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Asian Journal of Plant Science and Research, 2013, 3(5):20-27

ISSN : 2249-7412
CODEN (USA): AJPSKY

Genetic variation in natural populations of *Salvadora oleoides*: An important medicinal plant that needs conservation

Sushila Saini[1] and Jaya ParkashYadav[2*]

[1]*Department of Botany, J.V. M. G. R. R. College, Ch. Dadri, Bhiwani, Haryana, India*
[2]*Department of Genetics M.D. University, Rohtak, Haryana, India*

ABSTRACT

Evolutionary potential of a taxon is a function of the nature and amount of genetic variability occurring in it. Conservation genetic studies suggest that genetic diversity significantly influences the long-term viability and persistence of local population. The present investigation was undertaken to analyze genetic diversity in eleven natural populations of Salvadora oleoides by isozyme electrophoresis using seven enzyme systems. A total of 500 plants were studied for allozyme variation by means of acrylamide gel electrophoresis using seven enzyme systems. Parameters of genetic diversity and its partitioning were calculated. The genetic analysis demonstrated that S. oleoides maintain relatively high genetic diversity (p was 0.62, n_a was 1.75 and H_o and H_e were 0.184 and 0.199 respectively) when compared with other plant taxa. Genotypic proportions at most loci in most population's fit Hardy-Weinberg expectations. However, small heterozygote deficiencies were commonly observed. The coefficient of genetic differentiation among populations based on F_{ST} equaled 0.023. Genetic identities between population's pairs were high (mean I= 0.98). These values are high as compared with other widespread congener species. The levels of genetic diversity maintained within populations of S. oleoides indicate that an appropriate sampling design for ex situ safeguarding should capture the majority of genetic diversity found within this taxa to help ensure the long term viability of this species.

Keywords: Genetic diversity, *S. oleoides*, Allozyme, conservation, long lived perennial

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INTRODUCTION

India possesses a distinct identity, not only because of its geography, history and culture but also because of the great diversity of its natural ecosystems. The panorama of Indian forests ranges from evergreen tropical rain forests in the Andaman and Nicobar Islands, the Western Ghats, to dry alpine scrub high in the Himalaya to the north. Between the two extremes, the country has semi-evergreen rain forests, deciduous monsoon forests, thorn forests, subtropical pine forests in the lower montane zone and temperate montane forests [1]. *Salvadora persica* L. and *Salvadora oleoides* Decne. are two important medicinal plant species of western India. In Thar Desert, their wide ranging ecological, social and economic importance on the one hand and declining population on the other necessitates that the species are included in restoration programmes.

Salvadora oleoides Decen belonging to the family Salvadoraceae is a small, medicinal multipurpose perennial tree adaptable to arid conditions [2]. The leaves, root, bark, fruits and seeds are used for the treatment of cough, fever and asthma. Roots are also used for chest diseases, while latex is used for treating sores [3]. The plant holds strong antifungal [4], anti-parasitic, antiviral [5] and antibacterial [6] properties. The young branches and leaves are also

favorite fodder for camels because of the high water content (15-36%). Leaves and stem of both the species have shown significant hypoglycemic and hypolipidemic properties and is effective in rheumatic pains [7-8]. *S. oleoides* seed oil shows 100% toxicity to Anopheles stephensi at 0.01% [9]. The most vital aspect of oil is its constituency of low percentage of C_8 and C_{10} fatty acids that holds a great economic significance [10]. Fruits of *S. oleoides* Decne are also found to be rich sources of calcium containing about 15 times the amount of Ca present in wheat [11].

