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MORPHOMETRIC AND PHYLOGENETIC ANALYSES OF *ANABAENA* STRAINS (CYANOPROKARYOTA) FROM TERRESTRIAL HABITATS OF IRAN

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In present study, *Anabaena* isolates were collected from paddy field soils of seven main rice cultivation provinces situated in north, centre, south, west and east of Iran during 2 years from April 2008 to May 2010. Identification of taxa was carried out based on morphometric and molecular methods. Twenty one morphological characters and numerical taxonomic methods were used for classifying the several species of this genus. Numerical taxonomic studies were performed on 34 populations of 13 *Anabaena* morphospecies. A cluster analysis and principal component analysis performed using SPSS software and rate of resemblance among the species recognized. In the other section of this study phylogenetic relationships were determined by constructing 16S rRNA gene tree using the neighbor-joining algorithm. The results showed that populations of each species were placed close to each other and separate from the other species base on morphological characters. According to factor analysis, colonies form, filament structure, apoheterocytic or paraheterocytic form of filaments, position, shape and number of akinetes in filament, presence or absence of gelatinous sheath were the most variable characters which have been used for identification. Phylogenetic analysis based on 16S rRNA gene sequences also indicated that this gene site cannot separate genera such as *Anabaena*, *Trichormus* and *Wollea* which are morphologicaly close to each other.

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Key words: *Anabaena*; cluster analysis; factor analysis; morphospecies; numerical taxonomy

مطالعه مورفومتریک و فیلوژنتیک سویه‌های جنس *Anabaena* در اکوسیستم‌های خشکی ایران

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مزارع برنج از جمله اکوسیستم‌های خشکی هستند که از شرایط محیطی مناسبی برای رشد و انتشار سیانوباکتری‌ها برخوردارند. جنس *Anabaena* Bory ex Bornet et Flahault از جمله مهمترین جلبک‌های سبز–آبی رشته‌ای دارای هتروسیست در این اکوسیستم‌های خشکی به‌شمار می‌آید. در مطالعه حاضر، ایزوله‌های مختلف از این جنس، از خاک مزارع برنج ۷ استان دارای کشت برنج واقع در مناطق شمالی، جنوبی، شرقی، غربی و مرکزی کشور، از اردیبهشت ماه ۱۳۸۷ تا خرداد ماه ۱۳۸۹، جمع آوری و مطالعه شدند. شناسایی این تاکسون‌ها با استفاده از روش‌های مورفومتریک و فیلوژنتیک انجام شد. در مجموع ۲۱ صفت مورفولوژیک و روش‌های تاکزونومی عددی به منظور طبقه بندی ۳٤ جمعیت از ۱۳ ریخت‌گونه از این جنس مورد ارزیابی قرار گرفت. همچنین روابط فیلوژنتیک تاکسون‌های خالص سازی شده از طریق رسم درختچه فیلوژنتیک ١٦S rRNA و با استفاده از الگوریتم neighbor-joining مورد بررسی قرار گرفت. نتایج حاصل از تجزیه خوشه‌ای و تجزیه به مولفه‌های اصلی با استفاده از نرم افزار SPSS نشانگر گروه‌بندی مناسب جمعیت‌های متعلق به هر گونه و تفکیک آنها از دیگر گونه‌ها، تنها بر مبنای صفات ریختی ارزیابی شده بود. متغییرترین صفات ریختی ارائه شده در این بررسی عبارت بودند از: شکل کلنی؛ ساختار رشته؛ آپوهتروسیتیک یا پاراهتروسیتیک بودن رشته؛ جایگاه، شکل و تعداد اکاینت در رشته و نیز حضور یا عدم حضور غلاف ژلاتینی. نتایج حاصل از آنالیز فیلوژنتیک نیز حاکی از عدم کارایی آنالیزهای مبتنی بر سکانس‌های ژنی ١٦S rRNA در جداسازی تاکسون‌های متعلق به جنس‌های نزدیک به یکدیگر نظیر *Anabaena*, *Wollea* و *Trichormus* و نیز نشانگر قرابت ژنتیکی بسیار بالای این تاکسونها بود.

INTRODUCTION

Paddy fields are terrestrial ecosystems that represent a favorable environment for the growth of cyanobacteria. The genus *Anabaena* Bory ex Bornet et Flahault is the most important among filamentous heterocystous cyanobacteria in these terrestrial ecosystems. This genus is one of the filamentous, heterocystous, unbranched and not polarized cyanobacteria, classified traditionally in Nostocaceae family (Komárek 2010). In the most recent classification, the genus *Anabaena* has been classified under subsection IV family I (Rippka *et al.* 2001). Although there are different opinions regarding to the numbers of species, but for many years the 57 *Anabaena* species recognized by Geitler was the main reference for the classification in this genus (Zapomělová 2008). Discrepancy about the number of *Anabaena* species is due to taxonomic problems existing in this genus. Contemporaneous use of botanical and bacteriological codes in cyanobacteria nomenclature and lack of general nomenclature system for them is one of these problems (Zapomělová 2006).

