NCBI Data Base). These signatures help in determining the sequence that is unique to a given genus or species when compared to other reported genera or species.

RESULTS

This study provided a brief overview of the distribution of leaf endophytic bacteria found in citrus varieties, contributing significantly to the study of microbial populations in Pakistan's environment.

Morphological and Biochemical Identification of extracted endophytic bacterial strains

Extraction of bacteria from leaf midribs was accomplished using mince soaked techniques on NA medium, and identification based on colony morphology (shape, texture, color, elevations, margins and, transparency of colony) was accomplished using Bergey's Manual of Systematic Bacteriology Smibert and Krieg (1981), and findings have been summarized in (Table 1 and 2).

Table 1: Morphology based determination of bacterial endophytes from Leaf tissue of various citrus genotypes

Bacterial strain used in this study	Colony texture	Colony color	Margins	elevations	Opacity	shape
<i>Lysinibacillus sphaericus</i>	Smooth and shiny	White	Entire	Raised	Opaque	Round
<i>Pseudomonas aeruginosa</i>	Smooth	Dirty off white	Wavy	Concave	Opaque	Round
<i>Enterococcus casseliflavus</i>	Smooth	Creamy white	Irregular	Flat	Opaque	Round
<i>Paenibacillus pabuli</i>	Smooth and shiny	Dirty off-white	Wavy	Raised	Opaque	Round
<i>Comamonas terrigena</i>	Smooth and shiny	Creamy off white	Wavy margins	Concave	Opaque	Round
<i>Enterococcus faecalis</i>	shiny and smooth	Off white	Wavy margins	Flat	Dense or cloudy	Round
<i>Burkholderia cepacia</i>	Smooth	Off-white	Wavy margins	Slightly Elevated	Opaque	Round
<i>Brevibacillus borstelensis</i>	rough crust but slightly smooth	White	Irregular margins	Concave	Opaque	Round
<i>Brevibacterium halotolerans</i>	Rough crust	Dirty (off white) color	Irregular	Flat	Opaque	Asymmetrical
<i>Pseudo acidovorax</i>	smooth	Creamy white	Intact /entire	Slightly elevated	Transparent	Round
<i>Bacillus pumilus</i>	Smooth and shiny	Off white	Irregular	Slightly raised	Opaque	Round
<i>Psychrobacter pulmonis</i>	Smooth and	Dirty	Wavy	Smooth	Opaque	Round

	shiny	white				
<i>Klebsiella pneumoniae</i>	Smooth	Dirty white	Wavy	Raised	Opaque	Round
<i>Pantoea agglomerans</i>	Smooth	Pale yellow	Entire	Convex	Transparent	Round
<i>Proteus mirabilis</i>	Smooth and shiny	Dirty white	Wavy	Concave	Opaque	Round
<i>Bacillus subtilis</i>	Smooth and shiny	Dirty white	Wavy	Concave	Opaque	Round
<i>Yersinia mollaretii</i>	Smooth	Dirty white	Wavy	Flat	Opaque	Round
<i>Enterobacter cloacae</i>	Smooth	Dirty off white	Wavy	Raised	Opaque	Round
<i>Staphylococcus Scuiiri</i>	Smooth and shiny	Dirty off white	Irregular	Flat	Opaque	Round
<i>Escherichia coli</i>	Smooth	Dirty off-white	Slightly wavy	Raised	Opaque	Round
<i>Bacillus cereus</i>	Smooth	Dirty off white	Irregular	Flat	Opaque	Round

144 **Table 2: Biochemical assessment of endophytes extracted from leaf mid rib part of**
145 **different citrus cultivars**

