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Taxonomic study of cyanoprokaryotes from medicinal plants bed with emphasis on phylogeny of complex taxa using 16S rRNA marker

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Abstract

Cyanoprokaryotes are simple photosynthetic microorganisms which have an important role in the soil carbon and nitrogen cycle. The current study aimed to investigate the flora of cyanoprokaryotes from medicinal plants bed. Also phylogenetic analysis based on 16S rRNA marker was performed to investigate the phylogenetic relationships between different cyanoprokaryotic taxa and evaluate the efficiency of this marker in separation of taxonomic boundaries between taxa especially in the case of complex taxa, which their relations are not well-defined. For this purpose, after collection of soil, isolation and purification of strains were performed. The cyanoprokaryotic taxa were identified morphologically and 16S rRNA marker was used to approve the identifications. Phylogenetic analysis performed using Maximum Likelihood, Maximum Parsimony and Bayesian Inference. Totally, 42 cyanoprokaryotic taxa were identified and *Nostoc* was an abundant genus in the soil of medicinal plants bed. The phylogenetic tree revealed *Nostocales* as a monophyletic group. Also, *Wollea* together with *Anabaena*, and *Nostoc* together with *Desmonostoc* created monophyletic groups. Results revealed that, 16S rRNA is an effective phylogenetic marker in high classification rankings such as order, family and genus. However, 16S rRNA could not be an effective marker in separation of close genera such as *Nostoc* and *Desmonostoc*.

Keywords: Cyanoprokaryotes, *Desmonostoc*, phylogeny, *Wollea*, 16S rRNA

مطالعه تاکسونومیک سیانوپروکاریوت‌های بستر رویشی گیاهان دارویی با تاکید بر فیلوژنی آرایه‌های پیچیده با استفاده از مارکر 16S rRNA*

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خلاصه

سیانوپروکاریوت‌ها موجودات ریز و ساده فتوسنتزکننده‌ای هستند که نقش مهمی در چرخه‌های نیتروژن و کربن خاک ایفا می‌نمایند. هدف مطالعه حاضر، بررسی فلور سیانوپروکاریوت‌های موجود در بستر رویشی گیاهان دارویی است. همچنین، بررسی فیلوژنتیک به منظور ارزیابی روابط فیلوژنی میان آرایه‌های مختلف سیانوپروکاریوتی با استفاده از مارکر 16S rRNA و ارزیابی کارآمدی این مارکر در جداسازی مرزهای تاکسونومیک میان آرایه‌ها، به ویژه در موارد پیچیده که روابط میان آن‌ها به خوبی شناخته شده نیست، انجام گرفته است. به این منظور، پس از جمع‌آوری خاک، جداسازی و خالص‌سازی سویه‌ها انجام گرفت. آرایه‌های سیانوپروکاریوتی براساس خصوصیات ریخت‌شناختی شناسایی شدند و تایید شناسایی‌ها با کمک مارکر مولکولی 16S rRNA انجام گرفت. روابط فیلوژنی با استفاده از Maximum Likelihood، Maximum Parsimony و Bayesian Inference مورد ارزیابی قرار گرفت. در مجموع، ۴۲ آرایه سیانوپروکاریوتی شناسایی شد و نتایج نشان‌دهنده غالب بودن جنس گونه *Nostoc* در فلور بستر رویشی گیاهان دارویی بود. درخت حاصل از نتایج فیلوژنی نشان‌دهنده تک‌نیایی بودن *Nostocales* است. همچنین، جنس‌های *Wollea* و *Anabaena* با یکدیگر و نیز جنس‌های *Nostoc* و *Desmonostoc* با هم، گروه‌های تک‌نیا را تشکیل دادند. نتایج نشان‌دهنده کارآمدی مارکر16S rRNA در سطوح بالای سیستم رده‌بندی، اعم از راسته، تیره و جنس می‌باشد. با این وجود، این مارکر در جداسازی آرایه‌های نزدیک به یکدیگر نظیر *Desmonostoc* و *Nostoc* از کارآمدی لازم برخوردار نیست.

