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RESEARCH ARTICLE

Molecular identification of sex in *Hippophae rhamnoides* L. using isozyme and RAPD markers

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Abstract In many dioecious plants, gender affects economic value, breeding schemes and opportunities for commercial harvests. *Hippophae rhamnoides* L. is a dioecious plant species in which female genotypes are commercially preferred over male genotypes. Its berries have rich medicinal, nutritional and pharmaceutical properties because of their large amounts of vitamins, essential oils, proteins, fatty acids, free amino acids and flavanoids. Primary limitation for breeding *H. rhamnoides* L. is its dioecious nature, since gender cannot be identified by traditional methods. Therefore, some reliable and quick methods need to be developed. This communication deals with the development of isozyme and RAPD markers for early sex identification in this dioecious tree. The isozyme analysis was conducted with four enzyme systems, viz. peroxidase, esterase, malate dehydrogenase and catalase. The peroxidase enzyme system produced a female specific sex marker, which successfully differentiated between the staminate and pistillate genotypes of *H. rhamnoides* L. Thirty five random decamer primers were used in our study and one male sex linked marker was identified. OPD-20 (5′-ACTTCGCCAC-3′) displayed a band at 911 bp that expressed polymorphism between male and female genotypes. The staminate and pistillate genotypes could be distinguished using RAPD marker OPD-20_{911}. These results revealed the immense potential of peroxidase isozyme patterns and RAPD as genetic markers for sex identification in *H. rhamnoides* L.

Key words *Hippophae rhamnoides* L., sex markers, isozyme, RAPD, dioecism

1 Introduction

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 bases of gender determination in plants.

Seabuckthorn is a multipurpose tree belonging to the family Elaeagnaceae, with six species and twelve subspecies in the world (Ruan and Xie, 2001). Of the global area of seabuckthorn resources 90 per cent, i.e., more than 1.5 million hectare, is found in China. Natural seabuckthorn stands are also widespread in Asia, Europe and on riverbanks and coastal dunes along the Baltic coast of Finland, Poland and Germany. In Asia, seabuckthorn is distributed through the Himalayan regions including India, Nepal, Bhutan and Northern parts of Pakistan and Afghanistan. In Himachal Pradesh, *Hippophae* species are found in the valleys of Laddakh, Lahaul & Spiti and Kinnaur districts.

Seabuckthorn viz. *Hippophae rhamnoides* L. has received global attention owing to its rich medicinal properties, its role in environmental conservation, in afforestation programs and for greening hill regions. Recently, it has gained importance as a horticultural tree for berry production because of its high nutritional and medicinal properties. The berries are acidic in nature and the most prominent feature of its juice is its rich source of vitamins C, E and K. Nutritious products from seabuckthorn include tea, juice, wine, jams and snacks. Residues, after the juice and oil have been extracted, are used as fodder and food additives. The medicinal properties include prevention of cardiovascular diseases, cancers, skin problems, burns and digestive tract disorders and for anti-senilism, anti-inflammation, anti-radiation damage and for improvement of the immune system (Buhatel et al., 1991; Chen, 1991; Ruan and Li, 2000).

H. rhamnoides L. is a diploid species with a basic chromosome number 2n = 24, of which eleven pairs are autosomes and one pair consists of sex chromosomes (Lebeda, 2003). A unique feature of seabuckthorn is its dioecious nature, i.e., the tree is either male or female and the sex of saplings cannot be determined until flower buds appear, which may take 5–7 years (Synge, 1974). For economic reasons, the ratio of male to female plants is important in fruit bearing

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plants, i.e., their pistillate trees should be optimum and a proportion of 8–12% of staminate trees is considered adequate for pollination (Walf and Wegart, 1993). Singh (1998) has recommended a 9:1 ratio of female to male trees for seabuckthorn. Thus, correct identification of staminate and pistillate genotypes at its juvenile phase is important to maintain proper densities of pistillate and staminate bushes/trees in a plantation. Currently, there is no method to distinguish between male and female prior to the reproductive phase of this dioecious tree. Molecular markers can be utilized to diagnose and select a genotype based on linked DNA markers, long before its phenotype is apparent. Financial resources and valuable time and money can be saved if unfavorable genotypes could be discarded at an early stage of commercial plantations (Jeppsson, 1999).

