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Validation of 16S rRNA Sequences as a Tool for Taxonomy of Cyanobacteria with Reference to *Nostoc* and *Oscillatoria*

Shakena Fathima T.[1], Asraf Sithikka M. R.[2], Fayaazuddin T.[1], Dhanasekaran D.[1], Muralitharan G.*[1] and Thajuddin N.[3]

[1] National Repository of Microalgae and Cyanobacteria-Freshwater and Marine (NRMC-F&M), Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli - 620 024, Tamil Nadu, India
[2] School of Life Sciences, Bharathidasan University, Tiruchirappalli - 620 024, Tamil Nadu, India
[3] B. S. Abdur Rahman Crescent Institute of Science and Technology (Deemed to be University), Vandalur, Chennai - 600 048, Tamil Nadu, India

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Abstract

Blue green algae, or cyanobacteria, evolved over millions of centuries to withstand a wide range of conditions, including extremes in pH, salinity and temperature. Because of their diverse morphology and the presence of comparable phenotypic traits with variances in genotypic features, cyanobacteria can be difficult to identify. The most common issues with cyanobacterial identification are as follows: (1) morphological variation within the same taxa can vary in both natural and the lab conditions; (2) taxonomic modifications are common due to the constantly changing classification of cyanobacteria; (3) cryptic species exhibit morphological similarity but genetic distinctness; (4) cyanobacterial growth is impacted by environmental factors such as light, temperature, and pH, leading to phenotypic variations and absence of extensive reference materials. This study investigated the technique of utilizing 16S rRNA CYA primer to figure out the genetic links between twelve isolates of Oscillatoria and 8 Nostoc strains of cyanobacteria. Their phylogenetic tree, *in silico* restriction digestion analysis, and secondary structure prediction were used to estimate the taxonomic resolution. By using their genotypic and phenotypic differences, these techniques made it possible to discriminate between different cyanobacterial genera.

***Key words*:** Oscillatoriales, Nostocales, 16S rRNA, Molecular taxonomy, *In-silico*, Restriction digestion, Secondary structure, Phylogenetic tree

Cyanobacteria are an ancient class of prokaryotic photosynthetic organisms that are found all over the planet in soils, thermal springs, freshwater and marine habitats and the Polar Regions according to Whitton [25], Garcia-Pichel [6], Abed *et al*. [1] and others. As secondary metabolites, these organisms produce a wide range of physiologically active compounds that have antibacterial, carcinogenic, immunosuppressive, anticancer, antifungal and antitumor properties [8], [25]. This proved their enormous potential as suppliers of chemicals to the food, pharmaceuticals and biotech industries. Even though cyanobacteria are widely used, their nomenclature has changed significantly several times since the early 2000s due to the advancements in genome sequencing. This change started with their classification as prokaryotes and continued with a plethora of new taxonomic descriptions and reclassifications [3]. In fact, morphology seems to be lacking in recent identification systems; instead, their systematic grouping approach entirely depends on phenotypic (and infrequently ecological) traits. The taxonomy of cyanobacteria is complicated by its diverse evolutionary patterns, adaptable cyto-morphological features and rapid evolution across all families. Different higher taxa may differ in the stability and taxonomic importance of numerous criteria included in cyanobacterial genomes. The taxonomic importance of identical characteristics in different clades can therefore differ. Even the same species from different populations have diverse evolutionary sequences because of their non-overlapping molecular data [4], [17]. Amongst the few justifications that molecular information and the species phylogeny concept should be used together are the following [2]. Although DNA sequences are strict hereditary inheritances, environmental influences can affect biochemical and morphological characteristics [3]. While the nature of phenotypic traits is not always evident, molecular features can be clearly characterized [4]. Genomic data may be applied to quantitative techniques considerably more easily [14]. The comparison of extremely distant family organisms (i.e., across domains and phylum) is only possible with a limited number of techniques, because morphological features do not hold up at such distances [7].

To detect cyanobacteria and establish a phylogenetic tree, the most prevalent 16S rRNA gene was utilized [22-23]. These molecules are present in all prokaryotes, and since the strains differ in their sequence, it is possible to deduce evolutionary relationships from them. Furthermore, strain investigations are made possible by the comparatively easy alignment of the 16S rRNA gene sequences, which have

***Correspondence to:** Muralitharan G., E-mail: drgm@bdu.ac.in

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accumulated into a substantial library of sequences (about 11 lakh cyanobacterial sequences as of today) [16]. The diversity of cyanobacteria has been investigated using a number of markers, such as the *rpo* gene, ITS region, *nif* gene, 16S rRNA, phycocyanin locus and phosphoenol pyruvate carboxylase gene [24]. With the help of the use of bioinformatics, rapid advancements in genomic and alternative molecular research technologies along with information technology trends have combined to give an astounding amount of molecular biology-related information. Utilizing 16S rRNA gene sequences, this study investigated the genetic interference among different isolates of Oscillatoriales and Nostocales through *in-silico* techniques like phylogenetic tree, Restriction digestion analysis and their secondary structure to illustrating the taxonomic resolution. By using their genotypic and phenotypic distinctions, these techniques made it possible to discriminate between different cyanobacterial genera. Therefore, this work uses the 16s rRNA gene, which can be established as a molecular marker for cyanobacteria authentication, to determine the taxonomic interference between various *Oscillatoria* and *Nostoc* species.

