Volume 61(1):59-68, 2017
Acta Biologica Szegediensis
http://www2.sci.u-szeged.hu/ABS

ARTICLE

Efficiency of partial 16S rRNA gene sequencing as molecular marker for phylogenetic study of cyanobacteria, with emphasis on some complex taxa

Zeinab Shariatmadari[1]*, Farideh Moharrek[2], Hossein Riahi[1], Fatemeh Heidari[1], Elaheh Aslani[1]

[1]Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, G.C. Tehran, Iran
[2]Department of Plant Biology, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

ABSTRACT At present, the analysis of 16S rRNA gene sequences is the most commonly used molecular marker for phylogenetic studies of cyanobacteria. However, in many studies partial sequences is used. To evaluate the performance of this molecular marker, phylogenetic relationship of several taxa from this phylum, especially some intermixed taxa, was studied. We analyzed a data set consisting of three categories of cyanobacterial strains, traditionally classified in three orders, by morphological and phylogenetic analyses. The phylogenetic analyses were performed with an emphasis on partial 16S rRNA gene sequences (600 bp) and the phylogenetic relationships were assessed using Maximum Parsimony, Maximum Likelihood and Bayesian Inference. In morphometric study, numerical taxonomy was performed on several morphospecies, and cluster analysis was performed using SPSS software. Based on the findings of this study, unlike the morphological analysis which was useful in several taxonomic ranks, this molecular marker is recommended for use only in high taxonomic levels such as order and family, because, contrary to our expectations, using partial 16S rRNA gene sequencing in the lower taxonomic levels, even in the genus level, was not necessarily successful. Inefficiency of this molecular marker in taxonomy of some genera, especially intermixed taxa, was another finding of the present study, which represents the genetic similarity of these taxa. **Acta Biol Szeged 61(1):59-68 (2017)**

KEY WORDS

cyanobacteria
intermixed taxa
molecular phylogeny
taxonomy
16S rRNA gene

Introduction

Most widespread reports about the use of 16S rRNA gene sequencing in taxonomy of cyanobacteria (cyanoprokaryotes) indicate the importance of this molecular marker as a new mean to classifying this group. Although numerous studies have shown the efficiency of the sequence analysis of genes encoding small-subunit ribosomal RNA (16S rRNA) in taxonomy of cyanobacteria (Nübel et al. 1997; Komàrek 2005), not much attention has been paid to the performance of partial 16S rRNA gene sequencing for phylogenetic studies. Furthermore, not much attention has been paid to the performance of this marker in separating complex taxa. Complex cyanobacteria are defined as microorganisms that are not well-defined yet, and further investigations and research for new characters are needed which would clearly define these taxa (Palinska et al. 2011).

Submitted November 13, 2016; Accepted ?March 26, 2017
*Corresponding author. E-mail:z_shariat@sbu.ac.ir

Several reasons can be cited for this complexity. Morphological flexibility of these microorganisms is certainly one of these reasons. Many studies indicate the instability of morphological, biochemical and physiological characteristics of cyanobacteria in several habitats (Moisander et al. 2002; Bittencourt-Oliveira et al. 2012; Soares et al. 2013; Iranshahi et al. 2014). These diverse environmental responses create complexity in this group of prokaryotic microorganisms. The shape of colony, the presence or absence of gelatinous envelope, the width of envelope, and even traits such as the shape and size of cells are characters which are quite influenced by the environmental factors (Yamamoto and Nakahara 2009). However, many of these characters are the bases for classification and separation of several taxa. For example, the genus *Anabaena* is one of the nostocacean cyanobacteria with a special taxonomical history. This genus, which belongs to a group with diverse characteristics, can show phenotypic diversity in several habitats. The genera *Wollea* and *Trichormus* are other taxa placed in Nostocaceae family and are very similar to *Anabaena* species. It should be noted that some taxa of the latter genera were recently separated from Genus *Anabaena*. Therefore, despite all the differences,

similarities between the mentioned genera are striking. It is expected that using molecular markers such as 16S rRNA gene sequencing can be useful in the classification of these similar genera. However, can this molecular marker define the precise boundary of these taxa?

In response to this question it should be noted that, although numerous studies have introduced the efficiency of the 16S rRNA in all taxonomic levels above species, not much attention has been paid to the efficiency of this molecular marker in separating taxa with high complexity. The aim of this study was to investigate the efficiency of 16S rRNA gene sequences (partial 16S rRNA gene sequences) on separation and classification of cyanobacteria as well as its accuracy in several taxonomic ranks. In addition, in the present study this molecular marker was used for separating taxa with high complexity such as *Anabaena* and other intermixed taxa.

Materials and Methods

Isolation and purification of cyanobacterial strains

For isolating cyanobacterial strains, soil and water samples were collected from several terrestrial and aquatic ecosystems of Iran (Table 1). The samples were collected over three consecutive years (from 2008 until 2010) and in accordance with the methodologies of Rangaswamy (1966) as well as Hötzel and Croome (1999).