The present study was carried out to develop male and female specific isozymes and RAPD markers so that the gender of *H. rhamnoides* L. can be identified at its juvenile stage and material can be raised as staminate and pistillate populations. Sex-linked genetic markers are also useful in breeding programs and allow understanding of dioecism in *H. rhamnoides* L.

2 Materials and methods

2.1 Plant material

The study material was taken from the village of Sunnam in the Kinnaur District, India, where natural seabuckthorn stands occur at an elevation of about 2250 m.s.l. It falls in the dry temperate zone of the northwestern Himalayas (Himachal Pradesh), where the area is covered with snow from November to March. Five male and five female *H. rhamnoides* L. trees were marked at the time of fruiting, i.e., during August and September, when it was possible to differentiate between male and female trees. Fresh and young green leaves were excised from each of five randomly selected male and female trees in the month of April and samples were stored in liquid nitrogen until further analysis.

2.2 Isozyme studies

Four enzyme systems were investigated for gender specific banding patterns: catalase (EC 1.11.1.4), peroxidase (EC 1.11.1.7), malate dehydrogenase (EC 1.1.1.37) and esterase (EC 3.1.1.2). Enzyme extraction of male and female samples was carried out using a Tris citrate buffer, pH 8.3 (Trizma base 6.2 $g \cdot L^{-1}$ and citric acid 1.46 $g \cdot L^{-1}$) along with 0.75 $g \cdot L^{-1}$ of insoluble polyvinyl pyrolidone. To achieve resolution of the enzymes a single gel/tray buffer system of lithium borate, comprising 0.04 $mol \cdot L^{-1}$ lithium hydroxide and 0.19 $mol \cdot L^{-1}$ boric acid, pH 8.3, was used for all four enzymes. Different staining recipes were used for each enzyme, i.e., catalase was stained by the standard method of Conkle (1983), peroxidase by Lee (1973), esterase by Scandalios (1969) and the Shaw and Prasad (1970) method was used for malate dehydrogenase. Polyacrylamide gel electrophoresis was carried out to separate isozymes, due to their high power of resolution, transparency and chemical inertness. An anionic system (Davies, 1964; Ornstein, 1964) was used for all enzyme systems. Differences in isozyme banding patterns (phenotypes) were recorded as differences in relative mobility, the migration of each band relative to the protein front.

2.3 RAPD studies

2.3.1 Genomic DNA isolation

Genomic DNA from fresh, young and green leaves of the five male and five female sample trees was isolated by the CTAB method (Doyle and Doyle, 1987). The isolated DNA was purified by successive RNase and proteinase K treatments followed by phenol: chloroform: isoamyl alcohol (25:24:1, v/v) and chloroform: isoamyl alcohol (24:1, v/v) solutions.

2.3.2 DNA quantification

The concentration and purity of DNA was checked by taking absorption at A_{260} and A_{280} with dilution factor 10 using a spectrophotometer BIORAD Smartspec^{Tm}3000. In the end, the purified DNA was diluted to a final concentration of 25 $ng \cdot L^{-1}$ and subjected to RAPD amplification.

