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Molecular and ecological study of *Eryngium* species in Syria

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Abstract: *Eryngium* L. species growing in Syria were characterized using morphological, geographical and molecular analyses (IRAP and RAPD). Eight *Eryngium* L. species have been determined to exist in Syria. *E. glomeratum*, *E. campestre* and *E. falcatum* were found to grow in the mountain regions. *E. creticum* and *E. desertorum* were found to grow in variant environments: mountains, semidesert and saline environments, which indicate their wide range of adaptation and tolerance to abiotic stresses.
E. maritimum was the only species found to grow on the coastal sandy beaches suggesting that it is adapted to sandy, saline and humid environments. The two PCR-based techniques showed that *E. pussilum* and E. *billardieri* were the most distal to all other species. *E. pussilum* is found to grow in Leftaya region, which is a swamps area similar to the typical habitat for American Eryngiums. The most related species were *E. glomeratum* and *E. campestre*, which were mainly found in mountainous regions, followed by *E. desertorum*, *E. falcatum*, and *E. creticum*.

Key words: *Eryngium*; IRAP; RAPD

Introduction

The genus *Eryngium* represents a wide range of species adapted to harsh environmental conditions such as drought, salinity and non-optimal temperatures in what is categorized as abiotic stresses. Syria, located in the Eastern Mediterranean region, is well known for its diverse environmental conditions besides the fact that it is considered as a center of origin, and biodiversity (Zohary 1962, 1973) for many crops and fruit trees (wheat, barley, lentil, chickpea, olive, almond, pear, plum, Pistachio, ect). It is estimated that the Syrian flora includes about 3150 species arranged in 919 genuses in 133 families (Barkoudah et al. 2000).

Many studies have been carried out to investigate the genus *Eryngium* L. (Apiaceae-subfamily Saniculoideae) (O'Leary et al. 2004; Wörz 2004; Clausing et al. 2000; Gaudeul et al. 2000). The genus includes about 250 species in Eurasia, North Africa, North and South America and Australia (Pimenov & Leonov 1993; reviewed in Wörz 2004).

Eryngium L. species are known for their importance in the field of medicinal plants research. Species such as *E. expansum*, *E. pandanifolium*, *E. rostratum*, *E. vesiculosum* (Brophy et al. 2003) and *E. bourgatii* (Pala-Paul et al. 2005) have been studied to assess essential oils. Other *Eryngium* L. species have been studied to evaluate the anti-inflammatory and antinociceptive activity (Kupeli et al. 2006).

Genetic variation studies of *Eryngium* have also been conducted in several parts of the world (Clausing et al. 2000; Gadeul et al. 2000; Andrada et al. 2001). The Old World of *Eryngium* L. species that grow mostly in regions with a Mediterranean type climate have been investigated by Wörz (2004). According to Wörz (2004), there are two centers of diversity: one in the Western Mediterranean (Iberian Peninsula, Morrocco) and the other one in South-West Asia.

Eryngium L. species in Syria offer a rich source of genetic material regarding the adaptation to extreme environmental conditions and show great potential as medicinal plants. Investigating such species provides great source of genetic information regarding adaptation to harsh environments. This paper aims to investigate the geographical distribution, morphological characterization and the phylogeny of *Eryngium* L. species in Syria. The current work stamps *Eryngium* L. species in Syria on the geographical distribution map of *Eryngium* species in the world and enriches the molecular data available currently in the literature.

Material and methods

Regions of collection

Plants of the family *Apiaceae,* that belong to the genus *Eryngium,* were collected during the growing season in 2006 from road and field sides in central, Northern, Southern, Western, and coastal regions of Syria (Fig. 1). The main collection sites are summarized in Table 1.

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Table 1. Collection sites information and observed *Eryngium* L. species along with major plant genera.

