Indian Journal of Biotechnology
Vol 15, January 2016, pp 48-56

Morphological, biochemical and molecular characterization of *Evolvulus alsinoides* Linn.: A memory enhancing herb

Vikas Bandu Naikawadi[1,2], Mahendra Laxman Ahire[1,3], Vinayak Haribhau Lokhande[1,4], Ravi Parshuram Ghorpade[1] and Tukaram Dayaram Nikam[1]*

[1]Department of Botany, Savitribai Phule Pune University, Pune 411 007, India
[2]Department of Botany, C T Bora College, Shirur (Ghodnadi), Pune 412 210, India
[3]Department of Botany, Yashavantrao Chavan Institute of Science, Satara 415 001, India
[4]Department of Botany, Shri Shiv Chhatrapati College, Junnar, Pune 410 502, India

Received 19 September 2014; revised 5 February 2015; accepted 17 April 2015

Evolvulus alsinoides Linn. is one of the important plants used in Medhya rasayana drug of Ayurveda. In the present investigation, attempts were made to study morphological, biochemical and molecular diversity of *E. alsinoides* collected from different regions of Maharashtra. Significant morphological differences were observed when accessions were compared with accession from Pune (PU1). Dendrogram for morphological traits clustered *Evolvulus* accessions into two groups considering 90% similarity. Chemical constituents in alkaloidal extract from 14 *Evolvulus* accessions were analyzed using high performance thin layer chromatography (HPTLC). HPTLC profiles showed presence of 12 distinct compounds in the fraction. Dendrogram constructed on the basis of Rf values, grouped *Evolvulus* accessions in three major groups. Further, molecular diversity was studied using randomly amplified polymorphic DNA (RAPD). Of the total 387 RAPD loci amplified with 40 primers, 220 loci were polymorphic with 56.1% diversity. A dendrogram constructed with UPGMA, grouped the accessions into two major clades. Wide morphological, biochemical and genetic diversity observed among the *E. alsinoides* accessions could probably be because of differences in their geographic environmental conditions.

Keywords: Convolvulaceae, chemical fingerprinting, *Evolvulus alsinoides*, HPTLC, RAPD

Introduction

Evolvulus alsinoides Linn. (Family: Convolvulaceae) is a hairy perennial herb with spreading and prostrate branches. It occurs in mostly dry tropical and sub-tropical regions of the world. The drug prepared from the plant is known as Shankhpushpi. This drug is widely used to improve memory, intelligence and to treat various brain disorders and problems like insomnia, loss of memory, mental as well as physical fatigue[1,2]. In the treatise of Ayurveda like *Charakasamhita*, *Susrutasamhita* and *Ashtanga Hridaya*, it is mentioned as a medhya drug. *E. alsinoides* is also one of the ingredients of ayurvedic formulations like *Brahmi grihta*, *Vachadi grihta*, *Jeevanyadi grihta*, *Agastya rasayana*, *Brahma rasayana* and *Naladi grihta*[1]. Although Shankhpushpi is used worldwide on large-scale to treat various brain disorders, it is in the list of controversial drugs because of the different botanical sources suggested in ayurvedic and various literatures. *Convolvulus microphyllus* Sieb. ex Spreng. (Convolvulaceae) in North India, *E. alsinoides* Linn. (Convolvulaceae) and *Clitoria ternatea* Linn. (Fabaceae) in South India, and *Canscora diffusa* L. (Gentianaceae) in Eastern India are used for the ayurvedic and other preparations under the name of Shankhpushpi[1]. To remove the controversy, it is pre-requisite to study the morphological, biochemical and molecular characterization of these taxa.

Production of medicinally important secondary metabolites of plants varies with geographical origin and developmental stages. The components of intraspecific variation of plant secondary metabolism are found to be under genetic control[3]. The plants which produce large variety of metabolites have developed best defending mechanisms against biotic and abiotic stress factors[4]. To achieve this attribute, in course of evolution, the plants has developed predictable traits of increase in a number of diversified active secondary metabolites by retention and utilization of inactive chemicals produce in their metabolism. This tendency in plants helps to decrease the investment in development of completely

*Author correspondence:
Tel: +91-20-25601439; Fax: +91-20 25690498
tdnikam@unipune.ernet.in

new mechanism and increase the probability of biosynthesis of new active metabolites. For selection and identification of source plant genotype from regional population, quantification of medicinally active metabolites is prerequisite. WHO[5] accepted the chromatographic fingerprinting as a strategy for quality assessment of herbal medicines. HPTLC fingerprinting method is a modern and useful analytical technique to screen plants rapidly[6]. It is widely accepted technique for its high accuracy, precision, reproducibility of results, low cost per sample, easy sample preparation and short analysis time[7].

