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A Female Sex Associated Randomly Amplified Polymorphic DNA Marker in Dioecious *Hippophae salicifolia*

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ABSTRACT

In many dioecious plants, gender influences economic value, breeding schemes and opportunities for commercial harvest. In the plant kingdom, dioecism has arisen independently in different families and genera, and several distinct genetic mechanisms regulating dioecy have been found. Molecular studies are beginning to detail the genetic control of dioecy in several plant species. However, there is paucity of information on chromosomal and genetic basis of sex determination. In the present study, randomly amplified polymorphic DNA (RAPD) markers were used to differentiate between the pistillate and staminate genotypes of *Hippophae salicifolia*. Out of the 31 decamer primers used, 27 primers amplified the genomic DNA of all the 10 genotypes taken for study. 25 primers were found to be polymorphic. In total, 118 DNA bands were reproducibly obtained, out of which 95 (80.5%) were polymorphic. The polymorphism was scored and used in band-sharing analysis to identify genetic relationships. Cluster analysis based on Jaccard's similarity coefficient using unweighted paired group method of arithmetic averages (UPGMA) grouped all the 10 genotypes into two major groups. Similarity indices ranged from 0.26 to 0.82. A female-specific RAPD marker of 1190 bp was obtained with primer OPF-11. This RAPD marker specific for female genotype can be utilized in the future to develop sequence characterized amplified region (SCAR) marker. To our knowledge this is the first report on gender identification in *Hippophae salicifolia* using RAPD markers.

Keywords: dioecy, RAPD markers, seabuckthorn, sex determination

INTRODUCTION

Hippophae salicifolia D. Don, commonly known as seabuckthorn is a remarkable multipurpose forest tree species belonging to family Elaegnaceae and consists of seven species and eight sub species. *Hippophae salicifolia* is widely distributed in southern slopes of Himalayan mountains in range of 2000-3850m (Nepal *et al*. 2001), including Nepal, Bhutan, India and China. This plant has recently attributed the attention of scientists, policy makers, industrialists, foresters and farmers in India for its great economic and ecological uses. The fruits of the plant are in the form of berries, which are nutritious and have high vitamin content. These berries are reported to contain multivitamins such as A, B_1, B_2, C, K, E and P, fatty acids, amino acids, soluble sugars (glucose, fructose and xylose) and 27 trace elements (Singh 2004). It is also rich in proteins, organic acids, carotenoides, flavonoids, etc. and extremely rich in minerals like iron, cobalt, molybdenum, etc. Local traditional medical practitioners also produce medicines from the *Hippophae salicifolia* fruits for curing cough, lungs and digestive disorders and headaches (reviewed in Ruan *et al*. 2007). The fruit is utilized in Europe, Russia, China and other countries for industrial production of food products, medicines, and cosmetics (Singh 2001). It has extraordinary capacity to grow and survive under adverse climatic conditions (-43 to +40°C), has a highly developed root system with very good soil binding capacity, and is an excellent nitrogen fixer (180 kg N ha^{-1} yr^{-1}) (Akkermans *et al*. 1983). Seabuckthorn is a wonderful plant for overcoming afforestation and ecological rejuvenation of the cold desert areas of Himalayas where plantations of poplars, willows and robinia have not given very encouraging results. It has potential to economically transform these harsh and marginal areas plagued by low productivity (Sankhyan *et al*. 2005).

