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Characterization and identification of ISSR markers associated with oil content in sea buckthorn berries

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ABSTRACT. Bioactive oils extracted from sea buckthorn (*Hippophae rhamnoides*) berries contain highly nutritional and medicinal compounds; however, the oil contents of sea buckthorn berries are very low. Thirteen inter-simple sequence repeat (ISSR) primers were used to identify markers associated with oil content of dry pulp in 51 cultivars and lines, which clustered into three major groups based on 137 polymorphic markers. Dry pulp oil contents in 45 cultivars and lines in Group I ranged from 6.6 to 33.1%; these accessions belonged to *H. rhamnoides* ssp *mongolica* and its hybrids with *H. rhamnoides* ssp *sinensis*. Three lines (*H. rhamnoides* ssp *mongolica*) in Group II had high dry pulp oil contents (33.7 to 37.5%), whereas three lines of

hybrids in Group III had low dry pulp oil contents (10.9 to 17.5%). The dry pulp oil content of *H. rhamnoides* ssp *mongolica* (27.2 ± 0.9%) was higher than that of hybrids (12.0 ± 1.2%) ($P < 0.01$). Four ISSR markers (881_{340}, 825_{1000}, 817_{380}, and 807_{1100}) had positive association with high dry pulp oil content ($P < 0.01$) using stepwise multiple regression analysis. The use of these ISSR markers is a potential strategy to select genotypes with high dry pulp oil content and suitable parental combinations for improvement of sea buckthorn berries.

Key words: *Hippophae rhamnoides*; Oil content; ISSR marker; Association; Stepwise multiple regression analysis

INTRODUCTION

Sea buckthorn (*Hippophae rhamnoides*) is deciduous perennial shrub or small tree belonging to the Elaeagnaceae family. This species adapts very well to harsh environments, including conditions of drought, salinity and temperatures ranging from -40° to 40°C (Ruan et al., 2013). Bioactive oils extracted from sea buckthorn berries contain polyunsaturated fatty acids, vitamins, carotenoids and flavonol glycosides (Ranjith et al., 2006; Lehtonen et al., 2010; Li et al., 2013), although oil contents are only 6 to 12% in seeds and 1 to 6% in fresh pulp (Yang and Kallio, 2001; Ruan et al., 2007; Dulf, 2012).

Sea buckthorn is heterozygous and dioecious. All species are diploid (2n = 24) and wind pollinated. The genus *Hippophae* contains six species and 12 subspecies, all of which are naturally distributed in Asia and Europe (Ruan et al., 2013). Of these, the two subspecies *H. rhamnoides* ssp *sinensis* and ssp *mongolica* are distributed mainly in northern China, Russia, and Mongolia, and have substantial differences in morphological and physiological traits. Many cultivars of *H. rhamnoides* ssp *sinensis* are fast-growing and highly adaptable to harsh environments, although many have small fruit, many thorns and low oil contents, whereas many cultivars of *H. rhamnoides* ssp *mongolica* show promising agronomic and commercial traits including large fruit, fewer thorns and high oil contents (Ruan et al., 2007). For example, oil contents in seeds (12%) and fresh pulp (6%) of *H. rhamnoides* ssp *mongolica* from Russia were significantly higher than those in seeds (7.3%) and fresh pulp (2.0%) of *H. rhamnoides* ssp *sinensis* from China (Yang and Kallio, 2001, 2005). Many superior lines with no or fewer thorns, large fruit and high yield have been selected from hybrids between *H. rhamnoides* ssp *mongolica* and ssp *sinensis*, including 'Mengzeza14', 'Ezhongza4' and 'Zaciyou' (Ruan et al., 2013; Yang et al., 2015). However, there have been no reports concerning differences in pulp oil contents between *H. rhamnoides* ssp *mongolica* and its hybrids with *H. rhamnoides* ssp *sinensis*.

DNA markers linked to quantitative trait loci (QTL) can play a vital role in selective breeding programs and help to produce improved varieties (Chhotaray et al., 2015). However, sea buckthorn has a 3-5-year juvenile period during which fruits are not produced, and breeding techniques using marker-assisted selection (MAS) based on a QTL linkage map is time-consuming and labor-intensive (Ruan et al., 2013). As such, it is necessary to first use molecular tools to identify and classify genotypes with increased content of the functional edible oils with nutritional and health benefits.

