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A new strategy for complete identification of sea buckthorn cultivars by using random amplified polymorphic DNA markers

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ABSTRACT. DNA fingerprinting is both a popular and important technique with several advantages in plant cultivar identification. However, this technique has not been used widely and efficiently in practical plant identification because the analysis and recording of data generated from fingerprinting and genotyping are tedious and difficult. We developed a novel approach known as a cultivar identification diagram (CID) strategy that uses DNA markers to separate plant individuals in a more efficient, practical, and referable manner. A CID was manually constructed and a polymorphic marker was generated from each polymerase chain reaction for sample separation. In this study, 67 important sea buckthorn cultivars cultivated in China were successfully separated with random amplified polymorphic DNA markers using the CID analysis strategy, with only seven 11-nucleotide primers employed. The utilization of the CID of these 67 sea buckthorn cultivars was verified by identifying 2 randomly chosen groups of cultivars among the 67 cultivars. The main advantages of this

identification strategy include fewer primers used and separation of all cultivars using the corresponding primers. This sea buckthorn CID was able to separate any sea buckthorn cultivars among the 67 studied, which is useful for sea buckthorn cultivar identification, cultivar-right-protection, and for the sea buckthorn nursery industry in China.

Key words: Cultivar identification; New strategy; Sea buckthorn

INTRODUCTION

Sea buckthorn (*Hippophae rhamnoides*), a deciduous shrub or bush, belongs to the Elaeagnaceae family. It can provide an effective green protective windbreak and is a useful and durable pioneer plant that prevents soil erosion from wind and water because of its strong ecological adaptability and resistance to extreme conditions such as drought, heat or cold, salinity, and alkalinity. Additionally, its fruits and leaves are rich in vitamin C and many other bioactive substances with valuable nutritive and medical properties (Lian and Chen, 2000; Jin, 2002). The ecological benefits and economic value of sea buckthorn have led to its development into a major resource in China. Its distribution is widespread, ranging from 2-123°E and 27-69°N, throughout the temperate zone of Europe and Asia (Lian, 1988; Lian and Lian, 1996; Lian and Chen, 2000) and particularly in China, where the germplasm resources and reserves of sea buckthorn are the highest. It is widely distributed in the transition zone of forest-grassland or forest-meadow in the south-Western, north-Western, and northern regions of China (Yu et al., 1993; Chen and Lian, 1994).

Over the last few decades, methods for handling the large repertoire of sea buckthorn germplasm and proper identification of the different cultivars have become necessary. Among the techniques, classical approaches have been used to identify cultivars based on morphological, physiological, and agronomic traits. However, these traits have limitations as they can be easily influenced by the environment and require extensive observation of mature plants. In contrast, molecular markers are uniquely advantageous because they are not affected by the environment and are powerful tools in cultivar characterization. Although DNA-based molecular markers have been utilized in genetic studies, cultivar characterization and identification of sea buckthorn have not been conducted (Sheng et al., 2006). Information regarding genetic diversity levels and the separation of the plant individuals studied have not been reported, and no single report has identified a large number of sea buckthorn cultivars or developed methods that may be useful in future studies. There are currently no methods available that can provide a referable result for the practical utilization of DNA markers in plant cultivar identification. Methods such as DNA fingerprinting have not generated referable information for easy primer determination, and a polymorphic marker for identifying cultivars must be developed. Analysis techniques for DNA banding patterns such as cluster analyses cannot efficiently separate cultivars or species. Employing a strategy for verifying sea buckthorn cultivars in a reliable, easy, referable, and practical manner is crucial for the sea buckthorn nursery and farming industries, cultivar patent protection, and genetic resource conservation and evaluation.