Collection site	Location	Altitude (m)	Rainfall (mm)	Major plant genera	*Eryngium* species
1– Abdul Aziz mountain	North East	900	450	*Crataegus*, *Pinus*, *Echium*, *Hordium*, *Sinapis*	*E. creticum* *E. desertorum*
2 – Raka	North Center	650	450	*Convolvulus*, *Anchusa*, *Solanum*, *Astragalus*, *Alyssum*	*E. creticum* *E. desertorum*
3 – Aljaboul	North West	650	250–300	*Tamarix*, *Frankena*, *Artimisia*, *Phragmites*, *Peganum*	*E. creticum* *E. desertorum*
4 – Arab Al-shateh	North West	Sea shores	1000	*Plantago*, *Euphorbia*, *Kakile*, *Pancratum*,	*E. maritimum*
5 – Slunfa	West	1800	1000	*Quercus*, *Juniperus*, *Cedrus*, *Ruscus*, *Vibrum*	*E. falcatum* *E. creticum*
6– Leftaya	West Center	200	800	*Taraxacum*, *Anthemis*, *Geranium pharagmities urginia*	*E. pussilum* *E. creticum*
7 – Palmyra	Center	750	170	*Cotandia*, *Trigonella*, *Peganum*, *Achillea*, *Alhagi*	*E. creticum* *E. desertorum*
8 – Hush Arab	South West	1300	450	*Crataegus*, *Pronus*, *Anagalis*, *Cardaria*, *Euphorbia*	*E. creticum* *E. desertorum*
9 – Surghaya	South West	1300–1400	600	*Crataegus*, *Pronus*, *Malva*, *Aegilops*, *Trifolium*	*E. creticum E. glomeratum* *E. campestre*
10 – Erna	South West	1100	650	*Morus*, *Crataegus*, *Pronus*, *Ferula*, *Centauria*	*E. creticum* *E. billardieri*
11 – Aldumeer	Center	750	120	*Diplotaxis*, *Onobrychis*, *Alhagi*, *Achillea*, *Malva*	*E. desertorum*
12 – Bosra Al-hareer	South	800	250	*Echaballium*, *Echinopis*, *Centhauria*, *Sinapis*, *Bromus*	*E. creticum* *E. campestre*
13 – Sweida, Al-jabal	South	1100	450	*Carthamus*, *Trifolium*, *Lamium*, *Centauria*, *Aegilops*	*E. creticum* *E. desertorum*

Seed collection and specimen sampling

Eight *Eryngium* L. species have been investigated for morphological characterization and molecular studies. The species under study are: *E. maritimum, E. creticum, E. desertorum, E. glomeratum, E. campestre, E. billardieri, E. pussilum, E. falcatum*. Twenty plants from each species were characterized during May and July 2006 in the aforementioned regions of collection. Seeds were collected during August and September of 2006.

Identification of plants

The morphological characterization and identification of plants covered the following traits: plant height, morphology of basal leaves, head diameter, number of bracts, seeds length and shape, type of branching and flowering period. Final species identification was determined using the following references (Wörz 2004; Pimenove and Leonov 1993; Mouterede 1983; Zohary 1973).

Plant material and DNA Extraction

Total genomic DNA of collected plants was extracted from leaf material of each of the *Eryngium* L. species under study. DNA extraction was conducted following Leach et al (1986) protocol. Total DNA concentration was detected by Spec-