Several types of molecular markers are available for use in the identification of sea buckthorn genetic relationships including random amplified polymorphic DNA, inter-

simple sequence repeats (ISSR), amplified fragment length polymorphism, sequence-related amplified polymorphism, and simple sequence repeat. ISSR markers have been used widely in diverse species (Ruan et al., 2004, Ruan and Li, 2005; Ruan, 2006; Li et al., 2010; Zhao et al., 2014; Bentley, et al., 2015) due to an ability to identify high levels of polymorphism, to reliably differentiate between genotypes (Gramaje et al., 2014) and identify markers associated with desirable traits (Ruan et al., 2009). Using ISSR markers, Tian et al. (2004) found that total molecular variance of 11 *H. rhamnoides* populations mainly existed within populations. ISSR markers were also used to differentiate between different cultivars that were labeled identically and identical cultivars with different names, and to determine the genetic relationship of cultivars with unknown parentage to those with known parentage (Li et al., 2009). Similarly, four ISSR markers associating with resistance to dried-shrink disease of sea buckthorn were identified using stepwise multiple regression analysis (MRA) of resistance indices of 52 sea buckthorn accessions (Ruan et al., 2009). Association of ISSR markers with desirable nutritional and agronomic traits such as ρ-cymene, γ-terpinene, thymol, carvacrol, and borneol contents in forest savory (Khadivi-Khub et al., 2014); sugar and protein contents in mulberry (Kar et al., 2008); antioxidant activity and valerenic acid content in *Valeriana jatamansi* Jones (Jugran et al., 2013, 2015); and fruit skin color, harvest time and soluble solids in sweet cherry (Ganopoulos et al., 2011) have been detected using MRA methods.

In this study, we first analyzed the genetic relationships of 51 cultivars and lines of *H. rhamnoides* ssp *mongolica* and its hybrids with *H. rhamnoides* ssp *sinensis* using 13 ISSR primers. We then tested differences in oil contents of sea buckthorn berry dry pulp between *H. rhamnoides* ssp *mongolica* and its hybrids. Finally, we detected associations of ISSR markers with dry pulp oil contents using MRA methods. In addition, we discuss potential strategies for selecting genotypes with high dry pulp oil contents and parental combinations that may be ideal for improvement of sea buckthorn berries.

MATERIAL AND METHODS

Plant materials

Fifty-one cultivars and lines of sea buckthorn from China and other countries were selected for use in this study (Table 1). All accessions were grown in an orchard at the Institute

Table 1. Name, origin, sub-species, and pedigree of 51 *Hippophae rhamnoides* cultivars and lines.