The genetic structure of the plant population is difficult to estimate by agronomical and morphological characteristics due to the environmental conditions. DNA fingerprinting techniques have been widely used to analyze the genetic variation and to differentiate species or populations[8]. The randomly amplified polymorphic DNA (RAPD) fingerprinting method was proved to be an efficient technique as it surveys numerous loci in the genome and helps to detect the genetic variation in the species or population[8,9].

The present investigation was aimed to characterize morphological, chemical and molecular diversity in *E. alsinoides*. Genetic diversity analysis with morphological determinants, DNA-based markers and chemical fingerprinting would help in selection of divergent genotypes for the improvement of quality of the drug and on knowing the similarities and differences in profile for removal of controversy among the different species used under name Shankhpushpi.

Materials and Methods

Plant Material

E. alsinoides plant samples were collected from different agroclimatic zones representing 13 districts of Maharashtra (Table 1; Fig. 1) and plants were established in Botanical Garden of the Department of Botany, Savitribai Phule Pune University (formerly University of Pune), Pune (lat 18°31′ N; long 73°51′ E). At respective locations, leaf samples were immediately preserved in liquid nitrogen and brought to the laboratory. Plant identification and authentication was carried out at Botanical Survey of India, Pune and voucher specimen was deposited in the herbarium section of BSI (Ref.: BSI/WRC/Tech/2009/506).

Morphological characterization

Plants from different locations were visually observed for morphological characters (Table 2; Fig. 2). Leaf length, leaf width, branch length and internodal distance were measured at the field. Data were recorded from at least 15 plants from each location. The experiment was laid out in completely randomized design and data were analyzed by one-way analysis of variance using the SPSS statistical software (Version 9.0). Means were compared by using the post hoc least significant difference test. The term significant has been used to indicate the differences for which $P \leq 0.05$. The morphological data was sorted according to leaf length: 1 (1-30 mm), 2 (31-50 mm), 3 (51-70 mm); leaf width: 1 (1.0-5.0 mm), 2 (5.1-10.0 mm); leaf area: 1 (1-20 mm^2), 2 (21-40 mm^2), 3 (41-60 mm^2); internodal distance: 1

Table 1—Collection sites of *E. alsinoides* accessions from the state of Maharashtra

No.	Acc. no.	District	City/Village	Latitude (°N)	Longitude (°E)	Elevation (m)
1	AB1	Aurangabad	Chitegaon	19°45′	75°29′	568
2	TH1	Thane	Wadi Warhe	19°18′	73°06′	28
3	DH1	Dhule	Vinchur	20°43′	74°51′	320
4	NS1	Nasik	Wadali Bhoi	20°17′	74°06′	680
5	AH1	Ahmednagar	Karanji Ghat	19°07′	74°58′	768
6	AB2	Aurangabad	Jaikwadi	19°26′	75°21′	465
7	SN1	Sangli	Shirala	16°58′	74°09′	640
8	WD1	Wardha	Kharangana	20°44′	78°41′	253
9	YM1	Yavatmal	Yavatmal	20°24′	78°08′	413
10	AM1	Amarawati	Anjangaon	21°09′	77°19′	346
11	HN1	Hingoli	Basmath	19°19′	77°09′	384
12	ND1	Nanded	Mahurgarh	19°09′	77°18′	365
13	ST1	Satara	Anewadi	17°41′	74°01′	689
14	PU1	Pune	Pune	18°32′	73°49′	586

(1.0-5.0 mm), 2 (5.1-10 mm), 3 (10.1-15.0 mm); and branch length: 1 (1-150 mm), 2 (151-300 mm), 3 (301-450 mm). The morphological data was subjected to construct dendrogram based on similarity matrix using SPSS software, version 9.0.

Chemical Fingerprinting

Extraction

Dry powder (2 g) of aerial parts of each sample was extracted separately with 50 mL methanol overnight on rotary shaker at 80 rpm. Then the mixture was filtered and residue was re-extracted with 50 mL methanol as earlier for 2 times. The extracts were pooled and concentrated under reduced pressure and then cooled to room temperature. To this, cold 2N H_2SO_4 was added and then extracted with chloroform ($CHCl_3$). Chloroform and aqueous layers were separated. To the aqueous layer cold 2N NaOH was added till the pH increased to 11.0. Then the aqueous solution was washed with chloroform till the washings were neutral. Chloroform layer was dried over anhydrous sodium sulphate and filtered through 0.45 µ syringe driven filters and filtrate was used for HPTLC analysis.