In recent years, various DNA-based markers have been developed and used in genetic diversity, fingerprinting, and cultivar origin studies (Fang et al., 2006; Cheng and Huang, 2009; D'Onofrio et al., 2009; Elidemir and Uzun, 2009; Melgarejo et al., 2009; Papp et al., 2010). Among the DNA-based markers, the random amplified polymorphic DNA (RAPD) (William et

al., 1990) marker technique is useful for cultivar analysis and has advantages such as its simplicity, efficiency, and non-requirement of any previous sequence information. If optimization of the RAPD technique is conducted by choosing 11-nucleotide (nt) primers and strict screening of the polymerase chain reaction (PCR) annealing temperature for each primer before RAPD is employed in fingerprinting plants, this method can be used for plant cultivar identification. RAPD markers have been widely used in cultivar identification and genetic relationship analysis of a number of fruit species, such as apricot (Ercisli et al., 2009), pomegranate (Hasnaoui et al., 2010), cherry (Demirsoy et al., 2008), pistachio (Javanshah et al., 2007), and strawberry (Wang et al., 2007). Despite their popularity, few DNA markers are available for plant identification.

In this study, we developed a strategy for identifying sea buckthorn cultivars a practical, efficient, recordable, and referable manner, in which a cultivar identification diagram (CID) was constructed manually from RAPD banding patterns. Using our method, we obtained results that differed than those obtained using cluster analysis. The CID, which was used to identify 67 sea buckthorn cultivars, is a valuable service for the sea buckthorn industry in China.

MATERIAL AND METHODS

Plant materials

Leaf samples from 67 important sea buckthorn cultivars (Table 1) were collected from the Berries Research Institute, Heilongjiang Academy of Agricultural Sciences, Suiling, Heilongjiang, China. RAPD primers were synthesized by Invitrogen (Carlsbad, CA, USA).

Genomic DNA extraction

Total genomic DNA of each genotype was extracted from young sea buckthorn leaves using the modified cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). Extracted DNA was diluted to a final concentration of 30 ng/μL with 1X TE buffer and stored at -40°C until use.

RAPD analysis

For RAPD reactions, 54 random primers, synthesized by Generay Biotechnology (Shanghai, China), were initially tested with a few genotypes and only primers showing products with clear, unambiguous banding patterns for all genotypes tested. These primers were selected for use in genotyping. Eleven-nucleotide RAPD primers were used for screening in this study. To increase the reliability of our results, we used only primers that produced clear, unambiguous banding patterns. Seven primers (Table 2) showing well-resolved and reproducible bands were selected for assaying all genotypes, while the other primers were discarded. Reaction solutions contained 2.0 μL 10X buffer, 1.2 μL $MgCl_2$ (25 mM), 1.6 μL dNTPs (2.5 mM), 1.6 μL primer (1.0 μM), 0.1 μL rTaq Polymerase Dynazyme (5 U/μL) 10X buffer, $MgCl_2$, dNTPs, and rTaq (TaKaRa, Shiga, Japan), and 1 μL genomic DNA, for a total volume of 20 μL. Amplification reactions were performed based on the standard protocol described by Williams et al. (1990), with minor modifications. PCR was carried out in an Autorisierter Thermocycler (Eppendorf, Hamburg, Germany), programmed as follows: initial

Table 1. Cultivar name and origin of sea buckthorn used in this experiment.

No.	Cultivar	Origin	No.	Cultivar	Origin
1	Shen qiu hong	China	36	Za 4-2	China
2	Shou du	Russia	37	HS-19	China
3	Fen lan	Finland	38	A lie yi	Russia
4	Xiao la jiao	Russia	39	36	Russia
5	Wu ci feng	China	40	TF1-18	Russia
6	Sui 3	China	41	Za 56	China
7	HS-4	China	42	TF1-13	Russia
8	Za 4	China	43	TF2-31	Russia
9	You sheng	Russia	44	♂6	Russia
10	Xin e 3	Russia	45	TF2-23	Russia
11	Xin e 2	Russia	46	Wu ci xiong	Russia
12	Xin e 1	Russia	47	♂3	Russia
13	Chu yi	Russia	48	6	Russia
14	HS-23	China	49	♂5	Russia
15	Cheng se	Russia	50	♂4	Russia
16	HS-3	China	51	HS-9	China
17	Hun jin	Russia	52	♂1	Russia
18	Wu lan ge mu	Mongolia	53	Za 2-3	China
19	Xiang yang	Russia	54	Sui 1	China
20	Sui 4	China	55	Lv zhou 4	China
21	Sui 2	China	56	Lv zhou 1	China
22	A er tai	Russia	57	Feng chan	Russia
23	Za 14	China	58	HS-10	China
24	Jin se	Russia	59	HS-1	China
25	TF2-7	Russia	60	HS-22	China
26	TF1-19	Russia	61	HS-12	China
27	TF2-27	Russia	62	HS-15	China
28	TF2-13	Russia	63	HS-21	China
29	TF2-26	Russia	64	HS-20	China
30	2	Russia	65	Ju ren	Russia
31	Za 1-2	China	66	♂7	Russia
32	Za 54	China	67	♂2	Russia
34	Lv zhou 3	China	68	Ka tu ni	Russia
35	42	Russia			