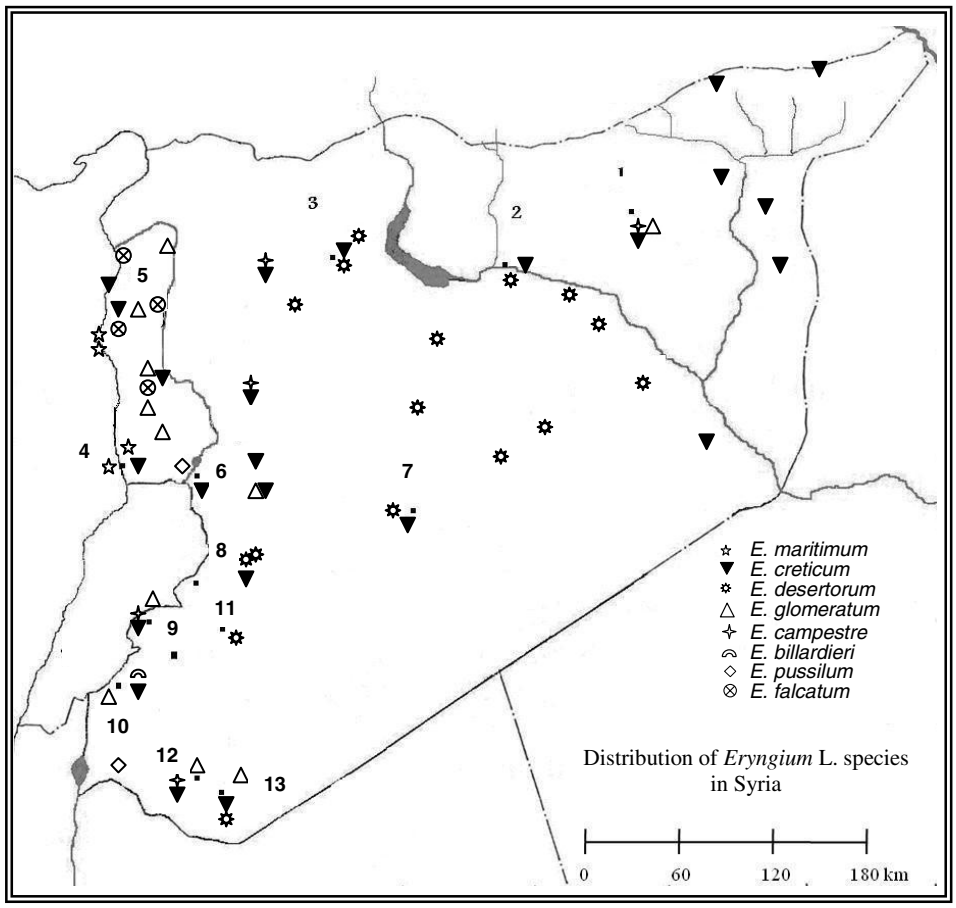

Fig. 1. The distribution map of *Eryngium* L. species in the Syrian Arab Republic. Numbers correspond to collection sites (refer to Table 1).

trophotometer (Gene quant, Amersham Biosciences, USA) and the concentration was adjusted to 20 ng/μL.

Inter-retrotransposon amplified polymorphism (IRAP) procedure

Nine IRAP primer combinations (Table 2) were synthesized using the PolyGen DNA synthesizer (PolyGen DNA-Synthesizer, Germany), and the iCycler PCR machine (BIO-RAD, USA) was used for the amplification of total genomic DNA. The amplification reactions of total genomic DNA were performed in 25μL reaction volumes, containing half volume of Bio-Rad super mix, 2.5 μM of each forward and reverse primers and 75 ng of DNA template.

The cycle program included an initial 2 min denaturation at 95℃, followed by 35 cycles consisting of 1 min at 95℃, 1 min at 40.5℃ or 45℃ depending on the tested primer combination (Table 2) over which the annealing time was increased 0.3 sec per cycle, and 2 min at 72℃ with a final extension at 72℃ for 10 min. IRAP fragments were separated on 2.5% agarose gels in 1X TAE buffer, stained with ethidium bromide (10 mg/mL) and subjected to a UV transilluminator using a (BIO-RAD Documentation System, USA.). Each primer combination was tested on the eight *Eryngium* L. samples and polymorphisms were compared.

RAPD procedure (Random Amplified Polymorphic DNA)

Thirty nine RAPD primers (Table 3) from Operon Technologies, Inc (OP; Almeda, California, USA) and the University of British Columbia (UBC; Vancouver, BC, Canada) were used for the amplification of total genomic DNA using the iCycler PCR machine (BIO-RAD, USA). The amplification reactions of total genomic DNA were performed in 25μL reaction volumes, containing half volume of Bio-Rad super mix, 2.5 μM of each primer and 50 ng of DNA template. The cycle program included an initial 4 min denaturation step at 94℃, followed by 45 cycles of 30 sec at 94℃, 60 sec at 36℃ and 90 sec at 72℃, and the program ends with a final extension at 72℃ for 6 min. RAPD fragments were separated electrophoretically on 1.5% agarose gels in 1X TAE buffer, stained with ethidium bromide (10mg/mL), and subjected to a UV transilluminator using a (BIO-RAD Documentation System, USA.). Each primer was tested on the eight *Eryngium* L. samples and polymorphisms were compared.

Data analysis

The presence or absence of each single fragment was coded by 1 or 0, respectively. IRAP and RAPD data were clustered and a dendograms based on percent disagreement values (PDV) were generated using the Unweighted Pair Group of Arithmetic Means (UPGMA) available in STATISTICA 6.1 package (StatSoft, 2003). Bootstrap values were obtained using PAST programme (1.94b version), available free online (Hammer et al. 2001).

Results

Morphological characterization

Eryngium L. species investigated in the current study were characterized morphologically by covering several traits: Plant height, type of basal leaves, stem branching, head diameter, number of bracts and seed length of each of the eight studied species are illustrated in

Table 2. IRAP primers pairs, sequences, literatures and corresponding annealing temperature (Ta).