Identified Bacterial Strains	Gram type	Cell shape	Staining of Capsule	Motility test	Spore type	Methyl red test	Citrate utilization test	Indole test	Hydrogen sulphide test	Oxidase test	Catalase test	Nitrate reduction test	Growth at 40 °C	Growth at 25°C	Growth at 2% NaCl
<i>Brevibacillus borstelensis</i>	Pos.	Rod	Neg.	Pos.	Pos.	Pos.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Pos.	Neg.
<i>Comamonas terrigena</i>	Neg.	Rod	Neg.	Pos.	Neg.	Neg.	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Neg.	Neg.	Neg.
<i>Burkholderia cepacia</i>	Neg.	Cocci	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
<i>Enterococcus casseliflavus</i>	Neg.	Rods	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.
<i>Paenibacillus pabuli</i>	Pos.	Rod	Neg.	Pos.	Pos.	Pos.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.
<i>Enterococcus faecalis</i>	Pos.	Cocci	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.
<i>Lysinibacillus sp.</i>	Pos.	Rod	Neg.	Pos.	Pos.	Neg.	Pos.	Neg.	Neg.	Pos.	Pos.	Neg.	Neg.	Pos.	Neg.
<i>Enterococcus faecalis</i>	Neg.	Rods	Neg.	Pos.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.
<i>Brevibacterium halotolerans</i>	Pos.	Rod	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.
<i>Enterobacter cloacae</i>	Neg.	Rod	Neg.	Pos.	Neg.	Neg.	Pos.	Neg.	Pos.	Pos.	Pos.	Neg.	Neg.	Neg.	Neg.
<i>Escherichia coli</i>	Neg.	Rod	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Neg.	Pos.	Pos.	Neg.	Neg.	Neg.
<i>Staphylococcus haemolyticus</i>	Pos.	Cocci	Neg.	Neg.	Neg.	Pos.	Pos.	Neg.	Neg.	Neg.	Pos.	Pos.	Neg.	Pos.	Neg.
<i>Yersinia mollaretii</i>	Neg.	Rods	Neg.	Pos.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Pos.	Neg.
<i>Bacillus pumilus</i>	Neg.	Rods	Neg.	Pos.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Pos.	Pos.	Neg.	Neg.	Neg.
<i>Pseudomonas aeruginosa</i>	Neg.	Rod	Neg.	Pos.	Neg.	Neg.	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Neg.	Neg.	Pos.
<i>Klebsiella sp.</i>	Neg.	Rod	Pos.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Pos.	Pos.	Neg.	Pos.	Neg.
<i>Proteus Sp.</i>	Neg.	Rod	Neg.	Pos.	Neg.	Pos.	Pos.	Neg.	Pos.	Neg.	Pos.	Pos.	Neg.	Neg.	Neg.
<i>Pseudoacidovorax</i>	Neg.	Cocci	Pos.	Pos.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.
<i>Psychrobacter pulmonis</i>	Neg.	Rods	Neg.	Pos.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Pos.	Neg.
<i>Bacillus cereus</i>	Neg.	Rods	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Pos.
<i>Pantoea Sp.</i>	Neg.	Rod	Neg.	Pos.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Pos.	Pos.	Neg.	Neg.	Neg.
<i>Bacillus subtilis</i>	Pos.	Rods	Neg.	Pos.	Pos.	Neg.	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Neg.	Pos.	Neg.

146 **Note:** Pos. and Neg. Abbreviations represent the positive and Negative respectively.

Diversity of Bacterial Endophytes

To get a representative sample of the cultivable endophytes living inside leaf tissues (Citrus), Single-cell and morphologically different colonies of bacteria were picked for 16S rDNA sequencing. A BLAST homology search resulted in the identification of 37 bacterial endophytic species. The overall diversity of the citrus endophytes is represented in the form of Pie graphs (Figure 1 & 2) which showed the distribution of bacterial groups and Genus based on their prevalence percentage in samples.

These bacterial endophytes were classified into two main groups: Firmicutes and Proteobacteria. About 73.59% of Firmicutes were found in citrus samples assessed. Proteobacteria was further comprised of three groups including (2.94%) of Alpha proteobacteria, (5.88%) of beta proteobacteria, and (17.64%) of gamma proteobacteria. The results showed that the majority of genera belonged to the phylum Firmicutes made up of genus (*Bacillus* accounts for 60%, *Enterococcus* for 6%, *Staphylococcus* for 7%, and *Brevibacterium* for 1%). Additionally, during this study, the phylum Proteobacteria (Alpha (*Rhizobium*) beta (*Burkholderia cepacia*; *Comamonas terigena*), gamma Proteobacteria including *Enterobacter hermachei* (1%), *P. aeruginosa* (5%), *Psychrobacter pulmonis* (1%), *P. mirabilis* (8%), *Yersinia molarretti* (1%), and *K. pneumoniae* (1%) were detected. These isolates had a high level of 16S rDNA gene similarity with the genera *Bacillus* (60%), *Staphylococcus* (7%), and *Proteus* (8%), all of which belong to the division Bacilli, while *Proteus mirabilis* (8%), a member of the Gamma Proteobacteria, displayed the highest level of diversity among all isolates. *Bacillus cereus* remained the frequently discovered and abundant endophyte in citrus

169 leaves extract, according to a research of endophytes prevalence both species and genus
170 levels as illustrated in (Figure 2).

