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Original Research

Morphological, genetic and pigment diversity of *Nerium indicum* Mill in Iran

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Abstract: The *Nerium indicum* Mill organs and parts of the 40 genotypes were sampled from 5 habitats of southand south east of Iran. In total, 15 morphological and pigmentvariables were measured. Analysis of variance was carried out based on completely randomized design and revealed significance differencesat $p \leq 0.05$, 0.01 and 0.001 for the most variablesindicating a large-scale diversity among the genotypes. Cluster analysis divided the genotypes into 3 main groups. The first and second principal components had 34.73% and 18.67% of the variance, respectively. The main factors had 76.79% accumulated eigenvalue. Random amplified polymorphic DNA (RAPD) markers used to assess the population structure and genetic variation. In total, 361 polymorphic band amplified from effective 14 chosen RAPD markers. Analysis of molecular variance (AMOVA) showed 67% and 33% within and between populations genetic variation respectively. Cluster analysis by using UPGMA method divided genotypes into 6 main groups. A high cophenetic correlation coefficient ($r = 0.90$) was obtained. The first and second principle coordinates had 29.31% and 25.78% of the variance, respectively.

Key words: *Nerium indicum* Mill; Morphological and phytochemical variables; Cluster analysis; RAPD marker.

Introduction

Nerium indicum Mill belong to the family apocynaceae. It is an evergreen shrub, which is cultivated all over the world, especially in south-west Asia. It is used asan ornamental shrub in the Mediterranean region and in southern Asia.Also, this plant is used as traditional medicine in different parts of the world(1).Their leaves contain two principle components: neriin and oleandrin. They are glycosides with properties similar to digitalin (2) which are useful in the treatment of diverse ailments such as cardiac illnesses. A-amyrin and b-sitosterol phytochemicals found in alcoholic extract of*N.indicum* root bark which have anti-inflammatory, antineoplastic, antipyretic, and immunomodulating activity (1).Agreen dye from the flower is used in the treatment of skin diseases and also possess wound healing and anti-inflammatory property (3). In Calabria, southern Italy,itis used for the treatment of malaria (4), and it is also used in the treatment of hypertension and diabetes in Morocco (2).

The plant genetic diversity represents heritable morphological, phytochemical and structure variation intra and inter plant species. Appropriate knowledge of geographical diversity pattern is necessary for choosing effective strategies with regard to plant genetic resources conservation and germplasm organization.The mating system, floral morphology and mode of reproduction significantly affect plant distribution and genetic diversity (5). In recent years, there has been a significant increase in the application of morphological and molecular markers for assessing plant diversity and conservation of plant genetic resources. Molecular techniques have critical roles in studies of phylogeny and species evolution. Molecular markers divided into two categories; Hybridization-based markers, and PCR-based markers. RAPD technique is quick and easy PCR-based molecular marker for diversity study of unknown sequence plant species. This technique does not require prior DNA base sequence information and detects nucleotide sequence polymorphism using a single primer of arbitrary nucleotide sequence (6). Himalayas and southern peninsular India are main origin centers of *N.indicum.* The south and south east of Iran are important last distribution centers of this threaten plant in the country. Because of the high medicinal value, limited growth regions in Iran, loss, degradation and fragmentation of its natural habitats and risk of genetic eradication because of climate changes, deep analysis on entire of its traits is necessary to have proper view on cultivation, breeding, active genetic conservation of this medicinal, ecological and economical useful plant. The objective of this research was to analyze the morphological, genetic and pigment diversity of 40 *N.indicum* genotypes from 5 important distribution centers of Iran for having proper programs on *N. indicum*, Millconservation and breeding in Iran.

Materials and Methods

Plant material

A total of 40 genotypes of *Nerium indicum* Mill. collected from five distribution centers of south and south east of Iran. The samples were collected during January in 2015 and 2016. Sampling regions include: Dashtestan, Fariab, Bandar Abbas, Rudan, and HajiAbad. The all distribution centers information include: Latitude, Longitude, and Altitude are registered in Table 1. Some information such as: leaf length, leaf width, petiole

Table 1 . Geographic distribution and continental information of collected *N.indicum* genotypes.