MATERIALS AND METHODS

Strain collection

Totally 12 strains of Oscillatoriacales and 8 different strains of Nostocales were collected from National Repository of Microalgae and Cyanobacteria – Freshwater (NRMC-F) and National Facility for Marine Cyanobacteria (NFMC) funded by DBT, Government of India, Microalgae and cyanobacterial collection centre located in Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. 16S rRNA CYA sequences (Table 1) for these strains were retrieved from NCBI bank.

Phylogenetic analysis of 16s rRNA gene

Using the retrieved sequence neighbour joining (NJ) procedure in MEGA 11 [15], the distance matrix has been adjusted for phylogenetic analysis, by removing multiple substitutions and gaps are avoided. The statistical significance of the tree branches was ascertained using bootstrap analysis [18].

Restriction digestion analysis

A version 2.0 of the NEB CUTTER program was utilized to determine the restriction sites in the 16S rRNA CYA gene Sequence of a particular Oscillatoriaceae family member (http://tools.neb.com/NEBCUTTER2/index.php). According to Lehtimäki *et al*. [15], the *in-silico* research made it possible to identify unique restriction endonucleases, allowing for the classification of cyanobacterial taxa across strains.

Secondary structure prediction

With RNA alifold server version 2.5.8, the secondary structures of 16S rRNA gene sequences were folded. Using these secondary structural traits, the significance of considerable variability in 16S rRNA gene sequence data was evaluated.

RESULTS AND DISCUSSION

16S rRNA CYA gene sequence for 12 Oscillatorial strains and 8 Nostoc strains of Cyanobacterial from NRMC-F and NFMC were deposited and collected from NCBI Genbank. The strain details and accession number were mentioned (Table 1). The 16S rRNA gene sequences of the examined cyanobacterial species were aligned with comparable sequences from the NCBI GenBank database using CLUSTAL-W. Utilizing the Neighbour-Joining methodology [20], the phylogenetic tree was built. It was evident that the examined Oscillatorial and *Noctoc* strains could be divided into at least four clusters (Fig 1).

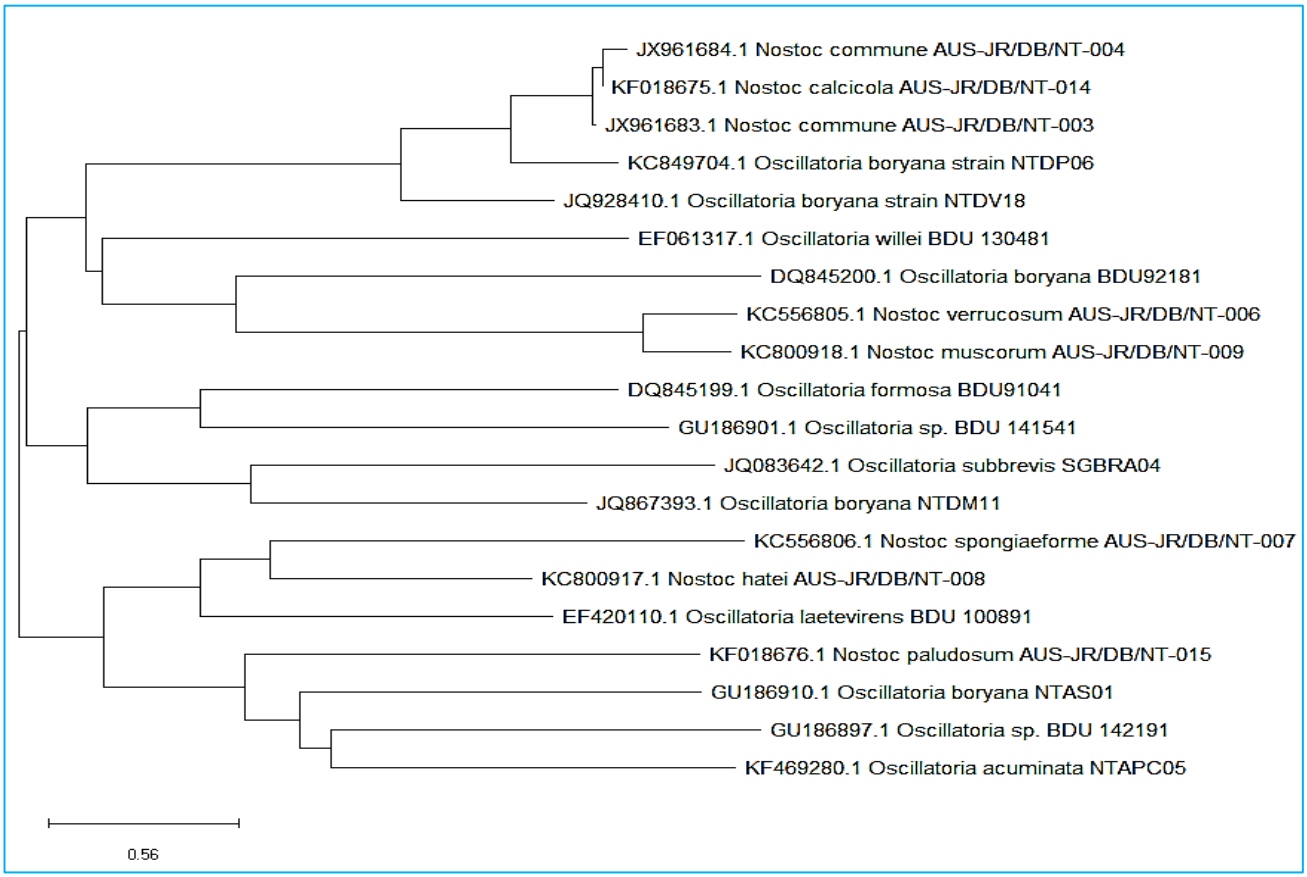

Fig 1 Phylogenetic *t*ree construction for 12 strains of Oscillatoriales and 8 strains of Nostocales