171 The findings of detection of bacterial species from the leaf of various citrus cultivars and
172 subsequent characterization of partial 16S rRNA gene sequences, such as host, location,
173 and Accession no., are presented in the table below (Table 3).

174 **Table 3. Molecular Characterization based on Partial 16S rRNA sequencing**
175 **of endophytes extracted from leaf mid rib part of different citrus cultivars**

Sr. no.	Identified bacterial strains	Cultivar names	Location	Accession numbers	% 16S rRNA identity
1	<i>Bacillus anthracis</i>	Casa grand (<i>Citrus sinensis</i>)	Sargodha	MF802839	99%
2	<i>Bacillus velenzensis</i>			MF957301	99%
3	<i>Comamonas terrigena</i>			LT844635	95%
4	<i>Bacillus subtilis</i>			MF962597	96%
5	<i>Bacillus pumilus</i>			MF972867	99%
6	<i>Enterococcus faecalis</i>			MF977352	99%
7	<i>Enterobacter hermachei</i>	Grape fruit (<i>Citrus paradisi</i>)	Mian chanu	LT745966	97%
8	<i>Bacillus hwajinpoensis</i>		Sahiwal	LT844644	99%
9	<i>Bacillus firmus</i>		Multan	LT745984	99%
10	<i>Bacillus subtilis</i>		Sahiwal	MF977360	99%
11	<i>Brevibacillus borstelensis</i>	Sweet orange (<i>Citrus sinensis</i>)	Mian chanu	LT745989	93%
12	<i>Bacillus cereus</i>		Faisalabad	MF977351	99%
13	<i>Bacillus cereus</i>	Westin (<i>Citrus sinensis</i>)	Sargodha	MF964937	99%
14	<i>Staphylococcus lentus</i>	Volka marina (<i>Citrus x limon</i>)	Sargodha	MF972869	99%
15	<i>Pseudomonas aeruginosa</i>		Sargodha	MF802282	98%
16	<i>Bacillus thuringiensis</i>		Sargodha	MF977364	99%
17	<i>Bacillus pumilus</i>	Lemon (<i>Citrus x limon</i>)	Mian chanu	MF977357	99%
18	<i>Bacillus cereus</i>		Mian chanu	MF966921	99%
19	<i>Staphylococcus scuri</i>		Multan	MF972865	99%
20	<i>Bacillus safensis</i>		Mian chanu	MF801628	99%
21	<i>Rhizobium sp.</i>		Faisalabad	LT844646	92%
22	<i>Bacillus cereus</i>		Multan	MF801630	97%
23	<i>Yersinia mollaretti</i>		Multan	LT745988	92%
24	<i>Bacillus tequilensis</i>	Chinese lemon (<i>Citrus limon</i>)	Multan	MF967252	99%
25	<i>Staphylococcus scuri</i>	Parson brown	Sargodha	LT745975	99%
26	<i>Staphylococcus haemolyticus</i>	Musambi (<i>Citrus sinensis</i>)		MF957708	91%
27	<i>Proteus mirabilis</i>		Sahiwal	MF957272	99%
28	<i>Staphylococcus haemolyticus</i>		Sahiwal	LT745967	92%
29	<i>Enterococcus caseliflavus</i>		Sahiwal	MF802859	97%
30	<i>Bacillus pumilus</i>		Sargodha	MF801624	97%
31	<i>Bacillus cereus</i>		Sahiwal	LT745965	96%
32	<i>Enterococcus gallinarum</i>		Faisalabad	MF972868	97%