واژه‌های کلیدی: سیانوپروکاریوت، 16S rRNA، فیلوژنی، *Wollea*، *Desmonostoc*

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Introduction

Cyanoprokaryotes (cyanophyta or cyanobacteria), special group of photosynthetic organisms, are known as major supplier of global oxygen (Beck *et al*. 2012). In soil crust these microorganisms play a major role in bonding soil particles; and they also provide part of soil carbon and nitrogen (Yeager *et al*. 2007). Many species of cyanoprokaryotes, especially those belong to orders *Nostocales* and *Stigonematales*, produce specialized cells which include: heterocyts and akinetes (Kumar *et al*. 2010, Adams & Duggan 1999). Heterocyts are specialized cells with capability of fixing the atmospheric nitrogen under nitrogen deficiency conditions (Kumar *et al*. 2010). Akinetes are the other specialized cells with having thickened cell wall are able to survive more than vegetative cells during the unfavorable conditions (Adams & Duggan 1999). The shape, size and position of these specific cells also facilitate morphological identification of these microorganisms.

Because of profound role of cyanoprokaryotes in carbon and nitrogen cycle and their symbiotic association with plants, fungi and algae, the study of flora of these microorganisms is of particular importance. During the first six decades of last century, taxonomic study of cyanoprokaryotes based on their morphological traits was widespread but over the past two decades it is alternated by molecular taxonomy (Anand *et al*. 2019). Molecular studies also is an appropriate way for clearance the relationships between complex taxa. Complex taxa are not clearly defined and more studies are need for well-definition of them (Palinska *et al*. 2011, Ahlesaadat *et al*. 2017). Phylogenetic study of cyanoprokaryotes using 16S rRNA has been performed by many of researchers (Nelissen *et al*. 1996, Honda *et al*. 1999, Robertson *et al*. 2001, Ezhilarasi & Anand 2009, Muralitharan & Thajuddin 2013). Some researchers suggested that, 16S rRNA gene sequencing could be as an important molecular marker in phylogenic study of cyanoprokaryotes (Anand *et al*. 2019). Also, over the past years it has been shown that, 16S rRNA has been beneficial marker in separation of taxa with similar morphology such as *Desmonostoc* and *Nostoc* (Hrouzek *et al*. 2013).

Despite all the studies done in the field of cyanoprokaryotes, there are few studies about the flora and phylogeny of cyanoprokaryotes in terrestrial habitats of Iran which performed on flora of cyanoprokaryotes isolated from agricultural fields of Iran (Shariatmadari *et al*. 2017, Nowruzi *et al*. 2017, Hokmollahi *et al*. 2015, Ahlesaadat *et al*. 2017). In the meantime, some researchers worked on flora of cyanoprokaryotes from the bed of medicinal plants, which are of particular importance (Hosseini 2016, Chookalaii 2015). Since some of cyanoprokaryotes produce plant growth promoting agents such as phytohormones, therefore, presence of these microorganisms in soil of medicinal plants bed cause an enhancement in plant growth and essential oil production (Shariatmadari *et al*. 2015).

As studies in the field of flora and phylogeny of cyanoprokaryotes are not enough, further studies are needed in this area. Because of the important role of cyanoprokaryotes especially heterocytous cyanoprokaryotes in terrestrial habitats, especially in the soil of medicinal plants bed we aimed to conduct a floristic study on heterocytous cyanoprokaryotes collected from medicinal plants bed. Also, we performed a phylogenetic analysis based on 16S rRNA marker to investigate the efficiency of this molecular marker in separation and clarifying the relationships between complex taxa such as *Nostoc* and *Desmonostoc*, as well as *Anabaena* and *Wollea*.

Materials and Methods

- Sampling sites and sample collection

Soil samples were collected from peppermint and chamomile plants fields located in Mazandaran, East Azarbaijan and Ardabil provinces (Iran). Soil samples were collected from eight sites from June 2015 to May 2016 according to Rangaswamy method (1996). The locations of soil samples collection sites are shown in table 1.

Table 1. Location of soil sample collection sites in Iran

Site	Province	Location	Coordinates	Altitude (m)
1	E Azarbaijan	Marand, Koshksaray	N: 38˚26′ 54 E: 45˚ 33′ 84	1220
2	E Azarbaijan	Marand	N: 38˚ 33′ 06 E: 45˚ 19′ 23	1047
3	E Azarbaijan	Ahar, Afil	N: 38˚ 23′ 10 E: 47˚ 19′ 04	1121
4	Ardabil	Meshgin Shahr	N: 38˚ 22′ 60 E: 47˚ 41′ 10	1484
5	Mazandaran	Savadkuh, Part Kola	N: 36˚ 09′ 34 E: 53˚ 21′ 29	806
6	Mazandaran	Galugah, Niala	N: 36˚ 37′ 18 E: 53˚ 49′ 48	1381
7	Mazandaran	Galugah, Vezvar	N: 36˚ 36′ 02 E: 53˚ 51′ 56	988
8	Mazandaran	Kiasar	N: 36˚ 14′ 23 E: 53˚ 30′ 28	1161