Hippophae salicifolia is a diploid species with basic chromosome number 2n=24, out of which eleven pairs are autosomes and one pair consists of sex chromosomes (Lbeda 2003). The male genotype is a heterogametic sex containing one X and Y chromosome each. It is predicted that there may be no constraints to make hybrids between *H. salicifolia* and other species of *Hippophae*, because all the species of *Hippophae* have the same number of chromosomes (2n=24) and similar karyotypes (Yalin 1988). An important feature of *H. salicifolia* is its dioecious nature viz., staminate and pistillate genotypes are born on different plants. Since it is a dioecious plant, and breeding aims at producing both female and male improved cultivars. For economical cropping, the ratio of pistillate to staminate plants is important, as the number of fruiting trees i.e., pistillate plants should be maximum. The sex cannot be determined in the seed, or prior to 3-4 years of growth, on or during budding of both vegetative and mixed (vegetative–generative) buds when initiated along shoots. The vegetative buds form primarily on plants which are not bearing fruit. On fruiting plants, the mixed buds are formed. These in appearance, according to the gender, on male plants the buds are larger, more protruding and have 6-8 covering scales. On female plants the buds are smaller, more elongated, sensile on the branch, and have only two covering scales. At appearance, transplanting may occur to facilitate the establishment of the correct ratio (male/female), required for optimum production in the orchard (Li and McLoughlin 1997). For economical cropping, the ratio of pistillate to staminate plants is important. Pistillate plants are fruit bearer thus is commercially more important than the staminate plants so the percentage of pistillate plants should be maximum. Plantation of seedlings of unknown

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sex may result into less number of pistillate plants and more number of staminate plants that are unproductive in terms of fruits and this situation can lead to long-term affect on the yield. It has been reported that 6-7% male trees are adequate for tree pollinations (Gakov 1980), whereas in *Hippophae rhamnoides* 8-12% is recommended (Walf and Wegart 1993). A 9:1 ratio of female to male plants as has been reported in commercial plantation of other dioecious plants such as kiwifruit (*Actinidia deliciosa* var. *deliciosa*) (Singh 1998). Thus, correct identification of staminate and pistillate genotypes at the juvenile stage is important to maintain proper density of pistillate and staminate bushes/ trees in commercial plantations, because morphologically the sexes are indistinguishable with regard to various phenomorphological features and staminate and pistillate trees can only be identified at the time of fruiting. *H. salicifolia* commences the difference in the male and female sexes after 3-4 years and currently there is no method to distinguish between males and females prior to the reproductive phase. Molecular markers can be utilized to diagnose and select a genotype based on linked DNA markers, long before the phenotype is apparent. Financial resources and valuable time and money could be saved if unfavorable genotypes could be discarded at an early stage of commercial plantations (Jeppsson 1999). Hence, present study was carried out to understand molecular basis of differentiation between male and female genotypes so that the gender of the plants can be identified at the juvenile stage and material can be raised as staminate and pistillate populations. Sex-linked genetic markers are also useful in breeding programmes and allow understanding of dioecism in *H. salicifolia*.

A large number of methodologies exist for the assessment of genetic diversity in plant species. The relative genetic diversity among the individuals or populations can be determined using morphological, biochemical and molecular markers. Phenotypic characters have a limited importance since they are considerably influenced by environmental factors and developmental stages of the plant and also due to the fact that in some species adequate levels of phenotypic polymorphism is not available (Tatineni *et al*. 1996). Use of biochemical markers has been restricted due to limited number of informative loci in many species. Their developmental stage and tissue dependency for expression has been another limiting factor (Bhattacharya *et al*. 1999). A major difficulty in using isozymes for genotype identification in various trees is that there may not be sufficient polymorphism for unambiguous identification of individual genotypes this may be because of inherent lack of polymorphism or due to the lack of genetic variability in the group of genotypes examined. Also, the method based on analysis of gene products are subject to environmental variation for the identification of genotype than the method based on direct analysis of the genome. Molecular markers, based on the DNA sequence polymorphism are independent of environmental conditions and show high levels of polymorphism (Choudhary *et al*. 2001).

