

Original Research Article

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Identification and Molecular Characterization of *Castellaniella ginsengisoli* Isolated from Sugarcane-Wheat Cropping System

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ABSTRACT

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An increasing interest has emerged with respect to the importance of microbial diversity in soil habitats. The extent of the diversity of microorganisms in soil is seen to be critical to the maintenance of soil health and quality, as a wide range of microorganisms is involved in important soil functions. Most soil microorganisms are still unknown. A Gram-negative, rod-shaped, non-spore-forming bacterium, designated as strain P2, was isolated from the soil of the wheat ratooning field of ICAR-Indian Institute of Sugarcane Research, Lucknow, 16S rRNA gene sequence analysis showed that the isolate was closely related to species of the genus *Castellaniella*. *Castellaniella ginsengisoli* DCY36 was shown to be the most closely related (99% 16S rRNA gene sequence similarity), followed by *Castellaniella ginsengisoli* strain MN ZOO (99%). *Castellaniella ginsengisoli*, RNA secondary structure prediction was also been performed by RNA Vienna RNA Web Services.

Introduction

Soils typically contain 10^9 to 10^{10} microorganisms per gram (dry weight), which may represent more than a million bacterial species. However, characterization of the small fraction of microbes that has been cultivated provides only a glimpse of their potential physiological capacity and influence on soil ecosystems. Soil is considered to be the richest environment, with a high diversity of microorganisms belonging to the three domains of life, Bacteria, Archaea and Eukarya. This diversity is extreme at the species level, with approximately 50,000 bacterial species found in one soil sample. Investigation of bacterial diversity is an important step to assess soil conditions due to

its importance in nutrient cycling, and consequently in crop productivity. Soil bacteria and, in particular, rhizosphere bacteria play an important role in many processes, such as decomposition, mineralization, biological nitrogen fixation, and denitrification. In addition, some bacteria associate with plants and promote growth, the so-called plant growth-promoting bacteria (Singh *et al.*, 2004). In the course of screening micro-organisms obtained from the soil of sugarcane-wheat cropping system, a Gram-negative strain, P2, was isolated. The following study was carried out for the identification of the bacteria by 16s RNA techniques.

Materials and Methods

The soil samples used for isolation of bacteria was collected from the root-free soil of rhizosphere from after wheat ratooning field of ICAR-Indian Institute of Sugarcane Research, Lucknow. The sample of each varietal rhizosphere soil was mixed thoroughly to make a composite soil. 10g of dry and highly pulverised soil sample is suspended in 90 ml of sterile distilled water considered as a stock solution then transferring 1ml of soil suspension into 9 ml sterile distilled water with the help of a sterile pipette to yield 10 dilution. Similarly, a series up 1 to 10 dilution was prepared under aseptic condition. Bacteria are isolated by employing serial dilution plate technique using nutrient agar. Then 0.1 ml soil suspension is introduced into sterilized nutrient agar media in Petri dishes and spread it thoroughly on the media incubated at 37°C for 24-48 hours and for each dilution the plates are taken in triplicates. After incubation period, visual morphological characterization of the bacterial colonies isolated on the agar petri plates is observed on the basis of colour, shape, size, elevation etc. of the bacterial colonies. Colonies exhibiting prolific growth are selected for further streaking on fresh agar plates for purification and multiplication of the isolates is done by streak plate methods.

The isolate was grown on nutrient agar plates at 37°C for 24-48 h and was maintained on nutrient agar slants and stored at 4°C as well as at -80°C by making their suspensions in 10% (v/v) glycerol.

Identification of bacterial strain was done using 16SrRNA gene sequencing. The DNA template was prepared by picking an individual colony of bacterial strain, and amplification of the 16S rRNA gene was carried out by the PCR. PCR amplification of DNA was performed using universal primers (9F: 5'-GAGTTTGATCCTGGC TCAG -3';

1510R: 5'-GGCTACCTTGTTACGA-3') in a reaction mixture (25 µl). The amplification program for the full-length 16S rRNA gene consisted of an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 2 min, primer annealing at 55 °C for 1 min and primer extension at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min, in a thermocycler. Amplified PCR products of the 16S ribosomal gene were separated on 1 % agarose gel in 0.5× TE (Tris-EDTA) buffer containing 2 µl ethidium bromide (20 mg/ml) (Chandra and Chandra, 2016). The purified PCR product samples were sent for sequencing using universal 16S rRNA sequencing primers.