Anabaena species are distributed in several habitats such as aquatic and terrestrial ecosystems. One of the favorable environments for distribution of this genus is paddy fields. Up to now several species from this genus have been reported from paddy field soils (Desikachary 1959, Komárek 2005). The number of *Anabaena* species reported from paddy soils of Iran is around 15 according to different authors (Nowruzi & Ahmadimoghadam 2006, Saadatnia & Riahi 2009, Shariatmadari *et al*. 2011, Shariatmadari *et al*. 2013). Available literatures dealing with systematic and biosystematic of *Anabaena* species also indicate the importance of these taxa (Komárek 2005, Nayak & Prasanna 2007, Komárek & Zapomělová 2008, Nayak *et al.* 2009, Tuji & Niiyama 2010), but no report is available on the biosystematic of *Anabaena* species and their populations from Iran. The present study therefore is considering numerical taxonomic study of 34 populations belonging to 13 *Anabaena* morphospecies and trying to reveal the inter-population morphological variation and inter-specific relationships. Phylogenetic analysis based on 16S rRNA gene sequences also was used to demonstrate the correct position of the other close genera such as *Trichormus* and *Wollea*.

MATERIALS AND METHODS

Isolation and identification of cyanobacteria: Soil samples were collected from 13 paddy fields from April 2008 to May 2010 according to Rangaswamy method (1996). The collected soil samples were transferred to sterile Petri dishes and sterilized nitrate free BG-11 medium (Stanier *et al.* 1971) was added and the pH adjusted in 8.1 after sterilization. The Petri dishes were placed in a culture chamber at 25±1°C and 12/12h light-dark cycle at artificial illumination (2000-2500 Lux) for two weeks. After colonization, taxonomic determination was carried out by light microscopy (Olympus, Model BH-2) and based on Desikachary (1959), Prescott (1970), Whitford & Schumacher (1973), Wehr *et al.* (2002), John *et al.* (2002), Komárek (2005) and Komárek & Zapomělová (2008) by prepared semipermanent slides. The stable vegetative and reproductive characters were used in the taxonomic determination.

Morphometric study on *Anabaena* strains: Morphometric studies were performed on 34 populations of 13 *Anabaena* morphospecies isolated from paddy soils of diverse geographic locations in Iran (Fig. 1, Table 1). In addition two morphospecies of *Nostoc* were also used as outgroup in this study. Ten filaments from each population were used for morphometric studies. In total 21 quantitative and qualitative morphological characters were studied (Table 3). Characters were selected based on those reported by Nayak & Prasanna (2007) and our own field observations.

Statistical analysis: In order to determine the species 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 average taxonomic distance and squared Euclidean distance were used as dissimilarity coefficient in cluster analysis of morphological data (Podani 2000). In this study, SPSS software was used for statistical analysis.

Culture condition and DNA extraction: 12 cultures of purified *Anabaena* isolates were used for phylogenetic study. The most important taxonomic details of isolates are listed in Table 4. DNA extraction was carried out by AccuPrep® genomic DNA extraction kit from Bioneer Inc.

Amplification of the 16S rRNA gene and sequencing: PCR amplification was performed according to Ezhilarasi and Anand (2009). Amplification of the 16S rRNA gene was carried out by PCR 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. Total reaction volume was 100 µl after an initial cycle consisting of 4 min at 95˚C, 35 cycles of amplification were started (1 min at 95˚C, 1 min at 59˚C and 2 min at 72˚C). The termination cycle was 8 min at 72˚C. The PCR products were migrated

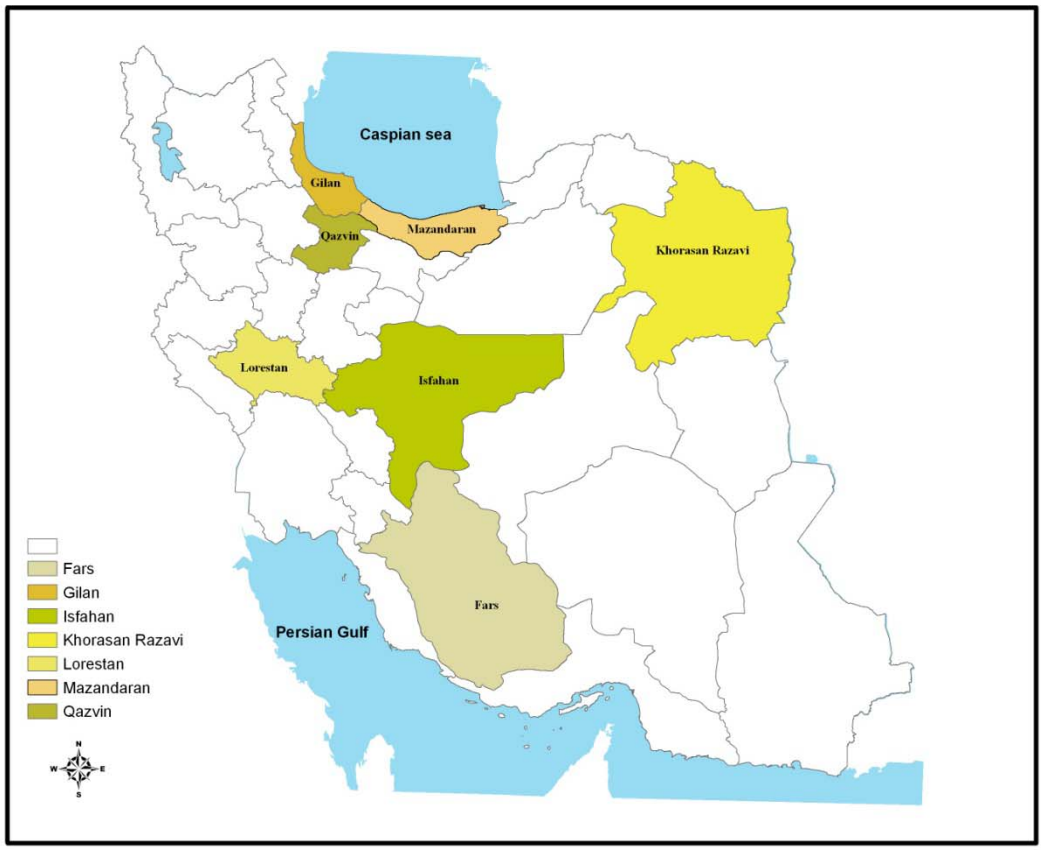

Fig. 1. Geographical distribution of studied area.

either on 1% (w/v) agarose gel and visualized by ethidium bromide. Proper PCR products of 16S rRNA were sequenced by Avicenna Research Institute, Tehran, Iran.