Since the advent of Random Amplified Polymorphic DNA (RAPD) markers in 1990 (Williams *et al*. 1990), their use in plant genetic analysis have increased in an exponential manner principally due to the ease of procedure. RAPDs are relatively quick, inexpensive and require no prior sequence information of the target genome. Low amounts of DNA are sufficient, require no radioactive elements and detect a good number of polymorphisms. Significant work has been carried out on differentiation of pistillate and staminate genotypes in various dioecious plant species using RAPD markers e.g., *Silene latifolia* (Mulcahy *et al*. 1992; Zhang *et al*. 1998; Nakao *et al*. 2002), *Pistachio vera* (Hormaza *et al*. 1994; Tang 2003), in the F1 hybrids resulting from crosses between *Populus trichocarpa* and *Populus deltoides* (Mclethchie and Tuskan 1994), *Actinidia chinensis* (Gill *et al*. 1995), *Cannabis sativa* L. (Sakamoto *et al*. 1995; Mandolino *et al*. 1999), *Humulus lupulus* (Polley *et al*. 1997), *Salix viminalis* (Alstrom *et al*. 1998; Gunter *et al*. 2003), *Hippophae rhamnoides* (Persson and Nybom 1998), *Atriplex garrettii* (Ruas *et al*. 1998), *Silene dioica* (DiStilio *et al*. 1998), *Gracilaria gracilis* (Martinez *et al*. 1999), *Piper longum* (Banerjee *et al*. 1999), *Distichlis spicata* (Eppley 2000), *Carica papaya* (Parasnis *et al*. 2000; Urasaki *et al*. 2002), *Myristica fragrans* Houtt. (Shibu *et al*. 2000), *Pistachio atlantica* (Kafkas *et al*. 2001), *Pistachio terebinthus* (Kafkas *et al*. 2001), *Pistachio eurycarpa* (Kafkas *et al*. 2001), *Trichosanthes dioica* Roxb. (Singh *et al*. 2002), *Actinidia deliciosa* var. *deliciosa* (Shirkot *et al*. 2002), *Eucommia ulmoides* (Xu *et al*. 2004) thus indicating the immense potential of RAPD in sex determination of dioecious plant species.

This is probably the first effort to develop RAPD markers for gender identification in *H. salicifolia*. We report here the application and reliability of RAPD markers to investigate the gender in male and female genotypes of *H. salicifolia*.

MATERIALS AND METHODS

Plant material

Very young, fresh, green leaves of 10 genotypes (five each of male and female) of *H. salicifolia* were collected from the Jeera Experimental Farm of Himachal Pradesh Krishi Vishavavidalaya (HPKVV) situated at Kuppa in Kinnaur district and frozen in liquid nitrogen, and stored at -80°C until used for DNA extraction. It falls in the dry temperate zone of Himachal Pradesh, as the area is covered with snow during November to March. Five trees each of male and female genotypes of *H. salicifolia* were marked at the time of fruiting i.e. during August/September, when it was possible to differentiate between the male and female genotypes. Fresh and young green leaves were excised from each of five selected male and female plants in April and samples were stored in liquid nitrogen until further analysis.

Genomic DNA isolation

All the chemicals used in this study were of molecular biology grade. Genomic DNA from fresh, very young and green leaves of each five male and female samples of *H. salicifolia* was isolated using the cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980) with some modifications (Yadav 2003). Three grams of leaf tissue were washed with sterile Milli-Q water and put into a mortar, dipped into liquid nitrogen and crushed to a fine powder with the help of prechilled pestle and mortar. Powdered leaf samples were mixed into 12.5 ml prewarmed (at 65°C) DNA extraction buffer [100 mM Tris-HCl: pH 8.0 (Plusone, Pharmacia Biotech, Amersham, England), 20 mM EDTA (ethylene diamine tetra acetic acid): pH 8.0 (Sigma-Aldrich, St. Louis, USA), 1.4 M NaCl (Plusone), 2% w/v CTAB (Sigma-Aldrich) contained in 50 ml centrifuge tubes (Tarsons, Kolkata, India; Model 541040) and just before adding the powdered sample, 0.2% β-mercaptoethanol (Sigma-Aldrich) was added to each tube and mixed quickly with the help of a vortex. The tubes were then incubated at 65°C in a water-bath (Neolab, Heidelberg, Germany) for 1 hr with gentle shaking, at least three times at about an interval of 15 min. Chloroform: isoamyl alcohol (24: 1, v/v) (Sigma-Aldrich) was taken in 30 ml centrifuge tubes (Tarsons; Model 541030) and samples from 50 ml centrifuge tubes (Tarsons) were poured into these tubes one by one, after incubation for 1 hr. These samples were gently inverted up and down for 15 min for thorough mixing and tubes were centrifuged (Sigma, Osterode am Harz, Germany; Model 3K18) for 15 min at 18,000 × *g* at room temperature to pellet debris. The upper aqueous layer was carefully transferred to fresh 50 ml centrifuge tubes containing 7.5 ml of isopropanol (Sigma-Aldrich) each. These were mixed by inverting the tubes gently and kept in a refrigerator for 2 hrs overnight until white fibrous nucleic acid was visible. Cloudy fibrous DNA that could be seen was pipetted out with the help of broad-mouth white tips and transferred into 2 ml centrifuge tubes (Axygen, Union City, California, USA) which were centrifuged for 5 min at 5000 × *g*. The supernatant was decanted off carefully so that pellets formed did not move down the tube. 500 µl of 70%

