Molecular phylogeny of the family Araceae as inferred from the nuclear ribosomal ITS data

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Abstract

The internal transcribed spacer regions of nuclear ribosomal DNA are widely used to infer phylogenetic relationships in plants. In this study, it was obtained the ITS sequences from 24 samples of Araceae in Iran, representing 3 genera: *Arum* L., *Biarum* Schott. and *Eminium* (Blume) Schott. Phylogenetic analyses were conducted by Bayesian inference and maximum Parsimony methods. Cladistic analysis of ITS dataset indicated that all species constituted a monophyletic clade, with no major subclades with robust support. *Eminium lehmani* Bge., *Eminium intortum* Banks and Sol. and *Eminium alberti* Rgl. were considered as outgroups. *Biarum* and *Arum* species were placed in a monophyletic clade as sister groups and *B. platyspatum* Bornm., *B. Carduchorum* Schott., and *B. straussi* Engl. were grouped in a monophyletic clade. *A. maculatum* L., *A. giganteum* Ghahreman, Some populations of *A. conophalloides* Schott and *A. virescense* Stapf. were placed in cluster I. Some populations of *A. virescence* and *A. conophalloides* were placed in cluster II, *A. kotschyi* Boiss. and *A. korolkowii* L. were placed in cluster III. All these three clades had poor supports. *Arum giganteum* Ghahreman was introduced as new species in Iran, but in *Arum* monograph it is mentioned as potentially equivalent to *Arum rupicola* Boiss. The data obtained from the molecular studies in this research could separate these two species and confirmed previous studies.

Key words: Cluster, Internal transcribed spacer, Iran, Monophyly, Phylogenetic relationship.

INTRODUCTION

The family Araceae includes 3790 species in 117 genera (Boyce and Croat, 2011). According to Chouteau *et al*. (2008), Araceae is one of the most important families of monocotyledons, and is found in a wide range of environments, from Arctic–Alpine (e.g., *Calla palustris* L.) to xerophytes (e.g., *Anthurium nizandense* Matuda). They are most diverse in the tropics, and have a large variety of life forms, from epiphytic to aquatic (Espindola *et al*., 2010).

Araceae has three genera in Iran, consisting of *Arum* L., *Biarum* Schott. and *Eminium* (Blume) Schott. Six species of *Arum* are: *Arum maculatum* L., *A. virescense* Stapf, *A. conophalloides* Schott., *A.kotschyi* Boiss. and Hohen., *A. korolkowii* L. and *A. giganteum* Ghahreman. *Biarum* consists of *Biarum cardochrum* Schott., *B. straussi* Engl. and *B. platyspathum* Bornm. *Eminium lehmani* Bge., *E. intortum* Banks and Sol. and *E. alberti* Rgl. belong to *Eminium*.

There is no doubt that the members of Araceae family had geographically great expansion in the Cretaceous (Friis *et al*., 2010). The nature of their fossils not only allows the strong calibration of DNA substitution rates, but also gives information about the previous ranges of certain clades (Nauheimer *et al*., 2012). The unique structure of the inflorescence is one of the most

significant features of this family, with small flowers emerging from the fleshy axis (spadix) subtended by a modified leaf (spathe) (Boyce, 1988). Cabrera *et al*. (2008) effectively settled the long-standing question of the relationships of the Araceae subfamily using a matrix of 102 aroid genera and 5188 aligned base pairs of chloroplast DNA. In order to convert such phylogeny information into a formal classification, it is ideal to compare and contrast them with phenotypic data so as to highlight the clades that are supported by distinctive morphological or anatomical synapomorphies and those that are supported by molecular synapomorphies. For example, Keating (2002) was able to interpret the morphological and anatomical data using the phylogeny of family presented by French *et al*. (1995), leading him to propose a new formal classification of the family. Bogner and Petersen (2007) introduced an updated interpretation of the classification of Mayo *et al*. (1997), which resulted from the comparison of morpho-anatomical data with French *et al*. (1995) molecular tree.