The isolation and purification of cyanobacterial strains were performed according to Stanier et al. (1971). Purified cultures of taxa were grown in BG11 medium (with and without nitrate). Incubation of cultures was performed in a culture chamber at 25 ± 2 °C for two weeks under artificial light illumination (74 µmol photons m^{-2} s^{-1}) with a 12/12 h light-dark cycle.

Morphological observation and morphometric studies

It is necessary to mention that morphometric study was performed only based on taxa isolated from several ecosystems of Iran (aquatic and terrestrial). Morphological observations were made on liquid as well as solid media. For taxonomic determinations, semi-permanent slides of colonies were prepared and the morphometric study was performed by light microscopy (Model BH-2, Olympus) according to previous studies (Desikachary 1959; Prescott 1970; Wehr et al. 2002; John et al. 2002; Komárek 2005; Komárek and Zapomělová 2008).

Characters were selected based on those reported by Nayak and Prasanna (2007) and our own field observations. The main morphological characteristics which separate several genera are listed in Table 2. Morphometric studies were done with emphasis on the population of several taxa from three orders of cyanobacteria. Ten filaments from each population were used for this purpose. In total, 19 quantitative and qualitative morphological characters were studied (Table 2).

Statistical analysis

In order to determine the taxa interrelationships, cluster analysis and principal component analysis (PCA) were performed. For multivariate analyses the mean of quantitative characters were used, while qualitative characters were coded as binary/multistate characters. Standardized variables (mean = 0, variance = 1) were used for multivariate statistical analyses. The Euclidean distance was used as dissimilarity coefficient in cluster analysis of morphological data (Podani 2000). In this study, SPSS software was used for statistical analysis.

DNA isolation, PCR amplification and sequencing

Genomic DNA was extracted from the fresh mass of 29 strains of cyanobacteria by Genomic DNA Extraction Kit (AccuPrep®, Bioneer). PCR amplification was performed as described in the literature (Ezhilarasi and Anand 2009). The 16S rRNA gene was amplified using primers A2 (AGAGTTTGATCCTGGCTCAG) and S8 (TCTACGCATTTCACCGCTAC). The PCR mixture contained 10 µl Taq commercial buffer, 10 µl purified DNA, 150 µM of each dNTP, 500 ng of each primer and 2.5 U Taq polymerase. The PCR reactions were carried out with a denaturation step of 4 min at 95 °C, followed by 35 cycles of 1 min denaturation at 95 °C, 1 min annealing at 59 °C, and 2 min extension at 72 °C, followed by a final extension step of 8 min at 72 °C. The PCR products were migrated on 1% (w/v) agarose gel and were visualized by ethidium bromide. Selected PCR products of 16S rRNA were sequenced by Avicenna Research Institute (Tehran, Iran).

Sequence alignment

Sequences were edited using BioEdit ver. 7.0.9.0 (Hall 1999) and aligned with MUSCLE under default parameters (Edgar 2004), followed by manual adjustment. Positions of indels were treated as missing data for all datasets. Pairwise genetic distances between sequences were calculated using the maximum composite likelihood model with pairwise deletions and gamma-distributed among-site rate variation, as implemented in MEGA version 1.5 (Tamura et al. 2011).

Table 1. Voucher information and GenBank accession number for 56 species and related taxa.