33	<i>Staphylococcus scuri</i>		Sargodha	MF977365	99%
34	<i>Bacillus cereus</i>	Futrell (<i>Citrus reticulata</i>)	Multan	MF972894	99%
35	<i>Brevibacterium halotolerans</i>		Faisalabad	LT745976	99%
36	<i>Bacillus cereus</i>		Mian chanu	MF972895	99%
37	<i>Bacillus cereus</i>		Sahiwal	MF977367	99%
38	<i>Enterococcus casseliflavus</i>	Kinnow (<i>Citrus reticulata</i>)	Sahiwal	MF804508	100%
39	<i>Proteus mirabilis</i>	Kinnow (<i>Citrus reticulata</i>)	Sahiwal	MF958504	99%
40	<i>Enterococcus casseliflavus</i>	Kinnow (<i>Citrus reticulata</i>)	Mian chanu	MF804505	100%
41	<i>Bacillus mycoides</i>		Multan	LT844640	99%
42	<i>Bacillus thuringiensis</i>		Multan	LT844641	100%
43	<i>Bacillus cereus</i>		Faisalabad	MF977355	99%
44	<i>Bacillus cereus</i>		Faisalabad	LT844655	99%
45	<i>Bacillus cereus</i>		Sahiwal	MF967250	96%
46	<i>Bacillus sonorensis</i>	Salustina (<i>Citrus sinensis</i>)	Sargodha	MF977361	99%
47	<i>Bacillus cereus</i>		Sargodha	MF962609	98%
48	<i>Bacillus cereus</i>	Rangpur lime (<i>Citrus jambhiri</i>)	Sargodha	MF967251	99%
49	<i>Bacillus velenzensis</i>		Sargodha	LT844636	94%
50	<i>Proteus mirabilis</i>		Sargodha	MF962605	99%
51	<i>Bacillus pseudomycoides strain</i>	Sour orange (<i>Citrus aurantium</i>)	Sargodha	LT844650	94%
52	<i>Enterococcus faecalis</i>		Lahore	LT844634	100%
53	<i>Bacillus cereus</i>		Lahore	LT844658	99%
54	<i>Bacillus cereus</i>	Pishawri mitha	Sargodha	LT745985	99%
55	<i>Bacillus thuriengiensis</i>		Sargodha	MF957274	99%
56	<i>Psychrobacterium pulmonis</i>	Natal	Sargodha	LT745968	97%
57	<i>Staphylococcus scuri</i>		Sargodha	MF804507	96%
58	<i>Proteus vulgaris</i>		Sargodha	MF957268	99%
59	<i>Bacillus cereus</i>		Sargodha	LT745970	99%
60	<i>Bacillus flexus</i>		Sargodha	LT745979	99%
61	<i>Staphylococcus sciuri</i>		Sargodha	LT745982	94%
62	<i>Proteus mirabilis</i>	Marse early (<i>Citrus sinensis</i>)	Sargodha	MF977359	100%
63	<i>Enterococcus casseliflavus</i>		Sargodha	MF959774	99%
64	<i>Bacillus oceanisediminis</i>		Sargodha	LT745983	99%
65	<i>Bacillus cereus</i>		Sargodha	MF957270	99%
66	<i>Bacillus anthracis</i>	CRI- 78 (<i>Citrus sinensis</i>)	Sargodha	LT844654	99%
67	<i>Proteus mirabilis</i>		Sargodha	MF957273	99%
68	<i>Bacillus subtilis</i>	Kozan (<i>Citrus sinensis</i>)	Sargodha	MF977356	96%
69	<i>Bacillus aerophilus</i>	Pine apple (<i>Citrus sinensis</i>)	Sargodha	LT844648	99%
70	<i>Proteus mirabilis</i>		Sargodha	LT745977	99%
71	<i>Comamonas terrigena</i>	Robble (<i>Citrus sinensis</i>)	Sargodha	LT844649	92%
72	<i>Bacillus megaterium</i>	Dancy (<i>Citrus reticulata</i>)	Sargodha	MF802485	94%
73	<i>Pseudomonas sp.</i>		Sargodha	MF973203	89%
74	<i>Brevibacterium halotolerans</i>	Chakotra (<i>Citrus maxima</i>)	Sargodha	MF977363	99%
75	<i>Pseudomonas aeruginosa</i>		Sargodha	LT844657	99%
76	<i>Pseudomonas aeruginosa</i>	Olinda valecia (<i>Citrus sinensis</i>)	Sargodha	MF802727	95%

