C. R. Biologies 339 (2016) 147–151

Contents lists available at ScienceDirect

Comptes Rendus Biologies

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Biodiversity/Biodiversité

Genetic diversity and relationships detected by ISSR and RAPD analysis among *Aethionema* species growing in Eastern Anatolia (Turkey)

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ARTICLE INFO

Article history:
Received 12 February 2016
Accepted after revision 29 February 2016
Available online 21 March 2016

Keywords:
Aethionema species
Genetic diversity
ISSR
RAPD

ABSTRACT

In this study, Random amplified polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) analysis were used to examine the genetic relationships among eight *Aethionema* species (*Aethionema caespitosum*, *A. arabicum*, *A. cordatum*, *A. fimnraitum*, *A. armenum*, *A. speciosum* supsp. *speciosum*, *A. memraneceum*, *A. grandiflorum* var. *grandiflorum*) growing in the wild in Eastern Anatolia, Turkey. Fourteen RAPD primers and 7 ISSR primers were used. The UPGMA cluster was constructed using a combination of data from RAPD and ISSR markers. The *Aethionema* species were classified into two major groups. The similarity matrix values of between 0.182 (*A. cordatum*, *A. speciosum* supsp. *speciosum*) and 0.927 (*A. grandiflorum* var. *grandiflorum*, *A. cordatum*). High genetic variations among *Aethionema* species growing in the wild in Eastern Anatolia, Turkey may reveal differences in their origin. The present study suggests that both RAPD and ISSR analysis are useful for the differentiation of the *Aethionema* species.

1. Introduction

Aethionema R. Br. (Brassicaceae) is a taxonomically complex genus, and a few macromorphological characters are available for species delamination. *Aethionema* is a genus of flowering plants, within the family Brassicaceae, subfamily Brassicoideae. They originate from limestone mountainsides in Europe and Western Asia, especially Turkey. Life duration (annular/perennial) and fruit morphology are of impotence at the species level in *Aethionema*. The genus has its center in Turkey, and outside Anatolia its population declines very rapidly [1]. The Turkish flora is comprised of about 41 *Aethionema* species, of which 20 species are endemic to Turkey [2–4].

Most species of the Brassicaceae develop fruits in which seeds are released through a process termed fruit dehiscence. Some genera in Brassicaceae develop indehiscent fruits that do not release ripe seeds. The genus *Aethionema* is the sister group to all other extant Brassiceceae, some species of *Aethionema* are heterocarpic, meaning that they develop as both dehiscent and indehiscent.

Earlier classifications and evaluations of the genus *Aethionema* were done primarily based on phenotypic expressions of the plants such as growth form, leaf morphology, fruit properties such as color, length of the styles in female flowers and other agronomical characters, but information from these environmentally influenced morphological and physiological characteristics are not

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http://dx.doi.org/10.1016/j.crvi.2016.02.006
1631-0691/

sufficient to identify *Aethionema* because the differences between them are often subtle and misleading. Hence, recent advances in the field of molecular biology and gene technology have been successfully used for the evaluation of the genetic relationship between plants species in addition to their morphological characters [5–10]. The RAPD and ISSR techniques, when compared to AFLP and SSR, are fast and easy, since they do not require knowledge of the sequences of the markers and can produce abundant polymorphic fragments. So far, there have been few attempts to study the genetic variation in *Aethionema species* using molecular analyses [11,12]. However, no studies have been conducted to assess the genotypic differences in most of *Aethionema species*, including those encountered in Eastern Anatolia (Turkey). Therefore, this study aimed at determining the genetic relationship among *Aethionema caespitosum, Aethionema arabicum*, *Aethionema cordatum*, *Aethionema fimnraitum*, *Aethionema armenum*, *Aethionema speciosum* supsp. *speciosum*, *Aethionema memraneceum*, *Aethionema grandiflorum* var. *grandiflorum species* growing in Eastern Anatolia using RAPD and ISSR technologies. The information obtained will be useful in the genetic analysis of *Aethionema species.*

