Development of EST-based new SSR markers in seabuckthorn

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Abstract EST-based SSR markers were developed by screening a collection of 1584 clustered ESTs of seabuckthorn (*Hippophae rhamnoides*). PCR primers were designed for the amplification of 30 microsatellite loci. Two to five allelic bands were displayed by nine primer pairs in *H. rhamnoides* genotypes and by eleven primer pairs in *H. salicifolia* genotypes. None of the thirty primer pairs detected polymorphism in *H. tibetana* genotypes. Considering the high polymorphism detected in the tested genotypes and their direct origin from the genic regions, these EST-SSR markers hold immense promise in seabuckthorn genome analysis, molecular breeding and population genetics.

Keywords *Hippophae rhamnoides* · Expressed sequence tags · Simple sequence repeats (SSRs) · Cross-amplification

Introduction

Common seabuckthorn (*Hippophae rhamnoides* L.), a member of family Elaeagnaceae, is a deciduous shrub distributed in many parts of Europe and Asia. The plant grows in semi-desert dry conditions, and also in mountains above tree line. The plant has an ability to grow in all kinds of soils and can withstand the extreme environmental conditions (−40°C to 40°C).

Seabuckthorn is valued both for ecological as well medicinal purposes. Due to its extensive root system, it is useful in reclaiming and conserving soil especially on fragile slopes (Wang et al. 2008). This plant promotes growth of poplars, pines and other tree species in mixed stands (Shi et al. 1987). Seabuckthorn not only prevents the loss of soil, but also improves the degraded soils due to its nitrogen-fixing capabilities. It is a rich source of Vitamin C (Zeb 2004, 2006). Its berries possess great potential as an antioxidant (Geetha et al. 2009), immuno-modulator (Mishra et al. 2008), anti-carcinogenic (Zeb 2006), and anti-bacterial agent (Chauhan et al. 2007; Jain et al. 2008). Unfortunately, despite its immense importance as an ecological restorer and medicinal plant, the number of single locus co-dominant markers available is very limited for this plant (Wang et al. 2008). The molecular markers best suited for detecting genetic diversity, should be easy and inexpensive to use and highly polymorphic within the population. Among different classes of molecular markers available for evaluating genetic diversity, microsatellites or simple sequence repeats (SSRs) are well known for their potentially high information content and versatility as molecular tools. To the best of our knowledge, only nine microsatellite markers derived from an enriched library of seabuckthorn are available (Wang et al. 2008) in the public domain. This is the first report of microsatellite markers

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derived from ESTs in seabuckthorn. Compared to genomic microsatellites, EST-SSRs are present at more strategic locations and can be more efficiently exploited in molecular mapping and gene tagging experiments, as they are more likely to show tight linkage with important traits.

Materials and methods

Leaves of common seabuckthorn (*H. rhamnoides*) were used for the construction of cDNA library for generating EST sequences. Microsatellites discovered in this study were assessed on 14 genotypes comprising of five *H. rhamnoides*, five *H. salicifolia* and four *H. tibetana* genotypes. All the plant samples were collected from the premises of Defense Institute of High Altitude Research (DIHAR), Leh, India. Genomic DNA was extracted from freeze dried leaf material using a modified CTAB protocol (Doyle and Doyle 1988). Total RNA was extracted using modified CTAB-based method for RNA isolation (Ghargal et al. 2009).

The ESTs were generated by sequencing 3000 clones from a cDNA library using MegaBACE Dye Terminator Kit (GE Biosciences, USA). The resulting sequences were

clustered using CAP3 (Huang and Madan 1999). Microsatellites were identified in ESTs using MISA (Thiel et al. 2003) with its default parameters except that mononucleotides were not included in the search parameters. The primers were designed using Primer 3 (Rozen and Skaletsky 2000). One of the primer pair (Hr06) described earlier by Wang et al. (2008) was also assessed on fourteen individuals analyzed in this study.