Name (Abbreviation)	Origin	Sub-species	Pedigree or background
Juren (JR), Jinse (JS), Aertaixinwen (AET), Chuyi (CY), Chengse (CS), Xiangyang (XY), Hunjin (HJ), Xiaolajiao (XLJ)	Russia	ssp *mongolica*	Plants introduced from Russia and developed in China
Yousheng (YS)	Russia	ssp *mongolica*	Hybrid of 'Xieerbingkayihao' x wild 'Katong' in Russia and developed in China
Shoudu (SD)	Russia	ssp *mongolica*	Hybrid of 'Lieninggele' x 'Kashiajiaka' in Russia and developed in China
Fenlan (FL)	Finland	ssp *mongolica*	Selected from seedlings of mixed seeds collected from Finland and developed in China
TF2-13 (TF2-13), TF2-23 (TF2-23), TF2-24 (TF2-24), TF2-25 (TF2-25), TF2-36 (TF2-36), TF2-ZF (ZF)	China	ssp *mongolica*	Selected female plants from mixed seedlings of 'Tefengerhao' from Russia
10-06 (06), 10-47 (47), 10-42 (42), 13-00 (13-00), 13-10 (13-10), 13-11 (13-11), 13-14 (13-14), 10-33 (33), 10-34 (34), HD-3 (HD3), Suiji-1 (SJ1), Suiji-3 (SJ3), Suiji-4 (SJ4), HS-1 (HS1), HS-4 (HS4), HS-10 (HS10), HS-12 (HS12), HS-14 (HS14), HS-15 (HS15), HS-18 (HS18), HS-20 (HS20), HS-22 (HS22), Xine-1 (XE1), Xine-2 (XE2), Xine-3 (XE3)	China	ssp *mongolica*	Selected from seedlings of the mixed seeds of ssp *mongolica* and developed in China
Ezhongza-4 (EZZ4)	China	Hybrid	Selected from hybrids of *H. rhamnoides* ssp *sinensis* x ssp *mongolica*
Za 05-21 (5-21), Za 1-2 (1-2), Za 05-20 (5-20), Za 05-06 (5-6), Za 13-25 (13-25)	China	Hybrid	Hybrid of 'Yousheng' (*H. rhamnoides* ssp *mongolica*) x MK88-01 (*H. rhamnoides* ssp *sinensis*)
Za 13-19 (13-19), Za 13-20 (13-20)	China	Hybrid	Hybrid of 'Aertai' (*H. rhamnoides* ssp *mongolica*) x HZ-87-12 (*H. rhamnoides* ssp *sinensis*)
Za 56 (56)	China	Hybrid	Hybrid of HS-22 (*H. rhamnoides* ssp *mongolica*) x MK88-01 (*H. rhamnoides* ssp *sinensis*)

All data are from the breeder's register.

of Berries, Heilongjiang Academy of Agricultural Sciences, Suiling, China (127°06'E, 47°14'N). The orchard has a mean annual rainfall of 551.5 mm and a mean annual temperature of 1.4°C, and the local weather conditions are described by the north-temperature continental monsoon climate group (Shan, 2008).

Eleven cultivars of *H. rhamnoides* ssp *mongolica* were introduced from Russia and Finland, and the remaining 31 lines of *H. rhamnoides* ssp *mongolica* and nine hybrids between *H. rhamnoides* ssp *mongolica* and ssp *sinensis* were selected and bred in the People's Republic of China. This study focused on testing genetic variation of dry pulp oil contents between *H. rhamnoides* ssp *mongolica* and its hybrids and identifying the association of ISSR markers with oil contents in dry pulp.

DNA extraction, ISSR amplification, and gel electrophoresis

Young leaves (30 g) were collected from one plant of each accession in June 2013. Total genomic DNA (gDNA) was extracted from 0.2 g fresh leaf tissue following the CTAB-based procedure of Ruan et al. (2004). The quality and concentration of gDNA were evaluated by 1% (w/v) agarose gel electrophoresis and measuring the A_{260}/A_{280} value (1.8-1.9) using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). PCR (20 μL) was conducted using 2 μL 10X PCR buffer and final concentrations of 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μM each primer, 30 ng gDNA template, and 2 U Taq DNA polymerase (TaKaRa, Dalian, China). The thermocycler program was set at 90 s at 94°C followed by 45 cycles of 45 s at 94°C, 45 s annealing at 53° to 54°C for the different primers (Table 2), and 90 s extension at 72°C, followed by a final extension step at 72°C for 7 min. Each reaction was stored at 4°C until gel analysis. The 13 primers were those reported by Ruan et al. (2009). Samples were amplified in a C1000 Touch™ Thermal Cycler PCR system (Bio-Rad Laboratories Inc., Foster, CA, USA). The PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels and visualized in a GelDoc-It² Imaging System (Ultra-Violet Products Ltd., Upland, CA, USA) after staining with Gold View I (Solarbio Science & Technology, Beijing, China).

Table 2. Primers used and number of ISSR bands obtained.