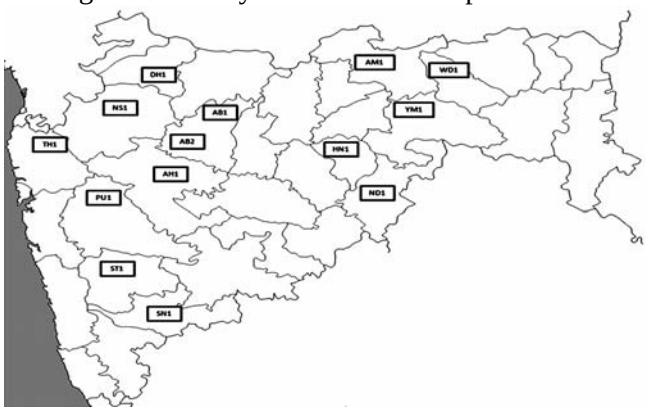

Fig. 1—Map of Maharashtra state showing geographical localities of different collection sites of *E. alsinoides* accessions. [Aurangabad (AB1 & AB2), Thane (TH1), Dhule (DH1), Nasik (NS1), Ahmednagar (AH1), Sangli (SN1), Wardha (WD1), Yavatmal (YM1), Amrawati (AM1), Hingoli (HN1), Satara (ST1) & Pune (PU1). Map is only representative and distances are not to the scale.]

Analytical Procedure

HPTLC was performed on 20×10 cm^2 aluminum foil plates coated with 200 µm layer of silica gel 60F254 (E. Merck, Germany). Samples (5 µL) were loaded with Linomat 5 semiautomatic applicator (CAMAG, Switzerland) fitted with 100 µL syringe as bands 8 mm in width. Distance between tracks was 13.0 mm and first application was 10.0 mm from the left edge of plate. From the lower edge, application distance was kept 8.0 mm. HPTLC plates were developed in twin-trough chamber (20×10×4 cm^3; CAMAG, Switzerland) containing toluene:ethyl acetate:formic acid (8:2:1 v/v/v). Plates were documented using CAMAG visualizer at 366 nm and scanned using CAMAG TLC Scanner III with winCATS software 1.4.3. Plates were scanned at

Table 2—Morphological variation among the *E. alsinoides* accessions

Acc. no.	Morphological characters				
	Leaf length (mm)	Leaf width (mm)	Leaf area (mm^2)	Branch length (mm)	Internodal distance (mm)
AB1	14.47	7.60*	57.67	150.13*	8.13
TH1	14.13	6.20	47.60*	277.33*	11.40*
DH1	12.53	6.87	52.07*	212.53	10.80*
NS1	11.00*	5.47*	36.13*	209.93	8.47
AH1	15.60*	5.87	37.53*	183.53*	8.93*
AB2	13.00	6.33	29.93*	124.97*	7.53
SN1	13.53	5.13*	30.47*	242.33*	6.73
WD1	11.47*	5.13*	31.80*	389.33*	7.33
YM1	13.20	5.87	41.80*	400.67*	8.20
AM1	15.07	3.53*	50.00*	408.67*	8.07
HN1	16.53*	5.93	39.73*	370.67*	5.93
ND1	13.93	5.87	37.53*	365.00*	8.40
ST1	10.93*	3.93*	26.33*	218.47	9.40*
PU1	14.13	6.80	58.79	204.67	6.87

The values shown here are the means of 15 replicates, entire experiment was repeated thrice
*Values are statistically significant at $P \leq 0.05$ when compared with the accession PU1

Fig. 2 (A-N)—Morphological variation in *E. alsinoides* accessions. [A, AB1; B, AB2; C, DH1; D, AH1; E, NS1; F, TH1; G, SN1; H, WD1; I, YM1; J, AM1; K, HN1; L, ND1; M, ST1; & N, PU1]

366 nm wavelength using Hg lamp at fluorescence mode. The Rf values of significant peaks were tabulated. Major significant and reproducible peaks were scored as (1) present or (0) absent and subjected to construct a dendrogram using SPSS.

DNA Fingerprinting

DNA Isolation

Genomic DNA was isolated from young leaves from 14 accessions using HiPurA^{TM} Plant Genomic DNA Miniprep Purification Spin Kit (Hi Media, India) by following instructions as given in manufacturer's protocol. DNA yield and quality were checked by electrophoresing on 1% agarose gel (SeaKem LE Agarose, USA) stained with ethidium bromide. DNA samples were diluted so as to get final concentration 50 ng/µL for diversity analysis. Different components of polymerase chain reaction (PCR) were optimized to get appropriate amplification products. Various concentrations of genomic DNA (50-200 ng/25 µL reaction mixture), $MgCl_2$ (1.0-3.0 mM), Taq DNA polymerase (0.5 or 0.6 U) (GeNei^{TM}, Banglore, India) were used. For primer annealing, different temperatures (37-40^{o}C) were tested for amplification of the template DNA with RAPD primers.