Despite its multipurpose utility *S. oleoides* has not received due attention of cytologists and geneticists to estimate the range and quantum of existing natural variation which is essential for framing meaningful genetic improvement programme, aimed at sustainable utilization. In the last ten years there has been a major decline in the populations of *S. oleoides.* The area under this genus is diminishing very rapidly and this will be a major threat for forest ecosystem. At many areas there is no seed setting on this plant, and if there is any seed setting, the germination power of the seeds is very low and after few weeks of their ripening, the viability of seed vanishes. Associated with decreasing population sizes are increased extinction risk from stochastic factors (e.g. food, drought), environmental factors (e.g. decreased pollinator service) and genetic factors (e.g. increased inbreeding and decreased genetic diversity) [12-13]. Knowledge of the level and distribution of genetic variation both within and among populations facilitates the conservation of gene resources and helps in developing strategies for conservation and tree improvement programmes [14-15]. For genetic variation studies, the choice of appropriate genetic markers assumes a great significance. Although morphological characters have been used traditionally to characterize levels and patterns of diversity, these traits alone represent only a small portion of plant genome and also influenced by the environmental factors [16-17]. In recent times use of molecular marker for study of genetic diversity is increasing [18-19]. But still isozymes are widely used because of their relative simplicity and cost effectiveness as compared to molecular markers, particularly in studies of intra- and inter-specific variation [20-21]. A large number of papers investigating the pattern and distribution of genetic variation with in plant species using isozyme electrophoresis have been published following the fast progress in the development of marker techniques [22-23].

In this paper, we report the level and pattern of genetic variability in eleven populations of *S. oleoides* in their natural range in western India. The objective is to provide valuable information for future conservation and breeding programmes on *S. oleoides*.

MATERIALS AND METHODS

Study species:

The study was performed on *S. oleoides* populations in western India, where this species in rapidly decreasing in size. The site is located in state of Haryana (27°37'- 30°35'N, 74°28'-77°36'E) in western India (Figure 1). Eleven populations (KH- Khidwali; RO- Rohtak; DH- Dhandlan; MT- Matanhail; KO- Kosli; DH- Dhoki; MG- Mohindergarh; RW- Rewari; AT- Atali; KN- Kanina; NC- Nangal Choudri) of *S. oleoides* (500 plants) were sampled across the species geographical range. Leaves were collected from 40-50 trees from April to June (separated from each other by at least 20 m to avoid samples of the same clone).

Electrophoresis:

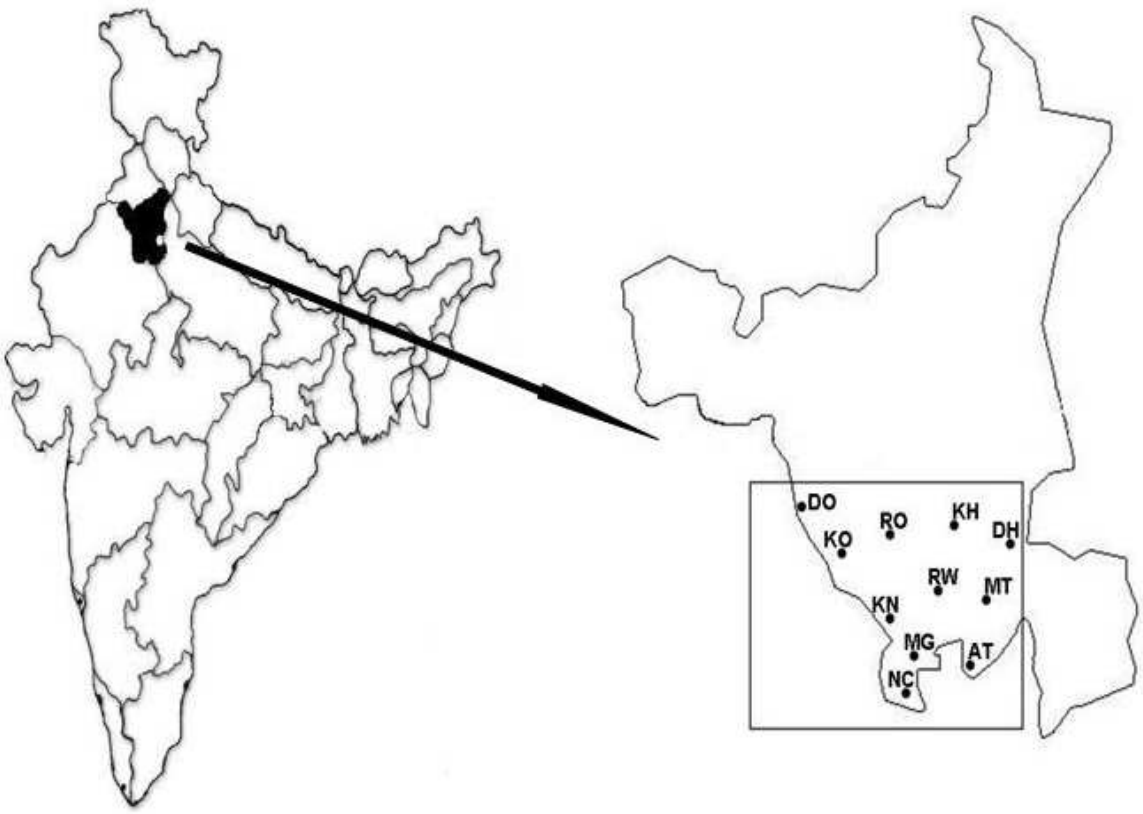
Electrophoresis was carried out on vertical 1.5% polyarylamide gels with a tris glycine, pH 8.3 buffer system. Enzymes extracts were prepared by crushing approximately 1g of leaf tissue in liquid nitrogen to which 2ml of extraction buffer [24] was added. The following seven enzymes were assayed as per standard methods [25-26] with some modifications to obtain better resolution – Esterase (Est-1, Est-2, Est-3, Est-4 Est-5), Acid phosphatase (Acph-1), Catalase (Cat-1, Cat-2), Malate dehydrogenase (Mdh-1, Mdh-2, Mdh-3, Mdh-4, Mdh-5), Peroxidase (Prx-1, Prx-2, Prx-3, Prx-4, Prx-5), Phosphoglucoisomerase (Pgi-1, Pgi-2) and Phosphoglucomutase (Pgm-1, Pgm-2) (loci are indicated in the parentheses). The allelic variants were designated as fast (F), medium (M) and slow (S).