Obs.: No. 33 is absent.

pre-denaturation step for 5 min at 94°C, followed by 42 cycles of a denaturation step for 30 s, an annealing step for 1 min at annealing temperature (Table 2), and an extension step for 2 min at 72°C. Amplification was terminated by a final extension in 72°C for 10 min. After amplification, DNA fragments were separated by gel electrophoresis on a 1.3% agarose (w/v) (Figure 1) in 1X 0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0, buffer at 100 V. The gels were stained with 0.5 μg/mL ethidium bromide and visualized under ultraviolet light. Polymorphic bands among the cultivars were observed in the photographs. Each amplification reaction was repeated at least 3 times to confirm the reproducibility of our results.

Table 2. Seven primers used for the separation of the 67 sea buckthorn genotypes.

Primer	Nucleotide sequence (5'-3')	Annealing temperature (°C)
Y-28	GTGTGCCCCAT	43.7
Y-36	AAGCCTCGTCC	44.4
Y-48	ACGACCGACAC	44.4
Y-42	AGCGTCCTCCC	44.4
Y-29	GTGTGCCCCAG	43.7
Y-35	AAGCCTCGTCG	43.7
Y-41	AGCGTCCTCCG	43.7

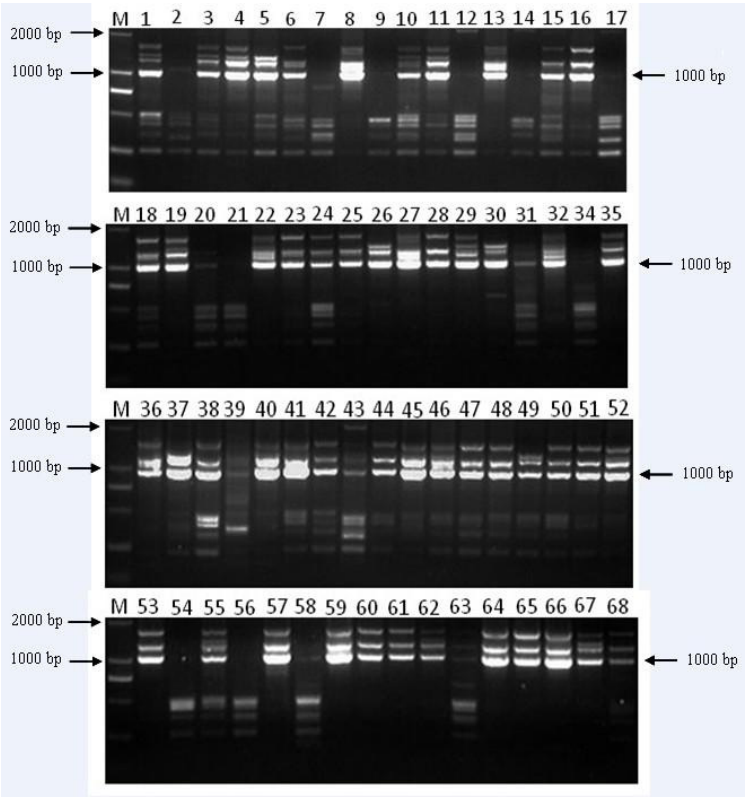

Figure 1. DNA banding patterns of 67 sea buckthorn cultivars amplified using primer Y36. *Lane M* = DL2000 plus DNA ladders; *lanes 1-32* and *34-68* = accession numbers of sea buckthorn cultivars listed in Table 1, which are the same as those in the following figures.