The sequence results were obtained from a BLAST search, and the sequences of all the related species were retrieved to determine the exact nomenclature of the isolates. The tree is created using Weighbor with alphabet size 4 and length size 1000.

Results and Discussion

The ribosomal operons mainly 16S rRNA has proven to be a stable and specific molecular marker for the identification of bacteria. The copy number of 16S rDNA genes may fluctuate from 1 to 15 among different bacterial genomes. The 16S rDNA is present in scattered form in the entire genome of bacteria. These ribosomal sequences are useful for the phylogenetic analysis and molecular taxonomy of bacteria. The 16S rDNA is a common target for the taxonomical purpose, mainly due to the mosaic composition of phylogenetically conserved and variable region within the gene (Pontes *et al.*, 2007). The aligned sequence data of isolate was 1382bp. 16S rRNA gene sequences were compared with the available sequences in the databank with help of BLAST homology search and the isolate was found to be *Castellaniella ginsengisoli* (Figure 1). Homology tree based on sequence

alignment of 16S rDNA of bacterial isolates permitted rapid phylogenetic analysis. However, strains isolated from different geographic location shared similar DNA homology. Phylogenetic analysis on the basis of 16S rDNA sequences provided better understanding in evaluation of genetic diversity of bacteria isolated from same and different ecological niche; phylogenetic analysis of 500 bp of terminal region of 16S rDNA from cultivated strain has been found to show existence of large bacterial diversity. A phylogenetic tree or evolutionary tree is a branching diagram or tree showing the inferred evolutionary relationships among various biological species or other entities based upon similarities and differences in their physical and/or genetic characteristics. The taxa joined together in the tree are implied to have descended from a common ancestor. Unrooted trees illustrate the relatedness of the leaf nodes without making assumptions about ancestry at all. In the case of unrooted trees, branching relationships between taxa are specified by the way they are connected to each other, but the position of the common ancestor is not (Mooers and Heard, 2004). The Microbe was found to be most similar *Castellaniella ginsengisoli* strain DCY36 16S ribosomal RNA gene, partial sequence Sequence ID: ref|NR_116482.1 and the next closest homologue was found to be *Castellaniella ginsengisoli* strain MNzoo 16S ribosomal RNA gene, complete sequence Sequence ID: gb|KM275476.1 (Table 1). The sequences of strain were submitted to NCBI Gene Bank database under accession numbers KY606683. The sequence is as follows:

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AGTAAAAGGCTTGGCTTTCTTGGGCGG
CGAGTGGCGAACGGGTGAGTAATGTA
TCGGAACGTGCCAGTAGCGGGGGAT
AACTGGCCGAAAGGTCAGCTAATACC
GCATACGCCCTACGGGGGAAAGGGGG
GGATCGCAAGACCTCTCACTATTGGAG
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CGGCCGATATCGGATTAGCTAGTTGGT
GGGGTAAAGGCCTACCAAGGCAACGA
TCCGTAGCTGGTTTGAGAGGACGACCA
GCCACACTGGGACTGGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTGGGG
AATTTTGGACAATGGGGGGCAACCCTGA
TCCAGCCATCCC GCGTGTGCGATGAAG
GCCTTCGGGTTGTAAAGCACTTTTGGC
AGGGAAGAAACAGCCCGGGCTAATAT
CCCGGGTCAATGACGGTACCTGCAGA
ATAAGCACCGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGGTGAAG
CGTTAATCGGAATTACTGGGCGTAAAG
CGTGCGCAGGCGGTTTCGGAAAGAAAG
GTGTGAAATCCCAGGGCTTAACCTTGG
AACTGCACTTTTAACTACCGGGCTAGA
GTACGTCAGAGGGGGGTAGAATTCCA
CGTGTAGCAGTGAAATGCGTAGAGAT
GTGGAGGAATACCGATGGCGAAGGCA
GCCCCCTGGGATGATACTGACGCTCAT
GCACGAAAGCGTGGGGAGCAAACAGG
ATTAGATACCCTGGTAGTCCACGCCCT
AAACGATGTCAACTAGCTGTTGGGGTT
TATTAACCTTAGTAGCGCAGCTAACGC
GTGAAGTTGACCGCCTGGGGAGTACG
GCGCAAGATTA AAAACTCAAAGGAATT
GACGGGGACCCGCACAAGCGGTGGAT
GATGTGGATTAATTCGATGCAACGCGA
AAAACCTTACCTACCCTTGACATGTCT
GGAATCCTTTAGAGATAGAGGAGTGCT
CGCAAGAGAACCGGAACACAGGTGCT
GCATGGCTGTCGTCAGCTCGTGTCTGTG
AGATGTTGGGTTAAGTCCC GCAACGAG
CGCAACCCTTGCCATTAGTTGCTACAT
TCAGTTGGGCACTCTAATGGGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGGA
TGACGTCAAGTCCTCATGGCCCTTATG
GGTAGGGCTTCACACGTCATACAATGG
TCGGGACAGAGGGTTGCCAAACCGCG
AGGTGGAGCCAATCTCAGAAACCCGA
TCGTAGTCCGGATCGCAGTCTGCAACT
CGACTGCGTGAAGTCGGAATCGCTAGT
AATCGCGGATCAGCATGTGCGGGTGA
ATACGTTCCC GGGTCTTGTACACACCG
CCCGTCACACCATGGGAGTGGGTTTCA
CCAGAAGTAGGTAGCCTAACACGACA
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Table.1 Table demonstrating percentage match between the bacteria with their accession no

S. No.	Organism Name	Accession No.	Percentage Match
1	<i>Castellaniella ginsengisoli</i> strain DCY36	NR_116	99%
2	<i>Castellaniella ginsengisoli</i> strain MN ZOO	KM275476.1	99%
3	Uncultured bacterium clone 7-5	JQ923845.1	99%
4	<i>Castellaniella</i> sp. Pyr2	GU951457.1	99%
5	<i>Castellaniella</i> sp. 528F1-2	EU851054	99%
6	<i>Castellaniella</i> sp. TCOB-5	KU257691.1	99%
7	<i>Alcaligenes</i> sp. A36	KT316405.1	99%
8	<i>Alcaligenes</i> sp. ZL3	JN085953	99%
9	Uncultured <i>Alcaligenes</i> sp. clone H2	FJ863103.1	99%
10	Beta proteobacterium C14 JRPA-2007	EF599312.1	99%