Taxon designation	Strain code	Origin	GenBank Accession Number
Wolleava ginicola	ISB26	Iran, Lorestan, Visan / paddy field soil	KM017086
Wollea vaginicola	ISB22	Iran, Lorestan, Visan / paddy field soil	KM017090
Wollea vaginicola	ISB24	Iran, Fars, Kamfiroz / paddy field soil	KM017088
Wollea vaginicola	ISB21	Iran, Esfahan, Jojil / paddy field soil	KM017091
Wollea saccata	-	Russia, Yenissei River / river basin	GU434226
Wollea ambigua	ISB17	Iran, Esfahan, Jojil / paddy field soil	KM035410
Anabaena iyengarii	-	India / paddy field soil	GQ466548
Anabaena torulosa	ISB20	Iran, KhorasanRazavi / paddy field soil	KM017092
Anabaena torulosa	ISB19	Iran, Mazandaran, Savadkoh / paddy field soil	KM017093
Anabaena sphaerica	ISB23	Iran, Esfahan, Falavarjan / paddy field soil	KM017089
Anabaena sphaerica	-	India / -	EF375612
Anabaena sphaerica f. *conoidea*	-	Italy, Umbria / -	FM177480
Anabaena sphaerica	-	- / -	DQ439647
Anabaena cylindrica	-	India / -	EF375611
Anabaena verrucosa	-	India / -	EF375614
Anabaena oscillarioides	-	India / paddy field soil	GQ466544
Trichormus variabilis	ISB27	Iran, Gilan, Rahimabad / paddy field soil	KM017085
Anabaena variabilis	-	- / -	EF488831
Anabaena aphanizomenoides	-	- / -	FJ830569
Wollea ambigua	-	India / paddy field soil	KP792338
Anabaena sp.	ISB54	Iran, Khorasan Razavi / paddy field soil	KT254261
Anabaena sp.	ISB 55	Iran, Khorasan Razavi / paddy field soil	KT254262
Trichormus variabilis	-	- / -	DQ234832
Trichormus variabilis	-	- / -	DQ234833
Trichormus variabilis	-	- / -	DQ234829
Trichormus azollae	-	- / -	AJ630454
Nostoc spongiaeforme	ISB50	Iran, Fars, Firozabad / paddy field soil	KT254257
Nostoc sp.	ISB49	Iran, Fars, Ebrahimabad / paddy field soil	KT254256
Nostoc muscorum	-	Brazil / -	AY218828
Cylindrospermum minutissimum	ISB48	Iran, Esfahan, Zarrinshahr / paddy field soil	KT254255
Cylindrospermum muscicola	ISB46	Iran, KhorasanRazavi / paddy field soil	KT254251
Cylindrospermum michailovscoense	ISB47	Iran, Mazandaran, Tazehabad / paddy field soil	KT254253
Cylindrospermum sp.	ISB57	Iran, Fars, Esmaeelabad / paddy field soil	KT254254
Cylindrospermum sp.	ISB53	Iran, KhorasanRazavi / paddy field soil	KT254260
Cylindrospermum sp.	ISB56	Iran, KhorasanRazavi / paddy field soil	KT254266
Cylindrospermum alatosporum	-	France / Soil	GQ287650
Cylindrospermum stagnale	-	- / -	AF132789
Cylindrospermum catenatum	-	Slovakia / -	KF052615
Calothrix sp.	ISB52	Iran, KhorasanRazavi / paddy field soil	KT254259
Calothrix elenkinii	-	India / -	GU292083
Calothrix sp.	-	- / -	HF678491.1
Tolypothrix sp.	-	Spain / running water	HM751850.1
Tolypothrix sp.	-	Spain / running water	AM230668.1
Oscillatoria minima	ISB29	Iran, Ramsar / hot spring water	KJ534024
Oscillatoria subbrevis	ISB30	Iran, Khamir / hot spring water	KJ534025
Oscillatoria subbrevis	ISB37	Iran, Geno / hot spring water	KJ546666
Oscillatoria angusta	ISB40	Iran, Chah Ahmad / hot spring water	KJ543481
Oscillatoria angusta	ISB38	Iran, Ramsar / hot spring water	KJ546665
Oscillatoria angusta	ISB35	Iran, Khamir / hot spring water	KJ546668
Oscillatoria sp.	-	- / -	EF150796.1
Synechocystis aquatilis	ISB33	Iran, Chah Ahmad / hot spring water	KJ546670
Synechocystis aquatilis	ISB32	Iran, Geno / hot spring water	KJ546671
Synechocystis sp.	-	- / -	HQ900668.1
Synechocystis sp.	-	- / -	AB039001.1
Synechococcus elongates	ISB34	Iran, Khamir / Hot spring water	KJ546669
Synechococcus elongatus	-	Iran, Ramsar / Hot spring water	JQ771323.1
Synechococcus sp.	-	- / -	AF448077
Bacillus subtilis	-	- / -	HQ232422
Bacillus amyloliquefaciens	-	- / -	HM016080

ISB: Shahid Beheshti University Algal Collection, Tehran, Iran

Table 2. Morphological characters and their character states in studied taxa.

Characters	Character state
Vegetative cell shape	0) Discoid; 1) Sub-quadrate; 2) Barrel shape; 3) Oblong; 4) Cylindrical
Apical cell shape	0) Rounded; 1) Conical with rounded apex
Heterocyst	0) Present; 1) Absent
Heterocyst shape	0) Sub-spherical; 1) Spherical; 2) Oblong with rounded apex; 3) Cylindrical; 4) Barrel shape
Heterocyst position	0) Only intercalary; 1) Only terminal; 2) Terminal & Intercalary
Akinet	0) Present; 1) Absent
Akinet position	0) At heterocyst; 1) Distant from heterocyst
Akinet shape	0) Oblong; 1) Long cylindrical with rounded ends; 2) Ellipsoidal; 3) Widely oval; 4) Sub-spherical
Akinet number	0) Single or two; 1) Several
Gelatinous sheath	0) Present; 1) Absent
Number of trichome in sheath	0) Single; 1) Several
Trichome colour	0) Blue-green; 1) Dark blue-green; 2) Yellowish brown
Colonial form	0) Mucilaginous; 1) Not mucilaginous
Colonial mass shape	0) Spreading; 1) Scattering; 2) Globose
Filaments structure	0) Entangled; 1) Not entangled
Thallus form	0) Filamentous; 1) Colony
Symmetry of filament	0) Symmetric; 1) Asymmetric
Trichome structure	0) Apoheterocytic; 1) Paraheterocytic
Division form	0) Binary division; 1) Hormogonium; 2) Akinet