Data analysis:

For each population, genetic diversity parameters were assessed in terms of mean number of alleles per locus (n_a), percentage of polymorphic loci (95% criterion) (p), expected heterozygosity (H_e) and observed heterozygosity (H_o). With in each population, single locus genetic structure was investigated by testing for deviations from Hardy-Weinberg (H-W) equilibrium. The extent and direction of the deviations from the H-W equilibrium with in each locus was quantified by calculating the weighted mean of F_{IS} (inbreeding coefficient) across all populations and by testing it for the significance of deviations from zero [27]. Total genetic diversity (H_T), with in population genetic diversity (H_S), and proportion of total genetic diversity occurring among populations (F_{ST}) were calculated for each

polymorphic locus. The heterogeneity of the allele frequencies over all eleven populations was examined using a contingency chi-square test [28]. Gene flow (Nm) was estimated from; Nm = 0.25 (1- F_{ST})/ F_{ST}. The genetic relationships among all populations were assessed by estimating Nei's genetic distances for all population pairs [29]. All the above calculations were performed using the program POPGENE ver 1.32 [30].

Figure 1: Map of studied populations of *S. oleoides*



DH- Dhoki; KO- Kosli; KN- Kanina; RO- Rohtak; MG- Mohindergarh; NC- Nangal Choudri; RW- Rewari; KH- Khidwali; MT- Matanhail; AT- Atali; DH- Dhandlan

RESULTS

Genetic variability:

Analyses of eleven populations (500 plants) of *S. oleoides* by isozyme electrophoresis lead to resolution of 22 genetic loci. Of these 22 genetic loci, 18 loci were polymorphic (Est-1, Est-2, Est-3, Est-4 Est-5, Acph-1, Cat-1, Cat-2, Mdh-2, Mdh-3, Mdh-4, Mdh-5, Prx-3, Prx-4, Pgi-1, Pgi-2, Pgm-1 and Pgm-2) while four loci showed monomorphism (Mdh-1, Prx-1, Prx-2 and Prx-5). The number of alleles observed at each polymorphic locus ranged from two (15 loci) to three (three loci). A total of 39 alleles were identified among the eleven *S. oleoides* populations sampled. One locus (Cat-2) showed a very low level of polymorphism, with the most frequent allele close to fixation in each category. Three Mdh loci (Mdh-3, Mdh-4, Mdh-5) showed moderate level of polymorphism (with one frequent allele) while rest of loci showed an expressive polymorphism and similar frequencies of the most common alleles. Eight loci showed significant linear alterations in allelic frequencies in eleven natural populations of *S. oleoides* (Table 1). The data on genetic indices indicate that mean number of alleles/locus

Table 1: Distribution of allelic frequencies for the species studied.