Phylogenetic analysis: Sequances were aligned using the CLUSTAL W multiple sequence alignment program and phylogenetic tree was constructed using the neighbor-joining method and according to the available cyanobacterial gene sequences. In this analysis, bootstrap analysis was used to evaluate the tree topologies by performing 1000 replications.

RESULTS

Morphometric study: In this research the morphological diversity of the genus *Anabaena* was investigated in three level, i. e., within a population, intraspecific (among different populations of each species) and interspecific (among different species).

In the cluster analysis based on all morphological characters, two major clusters were found. The first major cluster separated *Anabaena* species (Cluster A) from two representatives of the genus *Nostoc* (Cluster B). In the other words primary clustering clearly separated taxa of these two genera from each other (Fig. 2). The cluster A is divided into two sub-clusters or two groups. In the first of which (group 1), populations belonging to *A.variabilis* var. *ellipsospora*, *A. oryzae* and *A. fertillisima* and in the second sub-cluster (group 2), populations of *A. oscillarioides*, *A.*sp., *A. iyengarii*, *A. sphaerica*, *A. ambigua*, *A. viguieri*, *A. orientalis* and *A. vaginicola* are placed close to each other. Among these taxa, *Anabaena* species such as *A.variabilis* var. *ellipsospora, A. fertilissima* and *A. oryzae* are currently considered as synonyms of *Trichormus ellipsosporus, Trichormus fertilissimus* and *Nostoc oryzae*. Otherwise the first group of cluster A is comprised of populations of the species that are transfered to other genera such as *Trichormus* and *Nostoc*.

Two sections of taxa were observed in second group (Fig. 2). Akinete shape was the most important character for division of these two sections from each other. In the first section, taxa with cylindrical or sub-cylindrical akinetes and in the second section, taxa with spherical, sub-spherical, ellipsoidal or oblong akinetes were located. Shape and number of akinetes and