77	<i>Staphylococcus lentus</i>	Marse early (<i>Citrus sinensis</i>)	Sargodha	MF972864	99%
78	<i>Bacillus megaterium</i>	Olinda Valencia (<i>Citrus sinensis</i>)	Sargodha	MF977353	99%
79	<i>Bacillus subtilis</i>	Eureka lime (<i>Citrus × limon</i>)	Sargodha	MF962608	99%
80	<i>Bacillus mojavensis</i>	Kaghzi lime (<i>Citrus × aurantiifolia</i>)	Sargodha	MF962628	99%
81	<i>Bacillus cereus</i>	Hinkly (<i>Citrus sinensis</i>)	Sargodha	MF962815	96%
82	<i>Bacillus velezensis</i>	Murcot (<i>Citrus sinensis</i>)	Sargodha	MF966641	100%
83	<i>Klebsiella pneumoniae</i>	Gada dahi (<i>Citrus aurantium</i>)	Sargodha	MF966247	98%
84	<i>Bacillus amyloliquifaciens</i>		Sargodha	MF977354	100%
85	<i>Enterococcus gallinarum</i>	Robble (<i>Citrus sinensis</i>)	Sargodha	MF977366	99%
86	<i>Bacillus velezensis</i>	Peera rio (<i>Citrus sinensis</i>)	Sargodha	MF977358	99%
87	<i>Burkholderia cepacia</i>		Sargodha	LT844653	99%
88	<i>Bacillus cereus</i>		Lahore	LT844658	99%
89	<i>Proteus mirabilis</i>	Malta	Multan	MF977368	99%
90	<i>Staphylococcus lentus</i>		Multan	MF977362	100%

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177 For phylogenetic investigations, all isolates with a nucleotide sequence identity of 91-100
178 percent were selected. Using MEGA 6 software and a 1000 bootstrap value, a neighbor-
179 joining dendrogram was constructed. During sequence alignment analysis, 16S rDNA
180 bacterial sequences isolated from different citrus samples were used along with reported
181 species with identities greater than 97%. This phylogenetic analysis contains 90 bacterial
182 strains from the four classes described as (Bacilli, Alpha proteobacteria, Beta
183 proteobacteria, and Gamma proteobacteria) as shown in (Figure 3).

184 Identification Patterns/Signature Sequences of bacteria

185 16S ribosomal RNA gene (rRNA) sequencing is considered the gold standard in bacterial
186 identification and classification according to modern taxonomists, with more than
187 100,000 sequences available in public databases (NCBI). The 16S rRNA gene has
188 conserved regions that can be used to construct PCR primers that can amplify diverse

189 portions of the 16S rRNA gene from bacteria. Hypervariable sections with species-
190 specific signature sequences are included among the fragment, which can be used to
191 identify bacteria up to the species level. All identified bacterial endophytes' signatures
192 patterns are shown in (Table 4).

193 **Table 4: Descriptive analysis of isolated Bacteria at the genus or species level, demonstrating possible identification**
194 **patterns/signature sequences**

Sr. No.	Genus of identified bacterial strains	Signature	Base pair	Sequenc e Repeat	Species of identified bacterial strains	Identification pattern/ Signature Sequence	Identified Base pair from sequences
1	Enterobacter	CAGGCGGTCTGTCAAGTCGGATGTG	559-583	Not repeat	Enterobacter hormaechei	GTGAATTGACGGGGGTCC	902-918
		GGCGGTCTGTCAAGTCGGATGTGA AATC	561-588				
		AACACATGCAAGTCGAACGGTAG	1-22				
		AGTCGGA	1335-1341 598-604	731			
2	Psychrobacter	GGGAGTTGATTGCACCAGAAGT	1454-1475	Not repeat	Psychrobacter pulmonis	Not found	
		GACGAGCGGCGGACGGGTGAGTAAT ACTTA	88-117				
		AGTCCAGATTGGAGTCTGCAACTCG	1333-1357				
3	Proteus	GCGCACGCAGGCGGTCA	559-575	Not repeat	Proteus mirabilis	GTGGGAACTCAAAGGAGAC	1308-1326
		ATAATGTCTACGGACC	135-150				
		CTCTAGCTGGTCTGAGAGGATGA	252-274				
		AGCGGCGGACGGGTGAGTAATGTAT GGG	55-82				
4	Brevibacillus	ATGGTTCAAACATAAAAGGTGG	209-230	Not repeat	Brevibacillus halotolerans	TTTATGGAGCCAGCCGCC-GAAGGT	1454-1478
5	Rhizobium	CAGTTCGGATTGCACTCTGCAACTCG	1248-1272		Rhizobium sp.	GGAATCTACCTTTTGCTACGG	82-102
6	Yersinia	CGCCATCGGATGTGCCAGATGGGA TTAGCTAGT	177-210	Not repeat	Yersinia molretti	CATCCATGCCGCGTGTG	350-366
		AAGTAGTTT-ACTACTTTG	18-36				
		GCTTAACGTGGGAACTGCATTGA	581-604				
7		TCGTAGATCAGCATGCTAC	1348-1366		Staphylococcus haemolyticus	GAGCTAATACCGGATAATATTTTCG	123-152