RAPD Analysis

In total 40 random decamer primers of different sets (OPA procured from Operon Biotechnologies, Germany; F and H from IDT Inc., USA) were used. PCR amplification was performed using 25 µL reactions in thermal cycler (Eppendorf Cycler Gradient, Germany). PCR amplification was performed with initial denaturation at 94^{o}C for 5 min, followed by 40 cycles, each consisting denaturation (94°C) for 1 min, annealing (38.2°C) for 1 min, extension (72°C) for 2 min, with final extension for 5 min at 72°C followed by hold at 4°C. PCR products were loaded with 1 µL gel loading dye (6×), and separated on 2% agarose gel in 1× TAE buffer using submarine gel electrophoresis unit (GeNei^{TM}, India) at 70 V for 2 h. A 100 bp DNA ladder was used as molecular standard. Gels were visualized under UV-visible transilluminator and then documented.

Scoring and Data Analysis

The primers displayed reproducible and scorable bands were considered for analysis. Distinct and reproducible bands were scored as present (1) or absent (0). The band was considered to be 'polymorphic' if present in some of the accessions and absent in others. Data were used for computing Jaccard's and Simple Matching Similarity coefficients using NTSYS-Pc (Applied Biostatistics, Inc., USA, version 2.02e) developed by Rohlf[10]. The similarity coefficients were subjected to construct dendrogram by employing Unweighed Pair Group Method with Arithmetical Averages (UPGMA) and Sequential Hierarchical and Nested Clustering (SHAN) programme.

Results and Discussion

Morphological analysis

Morphologically, *Evolvulus* accessions revealed variation with respect to their length, width and area

of the leaf, branch length and internodal distance (Table 2; Fig. 2). The accessions showed significant morphological differences when compared with the accession PU1 for observed morphological characters. Among the morphological traits studied, internodal distance did not show significant difference when accessions AB1, NS1, AB2, SN1, WD1, YM1, AM1, HN1 and ND1 were compared with PU1 (Table 2). Among the morphological characters, leaf area was more significantly different in all accessions (Table 2). Based on the pattern of secondary branching, the accessions were grouped into four clusters (Fig. 3). The first cluster was the group of accessions WD1, ND1, AH1, SN1, NS1 and ST1. These accessions belong to climate with moderate rainfall (1130 mm), temperature (16 to 38°C) and humidity (35 to 72%). The second cluster was comprised of accessions TH1, DH1, YM1 and PU1. Compared to first cluster, this group was exposed to wide range of rainfall (700 to 2607 mm), temperature (21 to 41°C) and humidity (41 to 98%). While the accessions AM1 and HN1 were grouped into third cluster and the fourth cluster was comprised of accessions AB1 and AB2. Both 3rd and 4th cluster accessions were growing in the region having rainfall 700 to 900 mm, temperature range 21 to 41°C and humidity 41 to 75%. Along with these factors, elevation might also be one of governing factor for the cluster of the *E. alsinoides* (Table 1). Similarity coefficient obtained by morphological markers was ranged from 0.299 to 1.0. The highest pair wise phenotypic similarity was observed for the accessions WD1, ND1, AH1, SN1, and NS1 in the first cluster, while TH1 and DH1 in the second cluster (1.000).

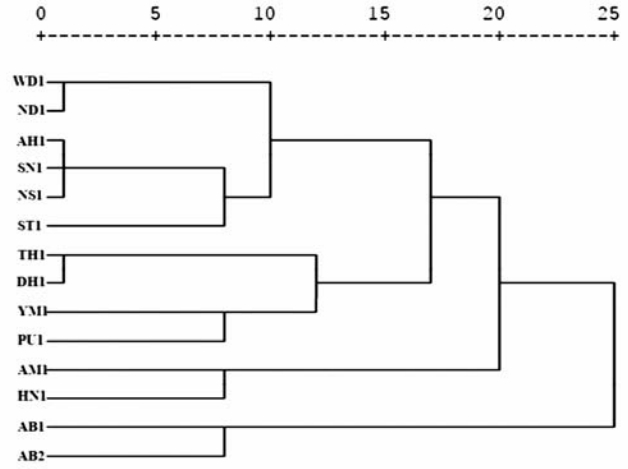

Fig. 3—Dendrogram showing phenotypic relationship among the *E. alsinoides* accessions generated by morphological markers.

Morphologically all the 14 accessions of *Evolvulus* showed wide diversity with morphological markers. The lowest pair wise phenotypic similarity was observed for the accessions WD1, ND1 and AB1 (0.299). This variation in morphological diversity in *Evolvulus* accessions was might be due the different climatic conditions and the edaphic factors. Similar variation in morphological data was recorded with variable environmental conditions in sago palm[11]. Lokhande *et al*[12] also reported the variation in morphological traits because of different environmental conditions.