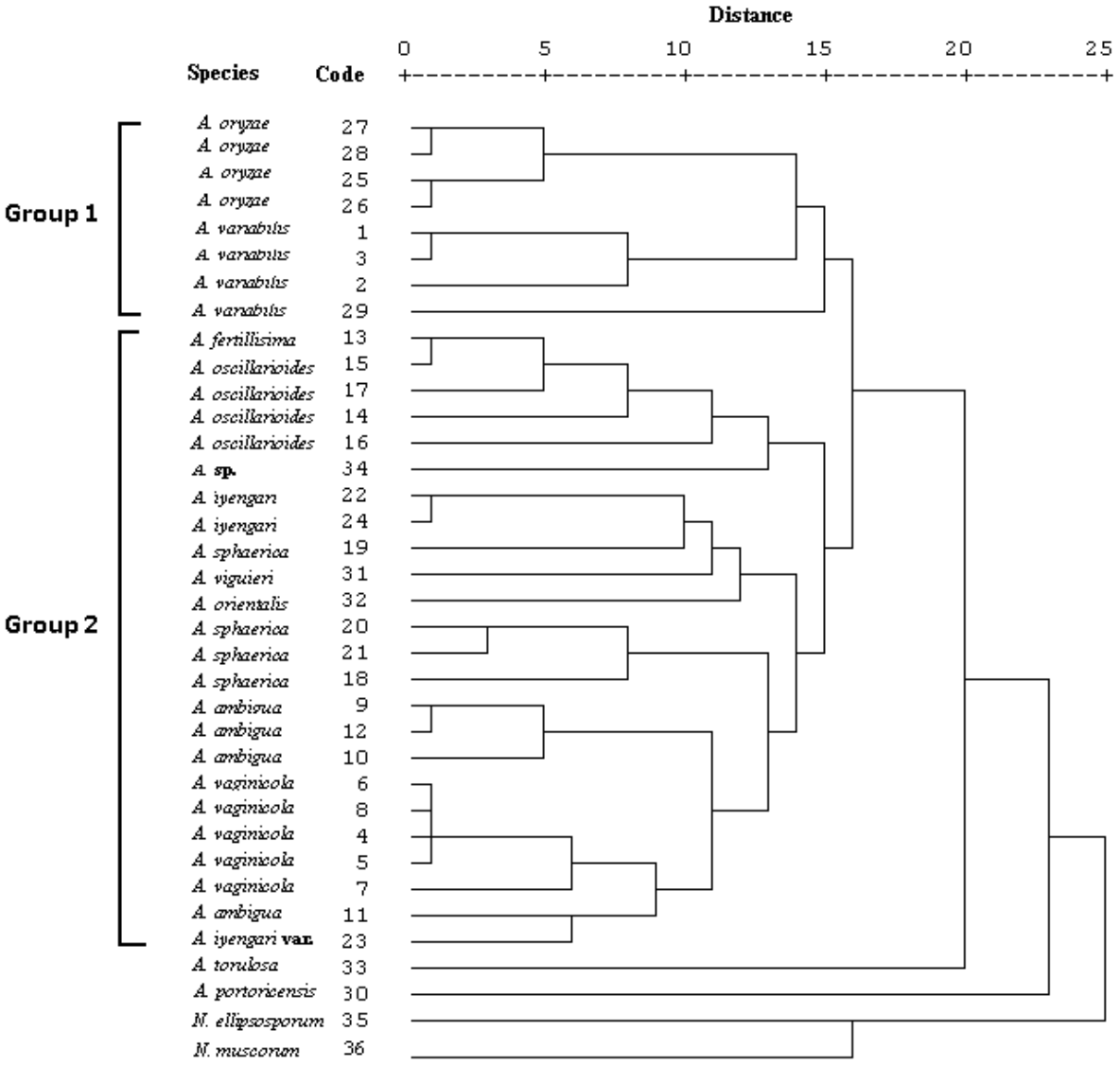

Fig. 2. Hierarchical cluster analysis dendrogram of *Anabaena* taxa based on morphological characters using UPGMA method (Species code as in Table 1).

presence or absence of gelatinous envelop were the determining characters for separation of taxa in secondary section. Akinete shape also was the most important character for isolation of *Anabaena torulosa* and *Anabaena portoricencis* in separated groups. Irregularly uneven size mature akinetes in *Anabaena portoricencis* and ellipsoidal akinetes with middle constriction in *Anabaena torulosa* separated these two taxa from the others. Result of clustering analysis showed that with the exception of three populations of *Anabaena* species (C20, C28 and C32), separation of other isolates was performed precisely. Different shape of vegetative cells in these isolates was the reason of this incorrect placement in the phenogram. For example, C20 represented one population of *Anabaena ambigua* with sub-quadrate vegetative cells against to discoid cells of other populations of this species. Soil salinity is one of the environmental factors that might affect the vegetative cells shape in this isolate (Table 2).

Table 1. Origins of 34 Anbaena taxa studied in this study.

Strain No.	Taxonomic designation	Origin
C10	*A.variabilis* var.*ellipsospora* (=*Trichormus ellipsosporus*)	Gilan: Rodsar, Rahimabad
C11	*A.variabilis* var.*ellipsospora* (=*Trichormus ellipsosporus*)	Esfahan: Falavarjan
C12	*A.variabilis* var.*ellipsospora* (=*Trichormus ellipsosporus*)	Khorasan Razavi: Kalat
C13	*A.vaginicola*	Gilan: Sangar, Omsheh
C14	*A.vaginicola*	Mazandaran: Tonkabon, Tazehabad
C15	*A.vaginicola*	Qazvin: Alamut
C16	*A.vaginicola*	Lorestan: Visan
C17	*A.vaginicola*	Fars: Marv dasht, Kamfiroz
C18	*A.ambigua*	Lorestan: Visan
C19	*A.ambigua*	Esfahan: Falavarjan
C20	*A.ambigua*	Fars: Firuzabad, Ebrahimabad
C21	*A.ambigua*	Esfahan: Lenjan, Zarrinshahr
C22	*A.oscillarioides*	Fars: Firuzabad, Ebrahimabad
C23	*A.oscillarioides*	Gilan: Rasht, Saravan
C24	*A.oscillarioides*	Mazandaran: Tonkabon, Tazehabad
C25	*A.oscillarioides*	Qazvin: Alamut,
C26	*A.oscillarioides*	Esfahan: Falavarjan, Jujill
C27	*A.sphaerica*	Qazvin: Alamut
C28	*A.sphaerica*	Lorestan: Visan
C29	*A.sphaerica*	Esfahan: Falavarjan, Jujill
C30	*A.sphaerica*	Gilan: Sangar, Omsheh
C31	*A.iyengari*	Qazvin: Alamut
C32	*A.iyengari* var.*tenuis*	Esfahan: Falavarjan, Jujill
C33	*A.iyengari*	Gilan: Sangar, Omsheh
C34	*A.oryzae* (=*Nostoc oryzae*)	Gilan: Sangar, Omsheh
C35	*A.oryzae* (=*Nostoc oryzae*)	Lorestan: Visan
C36	*A.oryzae* (=*Nostoc oryzae*)	Khorasan Razavi: Kalat
C37	*A.oryzae* (=*Nostoc oryzae*)	Fars, Marv dasht: Esmaeilabad
C38	*A.fertillisima* (=*Trichormus fertilissimus)*	Lorestan: Visan
C39	*A.portoricensis*	Khorasan Razavi: Kalat
C40	*A.viguieri*	Fars: Firuzabad, Ebrahimabad
C41	*A.orientalis*	Khorasan Razavi: Kalat
C42	*A.torulosa*	Khorasan Razavi: Kalat
C43	*A.*sp	Gilan: Rodsar, Rahimabad
C44	*N.ellipsosporum*	Gilan: Sangar, Omsheh
C45	*N.muscorum*	Mazandaran: Tonkabon, Tazehabad

Overall results of this study showed that morphological characteristics thoroughly define boundary between different species of this genus and also indicated the relative stability of morphological characteristics within the population and between populations of each species.