	Staphylococcus					AACCG	
		GATAATATTT TGAAC	160-174		<i>Staphylococcus scuri</i>	CTGCAAGTCGAGCGA	30-44
		GATTTGGGCTACACACGT	1212-1229	Not repeat	<i>Staphylococcus lentus</i>	GCGAACCCGCGAGGTCAAGCAAAT	1255-1278
		GCAAATCCCATAAAG	1273-1287				
8	Bacillus	AAGAGCT	1214-1220 24-30	1,184	<i>Bacillus subtilis</i>	ACCTGGGCTACACACGTGCTACAA TGGACAGAACAAAGGGCAGCGAA ACCG	1212-1262
		AGTCG	28-32 1412-1416	1380			
		AATGGA	1201-1206 16-20	1,181	<i>Bacillus cereus</i>	GTCGAGCGAATGGATT	25-40
		CAATGGACG	1200-1208 337-335	158	<i>Bacillus thuringiensis</i>	ATTTTGAAGTGCATGGTTCGAAATT GAA	84-111
					<i>Bacillus mycoides</i>	AGCGGCTGAAGGGTGA GGATAATATTTTGAAGTGCATAGT TCGAAATTGAA	70-85 77-111
					<i>Bacillus pseudomycoides</i>	AACATTTTGCACCGCATGGTGCG	81-103
					<i>Bacillus velenzensis</i>	ACCGCATGGTTCAGACATAAA	91-111
					<i>Bacillus flexus</i>	GAGCTAATACCGGATAACATTTTC	66-89
					<i>Bacillus amyloliquifaciens</i>	TAACCTTTTGGAGCCAGCCGCC	1463-1486
					<i>Bacillus pumilus</i>	ATAGTTCCTTGAACCGCATGGT	148-169
					<i>Bacillus oceanisediminis</i>	GCCGGTACCTTGACGGTAC AGGCTGCAACTCGCCTGG	464-482 1334-1351
					<i>Bacillus anthracis</i>	TGTAGGCTGCAACTCGCCTACA	1335-1356
					<i>Bacillus mojavenis</i>	CCCGAAGTCGGTGAGGTAACCTTT AT	1447-1472
					<i>Bacillus howanjipoensis</i>	GTAACACGTGGGCAACCTGCCCTG C	106-130
					<i>Bacillus firmus</i>	TGCAGTCGAGCGGACGATGGGAGC TTGCTCCC	27-58
					<i>Bacillus aerophilus</i>	CGTTTACGGCGTGACTAC	1241-1259
					<i>Bacillus tequilensis</i>	GTTTCGATCGCAGTCTGCAACTCG ACTGC	1332-1361
					<i>Bacillus megaterium</i>	AGACTGGGATAACTTCGGGAAACC	132-156