2. Materials and methods

2.1. Plant material

A sample collection of eight species of *Aethionema* (*A. caespitosum*, *A. arabicum*, *A. cordatum*, *A. fimnraitum*, *A. armenum*, *A. speciosum* supsp. *speciosum*, *A. memraneceum*, *A. grandiflorum* var. *grandiflorum*) was collected at the flowering stage from different locations in the vicinity of Erzurum, Bayburt, located in Eastern Anatolia, Turkey (Table 1). Plant materials were ground in a grinder equipped with a 2-mm diameter mesh. The powdered plant material was then used for DNA extraction. The voucher specimen has been deposited at the herbarium, Department of Biology, Atatürk University, Erzurum, Turkey (Table 1). Plants were collected around Erzurum in 2011–2012 and deposited at ATA (Atatürk University Herbarium).

2.2. DNA extraction

Genomic DNA was extracted from powdered plant materials using a method described by Sunar et al. [13]. The purity and quantity of genomic DNA was determined spectrophotometrically and confirmed using 0.8% agarose gel electrophoresis against known concentrations of unrestricted lambda DNA.

2.3. RAPD amplification

Forty-five primers had been attempted to generate RAPD profiles. Fourteen of these primers were selected: they produced amplicons with all of the *Aethionema* species tested, which were used in further studies based on the results of the preliminary tests (Table 2). PCR amplification reactions were carried out in a final reaction mixture volume of 30 μl, containing 10 × Buffer 3.0 μl, dNTPs (10 mM) 1.2 μl, magnesium chloride (25 mM) 1.2 μl, primer (5 μM) 2.0 μl, Taq DNA polymerase (5unit) 0.4 μl, water 19.2 μl sample DNA 3.0 μl (100 ng/μl). The thermal cycler (Eppendorf Company) was programmed as 2 min at 95 °C; 2 cycles of 30 s at 95 °C, 1 min at 37 °C, 2 min at 72 °C; 2 cycles of 30 s at 95 °C, 1 min at 35 °C, 2 min at 72 °C; 41 cycles of 30 s at 94 °C, 1 min at 35 °C, 2 min at 72 °C; followed by a final 5-min extension at 72 °C, then brought down to 4 °C.

2.4. ISSR amplification

A total of 34 ISSR primers were tested for DNA amplification. Seven primers were chosen for ISSR analyses of genetic diversity, based on band reproducibly (Table 2). PCR reactions were carried out using a single primer at a time, in a 25-mL reaction mixture containing 40 ng of template DNA, 1 × reaction buffer, 200 mM of each of the four dNTPs, 1 U of Taq DNA polymerase, 1.5 mM of magnesium chloride and 0.5 mM of primer. Amplification was performed using a thermal cycler programmed for an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 1 min at the specific annealing temperature and 1 min at 72 °C, ending with a final extension step of 7 min at 72 °C. The PCR products of ISSR markers were resolved by electrophoresis on 1.5% agarose gels.

2.5. Electrophoresis

The PCR products (27 μl) were mixed with a 6 × gel loading buffer (3 μl) and submitted to agarose (1.5% w/v) gel electrophoresis in a 0.5XTBE (Tris-Borate- EDTA) buffer

Table 1
Aethionema species tested in this study.

Aethionema species tested in this study	Herbarium number of the Vouchers	Locality	Status	Altitude
Aethionema caespitosum (Boiss.) Boiss.	9848	Kop mountain	End	2100
Aethionema arabicum (L.) Andrz. Ex DC	9849	Erzurum	–	2250
Aethionema cordatum (Desf.)	9850	Kop mountain	–	2180
Aethionema fimnraitum Boiss.	9851	Erzurum Hınıs	Ir–Tur	2200
Aethionema armenum Boiss.	9852	Erzurum Hınıs	Ir–Tur	2350
Aethionema speciosum Boiss. & A. Huet	9853	Kop mountain	Ir–Tur	2200
Aethionema memraneceum (Desu.) DC	9854	Erzurum Hınıs	–	2300
Aethionema grandiflorum Boiss. & Halenvar. *grandiflorum*	9855	Erzurum Tekman	–	2100

Table 2
Details of banding pattern revealed through RAPD and ISSR primers (R = A, G; Y = C, T).