Table 1 GenBank accession number for 12 Oscillatoriales and 8 Nostocales filamentous strains

S. No.	Strain name	Strain No.	Accession No.
1	*Oscillatoria boryana*	BDU91041	DQ845199
2	*Oscillatoria formosa*	BDU92181	DQ845200
3	*Oscillatoria willei*	BDU 130461	EF061317
4	*Oscillatoria laete-virens*	BDU100891	EF420110
5	*Oscillatoria boryana*	NTAS01	GU186910
6	*Oscillatoria sp.*	BDU 141541	GU186901
7	*Oscillatoria sp.*	BDU 142191	GU186897
8	*Oscillatoria sp*	BDU 142191	JQ083642
9	*Oscillatoria subbrevis*	SGBRA04	JQ867393
10	*Oscillatoria boryana*	NTDM11	JQ928410
11	*Oscillatoria boryana*	NTDV18	KC849704
12	*Oscillatoria acuminata*	NTAPC05	KF469280
13	*Nostoc commune*	AUS-JR/DB/NT-004	JX961684
14	*Nostoc commune*	AUS-JR/DB/NT-003	JX961683
15	*Nostoc spongiaeforme*	AUS-JR/DB/NT-007	KC556806
16	*Nostoc verrucosum*	AUS-JR/DB/NT-006	KC556805
17	*Nostoc hatei*	AUS-JR/DB/NT-008	KC800917
18	*Nostoc muscorum*	AUS-JR/DB/NT-009	KC800918
19	*Nostoc calcicola*	AUS-JR/DB/NT-014	KF018675
20	*Nostoc paludosum*	AUS-JR/DB/NT-015	KF018676

Table 2 16S rRNA gene sequence analysis of cyanobacterial strains -Restriction enzymes showing polymorphic bands by custom digestion with in silico experiments

S. No.	Strain Id	Strain name	Total Bps	Gc %	2 Cutter	3 Cutter	Restriction enzymes producing polymorphic fragments
1	*Oscillatoria Formosa* BDU91041	DQ845199	782	52%	23	18	*AatII, AflIII, AhdI, ApaI, BanII, BciVI, BfuAI, BsaAI, BsaHI, BseYI, BsmFI, BspEI, BspMI, BssSI, BstNI, BstUI, BtsCI, EcoO109I, FokI, HaeIII, Hpy188I, HpyAV, HpyCH4III, MseI, MwoI, NmeAIII, PspGI, PspOMI, SfaNI, TaqI, ZraI*
2	*Oscillatoria boryana* BDU92181	DQ845200	993	55%	26	29	*AatII, AflIII, AgeI, AhdI, AleI, ApaI, BanII, BccI, BfuAI, BmrI, BsaAI, BsaHI, BsaWI, BseYI, BslI, BsmFI, BspCNI, BspEI, BspMI, BsrFI, BsrI, BssSI, BstNI, Bsu36I, DpnI, DpnII, EcoNI, EcoO109I, HaeII, HhaI, HinP1I, Hpy188I, MboI, MboII, MluI, MslI, NmeAIII, NspI, PaqCI, PciI, PspGI, PspOMI, Sau3AI, Sau3AI, SgrAI, SphI, TaqI, ZraI*
3	*Oscillatoria willei* BDU 130461	EF061317	912	56%	26	17	*AatII, AfeI, AflIII, AgeI, AhdI, AleI, ApaI, ApoI, BaeGI, BanII, BbvCI, BccI, BcoDI, BfuAI, BmrI, BsaAI, BsaHI, BsaWI, BseYI, BsmAI, Bsp1286I, BspCNI, BspMI, BsrFI, BsrI, BssHII, BssSI, BstNI, EarI, EcoO109I, EcoRI, FokI, HaeII, HgaI, Hpy188I, HpyAV, MluI, MslI, MwoI, NdeI, NspI, PspGI, PspOMI, SgrAI, XcmI, ZraI*
4	*Oscillatoria laete-virens* BDU100891	EF420110	825	56%	17	21	*AatII, AflIII, AhdI, AleI, ApaI, AvaII, BaeGI, BanI, BanII, BmtI, BsaAI, BsaHI, BslI, BsmFI, Bsp1286I, BspCNI, BssSI, BstXI, Bsu36I, Bsu36I, BtsCI, EcoO109I, EcoP15I, EcoRV, FokI, Hpy188I, MluI, MslI, NheI, NruI, NspI, PciI, PflFI, PspOMI, RsrII,SexAI, SfaNI, SfcI, SphI, StuI, Tth111I, XcmI, ZraI*
5	*Oscillatoria boryana* NTAS01	GU186910	692	54%	21	9	*AgeI, AluI, AlwI, BfuAI, BsaHI, BsaWI, BslI, BsmFI, BsmI, BspEI, BspMI, BsrFI, BtsCI, BtsI, DpnI, DpnII, EcoNI, EcoP15I, EcoRI, Fnu4HI, FokI, HaeII, HaeIII, HgaI, HphI, Hpy99I, HpyCH4IV, HpyCH4V, MboI, MboII, MseI, MspA1I, PaqCI, Sau3AI, SfaNI, SfaNI, SfcI, SgrAI, TfiI, Tsp45I.*
6	*Oscillatoria* sp. *BDU 141541*	GU186901	639	54%	23	4	*ApoI, BceAI, BfuAI, BslI, BsmI, BspEI, BspMI, BtsCI, BtsI, DpnI, DpnII, EcoNI, EcoRI, FokI, HgaI, HphI, HpyCH4V, MboI, MluCI, PaqCI, Sau3AI, TaqI.*
7	*Oscillatoria* sp. BDU 142191	GU186897	646	55%	16	9	*ApoI, AvaI, AvaII,BcoDI, BmrI, BpmI, BseRI, BsiEI, BsmAI, BsoBI, BspEI, BsrDI, BtgI, BtsCI, BtsI, CviQI, DpnI, DpnII, EcoNI, EcoRI, FokI, HindIII, HinfI, Hpy166II, Hpy188III, Hpy99I, HpyAV, HpyCH4III, MboI, MluCI, MlyI, MspA1I, NmeAIII,*