ethanol (Merck, Darmstadt, Germany) was added to each tube and centrifuged at 5000 × *g* for 2 min. The supernatant was decanted off again and the pellets washed with 500 µl of 70% ethanol. Two or three washings with 70% ethanol were sufficient to remove salts from DNA. Pellets were air-dried at room temperature till the last drop of ethanol evaporated and DNA was finally dissolved in 500 µl of TE buffer (10 mM Tris-HCl, pH 7.4-8.0 at 25°C; 1 mM EDTA, pH 8.0).

DNA purification

The isolated DNA was purified to remove the RNA, proteins, polysaccharides and phenols (Murray and Thompson 1980). After dissolving the DNA in TE buffer, 10-15 µl of RNase (Sigma-Aldrich) (10 mg/ml) per 500 µl was added in DNA and incubated at 37°C for 1 hr to degrade RNA. An equal volume of 25: 24: 1 (v/v) phenol: chloroform: isoamyl alcohol mixture (pH 8.0) (Sigma-Aldrich) was added, gently mixed for 5 min and centrifuged for 10 min at 10,000 × *g*. Three layers formed and the upper layer was separated into 2 ml Eppendorf tubes and a double volume of 100%-chilled ethanol was added to precipitate DNA (DNA was seen as clumped). Precipitated DNA was then centrifuged at 10,000 × *g* for 10 min at room temperature and the supernatant was discarded. The pelleted DNA was washed thrice in 70% ethanol with gentle tapping to expel salts.

Quantification of purified DNA

The concentration and purity of DNA was checked by taking absorption at A_{260} and DNA was quantified by following the protocol given by Pharmacia Biotech DyNA Quant Tm200 fluorometer instruction manual.