Primer	Primer sequence (5'→3')	Annealing temperature (°C)	Total number of ISSR bands	Total polymorphic number of bands	Polymorphism (%)	PIC
UBC807	AGAGAGAGAGAGAGAGT	53	9	9	100	0.93
UBC808	AGAGAGAGAGAGAGAGC	53	12	12	100	0.95
UBC809	AGAGAGAGAGAGAGAGG	53	10	10	100	0.93
UBC811	GAGAGAGAGAGAGAGAC	53	10	10	100	0.94
UBC812	GAGAGAGAGAGAGAGAA	54	10	10	100	0.93
UBC816	CACACACACACACACAT	53	9	8	88.9	0.93
UBC817	CACACACACACACACAA	53	10	8	80	0.93
UBC823	TCTCTCTCTCTCTCTCC	53	10	10	100	0.94
UBC825	TCTCTCTCTCTCTCTCG	53	13	13	100	0.94
UBC827	ACACACACACACACACG	53	9	8	88.9	0.92
UBC835	AGAGAGAGAGAGAGAGYC	53	17	17	100	0.96
UBC881	GGGTGGGGTGGGGTG	53	14	14	100	0.95
UBC887	DVDTCTCTCTCTCTCTC	53	9	8	88.9	0.92
Total			142	137	96.5	

Y = (C, T); D = (A, G, T); V = (A, C, G); PIC = polymorphism information content.

Oil content in dry pulp

Sea buckthorn leaves and berries were collected from one plant of each accession in

June and August 2013, respectively. Fresh fruits were stored at -46°C in sealed plastic bags until analysis. Berries with seeds removed (non-seed tissues) were air dried according to the method of Gutierrez et al. (2008). The oil contents of these tissues (hereafter dry pulp) were measured with a HCY-10 NMR oil content analyzer (Top Instrument CO., LTD, HangZhou, China) according to the protocol of Dong et al. (2011) using three replicates. Dry pulp oil contents of 51 cultivars and lines were grouped into three classes: low (0.0-21.0%), moderate (21.0-32.0%), and high (> 32.0%). Analysis of variance of oil contents among accessions was performed using the SPSS Statistics 20.0 software (IBM Corp., Armonk, NY, USA).

Data analysis of ISSR markers

Fragments amplified by each ISSR primer were scored as either "1" (present) or "0" (absent). Polymorphism information content (PIC) per primer was calculated according the method of Anderson et al. (1993):

$$1-\sum P_{ij}^2 \qquad \text{(Equation 1)}$$

where the relative frequency of the j^{th} band for the i^{th} primer is summed across all bands for the primer over all cultivars and lines. Genetic similarities between pairs of cultivars and lines were calculated using DICE similarity coefficients based on ISSR matrix data using the software of NTSYSpc version 2.1. Cluster analysis of 51 cultivars and lines was conducted using unweighted pair group analysis (UPGMA) with the SAHN module. A phylogenetic tree was constructed after combining with a graphical heatmap to show dry pulp oil contents using the MeV Software. The goodness-of-fit of the cluster was tested using the MXCOMP (matrix comparison) program, which directly compared the original similarity matrix with the cophenetic value matrix.

Pearson's correlation coefficients were calculated for all ISSR markers and dry pulp oil contents, and were categorized into positive or negative correlation groups. The association of ISSR markers and dry pulp oil contents was estimated by the MRA method using SPSS Statistics 20.0. Oil content was considered a quantitative trait and dependent variable (Virk et al., 1996) whereas the negative and the positive ISSR markers were treated as independent variables. *F* values with P values between 0.045 and 0.099 were used to include and remove independent variables from the regression equation, respectively (Kar et al., 2008; Ruan et al., 2009). R^2 denotes the square of *r*, the correlation coefficient.

RESULTS

Oil contents in dry pulp of sea buckthorn berries

Among the 51 cultivars and lines, the highest dry pulp oil content (38.6 ± 0.1%) was measured in line TF2-36 (*H. rhamnoides* ssp *mongolica*) whereas the lowest (6.6 ± 0.1%) was measured in line 56 (hybrids between *H. rhamnoides* ssp *mongolica* and ssp *sinensis*) (Figure 1). The dry pulp oil contents of 42 cultivars and lines of *H. rhamnoides* ssp *mongolica* ranged from 12.3 to 38.6%, and that in nine lines of hybrids was 6.6 to 17.5%. The average oil content of *H. rhamnoides* ssp *mongolica* (27.2 ± 0.9%) was significantly higher than that in the hybrids (12.0 ± 1.2%) ($P < 0.01$; Figure 1).