Loci	Allele	KH	RO	DH	MT	KO	DO	MG	RW	AT	KN	NC
EST-1	**F**	0.05	0.07	0.06	0.10	0.14	0.13	0.21	0.18	0.23	0.27	0.30
	S	0.95	0.93	0.94	0.90	0.86	0.87	0.79	0.82	0.77	0.73	0.70
EST-2	**F**	0.95	0.85	0.90	0.92	0.89	0.91	0.89	0.84	0.89	0.91	0.91
	S	0.05	0.15	0.10	0.08	0.11	0.09	0.11	016.	0.11	0.09	0.09
EST-3	**F**	0.83	0.79	0.95	0.93	0.88	0.88	0.95	0.95	0.96	0.97	0.98
	S	0.17	0.21	0.05	0.07	0.12	0.12	0.05	0.05	0.04	0.03	0.02
EST-4	**F**	0.11	0.12	0.10	0.19	0.20	0.21	0.20	0.23	0.18	0.22	0.25
	S	0.89	0.88	0.90	0.81	0.80	0.79	0.80	0.77	0.82	0.78	0.75
EST-5	**F**	0.05	0.16	0.10	0.16	0.18	0.22	0.09	0.15	0.12	0.12	0.13
	S	0.95	0.84	0.90	0.84	0.82	0.78	0.91	0.85	0.88	0.88	0.87
ACPH-1	**F**	0.92	0.86	0.88	0.83	0.83	0.88	0.77	0.80	0.77	0.73	0.72
	S	0.08	0.14	0.12	0.17	0.17	0.12	0.23	0.20	0.23	0.27	0.28
CAT-1	**F**	0.14	0.14	0.11	0.17	0.16	0.20	0.20	0.23	0.17	0.19	0.26
	S	0.86	0.86	0.89	0.83	0.84	0.80	0.80	0.77	0.83	0.81	0.74
CAT-2	**F**	1	1	1	0.99	0.98	0.97	0.97	0.97	0.95	0.94	0.93
	S	0	0	0	0.01	0.02	0.03	0.03	0.03	0.05	0.06	0.07
MDH-2	**F**	0.26	0.20	0.20	0.16	0.19	0.14	0.16	0.11	0.10	0.12	0.11
	S	0.74	0.80	0.80	0.84	0.81	0.86	0.84	0.89	0.90	0.88	0.89
MDH-3	**F**	0.93	0.93	0.94	0.90	0.87	0.95	0.96	0.86	0.87	0.94	0.93
	S	0.07	0.07	0.06	0.10	0.13	0.05	0.04	0.14	0.13	0.06	0.07
MDH-4	**F**	0.06	0.11	0.06	0.13	0.15	0.16	0.11	0.07	0.06	0.08	0.11
	S	0.94	0.89	0.94	0.87	0.85	0.84	0.89	0.93	0.94	0.92	0.89
MDH-5	**F**	0.93	0.87	0.94	0.87	0.82	0.82	0.84	0.90	0.93	0.87	0.85
	S	0.07	0.13	0.06	0.13	0.18	0.18	0.16	0.10	0.07	0.13	0.15
PRX-3	**F**	0.21	0.26	0.19	0.22	0.26	0.19	0.16	0.14	0.07	0.14	0.09
	S	0.79	0.74	0.81	0.78	0.74	0.81	0.84	0.86	0.93	0.86	0.91
PRX-4	**F**	0.09	0.09	0.06	0.08	0.08	0.07	0.12	0.16	0.15	0.10	0.10
	M	0.11	0.11	0.09	0.07	0.10	0.15	0.13	0.11	0.11	0.10	0.11
	S	0.80	0.80	0.85	0.85	0.82	0.78	0.75	0.73	0.74	0.80	0.79
PGI-1	**F**	0.11	0.13	0.09	0.12	0.14	0.12	0.09	0.14	0.08	0.11	0.09
	M	0.75	0.73	0.72	0.71	0.69	0.68	0.72	0.69	0.77	0.69	0.74
	S	0.14	0.14	0.19	0.17	0.17	0.20	0.19	0.17	0.15	0.20	0.17
PGI-2	**F**	0.19	0.17	0.14	0.18	0.15	0.20	0.19	0.16	0.16	0.14	0.17
	S	0.81	0.83	0.86	0.82	0.85	0.80	0.81	0.84	0.84	0.86	0.82
PGM-1	**F**	0.06	0.06	0.03	0.08	0.08	0.09	0.12	0.09	0.06	0.09	0.13
	M	0.10	0.07	0.11	0.08	0.08	0.08	0.08	0.06	0.05	0.04	0.05
	S	0.84	0.87	0.86	0.84	0.84	0.83	0.80	0.85	0.89	0.87	0.82
PGM-2	**F**	0.11	0.13	0.12	0.14	0.14	0.14	0.13	0.11	0.12	0.12	0.15
	S	0.89	0.87	0.88	0.86	0.86	0.86	0.87	0.89	0.88	0.88	0.85
Population size		42	43	40	45	50	58	47	44	41	44	46