DNA amplification

In the present study, a total number of 31 random decamer primers (Operon Technology, Alameda, CA, USA) were used for RAPD-PCR to amplify the purified genomic DNA from five male and five female plants of *H. salicifolia* because RAPD markers are relatively quick, inexpensive and require no prior sequence information of the target genome. Low amounts of DNA are sufficient, require no radioactive elements and detect a good number of polymorphism and significant work has been carried out by different workers on differentiation of pistillate and staminate genotypes in various dioecious plant species using RAPD markers. DNA amplification of the 10 genotypes of *H. salicifolia* was carried out using RAPD-PCR as performed by Williams *et al*. (1990) with modifications (Yadav *et al*. 2006). PCR reactions were performed in 25 µl of reaction volume containing 1X PCR buffer (Bangalore Genei, Bangalore, India) (10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl and 0.01% gelatin), 200 µM of each dNTP, 10 pmol of random primers (Operon Technology), 0.75 U of *Taq* DNA polymerase (Bangalore Genei) and 25 ng genomic DNA. Amplification was performed in a MJ Research Inc., USA, Thermal Cycler (Model PTC-100). In total, 40 cycles were used, each cycle consisting of 1.0 min denaturation at 94°C, 1.0 min annealing at 36°C and 2.0 min. extension at 72°C. The reactions were maintained at 95°C for 3 min before the start of the first cycle and after the final cycle all amplification products were completed with an 8 min extension at 72°C. Amplification products were separated by electrophoresis on a 1.5% (w/v) agarose (Sigma-Aldrich, molecular biology grade) gel with 1 µg/ml ethidium bromide (EtBr) (Pharmacia Biotech, molecular biology grade) for 2 h at 4.5 V/cm constant voltage. The size of the fragments was estimated using a Gene Ruler 100 bp DNA ladder plus (MBI Fermentas, Vilnius, Lithuania) marker. Photographs of EtBr-stained gels were made using Syngene-gel documentation system with image analysis software programmes (Gene Snap and Gene Tools, Frederick, Maryland, Syngene). For RAPD analysis, the bands with same molecular weight and mobility were treated as identical fragments and amplification products were scored as present (1) or absent (0) for each primer-genotype combinations. Molecular data generated from all the primers for RAPD were entered into a binomial matrix. The bivariate 0-1 data were analysed using SIMQUAL route to generate Jaccard's similarity coefficients (Jaccard 1908) using NTSYS-PC software version 2.0 (Rohlf 1997). These similarity coefficients were then used to construct dendrograms depicting the genetic relationship employing the Unweighted Paired Group Method of Arithmetic Averages (UPGMA) algorithm and SAHN clustering using NTSYS software.

RESULTS AND DISCUSSION

Out of the 31 random decamer primers screened (**Table 1**), only 27 primers (**Table 2**) were found to produce intensely stained and reproducible polymorphic bands among 10 genotypes of seabuckthron after two RAPD-PCR reactions, while the rest of the primers resulted in either no amplification or smeared profiles. These 27 primers selected and yielded 118 fragments. The number of bands (fragments) per primer ranged from 1 (OPF-10) to 9 (OPF-01), the average number of bands per primer being 4.37 (**Table 3**). The size of the amplified DNA products separated by electrophoresis in 1.5% agarose gel ranged from 500 to 3200 bp. Of the 118 bands scored, 95 (80.5%) were polymorphic and the rest monomorphic. Maximum numbers of polymorphic bands (8) were obtained with the primer OPF-01 and OPF-14, however, no polymorphic band was observed with primer OPC-12 and OPF-10. The total number of monomorphic bands also varied from a minimum of 1 (OPD-20, OPF-01, OPF-03, OPF-04, OPF-08, OPF-10, OPF-13, OPF-15 and OPG-14) to a maximum of 3 (OPC-12 and OPF-12).

Only a single primer viz., OPF-11 with sequence 5′-TTGGTACCCC-3′ produced an 1190-bp band (**Fig. 1**) and resulted in a single gender specific marker. This 1190-bp RAPD marker, which was consistently and reproducibly present in female genotypes and absent in male genotypes, can be used for selection of staminate and pistillate genotypes of *H. salicifolia*. To check the stability and reliability of this DNA marker the experiment was repeated with individual male and female DNA samples.

A genetic similarity matrix was calculated on the basis

Table 1 Nucleotide sequence of decamer random primers (Operon) used for present study.