No.	Population	Province	Genotype number	Longitude (E)	Latitude (N)	Altitude (m)
1	Dashtestan	Bushehr	8	50° 25'	28° 30'	80
2	Fariab	Kerman	8	57° 41'	28° 36'	640
3	Bandar Abbas	Hormozgan	8	56° 27'	27° 18'	10
4	Rudan	Hormozgan	8	57° 12'	27° 25'	192
5	Haji Abad	Hormozgan	8	55° 55'	28° 19'	1200

length scored in situ. All of the samples were kept up separately in -20 ˚C on dry ice for another laboratory observations.

Morphological and pigment studies

In total 15 morphological and pigmentsvariables were measured. The leaf length and width (mm), petiole length (mm), inflorescence branch diagonal (mm) and Diagonal of branch in 30 cm (mm) were measured by a Caliper and length / width ratio was calculated.

Pigment studies

Extraction was performed with 80% aceton and the yielded solution was centrifuged at 8000 rpm for 10 min. Chlorophyll and Carotenoid contents were colorimetrically determined using the following formula (7):

Chl_a (mg g^{-1}) = [(12.7×A_{663})-(2.6×A_{645})]×ml acetone / mg leaf tissue

Chl_b (mg g^{-1}) = [(22.9×A_{645})-(4.68×A_{663})]× ml acetone / mg leaf tissue

Chl_T (mg g^{-1}) = [(20.2×A_{645})+(8.02×A_{663})]× ml acetone / mg leaf tissue

C_{x+c} =1000A_{470}-1.90Chl_a-63.14Chl_b / 214,
(x=xanthophylls and carotenes).

Were A is absorption value, V is ultimate volume of extract and W is leaf fresh weight.

Statistical analysis

Fifteen variableswere measured for multivariable Analysis. Cluster analysis was made using Xlstat 2016 statistical software by ward method. Analysis of variance (ANOVA) was performed with SAS software (SAS Institute, version 9.1.3 Cary, NC). The statistical significance was determined at the $p \leq 0.05$, 0.01 and 0.001. LSD test used to compare means. Factor analysis was performed with SPSS 16.0 software.

Genetic study

DNA extraction

Genomic DNA was extracted from young leaves of genotypes of *N.indicum* Mill using the modified CTAB method. The CTAB extraction buffer consists of 2.0 g cetyl trimethylammonium bromide, 1.0 g polyvinyl pyrrolidone, 10.0 ml Tris-base1 M (pH 8), 28.0 ml NaCl5 M, 4.0 ml EDTA0.5 M (pH 8), 40 ml H_2O,and added 100 µl 2-mercaptoethanol to each 20 ml of CTAB extraction buffer. Young leaves (500 mg) from each sample were cryogenically ground in a mortar and pestle after chilling in liquid nitrogen. 1.5ml pre heated-CTAB extraction buffer at 65°C was added to the fine homogenized leaf powder. The samples were then incubated at 65°C for 1 h in a water bath with slow shaking every 10 min. Subsequently the mixture centrifuged for 15 minutes at 12600 rpm. Then, supernatant transferred to clean microfuge tube and an equal volume of chloroform:isoamyl alcohol (24:1) was added to solution mixed the solution by inversion. The mixture centrifuged for 5 min at 12600 rpm. The upper aqueous phase only transferred to a clean microfuge tube. To each tube added 500 µl of ice cold absolute ethanol and 50 µl of 7.5 M sodium acetate. After incubation at - 20 °C overnight, the mixture centrifuged for 30 min at 12600 rpm and then supernatant removed and allowed the DNA pellet to dry. Finally re-suspended the DNA pellet in 50 µl sterile water and stored at - 20°C until using in PCR. The quality of the DNA was estimated on an agarose gel (1%).

RAPD-PCR

Sixty RAPD primers were chosen for preliminary amplification tests. In primer screening, DNA amplifications were repeated at least twice for each primer. Fourteen RAPD primer (Table 2) with reproducible and score able amplifications characters were selected for next investigations.