Code	Primer pair	Sequence	Source	Ta
1	IRAP-TDK1 F IRAP-TDK1 R	TCAATCGGACTTGTTCAAAAC CCCA TACAGACCAAATGCTCACCATCACT	Japanese persimmon (Guo et al. 2006)	45 ℃
2	IRAP-TDK2 F IRAP-TDK2 R	GAAGTTAGTGGGAGCAAAAGATGT TACCAATGTCGGGAGGCTTGTGTCA	Japanese persimmon (Guo et al. 2006)	45 ℃
3	IRAP-TDK10 F IRAP-TDK10 R	CTTTGTGATAGAACTTGGGTTTGCT AGACTTGGTCCATCCTTCCTTTAGA	Japanese persimmon (Guo et al. 2006)	40.5 ℃
4	IRAP-TDK11F IRAP-TDK11 R	AGGTATGGTTTCAAGATGATGGATG ACCCGCTGGTTGTGTCAGATAGATT	Japanese persimmon (Guo et al. 2006)	45 ℃
5	IRAP-TDK12 F IRAP-TDK12 R	ATACAACAGACTCAATGCCGACCCT ACCTGCCAACCAACTTCTTTTCCTC	Japanese persimmon (Guo et al. 2006)	45 ℃
6	IRAP-TDK13 F IRAP-TDK13 R	TCCTGATGGGAACTTCGTTGCTCGT CCTGACACCTCAAAACCTTCTGGCT	Japanese persimmon (Guo et al. 2006)	45 ℃
7	BREP F BREP R	TTCAAGATTTCTGACCTTTCG CCAGTGGCACATCAAAACAAAA	Japanese persimmon (Guo et al. 2006)	45 ℃
8	BREPI F BREPI R	AAGTATTCGGTGTCCAAAATC ACTCCCTGTTGAAAATTCTGA	Banana (Baurens et al. 1997)	45 ℃
9	5-LTR1 BARE-1 5-LTR2 BARE-1	TTGCCTCTAGGGCATATTTCCAACA ATCATTCCCTCTAGGGCATAATTC	Barley (Teo et al. 2005)	40.5 ℃

Table 3. RAPD primer sequences used in the amplification of genomic DNA of *Eryngium* L. species (* primers have been used for scoring and studying phylogenies).

No.	Code	Sequence 5'-3'	No.	code	Sequence 5'-3'
1	OPA-01	CAGGCCCTTC	21	OPF-18	GGCTCATGTG
2	OPA-09	GGGTAACGCC	22	OPH-10	CCTACGTCAG
3	OPA-11	CAATCGCCGT	23	OPI-18	TGCCCAGCCT
4	OPA-12	TCGGCGATAG	24*	OPJ-01	CCCGGCATAA
5	OPA-13	CAGCACCCAC	25*	OPJ-04	CCGAACACGG
6	OPA-14	TCTTGTCTGG	26	OPJ-05	CTCCATGGGG
7*	OPB-11	GTAGACCCGT	27*	OPJ-07	CCTCTCGACA
8*	OPB-12	CCTTGACGCA	28*	OPK-08	GAACACTGGG
9*	OPB-15	GCAGGGTGTT	29*	OPK-12	TGGCCCTCAC
10	OPB-17	AGGGAACGAG	30*	OPK-13	GGTTGTACCC
11	OPB-18	AGGTGACCGT	31*	OPK-17	CCCAGCTGTG
12	OPC-01	TTCGAGCCAG	32*	OPT-18	GATGCCAGAC
13	OPC-05	GATGACCGCC	33*	OPW-17	GTCCTGGGTT
14	OPC-06	GAACGGACTC	34*	OPY-10	CAAACGTGGG
15	OPC-07	GTCCCGACGA	35	OPZ-02	CCTACGGGGA
16*	OPD-08	GTGTGCCCCA	36	OPZ19	GTGCGAGCAA
17	OPD-16	AGGGCGTAAG	37*	UBC-132	AGGGATCTCC
18*	OPD-20	GGTCTACACC	38*	UBC-159	GAGCCCGTAG
19	OPF-10	GGAAGCTTGG	39*	UBC-702	GGGAGAAGGG
20*	OPF-16	GGAGTACTGG			

Table 4. Seed shape and general view of studied species are presented in Fig. 2.

The biology of propagation of the studied *Eryngium* L. species was also studied and showed that plants belonging to these species are perennials, except for *E. creticum*, which exist as biennial and *E. pussilum* as an annual plant. The flowering period ranges from two months in both *E. maritimum* (March–May) and *E. pussilum* (May–June) to six in *E. falcatum* (March–August). *E. creticum* has three months flowering period starting from April; whereas *E. campestre* has four months flowering period starting from June. *E. desertorum*, *E. glomeratum* and *E. billaridieri* share prolonged flowering period of five months starting May–June.

Eryngium L. species habitat in the current study were diverse, from sandy beaches such as *E. maritimum* to mountainous, slopes and rocky places such as *E. glomeratum*, *E. billaridieri*, *E. creticum*, *E. falcatum* and *E. campestre*. *E. desertorum* was found to grow in dry and semi-dried regions; where *E. pussilum* was

Table 4. Morphological characters of the eight studied *Eryngium* L. species in Syria.