Markers	Primer/primer combination	Sequence (5′–3′)	Length of amplified bands	No. of bands	No. of polymorphic bands	Polymorphism ratio (%)
RAPD	A-1	AGTCAGCCAC	400–1800	7	6	85.7
	B-20	GGACCCTTAC	750–2200	8	8	100
	C-10	TGTCTGGGTC	300–2000	5	5	100
	OPBA-03	GTGCGAGAAC	500–2500	11	10	90.9
	OPBB-03	TCACGTGGCT	500–1600	9	8	88.8
	OPA-4	AATCGGGCTG	400–2300	8	8	100
	OPA-13	CAGCACCCAC	600–2000	6	5	83.3
	OPK-04	CCGCCCAAAC	750–2400	9	7	77.7
	OPC-02	GTGAGGCGTC	250–2500	7	7	100
	OPK-19	CACAGGCGGA	400–2100	12	12	100
	OPL-15	AAGAGAGGGG	500–2600	6	6	100
	OPY-13	GGGTCTCGGT	300–2500	10	9	90
	OPD-20	ACCCGGTCAC	400–2800	7	7	100
	OPH-10	CCTACGTCAG	600–2300	9	8	88.8
	Total		250–2800	113	105	92.9
ISSR	UBC810	(GA8)T	400–2000	10	9	91.66
	UBC842	(GA)8YG	500–2400	7	7	100
	UBC868	(GAA)	300–1800	8	7	87.5
	UBC818	(CA)8G	600–3000	12	10	83.3
	UBC825	(CA)8T	500–2600	8	8	100
	UBC808	(AG)8C	400–2500	6	5	83.3
	UBC811	(GA)8C	750–2000	7	7	100
	Total		300–3000	58	53	91.4

at 70 V for 150 min. The gel was stained in an ethidium bromide solution (2 μl EtBr/100 ml 1 × TBE buffer) for 40 min and visualized under UV in Bio Doc Image Analysis System with Uvisoft analysis package (Cambridge, UK).

2.6. Data analysis

PCR products were scored as presence (1) and absence (0) of band for each genotype and analyzed. Data were used to calculate a Jaccard (1908) similarity index, from which a dendrogram was constructed on the basis of this matrix by the UPGMA (unweighted pair group method using arithmetic average) method. Statistical analyses were performed using SPSS. All of the experiments in this study were repeated at least twice.

3. Results

Forty-five RAPD primers were initially screened against *Aethionema* species. Fourteen primers could produce a total of 113 distinct reproducible bands with an average of 8.1 bands per primer. The sizes of the amplified products ranged from 250 to 2800 bp. Of the 113 bands obtained, 105 (92.9%) were polymorphic. The number of polymorphic bands detected with each primer ranged from 5 (primer C10) to 12 (primer OPK-19).

A dendrogram constructed according to RAPD data (Fig. 1) of *Aethionema* species allowed us to divide them into two main clusters. The first cluster included *A. caespitosum*, *A. cordatum*, *A. speciosum* supsp. *speciosum*, A. *armenum*. The second one included *A. arabicum*, *A. fimnraitum*, *A. memraneceum*, *A. grandiflorum* var. *grandiflorum*. The greatest similarity was observed between species *A. fimnraitum* and *A. memraneceum* (0.185), the greatest dissimilarity was observed between species *A. caespitosum* and *A. fimnraitum* (0.948).

Seven ISSR primers showing reproducible and polymorphic patterns were chosen for cultivar identification and generated a total of 58 bands, with an average of 8.3 bands per primer. The size ranged from 300 to 3000 bp. Of the 58 bands produced, 53 (91.4%) were polymorphic.

The number of polymorphic bands detected with each primer ranged from5 (primer UBC808) to 10(primer UBC818). A dendrogram that was constructed according to ISSR data of seven *Aethionema* species allowed us to divide them into two main clusters (Fig. 2). The first cluster included *A. memraneceum*, *A. fimnraitum*, *A. armenum*, *A. grandiflorum* var. *grandiflorum*, *A. arabicum*. The second cluster included *A. cordatum*, *A. speciosum* supsp. *speciosum*, *A. caespitosum*. The greatest similarity was observed between species *A. memraneceum* and *A. fimnraitum* (0.216), and the greatest dissimilarity was observed between species *A. memraneceum* and *A. caespitosum* (0.931).