							PflFI, PleI, PvuI, RsaI, SacII, Sau3AI, StyI, TfiI, Tth111I.
8	*Oscillatoria subbrevis* SGBRA04	JQ083642	1329	54%	20	14	*AatII, Acc65I, AfeI, AflIII, AhdI, AleI, AlwNI, ApaI, ApoI, AvaI, BaeGI, BanI, BccI, BcoDI, BfuAI, BpuEI, BsaAI, BsaHI, BsaWI, BseRI, BsmAI, BsmFI, BsoBI, BspEI, BspMI, BsrDI, BsrFI, BsrGI, BssSI, BtgI, BtsI, CviQI, DpnI, DpnII, EcoNI, EcoO109I, EcoRI, HaeII, HindIII, Hpy166II, KpnI, MboI, MluI, MlyI, MslI, MspA1I, MwoI, NcoI, NmeAIII, NruI, NspI, PaqCI, PciI, PleI,PspOMI, RsaI,SacII, Sau3AI, SmaI, SmlI, SphI, StuI, StyI, TaqI, Tsp45I, TspMI, XmaI, XmnI, ZraI.*
9	*Oscillatoria boryana* NTDM11	JQ867393	581	54%	25	5	*ApoI, BmrI, BslI, BtsI, EcoNI, EcoRI, Hpy166II, KpnI, MboI, MluI, MlyI, MslI, MspA1I, MwoI, NcoI, NmeAIII, NruI, NspI, PaqCI, PciI, PleI,PspOMI, RsaI,SacII, Sau3AI, SmaI, SmlI, SphI, StuI, StyI, TaqI, Tsp45I, TspMI, XmaI, XmnI, ZraI.*
10	*Oscillatoria boryana* NTDV18	JQ928410	639	53%	23	11	*Acc65I, ApaI, ApeKI,ApoI, AvaI, BaeGI, BanI, BanII, BbvI, BceAI, BcoDI, BfuAI, BmrI, BsaI, BsmAI, BsoBI, Bsp1286I, BspEI, BspMI, BspQI, BtsI, CviQI, EarI,EcoNI, EcoO109I, EcoP15I, EcoRI, HgaI, Hpy166II, HpyCH4V, KpnI, MluCI, MseI, MspA1I, PaqCI, PspOMI, RsaI, SacII, SapI, SmaI, TaqI, TseI, TspMI, XmaI, XmnI.*
11	*Oscillatoria boryana* NTDP06	KC849704	637	56%	19	13	*Acc65I, ApoI, BanI, BbvI, BceAI, BcoDI, BfuAI, BfuAI, BmrI, BseRI, BsiEI, BsiHKAI, BsmAI, BspEI, BspMI, BsrDI, Bsu36I, BtsI, CviQI, DpnI, DpnII, Eco53kI, EcoNI, EcoRI, HgaI, Hpy166II, HpyCH4V, KpnI, MboI, MluCI, MlyI, MspA1I, MwoI, PaqCI, PleI, PvuI, RsaI, SacI, SacII, Sau3AI, Sau96I, XmnI*
12	*Oscillatoria acuminata* NTAPC05	KF469280	431	54%	12	9	*Acc65I, ApeKI, BanI, BbvI, BceAI, BstUI, BtsCI, CviQI, EcoP15I, EcoRI, HgaI, HpyCH4III, HpyCH4V, KpnI, MspA1I, RsaI, SacII, TseI*
13	*Nostoc commune AUS-JR/DB/NT-004*	JX961684	630	53%	19	17	*AlwNI, AvrII, BanII, BceAI, BclI, BcoDI, BfuAI,BmrI, BsaJI, BsiHKAI, BsmAI, Bsp1286I, BspEI, BspMI, BsrDI, BsrFI, BsrI,BtgI, BtsI,CviQI, Eco53kI, EcoNI, HgaI, HhaI, HinP1I, Hpy166II,Hpy99I,HpyCH4V, MlyI, MspA1I, MwoI, PaqCI, PleI, RsaI, SacI, SacII, StyI*
14	*Nostoc commune AUS-JR/DB/NT-003*	JX961683	638	53%	21	14	*AlwNI, ApoI, BanII,BceAI, BclI, BcoDI, BfuAI, BmrI, BsiHKAI, BsmAI, Bsp1286I, BspEI, BspMI, BsrDI, BsrI, BtgI, BtgZI, BtsCI, BtsI, CviQI, Eco53kI, EcoNI, EcoRI, FokI, HgaI, Hpy166II, Hpy99I, HpyCH4V, MluCI, MlyI, MspA1I, MwoI, PaqCI, PleI, PspGI, RsaI, SacI, SacII,Sau96I, StyD4I, StyI*
15	*Nostoc spongiaeforme AUS-JR/DB/NT-007*	KC556806	557	54	28	7	*AlwNI, AvrII, BanII, BclI, BcoDI, BfuAI, BmrI, BsaJI, BsiHKAI, BsmAI, Bsp1286I, BspEI, BspMI, BsrDI, BtgI, CviQI, Eco53kI, EcoNI, HaeIII, Hpy166II, Hpy188III, Hpy99I, HpyAV, HpyCH4V, PaqCI, RsaI, SacI, SacII, Sau96I, StyI, Tsp45I*
16	*Nostoc verrucosum AUS-JR/DB/NT-006*	KC556805	601	54	21	10	*Acc65I, AlwNI, AvrII, BanI, BanII, BceAI, BclI, BcoDI, BfuAI, BmrI, BsaJI, BsiHKAI, BsmAI, Bsp1286I, BspEI, BspMI, BsrDI, Bsu36I, BtgI, BtsCI, BtsI, CviAII, CviQI, Eco53Ki, EcoNI, FatI, FokI, Hpy166II, Hpy99I, HpyAV, HpyCH4V, KpnI, MlyI, MseI, MspA1I, NlaIII, NlaIV, PaqCI, PleI, RsaI, SacI, SacII, Sau96I, StyI*
17	*Nostoc hatei AUS-JR/DB/NT-008*	KC800917	430	54	27	4	*BfuAI, BsaJI, BspEI,BspMI,BstUI,BtgI,BtgZI, CviQI, HgaI, Hpy166II, HpyAV, HpyCH4III, HpyCH4V, MseI, MspA1I, PaqCI, RsaI, SacII, TfiI*
18	*Nostoc muscorum AUS-JR/DB/NT-009*	KC800918	582	52	23	17	*AcuI, AlwNI, ApeKI, AvaII, BanII, BbvI, BceAI, BclI, BcoDI, BfuAI, BmrI, BsaJI, BsiHKAI, BsmAI, Bsp1286I, BspEI, BspMI, BsrDI, BsrI, BtgI, Eco53kI, EcoNI, HaeIII, HhaI, HinP1I, Hpy166II, Hpy99I, HpyCH4V, MlyI, MspA1I, NlaIV, PaqCI, PleI, SacI, SacII, Sau96I, StyI,TseI*