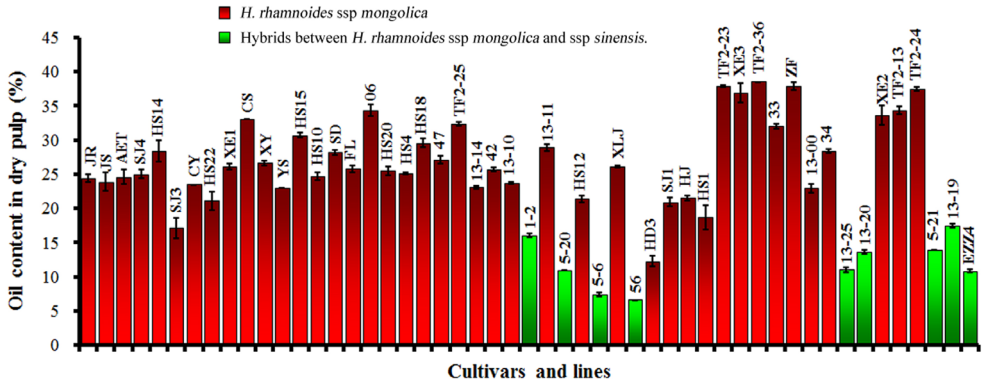

Figure 1. Dry pulp oil contents of 51 sea buckthorn (*Hippophae rhamnoides*) cultivars and lines. Abbreviations used for the names of cultivars and lines are: JR = Juren; JS = Jinse; AET = Aertaixinwen; CY = Chuyi; CS = Chengse; XY = Xiangyang; HJ = Hunjin; XLJ = Xiaolajiao; YS = Yousheng; SD = Shoudu; FL = Fenlan; TF2-13 (TF2-13); TF2-23 (TF2-23); TF2-24 (TF2-24); TF2-25 (TF2-25); TF2-36 (TF2-36); TF2-ZF (ZF); 10-06 (06); 10-47 (47); 10-42 (42); 13-00 (13-00); 13-10 (13-10); 13-11 (13-11); 13-14 (13-14); 10-33 (33); 10-34 (34); HD-3 (HD3); Suiji-1 (SJ1); Suiji-3 (SJ3); Suiji-4 (SJ4); HS-1 (HS1); HS-4 (HS4); HS-10 (HS10); HS-12 (HS12); HS-14 (HS14); HS-15 (HS15); HS-18 (HS18); HS-20 (HS20); HS-22 (HS22); Xine-1 (XE1); Xine-2 (XE2); Xine-3 (XE3); Ezhongza-4 (EZZ4); Za 05-21 (5-21); Za 1-2 (1-2); Za 05-20 (5-20); Za 05-06 (5-6); Za 13-25 (13-25); Za 13-19 (13-19); Za 13-20 (13-20); Za 56 (56).

ISSR analysis

Thirteen ISSR primers generated 142 bands among the 51 cultivars and lines, 137 of which (96.5%) were polymorphic (Table 2). The total number of bands generated by each primer ranged from 9 (UBC807, UBC816, UBC827, and UBC887) to 17 (UBC835), with a mean of 10.9 bands per primer. Nine primers (those not including primers UBC816, UBC817, UBC827, and UBC887) generated 100% polymorphism. The PIC per primer ranged from 0.92 to 0.96 with a mean of 0.94.

Cluster analysis

DICE coefficients between pairs of the 51 cultivars and lines ranged from 0.302 to 0.935 with a mean value of 0.636 ± 0.094. The highest DICE coefficient found (0.935) was between Russian cultivar CY (*H. rhamnoides* ssp *mongolica*) and line HS22 (*H. rhamnoides* ssp *mongolica* generated from progeny of Russian seeds) (Table 1). The minimum DICE coefficient found (0.302) was between line TF2-24 (selected from mixed female seedlings of *H. rhamnoides* ssp *mongolica* 'Tefengerhao' from Russia) and line 13-19 (selected from seedlings of *H. rhamnoides* ssp *mongolica* 'AET' and *H. rhamnoides* ssp *sinensis* line HZ-87-12 from China) (Table 1).

