Pak. J. Bot., 41(5): 2419-2426, 2009.

EVALUATION OF PHYLOGENETIC RELATIONSHIP AMONG SEA BUCKTHORN (*HIPPOPHAE RHAMNOIDES* L SPP. *TURKESTANICA*) WILD ECOTYPES FROM PAKISTAN USING AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)

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Abstract

Sea buckthorn (*Hippophae rhamonides* L) is a very potent medicinal and multipurpose plant which has gained global significance due to its medicinal and multipurpose utility. It bears yellow to orange berries which are known to human beings from centuries for their effects on health. The plant is wildly distributed throughout Northern Areas of Pakistan. The phylogenetic relationship among these natural Sea buckthorn ecotypes from Northern Areas of Pakistan is not established so for using reliable molecular markers. AFLP has been proved to be an effective tool for the determination of phylogenetic relationship among closely related species. To provide a population level genetic profile for investigation and exploitation of genetic diversity of Sea buckthorn (*Hippophae rhamnoides* sub spp. *turkestanica*), 25 plant samples selcted from natural populations of Sea buckthorn in Pakistan were analyzed using AFLP (Amplified Fragment Length Polymorphisim) markers the University of Sussex, UK. Phylogenetic distance estimated revealed that the ecotypes expressed common heritage for their phylogenetic relationship with a considerable genetic diversity among them as well. Quite a few ecotypes showed close relationship irrespective of their geographic distances and morphological attributes. The research evolved a significant outcome to start a breeding program for the evolution of sea buckthorn varieties for the mountain areas of Pakistan and Azad Kashmir.

Introduction

Sea buckthorn (*Hippophae rhamnoides* L.) of the family Elaeagnaceae is a multipurpose shrub distributed in Asia, Europe and North America (Ercisli *et al*., 2008). In Pakistan Sea buckthorn has natural populations found throughout Northern Areas (Shah *et al.,* 2007).

The broad geographical distribution and diversified ecological conditions may contribute to the extensive diversity of Sea buckthorn (Ercisli *et al*., 2008). Sea buckthorn ecotypes of Pakistan do not only exhibit great diversity in plant morphology, physical attributes but also express diversity in biochemical constituents (Shah *et al*., 2007; Sabir *et al.,* 2005). The morphological, physical and biochemical diversity of naturally occurring ecotypes requires a credible evidence to be declared as genetically diverse germplasm before exploiting them to start any breeding program for crop improvement.

The knowledge of genetic relationships among the germplasm available, helps in great deal to achieve desirable objectives (Vijayan *et al*., 2005; Le *et al*., 2000) .The molecular approach for the estimation of genetic diversity has improved increasingly valuable in identification of germplasm (Singh *et al*., 2006; Congiu *et al.,* 2000).

Different Molecular Markers have been used to assess phylogenetic relationships and genetic diversity in Sea buckthorn including isozymes (Yao & Tigersted, 1993), randomly amplified polymorphic DNA (RAPD) (Ercisli *et al*., 2008, Ercisli *et al*., 2007, Sheng *et al*., 2006; Singh *et al*., 2006; Raun *et al.,* 2004; Bartish *et al.,* 2002; Sun *et al.,* 2002). In addition to RAPD and other molecular markers, AFLP (Amplified fragment length polymorphism) is a whole genome approach to study the genetic diversity which is gaining the popularity for lower level systematics (Brussell *et al.,* 2005).The use of restriction enzymes and involving PCR rounds proved to be extremely useful, as no prior sequence information is required for analysis. The AFLP technique has recently illuminated the details of phylogenetic relationships within different plant varieties (Mc Kinnon *et al*., 2008, Bousios *et al.,* 2007, Pellmyr *et al.,* 2007, Joly & Bruneau 2007). The technique is very useful for the detection of genetic relationship among sea buckthorn cultivated varieties as well (Raun & Li. 2005).