The combined data generated from RAPD and ISSR marker analyses a dendrogram was constructed (Fig. 3). Eight *Aethionema* species were separated into two distinct clusters, each one with two species. The first cluster included *A. cordatum*, *A. speciosum* supsp. *speciosum*, *A. caespitosum*, *A. arabicum*. The second cluster included *A. fimnraitum*, *A. memraneceum*, *A. grandiflorum* var. *grandiflorum*, *A. armenum*. The combined analysis revealed that similarities of the species ranged from 0.182 (*A. cordatum*, *A. speciosum* supsp. *speciosum*) to 0.927 (*A. grandiflorum* var. *grandiflorum*, *A. cordatum*).

4. Discussion

The species of *Aethionema* tested in this study showed the presence of two clusters. Each cluster is represented by a different species of *Aethionema*, except for one cluster, which is divided into two subclusters and includes

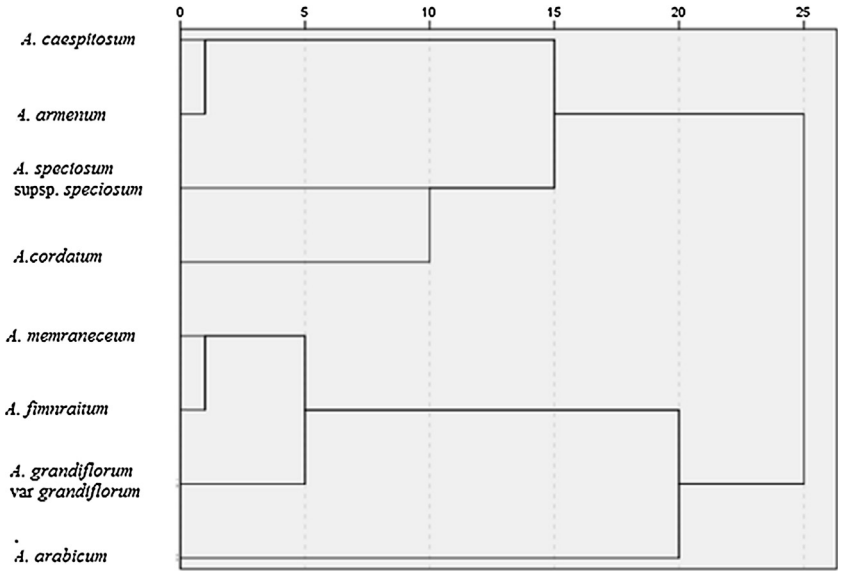

Fig. 1. RAPD marker-based UPGMA clustering for eight *Aethionema* species.

A. caespitosum, *A. cordatum*, *A. speciosum* supsp. *speciosum*, *A. armenum* and *A. arabicum*, *A. fimnraitum*, *A. memraneceum*, *A. grandiflorum* var. *grandiflorum*, which were the most genetically related species.

Both ISSR and RAPD profiles have been used to study phenotypic and genetic diversity in many plant species [11]. There have been a few attempts to study the genetic variations among the *Aethionema* species using molecular analysis [12,14], but none with the species tested in this study, except *A. arabicum*, *A. grandiflorum.*

Earlier studies using nuclear ribosomal DNA sequences (rDNA ITS) and ribosomal protein S 16 in the chloroplast genome (rps16) techniques showed genetic variations among *A. carneum*, *A. semanensis*, *A. umbellatum*, *A. arabicum* Andrz. Ex DC., *A. elongatum* Boiss., *A. grandiflorum* Boiss. & Hohen., *A. saxatile R. Br.*, *species*. Khosravi et al. [12] reported little genetic variations among these species. However, molecular markers such as RAPD, ISSR, AFLP, SSR show considerable differences among species and genotypes. The RAPD and ISSR techniques when compared to AFLP and SSR are fast and easy, since they do not require knowledge of the sequences of the markers and can produce abundant polymorphic fragments Thus, RAPD- and ISSR-based molecular markers were able to distinguish between different species. This paper is the first report indicating the relationships between *Aethionema* species growing in Eastern Anatolia using RAPD and ISSR techniques. In