19	*Nostoc calcicola AUS-JR/DB/NT-014*	KF018675	596	54	19	10	*Acc65I, AlwNI, AvrII, BanI, BanII, BceAI, BclI, BcoDI, BfuAI, BmrI, BsaJI, BsiHKAI, BsmAI, Bsp1286I, BspEI, BspMI, BsrDI, Bsu36I, BtgI, CviQI, Eco53kI, EcoNI, HaeIII, HgaI, Hpy166II, Hpy99I, HpyCH4V, KpnI, MlyI, MseI, MspA1I, NlaIV, PaqCI, PleI, RsaI, SacI, SacII, Sau96I, StyI*
20	*Nostoc paludosum AUS-JR/DB/NT-015*	KF018676	607	54	19	18	*Acc65I, AlwNI, ApeKI, BanI, BanII, BceAI, BclI, BcoDI, BfuAI, BmrI, BsaHI, BsaJI, BsiHKAI, BsmAI, Bsp1286I, BspEI, BspMI, BsrDI, Bsu36I, BtgI, BtgZI, BtsI, Eco53kI, EcoNI, HindIII, Hpy166II, Hpy99I, HpyCH4V, KpnI, MlyI, MspA1I, NlaIV, PaqCI, PleI, PshAI, SacI, SacII, Sau96I, StyI, TaqI, TseI*

Table 2 Secondary structural features of 16S rRNA genes from the tested cyanobacterial strains

S. No.	Name of the strain	No. of stems	No. of loops	Thermodynamic ensemble (Kcal/Mol)	Minimum free energy (Kcal/Mol)
1.	*Oscillatoria Formosa* BDU91041	39	41	-203.32	-189.64
2.	*Oscillatoria boryana* BDU92181	56	58	-358.77	-344.70
3.	*Oscillatoria willei* BDU 130461	52	55	-349.43	-335.60
4.	*Oscillatoria laete-virens* BDU100891	50	52	-306.28	-295.10
5.	*Oscillatoria boryana* NTAS01	38	42	-255.50	-243.40
6.	*Oscillatoria* sp. *BDU 141541*	36	37	-238.11	-226.50
7.	*Oscillatoria* sp. BDU 142191	31	35	-246.32	-236.30
8.	*Oscillatoria subbrevis* SGBRA04	77	80	-504.02	-483.90
9.	*Oscillatoria boryana* NTDM11	34	35	-219.19	-209.80
10.	*Oscillatoria boryana* NTDV18	40	43	-225.99	-214.40
11.	*Oscillatoria boryana* NTDP06	37	39	-250.29	-238.90
12.	*Oscillatoria acuminata* NTAPC05	22	26	-161.04	-154.30
13.	*Nostoc commune AUS-JR/DB/NT-004*	31	32	-231.35	-222.70
14.	*Nostoc commune AUS-JR/DB/NT-003*	35	33	-228.79	-219.60
15.	*Nostoc spongiaeforme AUS-JR/DB/NT-007*	31	29	208.50	-200.30
16.	*Nostoc verrucosum AUS-JR/DB/NT-006*	55	50	-390.96	-376.90
17.	*Nostoc hatei AUS-JR/DB/NT-008*	60	64	-498.79	-479.90
18.	*Nostoc muscorum AUS-JR/DB/NT-009*	76	81	-552.30	-532.10
19.	*Nostoc calcicola AUS-JR/DB/NT-014*	35	33	-227.88	-218.60
20.	*Nostoc paludosum AUS-JR/DB/NT-015*	38	37	-218.81	-208.50