Phylogenetic analyses

Fifty-seven taxa of cyanobacteria were used in phylogenetic analyses. It is necessary to mention that some sequences were obtained from GenBank (Table 1). Phylogenetic relationships were assessed using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI). MP was conducted using PAUP* version 4.0b 10 (Swofford 2002). The heuristic search option was employed for the dataset, using tree bisection-reconnection (TBR) branch swapping, with 100 replications of random addition sequence and an automatic increase in the maximum number of trees. Branch supports were assessed by 1000 bootstrap replicates (yielding bootstrap percentages, BP; Felsenstein 1985) with the same settings as for the heuristic searches.

The substitution model was obtained using the program MrModeltest version 2.3 (Nylander 2004) based on the Akaike information criterion (AIC) (Posada and Buckley 2004). GTR + G + I (six substitution types with rate variation across sites were modelled using a gamma distribution, with a proportion of invariant sites) was identified as the best model for the dataset.

ML analysis was performed for the dataset in raxmlGUI ver. 1.3. (Silvestro and Michalak 2012). The model of evolution employed for the dataset was the same as that of BI. Bootstrap values for maximum likelihood (ML BS) was calculated in raxmlGUI based on 1000 replicates in a single run.

The program MrBayes version 3.2 (Ronquist and Huelsenbeck 2003) was used for the Bayesian reconstruction. Two simultaneous analyses with eight Metropolis-coupled Markov chain Monte Carlo (MCMCMC) chains with incremental heating of 0.2 were run for 10 million generations and sampled every 100 generations. TRACER v.1.5 was used to evaluate mixing of chains and to determine burn-in. The first 25% of trees were discarded as the burn-in. The remaining trees were then used to build a 50% majority rule consensus tree, accompanied with posterior probability (PP) values. Tree visualisation was carried out using TreeView version 1.6.6 (Page 2001).

Results

Morphological study

In morphometric study, the morphological diversity of cyanobacteria was investigated among several species and genera from several families. A list of cyanobacteria, identified and used in this study, is given in Table 1. Six morphotypes corresponding to the genera *Anabaena*, *Trichormus*, *Wollea*, *Nostoc*, *Cylindrospermum* and *Calothrix* from Nostocales, as well as several taxa from Oscillatoriales (filamentous cyanobacteria without heterocytes and akinetes) and Synechococcales (coccoid and colonial cyanobacteria with binary fission of cells) were presented among the studied strains. The most important characteristics of studied genera is summarized in Table 2. This study was conducted with an emphasis on similar genera from nostocacean cyanobacteria such as *Anabaena*, *Trichormus* and *Wollea.*