Chemical Fingerprinting

The phytochemical diversity of alkaloidal fraction of *E. alsinoides* was evaluated using HPTLC at wavelength 366 nm (Fig. 4). HPTLC profile showed presence of 12 distinct compounds. The compound 1, 2 and 12 were commonly found in all the accessions, while others were specific to certain locations and compound 3-11 showed the diversity in all accessions (Table 3).

Among the different accessions, WD1 showed the presence of 12 of compounds. Further, accessions TH1, DH1, AH1, SN1 and HN1 showed the presence of 11 compounds, while rest of the accessions varied in the presence of compounds (Table 3). Based on the similarity matrix, a dendrogram was constructed, which showed the distribution of 14 accessions of *E. alsinoides* in two distinct clades (Fig. 5). Clade I is comprised of a single accession ND1 and Clade II consisted of rest 13 accessions distributed in two clusters; the first cluster comprised of accession AB1 and YM1, while cluster II includes two groups. In Group I, the 1st subgroup includes accessions NS1, AB2 and PU1, while 2nd subgroup consists of only accession ST1. In Group II, accession AM1 formed the 1st subgroup and accessions TH1, AH1,

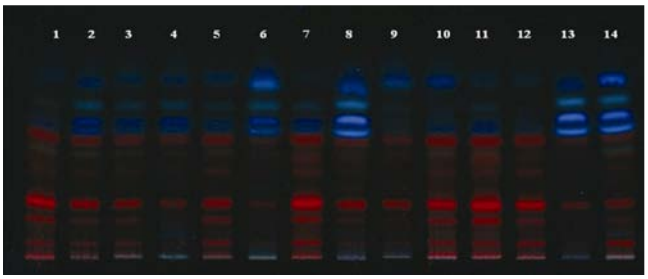

Fig. 4—Chemical fingerprint profiles of *E. alsinoides* accessions on HPTLC plates detected at = 366 nm. [Track 1: AB1; 2: TH1; 3: DH1; 4: NS1; 5: AH1; 6: AB2; 7: SN1: 8: WD1; 9: YM1; 10: AM1; 11: HN1; 12: ND1; 13: ST1; & 14: PU1]

Table 3—Rf values of significant peaks detected in aerial parts of *E. alsinoides* accessions

Peak no.	AB1	TH1	DH1	NS1	AH1	AB2	SN1	WD1	YM1	AM1	HN1	ND1	ST1	PU1
1	0.07	0.06	0.07	0.06	0.06	0.07	0.07	0.07	0.07	0.07	0.06	0.06	0.07	0.06
2	0.12	0.12	0.14	0.13	0.14	0.12	0.14	0.13	0.14	0.14	0.14	0.14	0.12	0.15
3	0.25	0.24	0.24	-	0.25	-	0.25	0.23	-	0.24	0.24	0.24	-	0.25
4	-	0.35	0.35	0.34	0.36	-	-	0.34	0.35	0.36	-	0.36	0.34	0.34
5	0.41	-	-	-	-	0.43	0.40	0.42	-	-	0.40	-	-	-
6	0.51	0.50	0.51	-	0.51	-	0.50	0.49	-	0.51	0.49	0.51	-	0.52
7	-	0.59	0.59	-	0.59	-	0.59	0.58	0.59	0.59	0.58	0.58	-	-
8	0.61	0.65	0.66	0.66	0.67	0.64	0.67	0.64	0.68	0.68	0.66	-	-	0.61
9	-	0.71	0.71	0.71	0.72	0.71	0.71	0.70	-	0.73	0.71	0.73	0.72	0.74
10	0.81	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.77	-	0.76	-	0.80	0.81
11	0.92	0.88	0.89	0.89	0.89	0.87	0.88	0.86	0.85	0.85	0.87	-	0.89	0.90
12	0.99	1.00	1.00	1.00	1.00	1.00	0.99	0.99	1.00	1.00	1.00	1.00	1.00	1.00

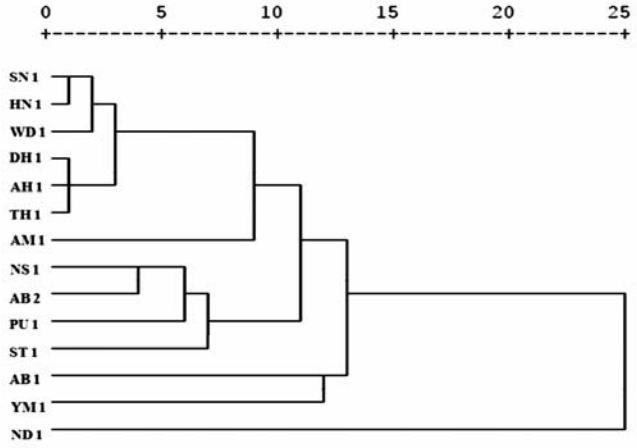

Fig. 5—Dendrogram showing relationship among *E. alsinoides* accessions generated by chemical fingerprinting of alkaloid fraction using HPTLC. [Only the major significant peaks were considered for analysis]