Species	Height	Basal leaves	Stem	Head diameter (cm)	Number of bracts
E. maritimum	60	undivided	much branched	2–4	4–5
E. creticum	10–60	trifid	highly branched	0.5–1.2	4–5
E. desertorum	15–40	tricospedate	2–3 branches	1	4–6
E. glomeratum	25–100	oblong-lanceolate	sparingly branched	0.8–1.2	5
E. campestre	80	tricospedate	highly branched	0.4–1	6–7
E. billaridieri	40–100	pinnate	sparingly branched	0.4–1.3	6–8
E. pussilum	7–35	lanceolate	dichotomously branched	1.5–2	Many
E. falcatum	35–100	undivided or trifid	dichotomously branched	1.2–1.6	5–6

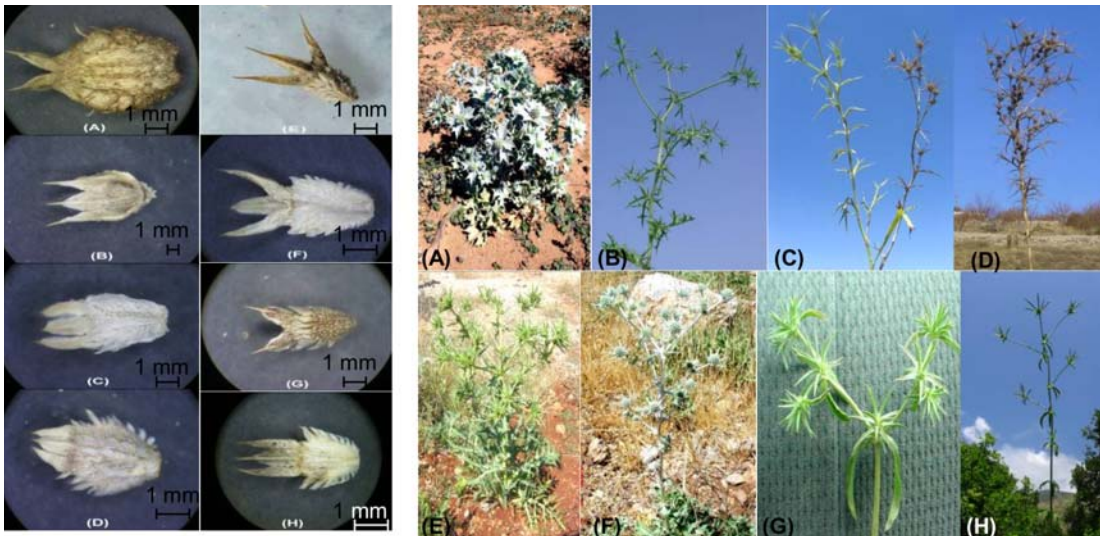

Fig. 2. Seed shape and morphology of the eight *Eryngium* L. species studied in Syria is illustrated on the left and the general view of plants of each species is presented on the right; where: (A) – *E. maritimum*, (B) – *E. creticum*, (C) – *E. desertorum*, (D) – *E. glomeratum*, (E) – *E. campestre*, (F) – *E. billardieri*, (G) – *E. pussilum* and (H) – *E. falcatum*.

found growing bay water and swamps. Interestingly, *E. creticum* and *E. desertorum* were also observed in saline soils tolerating high levels of salt.

Amplification of genomic DNA using IRAP primers
All nine primer combinations generated multiple bands from the genomic DNA of all studied*Eryngium* L. species (Fig. 3A). The banding pattern of amplification using IRAP primers is summarized in Table 5. Amplification of genomic DNA using IRAP primers was conducted twice to ensure repeatability and reproducibility.

Amplification of genomic DNA using RAPD primers
The DNAs of the eight *Eryngium* L. species were amplified using 39 RAPD primers. However, 19 of these primers (Table 3) were highly polymorphic (Fig. 3B), and were used for studying *Eryngium* L. species phylogeny. The banding pattern of amplification using RAPD primers is summarized in Table 5. Amplification of genomic DNA using RAPD primers was conducted twice to ensure repeatability and reproducibility.

Phylogenetic analysis of genome diversity
Dendograms generated using UPGMA and percent disagreement values (PDV) of STATISTCA 6 program were used to estimate the degree of relatedness among the eight studied *Eryngium* L. species. Results show great deal of similarity between IRAP and RAPD data dendograms (Figs 4A, 4B), which is confirmed in combined IRAP and RAPD data dendogram (Fig. 4C). The three dendograms group *E. glomeratum* and *E. campestre* in one cluster that makes a sub-cluster with *E. desertorum*. *E. pussilum* and *E. billardieri* are the most distant to all other *Eryngium* L. species investigated in the current study and is confirmed in the three resultant dendograms (Fig. 4).