In the cluster analysis based on all morphological char-

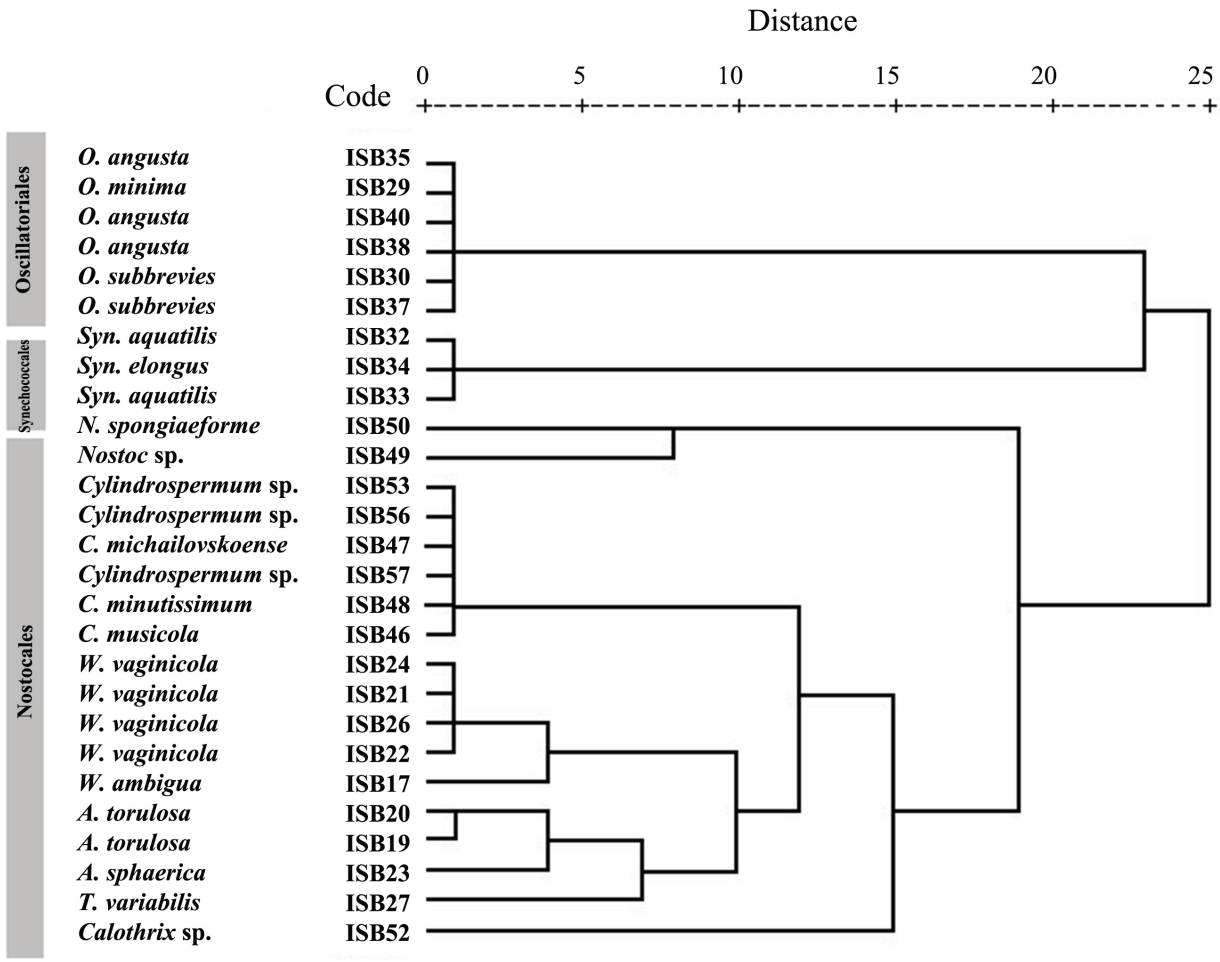

Figure 1. Hierarchical cluster analysis dendrogram of cyanobacterial taxa based on morphological characters using UPGMA method.

acters, two major clusters were found. The first major cluster separated nostocacean taxa from Oscillatoriales and Synechococcales. In other words, primary clustering clearly separated heterocystous taxa from the others (Fig. 1). Two sub-clusters or two groups can be seen in the cluster which belongs to Nostocales. In the first group, apoheterocytic cyanobacteria except *Trichormus ellipsosporus*, and in the second one paraheterocytic taxa are presented. Among taxa presented in this cluster, *Anabaena* species such as *A. variabilis* var. *ellipsospora* and *A. ambigua* are currently considered as synonyms of *Trichormus ellipsosporus* and *Wollea ambigua*. The results of morphometric analysis indicate a high morphological similarity between *Anabaena* species and these taxa (Fig. 1). Therefore, these genera were placed in the same sub-cluster with *Anabaena* species.

In order to determine the most variable morphological characters which separated studied taxa, PCA analysis was performed. The analysis revealed that the first four factors comprise about 95% of total variance. In the first factor with about 48% of total variance, characters such as apoheterocytic or paraheterocytic form of filaments, heterocyst and akinet shape, akinet number in filament, position of akinet with regard to heterocyst and the form of thallus possessed the highest positive correlation. In the second factor with about 22% of total variance, characters like vegetative cells shape, filament structure (entangled or not) and symmetry of filament possessed the highest positive correlation. Therefore, these are the most variable morphological characters among the studied taxa, especially nostocacean cyanobacteria.

Phylogenetic study

Sequence analyses

Sequences characteristics, tree statistics and model choice of data set are summarized in Table 3.

Phylogenetic analyses

Maximum Parsimony, Likelihood analyses, and Bayesian inference gave very similar results. However, support and resolution were improved using the latter approach. Hence, we here show the BI tree along with PP, ML BS and BP (Fig. 2). In all gained trees, Nostocales and Synechococcales were each recovered as monophyletic (PP = 0.88, ML BS = 78, BP = 77 and PP = 0.90, ML BS = 82, BP = 89, respectively), and sister relationship among them was supported (PP = 0.84, ML BS = 80, BP = 86). Species of *Oscillatoria* were recovered as the paraphyletic taxa in the base of the trees.

The inferred phylogenies indicated that the resolution within the Nostocales clade was rather poor, but some small groups with low to high supports were found across this clade. This lack of resolution is a reflection of the low sequence divergence values across the Nostocales clade, less than 0.1 (0.02-0.059) substitutions per site for pairs of taxa. Many species, represented by multiple accessions, were poorly resolved. The genus *Anabaena* was found to be paraphyletic by the nested inclusion of *Wollea* and *Trichormus*. *Anabaena oscillarioides* and *A. iyengarii* made a separate subclade which is close to the *Cylindrospermum* species.