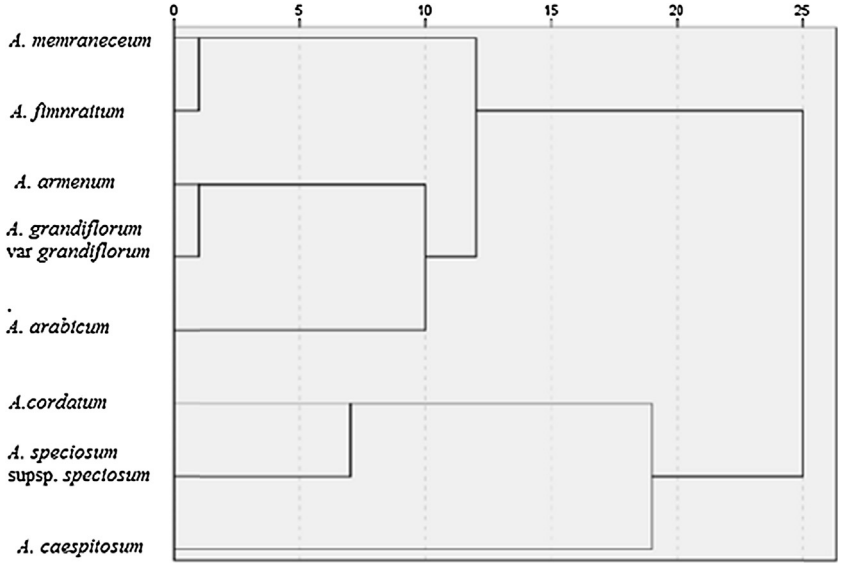

Fig. 2. ISSR marker-based UPGMA clustering for eight *Aethionema* species.

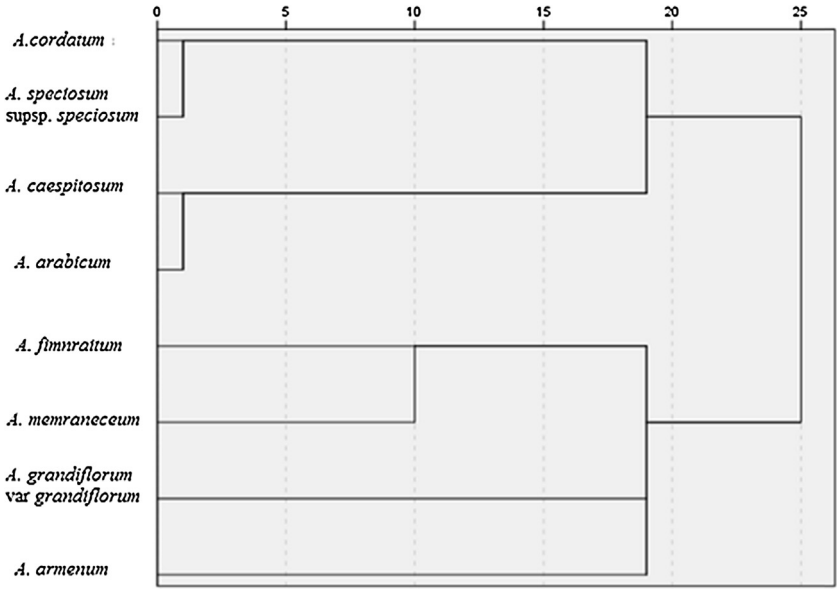

Fig. 3. RAPD and ISSR marker-based UPGMA clustering for eight *Aethionema* species.

conclusion, the results demonstrated that RAPD and ISSR analyses are useful for the differentiation of the *Aethionema* species tested in the present study.

However, additional phylogenetic studies using chloroplast or mitochondrial gene sequences or appropriate nuclear genes like ITS of nrDNA sequences can be helpful to reevaluate the systematic positions of these species.

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