						GA	
					<i>Bacillus sonorensis</i>	GAGGCTAAGCCAATCCCACAAATC T	1194-1218
9	Pseudomonas	TCCTACGGGA	354-364 1368-1378	1,004	<i>Pseudomonas aeruginosa</i>	GAAGGGAGCTTGCTCCTGGATTC	19-41
		GGAATT	571-576 687-692	111		GTCCTGAGGGAGAAAGTGGGGGAT CTT	146-172
		CAGCAG	366-371 533-538	162		GGGGATAACGTCCGAAACGGGC G	93-116
		GTAAAGC	442-448 584-590	136		GTCCTGAGGGAGAAAGTGGGGGAT CTT	146-172
10	Klebsiella	<i>GTGACGAGCGGCGGACGGGTGAGTA A</i>	85-110	Not repeat	<i>Klebsiella pneumoniae</i>	Not found	
		<i>GAAGTGTGAGACAGGTGCTGCAT</i>	1066-1088				
		<i>ACACGTGCTACAATGGCATATA</i>	1261-1282				
11	Enterococcus	ATAAACT	117-124 147-154	23	<i>Enterococcus caseliflavus</i>	Not found	
		TTGACCACTCTAGAGATAGAGC	1018-1039	Not repeat	<i>Enterococcus faecialis</i>		
		GAGTAAAATGTTTCATCCC	453-470		<i>Enterococcus gallinarum</i>		
		AGCGAGACTGCCGGTGACAAACCG	1172-1195				
12	Comamonas	TGGGGGATAACTACTCGAAAAGAGT	167-190	Not repeat	<i>Comamonas terigena</i>	TACTGCGG	1299-1306
		CCACACTGGGACTGAGACACGGCC CA	346-371				
		TGGGGGATAACTACTCGAAAAGAGT	156-179				
13	Burkholderia	<i>AAACCTTACCTACCCCTTGACA</i>	1033-1053	Not repeat	<i>Burkholderia cepacia</i>	TTTGTAACACCCGAAGTCGG	1487-1506
		<i>GGGTAGGGCTTCACACGTCATACAAT GGTC</i>	1279-1308			CTTATGGATGGACCCGCGT	256-274
		<i>GAGTGGGTTTTACCAGAAGTG</i>	1484-1504			CTTATGGATGGACCCGCGT	256-274

Discussion

Diversity of bacteria from Citrus

Microbial community research can help towards a more comprehensive understanding of the complex structure and function of microbial flora, and it may lead to rapid identification of novel bacterial strains [33, 34]. This study provides a thorough examination of the diversity and makeup of microbial communities within the leaf tissue of citrus. Endophytes are important in food safety, agricultural production, and phytoremediation research. The complexity of endophytic microbial flora and the processes that affect their formations in non-cultivated plants, on the other hand, is indeed unexplored. One of the really interesting and relatively unknown fields of agriculture in Pakistan is the exploration of the epiphytic microbial biodiversity of citrus. It has been found that the population of endophytic bacteria within leaf tissue is approximately proportional to the biological influence imparted on the hosts. This is a fascinating theory that needs to be tested on endophytes in different plants. Furthermore, the number of bacterial endophytes inhabiting plant inner parts may be dynamic and depends on a variety of factors such as the growth stage of the plant, species, and tissue type examined [35]. Therefore this study was complemented by an exploration of the endophytic bacterial species inhabited in the citrus phyllo sphere. Thirty-seven distinct strains of bacteria, including Firmicutes and Proteobacteria, were found. *Bacillus* sp. was the most prevalent genus observed in citrus.

Endophytes have been identified from approximately all kinds of Vegetation [6, 36, 37]. Numerous studies have also reported the isolation of indigenous endophytes from other hosts,

like potatoes [25], maize [38], wheat [39], rice, banana, carrot, sugarcane, tomatoes [28], and peppers [40], soybean plants and among others [41]. To the best of our knowledge, this is the first study to characterize indigenous bacterial endophytes extracted from different citrus varieties.

This remains a substantial difference in the kinds of indigenous microorganisms extracted from distinct different host plants. Bacterial isolates are distinguished by their colors and colony shape [42, 43]. A previous study was revealed the identification of BE from tomato [44], common bean (*Phaseolus vulgaris*) [19], and Alien Weed (*Mikania micrantha*) [21]. Endophytes have also been isolated from the roots, stems, and leaves of numerous rice varieties [45].

A variety of bacteria have already been identified from the vascular tissue of lemon roots, such as *Alcaligenes-Moraxella*, *Acinetobacter baumannii*, *Acromobacter spp.*, *Bacillus spp.*, *Acinetobacter iwoffii*, *Arthrobacter spp.*, *Corynebacterium spp.*, *Burkholderia cepacia*, *Citrobacter freundii* [46]. Endophytes such as (*Citrobacter freundii*, *Achromobacter*, *Acinetobacter*, *Arthrobacter*, *Burkholderia cepacia*, *Alcaligenes-Moraxella*, *Enterobacter*, *Bacillus*, *Corynebacterium*, and *Pseudomonas*) have also been found in the vascular tissue of lemon roots in Florida [47]. Endophytic bacteria *Pantoea agglomerans* and *Bacillus pumilus* were also identified from citrus rootstocks from Brazil reported by [48].