Most specimens of the genus *Cylindrospermum* were grouped in a separate subclade which also included *Trichormus azollae*. But, two taxa, *C. michailovskoense* and *C. catenatum,* were separated from others. Species of the genus *Nostoc* were found to be monophyletic with a high nodal support (PP = 0.98, ML BS = 90, BP = 92). *Tolypothrix* and *Calothrix* grouped together with a low nodal support (PP = 0.78, ML BS = 72, BP = 73), but adjusent to other para-heterocytic taxa.

Discussion

The purpose of the study was to investigate the efficiency of partial 16S rRNA gene sequencing, as a common molecular marker, in several taxonomic ranks. In phylogenetic study, a data set, consisting of cyanobacterial strains from three orders, Nostocales, Oscillatoriales and Synechococcales, were analyzed. In all analyses (BI, ML and MP), Nostocales taxa formed a monophyletic group. According to Komàrek et al. (2014), Nostocales represents a large and monophyletic cluster of filamentous cyanobacteria with special cells such as heterocysts and akinetes. Other studies also emphasize on monophyly of this order (Wanigatunge et al. 2014; Valério et al. 2009; Ishida et al. 2001). This order contains several families with a range of diversity from isopolar to heteropolar structures. From the isopolar families, *Nostocaceae* family and from heteropolar of them *Rivulariaceae* and *Tolypothrichaceae* can be noted.

Table 3. DNA sequence characteristics and phylogenetic statistics of data partition.

Number of sequences	59
Number of characters	600
GC contents (%)	53.1
Number of variable characters	257
Number of PI characters	212
ASD, all sequences (%)	0.118
ASD, between *Anabaena* spp. and *Wollea* spp. (%)	0.059
ASD, between *Anabaena* spp. and *Cylindrospermum* spp. (%)	0.065
ASD, between *Anabaena* spp. and *Trichormus* spp. (%)	0.061
ASD, between *Anabaena* spp. and *Tolypothrix* spp. (%)	0.062
ASD, between *Anabaena* spp. and *Calothrix* spp. (%)	0.062
ASD, between *Anabaena* spp. and *Nostoc* spp. (%)	0.063
ASD, between *Tolypothrix* spp. and *Calothrix* spp. (%)	0.028
ASD, between the Nostocales species	0.064
ASD, between *Synechocystis* spp. and *Synechococcus* spp. (%)	0.061
Number of MPTs	806
Length of MPTs	382
C.I. of MPT	0.474
R.I. of MPT	0.784
Evolutionary model selected (under AIC)	GTR+I+G

*ASD: Average sequence divergence

The Nostocaceae is an important family which consists of unbranched heterocystous cyanobacteria with isopolar or heteropolar filaments (Komàrek et al. 2014). In our study, most of the genera from this family were separated relatively by partial 16S rRNA gene sequencing, but it seemed ineffective in some cases. For example, taxa such as *Wollea vaginicola* (= *Anabaena vaginicola*) showed a close relationship with *Anabaena torulosa*; also, *Anabaena sphaerica* and *Wollea saccata* were placed in one group. *Trichormus variabilis* (= *Anabaena varibilis*) is another taxon of this family which was placed adjacent to *Anabaena* species such as *A. verrucosa* and *A. cylindrica* (PP = 0.97, ML BS = 90, BP = 90).

Recent studies indicate that several nostocacean cyanobacteria such as genera *Anabaena, Trichormus* and *Wollea* are polyphyletic and can be noted as intermixed taxa (Rajaniemi et al. 2005; Kozhevnikov and Kozhevnikova 2011). Inefficiency of this molecular marker in taxonomy of these taxa may be due to simillarity of genes encoding small-subunit ribosomal RNA in these genera. Previous studies also confirm the presence of this similarity. For example, the results of the present study are in agreement with those of Kozlíková-Zapomělová et al. (2016). According to this study, separation of these genera (*Anabaena*, *Wollea* and *Trichormus*) is not well suported by the partial 16S rRNA gene analysis. Kozhevnikov and Kozhevnikova study (2011) also confirm the phylogenetic similarity of these genera, especially genera *Wollea* and *Anabaena*.