Similar to cluster analysis, PCA ordination of these isolates based on all morphological characters can separate *Anabaena* spp. and *Nostoc* spp. (Fig. 3). In order to identify the most variable morphological characters among the studied species, PCA analysis was performed. The analysis revealed that the first seven factors comprise about 83% of total variance. In the first factor with about 25% of total variance, characters like colonial mass shape, filament structure (entangled or not), apoheterocytic or paraheterocytic form of filaments possessed the highest positive correlation. In the second factor with about 14% of total variance, characters like position of akinete with regard to heterocyst, presence or absence of gelatinous sheath and akinete number in filament structure possessed the highest positive correlation. In the third and fourth factors with about 13% and 10% of total variance, characters like epispore colour, number of filaments in gelatinous sheath, akinetes and vegetative cells shape possessed the highest positive correlation. Therefore these are the most variable morphological characters among the studied species. Result of this study also showed that traditional characters commonly used in description of the genus *Anabaena* are not sufficient for recognition of taxa such as *Trichormus ellipsosporus, Trichormus fertilissimus* and *Nostoc oryzae*. In other words, characters such as apoheterocytic or paraheterocytic form of trichomes are essential for correct identification and for making coordination between previous and modern nomenclature systems.

Table 2. Geographical data and some ecological details of the sampling locations.

Location	Latitude/Longitude	pH	EC (dS/m)
Mazandaran: Tonkabon,Tazehabad village	36˚39′ N 51˚25′ E	8.1	1.16
Gilan: Sangar, Omsheh village	37˚16′ N 49˚35′ E	8.2	2.39
Gilan: Rodsar, Rahimabad village	36˚51′ N 50˚13′ E	8	1.47
Gilan: Rasht, Saravan village	37˚05′ N 49˚24′ E	8.1	2.79
Qazvin: Alamut village	36˚23′ N 50˚33′ E	8.1	2.47
Lorestan: Visan village	33˚49′ N 48˚07′ E	8.4	1.03
Fars: Firuzabad, Ebrahimabad village	29˚00′ N 52˚56′ E	8.1	9.55
Fars: Marv dasht, Esmaeilabad village	28˚85′ N 53˚83′ E	8.3	2.38
Fars: Marv dasht, Kamfiroz village	30˚15′ N 52˚17′ E	8	2.50
Khorasan razavi: Kalat village	36˚59′ N 59˚47′ E	8.1	2.93
Esfahan: Flavarjan village	32˚32′ N 51˚30′ E	8.4	2.48
Esfahan: Lenjan, Zarrinshahr village	32˚22′ N 51˚22′ E	8.3	3.31
Esfahan: Falavarjan, Jujil village	32˚34′ N 51˚28′ E	8.3	1.26

Table 3. Morphological characters and their character states in studied taxa of *Anabaena*.

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 shape	0) Sub-spherical, 1) Spherical, 2) Oblong with rounded apex, 3) Cylindrical, 4) Barrel shape
Heterocyst length	0) Lower than 9µ, 1) Higher than 9µ
Apical heterocyst	0) Present, 1) Absent
Position of akinet with regard to heterocyst	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
Akinete number	0) Single or two, 1) Several
Akinete position	0) In one side of heterocyst, 1) In two sides of heterocyst
Akinete middle constriction	0) Present, 1) Absent
Akinetes similarity	0) Even size, 1) Uneven size
Akinete length	0) Lower than 14µ, 1) higher than 14µ
Gelatinous sheath	0) Present, 1) Absent
Gelatinous sheath colour	0) Colourless, 1)Yellowish brown
Number of trichome in sheath	0) Single, 1) Several
Epispore colour	0) Brown, 1) Colorless
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 form	0) Entangled, 1) No entangled
Trichome structure	0) Apoheterocytic, 1) Paraheterocytic

Phylogenetic study: Phylogenetic relationship were determined for several taxa of *Anabaena* such as *Anabaena vaginicola* F. E. Fritsch & Rich, *A. iyengarii* Bharadwaja, *A. torulosa* Lagerheim ex Bornet & Flahault, *A. sphaerica* Bornet & Flahault, *A. verrucosa* J.B.Petersen, *A. cylindrica* Lemmermann, *A. ambigua* C.B. Rao, *A. oscillatorioides* Bory de Saint-Vincent ex Bornet & Flahault., *A. subtropica* Gardner, *A. oryzae* F.E.Fritsch and *A. variabilis* var. *ellipsospora* Fritsch. Among these taxa, *A. variabilis* var. *ellipsospora, A. oryzae*, *A. vaginicola* and *A. ambigua* are currently considered as synonyms of *Trichormus ellipsosporus* (F.E.Fritsch) Komárek & Anagnostidis, *Nostoc oryzae* (F.E.Fritsch) J.Komárek & K.Anagnostidis, *Wollea ambigua* (C.B.Rao) R.Y.Singh and *Wollea vaginicola* (Fritsch et Rich) R.N.Singh (Kozhevnikov and Kozhevnikova 2011, http://www.algaebase.org/, http://www.cyanodb.cz/). The sequences obtained from the present study were compared with those of representative heterocytic cyanobacteria from these genera which are available in GenBank, and additionally *Hapalosiphon* sp. was used as the outgroup (Table 4). The most probable phylogenetic tree is shown in Fig. 4.