- Isolation, purification and identification of cyanoprokaryotic strains

Isolation and purification of the cyanoprocaryotic strains from soil samples was performed using BG-11 and nitrogen free BG-11 medium following Stanier *et al*. (1971). Solid medium containing cyanoprokaryotes colonies were maintained at culture chamber with 74 µmol photon $m^{-2}s^{-1}$ artificial illumination and 25±2 °C temperature at Shahid Beheshti University (Tehran, Iran). Identification of isolated strains was performed using light microscopy (Olympus, Japan) and available references (Desikachary 1959, Prescott 1970, Wehr *et al*. 2002, John *et al*. 2002, Komárek 2013, Komárek & Hauer 2013). Identification of cyanoprokaryotic strains performed based on morphological properties such as: colonial form and color, thallus form, presence or absence of heterocyts and akinetes, the shape and position of heterocyts and akinetes, presence or absence of gelatinous sheath, form of apical and vegetative cells.

- Molecular study

Molecular studies were carried out using 43 cyanoprokaryotic taxa, among them 17 taxa were isolated from the bed soil of medicinal plants (Table 2). DNA extraction from cyanoprokaryotic isolates was carried out by using a Genomic DNA extraction kit (AccuPrep®, Bioneer) and based on factory manual. In order to amplification of 16S rRNA region of cyanoprokaryotic DNA, polymerase chain reaction (PCR) was carried out using A2 (AGAGTTTGATCCTGGCTCAG) and S8 (TCTACGCATTTCACCGCTAC) as primers (Shariatmadari *et al*. 2014). PCR amplification was based on Shariatmadari *et al*. (2017) method. Sequencing the PCR products was carried out at Pishgam Biotech Company (Tehran, Iran) following Sanger Sequencing Method (1975). Sequences of cyanoprokaryotic DNA recorded in GenBank.

- Alignment of sequences and phylogenetic analysis

In order to compare the resulted sequences with sequences in GenBank and find similar sequences, nucleotide BLAST was performed in NCBI. BioEdit software Ver. 7.0.9.0 (Hall 1999) was used to edit the sequences used for phylogenetic analysis. Alignment of sequences was carried out by using MUSCLE (Edgar 2004) and manually adjustment. In all of datasets, positions of insertions or deletions were considered as missing data. MEGA software Ver. 5.1 (Tamura *et al*. 2011) by using maximum likelihood method used to calculate the genetic distances between sequences.

Phylogenetic analysis of cyanoprokaryotic taxa was carried out using 43 taxa. List of cyanoprokaryotic strains used for analysis has been shown in table 2 out of which, 26 taxa were selected from GenBank (NCBI). Evaluating the phylogenetic relationships used Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian Inference (BI).