Primers	Nucleotide sequence (5′- 3′)	GC ratio	Tm value (°C)
OPC-11	AAAGCTGCGG	60	32
OPC-12	TGTCATCCCC	60	32
OPD-20	ACCCGGTCAC	70	34
OPF-01	ACGGATCCTG	60	32
OPF-02	GAGGATCCCT	60	32
OPF-03	CCTGATCACC	60	32
OPF-04	GGTGATCAGG	60	32
OPF-05	CCGAATTCCC	60	32
OPF-06	GGGAATTCGG	60	32
OPF-07	CCGATATCCC	60	32
OPF-08	GGGATATCGG	60	32
OPF-09	CCAAGCTTCC	60	32
OPF-10	GGAAGCTTGG	60	32
OPF-11	TTGGTACCCC	60	32
OPF-12	ACGGTACCAG	60	32
OPF-13	GGCTGCAGAA	60	32
OPF-14	TGCTGCAGGT	60	32
OPF-15	CCAGTACTCC	60	32
OPF-16	GGAGTACTGG	60	32
OPF-17	AACCCGGGAA	60	32
OPF-18	TTCCCGGGTT	60	32
OPF-19	CCTCTAGACC	60	32
OPF-20	GGTCTAGAGG	60	32
OPG-01	CTACGGAGGA	60	32
OPG-02	GGCACTGAGG	70	34
OPG-03	GAGCCCTCCA	70	34
OPG-04	AGCGTGTCTG	60	32
OPG-06	GTGCCTAACC	60	32
OPG-11	TGCCCGTCGT	70	34
OPG-12	CAGCTCACGA	60	32
OPG-14	GGATGAGACC	60	32

Table 2 Number of RAPD-PCR amplified bands generated by 27 decamer primers in five male and five female genotypes of *Hippophae salicifolia.*

Primer name	Total no. of bands amplified	Total no. of monomorphic bands amplified	Total polymorphic bands amplified	Range of amplified bands in bp
OPC-11	7	0	7	600-3100
OPC-12	3	3	0	675-950
OPD-20	7	1	6	650-2100
OPF-01	9	1	8	650-3200
OPF-02	3	0	3	650-1750
OPF-03	5	1	4	750-1190
OPF-04	6	1	5	500-2100
OPF-05	4	0	4	750-1750
OPF-08	4	1	3	750-2600
OPF-09	3	2	1	550-1725
OPF-10	1	1	0	1195
OPF-11	2	0	2	1190-1500
OPF-12	5	3	2	750-2750
OPF-13	4	1	3	1031-1510
OPF-14	8	0	8	825-2900
OPF-15	2	1	1	690-750
OPF-16	6	0	6	670-2500
OPF-17	3	2	1	725-1600
OPF-18	2	0	2	650-825
OPF-19	3	0	3	600-2000
OPF-20	4	0	4	850-1600
OPG-02	8	2	6	900-2400
OPG-03	3	2	1	940-1580
OPG-04	6	0	6	900-2450
OPG-11	2	0	2	1700-2100
OPG-12	5	0	5	525-1900
OPG-14	3	1	2	800-1800
Total	118	23	95	-

Table 3 Summary of RAPD amplified products obtained from 10 genotypes of *Hippophae salicifolia* examined in the study.

Total number of primers examined	31
Total number of primers amplified the genomic DNA	27
Number of polymorphic primers	25
Total number of marker bands amplified	118
Total number of polymorphic marker bands	95
Size range	500-3200 bp
Average number of marker bands per primer	4.37
Average number of polymorphic marker bands per polymorphic primer	3.80
Percentage of polymorphic bands	80.5%
Total number of amplified products (amplicons) obtained (27 primers)	672
Number of amplicons per primer	24.89

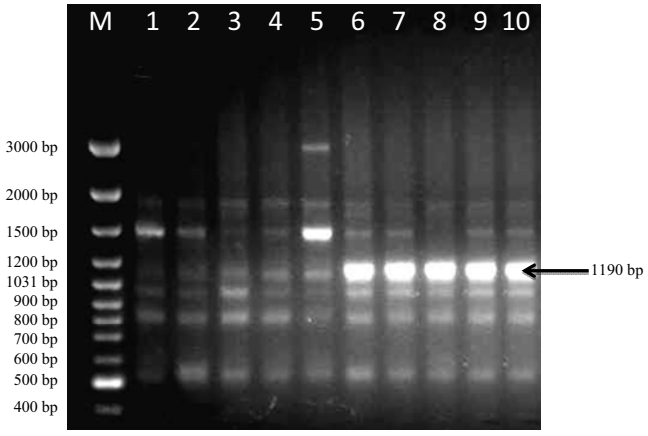

Fig. 1 RAPD profile generated by primer OPF-11 for five male (1-5) and five female (6-10) genotypes of *Hippophae salicifolia.* M: Gene Ruler^{TM} 100 bp DNA Ladder plus.