F, M and S represent fast, medium and slow electromorphs respectively. (KH- Khidwali; RO- Rohtak; DH- Dhandlan; MT- Matanhail; KO- Kosli; DH- Dhoki; MG- Mohindergarh; RW- Rewari; AT- Atali; KN- Kanina; NC- Nangal-Choudri) ranged from 1.72 to 1.77 with an average of 1.75. The percentage of polymorphic loci (p, 0.95 criterion) ranged from 64% to 77%, with an average of 72%. The expected hetrozygosities (H_e) and observed heterozygosities (H_o) were relatively high and varied from 0.169 to 0.220 and from 0.162 to 0.200 with an average of 0.199 and 0.184 respectively (Table 2). 193 Fixation indices were tested for deviations from Hardy Weinberg expectations with 13 significant ($P<0.05$) results. Twelve of the significant fixation indices were positive; indicating heterozygote deficits and one was negative, indicating an excess of heterozygote. Despite these statistically significant results, the overall F_{IS} values were nearly equal to zero, indicating that the populations were in Hardy-Weinberg equilibrium.

Table 2: Parameters of genetic variability for the species studied

Population	A	P_{95}	H_o	H_e
KH	1.72	0.64	0.162	0.169
RO	1.72	0.77	0.187	0.200
DH	1.72	0.73	0.162	0.161
MT	1.77	0.77	0.186	0.198
KO	1.77	0.77	0.194	0.220
DO	1.77	0.73	0.200	0.216
MG	1.77	0.68	0.188	0.211
RW	1.77	0.68	0.187	0.209
AT	1.77	0.68	0.173	0.189
KN	1.77	0.77	0.188	0.203
NC	1.77	0.77	0.195	0.212
Mean	1.75	0.72	0.184	0.199

A- average number of allele per locus; P_{95}- proportion of polymorphic loci at 95 % criteria; H_o- mean observed heterozygosity per locus; H_e- mean expected heterozygosity per locus.

Genetic differentiation:

Amount of genetic differentiation at 18 polymorphic loci in eleven natural populations of *S. oleoides* was calculated in terms of Wright's Fixation Index (F_{ST}). The heterozygosity at the polymorphic loci was partitioned with in population as well as between population components. The value of total heterozygosity (H_T) ranged from 0.052 at CAT-2 to 0.446 at PGI-1. The value of F_{ST} at 18 polymorphic loci ranges from 0.005 (Pgm-2) to 0.056 (Est-1) with average value equaling 0.023. Some of the loci (Est-1 and Est-3) have revealed modest genetic differentiation (F_{ST} value more than 0.05; 0.056 at Est-1 and 0.054 at Est-3) while all other loci have depicted lower amounts of genic differentiation. The allelic frequency distribution patterns at polymorphic loci were analyzed on the basis of contingency chi square test. The populations revealed allelic heterogeneity at Est-1, Est-3, Acph-1 and Prx-3 loci out of 18 loci. Since the allelic frequency patterns were largely similar at all the polymorphic loci, there seems to be little interpopulation heterogeneity with respect to allelic frequency distribution in *S. oleoides* (Table 3). The overall