One of the most effective phylogenetic tools in utilizing the genomic region, which has proven as a useful character for the phylogenetic data analysis in angiosperms, is the internal transcribed spacer (ITS) region of 18S-26S nuclear ribosomal DNA (nrDNA). The length of the ITS region of flowering seed plants is highly uniform and effective to establish the taxonomic composition of numerous families and genera (Baldwin *et al*., 1995; Slugina *et al*., 2014). Mayo *et al*. (1997) discussed the most detailed modern taxonomy of the Araceae as a distinct family, excluding the duckweeds (the former Lemnaceae, now Araceae subfamily Lemnoideae). Cusimano *et al.* (2010) examined 113 aroid genera and 4494 aligned nucleotides which resulted from adding 11 genera to the 2008 molecular matrix presented by Cabrera *et al*. (2008) including sequences of six chloroplast DNA regions; *rbcL, matK,* partial *trnK* intron, partial *tRNA-Leu* gene, *trnL – trnF* spacer, and partial *tRNA-Phe* gene. They also investigated 81 morphological characters with regard to the molecular phylogeny, utilizing a developed version of the 1997 morpho-anatomical data set.

Expanding on this line of research, in the present study, we mainly aim to provide a phylogenetic hypothesis for Araceae family based on the sequences of Internal transcribed spacer region (ITS) of nrDNA which is suitable to display relationships at the infrageneric level. This would allow us to assess the validity of the current classification, and identify the phylogenic and taxonomic relationships within different species of Araceae in Iran.

MATERIALS AND METHODS

Taxon sampling

Plant material of 24 specimens, including 3 genera and 12 species of Araceae were collected from different localities across Iran, and some of them were chosen from herbaria in Iran (TARI, IAUH) (Table1). Table 2 lists all taxa used in this study, and summarizes sources, voucher specimen data, and GenBank accession numbers. *Eminium* was selected as the outgroup according to a previous study on the Araceae family conducted by Mansion *et al*. (2008).

Molecular analysis

Phylogenetic reconstructions were performed for 24 samples of Araceae (*Arum*, *Biarum*, and *Eminium*) in six regions of Iran plus accessions from the Genbank. In this study, we also used the ITS sequences of 23 species of *Arum* from the GenBank. The list of non-Iranian taxa used in our analysis along with the GenBank accession numbers are indicated in Table 2. Also, we used the ITS sequences of *Eminium* as outgroup. Total genomic DNA was extracted from either silica-gel dried leaves directly collected from the plants in wild, or herbarium specimens. Total DNA was extracted using the DNeasy Plant Mini kit (Qiagen, Germany). The entire ribosomal ITS region (ITS1+ 5.8 S + ITS2) was amplified using the primer pairs AB 101 (forward, 5'- ACG AAT TCA TGG TCC GGT GAA GTG TTC G- 3') and AB 102 (reverse, 5' - TAG AAT TCC CCG GTT CGC TCG CCG TTA C- 3') (Douzery *et al*. 1999), using the following PCR protocol: 35 cycles of 1 min denaturation (95 °C), 1 min annealing (51.5 °C), and 72 °C for 1.5 min, followed by 7-10 min final extension at 72 °C for the completion of primer extension.

Forward and reverse sequences were visually compared and edited, and then they were initially aligned, using Sequencer 4 software (Gene Codes Corporation, Ann Arbor, MI USA). In addition to our sequences, 23 ITS sequences from other taxa were taken from the Genbank (Table 2). All ITS sequences were assembled and aligned using MacClade 4 (Maddison and Maddison, 2010).

Maximum parsimony analysis (MP)

Parsimony analyses were implemented by employing PAUP version 4.0 (Swofford, 2002) using the following criteria: 100 heuristic search replicates, random stepwise addition of taxa, and tree-bisection reconnection (TBR) branch swapping. These parsimonious trees were used to calculate the consensus tree. Bootstrap analyses (BS) were applied to determine the clade support. BS of clades was calculated using PAUP with 100 replicates of heuristic searches, and randomly stepwise

Table 1. List of taxa investigated and voucher specimens.