of Jaccard's algorithm for RAPD data. Similarity indices estimated on the basis of 27 primers ranged from 0.26 to 0.82 (**Table 4**). One of the major contributory factors to the high degree of polymorphism may be due to its evolutionary status as an out-crossing angiosperm (Kundu 1999; Dhillon *et al*. 2005). The high diversity revealed by RAPD is in agreement with the conclusion that out-breeding woody plants retain considerable variability (Hamrick 1990). This was further supported by molecular marker studies (Hamrick and Godt 1989). The maintenance of a high genetic variance within population was favoured by genetic system of the species like effective gene flow, out breeding, mutation, high genetic load, etc (Matyas 1996). The highest value of similarity coefficient (0.82) was detected between Female-1 and Female-3 genotypes. Maximum RAPD diversity (74%) was evident between Male-5 and Female-5 genotypes. Ecological and geographical differentiation are important factors, which influence breeding and sampling strategies of tree crops (Namkoong 1986), which further help in understanding the population structure. Variation in genetic diversity within species is usually related with geographic range, mode of reproduction, mating systems, seed dispersal and fecundity (Loveless 1992). Studies on genetic variations in various coniferous and angiospermic tree species observed that species with geographically distinct ranges tend to show a moderate to high-inter and intra-populations genetic variations (Muller-starck *et al*. 1992). High genetic diversity detected in the present study may be due to all these prevalent background factors as *H. salicifolia* is dioecious in nature thus is an essential out breeder.

The clustering of RAPD based genetic similarity values using the UPGMA method presented in dendrogram (**Fig. 2**). The first major bifurcation in the dendrogram divided the 10 genotypes of *H. salicifolia* into two groups.

Here, in this study, only one random primer was able to differentiate between the staminate and pistillate genotypes of *H. salicifolia* and only one gender specific marker was identified. One female specific marker i.e., OPF-11 of 1190 bp has been identified and no male specific RAPD band has been identified. RAPD-PCR technology has also been successfully used to differentiate in *H. rhamnoides*. Persson and Nybom (1998) used 78 primers to screen pools of male and female plants from two crosses of dioecious seabuckthorn. Only one marker OPD-15 of 600 bp was identified and found specific for all the males of Leikora X Pollmix cross. Hormaza *et al*. (1994) used RAPD technology for finding markers linked to sex determination in *Pistachio vera.* They used 14 males and 10 females from a 'Lessen' X 'Peters' cross and nine male and 12 females from a 'Kermen' X 'Peters' cross for their study and tested 700 decamer primers. Only one primer OPO-08 was able to produce an amplification pattern that could differentiate male and female samples of the two crosses. A 945-bp female-specific band was obtained from the pooled female DNA that was absent in males. In contrast, in the present study, we were able to identify only a single female-specific marker in the five female and five male selections belonging to *H. salicifolia* and no male-specific marker was identified. One 1190 bp RAPD band amplified by random primer OPF-11 (5′-TTGGTACCCC-3′) from the genomic DNA of all five pistillate genotypes were absent in PCR products from five staminate genotypes of *H. salicifolia*. Thus, within the limit of male and female genotypes available in the study, one RAPD band of 1190 bp amplified by OPF-11 appeared to be female sex-related DNA marker in *H. salicifolia*. This analysis of sex-linked inheritance pattern that was necessary to confirm the male/female specificity of these DNA markers has been initiated in *H. salicifolia*. However, to confirm these RAPD bands in all available female entries, the genomic DNA needs to be examined in the unknown seedling population of *H. salicifolia* and the RAPD profile developed with this prime needs to be analyzed. Absence of this specific band in male genotypes would confirm this marker to be female specific which will be useful to identify seedlings in a breeding program enabling their removal before plantation is carried out at a commercial level. Thus, the results of the present study confirm the potential of

Table 4 Genetic similarity matrix for five males (M_1-M_5) and five females (F_1-F_5) genotypes of *Hippophae salicifolia* using Jaccard's similarity coefficient.