Table 2. Information of taxa used in phylogenetic analysis

No.	Taxon	Strain No.	GenBank code	Locality
1	*Anabaena sphaerica*	ISB23	KM017089	Iran: Esfahan prov., Falavarjan, Paddy field soil
2	*A. torulosa*	ISB19	KM17093	Iran: Mazandaran prov., Savadkoh, Paddy field soil
3	*A. torulosa*	ISB20	KM017092	Iran: Khorasan Razavi prov., Paddy field soil
4	*Anabaena* sp.	ISB54	KT254261	Iran: Khorasan Razavi prov., Paddy field soil
5	*Anabaena* sp.	ISB55	KT254262	Iran: Khorasan Razavi prov., Paddy field soil
6	*Anabaena* sp.*	ISB105	MK771148	Iran: E Azarbaijan prov., Ahar, Afil, Chamomile bed soil
7	*Desmonostoc muscorum**	ISB94	MK764761	Iran: Mazandaran prov., Galugah, Niala, Peppermint bed soil
8	*D. muscorum**	ISB102	MK762748	Iran: Ardabil prov., Meshgin Shahr, Chamomile bed soil
9	*Nostoc calcicola**	ISB95	MK771143	Iran: Mazandaran prov., Kiasar, Peppermint bed soil
10	*N. calcicola**	ISB98	MK771140	Iran: Mazandaran prov., Galugah, Vezvar, Peppermint bed soil
11	*N. carneum**	ISB92	MK771137	Iran: Mazandaran prov., Galugah, Vezvar, Peppermint bed soil
12	*N. commune**	ISB103	MK762743	Iran: E Azarbaijan prov., Marand, Chamomile bed soil
13	*N. edaphicum**	ISB101	MK762744	Iran: Ardabil prov., Meshgin Shahr, Chamomile bed soil
14	*N. edaphicum**	ISB104	MK762747	Iran: E Azarbaijan prov., Marand, Koshksaray, Chamomile bed soil
15	*N. muscorum*	-	AY218828	Brazil: ?
16	*N. spongiaeforme*	ISB50	KT254257	Iran: Fars prov., Firozabad, Paddy field soil
17	*N. spongiaeforme* var. *tenue**	ISB97	MK764996	Iran: Mazandaran prov., Savadkuh, Part Kola, Peppermint bed soil
18	*Nostoc* sp.	ISB49	KT254256	Iran: Fars prov., Ebrahimabad, Paddy field soil
19	*Nostoc* sp.*	ISB100	MK762742	Iran: Ardabil prov., Meshgin Shahr, Chamomile bed soil
20	*Nostoc* sp.*	ISB107	MK771145	Iran: Ardabil prov., Meshgin Shahr, Chamomile bed soil
21	*Nostoc* sp.*	ISB93	MK771139	Iran: Mazandaran prov., Kiasar, Peppermint bed soil
22	*Nostoc* sp.*	ISB106	MK771142	Iran: E Azarbaijan prov., Marand, Koshksaray, Chamomile bed soil
23	*Nostoc* sp.*	ISB108	MK771150	Iran: E Azarbaijan prov., Marand, Koshksaray, Chamomile bed soil
24	*Nostoc* sp.*	ISB96	MK771136	Iran: Mazandaran prov., Galugah, Vezvar, Peppermint bed soil
25	*Oscillatoria angusta*	ISB35	KJ546668	Iran: Hormozgan prov., Khamir, Hot spring water
26	*O. angusta*	ISB38	KJ546665	Iran: Mazandaran prov., Ramsar, Hot spring water
27	*O. angusta*	ISB40	KJ543481	Iran: Hormozgan prov., Chah Ahmad, Hot spring water
28	*O. minima*	ISB29	KJ534024	Iran: Mazandaran prov., Ramsar, Hot spring water
29	*O. subbrevis*	ISB30	KJ534025	Iran: Hormozgan prov., Khamir, Hot spring water
30	*O. subbrevis*	ISB37	KJ546666	Iran: Hormozgan prov., Geno, Hot spring water
31	*Synechocystis aquatilis*	ISB32	KJ546671	Iran: Hormozgan prov., Geno, Hot spring water
32	*S. aquatilis*	ISB33	KJ546670	Iran: Hormozgan prov., Chah Ahmad, Hot spring water
33	*S. elongatus*	ISB34	KJ546669	Iran: Hormozgan prov., Khamir, Hot spring water
34	*S. elongatus*	-	JQ771323.1	Iran: Mazandaran prov., Ramsar, Hot spring water
35	*Synechocystis* sp.	-	AF448077	Japan: ?
36	*Synechocystis* sp.	-	AB039001.1	Japan: ?
37	*Synechocystis* sp.	-	HQ900668.1	India: Tamilnadu, Namakkal, Salem
38	*Wollea ambigua*	ISB17	KM035410	Iran: Esfahan prov., Jojil, Paddy field soil
39	*W. vaginicola*	ISB21	KM017091	Iran: Esfahan prov., Jojil, Paddy field soil
40	*W. vaginicola*	ISB22	KM017090	Iran: Lorestan prov., Visan, Paddy field soil

Table 2 (contd)

41	*W. vaginicola*	ISB24	KM017088	Iran: Fars prov., Kamfiroz, Paddy field soil
42	*W. vaginicola*	ISB26	KM017086	Iran: Lorestan prov., Visan, Paddy field soil
43	*W. vaginicola**	ISB42	MK771138	Iran: Kermanshah prov., Wheat bed soil

∗ shows the taxa isolated from the soil in current investigation.

PAUP* software Ver. 4.0b 10 was used to run the MP searches (Swofford 2002). Tree bisection reconnection branch swapping (TBR) was applied as heuristic search method with addition of 100 random sequences. Bootstrap supports, with the same settings for heuristic searches, were evaluated using 1000 replicates (Felsenstein 1985). MrModeltest 2.3 program (Nylander 2004) based on Akaike information criterion (AIC) (Posada & Buckley 2004) was used as nucleotide substitution model. GTR+I+G model were selected as an appropriate model.

Analysis of ML for dataset was carried out using raxml GUI Ver. 1.3 (Silvestro & Michalak 2012) and bootstrap values were calculated based on 1000 replicates.