Sea buckthorn is a potent multipurpose plant for the mountain areas of Pakistan and Azad Kashmir. The wild germplasm can be used as source of gene pool to start a breeding program to evolve new Sea buckthorn varieties for economical development in mountain areas of Pakistan. The advent of modern molecular techniques like AFLP has opened the new window of opportunity to know about the genetic diversity of existing gene pool to help in improving the efficiency and validity of breeding program. Sea buckthorn of Northern Areas of Pakistan has not been characterized so for, using any DNA based molecular marker. In present study we selected twenty-five (25) ecotypes of Sea buckthorn collected from different localities of Northern Areas of Pakistan, showing morphological, physical and biochemical diversity among them, for AFLP analysis to determine the phylogenetic relationship among them.

Materials and Methods

Twenty-five Sea buckthorn ecotypes were used in this study. The leaf samples were taken from wild Sea buckthorn populations from Skardu, Hunza Gilgit, Hussainabad, Murtazabaad, Nagar, Shigar, Sadpara, Khaploo, Sust and Jaglot areas of Northern Pakistan represented as dots in Fig. 1. All these ecotypes were showing diversity in some morphological parameters like tree shape, fruit color and tree height. The already frozen leaf samples were immediately subjected to -80^{o}C before transporting them to the University of Sussex, School of Life Sciences United Kingdom for further studies. The experiment was conducted in Plant Molecular Biology laboratory, John Maynard Smith Building, University of Sussex, UK.

DNA extraction: Genomic DNA was extracted from frozen leaf tissue using *DNeasy* Plant Mini Kit (Qiagen). The purity and quantity of extracted DNA were measured with the help of spectrophotometer in 260 and 280 nm wavelengths (Thermo Nicolet 100 UV, London,UK). The quantity of DNA was in between 1.3 (E7) and 1.6 (E15). Extracted DNA is shown in Fig. 2.

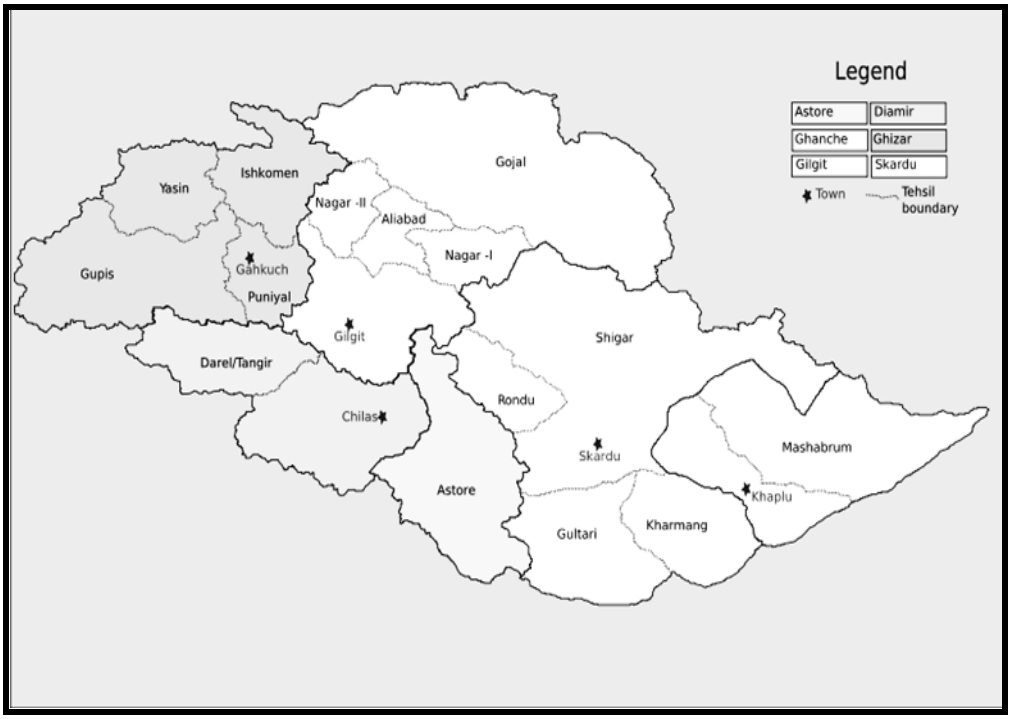

Fig. 1. Sampling sites from Northern areas of Pakistan.