No.	Species	Herbarium	Locality
1	*Arum maculatum* 69216	TARI	Iran, Prov. N. Mazandaran, ca 40 km on the road from Amol to Polur, 300m. Assadi & Shahsavari. 1991.
2	*A. maculatum* 19123	TARI	Iran, Prov. N. Gilan, Ardabil to Astara, east side Gardaneh Heyran,200-300m. Joudi. 2015.
3	*A. kotschyi* 27355	TARI	Iran, Prov. N. Mazandaran, Chalus-Vissar, 1600m. Mozaffarian. 1978.
4	*A. kotschyi* 27808	TARI	Iran, Prov. Azarbaijan, Assalem to Khalkhal after the pass near to Khalkhal, 2000m. Wendelbo & Assadi. 1987.
5	*A. kotschyi* 27910	TARI	Iran, Prov. Azarbaijan, ca20 km the pass to Ahar on road to Tabriz, 1700-1800m. Wendelbo & Assadi. 1978.
6	*A. kotschyi* 69151	TARI	Iran, Prov. N. Gorgan, Golestan forest, between Tangerah and Tangegol, 400m. Assadi & Shahsavari. 1991.
7	*A. virescens* 36826	TARI	Iran, Prov. Hamadan, Kuh-e-Alvand, 2700m. Assadi & Mozaffarian. 1981.
8	*A. virescens* 55285	TARI	Iran, Prov. N. Mazandaran, Pol-e-sefid, Bigining of the road, Sangdeh, 670m. Assadi & Maasoumi. 1986.
9	*A. virescens* 60066	TARI	Iran, Prov. N.Gilan, Manjil, Amarloo area, near Baresar, 1100m. Assadi & Shah Mohammadi. 1987.
10	*A. virescens* 64555	TARI	Iran, Prov. Hamadan, Kabodar-Ahang, Ghonairejeh, Yarumjeh bagh, Kuh-e-Boughati, 2200-2800m. Mozaffarian. 1988.
11	*A. conophalloides* 22222	IAUH	Iran, Prov. Hamadan, ca 20 km Nahavand, Kuh-e-Garo, 2600m. Joudi. 2015.
12	*A. conophalloides* 59955	TARI	Iran, Prov. W.Ghaharmahal-e-Bakhtiari, Sabz kuh, Ghahartag, 2350m. Mozaffarian. 1987.
13	*A. giganteum* 2558	TARI	Iran, Prov. Ilam. Islamabad-e-Gharb and Ilam,63 km to Ilam. Ghareman. 1983.
14	*A. giganteum* 68100	TARI	Iran, Prov. Isfahan, Pishkoh, 120km from Isfahan to Makkedin, 2500m. Hamzehee. 1990.
15	*A. giganteum* 71847	TARI	Iran, Prov. Ilam, Islam Abad to the West, 1600m Joudi. 2014.
16	*A. korolkowi* 50656	TARI	Iran, Prov. Khorasan, between Ghoochan and Darreh-Gaz, Tandooreh National Park, Shekarab, 2300m. Assadi & Maasoumi.1984.
17	*A. korolkowi* 55870	TARI	Iran, Prov. Khorasan,74km to Mashhad from Kalate-Naderi, 950m. Assadi & Maasoumi. 1986.
18	*Biarum platyspatum* 33333	IAUH	Iran, Prov. Lordgan, Javanmardi, Bag-e-Behzad, 1900-2000m. Assadi & Maasoumi.1991.
19	*B. carduchorum* 59948	TARI	Iran, Prov. Chaharmahal-e-Bakhtiari, Brojen Ardall, between Doupolan and Gandomkar, Gardaneh Kas-e-Kase, 2150m. Mozaffarian. 1987.
20	*B. straussi* 16447	TARI	Iran, Prov. Lorestan, Khalitabad, ca 40km S.E. or Aligodarz, 2300-2450m. Wendelbo & Assadi. 1978.
21	*Eminium lehmani* 28436	TARI	Iran, Prov. Semnan, Touran, Proteeted area, 3km from Chajan to Torud, 1100m. Ferintag & Mozaffarian.
22	*E. intortum* 16696	TARI	Iran, Prov. Lorestan, ca 40 km east of Kuh-e-Dasht, 1350m. Wendelbo & Mozaffarian. 1975.
23	*E. alberti* 55559	TARI	Iran, Prov. N. Gorgan, East of Maravetappe, Ghazanghayeh, 300m. Assadi & Massoumi. 1986.
24	*E. alberti* 96104	TARI	Iran, Prov. Khorasan, Esfarayen, Pelmis Mountain, 1450m. Emani. 2007.