PCR amplifications of microsatellite loci were performed in a 25 µL reaction mixture that contained 10 pmol of each primer, 100 µM of each of dNTPs, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.5 U of *Taq* polymerase (Intron Biotechnology, South Korea), and approximately 50 ng of template DNA. Amplifications were carried out in Mastercycler (Eppendorf, Germany) with cycle conditions being 5 min of pre-amplification denaturation at 95°C followed by 35 cycles of 45 s at 95°C, 40 s at a primer-specific annealing temperature (Table 1), and 1 min at 72°C. As a final step, products were extended for 5 min at 72°C. PCR products were resolved on electrophoresis on 4% metaphor agarose (Lonza, Switzerland). The amplicons were visualized after staining with ethidium bromide and recorded using gel documentation system (DNR BioImaging System, Israel).

Table 1 Amplification profile of polymorphic EST-SSRs in seabuckthorn

Locus	Primer sequence (5'–3')	Repeat	Location	T _m (°C)	Number of alleles		
					<i>H. rhamnoides</i>	<i>H. salicifolia</i>	Total alleles
HrMS003	F: GCT CTC ATC CGA TTT GAT CC R: GTC GCA GTC TTC TTG GGT TC	(TCA) ₆	CDS	56.0	4	4	5
HrMS004	F: GTT TGA GGT CGG TGC TGA GT R: GGG TAA CCC AAC TCC TCC TT	(TC) ₈	3'-UTR	57.0	2	2	2
HrMS010	F: GGA AGC GTG AGG AAA TGT C R: GAA CAG ACA GAC CAT TAG AGA AC	(TGG) ₅	3'-UTR	56.0	2	2	2
HrMS012	F: CTC CAT CTC AAT CAT CAC TGC R: TTA GGG ATC CGG ATG AAG	(CTT) ₁₁	CDS	58.0	4	4	5
HrMS014	F: ATA CCT AGC TCG GCA ACA AG R: ACG ACC CAT GGC ATA ATA GTA C	(TG) ₆ (TA) ₈	3'-UTR	57.0	3	3	3
HrMS018	F: CAA CAT TGT TTC GTG CAG R: ACT CAC ATA ATC GAT CTC AGC	(ATG) ₅	3'-UTR	51.0	2	2	2
HrMS021	F: CCC AAT GTA CTA CAC TTA CGG R: CCA GAA GCA GTT CCA CAA G	(GAA) ₅	CDS	57.0	1	3	3
HrMS023	F: TCA GTT GGT AGG ATG CTT C R: ACA CCA CTC TGT CCT CAA TC	(AAAAT) ₄	3'-UTR	53.0	2	2	2
HrMS025	F: GTA CTG TGA CCA CGC TGC R: GGG TTC AAA GTA ATG GCA AG	(AG) ₈	3'-UTR	53.0	3	3	4
HrMS026	F: ATG ATG ACG ACG ACA ACG R: AGT GGT GGT GAC GAT AGT ATC	(CAC) ₅	5'-UTR	53.0	3	3	3
HrMS028	F: CTT GCT GCC ACG TAT TTA CAC R: TGG CTT TGC TCT TCT GCT AG	(TTG) ₅	CDS	56.0	1	2	2