At the 0.59 DICE coefficient levels, 51 cultivars and lines were clustered into three groups (Figure 2). The cophenetic correlation (*r*) was 0.858 using the MXCOMP program, thus indicating a good fit between the dendrogram and the original similarity matrix. At the 0.63 DICE coefficient level, Group I was sub-divided into three sub-groups (Ia, Ib, and Ic) (Figure 2). Nine cultivars and 17 lines of *H. rhamnoides* ssp *mongolica* and one hybrid were

clustered into Group Ia; their dry pulp oil contents ranged from 16.1 to 34.4%. In this subgroup, 23 of 27 cultivars and lines had moderate oil contents, three had high oil contents, and one had low oil content. Thirteen cultivars and lines of *H. rhamnoides* ssp *mongolica* and three lines of hybrids were clustered into Group Ib. Dry pulp oil contents of hybrid lines 5-20, 5-6, and 56 were low, where *H. rhamnoides* ssp *mongolica* lines TF2-23, XE3, TF2-36, 33, and ZF had high oil contents. In Group Ic, the dry pulp oil contents of hybrid lines 13-25 and 13-20 were 11.0 and 13.7%, respectively. In Group II, *H. rhamnoides* ssp *mongolica* lines XE2, TF2-13, and TF2-24 had high oil contents (33.7, 34.3, and 37.5%, respectively). In Group III, hybrid lines 5-21, 13-19, and EZZ4 had low oil contents (14.0, 17.5, and 10.9%, respectively).

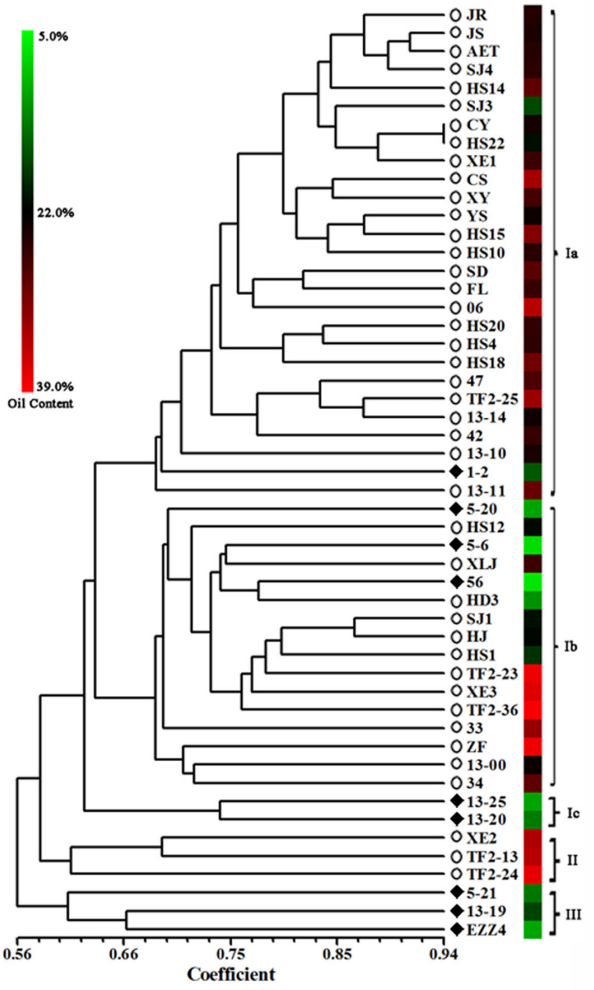

Figure 2. Dendrogram and dry pulp oil contents of 51 sea buckthorn cultivars and lines revealed by cluster analysis of genetic similarity estimates generated by DICE coefficient based on 137 polymorphic ISSR markers. Ia, Ib, Ic, II, and III are the number of groups. Open circles indicate *H. rhamnoides* ssp *mongolica* cultivars or lines. Filled diamonds indicate hybrids between *H. rhamnoides* ssp *mongolica* and ssp *sinensis*. Abbreviations are the same as in Figure 1.