Molecular characterization of bacterial endophytes

The search for novel bacterial endophytes may contribute to the discovery of novel processes that boost plant growth and disclose interesting interactions between plants and bacterial endophytes. Though the use of 16S rDNA sequencing to identify bacterial diversity has resulted in the recognition and characterization of many previously unknown bacteria,

determining pathogenic microbes and correlating them to disease still requires bacterial cultures. [49] Conducted similar studies on the 16S rRNA from the soya bean. However, in beans, 16S rRNA sequencing was performed, and a taxonomic study was applied to determine the evolutionary pattern between bacterial strains [50].

Signature Sequences

Signature patterns of all isolated genera were identified as signature patterns are critical characteristics for recognizing bacteria. There have been few studies to identify the distinctive signatures for another bacterial genus. The signature patterns of 15 genera were detected in this study, both at the species level levels. Every bacteria has a distinctive pattern that is genetically present in the 16S rRNA region. This Unique Pattern may perhaps be advantageous in the diagnosis of specific bacterial strains and also help to determine the biodiversity of bacteria in different environmental samples. By comparing all of the isolates to other reported strains in the database, signatures at the genus and species level were discovered in this study. Target-specific patterns could be determined based on dinucleotide composition to discriminate one group of bacteria from another [51]. These dinucleotides could be used to construct distinct sequence patterns to determine specificity against standard databases. However, the repeating patterns are conserved across different sequences of *Pseudomonas* that have been used to pinpoint a mismatched region and signature. In this research signatures of *Bacillus*, *Lysinibacillus*, *Enterobacter*, *Pseudomonas*, *Enterococcus*, *Brevibacterium*, and some other genera have been used to identify genus-specific patterns and primers. The 4 detected repeats show a pattern of repeating parts that are highly prevalent across the *pseudomonas* 16S sequences. At the genus level, the signature sequence revealed a repetition in a sequence because it was not the same from species, which indicated just a single unique sequence could be distinct from other reported

species of the same genus. No repeats of the sequence were discovered in certain taxa, as mentioned in (Table 4).

Except for *Pseudomonas*, the incidence of four repeats in a row was not seen in some other groups. This indicates that in other genera, these patterns have undergone substitutions/changes at one or more corresponding base locations, resulting in repeats not being formed, or that these four conserved repeating patterns in *Pseudomonas* are the consequence of evolution. Previously finding of identical repeat units throughout vertebrate species, some studies have proposed the evolutionary importance of repeat elements (Wilkinson et al. 1997).

Furthermore, in *Pseudomonas*, the discovered consistent repeats may have some relevance that should be examined. Another type of positional identifiers, such as non-duplicating patterns, requires the same distances between such sequence patterns. Although the approach was effective for *Pseudomonas*, it may have limitations when applied to other genera. In other cases, for instance, subsequences bordered by regular repetitions might indicate reduced variation throughout the length of the fragment or the variation may be uniform during its length; in certain cases, identifying a stretch to select patterns would be difficult. Furthermore, it's indeed possible that the patterns belonging to the most variable region do not produce enough hits to be considered target-specific. It's unclear to what extent various bacterial endophyte populations can be found in different plant tissues and species. Their characteristics and functional responsibilities, on the other hand, can vary. The hunt for novel bacterial endophytes, on the other hand, may aid in the discovery of novel mechanisms that enhance plant growth, as well as disclose intriguing interactions between plants and their endophytes, as well as between endophyte strains of the plant.

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Figure Legends:

Fig.1: Abundance of endophytes extracted from different citrus genotypes based on Phylum

Fig.2: The abundance of endophytic bacteria extracted from various citrus genotypes based on Genus

Fig.3: Illustrate the evolutionary relationships of bacterial endophytes obtained from different citrus genotypes. This evolutionary tree contains 22 bacterial endophytes constitute three classes

(Bacilli, Beta Proteobacteria, and Gamma Proteobacteria). All of the analysed samples by 16S rDNA were 97 percent identical to known bacterial strains.