Fig.1 Phylogenetic tree of the isolate

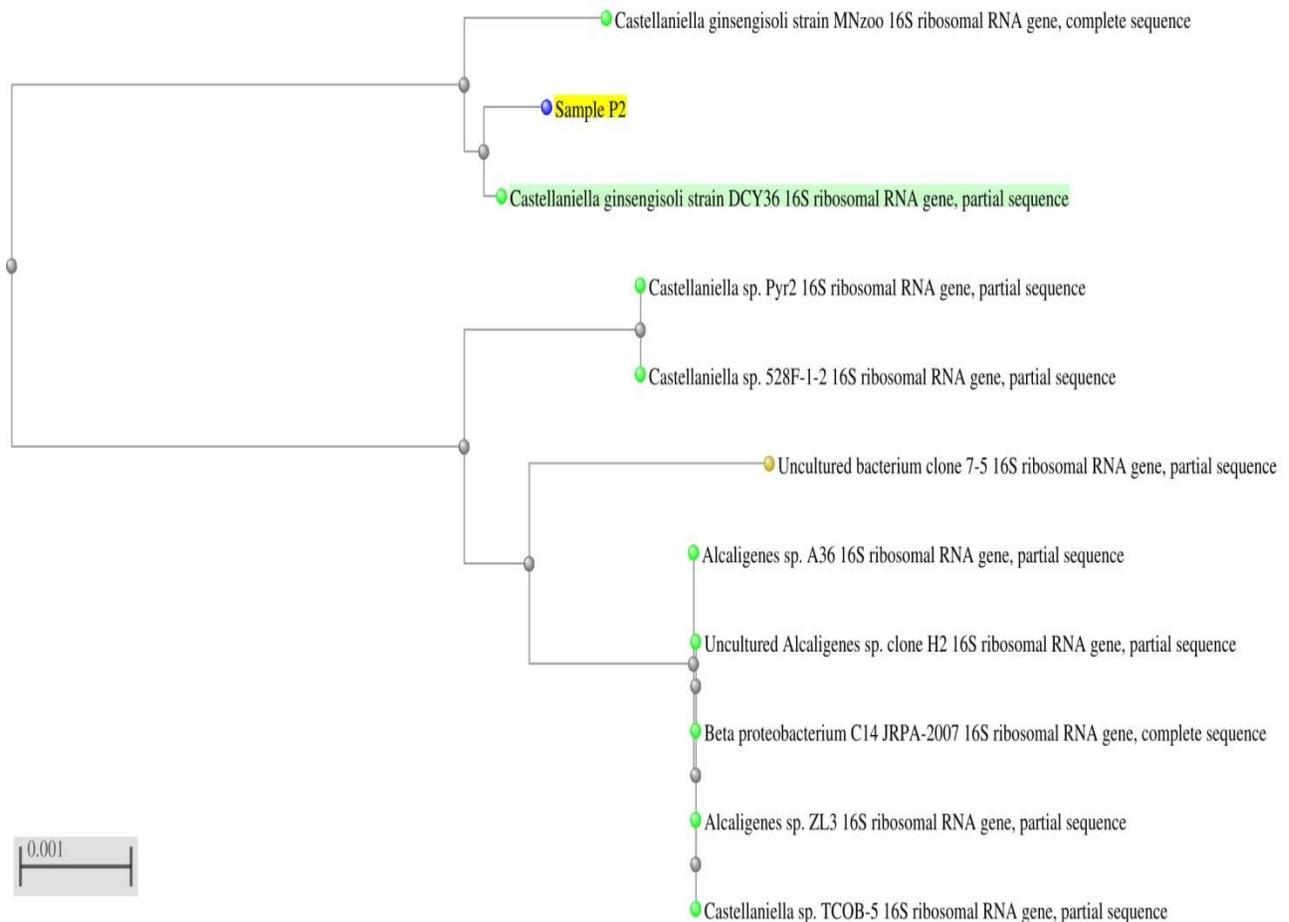


Fig.2 Secondary structure