Based on the matrix obtained using combined IRAP and RAPD data (data not shown), *E. pussilum* (swamps species) had the highest PDV (0.55) with *E. billardieri* (mountainous species). Whereas, *E. glomeratum* had the lowest PDV (0.25) with *E. campestre* followed by *E. desertorum* (0.28), *E. falcatum* (0.32) and *E. creticum* (0.33). *E. maritimum* (sea shore species, saline environment) was the most distant to *E. pussilum* (0.50) and the closest to *E. falcatum* (0.37).

Percent disagreement values of combined IRAP and RAPD data are consistent with the resultant dendogram (Fig. 4C). The aforementioned dendogram groups *E. glomeratum* and *E. campestre* (mainly moun-

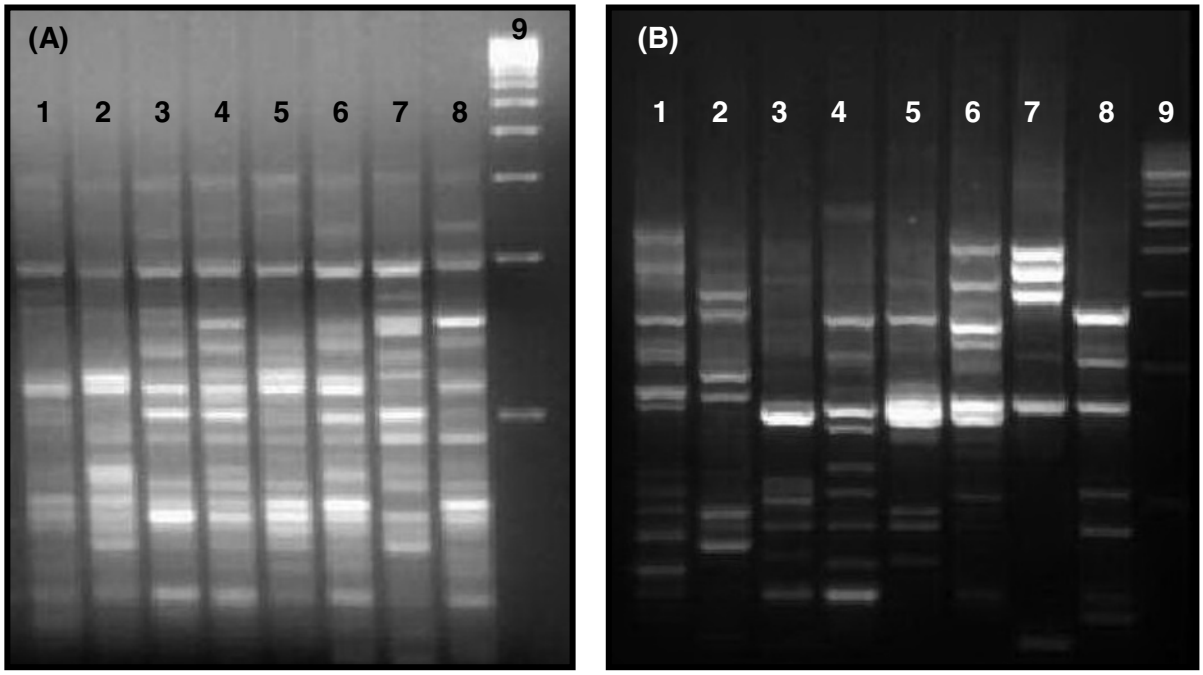

Fig. 3. Amplification of genomic DNA using (A) IRAP primers: TDK2F and TDK2R, (B) RAPD primer: OPB-11; where: 1 – *E. maritimum*, 2 – *E. creticum*, 3– *E. desertorum*, 4 – *E. glomeratum*, 5 – *E. campestre*, 6 – *E. billardieri*, 7 – *E. pussilum* and 8 – *E. falcatum*. 100 bp ladder (9–A) and 1Kb ladder (9–B).

Table 5. The banding pattern of IRAP and RAPD amplification products.