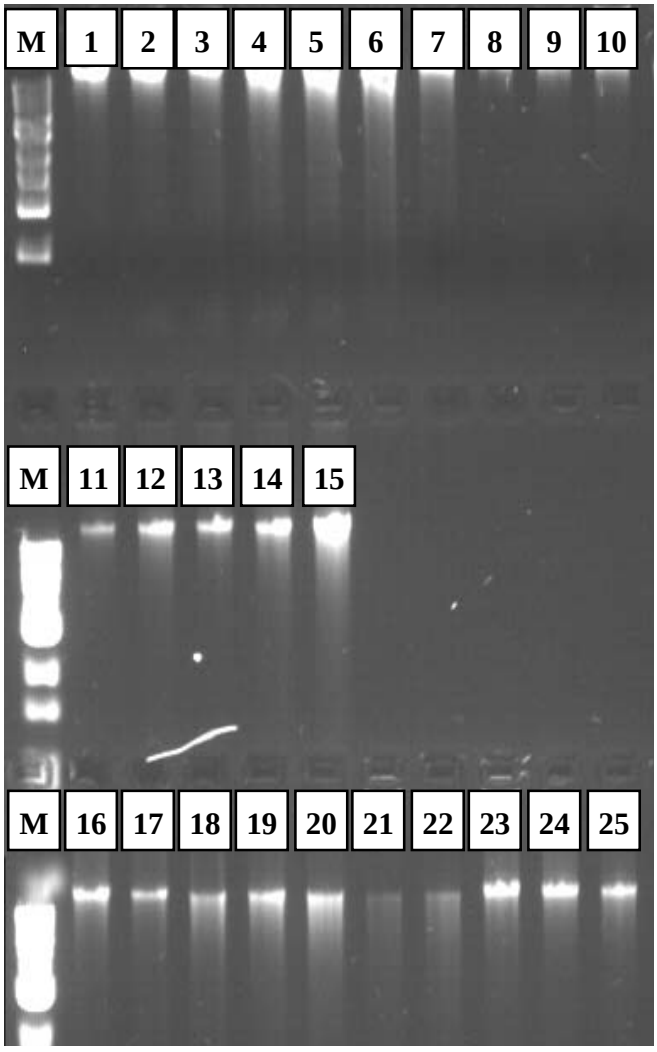

Fig. 2. Genomic DNA of sea buckthorn ecotypes.

AFLP analysis

Digestion of genomic DNA: Genomic DNA was digested with reagents and enzymes in the ratio as follows. Water 37µl, genomic DNA 2µl, 10 times Ligase buffer 5µl, MSe I adopter (50µM): 1µl, EcoRI adopter (5µM) 1µl, Mse I (10µ/µl) 1µl, EcoRI 1 µl, Ligase 2µl to make the final volume 50 µl. The mixture was vortexed and kept overnight at 50^{o}C for over night.

Preamplifications: For preamplification reaction reagents were mixed as follows: Water 15.3µl, 10 X PCR buffer 2.5 µl, dNTPs (1.25 mM each) 4µl, EcoRI(+A) 5′CTCGTAGACTGCGTACC-3′0.15g/µl)1µl,MseI(+C) ′3 CATCTGAGACGCATGG TTAA- ′5 (0.15µg/lµl), template ligation 1µl, taq polymerase 5u/µl 0.2µl and the total volume was made up to 25ul. The PCR conditions were 50^{o}C for 2 minutes to get infill and at 94^{o}C for 30 seconds, 56^{o}C for 60^{o}C and 72^{o}C for 60 seconds.

Labeling of primers: Water 6.25ul, Radiolabel (ICN 58404.2) 1ul, 10X kinase buffer 1ul, EcoRI (+AGG) primer 5′- GACTGCGTACCAATTCAGG-3′ (0.05ug/ul) 1.5ul, T4 polynucleotide kinase (10u per µl) 0.25 µl. The mixture was incubated for one hour in hot block then 70^{o}C for ten minutes to stop reaction.