Table 3: Partitioning of the total genetic variability

Loci	H_T	H_S	F_{ST}
EST-1	0.270	0.255	0.056
EST-2	0.184	0.183	0.008
EST-3	0.155	0.147	0.054
EST-4	0.300	0.292	0.025
EST-5	0.236	0.227	0.036
ACPH-1	0.300	0.293	0.023
CAT-1	0.296	0.291	0.017
CAT-2	0.052	0.054	0.036
MDH-2	0.266	0.262	0.012
MDH-3	0.152	0.151	0.007
MDH-4	0.183	0.176	0.038
MDH-5	0.223	0.214	0.039
PRX-3	0.290	0.281	0.030
PRX-4	0.349	0.346	0.008
PGI-1	0.446	0.442	0.008
PGI-2	0.280	0.279	0.007
PGM-1	0.275	0.272	0.011
PGM-2	0.226	0.224	0.005
Mean	0.249	0.243	0.023

H_T - total genetic diversity; H_S - mean genetic diversity within populations; F_{ST} - coefficient of genetic differentiation between populations.

Gene flow (Nm) among populations equaled 10.61, which gives an estimate of the average number of migrants between all studied populations per generation. The observed value indicated that gene exchange between populations is high and populations are genetically connected. The genetic distance, based on the allelic frequencies of the allozyme markers, were calculated for each pair of populations to estimate the extent of their divergence. The average genetic distance between populations equaled to 0.02. The lowest genetic distance (0.001) was found between populations of Matanhail and Kosli, and the greatest genetic distance (0.06) was found between populations of Rohtak and Dhandlan. Nei genetic identity values ranged from 0.94 to 0.99 with average value equaling 0.98, indicating that allele frequencies were fairly similar among populations (Table 4).

Table 4: Genetic distances and genetic identities among populations of *S. oleoides*

Popul-ation	KH	RO	DH	MT	KO	DO	MG	RW	AT	KN	NC
KH	***	0.972	0.969	0.995	0.995	0.993	0.992	0.986	0.989	0.988	0.984
RO	0.028	***	0.940	0.973	0.973	0.972	0.967	0.959	0.963	0.962	0.959
DH	0.031	0.06	***	0.973	0.971	0.971	0.970	0.967	0.968	0.969	0.967
MT	0.005	0.027	0.027	***	0.999	0.998	0.996	0.992	0.994	0.995	0.992
KO	0.005	0.027	0.029	0.001	***	0.996	0.996	0.991	0.993	0.994	0.991
DO	0.007	0.028	0.03	0.002	0.004	***	0.996	0.991	0.992	0.994	0.992
MG	0.008	0.033	0.03	0.004	0.004	0.004	***	0.994	0.997	0.998	0.997
RW	0.014	0.041	0.033	0.008	0.009	0.009	0.006	***	0.994	0.992	0.992
AT	0.011	0.037	0.032	0.006	0.007	0.008	0.003	0.006	***	0.998	0.997
KN	0.012	0.038	0.031	0.005	0.006	0.006	0.002	0.008	0.002	***	0.999
NC	0.016	0.041	0.033	0.008	0.009	0.008	0.003	0.008	0.003	0.001	***

Above diagonal: Genetic identities; Below diagonal: Genetic distances

DISCUSSION

Many studies using enzyme polymorphism in forest trees have shown the occurrence of very high genetic diversity, especially with in populations, whereas low differentiation has been observed among populations [31]. Isozyme polymorphism in eleven populations of *S. oleoides* collected from different locations of Haryana was observed for estimating the genetic variability present in this species. Out of 22 loci, 18 loci exhibited polymorphism. *S. oleoides* showed high genetic diversity as indicated by percent polymorphic loci (p = 72%), mean number of alleles per locus (n_a = 1.75) and mean heterozygosities (H_o = 0.184 and H_e = 0.199). Values of percent polymorphic loci were higher than those reported previously for species of similar taxonomic status (woody perennial p = 64.7), geographic range (widespread, p = 58.9), mating system (out-crossing, p = 66.1), pollination mechanism (mixed animal p = 57.4) and seed dispersal (wind p =55) [32-33]. *S. oleoides* had a mean expected heterozygosity (H_e= 0.199) higher than that of other widespread tree species such as: *Narcissus longispathus*, H_e = 0·139 [34] *Alnus maritima* H_e = 0.180 [35]; *Ulmus laevis*, H_e = 0.088 [36] and *Jatropha curcas* H_e = 0.0993 [37].