Note: TARI= Herbarium of Research Institute of Forests and Rangelands, IAUH= Islamic Azad University Avicennia herbarium.

addition of taxa. Clades with a bootstrap value of 70% or more were considered as well supported clades.

Bayesian analysis (BA)

The BA analyses of the ITS datasets were performed using MrBayes version 3.0 (Huelsenbeck and Ronquist, 2001). In order to find the appropriate model of DNA substitution, the Maximum Likelihood criteria for datasets were determined by the Akaike information criterion (AIC; Akaike 1974) as implemented in the software ModelTest version 3.7 (Posada *et al*., 1998).

Modeltest

For the Maximum Likelihood (ML) and Bayesian Analysis (BA), the best fit of DNA substitution model was found. The Akaike information criterion and hierarchical likelihood ratio test (hLRT) were calculated based on the log likelihood scores of 56 models using the Modeltest 3.7 (Posada *et al*. 1998). In general, AIC

Table 2. List of non-Iranian taxa used in the analysis and their GenBank accession numbers.

Species	ITS GenBank accession number	Species	ITS GenBank accession number
Arum cylindraceum	GU067540.1	*A .hygrophilum*	GU067550.1
A. apulum	GU067541.1	*A. idaeum*	GU067553.1
A. balansanum	GU067542.1	*A. italicum*	GU067554.1
A. byzantium	GU067543.1	*A. jacquemontii*	GU067555.1
A. concinnatum	GU067544.1	*A. korolkowii*	GU067556.1
A. creticum	GU067545.1	*A. maculatum*	GU067558.1
A. cyrenaicum	GU067546.1	*A. palaestinum*	GU067551.1
A. dioscoridis	GU067547.1	*A. pictum*	GU067560.1
A. euxinum	GU067548.1	*A. purpureospathum*	GU067561.1
A. elongtum	GU067549.1	*A. rupicola*	GU067562.1
A. gratum	GU067552.1	*A. sintenisii*	GU067563.1
A. cylindraceum	GU067557.1		

was chosen (Posada 2008). For ITS spacer dataset, Likelihood settings from the best-fit model (TVM+G) were selected by AIC in the Modeltest 3.7 with the nucleotide frequencies as A = 0.2122, C = 0.3121, G = 0.3156, T = 0.1601, a gamma shape parameter of 0.7197, and an assumed proportion of invariable sites of 0.5106.

Maximum likelihood

Maximum likelihood analysis was performed on the basis of the results of the Modeltest in PAUP. The parameters of the best model, such as the base frequency, the mean relative substitution rates, proportion of invariable sites, and Gamma distribution shape were employed. The heuristic search and bootstrap were implemented as in parsimony analysis in PAUP, mentioned above.

Bayesian inference

Bayesian inference of the phylogenetic trees was analyzed by some parameters of the Modeltest, and was included in the analysis. The option was set up using 5,000,000 generations of Markov Chain Monte Carlo (MCMC) searches and a sample frequency of 1000. Saturation was reached after a burn-in of 1000 generations. The clade support was assessed using Bayesian posterior probabilities employing the Mr Bayes version 3.0 (Huelsenbeck and Ronquist, 2001).