Association of ISSR markers with oil contents in dry pulp

The MRA results indicated that there was a significant correlation between four ISSR markers and dry pulp oil content (Table 3). Specifically, four markers (881_{340}, 825_{1000}, 817_{380}, and 807_{1100}) (Table 4) showed positive correlation with high dry pulp oil content. Of these, marker 881_{340} had substantial positive correlation (r = 0.489, P = 0.000, t = 3.929) with high oil content in dry pulp. Conversely, four markers 808_{1050}, 807_{380}, 827_{370}, and 809_{300} (Table 4) showed negative correlation with dry pulp oil content. Of these, marker 808_{1050} had a substantial negative correlation (r = -0.707, P = 0.000, t = -7.000) with high oil content in dry pulp.

Table 3. Coefficients for dependent variable in the stepwise multiple regression analysis (MRA) for association of ISSR markers with dry pulp oil content.

Correlation	Dimensions†	Markers	Un-standardized coefficient beta	SE	Standardized coefficient beta	*t* value	P value	*r*
Positive	I	881_{340}	14.645	3.727	0.489	3.929	0.000	0.489
	II	881_{340}	13.794	3.380	0.461	4.081	0.000	0.460
		825_{1000}	13.327	3.862	0.390	3.451	0.001	0.389
	III	881_{340}	12.990	3.054	0.434	4.254	0.000	0.432
		825_{1000}	13.640	3.480	0.399	3.919	0.000	0.398
		817_{380}	5.901	1.694	0.355	3.483	0.001	0.354
	IV	881_{340}	13.669	2.801	0.457	4.881	0.000	0.453
		825_{1000}	13.029	3.188	0.381	4.086	0.000	0.379
		817_{380}	5.086	1.570	0.306	3.240	0.002	0.301
		807_{1100}	12.495	3.911	0.302	3.195	0.003	0.297
Negative	I	808_{1050}	-15.640	2.234	-0.707	-7.000	0.000	-0.707
	II	808_{1050}	-14.421	2.051	-0.652	-7.032	0.000	-0.642
		807_{380}	-7.489	2.167	-0.320	-3.456	0.001	-0.316
	III	808_{1050}	-12.046	2.168	-0.545	-5.556	0.000	-0.482
		807_{380}	-7.014	2.067	-0.300	-3.393	0.001	-0.294
		827_{370}	-5.700	2.286	-0.244	-2.493	0.016	-0.216
	IV	808_{1050}	-11.384	2.093	-0.515	-5.439	0.000	-0.451
		807_{380}	-5.511	2.080	-0.236	-2.649	0.011	-0.220
		827_{370}	-6.601	2.221	-0.282	-2.973	0.005	-0.247
		809_{300}	-3.427	1.476	-0.206	-2.321	0.025	-0.193

†Denotes the dimensions for different steps under stepwise MRA, where the marker(s) of previous step(s) are included in the succeeding step.

Table 4. Markers associated with sea buckthorn dry pulp oil content as revealed by stepwise multiple regression analysis (MRA).

Correlation	Markers†	R^2	R^2 change	F change	P value of F change
Positive	881_{340}	0.240	0.240	15.439	0.000
	$+825_{1000}$	0.391	0.151	11.910	0.001
	$+817_{380}$	0.516	0.125	12.134	0.001
	$+807_{1100}$	0.604	0.088	10.208	0.003
Negative	808_{1050}	0.500	0.500	48.994	0.000
	$+807_{380}$	0.600	0.100	11.941	0.001
	$+827_{370}$	0.646	0.047	6.214	0.016
	$+809_{300}$	0.683	0.037	5.389	0.025

†"+" denotes the inclusion of marker(s) in the preceding step(s) in the stepwise MRA.

DISCUSSION

According to ISSR marker analysis, 51 cultivars and lines of *H. rhamnoides* ssp *mongolica* and its hybrids with *H. rhamnoides* ssp *sinensis* were clustered into three major groups that were in good agreement with taxonomic classifications and available pedigree and genetic background information. The coefficient of gene differentiation between *H. rhamnoides* ssp *mongolica* and its hybrids was 0.059, which indicated that 5.9% of total

genetic difference occurred between one subspecies and its hybrids with another subspecies, whereas 94.1% existed within subspecies (Ding et al., 2015).