MrBayes program Ver. 3.2 (Ronquist & Huelsenbeck 2003) was applied for Bayesian reconstruction. Parallel Metropolis Coupled Markov Chain Monte Carlo (MCMCMC) with proportion temperature of 0.2 was applied for ten million generations. TRACER Ver. 1.5 was applied to assess the mixing of chains and burn-in. As burn-in, 25% of trees were discarded and the left trees were used to construct the consensus tree with 50% of majority rule. TreeView 1.6.6 (Page 2001) was used to visualize the modeled tree.

Results

In current investigation, totally, 42 cyanoprokaryotic taxa including 31 heterocytous and 11 non-heterocytous species were identified. These species belong to 16 genera, 10 families and four orders. *Nostocales* with four families and 33 species is the dominant order in studied sites (Table 3).

Table 3. List of cyanoprokaryotic species identified from medicinal plants bed soil

No.	Taxon	Family	Location
	Nostocales		
1	*Anabaena* sp.$_1$	*Nostocaceae*	1
2	*Anabaena* sp.$_2$	*Nostocaceae*	2
3	*Aulosira* sp.	*Fortieaceae*	4
4	*Calothrix elenkinii* Kossinskaja	*Rivulariaceae*	8, 9
5	*Calothrix* sp.	*Rivulariaceae*	7
6	*Cylindrospermum michailovskoense* Elenkin	*Nostocaceae*	6
7	*C. muscicola* Kützing ex Bornet & Flahault	*Nostocaceae*	8
8	*Desmonostoc muscorum* (C. Agardh ex Bornet & Flahault) Hrouzek & Ventura	*Nostocaceae*	2, 6
9	*Nodularia harveyana* Thuret ex Bornet & Flahault	*Aphanizomenonaceae*	7
10	*N. spumigena* Mertens ex Bornet & Flahault	*Aphanizomenonaceae*	7
11	*Nostoc alatosporum* Sant'Anna *et al*.	*Nostocaceae*	6
12	*N. calcicola* Brébisson ex Bornet & Flahault	*Nostocaceae*	7, 9
13	*N. carneum* C. Agardh ex Bornet & Flahault	*Nostocaceae*	6, 7, 8
14	*N.* cf. *punctiforme*	*Nostocaceae*	2, 4
15	*N. commune* Vaucher ex Bornet & Flahault	*Nostocaceae*	5
16	*N. edaphicum* Kondrateva	*Nostocaceae*	2,3
17	*N. linckia* Bornet ex Bornet & Flahault	*Nostocaceae*	7
18	*N. paludosum* Kützing ex Bornet & Flahault	*Nostocaceae*	7

Table 3 (contd)

19	*N. punctiforme* Hariot	*Nostocaceae*	8
20	*N. sphaericum* Vaucher ex Bornet & Flahault	*Nostocaceae*	7
21	*N. spongiaeforme* var. *tenue* C.B. Rao	*Nostocaceae*	8, 9
22	*N. verrucosum* Vaucher ex Bornet & Flahault	*Nostocaceae*	9
23	*Nostoc* sp.$_1$	*Nostocaceae*	1
24	*Nostoc* sp.$_2$	*Nostocaceae*	2
25	*Nostoc* sp.$_3$	*Nostocaceae*	2
26	*Nostoc* sp.$_4$	*Nostocaceae*	2
27	*Nostoc* sp.$_5$	*Nostocaceae*	3
28	*Nostoc* sp.$_6$	*Nostocaceae*	3
29	*Nostoc* sp.$_7$	*Nostocaceae*	9
30	*Nostoc* sp.$_8$	*Nostocaceae*	7
31	*Trichormus fertilissimus* (C.B. Rao) Komárek & Anagnostidis	*Nostocaceae*	7
32	*T. variabilis* (Kützing ex Bornet & Flahault) Komárek & Anagnostidis	*Nostocaceae*	7
33	*Wollea vaginicola* (F.E. Fritsch & Rich) R.N. Singh	*Nostocaceae*	6, 9
	Synechococcales		
34	*Jaaginema angustissimum* (West & G.S. West) Anagnostidis & Komárek	*Synechococcaceae*	9
35	*J. pallidum* (Böcher) Anagnostidis & Komárek	*Synechococcaceae*	8, 9
36	*Jaaginema* sp.	*Synechococcaceae*	6, 7
37	*Leptolyngbya foveolara* (Gomont) Anagnostidis & Komárek	*Leptolyngbyaceae*	9
38	*Limnococcus limneticus* (Lemmermann) Komárková, Jezberová, O. Komárek & Zapomelová	*Merismopediaceae*	8
39	*Pseudanabaena* sp.	*Pseudanabaenaceae*	1
	Oscillatoriales		
40	*Oscillatoria limosa* C. Agardh ex Gomont	*Oscillatoriaceae*	8
41	*Phormidium retzii* Kützing ex Gomont	*Oscillatoriaceae*	6
	Chroococcales		
42	*Chroococcus turgidus* (Kützing) Nägeli	*Chroococcaceae*	9