The products of preamp reaction were analyzed visually in agarose gel and strong preamps were selected for further reaction in the following order. Water 387.2 µl, PCR buffer 64µl, dNTPs102.4µl Mse (+CAG primer 5′- GATGAGTCCTGAGTAACAG-3′) 0.05µg/µl) 19.2µl, Taq Polymerase 5u/µl) 3.2µl, labeled primer 3.2µl and diluted preamp with PCR conditions 94^{o}C for 30 seconds, 65^{o}C for 30 seconds, 72^{o}C for 60 seconds, (30 cycles) and 94, 56 and 72^{o}C for 30, 30 and 60 seconds respectively with 27 cycles.

Preparation of acrylamide gel solution (6%): Following reagents were mixed as follows:

Urea 306g, 5 X TBE 66.6ml, and 500 ml water to dissolve urea.

Acylamide stock 40% was taken and water was added to 666 ml volume. The stock was kept in room temperature in the dark.

Making of gel: To make one gel, 80 ml of gel solution, 90µl of 25% ammonium persulphate (made fresh) and 90µl TEMED were mixed with each other.

Making of gel case: Plates were washed thoroughly with detergent under hot running water and dried with towels. Gel was pored in the spaces of plates and comb was inserted in 4mm depth. The gel was left to set for one hour. The bulldog clips and cling film were removed and the gel was put into the electrophoresis apparatus. The tank was filled with 0.5% TBE. Comb was removed by pulling it upwards. The top was washed of the gel with hypodermic needle and syringe. The comb was replaced with shark's teeth pointing down. It was pushed down until the teeth just touched the surface of the gel and made small indents. Gel was run till the dye reached to the end of the gel. Drained the gel and was placed in 10% methanol and 10% acetic acid. Gel was allowed to fix for at least 30 minutes. The acrylamide was with clingfilm and dried in gel drier.

Autoradiography: The Clingfilm was removed and the gel was put in automated cassette. A piece of X-Ray film was put on the gel and closed the cassette down firmly. The gel was exposed for overnight in darkroom.

Results and Discussion

The DNA profile among 25 populations of Sea buckthorn is compared in Fig. 3. Maker data for AFLP were scored and phylogenetic relationships were determined. The UPGMA dendrogram based on genetic distances among populations shows very clear picture of species evolutionary pattern in relationship and variability shown in Fig. 4. The dendrogram shows that three groups of the populations separated very early from each other in past to form discrete populations. The ecotypes E1, E2, E8, E9, E15, E18, E21, E24 and E25 were observed in a unique cluster, providing some common phylogenetic relationship among them. Ecotypes E1 and E2 originating from same ancestors but separated in near past to make a discrete cluster. Similarly E9 and E19 also grouped them in a separate group. Sea buckthorn ecotypes E24 and E18 were found be close in phylogenetic relationship. The ecotypes E9 and E19 were found to be in a distinct relation showing that they were separated from their ancestor in near past. E21 and E15 also behaved differently among the other phylogentic relatives. The ecotypes E3, E4, E5, E6, E14, E16, E22 and E23 were genetically similar and clustered together. E22 and E23 are most similar in their genetic make up. Same behavior of genotypic similarity was expressed by ecotypes E5 and E6, E4 and E16 and E3 and E14 who have some common genetic make up respectively.

The third cluster includes E7, E10, E11, E12, E13, E17 and E20. These ecotypes expressed genetically common behavior. The ecotypes E7 and E17 expressed close genetic relationship along with E11 and E12 who are very similar in their genetic make up. E10 and E13 were separated from E20 to express distinct genetic behavior.

The common ancestor of all these ecotypes justifies the reporting of (Rongsen, 1992) that Sea buckthorn in Pakistan belongs to only one sub specie i.e., *turkestanica*. The genetic diversity and similarity among the ecotypes under this research further confirms that geographic distances and diverse climatic conditions along with different altitudes had no significant effect on the phylogenetic relationship of plant populations. Tian *et al.,* (2004) also reported that geographic distances had no visible effect in genetic differentiation. In an other studies for Autumn olive plant populations, Ahmed *et al.,* (2008) found that Autumn olive natural stands behaved quite differently when compared in phylogenetic relationship irrespective of their geographic distance.