of rRNA of the isolate

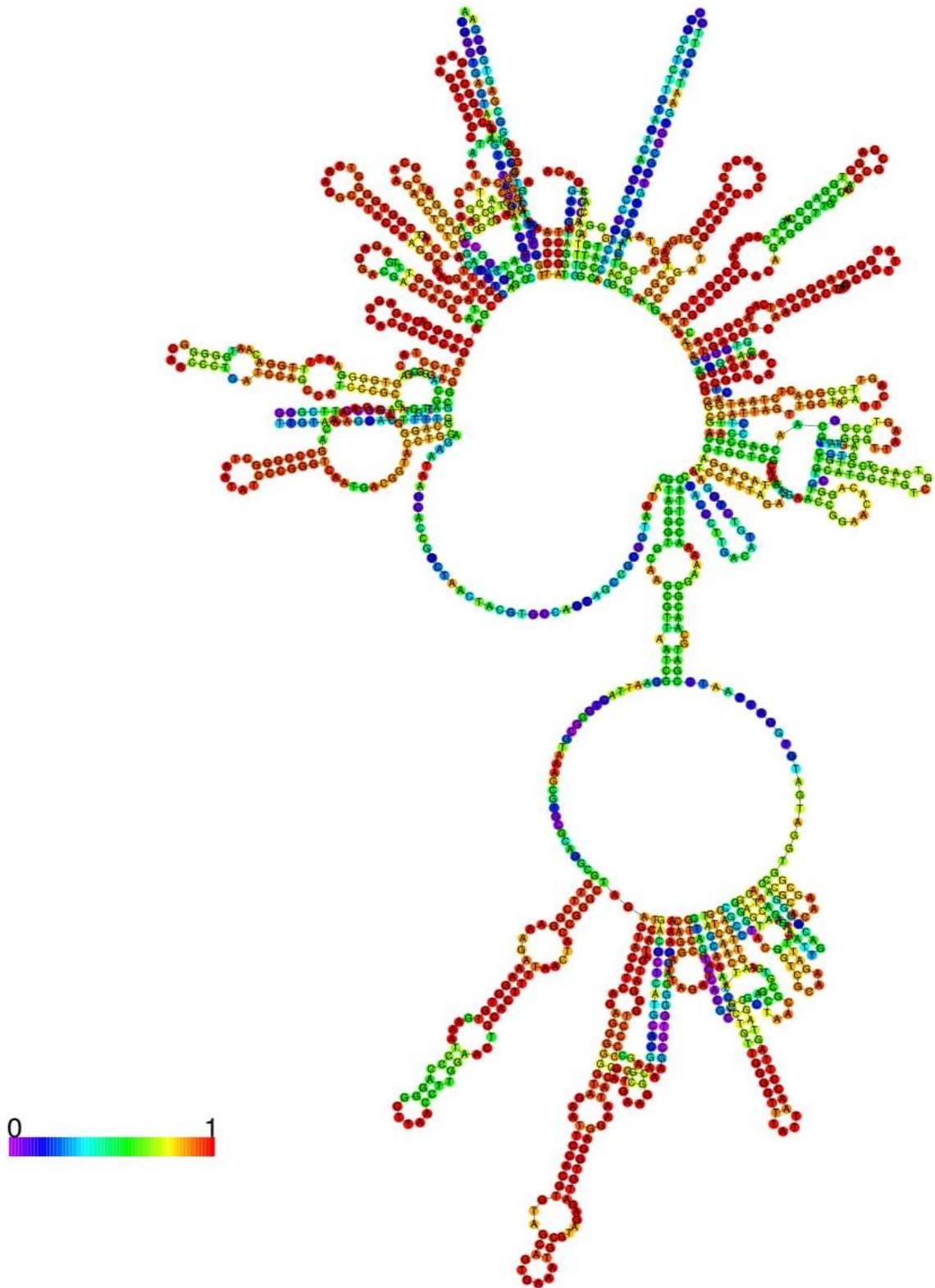
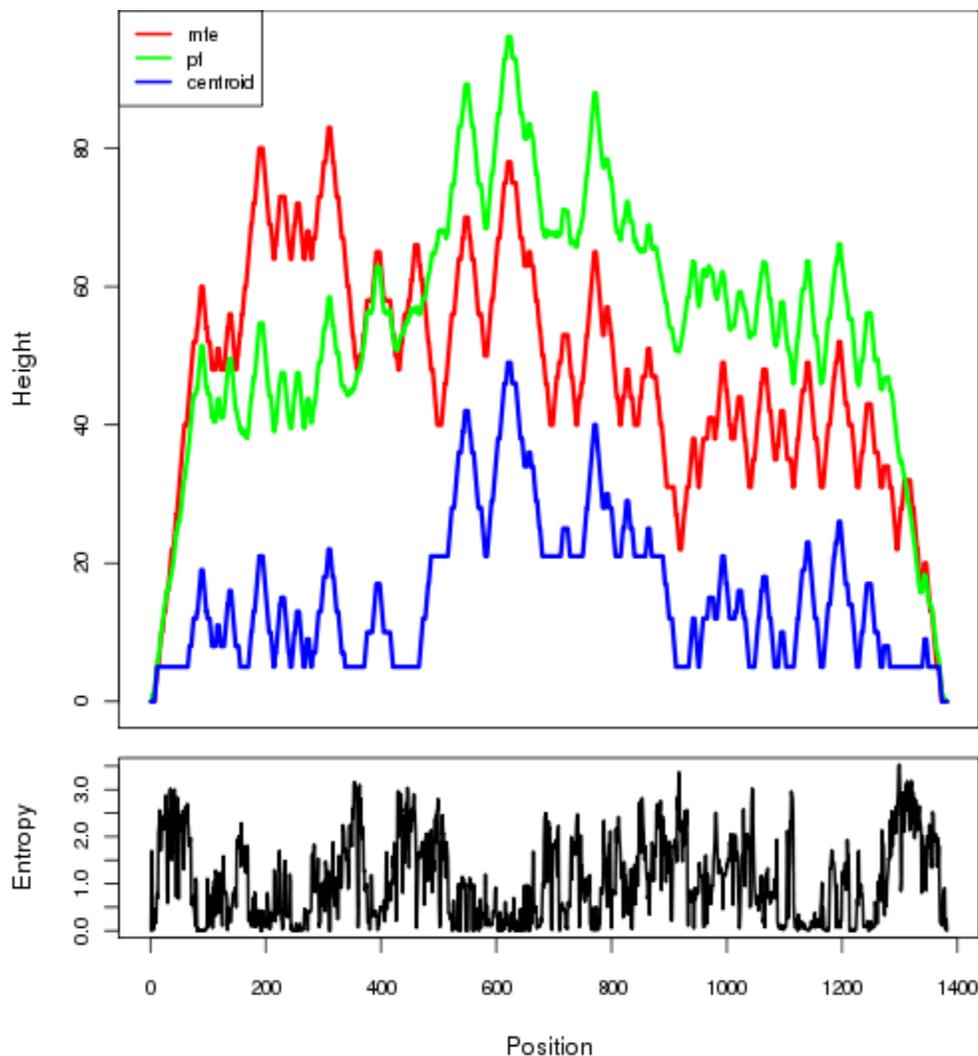