By traditional classification, the genus *Wollea* were placed

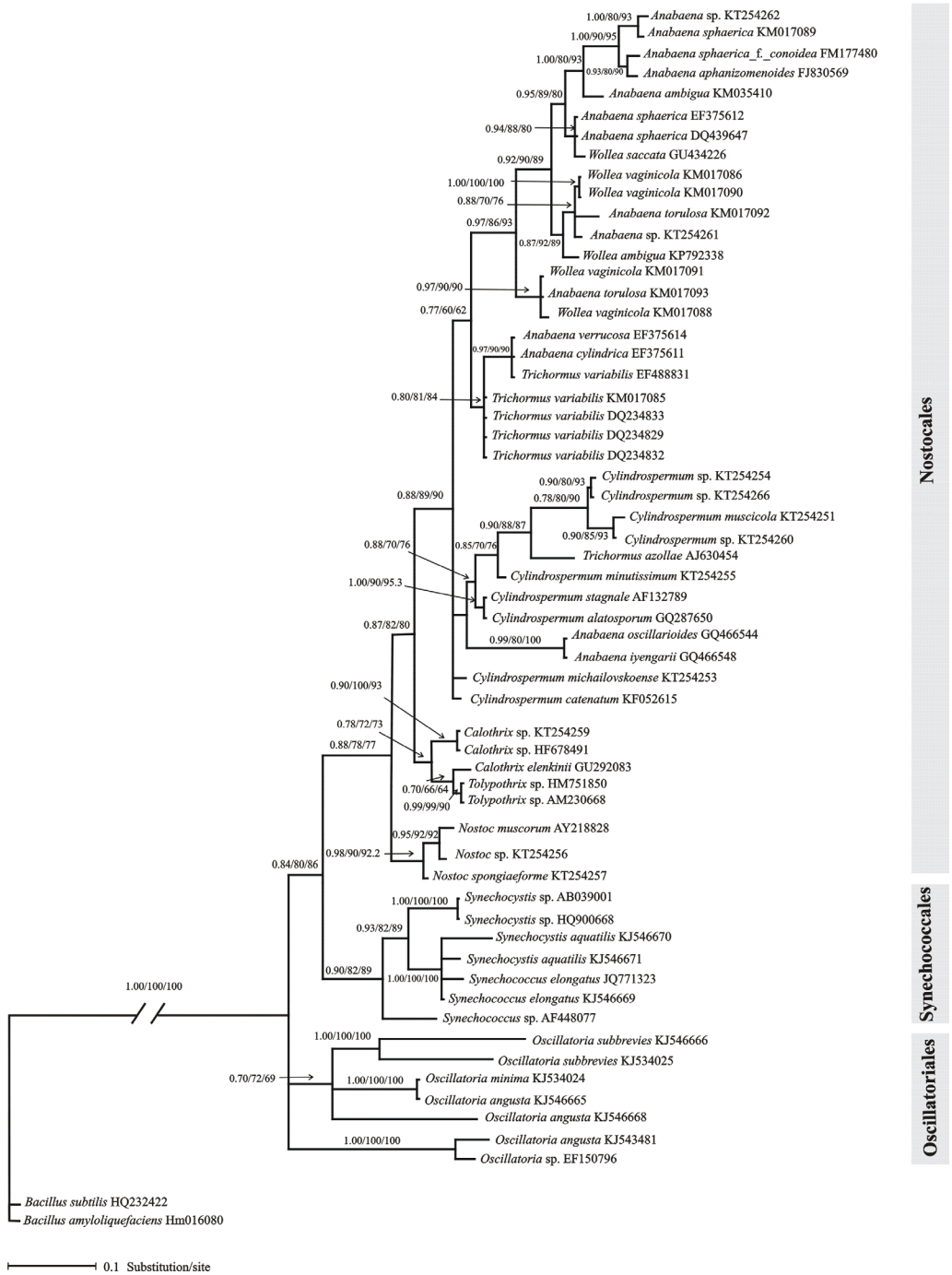

Figure 2. Fifty percent majority rule consensus tree resulting from Bayesian analysis of the 16S rRNA dataset. Numbers above branches are posterior probability and likelihood as well as parsimony bootstrap values, respectively. Values <50% were not shown.

in Nostocaceae family (Nostocales) and some of its taxa were recently separated from genus *Anabaena*. The similarity of these two genera is much more than we expected. For example, the *Wollea ambigua* is very similar to *Anabaena* species such as *Anabaena sphaerica*; also *Wollea vaginicola* is very similar to *Anabaena torulosa*. This similarity was clearly shown in our phylogenetic analyses (Fig. 2).

It is necessary to mention that *Wollea* specimens were separated from *Anabaena* species with characters such as macroscopic gelatinous colonies (which sometimes appear

tube-like), trichomes which are irregularly (or more or less) parallel and densely arranged in common and diffluent mucilage, and the absence of sheaths around trichomes (Kozhevnikov and Kozhevnikova 2011). But, according to several authors, these characters are not sufficient for the taxonomical separation of these two genera (Komàrek 1975; Shariatmadari et al. 2014).

Trichormus variabilis is another nostocacean cyanobacteria which was recently separated from the traditional genus *Anabaena* based on akinete development or apoheterocytic form of trichomes (Rajaniemi et al. 2005). The similarity of these two genera is also very much, especially before akinetes formation. Our results support this similarity, in both morphological and phylogenetic analyses (Fig. 1 and 2).