DH1 WD1, HN1 and SN1 were included in the 2nd subgroup (Fig. 5). Thus the alkaloidal fraction of *Evolvulus* accessions showed higher diversity. This might be possible in response to the changing environmental conditions to the different accessions. Secondary metabolites plays an important role in adaptation of plants to the changing environment and environmental factors, *viz.*, concentration of CO_2, supply of water, minerals, pH, temperature, humidity and light intensity[13]. Similar observations were recorded in methanol extracts of *Lawsonia inermis* leaves[6]. Alkaloids appeared to be quite variable among the forms of *Cissampelos capensis* from different provenances[14]. Standardized plant extracts or the pure compounds isolated from the plants provide unlimited opportunities for designing new drug because of the unmatched availability of chemical diversity[15, 16].

RAPD Analysis

Optimization of PCR condition is a prerequisite in molecular marker analysis for assessing genetic diversity. Among the different components of PCR reaction, $MgCl_2$ concentration is an important component. Mg^{2+} ions form a soluble complex with dNTPs, which is essential for dNTP incorporation. They also stimulate the polymerase activity and increase the Tm of double-stranded DNA and primer/template interaction. The concentration of $MgCl_2$ can have a dramatic effect on the specificity and yield in PCR. In the present study, 5.0 mM $MgCl_2$ produced scorable banding pattern, whereas the concentration below 5.0 mM produced faint bands or no bands due to increase in stringency with decrease in the concentration. Of the different concentrations of genomic DNA used (50-200 ng/25 µL reaction mixture), 100 ng/25 µL reaction was found to be the most suitable. The lower DNA quantity yielded less intense bands, whereas the higher concentrations added background effect. Further, Taq DNA polymerase (0.5 U) yielded good amplification of *Evolvulus* genomic DNA. Among the different primer annealing temperatures (37-40°C) used for the amplification, 38.2°C temperature was found to be the optimum. RAPD primers OPA1 and OPA2 were used for the optimization of the PCR. Same conditions found suitable for other primers as well as DNA samples were adopted for the rest of experiments.

Genetic differences are regarded as more discrete, standard and definite means of botanical identification. The morphological and chemical characters are influenced by environmental factors; while DNA markers are least affected by environment but gets linked to genotypes for their genomic constitution[12]. RAPD markers are most frequently

used molecular markers to evaluate genetic diversity of most of the crops as well as medicinal plants because they do not require prior genomic information and they are less costly and easy to handle[9,17]. RAPD analysis proved to be an effective technique to measure the magnitude of diversity and discriminate between the genotypes in supplementation of morphological characterization[12].

For the amplification of 14 *Evolvulus* accessions, 40 RAPD primers were used in the present investigation. As a result 387 scorable bands were amplified, of which 167 bands were found to be monomorphic, while 220 bands were polymorphic exhibiting 56.1% polymorphism (Table 4; Fig. 6). On an average each primer produced 9.68 bands and amplification products were ranged 0.1 to 2.0 kb.

Table 4—Summary of RAPD primers used in detecting genetic diversity in *E. alsinoides* accessions

Primer	Primer sequence (5′ to 3′)	Total no. of bands scored	No. of monomorphic bands	No. of polymorphic bands	% polymorphism
OPA-01	CAGGCCCTTC	06	04	02	33.30
OPA-02	TGCCGAGCTG	09	06	03	33.30
OPA-03	AGTCAGCCAC	09	05	04	44.40
OPA-04	AATCGGGCTG	09	02	07	77.80
OPA-05	AGGGGTCTTG	12	01	11	91.70
OPA-06	GGTCCCTGAC	10	02	08	80.00
OPA-08	GTGACGTAGG	09	03	06	66.70
OPA-09	GGGTAACGCC	11	02	09	81.80
OPA-13	CAGCACCCAC	08	02	06	75.00
OPA-14	TCTGTGCTGG	09	01	08	88.90
F-01	ACGGATCCTG	12	01	11	91.70
F-02	GAGGATCCCT	15	01	14	93.30
F-03	CCTGATCACC	16	00	16	100.0
F-04	GGTGATCAGG	07	07	00	00.00
F-05	CCGAATTCCC	08	03	05	62.50
F-06	GGGAATTCGG	09	09	00	00.00
F-07	CCGATATCCC	12	12	00	00.00
F-08	GGGATATCGG	08	08	00	00.00
F-09	CCAAGCTTCC	09	09	00	00.00
F-10	GGAAGCTTGG	09	02	07	77.80
F-11	TTGGTACCCC	08	00	08	100.0
F-12	ACGGTACCAG	09	03	06	66.70
F-13	GGCTGCAGAA	09	01	08	88.90
F-14	TGCTGCAGGT	09	09	09	00.00
F-15	CCAGTACTCC	08	02	06	75.00
F-16	GGAGTACTGG	10	01	09	90.00
F-17	AACCCGGGAA	07	03	04	57.10
F-18	TTCCCGGGTT	11	00	11	100.0
F-19	CCTCTAGACC	10	00	10	100.0
F-20	GGTCTAGAGG	10	00	10	100.0
H-01	GGTCGGAGAA	10	10	00	00.00
H-02	TCGGACGTGA	11	11	00	00.00
H-03	AGACGTCCAC	07	07	00	00.00
H-04	GGAAGTCGCC	12	12	00	00.00
H-05	AGTCGTCCCC	06	02	04	66.70
H-06	ACGCATCGCA	13	13	00	00.00
H-07	CTGCATCGTG	13	13	00	00.00
H-08	GAAACACCCC	09	00	09	100.0
H-09	TGTAGCTGGG	08	00	08	100.0
H-10	CCTACGTCAG	10	00	10	100.0
Total	Primers 40	387	167	220	56.1