PCR reactions were performed in a Thermal Cycler (from BIO-RAD) with a total volume of 25 µL containing 1× PCR reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmol primer, 10 ng template DNA and 1U of *Taq* DNA polymerase.PCR condition was set at 94 °C for 5 min, following 40 cycles of 94 °C for 1 min, 33 °C for 1 min, 72 °C for 2 min,which followed by a final extension of 5 min at 72 °C. Samples were run in 1.5% agarose gel in 1× Tris-borate-EDTA (TBE, pH 8.3) buffer. Gels were run at voltage 90 for 120 min and photographed under UV light using Gel Documentation system. The nucleic acid markers 100 bp and 3 kb (Pishgam Company, Tehran, Iran) were used to compare the amplification product sizes.

RAPD analysis

The RAPD bands were scored for their presence (1) or absence (0) and then transformed into a binary matrix. Polymorphic information content (PIC) was calculated, using the formula described by Powell *et al*. 1996 (13). Cluster analysis based on genetic similarities was performed by using NTSYS software version 2.02 (14). Principal coordinates analysis (PCoA) and genetic variation within and among populations was estimated by AMOVA, using GeneAlex software version 6.5 (15). Nei's gene diversity (H), Shannon's information index (I), Genetic diversity index within populations (H_S), Gene flow (Nm) and Genetic differentiation coefficient between populations (G_{ST}) were obtained by using the Population Genetic Analysis (POPGENE version 1.32) (16).

Table 2. The RAPD primers which used for genetic diversity study of five populations of *N. indicum* in Iran.

Reference	Melt temp	GC %	Sequences of primers	Primer name
(8)	32	60	5'-CCACGCATCA-3'	BA16
(8)	34	70	5'-CCTGGAACGG-3'	BD13
(8)	34	70	5'-GTTCGCTCCC-3'	BD17
(8)	34	70	5'-AAGCGGCCCT-3'	BE06
(9)	32	60	5'-AGGGCGTAAG-3'	D16
(8)	32	60	5'-TGCTAGCCTC-3'	J3
(9)	34	70	5'-CTCCATGGGG-3'	J5
(10)	34	70	5'-TGCGCGATCG-3'	MAP04
(10)	32	60	5'-GCGAATTCCG-3'	MAP10
(10)	34	70	5'-AGCCTGACGC-3'	MAP20
(11)	34	70	5'-CAGGCCCTTC-3'	OPA-01
(11)	32	60	5'-GTGACGTAGG-3'	OPA-08
(12)	34	70	5'-GGGTAACGCC-3'	OPA-09
(9)	30	50	5'-TTGAGACAGG-3'	U532

Results

Morphological and pigments study

Anova analysis

The analysis of variance showed significant differences between different genotypes for most studied variables at the $p \leq 0.05$, 0.01 and 0.001 (Table 3). The analysis of variance were non-significant for traits of inflorescence branch diagonal, number of leaf in 15 cm and diagonal of branch in 30 cm (Table 3).

Mean comparison analysis was performed on studied variables according to Duncan's Multiple-Range Test (SSR test) (Table 4). Results showed Rudan and Dashtestan genotypes had the highest leaf length with 132.872 and 128.471 mm, respectively. Also, these two genotypes had the biggest leaf width with 24.64 and 23.66 mm, respectively. The lowest length/width ratio was belong to Haji Abad genotype and the highest petiole lengths were belong to Haji Abad and Dashtestan genotypes with 9.58 and 9.57 mm, respectively. Bandar Abbas genotype had the most number of inflorescence per branch 4.87. The highest number of flower per inflorescencewas belong to Haji Abad 15.25. Bandar Abbas genotype had the most number of flower in branch 57.75. Rudan genotype had the lowest number of branches in 50 cm, 4.75 and Fariab genotype had highest Chlorophyll a, b, and Carotenoid content (Table 4).

Leaf width, leaf length, petiole length, number of flower in branch, carotenoid and total chlorophyll content variables had heredity higher than 60% (Table 5). Inflorescence branch diagonal, Diagonal of branch in 30 cm and Number of leaf in 15 cm variables had heredity less than 20 % (Table5).