The evolutionary tree shows that the 20 strains are trachomatous types. The strains gathered for this inquiry had high bootstrap values for their gene sequences, even if a bootstrap evaluation showed a meager degree of confidence at different nodes. *Nostoc commune* NT-004, *Nostoc commune* NT-003 and *Nostoc calcicola*NT-014; *Nostoc verrucosum* NT-006 and *Nostoc muscorum* NT-009; *Nostoc spongiaeforme* NT-007 and *Nostoc hatei* NT-008; *Oscillatoria Formosa* BDU92181 and *Oscillatoria* sp. BDU 141541; *Oscillatoria subbrevis* SGBRA04 and *Oscillatoria boryana* NTDM11 showed 100% bootstrap value. Ishida *et al*. [11] state that the topology of the phylogenetic tree was determined by matching many nucleotide sequences of different cyanobacteria and counting the partial 16S rRNA gene sequence that was consistent with all species. Studies have shown that taxa belonging to the Nostocales, such as *Anabaena*, *Cylindrospermopsis*, *Nodularia*, *Aphanizomenon* and *Anabaenopsis*, are polyphyletic based on the 16S rRNA gene [2], [12], [19]. The consistent 16S rRNA phylogeny was effective in determining the monophyletic clade formation for cyanobacteria, as opposed to alternative gene analyses that used the *rbcL*, *nif H*, or *nif D* phylogenies.

Cyanobacterial 16S CYA gene sequences were characterized by *in silico* restriction digestion using the NEB cutter V2.0 program. The (Table 2) lists the overall sums of cutters two and three as well as the restriction enzymes that produce polymorphic fragments of DNA. On the basis of their commercial availability and capacity for recognizing a certain sequence (4, 6, or 8 bp), the restriction digestions were determined. Most enzymes either didn't cut the 16S rDNA at all or just cut extremely little portions of the ends. Additionally, employing specialized digestion, restriction fragments larger than 100 bp were identified. Some enzymes resulted in partial digests and pieces that were too small to see, making an accurate and consistent analysis impossible. Enzymes such as *AlwNI, BanII, BclI, BcoDI, BfuAI, BsaJI, BsiHKAI, BsmAI, MspA1I* are very commonly found in all tested Nostocales strains, whereas enzymes like *BciVI, HhaI, HinP1I, BbvCI, BssHII, NdeI, BmtI, BstXI, EcoRV, NheI, RsrII, SexAI, AluI, AlwI, Fnu4HI, HpyCH4IV, BsiHKAI, Eco53kI, SacI, Sau96I* revealed a potential a distinction among the tested Oscillatorial species. The restriction digesting enzymes of *Oscillatoria* and *Nostoc* species differ significantly from one another.

In order to differentiate between organisms based on structural differences in the sequences of 16S rRNA genes and to calibrate the sequence alignment for tree study, secondary structures were built [5]. The 16S rRNA gene is widely used for phylogenetic analysis and classification of bacteria and archaea due to its conserved regions and variable regions that can be used for species differentiation. The (Fig 2) showed the rRNA secondary structures of the cyanobacterial species under investigation. The secondary structures displayed different branching patterns despite the significant variations in complementary and uncomplimentary indicating the stem and loop sequence lengths. There is an obvious difference between all the species that have been researched thanks to their stem and loop traits.