2.3.3 RAPD amplification

DNA amplification of purified DNA for RAPD analysis was conducted using 35 random primers (Operon Technology, USA). PCR amplification reactions were carried out as suggested by Williams et al. (1990) with some modifications. The 25 L reaction mixtures contained 50 ng genomic DNA, 10 $mmol \cdot L^{-1}$ Tris-HCl, 1.5 $mmol \cdot L^{-1}$ $MgCl_2$ and 200 $\mu mol \cdot L^{-1}$ each of dATP, dGTP, dCTP, dTTP (Genie, Bangalore), 25 $pmol \cdot L^{-1}$ of random decamer primers (Operon Technologies, USA) and 1U of Taq DNA polymerase (Genie, Bangalore). The PCR tubes were placed in a Perkin Elmer Thermal Cycler (Gene Amp PCR System 9700) and programmed for the following cycling parameters: one cycle of five min at 94°C followed by 45 cycles of one min each at 94°C for denaturation, one min at 37°C primer annealing and a two min extension at 72°C followed by 10 min cycle at 72°C; at the end, the

equipment was held at 4°C until the mixtures were analyzed. Amplification products were analyzed by gel electrophoresis on 1.6% agarose gel incorporated with 0.01% ethidium bromide in a 0.5 × TBE buffer. Gels were scanned in Fluors MultiImager (Bio-Rad) gel documentation system.

3 Results and discussion

3.1 Isozyme analysis

In the present investigation five male and five female samples of *H. rhamnoides* L. were examined with four enzyme systems, viz. peroxidase (PER), esterase (EST), catalase (CAT) and malate dehydrogenase (MDH). The peroxidase enzyme system produced three anodal bands at RM values of 0.23, 0.32 and 0.47 (Fig. 1). The two slower moving bands at RM values of 0.23 and 0.32 were found to be monomorphic, since they were observed in all the male and female genotypes, whereas the fastest moving band at RM value of 0.47 was observed only in five female genotypes (Fig. 1). Thus a female specific sex marker was identified, which could successfully differentiate between the staminate and pistillate genotypes of *H. rhamnoides* L., whereas the esterase enzyme system produced two bands at RM values 0.36 and 0.56. Both bands were found to be monomorphic, since they were observed in all staminate and pistillate genotypes, i.e., the esterase enzyme system could not be used for discrimination of gender in *H. rhamnoides* L. Even in repeated attempts, the catalase and malate dehydrogenase enzyme systems showed no activity in any of the staminate and pistillate genotypes.

3.2 RAPD analysis

Genetic differences among five staminate and five pistillate genotypes were investigated using 35 RAPD primers. Successful amplification of RAPD bands from all genotypes were obtained from all primers. One primer OPD-20 (5′-ACTTCGCCAC-3′) expressed polymorphism between male and female genotypes and was found to be male specific at a 911 bp (Fig. 2). This DNA marker was found to be reproducible. The experiment was repeated twice with both male and female DNA samples in order to check the reliability and stability of the marker. OPD-20_{911} has been found completely linked to the male sex and can be used for screening seedlings with the objective of sex determination in *H. rhamnoides* L.

Isozyme polymorphism is widespread in many plants and has been used for cultivar and sex identification in many horticultural plant and tree species. Peroxidase has been reported to be useful in sex determination of plants, because this enzyme system is involved in the hormonal sex control of many dioecious plants such as the kiwi fruit (Hirsch et al., 1997; Shirkot, 2000). In organogenesis, the role of peroxidase is suggested in auxin catabolism (Legrand and Bouazza, 1991). Peroxidase induces auxin modulation of morphogenesis and has an indirect role in sex determining mechanisms in many monoceious and dioecious plants and makes an important contribution to sex expression. Our studies are consistent with the studies carried out in *Mercurialis* genus by Kahlem (1976), where peroxidase profiles were emphasized for female and attenuated for male plants.

To obtain a marker linked to a gene or genomic region through RAPD analysis depends to a large extent on chance, because random sequences are used as PCR primers. In the present study, out of the 35 random primers only one gender specific marker, i.e., OPD-20_{911} was identified which was able to differentiate between staminate and pistillate genotypes of *H. rhamnoides* L. RAPD-PCR technology had also been successfully used by Persson and Nybom (1998) to differentiate gender in *H. rhamnoides*. They screened 78 primers and only one male specific marker OPD-15_{600} was identified. Hormaza et al. (1994)

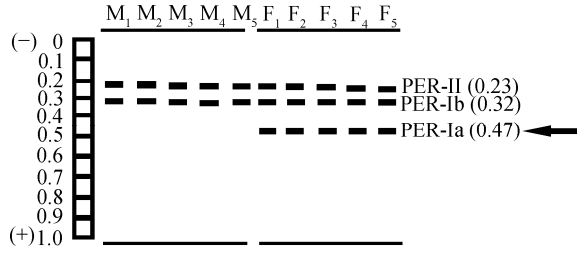

Fig. 1 Peroxidase isozyme patterns in five male and five female genotypes of *H. rhamnoides* L. The arrow indicates the female specific peroxidase activity.