Clustering analysis based on morphological and phytochemical variables

Cluster analysis divided populations in 3 main

Table 3. Mean squares, coefficient of variation (C.V), minimum, maximum and mean of studied variables in *N. indicum* genotypes.

	Mean squares			Domain		
Variables	**Model**	**Error**	**C.V**	**Min**	**Max**	**Mean**
Leaf length(mm)	3992.50***	52.45	6.74	71.40	143.30	107.42
Leaf width(mm)	136.02***	1.46	6.05	13.33	26.50	19.97
Length/Width	0.066**	0.01	2.21	5.01	5.60	5.37
Petiole length(mm)	19.04***	0.40	7.32	5.10	10.50	8.66
Inflorescence branch diagonal	0.53	0.28	37.53	1.00	3.00	1.95
Number of inflorescence per branch	12.22***	0.72	23.06	1.00	6.00	3.70
Number of flower per inflorescence	111.41***	3.60	15.89	4.00	18.00	11.95
Number of flower in branch	2101.58***	48.3	16.81	16.00	66.00	41.35
Number of leaf in 15 cm	61	25	36.77	16	43	29.5
Diagonal of branch in 30 cm(mm)	2.65	1.30	26.76	3.00	8.00	5.15
Number of branches in 50 cm	6.60*	2.17	25.40	2.00	8.00	5.80
Chlorophyll a	0.892***	0.028	18.6	1.4	1.7	1.55
Chlorophyll b	0.345***	0.017	15	0.5	0.8	0.65
Total Chlorophyll	0.980***	0.027	15.36	1.9	2.3	2.1
Carotenoid	0.293***	0.007	17.3	0.35	0.62	0.48

* ,** ,*** Significantly at a probability level of 5, 1 ,0.1 percentage, respectively. Degrees of freedom of model and error are 4 and 35, respectively.

Table 4. Mean comparison of studied variables in *N. indicum* genotypes (Duncan test, P ≤ 5% and P≤1%).

Variables	Dashtestan	Fariab	Bandar Abbas	Rudan	Haji Abad
Leaf length(mm)	128.471[a]	91.879[c]	82.338[d]	132.872[a]	101.538[b]
Leaf width(mm)	23.66[a]	16.90[c]	15.19[d]	24.64[a]	19.45[b]
Length/Width	5.42[a]	5.43[a]	5.42[a]	5.38[a]	5.21[b]
Petiole length(mm)	9.57[a]	8.86[b]	5.59[c]	9.32[ab]	9.58[a]
Inflorescence branch diagonal(mm)	1.87[a]	1.75[a]	1.87[a]	2.00[a]	2.25[a]
Number of inflorescence per branch	4.37[a]	1.87[c]	4.87[a]	4.37[a]	3.00[b]
Number of flower per inflorescence	5.62[c]	13.87[ab]	12.00[b]	13.00[b]	15.25[a]
Number of flower in branch	23.50[c]	25.25[c]	57.75[a]	55.12[a]	45.12[b]
Number of leaf in 15 cm	2.25[a]	1.87[a]	2.25[a]	2.00[a]	2.25[a]
Diagonal of branch in 30 cm(mm)	4.87[a]	4.50[a]	4.87[a]	5.62[a]	5.87[a]
Number of branches in 50 cm(mm)	5.50[ab]	5.25[ab]	6.75[a]	4.75[b]	6.75[a]
Chlorophyll a	1.42[d]	1.67[a]	1.54[b]	1.48[c]	1.52[bc]
Chlorophyll b	0.54[c]	0.73[a]	0.59[b]	0.56[c]	0.59[b]
Total Chlorophyll	1.94[d]	2.26[a]	2.13[b]	2.02[c]	2.11[b]
Carotenoid	0.38[d]	0.59[a]	0.47[c]	0.43[cd]	0.52[b]

Different letters show significant differences.