RESULTS AND DISCUSSION

The data set of the ITS region included 776 characters with 637 constant positions within the ingroup while 122 characters were parsimony informative. The Bayesian 50% majority-rule consensus tree for ITS contained 11 internal nodes with a posterior probability (PP) of 1.0 (Figure 1). Strict consensus phylogeny trees with 256 steps resulted in the consistency index (CI) of 0.697 and retention index (RI) of 0.846. Using the data of Figure 2, *E. Intortum* 16696, *E. alberti* 55559, and *E. alberti* 96104 were chosen as the outgroup to form a separate clade, and *E. lehmani* 28436 was taken from another group, which was sister to other species. The ingroup included two main clades, labeled A and B (PP=1; BS 100%). Clade A was divided into 2 subclades (PP=1; BS 100%), including subclade AII with *Biarum platyspatum* 33333 and subclade AI, which involved 2 species of *B. Straussi* 16447 and *B. Carduchorum* 59948. Clade B consisted of the *Arum* species from Iran and the GenBank. Clade B was divided into 2 main groups. Most of the Iranian species involved in the ITS sequence data set were classified in cluster I, II, and III with poor PP support. Cluster I consisted of *A. maculatum*, *A. conophalloides*, *A. giganteum* and *A. virescence*. *A. maculatum* 19123 and *A. maculatum* 69216 formed sister groups (PP = 0.87), but *A. maculatum* from the GenBank was not placed in the same clade with the *A. maculatum* from Iran. In this cluster, *A. giganteum* with its own unique characters was placed in different locations. Cluster II had some species, including *A. virescence* 36826*, A. virescense* 55285 and *A. Conophaloides* 59955. Posterior probability value of 0.78 supports the classification presented in cluster II (Fig. 2) and indicate that these species are closely related together. Cluster III consisted of *A. kotschyi* and *A. korolkowii* from Iranian species along with *A. rupicola*, *A. korolkowii,* and *A. jacquemonti* from the GenBank. Cluster B III was divided into three groups: group BIII1 with a single species of *A. rupicola,* and group BIII2 with two species *A. korolkowii* and *A. jaqumonti* (PP = 0.61 BS 55%) from the Genebank and group B III3 with *A. kotschyi* and *A. korolkowii*

Figure 1. Iran map showing the distribution of Araceae plants from 24 locations in Iran. (Google map, 2015).

from Iran (PP=0.74). Grade IV included the Gene bank species and had a variety of sub-clusters and different divisions.

Among species of BIV1 are 2 monophyletic subclades: BIV1a: (*A. cylindraceum*, *A. purpureospathum,* and *A. balansanum* (PP = 0.99; BS 82%) and *A. euxinium* and *A. cyrenaicum* from the GenBank), while BIV1b*: A. creticum* and *A. idaeum* were placed in a single group (PP = 0.94; BS 84%). *Arum elongatum* was placed in an independent group. All species of BIV2, including *A. concinnatum, A. italicum, A. maculatum, and A. byzantinum* from the GenBank diverged from each other in several situations. It seems that this group is not a monophyletic group. Whereas *A. concinnatum* is a sister group with all *Arum* species. *A. hygrophilum* and *A. gratum* together lied in a group. Best support was observed for relationship between clades A and B as two sister-groups (PP = 1; BS = 100%). It means that the species of clade A (Biarum species) and clade B (Arum species) have formed monophyletic groups. Subclades AI and AII were also well supported (PP = 1; BS 100%) and all speces of Biarum in clade I were placed in a monophyletic clade.

We provided the first phylogenetic analysis of the Araceae from Iran. Phylogenetic relationships between the Araceae genera in this study intensely organized the species of *Arum*, *Biarum* into a supported monophyletic group, and *Eminium* was as an outgroup (except *E. lehmani*). The results of cladistic analysis of the phylogenetic relationships among *Arum* species showed its monophyletic origin, and that *Biarum* was its sister group. According to the earlier studies, all species examined in our research were also nested inside Arae clade within the Mediterranian clade, comprising *Arum*, *Dracunculus*, *Biarum*, *Helicodiceros,* and *Eminium*, which diverged allopathically in a region encompassing tropical Asia and Anatolia during the Late Eocene (Mansion *et al*., 2008). There are two subgenera: *Gymnomesium* and *Arum*. *Gymnomesium* has only one species: *A. pictum* and is the first branching lineage of *Arum* (as shown previously by Mansion *et al*., 2008), and *Arum* which includes two sections and six subsections (Boyce, 1988) (Table 3). All species of this study were placed in the subgenera of *Arum*.

The results of the present study are consistent with those of Espindola *et al*. (2010), showing that *A. jacquemontii* and *A. korolkowii* were nested within the *A. rupicola*. Therefore, our study further supported their findings. In Iran, *A. korolkowii* and *A. kotschyi* are similar in their morphological characteristics and molecular analyses (Table 3).