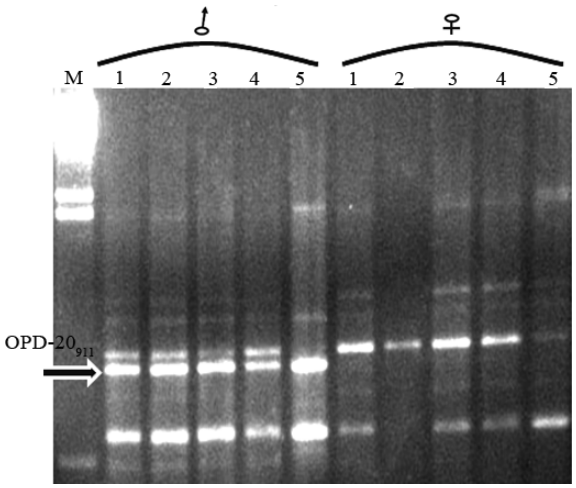

Fig. 2 RAPD profile generated by primer OPD-20 for five male and five female genotypes of *H. rhamnoides* L. M: λ size marker. The arrow indicates the unique male specific band.

tested 1000 decamer primers in *Pistachio vera* for sex determination and only one female specific marker OPO-08_{945} was obtained. Banerjee et al. (1999) obtained OPA-10_{908} and OPC-12_{757} male specific markers in *Piper longum.* Alstrom-Rapaport et al. (1998) reported RAPD band UBC-354_{560} linked to sex determination locus in *Salix viminalis*. Similarly, a female specific DNA marker was identified in nutmeg by Ganeshaiah et al. (2000) after screening 60 RAPD primers. Singh et al. (2002) identified a female sex associated marker OPC-07_{567} in *Trichosanthes dioica* after screening 100 decamer primers. Shirkot et al. (2002) identified eight sex linked markers in *Actinidia deliciosa* using 34 random primers. Aggarwal et al. (2007) ascertained sex of *Simmondsias chinensis* (jojoba) by testing 72 primers and only one male specific marker OPG-05_{1400} was obtained. OPA-06_{600} was found to be a male specific marker in *Borassus flabellifer* L. after testing 108 random decamer primers by George et al. (2007).

DNA based markers provide a useful tool to indicate dimorphism of the genome corresponding to its dimorphic gender. Random Amplified Polymorphic DNA (RAPD) is an inexpensive and rapid method not requiring any information regarding the genome of plants. Instead, it is based on the use of small arbitrary sequences, which are employed to amplify the genomic DNA of a plant. Whereas isozyme markers suffer from certain disadvantages and are usually affected by environmental conditions, their expression varies from tissue to tissue, plant phenological stages and post transcriptional modification. Hence the results from the different laboratories may not be compared. Because of these disadvantages, the use of protein profiles as marker systems to study phylogeny or diversity is limited. On the other hand, DNA molecular markers are not subject to these limitations and appear to be the best option for genetic analysis.

In this study we have reported the finding of peroxidase isozyme markers associated with females and one RAPD marker linked with males of *H. rhamnoides* L. The importance of this finding is the early assessment of gender in *H. rhamnoides* L. before the reproductive stage, as well as understanding the molecular basis of sex determination in this dioecious species. Additional markers will need to be identified and we are continuing to identify additional markers in an effort that the availability of markers, linked to sex determination genes, would allow to clone gene(s) involved in the process to generate SCAR markers.

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