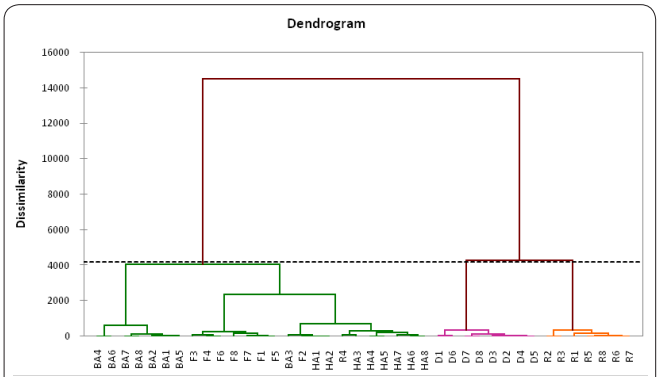

Figure 1. Clustering of *N.indicum* Mill. genotypes by morphological and phytochemical variables.

groups. The first main group consisted of 25 genotypes. The second main group was comprised of 7 genotypes and third group consisted of 8 genotypes "Figure 1". The first group consist: Bandar Abbas 1, 2, 3, 4, 5, 6, 7 and 8, Haji Abad 1, 2, 3, 4, 5, 6, 7 and 8, Fariab 1, 2, 3, 4, 5, 6, 7 and 8 and Rudan 4, The second group consist: Rudan 1, 2, 3, 5, 6, 7 and 8, The third group consist: Dashtestan 1, 2, 3, 4, 5, 6, 7 and 8. According cluster analysis. The main clusters cleave to tributary clusters which close genotypes was in same cluster.

Relationship analysis between variables was performed base on simple correlation coefficients. Maximum significant correlation observed between leaf length and leaf width (r = 0.99) and chlorophyll a to total chlorophyll (r = 0.99), in p ≤0.001. Also, there were significant correlations between, chlorophyll a and carotenoid (r = 0.87), number of flower per inflorescence and carotenoid (r = 0.66), leaf length and number of branch in 50 cm (r = -0.30). There was not significant correlation between numbers of branch in 50 cm with more variables.

PCA analysis

Principle component analysis (PCA) was evaluated and the first and second principal components which were a result of the linear combination of the 15 studied variables, were indicated. These two principal components explained 34.733% and 18.675% of the variance, respectively. The main four factors had 76.796% accu-

Table 5. Variance analysis, heredity and coefficient of variation of studied variables in *N. indicum* genotypes.

Morphological and phytochemical variables	Variance analysis		Coefficient of variation		Heredity
	genotypic	phenotypic	genotypic	phenotypic	
Leaf length	492.50	544.95	20.6	21.7	90.37
Leaf width	16.82	18.28	20.5	21.4	92.01
Length/Width	0.006	0.02	1.4	2.6	30
Petiole length	2.33	2.73	17.6	19	85.34
Inflorescence branch diagonal	0.031	0.318	9.02	28.9	9.74
Number of inflorescence per branch	1.43	2.15	32.3	39.6	66.51
Number of flower per inflorescence	13.47	17.07	30.7	34.5	78.91
Number of flower in branch	256.65	305.01	38.7	42.2	84.14
Number of leaf in 15 cm	4.5	29.5	13.57	34.74	15.25
Diagonal of branch in 30 cm	0.16	1.46	7.7	23.4	10.95
Number of branches in 50 cm	0.55	2.72	12.7	28.4	20.22
Chlorophyll a	0.108	0.136	21.20	23.79	79.41
Chlorophyll b	0.041	0.058	31.15	37.05	70.68
Total Chlorophyll	0.119	0.146	16.42	18.19	81.50
Carotenoid	0.035	0.042	38.97	42.69	83.33

Table 6. Eigenvalue and accumulated proportion of principal component analysis.

Factor	Eigenvalue	Variability (%)	Cumulative
1	5.210	34.733	34.733
2	2.801	18.675	53.408
3	1.951	13.007	66.415
4	1.557	10.381	76.796

mulated eigenvalue (Table 6).