Location 1: E Azarbaijan prov., Ahar, Aphil. Location 2: Ardabil prov., Meshgin Shahr. Location 3: E Azarbaijan prov., Marand, Koshksaray. Location 4: E Azarbaijan prov., Miyaneh to Hashtroud. Location 5: E Azarbaijan prov., Marand. Location 6: Mazandaran prov., Galugah, Niala. Location 7: Mazandaran prov., Galugah, Vezvar. Location 8: Mazandaran prov., Savadkuh, Part Kola. Location 9: Mazandaran prov., Kiasar

The results obtained from phylogenetic analyses of 16S rRNA, including Maximum Likelihood, Maximum Parsimony and Bayesian Inference, revealed similar results. Bayesian Inference tree using 16S rRNA dataset has been shown in figure 1. The numbers above the branches, with representing the Posterior Probability (PP), Bootstrap Values for Maximum Likelihood (ML BS) and Bootstrap Proportions (BP), reveal the strength of clade.

Evaluation of obtained tree shows the monophyly of *Nostocales* as a taxonomic group with heterocytous taxa (PP=0.98, ML BS=94 and BP=70) (Fig. 1). In clade belong to *Nostocales*; morphologically similar taxa such as *Desmonostoc* (Fig. 2a) and *Nostoc* (Fig. 2b), create a monophyletic group. This result has also supported by morphological similarity of these genera, and indicates the strong phylogenetic relationships between these heterocytous taxa. Moreover, other genera such as *Wollea* (Fig. 2c) and *Anabaena* (Fig. 2d) which are morphologically similar create a separate group (Fig. 1). The other monophyletic group, with strong support (PP=1.0, ML BS=100, and BP=76), belongs to *Synechococcales*, but members of genus *Oscillatoria* from *Oscillatoriales* have created a paraphyletic group (Fig. 1).