Among the 40 RAPD primers used, primer F3 showed the highest number of polymorphic bands (16) with 100% polymorphism in comparison to primers from the Set OPA, H and F (Table 4).

In the present study, the RAPD data revealed 56.1% genetic diversity among *Evolvulus* accessions with the similarity coefficient ranged 0.30 to 0.80. This might be due to the random primers that surveyed genome randomly involving the non-coding regions of the genome. Dendrogram constructed based on RAPD data grouped all the *Evolvulus* accessions in two major clades across the geographic regions (Fig. 7). Clade I includes in total eight accessions (AB1, TH1, DH1, NS1, AH1, AB2, SN1 & WD1) and formed two groups (Group I: accessions WD1 & SN1; Group II: accessions AB1, TH1, DH1, NS1, AH1 & AB2). In the group II of Clade I, accessions AH1 and AB2 formed the first subgroup with the highest similarity percentage (80%) and accessions AB1, TH1, DH1 and NS1 formed the second subgroup. Clade II included six accession (YM1, AM1, HN1, ND1, ST1 & PU1) and formed two groups (Group I: YM1 & AM1; Group II: HN1, ND1 ST1 & PU1) (Fig. 7). Accessions of Clade II were highly distant from the accessions AH1 and AB1 of Clade I. These genetic similarity and differences occurred among the 14 accession of *E. alsinoides*, which might be due to climatic, edaphic and environmental factors as well as the varied rainfall conditions prevailed among the distant geographical regions of Maharashtra state, from where the plant material collection of *E. alsinoides* was made. The lower range of rainfall in the eastern parts of Maharashtra in comparison to Western region was indirectly responsible for the variations in the phenotype of *Evolvulus*, thereby showing the genetic diversity among these accessions. Similar results with the genetic and morphological diversity were observed among the accessions of *Sesuvium portulacastrum*[12] and *Halodule wrightii*[18] by the other workers.

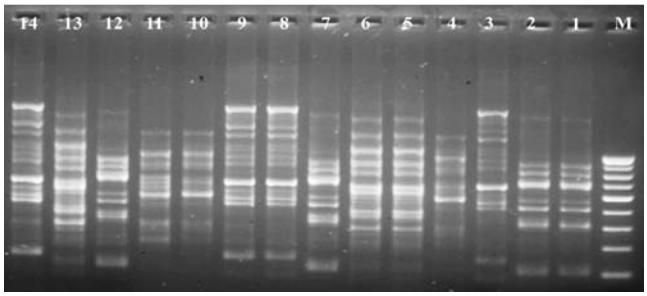

Fig. 6—Banding pattern of *E. alsinoides* accessions generated by RAPD primer F3. [M: 100 bp DNA ladder, 1: AB1; 2: TH1; 3: DH1, 4: NS1; 5: AH1; 6: AB2; 7: SN1; 8: WD1; 9: YM1;10: AM1; 11: HN1; 12: ND1; 13: ST1; & 14: PU1]

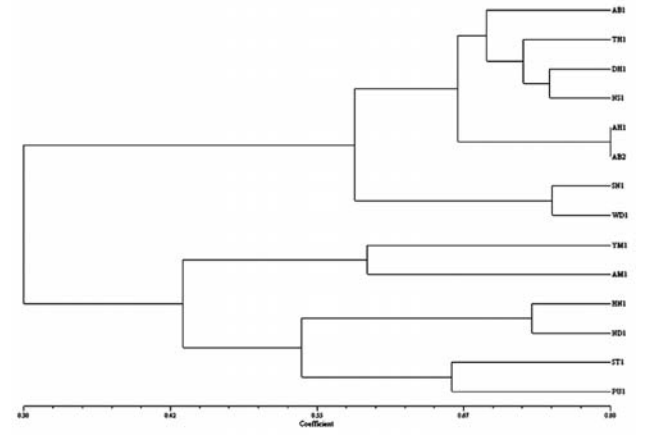

Fig. 7—Dendrogram showing genetic relationship among the *E. alsinoides* accessions based on Jaccard's similarity coefficient generated by RAPD markers.