Genetic study

RAPD polymorphism

Fourteen RAPD primers generated 361 bands, which all were polymorphic (100%). The number of band varied from 20 (primers BA16 and U532) to 33 (primer J3) (Table 5). The polymorphic level, calculated as the number of polymorphic band per primer, was 100% among all 14 primers.

Population genetic diversity, differentiation (G_{ST}), and gene flow (Nm)

Between applied RAPD markers, the polymorphism information content (PIC) varied from 0.82 (primer BA16) to 0.95(primer D16), with an average of 0.87. The Nei's gene diversity index (H) varied from 0.12 (D16) to 0.32(BA16), with an average of 0.22. The Shannon's information index (I) ranged from 0.22 (D16) to 0.49 (BA16), with an average of 0.36. The genetic diversity index within populations (H_S) varied from 0.09 (U532) to 0.22 (BA16), with an average of 0.15. The genetic differentiation coefficient between populations (G_{ST}) ranged between 0.08 (D16) and 0.38 (J5), with an average of 0.33. The gene flow (Nm) ranged between 2.34 (J5) to 8.79 (D16), with an average of 4.58, which indicated that there was a high gene exchange between *N.indicum* Mill. populations (Table7).

The Nei's gene diversity (H) and the Shannon's information index (I) were highest in the Dashtestan populations (H = 0.18; I = 0.28) followed by Haji Abad populations (H = 0.17; I = 0.26). Fariab populations had the lowest Nei's gene diversity and Shannon's information index (H = 0.11; I = 0.18) (Table 8).

Genetic identity and genetic distance between N.indicum populations

To further elucidate the gene differentiation between populations, Nei's original measure of genetic identity (I_N) and genetic distance (D) were evaluated (Table9). I_N ranged from 0.8377 to 0.9328 and D ranged from 0.0696 to 0.1771, in which the Rudan population and the Dashtestan population were the highest in the genetic identity (I_N = 0.9328) and closest in the genetic distance (D = 0.0696). The Haji Abad and Fariab populations were the lowest in genetic identity (I_N = 0.8377) and the furthest in genetic distance (D = 0.1771).

Clustering analysis of 40 N.indicum genotypes

Cluster analysis revealed six main clusters. Almost,

Table 7. Polymorphism characteristics of each RAPD maker in genetics diversity study of the*N.indicum* Mill. populations.

Nm	G_{ST}	H_S	I	H	PIC	P	N	Primer
2.95	0.26	0.22	0.49	0.32	0.82	20	20	BA16
4.96	0.13	0.18	0.35	0.21	0.86	25	25	BD13
3.71	0.26	0.12	0.33	0.20	0.88	28	28	BD17
4.32	0.33	0.10	0.35	0.22	0.87	28	28	BE06
8.79	0.08	0.11	0.22	0.12	0.95	32	32	D16
6.06	0.09	0.16	0.30	0.18	0.91	33	33	J3
2.34	0.38	0.17	0.48	0.31	0.85	22	22	J5
3.17	0.27	0.13	0.33	0.20	0.86	22	22	MAP04
8.33	0.11	0.15	0.31	0.18	0.92	28	28	MAP10
5.39	0.19	0.19	0.41	0.27	0.83	24	24	MAP20
3.00	0.30	0.14	0.38	0.24	0.87	29	29	OPA.09
3.38	0.24	0.17	0.39	0.25	0.84	29	29	OPB01
3.22	0.25	0.19	0.44	0.28	0.83	21	21	OPB08
4.52	0.34	0.09	0.34	0.22	0.90	20	20	U532
-	-	-	-	-	-	361	361	Total
4.58	0.33	0.15	0.36	0.22	0.87	-	25.78	Average

N: total number of bands; P: number of polymorphic bands; H: Nei's gene diversity; I: Shannon's information index; H_S: Genetic diversity index within populations; G_{ST}: Genetic differentiation coefficient between populations; Nm: Gene flow.

Table 8. Genetic variation and polymorphic features estimated in the populations of *N.indicum* Mill.