Arum conophaloides 59955 and two population of

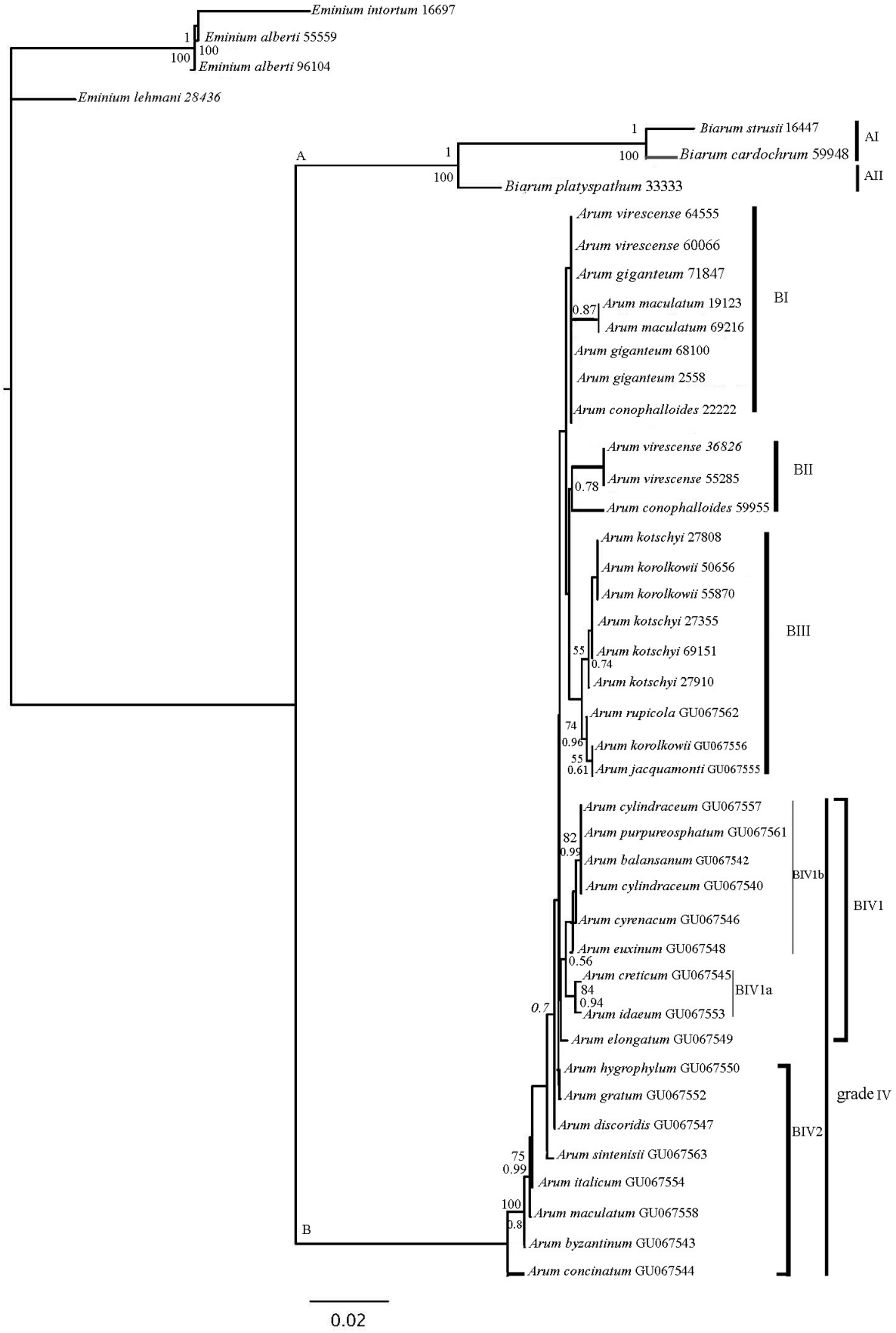

Figure 2. Bayesian consensus tree for combined data (Numbers above branches are Bayesian posterior probabilities and Numbers below branches are maximum likelihood percentage of the bootstrap values).