Table 4. Strains used in phylogenetic analysis

Taxon and Strain designation	Origin	Gen Bank accession no.
Anabaena vaginicola (A.vag$_1$)	Iran, Gilan, Rostamabad	KM017087
Anabaena vaginicola (A.vag$_2$)	Iran, Gilan, Omsheh	JN873351.1
Anabaena vaginicola (A.vag$_3$)	Iran, Lorestan, Visan	KM017086
Anabaena vaginicola (A.vag$_4$)	Iran, Lorestan, Visan	KM017090
Anabaena vaginicola (A.vag$_5$)	Iran, Fars, Kamfiroz	KM017088
Anabaena vaginicola (A.vag$_6$)	Iran, Esfahan, Jojil	KM017091
Anabaena vaginicola (A.vag$_7$)	India, Soil from rice field	GQ466533
Anabaena iyengarii (A.iyen$_1$)	India, Soil from rice field	GQ466528
Anabaena iyengarii (A.iyen$_2$)	India, Soil from rice field	GQ466529
Anabaena iyengarii (A.iyen$_3$)	India, Soil from rice field	GQ466530
Anabaena iyengarii (A.iyen$_4$)	India, Soil from rice field	GQ466531
Anabaena iyengarii (A.iyen$_5$)	India, Soil from rice field	GQ466532
Anabaena iyengarii (A.iyen$_6$)	India, Soil from rice field	GQ466548
Anabaena torulosa (A.tor$_1$)	Iran, Khorasan Razavi, Kalat	KM017092
Anabaena torulosa (A.tor$_2$)	Iran, Mazandaran, Savadkoh	KM017093
Anabaena sphaerica (A.spha$_1$)	Iran, Esfahan, Falavarjan	KM017089
Anabaena sphaerica (A.spha$_2$)	ICC, U.S.A (Ezhilarasi and Anand, 2009)	EF375612
Anabaena sphaerica (A.spha$_3$)	India, Soil from rice field	GQ466541
Anabaena sphaerica (A.spha$_4$)	-	DQ439647
Anabaena cylindrica (A.cyl)	ICC, U.S.A (Ezhilarasi and Anand, 2009)	EF375611
Anabaena subtropica (A.sub)	ICC, U.S.A (Ezhilarasi and Anand, 2009)	EF375613
Anabaena verrucosa (A.verr)	ICC, U.S.A (Ezhilarasi and Anand, 2009)	EF375614
Anabaena kisseleviana (A.kisse)	-	AY701558
Anabaena sp. (A.sp)	India, Pond	JN197411
Anabaena oscillarioides (A.oscill)	India, Soil from rice field	GQ466544
Anabaena oryzae (A.oryz)	India, Dried water body	JN197410
Anabaena ambigua (A.amb)	Iran, Esfahan, Jojil	KM035410
Anabaena variabilis (A.var$_1$)	Iran, Gilan, Rahimabad	KM017085
Anabaena variabilis (A.var$_2$)	CCAP, UK (Ezhilarasi and Anand, 2009)	EF488831
Anabaena variabilis (A.var$_3$)	India, Soil from rice field	GQ466540
Anabaena variabilis (A.var$_4$)	India, Soil from rice field	GQ466542
Trichormus variabilis (T.var$_1$)	-	DQ234832
Trichormus variabilis (T.var$_2$)	-	DQ234833
Trichormus variabilis (T.var$_3$)	-	DQ234829
Trichormus azollae (T.azo)	-	AJ630454
Trichormus doliolum (T.doli)	-	AJ630455
Wollea saccata (W.sacc)	Yenissei River basin (Eastern Siberia, Russia)	GU434226
Hapalosiphon sp. (H. sp)	Iran, Mazandaran, Gharakheil	KM017094

The strains studied here were divided into three branches. One of the branches separated outgrroup from other taxa. Other taxa also were distributed in two groups. In these two groups *Anabaena* spp. and taxa which are transfered to other genera, such as *Trichormus* and *Wollea*, accompanied with them in phylogenetic tree. Therefore in present study, phylogenetic tree based on 16S rRNA sequences is unable to separate *Anabaena* spp. from *Trichormus* spp. and *Wollea* spp. In other words, this molecular marker is not sufficient for separation of these taxa.

DISCUSSION

Morphometric study: Numerical techniques were recommended to solve problems presented in the taxonomy of cyanobacteria (Whitton 1969). The genus *Anabaena* is one of the nostocacean cyanobacteria of which numerous morphospecies were described. This group is a very diverse and variable genus of cyanoprokaryotes (Komárek 2005) and their

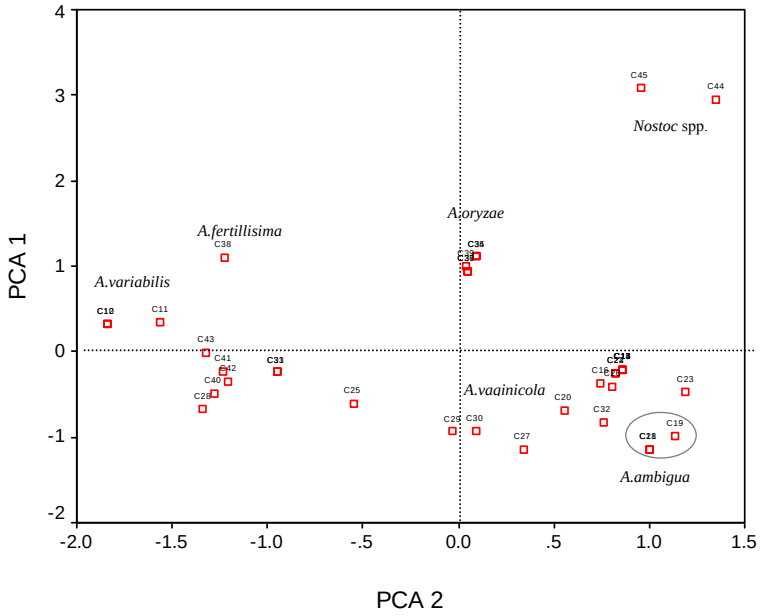

Fig. 3. PCA ordination of the *Anabaena* species based on all morphological characters (Species code as in Table 1).

phenotypic diversity in several habitats have been reported.