The common heritage of Sea buckthorn populations collected from diverse locations of Northern Areas of Pakistan could be due to the fact that birds consume Sea buckthorn berries and thus play a major role in seed dispersal at variable altitudes and distant areas. Moreover Sea buckthorn is mostly found near the river belts of Northern Areas and seed dispersal is also facilitated by rivers coming from higher altitudes to valleys downwards. The study provides evidence that geographic distances and locations have least effect on the genetic behavior of plant populations. The diversity in Sea buckthorn ecotypes on the basis of biochemical constituents (Shah *et al.,* 2007) has to be linked with genetic diversity. Sea buckthorn has tremendous potential to fulfill the objectives of nutritional, economical and environmental significance. It is full of nutritional and biochemical constituents and becoming very popular around the globe due to its multipurpose utility (Yao & Tigerest, 1993). The naturally occurring Sea buckthorn populations of Northern Pakistan require to be improved by evolving new multipurpose varieties. The knowledge of distribution of genetic diversity provides a guide to the wise management of the genetic resources (Barret & Kohn, 1991). This study provided the base to launch a breeding program for sea buckthorn improvement to exploit the potential of this magic shrub for sustainable economic development in mountain environments of the country in particular and for the world in general.

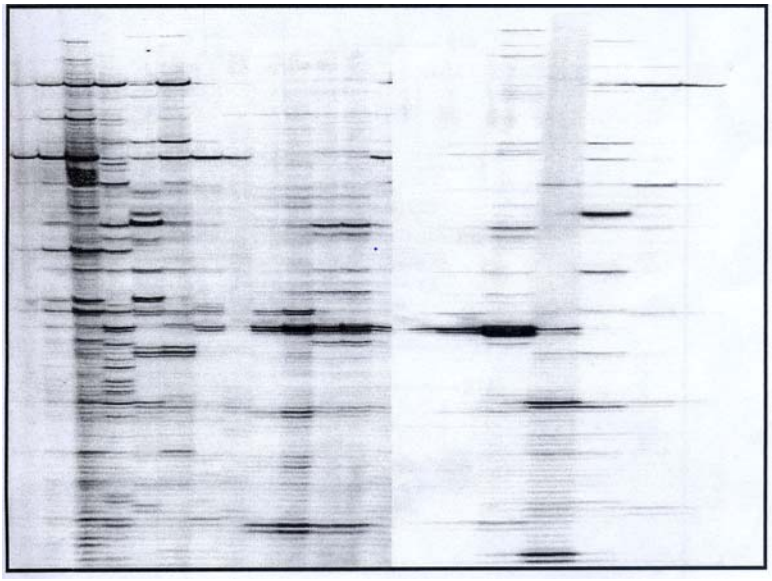

Fig. 3. AFLP profile of genomic DNA of 25 ecotypes separated on Polyacrylamide gel.