Primer	Unique Bands	Polymorphic Bands	Total band	% of Polymorphic Bands	Marker
TDK1F+TDK1R	8	38	46	82.61	
TDK2F+TDK2R	2	97	131	74.04	
TDK10F+TDK10R	5	19	24	79.16	
TDK11F+TDK11R	4	45	49	91.38	
TDK12F+TDK12R	1	84	93	90.32	
TDK13F+TDK13R	3	44	79	55.69	IRAP
BREP F+BREP R	4	13	17	76.47	
BREPI F+BREPI R	2	18	20	90	
5-LTR1 BARE-1 + 5-LTR2 BARE-1	13	72	85	84.70	
OPB-11	5	52	57	91.22	
OPB-12	9	15	32	46.87	
OPB-15	6	57	78	73.07	
OPD-08	1	40	41	97.56	
OPD-20	7	72	79	79.13	
OPF-16	5	48	61	78.68	
OPJ-01	6	68	90	75.55	
OPJ-04	7	58	65	89.2	
OPJ-07	4	16	28	57.14	
OPK-8	9	9	18	50	RAPD
OPK-12	4	60	80	75	
OPK-13	5	63	68	92.64	
OPK-17	6	58	72	76	
OPT-18	6	20	26	76.92	
OPW-17	10	57	75	73.07	
OPY-10	4	24	28	85.71	
UBC-132	3	62	65	95.38	
UBC-159	3	36	47	76.59	
UBC-702	4	39	51	76.47	

tainous species) in one cluster which forms a sub-cluster with *E. desertorum*. The dendogram clusters *E. falcatum* with *E. desertorum* and sub-cluster of *E. glomeratum* and *E. campestre*. The two PCR-based data show that *E. billardieri* and *E. pussilum* are the most distal to all other investigated *Eryngium* L. species. This is supported by PDV matrix where all PDVs of both *E. billardieri* and *E. pusillum* with the other studied species are higher than the mean average of PDVs of all species (0.35).