Population	Monomorphic bands	Polymorphic bands	Polymorphism (%)	H	I
Dashtestan	142	219	60.66	0.18	0.28
Fariab	217	144	39.88	0.11	0.18
Bandar Abbas	182	179	49.58	0.14	0.23
Rudan	173	188	52.07	0.15	0.23
Haji Abad	151	210	58.17	0.17	0.26
Average	173	188	52.07	0.15	0.23

Table 9. Nei 's original measures of genetic identity and genetic distance among five *N.indicum* populations with RAPD markers.

Population ID	BA	R	HA	D	F
BA	****	0.8689	0.9182	0.9161	0.8665
R	0.1405	****	0.8682	0.9328	0.8468
HA	0.0854	0.1413	****	0.9249	0.8377
D	0.0876	0.0696	0.0781	****	0.8846
F	0.1433	0.1662	0.1771	0.1226	****

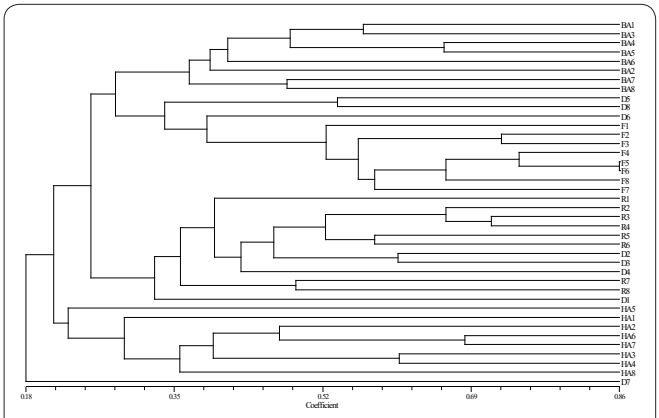

Figure 2. Dendrogram of 40 genotypes of *N.indicum* by RAPD analysis based on unweighted pair-group method with arithmetic average (UPGMA).

all genotypes from the same population were clustered into the same group "Figure 2". Forty *N.indicum* genotypes in Iran can be divided into 6 groups, of which all 8 genotypes from the Bandar Abbas population were clustered together; genotypes from the Dashtestan population and all 8 genotypes from the Fariab population were clustered together; genotypes from the Dashtestan population and all 8 genotypes from the Rudan population were clustered together. In the Dashtestan population, 12.5% genotype(one genotype) was clustered alone, 37.5% genotypes were clustered with genotypes of the Fariab population, and 50% genotypes were clustered with genotypes of the Rudan population. The majority of Haji Abad population genotypes, 87.5%, were clustered together, and the 12.5% of genotype was clustered alone.The cophenetic correlation coefficient indicated high correlation between the similarity matrix and the cophenetic matrix obtained from the UPGMA dendrogram (r=0.90), indicating a good representation of the molecular relationships among genotypes.

AMOVA and PCoA analysis

Analysis of molecular variance (AMOVA) showed that variance within the populations (67%) was higher than variance among the populations (33%) (Table 10).

Principle coordinates analysis (PCoA) was calculated to display genetic relationships among forty the*N. indicum* genotypes. The first and second principle coordinates had 29.31% and 25.78% of the variances, respectively. The first three coordinates derived from this

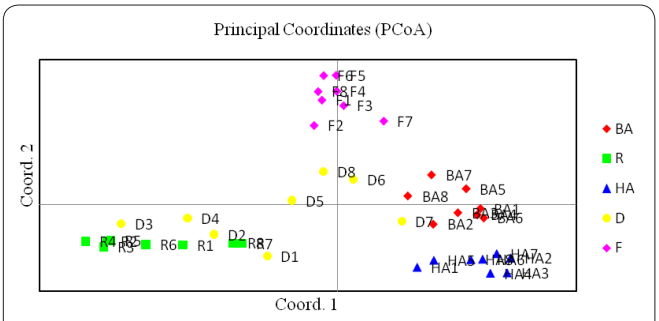

Figure 3. Two-dimensional scatter diagram of five the*N.indicum* populations based on principal coordinate analysis performed by GenAlex 6.5 software.

analysis could explained 70.79% of the variation. The two-dimensional plot also confirmed the cluster analysis results "Figure 3".