Anabaena spp. and *Nostoc* spp. are distinguished from each other based on morphological characters such as presence or absence of mucilaginous envelop and entangled form of trichome. Definite shapes of mucilaginous envelop and colony is one of the most important characters of *Nostoc* species, but thick mucilaginous envelop without a definite shape might be present in some species of genus *Anabaena* (Prescott 1970). McGuire (1984) considered that morphological characters such as shape and size of akinetes, vegetative cells and heterocysts; colour and shape of plant mass in nature are the most useful characteristics for separation of these two genera. Apoheterocytic or paraheterocytic form of trichome is another important character that separates these taxa at generic level. This character can also be used for identification of the genera such as *Anabaena* and *Trichormus*. The first and second groups in cluster A have been separated on the basis of trichome form and inattention to this character precludes correct identification. In other words, apoheterocytic taxa such as *Trichormus ellipsosporus*, *Trichormus fertilissimus* and *Nostoc oryzae* located in first group (group 1) and paraheterocytic taxa or *Anabaena* isolates entered into group second (group 2).

In separation of *Anabaena* species morphological characteristics such as akinete (Shariatmadari *et al.* 2011) vegetative cell and heterocyst shapes are the most important characters. At lower levels, other morphological characters such as number of akinetes and their distance from heterocysts as well as presence or absence of mucilaginous sheath can separate different populations of each species.

According to this clustering, most variable morphological characters such as colonial mass shape, entangled form of filaments and apoheterocytic or paraheterocytic form of filaments in the first axis separated *Nostoc* species from *Anabaena* species. Among these characters apoheterocytic or paraheterocytic form of filaments also separated first group of cluster A. In other words apoheterocytic form of filaments is the most important character for breaking down of *Anabaena* and transferring some of its taxa to the genera such as *Trichormus* and *Nostoc* (Fig. 3). However, these taxa show the highest similarity to *Anabaena* species and located with them in shared cluster (Cluster A). For instance, *Nostoc oryzae* (= *A. oryzae*) populations instead of being in cluster B, were placed in cluster A and being with other isolates of *Anabaena* species. In other words, according to all qualitative and quantitative morphological characters, these are more similar to *Anabaena* species in comparison with *Nostoc* species.

In cluster A, morphological characters as position of akinetes with regard to heterocyst, akinete number, presence or absence of gelatinous sheath, number of filaments in gelatinous sheath, akinetes and vegetative