Results and discussion

Sequencing of the cDNA clones generated 2787 single pass ESTs, which were further clustered into 1584 sequences using CAP3. Simple sequence repeats (SSR) were present in 56 (3.5%) of these sequences, a significantly lesser frequency than that reported for other plants (Varshney et al. 2005). More than 50% of the SSRs were dinucleotide repeats; trinucleotide repeats being next most abundant category of repeats. AT and AG motifs were most abundant among these repeats (12 each). Among trinucleotide repeats, AAG repeats were most abundant. Both AG and AAG repeats are known to occur near or within genes and have been reported as the most abundant classes in plant ESTs (Fujimori et al. 2003; Grover and Sharma 2004; Varshney et al. 2005; Grover and Sharma 2007; Grover et al. 2007). Primers could be designed for 30 of the microsatellites identified in this study. In the remaining 26 cases, the microsatellite was either too close to 5' or 3' end of the EST, or the sequence was highly AT-rich that hindered the successful primer design. Out of these thirty, twenty four primer pairs successfully amplified the targets in *H. rhamnoides*. However, only nine primer pairs generated polymorphic amplicons within *H. rhamnoides* accessions, and two additional primer pairs (SBT021 and SBT025) were found polymorphic on cross-amplification to *H. salicifolia* and *H. tibetana* (Table 1). Thus, the overall success rate of developing SSR markers from the EST library came out to be 0.69%, which is comparable to screening genomic libraries for development of microsatellites and lower than screening of microsatellite enriched libraries (Zane et al. 2002; Grover et al. 2010). All the eleven loci found polymorphic in this study were annotated to either CDS or 3'-UTR.

Number of alleles obtained in this study ranged from 2–4 within *rhamnoides* genotypes, and 2–5 in 14 genotypes belonging to three *Hippophae* species used in this study. Interestingly, all the loci were polymorphic in *H. salicifolia* but all of these were monomorphic in *H. tibetana* (Fig. 1). A possible reason for such unusual observations might be the fact that genotypes were collected from the fields of DIHAR, and the individuals collected in case of *H. tibetana* might be the grafts of the same plant, and thus might be representing the same genotypes. Most of the loci were

either scored as homozygous, when only one band was visible, or as heterozygous, when both the alleles could be scored (Fig. 1). In case of SBT023 and SBT025, however, three bands could be seen within the expected size range in some of the genotypes. The source of the third band was not clear, even though it was consistently obtained on repeating the experiment 3–4 times. It is quite common to get a third band in the heterozygote plants with many SSR markers. Microsatellite marker *Hr06* developed earlier by Wang et al. (2008) revealed 12 alleles in 12 individuals collected from distant geographic locations. In contrast, the same primer pair revealed only two alleles in our study (Table 1), which can be explained by the fact that individuals selected in this study were picked up from the same site. Wang et al. (2008) reported successful cross-amplification of *Hr06* in *H. gyantsensis*, *H. neurocarpa* and *H. goniocarpa*. Further, we demonstrated its cross-amplification in *H. salicifolia* and *H. tibetana*, Wang et al. (2008) reported successful cross-amplification of all the nine SSR markers in at least one of *H. gyantsensis*, *H. neurocarpa* and *H. goniocarpa*. Similarly, we found all the eleven new microsatellite markers cross-amplifying in *H. salicifolia* and *H. tibetana*. These two studies together indicate that marked genetic diversity exist in seabuckthorn. Further different species of seabuckthorn are genetically close to each other, as all the locus-specific microsatellite markers isolated from seabuckthorn successfully cross-amplify in the related species within the expected size range.

To the best of our knowledge, this is the first report of EST-SSR markers in seabuckthorn. EST-SSRs provide valuable tools for gene tagging and molecular breeding. These markers have also been shown to have high allelic value, the markers reported here are likely to be useful in understanding genetic structure in seabuckthorn required to formulate the conservation studies in this important medicinal plant. The transferability of these markers makes them important tools for comparative genomics, and analysis of evolutionary and ecological trends in seabuckthorn. Transferability of EST-SSRs is their inherent property as they occur in the conserved genic regions of the genomes. EST-SSRs are also viable options for studies aiming to detect synteny in related species. In future, we can expect development of many more genomic and genic microsatellites in seabuckthorn so as to facilitate the

Fig. 1 Polymorphism and cross-amplification displayed by microsatellite locus *HrMS012*. **M & L:** Molecular weight marker **R1–R5:** Accessions of *H. rhamnoides* **S1–S5:** Accessions of *H. salicifolia* **T1–T4:** Accessions of *H. tibetana*

construction of first-ever high density genomic map of seabuckthorn.

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