Data analysis

Only clear unambiguous bands in the photographs of gels were scored for cultivar identification. Some cultivars showed a specific band in the fingerprint generated from one primer and could be separated singly, while cultivars sharing the same banding pattern were separated into the same sub-group. Based on this strategy, all sea buckthorn cultivars were separated from one another as more primers were employed.

Utilization and workability of the CID

Two groups of sea buckthorn cultivars, which were randomly chosen from the inter- and intra-groups, were used to verify the utilization of the diagram showing the separation of the 67 sea buckthorn cultivars. The 2 groups of cultivars were marked as "A" and "B" and the corresponding primers for separating each group were easily located on the diagram. These cultivars were distinguished accurately and quickly based on the CID, and this method is efficient and can be used with molecular markers to identify other fruit crop cultivars and seed samples of field crops.

RESULTS

Cultivar identification

To establish a stable RAPD system with high reproducibility, longer primers (11 nt) were used and the annealing temperatures for each primer were screened based on the quality and reproducibility of the banding patterns. Primers were randomly screened from a stock of 60 11-nt primers, and once a primer that could produce reproducible polymorphic bands was screened, it was utilized to identify sea buckthorn cultivars. An example is the RAPD pattern obtained using primer Y36, which was the first primer used to amplify the 67 sea buckthorn cultivars examined in this study. The electrophoresis results showed that primer Y36 generated uniform and reproducible band patterns in 14 sea buckthorn cultivars assigned the sample codes 2, 7, 9, 12, 14, 17, 20, 21, 34, 39, 54, 56, 58, and 63 (Table 1). This group of cultivars was easily differentiated from the other 53 cultivars based on the presence or absence of a distinct 1000-bp band; the 67 cultivars were separated into 2 groups. The second primer (Y28) could then separate the 2 groups of cultivars into smaller groups or singly as "50". This separation was conducted sequentially using another 5 primers (Table 2) that were screened and chosen to differentiate sea buckthorn cultivars in the sub-groups composed of 2 or more cultivars. Using primer Y41 (Figure 1), all 67 cultivars were completely separated. The total set of 7 primers (Table 2) could identify all 67 sea buckthorn cultivars as shown in Figure 2.