According to the cluster analysis (Figure 2) and oil contents of 51 cultivars and lines (Figure 1), accessions that clustered into the same sub-groups had similar dry pulp oil contents, and that the oil content of *H. rhamnoides* ssp *mongolica* was significantly higher than that of the hybrids. Lines 13-25 and 13-20 in Group Ic and lines 5-21, 13-19, and EZZ4 in Group III had low oil contents (11.1, 13.7, 14.0, 17.5, and 10.9%, respectively). These lines were selected from hybrids between *H. rhamnoides* ssp *mongolic*a and ssp *sinensis*. In contrast, lines XE2, TF2-13, and TF2-24 in Group II had high oil contents (33.7, 34.3, and 37.5%, respectively) and all belonged to *H. rhamnoides* ssp *mongolica*. These results indicated that the cluster analyses based on ISSR markers could provide useful taxonomic information regarding pre-selection of cultivars and lines with high dry pulp oil contents.

The four ISSR markers associated with high oil content in dry pulp, which were first identified in this study, could also be used to identify sea buckthorn germplasm with high oil contents before maturity, and the four markers associated with low oil content in dry pulp could be used to eliminate germplasm with low oil content before entering the maturity. Although most ISSR markers are dominant markers and not appropriate for analysis of heterozygosity (Bentley et al., 2015), the most important application of this study was that the four ISSR markers could not only provide potential strategies for selecting genotypes with high oil contents and parental combinations for improving desirable agronomic traits in a sea buckthorn breeding program, but could also provide the foundation for the conversion of specific ISSR fragments into sequence-characterized amplified region (SCAR) markers, which could be directly used in early molecular diagnosis of oil content in sea buckthorn germplasm. For example, three reliable SCAR markers that were derived from six species-specific ISSR fragments were subsequently used to identify five necrophagous fly species (He et al., 2007).

Fresh and dry pulp oil contents of *H. rhamnoides* ssp *mongolica* were previously shown to be higher than those of *H. rhamnoides* ssp *sinensis* (Ruan and Li, 2001; Yang and Kallio, 2002, 2005). In this study, our results first indicated that the dry pulp oil content of *H. rhamnoides* ssp *mongolica* was significantly higher than those of hybrids (27.2 *vs* 12.0%). Whereas pulp traits of sea buckthorn berries have genetic characteristics of the female parent genotype (Oomah, 2005), oil contents may also be affected by the male parent genotype due to pollen xenia. For example, 'Yousheng' (*H. rhamnoides* ssp *mongolica*) has high dry pulp oil content (23.0%) whereas line MK88-01 (*H. rhamnoides* ssp *sinensis*) has low dry pulp oil content (6.0%) (Ruan and Li, 2001). However, the dry pulp oil contents of lines 5-20 and 5-6, which were hybrids between 'Yousheng' and MK88-01, were only 10.98 and 7.39%, respectively. Pollen xenia, which is defined as the effect of pollen sources on fruit quality and quantity, has been reported in pistachio (Cran and Iwakiri, 1980), hazelnut (Weingartner et al., 2004) and maize (Fattahi et al., 2014). For example, the use of pollen from small-seeded pistachio cultivars was shown to generate small seeds in cultivars that typically produce large seeds (Cran and Iwakiri, 1980).

As sea buckthorn is highly heterozygous and has a 3-5-year juvenile period before fruit production, MAS breeding based on a QTL linkage map to select materials with high oil contents in a conventional breeding program is overly time-consuming. As such, the results of clustering analysis and ISSR markers that are associated with high oil contents will play a vital and time-saving role in identifying and classifying genotypes that have high oil contents. The present study also presents the possibility of converting specific ISSR markers into SCAR

markers in future marker-assisted breeding programs, to greatly improve oil contents of sea buckthorn.

Conflicts of interest

The authors declare no conflict of interest.

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