Fig.3 Mountain plot of secondary structure of rRNA of the isolate, is representation of the minimum free energy (MFE) structure, the thermodynamic ensemble of RNA structures, and the centroid structure and the positional entropy for each position



In order to understand the significance in predicting the stability of chemical or biological molecules or entities of *Castellaniella ginsengisoli*, RNA secondary structure prediction has been performed. The 16S RNA gene sequence obtained was used to deduce the secondary structure of RNA ViennaRNA Web Services (Figure 2). This server provides programs, web services, and databases, related to our work on RNA secondary structures. For thermodynamic structure prediction, RNAfold web server was used. The RNAfold web server will predict secondary structures of

single stranded RNA or DNA sequences and current limits are 7,500 nt for partition function calculations and 10,000 nt for minimum free energy only predictions. The free energy of secondary structure rRNA of *Castellaniella ginsengisoli* was -505.30 kcal/mol. Mountain Plot was also drawn by the RNAfold web server which helps in predicting the hierarchical organization of RNA secondary structure, as nested helices translate into stacking mountains, easing the visual segmentation into domain. Here the sequence is drawn linearly, but this representation also presents, at each position i ,

the number of base-pairs nesting the position, i.e. involving bases respectively before and after i. In this setting, helices give rise to mountains while terminal loops translate into peaks (Figure 2).

Castellaniella ginsengisoli is a Gram-negative, oxidase- and catalase-positive, rod-shaped, motile, beta-glucosidase-producing bacterium from the genus *Castellaniella* which has been firstly isolated from soil of a ginseng field in South Korea (Kim *et al.*, 2009). Colonies of *Castellaniella ginsengisoli* are yellow coloured. The genus *Castellaniella* was created by the reclassification of *Alcaligenes defragrans* DSM 12141T to the type species of the genus *Castellaniella*.

The genus contains these species, *Castellaniella defragrans*, *Castellaniella denitrificans* (Kampfer *et al.*, 2006) and *Castellaniella caeni* (Liu *et al.*, 2008), *Castellaniella ginsengisoli* (Kim *et al.*, 2009) and *Castellaniella daejeonensis* (Lee *et al.*, 2010) *Castellaniella hirudinis*. In 2006, the genus *Castellaniella* was proposed (Kampfer *et al.*, 2006) for Gram-negative organisms characterized by short motile rods with a facultatively anaerobic and denitrifying metabolism. All species described so far contain ubiquinone Q-8 as the major quinone, phosphatidylethanolamine and phosphatidyl -glycerol as the major polar lipids and C16: 0, C16: 1v7c and C17: 0 cyclo as the major fatty acids (Kim *et al.*, 2009).

In conclusion, molecular techniques utilizing polymerase chain reaction (PCR), alone or in combination with DNA sequence analysis, have become increasingly popular in determining the evolutionary relationships of bacteria which are helping in the identification of novel bacteria.

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