Eight loci showed significant linear alteration in the allelic frequencies in eleven natural populations of *S. oleoides*. There are two main hypotheses to explain the observed changes in the allelic frequencies. The first is that natural selection acts directly on the allozymic loci in such a way as to favour certain alleles. Second is that linkage disequilibrium occurs, in which some allozymic loci might be linked to other loci that are under the action of natural selection. However considering the sparse evidence of linkage disequilibrium for the allozyme markers in natural populations, the first hypothesis seems to be more appropriate to explain such a phenomenon [38]. Prediction of genotypic frequency was compared to observe genotypic distribution and significance of the deviation were tested by Chi-square Goodness of Fit statistics. Only thirteen out of the 193 chi square tests were significant at 0.05 levels, thus the observed genotypic proportions were in accordance with Hardy-Weinberg expectations suggesting that populations were randomly mating. Another measure of conformation to equilibrium conditions was Wright's fixation index, which could be interpreted as the proportional increase or reduction in heterozygosity as compared to panmictic expectations. The value of F_{IS} ranges from –1.0 to +1.0; +ve values indicate a deficit of heterozygotes and –ve values indicate excess of heterozygotes. For majority of loci the average fixation index was remarkably close to zero, thus corroborating the results of chi-square analysis. However at many loci the value of F_{IS} comes out to be +ve indicating heterozygote deficiencies at these loci. The value of observed heterozygosity (H_o = 0.184) was lower than expected heterozygosity (H_e = 0.199) again indicating a deficit of heterozygotes. Small heterozygote deficiencies are commonly observed in out breeding plant population and the factors responsible for this are partial selfing, population structuring due to consanguineous mating and the Wahlund effect [39].

S. oleoides exhibited high total genetic (H_T = 0.249) diversity then reported for other woody species; *Phragmites australis*, H_T = 0.22 [40] and *Bixa orellana*, H_T = 0.064 [41]. F_{ST} measures the proportion of variation among populations relative to the total genetic diversity (H_T). Lower F_{ST} values of *S. oleoides* indicate little genetic differentiations between populations of *S. oleoides* collected from Haryana. Low F_{ST} values (F_{ST} = 0.024) were found in *S. oleoides* which are in agreement of F_{ST} values found in long lived woody perennials: *Pinus aristata*, F_{ST} = 0.131 [42] and *Ballota* species, F_{ST} = 0.045-0.099 [43]. The mean genetic identity for *S. oleoides* (I = 0.98) was higher indicating little genetic divergence among eleven populations of *S. oleoides*. The value of genetic identity was similar to that of other out crossing woody species; *Tillandsia achyrostachys*, I = 0.935 [44] and *Castanopsis carlesii*, I = 0.967 [45]. Further the range of genetic identities between population pairs of *S. oleoides* was small (I =

0.94 to 0.99). Genetic identities are influenced both by polymorphic loci and the number of monomorphic loci; F_{ST} values based on polymorphic loci also provide an additional perspective on population divergence. It was found that 98% (F_{ST} = 0.023) of the genetic variations at polymorphic loci was found with in *S. oleoides* populations thus indicating very little divergence. High value of gene flow (Nm) and low level of population divergence suggests that genetic drift is not currently of great concern for this species. Thus an examination of the genetic diversity values obtained in the population revealed a great capacity of this species to restore the levels of diversity. In fact, in spite of having been subjected to exploitation in the past, these populations present diversity levels compatible with undisturbed populations. The maintenance of this level of genetic diversity should allow this species to maintain its ability to adapt to novel environmental changes. Finally, the great homogeneity of the diversity indices suggests that the species has sufficient capacity to oppose the natural loss of genetic variability by drift. Reestablishment of populations at protected sites within the historic range of the species should be considered to help ensure the long-term viability of the species.

Acknowledgement
Authors are thankful to Department of Science and Technology, New Delhi for the award DST-FIST programme to the Department of Genetics, M.D. University, Rohtak, India

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