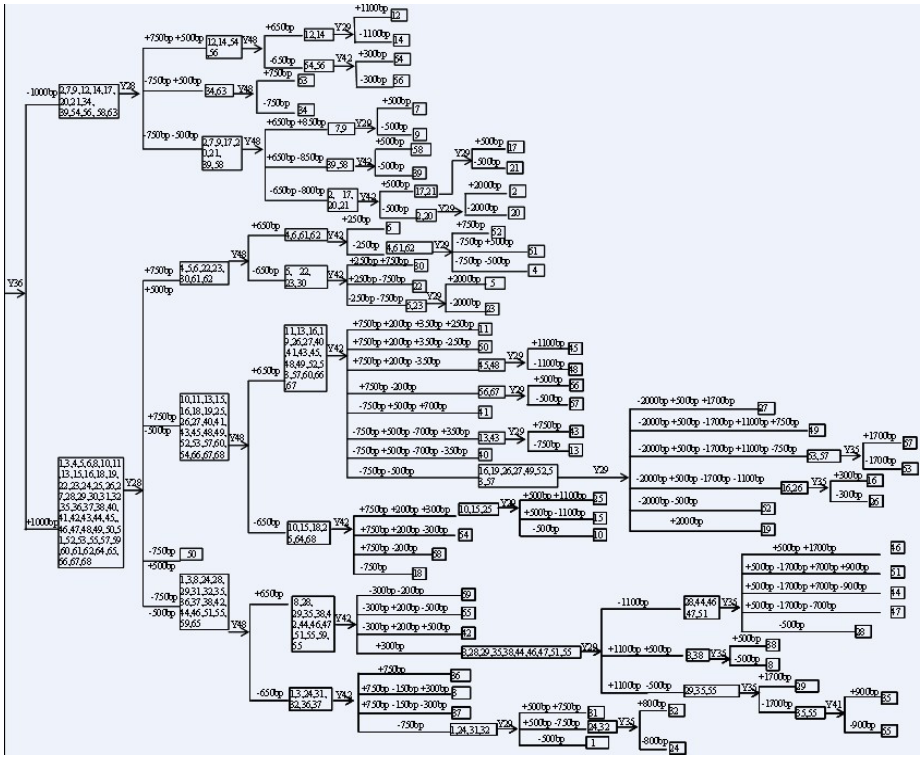

Figure 2. Identification results of sea buckthorn cultivars used by 7 primers and detailed fingerprints. All numbers marked in this chart indicated different size fingerprints, in units of bp; (+) = presence of bands in different sizes; (-) = absence of bands in different sizes.

Only the clear polymorphic bands generated from each primer were used to differentiate cultivars. The sizes and the presence/absence of polymorphic bands used in the CID are shown in Figure 2, and the CID is very useful for sea buckthorn cultivar identification. At each separation step in the CID, the primer and polymorphic bands amplified by the primer can be considered as a scale-like scheme for separating sea buckthorn cultivars. Therefore, the CID strategy is more useful for sea buckthorn cultivar identification than the currently used cluster analysis.

Test of the utilization and workability of the diagram in cultivar identification

Although a goal of this study was to develop a technique utilizing RAPD markers to distinguish 67 sea buckthorn cultivars, the larger aim was to generate a referable CID of sea buckthorn cultivars and polymorphic markers that would make it easier to separate and identify sea buckthorn cultivars using the CID. Previous studies focused on the genetic analysis and presence of some phylogenetic trees without referable information for practical plant sample identification. Our findings will benefit the sea buckthorn nursery industry and facilitate cultivar-right-protection.

To identify some of the sea buckthorn cultivars among those examined in this study, the primers can be easily located and the target polymorphic PCR product on the CID can be used for further identification. To confirm this, verification of the effectiveness and efficiency of the sea buckthorn CID was necessary. Three groups of cultivars comprising “12”, “13”, “14”, and “15”; “35” and “55”; “49” and “50”, which came from the inter- and intra-groups or sub-groups in the CID, were randomly chosen and used for verification. Based on the location of these cultivars in the CID, primers that could be used to separate these 3 groups of cultivars were chosen, including Y36, Y29, Y28, and Y41. The PCR results showed the anticipated banding patterns, and all cultivars in these 3 groups could be disjoined and separated using specific polymorphic bands marked in the CID. The 4 sea buckthorn cultivars in the first group were separated by the PCRs using the primers Y36 and Y29 (Figure 3A). The 1000-bp band generated by Y36 was first separated into 2 groups, from which the group including “12” and “14” could be further disjoined using the primer Y29 based on the 1100-bp band, while another group that included “13” and “15” was divided by primer Y29 with a band of approximately 1100 bp in size. The group of “35” and “55” was separated by the 900-bp long polymorphic band derived from primer Y41 (Figure 3B). A band that was approximately 800 bp in length generated using primer Y28 disjoined the group of “49” and “50” (Figure 3C). This validation of separation using randomly chosen groups of cultivars indicates that this sea buckthorn cultivar identification strategy is practicable, workable, effective, and referable, and can be used in the sea buckthorn industry. The data for cultivar separation from the CID can also be placed into a database for future use *in silico*.