In conclusion, the diversity studies based on morphological, biochemical and molecular characterization suggested that major variations in *E. alsinoides* were because of agro-climatological factors. Further, a method based on RAPD and HPTLC analyses could be used in authentication of *E. alsinoides*.

Acknowledgment

Authors are grateful to the Board of College and University Development (BCUD), Savitribai Phule Pune University, Pune; UGC-SAP-DRS III, DSA-I and ASIST; and DST-FIST, PURSE programme of Government of India, for their financial support.

References

1 Madhavan V, Yoganarasimhan S N & Gurudeva M R, Pharmacological studies on Shankhapushpi (*Convolvulus microphyllus* Sieb. ex Spreng. and *Evolvulus alsinoides* (L.), *Indian J Tradit Knowl*, 7 (2008) 529-541.

2 Kothiyal P & Rawat M S M, Comparative nootropic effect of *Evolvulus alsinoides* and *Convolvulus pluricaulis*, *Int J Pharm Bio Sci*, 2 (2011) 616-621.

3 Berenbaum M R & Zangerl A R, Genetics of secondary metabolism and herbivore resistance in plants, in *Herbivores*: *Their interactions with secondary plant metabolites*, vol II, edited by G A Rosenthal & M R Berenbaum (Academic Press, San Diego, USA) 1992, 415-438.

4 Jones C G & Firn R D, On the evolution of plant secondary chemical diversity, *Phil Trans R Soc Lond B*, 333 (1991) 273-280.

5 WHO, *General guidelines for methodologies on research and evaluation of traditional medicines* (World Health Organization, Geneva) 2000.

6 Gallo F R, Multari G, Giambenedetti M & Federici E, Chemical fingerprinting of *Lawsonia inermis* L. using HPLC, HPTLC and densitometry, *Phytochem Anal*, 19 (2008) 550-559.

7 Rumalla C S, Avulaa B, Zhao J, Smillie T J & Khan I A, Quantitative determination of phenolic acids in *Lonicera japonica* Thunb. using high performance thin layer chromatography, *J Liq Chromatogr Rel Technol*, 34 (2011) 38-47.

8 Rodriguez J M, Berke T, Engle L & Nienhuis J, Variation among and within *Capsicum* species revealed by RAPD markers, *Theor Appl Genet*, 99 (1999) 147-156.

9 Williams J G K, Kubelik A R, Livak K J, Reafalski J A & Tingey S V, DNA polymorphisms amplified by arbitrary primers are useful as genetic markers, *Nucleic Acid Res*, 18 (1990) 6531-6535.

10 Rohlf F J, *NTSYS-pc*: *Numerical taxonomy and multivariate analysis system*, version 2.02e, (Exeter Software, New York, USA) 1997.

11 Kjaer A, Barfod A S, Asmussen C B & Seberg O, Investigation of genetic and morphological variation in the sago palm (*Metroxylon sagu*; Arecaceae) in Papua New Guinea, *Ann Bot*, 94 (2004) 109-117.

12 Lokhande V H, Nikam T D, Patade V Y & Suprasanna P, Morphological and molecular diversity analysis in Indian clones of *Sesuvium portulacastrum* L., *Genet Resour Crop Evol*, 56 (2009) 705-717.

13 Ramakrishna A & Ravishankar G A, Influence of abiotic stress signals on secondary metabolites in plants, *Plant Signal Behav*, 6 (2011) 1720-1731.

14 de Wet H, van Heerden F R & van Wyk B E, Alkaloidal Variation in *Cissampelos Capensis* (Menispermaceae), *Molecules*, 16 (2011) 3001-3009.

15 Cos P, Vlietinck A J, Berghe D V & Maes L, Anti-infective potential of natural products: How to develop a stronger *in vitro* 'proof-of concept', *J Ethnopharmacol*, 106 (2006) 290-302.

16 Sharma A, Patel V K & Ramteke P, Identification of vibriocidal compounds from medicinal plants using chromatographic fingerprinting, *World J Microbiol Biotechnol*, 25 (2009) 19-25.

17 Bantawa P, Das A, Ghosh P D & Mondal T K, Detection of natural diversity of *Gaultheria fragrantissima* landraces by RAPDs: An endangered woody oil bearing plant of Indo-China Himalayas, *Indian J Biotechnol*, 10 (2011) 294-300.

18 Angel R, Genetic diversity of *Halodule wrightii* using random amplified polymorphic DNA, *Aquat Bot*, 74 (2002) 165-174.