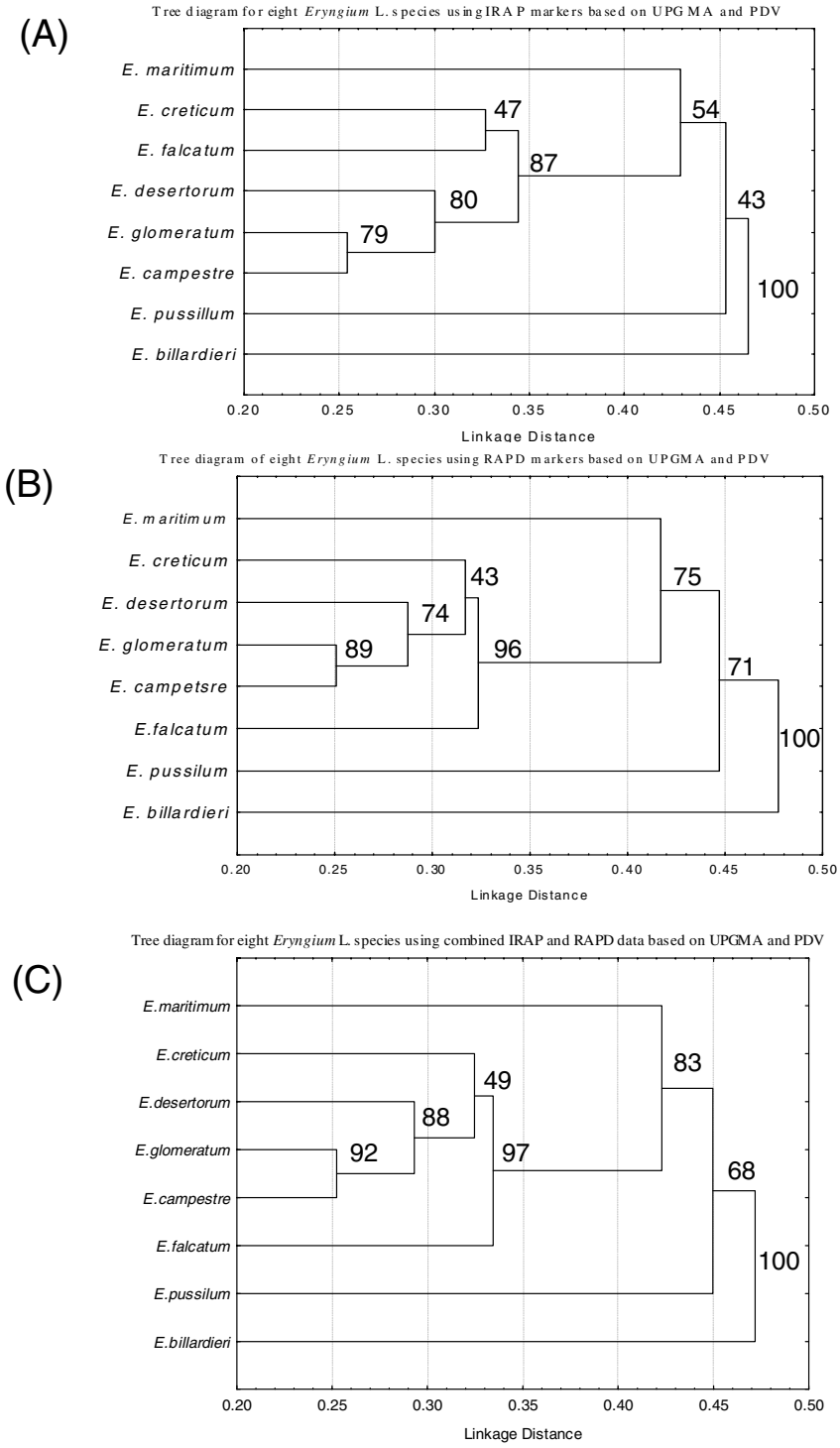

Fig. 4. Cluster analysis based on the percent disagreement values (PDV) of UPGMA, STATISTICA using (A) IRAP data, (B) RAPD data, and (C) combined IRAP and RAPD data on eight *Eryngium* L. species. Bootstrap values (%) are calculated using 500 simulations.

Discussion

This study utilized combined IRAP and RAPD data in an attempt to correlate the genetic makeup with the geographical distribution of *Eryngium* species that are widely spread in Syria. IRAP markers have been lately utilized for characterization of plants such as *Musa* and *Brassica* species (Muhammad & Othman 2005; Nair et al. 2005; Alix & Heslop-Harrison 2004). In the current study, IRAP data supports highly RAPD data analysis and they both come into agreement with the geographical facts of studied *Eryngium* L species in Syria.

The combined data grouped *E. campestre* and *E. glomeratum* in one cluster and so did the analysis of IRAP and RAPD data separately. Geographically speaking, the two species *E. campestre* and *E. glomeratum* grow mainly in mountainous regions with 800–1400 m a.s.l., along with *E. creticum* in the Northern and Southern regions of Syria. This suggests the adaptability of these species to cold weather conditions. *E.*

creticum was also found to grow in other mountainous regions, Hush Arab and Swaida Al-jabal along with *E. desertorum*. The molecular data indicate that *E. desertorum* is closely related to both mountainous *Eryngium* species *E. campestre* and *E. glomeratum*. This indicates the potential adaptation of *E. desertorum* to mountainous harsh environments.

E. desertorum was also found to grow along the Euphrates, and in the Syrian semidesert. It was also found to grow in Al-Jabool region which is famous for its high salinity levels along with *E. creticum*. This imply the wide spectrum of adaptation of the two species *E. desertorum* and *E. creticum* to harsh environments which range from mountainous to semidesert and saline environments.

E. falcatum was found to grow in the coastal region (Slunfa-1100 m a.s.l.) along with *E. creticum* and *E. glomeratum*. The combined data imply that *E. falcatum* is genetically close to both mountainous species *E. glomeratum* (PDV is 0.32) and *E. campestre* (PDV is 0.34) which supports its mountainous nature.

E. maritimum is uniquely found to grow on sandy beaches in the coastal regions of Syria. The molecular data clearly indicate the fact that *E. maritimum* is genetically distant from mountainous adapted species and wide range adapted *Eryngium* species. This genetical distance can be attributed to its unique tolerance to saline environments and high humidity levels.

The combined data show that *E. pussilum* lies distal from all other species on the cluster tree (Fig. 4C). A support for this finding comes from the work of Wörz (2004) who reported a preliminary new classification of the genus *Eryngium* L. and includes *E. pussilum* under subgenous C along with *E. atlanticum*, *E. corniculatum*, *E. galioides* and *E. viviparum*. These five species are considered the only Eurasian and North African species which do not belong to *Eryngium* subgenous *Eryngium* and are much more closely related to North American species. All of these species grow in lakes that dry out in summer, a typical habitat for American Eryngiums (Wörz, 2004). Indeed, *E. pussilum* in Syria was found to grow in Leftaya region, which is a swamps area with heavy clay soil and an altitude of 200 m a.s.l. The area receives relatively good rainfall of about 800 mm per year.

As for *E. billardieri*, it was also shown to be distant from all other studied Eryngiums (Fig. 4C) and that it is the closest to *E. glomeratum*, the mountainous species. The geographical information indicates that *E. billardeiri* is uniquely found in Erna, a mountainous region with around 1100 m a.s.l.

According to our knowledge the current study provides the first molecular, morphological and geographical data regarding *Eryngium* L. species in Syria. Our study has also indicated the potential of both *E. creticum* and *E. desretorum* species for adaptation to diverse environments. The two species were observed on mountains, in semidesert regions, and in saline environments. The two species must be investigated as candidate abiotic stress tolerant plants.

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