DISCUSSION

Sea buckthorn (*H. rhamnoides*) is environmentally important and is a new commercial berry crop. Commercially sea buckthorn is a hardy, multi-purpose plant that produces orange, red, or yellow berries. This plant fixes large amounts of atmospheric nitrogen, and rapidly develops an extensive root system and canopy, quickly covering large areas of soil. These properties make it an ideal candidate for soil and water conservation in extreme or mar-

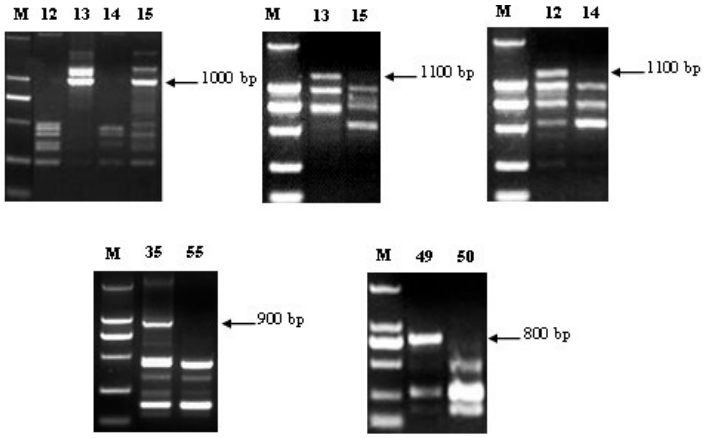

Figure 3. Verification result of several cultivars selected randomly by the corresponding primers. *Lane M* = DL2000 plus marker; other *lanes* = accession number of the cultivars used as listed in Table 1.

ginal areas (Ruan and Li, 2005). To improve the development of sea buckthorn research and its related industry, identifying sea buckthorn cultivars and germplasm resources is necessary. Therefore, this study is important for promoting genetic resource conservation and utilization as well as plant variety protection. The development and use of molecular markers in sea buckthorn and related *Hippophae* species have been previously reported using inter-simple sequence repeats (Jain et al., 2010) and amplified fragment length polymorphism (Ruan and Li, 2005). However, no efficient strategy for the easy application of DNA markers for sea buckthorn variety identification has been reported. The major goal of this study was not only to use RAPD markers to distinguish the 67 sea buckthorn cultivars based on the principle of DNA fingerprinting, but also to develop a new strategy for properly utilizing DNA markers as a universal strategy for distinguishing other plant and seed samples. An optimized RAPD method maybe can make this strategy more efficient and easier to adapt.

Another result of this strategy is that a readable and referable cultivar identification diagram can be constructed for identifying related plant species in a manner similar to the use of the periodic table of elements, i.e., to provide basic information for each cultivar in a central, universally accessible fashion. This method will be useful in the nursery industry and provide valuable information regarding cultivar-right-protection.

This strategy is efficient for plant identification because few primers and PCRs are needed, and polymorphic bands and DNA fingerprints from various primers can be jointly utilized for further specific identification. In this study, 7 RAPD primers were sufficient to distinguish all 67 sea buckthorn cultivars evaluated. Verification of the accuracy of cultivar identification and the workability of the diagram demonstrated that all groups of cultivars could be distinguished using the specific primers. The present study offers a new method for accurate and reliable identification of sea buckthorn varieties, as well as a theoretical foundation for identifying new cultivars and protecting intellectual property rights (Wang et al., 2009).

This method can be applied to other plant and seed samples, which are important for plant genetic germplasm conservation, cultivar-right-protection, provision of genetically uniform seedlings in production, and the seed industry. Advantages of this method include that only a few primers are necessary and all cultivars can be identified through PCR using

the corresponding primers found on the diagram. CID information can be transferred to a database *in silico* and made available to scientists and farmers worldwide. We have initiated additional studies to examine the most important fruit crop cultivars in China for cultivar-right-protection, the nursery industry, and genetic resource conservation. We hypothesize that this new method can be used to draw the CIDs for various species, providing information for separating cultivars or varieties as desired.

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REFERENCES

Chen XL and Lian YS (1994). The geographical distribution pattern and its formative factors on the genus *Hippophae* L. *Acta Bot. Boreal* 14: 105-110.

Cheng Z and Huang H (2009). SSR fingerprinting Chinese peach cultivars and landraces (*Prunus persica*) and analysis of their genetic relationships. *Sci. Horticult*. 120: 188-193.

D'Onofrio C, Lorenzis G, Giordani T, Natali L, et al. (2009). Retrotransposon-based molecular markers in grapevine species and cultivars identification and phylogenetic analysis. *Acta Horticult.* 827: 45-52.