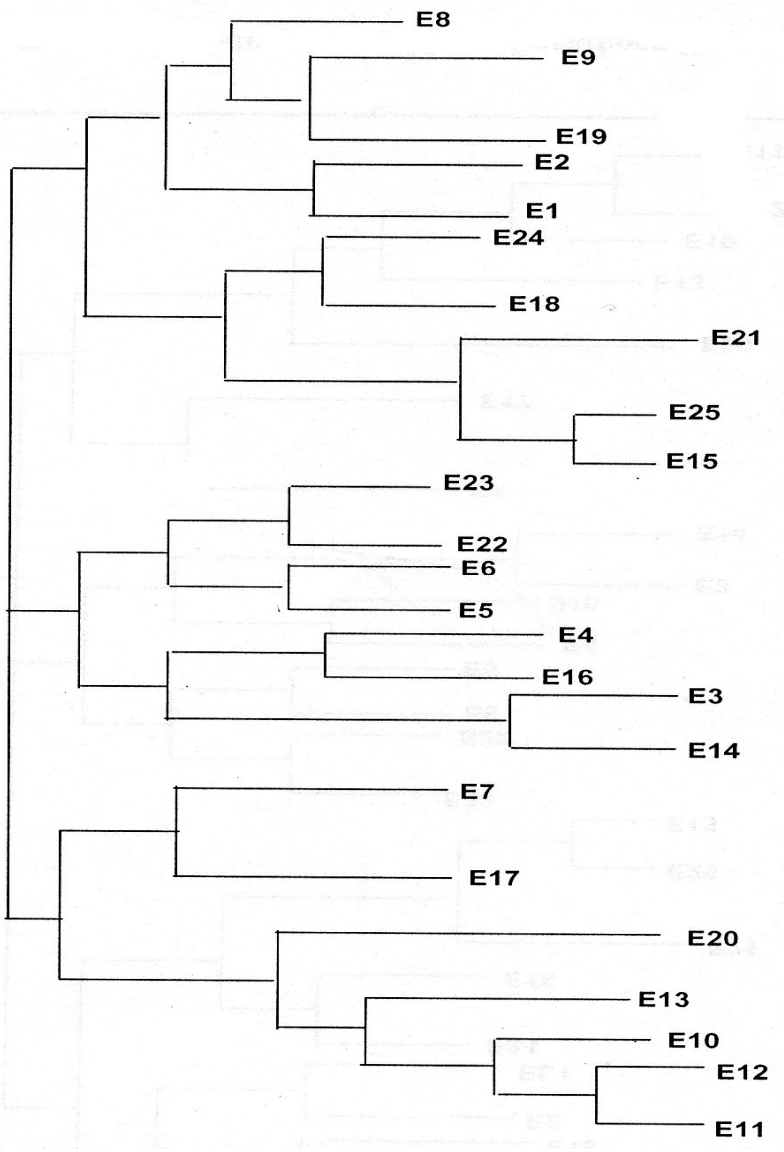

Fig. 4. Dendrogram of 25 Sea Buckthorn Ecotypes.

Acknowledgements

Higher Education Commission of Pakistan is duly acknowledged for providing the funds to complete this research under International Research Support Initiative Program. The authors also wish to thank Mr. Ghulam Nabi Shigri, consultant Pak. Sea buckthorn International for his help in survey and collection of Sea buckthorn samples.

References

Ahmad, S.D., S.M. Sabir, H.M. Saud and Yousaf Salihuddin. 2008. Evolutionary relationship and divergence based on SDS-PAGE of *Eleagnus umbellate* Thunb populations, a multipurpose plant from Himalayas. *Turkish J. Biol.,* 32: 31-35.

Barret, S.C.H. and J.R. Kohn. 1991. Genetic and evolutionary consequences of small population size in plants. Implications for conservation. *Genetics and Conservation of Rare Plants. Oxford University Press:* 3-30.

Bartish, J.V., N. Jeppson, H. Nybom and U. Swenson. 2002. Phylogeny of *Hippophae* (Eleagnaceae) infrared from parsimony analysis of chloroplast DNA and morphology. *Syst. Bot.,* 27: 41-54.

Bousios, A., I.S. Oyarzabal, A.G.V. Zapata, C. Wood and S.T. Pearce. 2007. Isolation and charecterazation of Ty1-copia retrotransposon sequences in the blue agave (*Agave tiquilana* Webber var. Azul) and their development as SSAP markers for phylogenetic analysis. *Plant Sci.,* 72: 291-298.

Brussell, J.D., M. Woycott and J.A. Chappill. 2005. Arbitrarily amplified DNA markers as characters for phylogenetic inference. *Prosp. Plant Eco. Evol. and System*, 7: 3-26.

Congiu, l., M. Chicca, R. Cella, R. Rossi and G. Bernacchia. 2000. The use of random amplified polymorphic DNA (RAPD) markers to identify strawberry varieties: a forensic application. *Mol. Ecol.,* 9: 229-232.

Ercisli, S., E. Orhan, N. Yildirim and G. Agar. 2008. Comparison of Sea buckthorn Genotypes (*Hippophae rhamonides* L.) based on RAPD and FAME data. *Turk. J. Agri.,* 32: 363-368.

Ercisli, S., E.Orhan, O. Ozdemir and M. Sengul. 2007. The genotypic effects on chemical composition and antioxidant activity of sea buckthorn (*Hippophae rhamnoides* L.) berries grown in Turkey. *Scientia Hort.,* 115: 27-33.