	M_1	M_2	M_3	M_4	M_5	F_1	F_2	F_3	F_4	F_5
M_1	1.000									
M_2	0.726	1.000								
M_3	0.704	0.667	1.000							
M_4	0.731	0.714	0.667	1.000						
M_5	0.532	0.516	0.396	0.523	1.000					
F_1	0.747	0.671	0.688	0.737	0.484	1.000				
F_2	0.652	0.585	0.596	0.675	0.495	0.753	1.000			
F_3	0.671	0.635	0.714	0.720	0.438	0.829	0.698	1.000		
F_4	0.689	0.693	0.690	0.674	0.495	0.753	0.677	0.718	1.000	
F_5	0.482	0.463	0.638	0.486	0.266	0.538	0.512	0.603	0.549	1.000

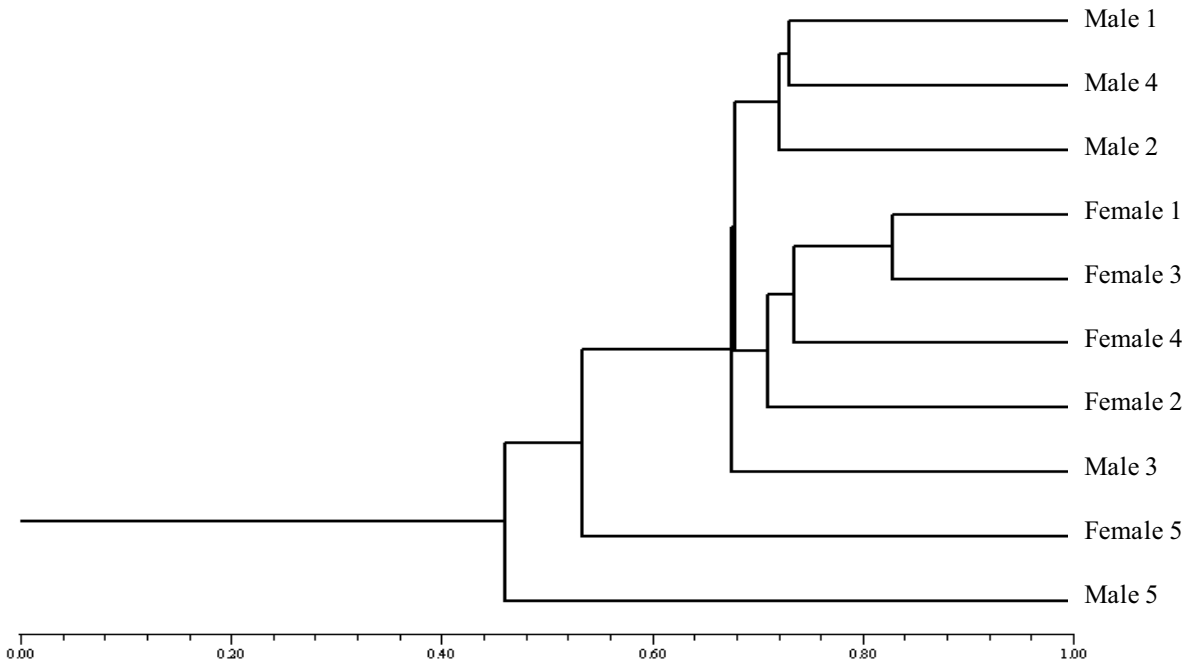

Fig. 2 Dendrogram showing genetic relationship among 10 genotypes of *Hippophae salicifolia* based on 95 polymorphic fragments obtained with 27 random decamer primers in RAPD.

RAPD technique to differentiate between the pistillate and staminate genotypes of *H. salicifolia*. This RAPD marker specific for female genotype can also be utilized in the future to develop a sequence characterized amplified region (SCAR) marker.

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