Discussion

Morphological and phytochemical study

Variance analysis results showed that there are significant differences between the genotypes for most studied variables. It indicated that high diversity exist between the genotypes which is first equipment for breeding program. The leaf width, leaf length and petiole length traits had highest genotypic to phenotypic variances ratio 92.01, 90.37 and 85.34 respectively. These results indicated that these three variables have higher selection efficiency in breeding programs. Obtained dendrogram based on morphological and phytochemical characteristics of the *N.indicum* genotypes illustrated that they clustered in three main groups. First cluster contains genotypes from more than one population including Bandar Abbas, Haji Abad, Fariab and one genotype from Roudan. Second and third cluster contain genotypes just from one population, Roudan genotypes in cluster 2 and Dashtestan genotypes in cluster 3. It means that there was not conformity between geographical and morphological diversity of the*N.indicum* genotypes, at least for cluster one.

Genetic study

In this study, the results of RAPD marker analysis on the*N.indicum* genotypes showed that high level of polymorphism for each RAPD primer (100 %) which illustrate that Iran has a rich *N.indicum.* Mill. germplasm. The percentage of polymorphic bands of *N.indicum* in the Dashtestan population was the highest, and followed by Haji Abad, Rudan, Bandar Abbas, and Fariab populations. These results illustrated that Dashtestan population and Haji Abad populations have more extensive application prospect in *N.indicum*Mill. breeding. Based on molecular analysis the*N.indicum* genotype clustered in main six groups.Although, genotypes from almostequal geographic characteristics and atmosphere with close distance placed in the same cluster. But, some geno-

Table 10. Analysis of molecular variance (AMOVA) among and within the *N.indicum* populations.

Source of variation	df	MS	Est. Var.	% of variation	
Among populations	4	184.538	18.342	33%	0.327*
Within populations	35	37.800	37.800	67%	
Total	39		56.142	100%	

df: degree of freedom, MS: Mean of squares, Est. Var: Estimated variance,* ;significant in p≤0.05.

types like Dashtestan 7 placed in separated cluster. This can be from migration or genetic exchanges current. Variable genotypes in a population could be because of wild type plants adaptation in a special environment during long-term natural selection pressure. However, further systemic studies are required to determine main cause of the inconsistency of genetic distance and geographical distance. This result is in agreement with the results of the Yuan *et al*. (17) and Qiu *et al*.(18) which showed that the geographical distance was not the main reason of gene exchange between pomegranate populations.

Gene differentiation and gene flow are important indexes to evaluate the population genetic structure. The value of G_{ST} is 0.33, based on the RAPD marker for *N. indicum*Mill. in Iran, indicating that the gene differentiation was higher within the population (67%) than between the populations (33%). Mean of Nm which is higher than Nm of gene differential threshold (>1) indicated that there were high gene exchange between the*N. indicum* Mill. Populations. Also, the range of genetic identity from 0.8377 to 0.9328 and genetic distance range from 0.0696 to 0.1771, indicated the presence of variability among the five populations of *N.indicum*.

However, The Result of morphological and genetic diversity studies on the*N.indicum* Mill. Populations were not completely identical. But, they described that the geographical distance was not the main reason of gene exchange among the*N.indicum* Mill. Populations. In conclusion, it should mentioned that this research denoted high diversity among and within of the*N.indicum* Mill. Populations which could be applied in following breeding and gene bank conservation programs.

Potential conflicts of interest

The 40 genotypes of *Nerium indicum* Mill were diverse based on genetics, phytochemical, and morphology.
Fourteen RAPD primers generated 361 bands.
The Nei's gene diversity and the Shannon's index were highest in the Dashtestan populations.
The value of G_{ST} is 0.33, based on the RAPD marker for *N. indicum* in Iran.

Authors' contributions

Sharifi-Sirchi, G. R., and Mohammadi, F conceived of the presented idea. Sharifi-Sirchi, G. R., and Samsampour, D developed the theory and performed the computations. Sharifi-Sirchi, G. R., and Mohammadi, F verified the analytical methods. Sharifi-Sirchi, G. R., supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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