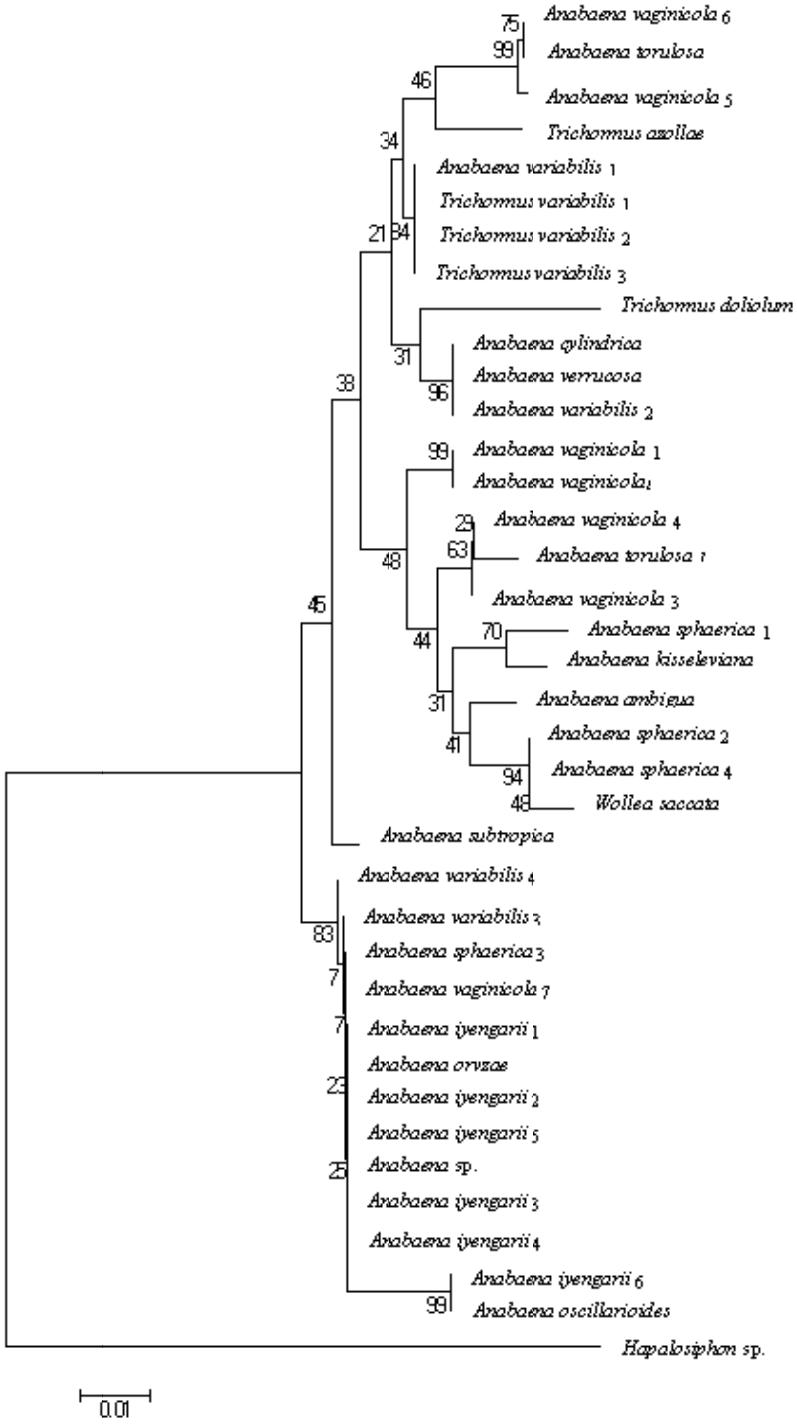

Fig. 4. Phylogenetic tree from 16S rRNA gene sequences of *Anabaena* strains using the neighbor-joining method.

cells shape as well as epispore colour are the most variable morphological characters among different *Anabaena* species and these are more effective characters for separation of these taxa. In principle, PCA analysis revealed that qualitative characters are the main characters for identification and separation of several species of this genus. With respect to instability of some quantitative characters and the influence of

environmental factors such as temperature, pH and electrical conductivity (EC), we consider that quantitative characters such as size of vegetative cells or heterocysts and akinetes solely cannot be sufficient for identification of these taxa, but utilization of these characters with qualitative characters can improve the results of morphological identification and clustering of *Anabaena* species.

In this study the interpopulation and intrapopulation stability of morphological characteristics was observed in several taxa. Soil properties such as temperature, light intensity, humidity and physiochemical properties such as pH and EC are the main environmental factors that can affect the morphological characteristics and the frequency of cyanobacteria in terrestrial habitats. Several studies on response of cyanobacteria to these factors have been done in laboratory conditions (Kellar & Paerl 1980, Kaplan 1981, Stockner & Shortreed 1988, El-Gamal *et al.* 2008). Considering that all isolates in this study were land-based, they were exposed to low levels of temperature changes. Furthermore microorganisms in depth of topsoil are not exposed to direct light, therefore light intensity changes in different sites has no effect on morphological variation.

The physicochemical properties of collection sites were diverse in terms of EC and pH (Table 2). Among soil properties, pH is the most important factor determining soil floristic composition (Nayak & Prasanna 2007). Due to the limited range of pH in the studied sites (8-8.4), soil pH as an influencing factor that affects the morphological diversity of *Anabaena* populations has no significant impact. Electrical conductivity is another environmental factor that can affect on morphological diversity of *Anabaena* strains. The soil sample of Ebrahimabad exhibited a high EC, while the other soil samples exhibited low or moderate EC. So here the interpopulation and intrapopulation stability of morphological characteristics might be related to the uniform environmental conditions of habitats from which the strains of each species were collected.

In phylogenetic section of this study, 16S rRNA gene sequences were applied. At all taxonomic levels above species, such as generic level, the sequence analysis of genes encoding small-subunit ribosomal RNA (16S rRNA) are currently the most promising approach for the phylogenetic classification of cyanobacteria (Nübel et al. 1997). Independence of 16S rRNA genes from cultivation or growth conditions is one of the most important reasons of the study on this region. In present study, with due attention to efficiency of this marker at generic level, relativity of *Anabaena* species with taxa which have been recently transferred to other genera such as *Wollea*, *Trichormus* and *Nostoc* were considered.

Rajaniemi et al. (2005) showed that molecular studies can separate the genera *Anabaena* and *Nostoc* carefully. But in this study 16S rRNA gene sequences did not separate *Nostoc oryzae* (= *A. oryzae*) from other *Anabaena* species. *Wollea ambigua* was another taxa which were exposed to the same morphologically taxa such as *A. sphaerica*. Placement of *Wollea ambigua* and *Wollea vaginicola* among *Anabaena* species also showed affinity of these taxa and inability of this molecular marker to separate them.

Wollea Bornet et Flahault is a poorly known genus which is most morphologically similar to genera *Anabaena* and *Nostoc* (Komárek 2010, Kozhevnikov & Kozhevnikova 2011). Kozhevnikov and Kozhevnikova (2011) showed that the phylogenetic placement of *Wollea* based on 16S rRNA gene sequence was distinct from *Nostoc* and the most closely related to taxa in benthic *Anabaena*, *Sphaerospermopsis*, *Cylindrospermopsis* and *Raphidiopsis*. Due to morphological and molecular similarity of *Wollea ambigua* and *Wollea vaginicola* to *Anabaena* specimens, transferring them to other genera need more studies and stronger evidence.

Unlike *Wollea, Trichormus* and *Nostoc* are the well defined and genetically confirmed genera with apoheterocytic formation of akinetes (Komárek 2010). *Trichormus* taxa in vegetative phase of growth and colonial shape are very similar to *Anabaena* species. 16S rRNA sequencing also supports this similarity (Fig. 4).

In conclusion we propose that, according to morphological and molecular data, transferring taxa such as *A. variabilis* var. *ellipsospora, A. oryzae*, *A. vaginicola* and *A. ambigua* to other genera need more studies and evidence. It may be better to change their taxonomic status to lower levels rather than moving them to different genera.

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