In addition to the *Nostocaceae* family, the taxa of heteropolar families such as *Rivulariaceae* and *Tolypothrichaceae* were analyzed. The *Rivulariaceae* is characterized by tapered trichomes, a part from short phases of hormogonium formation, and mostly has a terminal heterocyst in the maturity (Berrendero 2008). The *Tolypothrichaceae* is also characterized as a heteropolar cyanobacteria with non-attenuated, false branching trichomes (Komàrek et al. 2014).

The results of the present study indicate the close affinity of these two families. In all analyses (BI, ML and MP), heteropolar taxa, *Calothrix* and *Tolypothrix*, were placed in a separated clade. Heteropolarity is one of the most important properties of taxa which are placed in this clade and the taxonomic complexity is one of the dificulties seen in this group. For example, several families have been proposed for some taxa of this heteropolar group such as *Tolypothrix*. The *Rivulariaceae*, *Microchaetaceae* and *Tolypotrichaceae* are from these proposed families (Hauer et al. 2014; Sihvonen et al. 2007; Rippka et al. 1979; Desikachary 1959). Althought *Tolypothrix,* according to the Botanical code, was classified in family *Rivulariaceae*, nowadays this genus is separated from *Calothrix* and is placed in *Tolypotrichaceae* family (Hauer et al. 2014). In the present study and according to partial 16S rRNA gene sequencing, these two genera (*Calothrix* and *Tolypothrix*) showed a close relationship and placed as the sister taxa**.** Thus, although this molecular marker could separate heteropolar taxa such as *Calothrix* and *Tolypothrix* from the others, it does not seem appropriate for separation of *Rivulariaceae* and *Tolypothrichaceae* taxa.

The results of the present study also showed that Synechococcales, similar to Nostocales, was monophyletic and several taxa of this order were placed in one group with high support (PP = 0.90, ML = 82, MP = 89) (Fig. 2). In other words, the Synechococcales taxa such as genera *Synechocystis* and *Synechococcus* are well separated from the filamentous taxa (Nostocales and Oscillatoriales taxa) based on the information given by the partial 16S rRNA gene sequencing. It should be noted that some algologists placed this genera in Chroococcales. The Chroococcales was known as an order of the cyanobacteria with unicellular or colonial taxa, sometimes forming a pseudofilamentous colony and never differentiated into base and apex. Polyphyly of this order was reported in previous studies (Wanigatunge et al. 2014). Unlike Chroococcales, the order Synechococcales is introduced as a monophyletic family. According to Thomazeau et al. (2010), Synechococcales is also a large and monophyletic group of cyanobacteria with both unicellular (plus colonial) and filamentous structure. In addition to studies that show monophyly of this order, some studies emphasize the polyphyly of Synechococcales (Komárek et al. 2014). Even some genera of this order have been reported as a polyphyletic taxa. For example, according to Dvořák et al. (2014), the genus *Synechococcus* Nägeli from Synechococcales represents an enigmatic group of cyanobacteria with polyphyletic evolutionary origin. The studies conducted by Haverkamp et al. (2009) also represent the polyphyly of this genus according to several molecular markers. The polyphyletic origin of *Synechococcus* was also confirmed in the present study (Fig. 2). In this study it was observed that this molecular marker could not separate morphologicaly similar genera such as *Synechocystis* and *Synechococcus*. It should be noted that the genetic similarities of these genera are supported with morphological similarities.

Polyphyly of Oscillatoriales is another result that can be derived from the results of the present study. This order includes filamentous taxa with more complicated cytology (Komárek et al. 2014). In previous classifications, Oscillatoriales taxa were placed in Nostocales. But, later studies transferred these taxa to several families of Oscillatoriales. The uniseriate filaments as well as non-heteterocystous structure of them are among the important characteristics of this order.

In the present study, using partial 16S rRNA gene sequencing could not separate the boundry of Oscillatoriales taxa completely. In other words, all samples belonging to this order were not observed in a single clade of phylogenetic tree. Our results confirmed the results of the previous studies which emphasized the polyphyly of Oscillatoriales (Ishida et al. 2001; Lokmer 2007; Valério et al. 2009).

In conclusion, our results revealed the efficiency of partial 16S rRNA gene sequencing as a molecular marker, specially in high taxonomic levels such as order and family. In contrast, our results did not support the efficiency of this molecular marker in the taxonomy of lower taxonomic ranks such as genera. This inefficiency, particularly in the complex taxa such as *Anabaena*, *Wollea* and *Trichormus,* was considerable. It seems that the genetic similarity of these taxa prevents their separation by the described molecular marker. This genetic similarity, which is also supported by the morphological similarity, indicates that the taxonomic status of intermixed taxa such as *Anabaena*, *Wollea* and *Trichormus* needs to be revised further.

Acknowledgment

The authors wish to thank University of Shahid Beheshti for funding this project. Thanks are also due to Dr. Somayeh Keypour for her kind assistance during the research and help in editing and reviewing this paper.

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