Demirsoy L, Demir T, Demirsoy H, Okumus A, et al. (2008). Identification of some sweet cherry cultivars grown in Amasya by RAPD markers. *Acta Horticult.* 795: 147-152.

Elidemir AY and Uzun I (2009). Assessment of genetic diversity of some important grape cultivars, rootstocks, and wild grapes in Turkey using RAPD markers. *Acta Horticult.* 827: 275-278.

Ercisli S, Agar G, Yildirim N, Esitken A, et al. (2009). Identification of apricot cultivars in Turkey (*Prunus armeniaca* L.) using RAPD markers. *Romanian Biotechnol. Letter* 14: 4582-4588.

Fang J, Twito T, Zhang Z and Chao CT (2006). Genetic relationships among fruiting-mei (*Prunus mume* Sieb. et Zucc.) cultivars evaluated with AFLP and SNP markers. *Genome* 49: 1256-1264.

Hasnaoui N, Messaoud M, Jemni C and Mokhtar T (2010). Molecular polymorphisms in Tunisian pomegranate (*Punica granatum* L.) as revealed by RAPD fingerprints. *Diversity* 2: 107-114.

Jain A, Ghangal R, Grover A, Raghuvanshi S, et al. (2010). Development of EST-based new SSR markers in seabuckthorn. *Physiol. Mol. Biol. Plants* 16: 375-378.

Javanshah A, Tajabadipour A and Mirzaei S (2007). Identification of a new phenotype (Siah Barg) of pistachio (*Pistacia vera* L.) with shiny-blackish green leaves using RAPD assay. *Int. J. Agri. Biol.* 9: 307-310.

Jin XJ (2002). Analysis of effective composition of *Hippophae rhamnoides* L. *Chem. Res. Chinese U*. 18: 274-276.

Lian YS (1988). New discoveries of the genus *Hippophae* (Elaeagnaceae). *Acta Phytotax. Sin*. 26: 235-237.

Lian YS and Lian H (1996). Systematic classification of the genus *Hippophae* L. *Hippophae* 9: 15-24.

Lian YS and Chen XL (2000). The regular patterns of distribution on the natural components in plants of the genus *Hippophae* L. *J. Northwest Normal Univ. (Nat. Sci. Edn.)* 36: 113-128.

Melgarejo P, Martínez JJ, Hernández F, Martínez R, et al. (2009). Cultivar identification using 18S-28S rDNA intergenic spacer-RFLP in pomegranate (*Punica granatum* L.). *Sci. Horticult.* 120: 500-503.

Murray GC and Thompson WF (1980). Rapid isolation of high molecular weight DNA. *Nucleic Acids Res*. 8: 4321-4325.

Papp N, Szilvássy B, Abrankó L, Szabó T, et al. (2010). Main quality attributes and antioxidants in Hungarian sour cherries: identification of genotypes with enhanced functional properties. *Int. J. Food Sci. Tech.* 45: 395-402.

Ruan C and Li D (2005). AFLP fingerprinting analysis of some cultivated varieties of sea buckthorn (*Hippophae rhamnoides*). *J. Genet*. 84: 311-316.

Sheng HM, An LZ, Chen T, Xu SJ, et al. (2006). Analysis of the genetic diversity and relationships among and within species of *Hippophae* (Elaeagnaceae) based on RAPD markers. *Plant Syst. Evol*. 260: 25-37.

Wang C, Fang J, Zhang Z, Cao S, et al. (2009). Brief introduction of fruit cultivar-right-protection in China and its ongoing situation. *Chin. Agric. Sci. Bull.* 25: 298-302.

Wang ZG, Zhang ZH, Li H, Gao XY, et al. (2007). Identification of strawberry cultivars by RAPD and SCAR markers.

Acta Horticult. Sin. 34: 591-596.
Williams JG, Kubelik AR, Livak KJ, Rafalski JA, et al. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18: 6531-6535.
Yu ZD, Ao F and Lian YS (1993). The origin, classification, populations and resource of the genus *Hippophae* L. in China. *Hippophae* 3: 19-24.