Table 3. Infrageneric classification of *Arum* (*A. giganteum, A. kotschyi* and *A. virescence* have not neen reported as separated species and introduced as *A. rupicola* varieties).

Subgenus	Section	Subsection	Species
Gymnomesium			*A. pictum* L.f.
			A. byzanyinum Blume.
			A. concinnatum Schott.
Arum	Arum		*A. italicum* Mill.
			A. maculatum L.
			A. megoberibi Lobin., M.neumann., Bogner. & P. C. Boyce.
	Dioscoridea	Alpina	*A. cylindraceum* Gasp.
			A. lucanum Gavara. & Grande.
		Discroochiton	*A. apulum* (Carano) P.Boyce
			A. balansanum R. R. Mill.
			A. besserianum Schott.
			A. cyrenacium Hruby.
			A. elongatum Steven.
			A. gratum Schott.
			A. hainessi Reidl.
			A. nigrum Vell.
			A. orientale M.bieb.
			A. purpureospathum P. C. Boyce.
			A. sintenissi P. C. Boyce.
		Tenuifila	*A. jacquemontii* Blume.
			A. korolkowii Reidl.
			A. rupicola Boiss.
		Hygrophila	*A. euxinum* R. R. Mill.
			A. hygrophylum Boiss.
		Poiciloporphyrochiton	*A. dioscoridis* Sm.
			A. palastinum Boiss.
		Cretica	*A. creticum* Boiss. & Heldr.
			A. idaeum Coust. & Gand.

Note: Adopted from Boyce (1988).

A. virescense formed a monophyletic clade, which was supported by morphometric research findings (Joudi *et al*., 2016). Accordingly, *A.virescens, A. conophaloides*, *A. kotschyi,* and *A. korolkowii* constructed a monophyletic group that supported the monophyly of *Arum* species in the Arae clade.

Arum giganteum is separated from *A. rupicola* phylogenetically, and is widely distributed in Iran. Studies on morphological differences have separated this species, and confirmed earlier findings by identifying the synapomorphies (Joudi *et al*., 2016).

The placement of *A. maculatum* samples in cladeI was unexpected while according to Boyce, *Arum* subsection *A. maculatum* was placed with *A. concinnatum, A.italicum* and*, A. byzantinum* (BPP=0.99). In another research conducted by Espindola *et al.* (2010), *A. maculatum* was placed in different positions. Also, *A. maculatum* was placed in different locations based on the trnK, trnL, ndh, and trnT molecular markers data, which confirmed our results (Espindola *et al*., 2010). These results can explain that *A. maculatum* possesses some characters that caused it to be placed in various positions in the phylogenetic tree.

CONCLUSION

The internal transcribed spacer of the nuclear region is a widely used molecular marker for the reconstruction of evolutionary patterns in the plant kingdom. This research indicated that this marker can potentially be a valuable tool to identify the genus and species. Based on the results of cladistics analysis, the phylogenetic relationships among Araceae family confirmed the morphological investigations with a high level (Joudi *et al*., 2016). Furthermore, *Arum giganteum* has been introduced as a new species in Iran (Ghahreman, 1983) with its exclusive morphological features e.g. the whole size of the plant, size of the leaves and inflorescence that could not be seen in other *Arum* species. In Arum Monograph it is mentioned as potential equivalent to *A. rupicola.* Therefore, more research is needed to separate these two species. Boyce (1988) had report-

ed (in a Monograph) if any features can separate these two species, then *A. giganteum* could be reported as a new species from Iran. Finally, *A. giganteum* can be introduced as a new species with morphological (Joudi *et al*., 2016) and molecular features and confirmed Ghahreman´ s findings (1983).The limiting factor is that this marker is not able to diverge some groups. Therefore, a more differentiating molecular marker such as low copy number nuclear genes and intergenic nuclear spacers could potentially be more helpful. However, ITS marker could detect the relationships between some of the major groups, although the position of some groups in phylogenetic tree remained unresolved. According to our findings, we assume that further research regarding the unresolved nodes within the *Arum* genus would provide important insights into the relations between the *Arum* species. The combined approaches, including the application of different markers, would increase the resolutions and support the *Arum* clades.

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