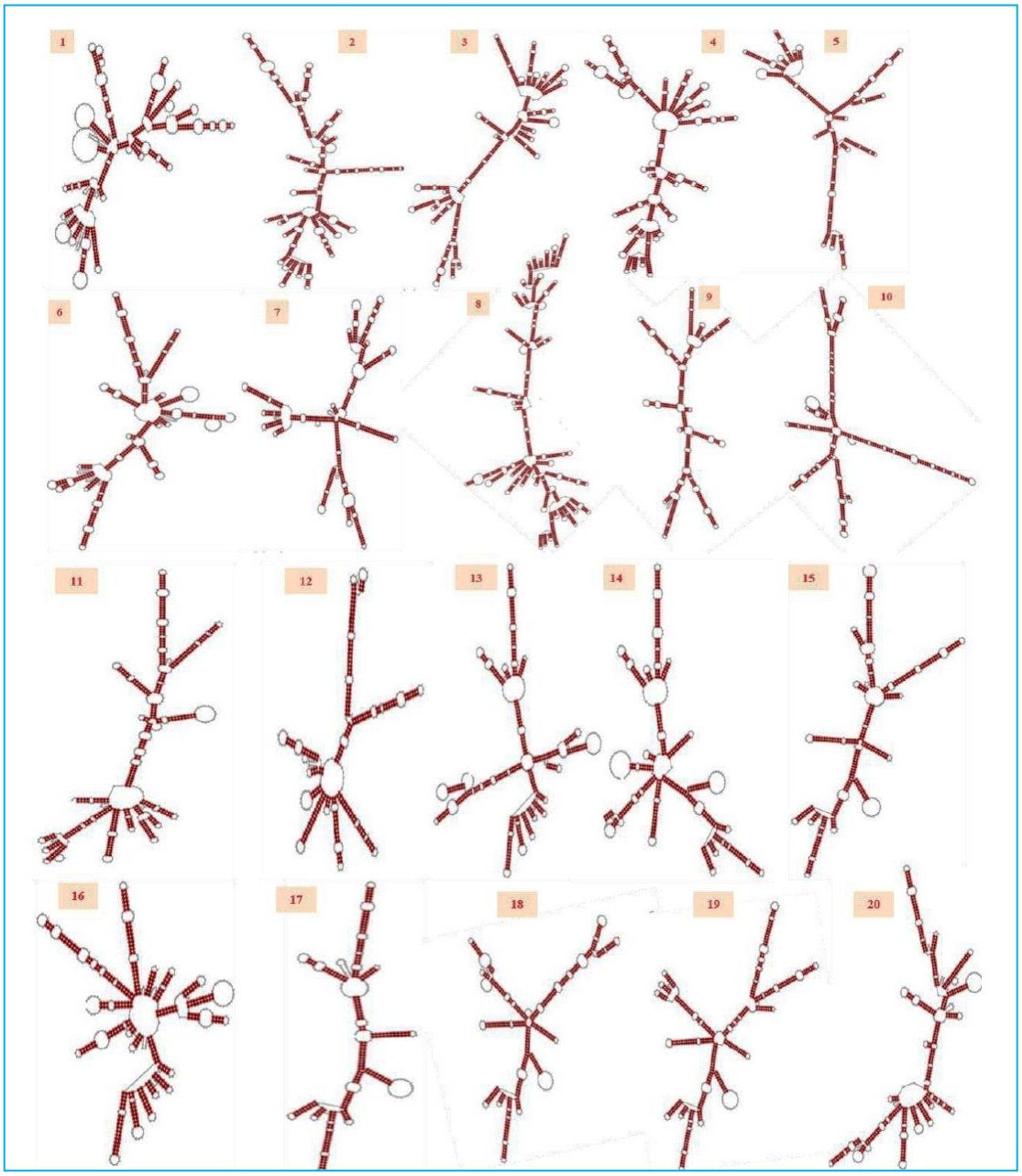

Fig 2 Secondary structure analysis of 16S rRNA

1.*Oscillatoria Formosa* BDU91041; ***2.*** *Oscillatoria boryana* BDU92181; ***3.*** *Oscillatoria willei* BDU 130461; ***4.*** *Oscillatoria laete-virens* BDU100891; ***5.*** *Oscillatoria boryana* NTAS01; ***6.*** *Oscillatoria sp. BDU 141541;* ***7.*** *Oscillatoria* sp. BDU 142191; ***8.*** *Oscillatoria subbrevis* SGBRA04; ***9.*** *Oscillatoria boryana* NTDM11; ***10.*** *Oscillatoria boryana* NTDV18; ***11.*** *Oscillatoria boryana* NTDP06; ***12.*** *Oscillatoria acuminata NTAPC05;* ***13.*** *Nostoc commune AUS-JR/DB/NT-004;* ***14.*** *Nostoc commune AUS-JR/DB/NT-003;* ***15.*** *Nostoc spongiaeforme AUS-JR/DB/NT-007;* ***16.*** *Nostoc verrucosum AUS-JR/DB/NT-006;* ***17.*** *Nostoc hatei AUS-JR/DB/NT-008;* ***18.*** *Nostoc muscorum AUS-JR/DB/NT-009;* ***19.*** *Nostoc calcicola AUS-JR/DB/NT-014;* ***20.*** *Nostoc paludosum AUS-JR/DB/NT-015*

By analyzing secondary structures, differentiation of the cyanobacterial 16S CYA rRNA gene sequences was streamlined. The structural features that are evident in the predicted structures may be important traits of cyanobacterial clades and aid in explaining the differences in 16S rRNA gene sequences between them. The colours of the structure represent base-pair probability. For every examined strain, there were differences in the structural type, minimum free energy (MFE), and thermodynamic ensemble free energy. By utilizing the sequencing information, this structure predictions approach proven to be an effective tool for comparing and differentiating *Oscillatoria* strains and *Nostoc* at the species and strain level. Additionally, it was shown that the studied *Oscillatoria* and *Nostoc* strains differed in terms of the total number of stem and loop formation in terms of 16S rRNA gene. This characteristic may be utilized as well to display differences between isolated members of similar species in different habitats.

Numerous studies demonstrated the inadequacy of classifying taxa solely on the basis of morphological traits; 16S rRNA gene sequencing was used to enhance culture identification [10]. As a contemporary method of cyanobacterial taxonomy, a polyphasic approach was used for the distinction of morphologically confusing isolates. Using both molecular 16S rRNA sequence-based and traditional morphology-based techniques, 25 marine cyanobacteria were identified in Sheils *et al.* [23]. Due to differences in sequences of genes between two similar species from a single genus that were isolated from different locations and resulting in two different strains of the same species, genetic diversity is growing of greater significance [17], [21]. In order to analyze the genetic variability of the Oscillatoriacales and Nostocales, which was gathered from several environments, 16S rRNA ribosomal conserved sequences were used.

CONCLUSION

16S rRNA CYA gene sequencing was employed to improve Cyanobacterial identification since a number of studies have shown the drawbacks of categorizing species purely on the basis of physical traits. The genetic variation of the Oscillatoriacales and Nostocales retrieved from NRMC-F and NFMC was investigated using the CYA gene Marker. The strain level differences were analyzed with the help of *in-silico* techniques like Phylogenetic tree construction, secondary structure prediction and restriction digestion analysis.