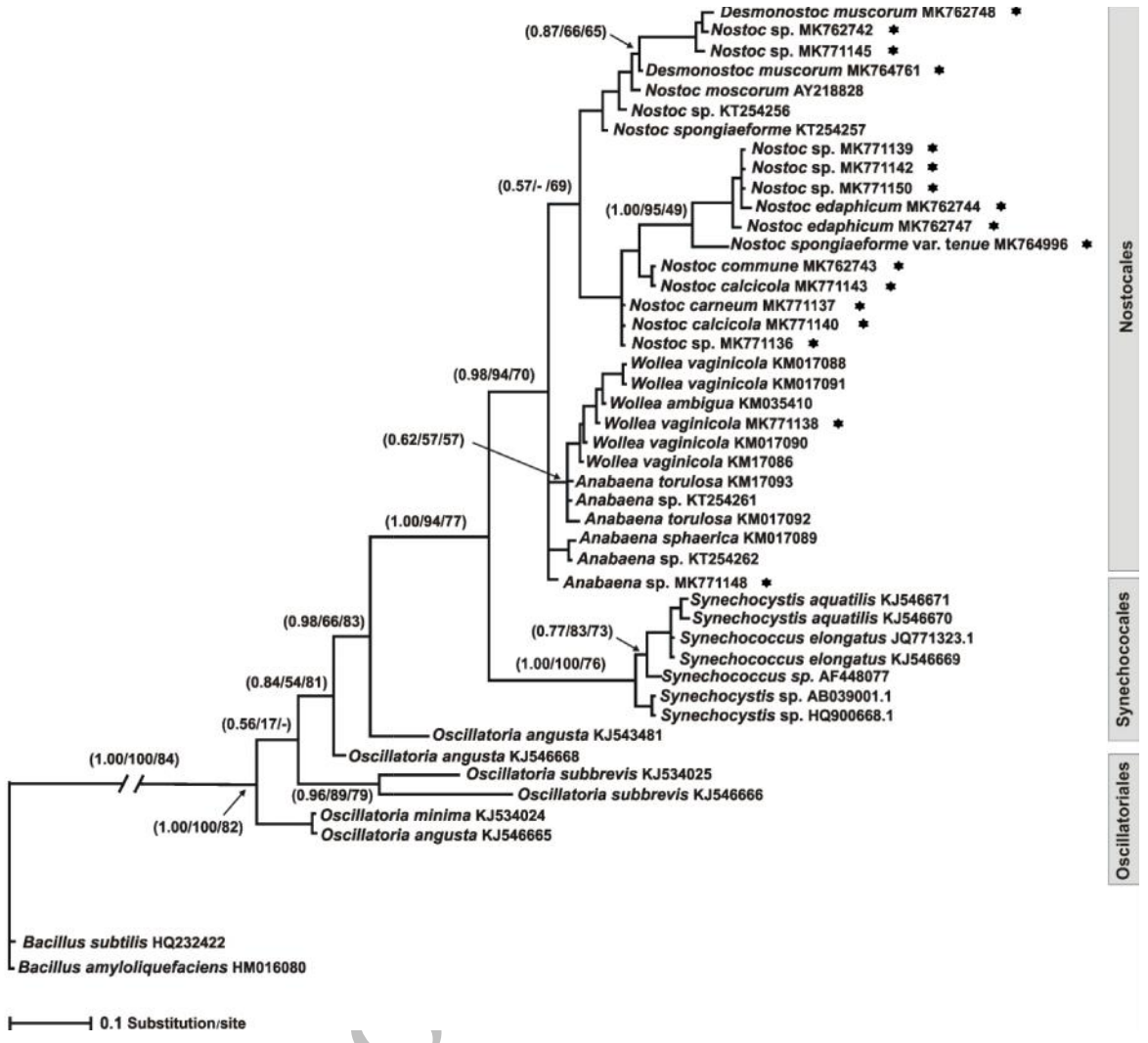

Fig. 1. 50% majority rule consensus tree shows the result of Bayesian analysis used cyanoprokaryotic 16S rRNA dataset. Values above the clades show posterior probability, bootstrap values for maximum likelihood and bootstrap percentage, respectively. Only values >50% has been shown (* shows the taxa isolated from soil in current investigation).

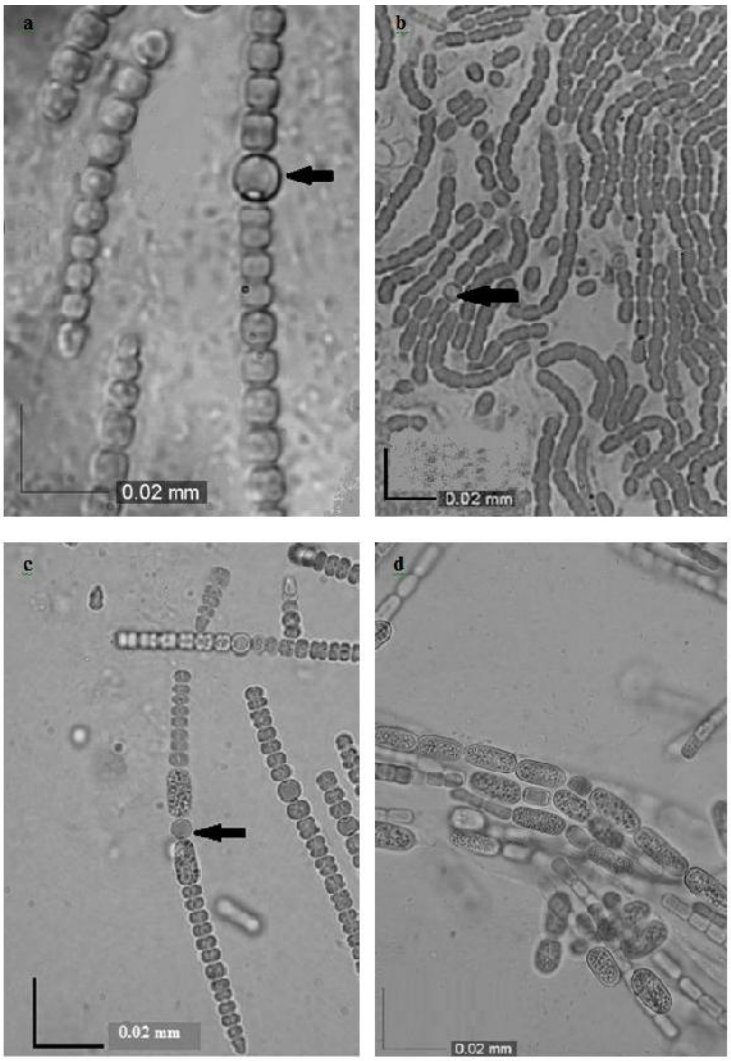

Fig. 2. a. Filament of *Desmonostoc muscorum*, b. *Nostoc* sp., c. *Wollea vaginicola*, d. *Anabaena* sp. (arrow shows heterocyte).