Joly, S. and A. Bruneau. 2007. Delimiting species boundaries in *Rosa* sect. *Cinnamomeae* (Rosaceae) in eastern North America. *Syst. Bot.,* 32: 819-836.

Le, E.T., O. Panau and B. toupance. 2000. Assesment of genetic relationships between *Setaria italica* and its wild relative *S. viridis* using AFLP markers. *Theoretical and Applied Genetics*, 100: 1061-1066.

McKinnon, G.E., R.E. Vaillancoat, D.A. Steane and B.M. Potts. 2008. An AFLP marker approach to lower-level systematics in *Eucalyptus* (Myrtaceae) *Amer.J. Bot.,* 95: 368-380.

Morell, M.K., R. Peakhall, R. Apples, R.L. Preston and H.L. Lloyd. 1995. DNA profiling techniques for plant variety identification. *Aust. J. Exp. Agr*., 32: 807-819.

Pellmyr, O., K.A. Segraves, D.M. Althoff, M. Balcazar-Lar and J. Leebens-Mack. 2007. The phylogeny of yuccas. *Mol. Phylogene. Evol.,* 43: 493-501.

Raun, C. and Li Diaqiong. 2005. AFLP fingerprinting analysis of some cultivated varieties of sea buckthorn (*Hippophae rhamnoides*). *J. Gene.,* 4:(3): 2005.

Raun, C., P. Qin, J. Zheng and Z. He 2004. Genetic relationships among some cultivars of sea buckthorn from China, Russia and Mongolia based on RAPD analysis. *Sci. Hortic*., 101: 417-426.

Rongsen, L. 2004. *Proceedings of the 1st Congress of the International Sea Buckthorn Association, Berlin, Germany,* 4: 20-35.

Sabir, S.M., H. Maqsood, S.D. Ahmad, A.H. Shah and M.Q. Khan. 2005. Chemical and nutritional constituents of sea buckthorn (*Hippophae rhamnoides* spp. *tuerkestanica*) berries from Pakistan. *Ital. J. Food Sci*., 17: 455-462.

Shah, A.H., S.D. Ahmad, S.M. Sabir, S. Arif, I. Khaliq and F. Batool. 2007. Biochemical and nutritional evaluations of sea Buckthorn (*Hippophae rhamnoides* L. spp. *turkestanica*) from different locations of Pakistan. *Pak. J. Bot*., 39(6): 2059-2065.

Sheng, H.M., L.Z. An, T. Chen, S.J. Xu, G.X. Lu, X.L. Zheng, L.L. Pu, Y.J. Lu and Y.S. Lian. 2006. Analysis of genetic diversity and relationships among and within species of *Hippophae* (Elaeagnaceae) based on RAPD markers. *Plant Syst. And Evol*., 260:25-37.

Singh, R., S.N. Mishra, S.K. Diwiedi and Z. Ahmad. 2006. Genetic variation in Sea buckthorn (*Hippophae rhamnoides* L.) populations of cold arid Ladakh (India) using RAPD markers. *Curr. Sci.*, 91 (10): 1321-1322.

Sun, K., X. Chen, R. Ma, C. Li, Q. Wang and S. Ge. 2002. Molecular pylogenetics of *Hippophae* (Eleagnaceae) based on internal transcribed spacer (ITS) sequence of nrDNA. *Plant Syst. Evol*., 235: 131-134.

Tian, C., P. Nan, S.Suhua, C. Jiakuan and Y. Zhong. 2004. Molecular variation in Chinese populations of three subspecies of (*Hippophae rhamnoides*). *Bioche. Gene.,* 42: 259-266.

Vijayan, K., C.V. Nair and S.N. Chatterjee. 2005. Molecular charecterization of Mulbery genetic resources indigenous to India. *Genet. Resou. Crop Ev*., 52: 77-86.

Yao, Y.M. and P.M.A. Tigerstedt. 1993. Isozyme studies of genetic diversity and evolution in *Hippophae*. *Genet. Resou. Crop. Ev.,* 40: 153-164.

(Received for publication 19 January 2009)