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LITERATURE CITED

1. Abed RM, Dobretsov S, Sudesh K. 2009. Applications of cyanobacteria in biotechnology. *Journal of Applied Microbiology* 106(1): 1-12.
2. Akiko T, Andrew HK, Colleen MC, Terufumi O. 2006. The evolutionary diversification of cyanobacteria: Molecular–phylogenetic and paleontological perspectives. *PNAS* 103(14): 5442-5447.
3. Alvarenga DO, Fiore MF, Varani AM. 2017. A metagenomics approach to cyanobacterial genomics. *Frontiers in Microbiology* 8: 809.
4. Anand N, Thajuddin N, Dadheech PK. 2019. *Cyanobacterial Taxonomy: Morphometry to Molecular Studies*. Cyanobacteria. From Basic Science to Applications. pp 43-64.
5. De Rijk P, De Wachter R. 1997. RnaViz, a program for the visualization of RNA secondary structure. *Nucleic Acids Research* 25: 4679-4684.
6. Garcia-Pichel F. 2009. *Cyanobacteria*. In Encyclopedia of Microbiology. pp 107-124.
7. Graur D, Li WH. 2000. *Fundamentals of Molecular Evolution*. Sinauer Associates, Sunderland, Massachusetts. pp 443.
8. Gupta T, Singh J, Kaur S, Sandhu S, Singh, Kaur IP. 2020. Enhancing bioavailability and stability of curcumin using solid lipid nanoparticles (CLEN): A covenant for its effectiveness. *Frontiers in Bioengineering and Biotechnology* 8: 879.
9. Heidari F, Riahi H, Yousefzadi M, Shariatmadari Z. 2013. Morphological and phylogenetic diversity of cyanobacteria in four hot springs of Iran. *Iranian Journal of Botany* 19: 162-172.
10. Heidari F, Riahi H, Yousefzadi M, Shariatmadari Z. 2013. Morphological and phylogenetic diversity of cyanobacteria in four hot springs of Iran. *Iranian Journal of Botany* 19: 162-172.
11. Ishida T, Yokota A, Sugiyama J. 1997. Phylogenetic relationships of filamentous cyanobacterial taxa inferred from 16S rDNA sequence divergence. *Journal of General and Applied Microbiology* 43: 237-241.
12. Iteman I, Rippka R, Tandeau DN, Herdman M. 2002. rDNA analyses of planktonic heterocystous cyanobacteria, including members of the genera *Anabaenopsis* and *Cyanospira*. *Microbiology* 148: 481-496.
13. Kumar S, Tamura K, Nei M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* 5: 150-163.
14. Kunnumakkara AB, Newman RA, Aggarwal BB. 2007. Bioavailability of curcumin: Problems and promises. *Molecular Pharmaceutics* 4: 807-818.
15. Lehtimaki J, Lyra C, Suomalainen S, Sundman P, Rouhiainen L, Paulin L, Salkinoja Salonen M, Sivonen K. 2000. Characterization of *Nodularia* strains, cyanobacteria from brackish waters, by genotypic and phenotypic methods. *International Journal of Systematic and Evolutionary Microbiology* 50: 1043-1053.
16. Ludwig, W. and Klenk, H. P. (2005). Overview: A phylogenetic backbone and taxonomic framework for prokaryotic systematics. *Bergey's manual of systematic bacteriology*. 49–66.
17. Muralitharan G, Thajuddin N. 2011. Rapid differentiation of phenotypically and geno-typically similar *Synechococcus elongates* strains by PCR finger printing. *Biologia* 66(2): 238-243.
18. Muralitharan G, Thajuddin N. 2013. Genetic heterogeneity of marine Oscillatoriales revealed by 16S rRNA gene sequencing. *Phykos* 43(1): 8-21.
19. Rajaniemi WP, Rantala A, Mugnai MA, Turicchia S, Ventura SKJ, Lepisto L, Sivonen K. 2005. Correspondence between phylogeny and morphology of *Snowella* spp. and *Woronichinia naegelian*a, cyanobacteria commonly occurring in lakes. *Journal of Phycology* 42: 226-232.
20. Saitou M, Nei M. 1987. The neighbour joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406-425.
21. Shakena Fathima T, Fayaazuddin T, Dhanveer AB, Prakash P, Akilan E, Asraf Sithikka MR, Muralitharan G, Thajuddin N. 2023. Suitability of phycocyanin gene sequences for the identification of cyanobacterial taxa belonging to Oscillatoriales. *Biological Forum – An International Journal* 15(6): 491-498.
22. Shakena T, Adams SJ, Majeed A. 2022. Study on the bioconversion of curcumin to calebin-A using *Spirulina subsalsa* and its taxonomic resolution using 16S rRNA analysis. *Applied Biochemistry Biotechnology* 195: 2933-2946.
23. Shiels K, Browne N, Donovan F, Murray P, Saha SK. 2019. Molecular characterization of twenty-five marine cyanobacteria isolated from coastal regions of Ireland. *Biology* 8(3): 59.
24. Smith AA, Coomes MW, Smith TE. 2008. Isolation and sequence of the phosphoenolpyruvate carboxylase gene of the marine cyanobacterium *Synechococcus* PCC 7002. *Journal of Biological Sciences* 8: 1261-1270.
25. Whitton BA, Potts M. 2012. Introduction to the cyanobacteria. *Ecology of Cyanobacteria II: Their Diversity in Space and Time*. Published Springer Dordrecht. pp 1-13.