Discussion

It has been shown that, cyanoprokaryotes have wide distribution in agricultural fields of Iran (Shariatmadari *et al*. 2013, Aslani *et al*. 2014, Ahlesaadat *et al*. 2017). Our investigation revealed that, these microorganisms also occupied the medicinal plants bed, where heterocytous cyanoprokaryotes including *Nostocaceae* family, are widely distributed (Table 3).

As morphological characteristics alone are not sufficient for classification of cyanoprokaryotes, especially in complex taxa, we also performed a phylogenetic analysis based on 16S rRNA gene sequencing. 16S rRNA is a key marker in phylogenetic study of cyanoprokaryotes (Korelusová 2005). Although, it is suitable in high ranks of classification, but having conservative sequence, in most of cases make it unsuitable for evaluating the relationships at species level (Sentausa & Fournier 2013).

The phylogenetic tree in our investigation separated *Nostocales* and *Synechococcales* as monophyletic orders (Fig. 1) indicating 16S rRNA act as an effective molecular marker in separation of these orders. The monophyly of order *Nostocales* using 16S

rRNA have been shown previously (Giovannoni *et al.* 1988, Nelissen *et al*. 1966, Wanigatunge *et al*. 2014).

From our phylogenetic tree, it is concluded that, taxa belong to *Oscillatoriales* are paraphyletic. There are some investigations based on 16S rRNA marker that reveal order *Oscillatoriales* is not monophyletic and claim that, 16S rRNA is not an effective marker in separation of members of this order (Ishida *et al*. 2001, Shariatmadari *et al.* 2017, Casamatta *et al.* 2005). Some of these studies consider *Oscillatoriales* as polyphyletic (Ishida *et al.* 2001, Shariatmadari *et al*. 2017) while others, such as current investigation, consider the *Oscillatoriales* as paraphyletic group (Casamatta *et al.* 2005).

The phylogenetic tree also revealed that, 16S rRNA effectively separate apoheterocytic species including *Nostoc* and *Desmonostoc* from paraheterocytic species i.e. *Anabaena* and *Wollea*.

In current investigation, there are some taxa, despite the morphological similarity, currently changed their taxonomic position and separated from their previous taxon (Hrouzek *et al*. 2013). These taxa can be considered as complex taxa. For example, *Nostoc muscorum* which formerly considered as genus *Nostoc*, separated from this genus; and known as *Desmonostoc muscorum*. According to Hrouzek *et al*. (2013), both of these genera with similar morphological behavior, represent their most important character that is production of mucilaginous colony. The other important diagnostic key is heterogeneity of life cycle which means they have hormogenic cycle and sporogenic cycle (Hrouzek *et al.* 2013). In hormogenic cycle fragmentation of filaments in vicinity of heterocytes creates new filaments; while in sporogenic cycle new filaments are created from akinetes (Lazaroff 1966). The only difference in morphology of these genera is that, filaments in *Nostoc* are closely coiled with dense trichomes while this character is never seen in *Desmonostoc* (Hrouzek *et al*. 2013). *Anabaena* and *Wollea* are the other example of complex taxa in this study. Some species of *Wollea* currently separated from *Anabaena*. The most important diagnostic characters of these species are gelatinous colonies as well as unsheathed trichomes which densely placed in diffluent mucilage (Kozhevnikov & Kozhevnikova 2011). Although, some researchers believe that, these characters are not enough to separate the species of *Wollea* from *Anabaena* (Komàrek 1975, Shariatmadari *et al*. 2014), the phylogenetic tree in current study separated species of *Wollea* as monophyletic group.

Based on current investigation, it seems 16S rRNA marker in separation of *Desmonostoc* and *Nostoc* was weak; however, the marker was effective in separation of *Wollea* from *Anabaena*.

Although previous phylogenetic investigations (Hrouzek *et al*. 2013) show *Desmonostoc* as monophyletic group, our phylogenetic tree revealed that, some species of *Nostoc* have close affinity with this genus and together they form a monophyletic group. Also, in current investigation 16S rRNA separated species belong to *Wollea* as monophyletic group.

Finally, in this study genus *Nostoc* was an abundant genus in medicinal plants bed. Also, based on phylogenetic analysis we concluded that, 16S rRNA could be an effective phylogenetic marker in high classification ranking including *Nostocales* and *Synechococcales* orders. The marker could not effectively separate the genera with more affinity such as *Nostoc* and *Desmonostoc*.

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