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Full Length Research Paper

Development of genomic SSR and potential EST-SSR markers in *Bupleurum chinense* DC.

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Nineteen genomic SSR markers were developed using inter-simple sequence repeat (ISSR)-suppression PCR technique in *Bupleurum chinense* DC., a widely used Chinese medicinal plant. A total of 126 alleles were detected across 22 individual plants of *B. chinense* DC. f. *octoradiatum* (Bunge) Shan et Sheh, with an average of 3 - 13 alleles per locus. The observed heterozygosity (H_O) and the expected heterozygosity (H_E) values ranged from 0.23 to 1.00 and from 0.29 to 0.92, respectively. Nine loci deviated from Hardy-Weinberg equilibrium (HWE) ($P < 0.05$) and eight pairs of loci showed significant linkage disequilibrium (LD) (Fisher's exact test, $P < 0.01$). The species transferability of these genomic SSR markers was also detected in seven other *Bupleurum* species. Eight SSR markers were successfully amplified in all tested species. In addition, forty four EST-SSRs which can be amplified with expected sizes were identified from a *B. chinense* root cDNA library. The genomic SSR markers and potential EST-SSR markers developed in the present study should be useful for genetic diversity and molecular marker assistant selection breeding research in *Bupleurum* species.

Key words: *Bupleurum chinense* DC., Simple sequence repeat (SSR), Inter-simple sequence repeat (ISSR)-suppression PCR.

INTRODUCTION

Radix bupleuri (Chaihu; Chinese Thorowax Root), sourced from the dried roots of *Bupleurum* species (*Umbelliferae* family), has been used in indications such as common cold with fever, alternate chills and fever such as malaria, distending pain in the chest and hypochondriac regions, menstrual disorders, prolapse of the uterus, prolapse of the rectum with the action to release both exterior and interior, soothe the liver and elevate yang (Chinese Pharmacopoeia Commission, 2005). It is widely used in China, Japan, Korea and other south Asian countries. In China, although *Bupleurum chinense* DC. and *B. scorzonerifolium* Willd. are the two official *R. bupleuri* source species (Chinese Pharmacopoeia Commission, 2005), other *Bupleurum* species can be often found in the medicinal materials markets of some Chinese districts, such as *B. yinchowense* Shan et Y. Li, *B. smithii* Wolff, *B. smithii* Wolff. Var. *parvifolia* Shan et Y. Li, *B. marginatum* Wall. ex DC., *B. bicaule* Helm. and *B. scorzonerifolium* Willd. var. *angustissimum* (Franch.) Huang (Song, 2002). Many studies have shown the active component, such as saikosaponins, volatile oils and polysaccharides, varied remarkably between different *Bupleurum* species and also regional populations of the same species (Zhang et al., 2006; Shon et al., 1997; Shon et al., 2008; Chen et al., 2006). So, to use the species correctly demands the authentication of the *R. bupleuri* sourced species and learning their phylogenetic relationship. The great difficulty on distinguishing *Bupleurum* species through morphologic appearance has been noticed (Neves and Watson, 2004; Yang et al., 2007). A few attempts had been done with molecular methods to identify species and detect geographic variations, such as RFLP (Restriction Fragment Length Polymorphism) (Mizukami et al., 1993), chloroplast DNA restriction site analysis (Matsumoto et al., 2004) and rDNA ITS (Internal Transcribed Spacer) sequence analysis (Yang et al., 2007). SSR is the kind of preferred marker rather than several others such as RAPD, ISSR and AFLP. Recently, they

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have been developed and used for authentication and population structure analysis in some medicinal plants (Kumar et al., 2007; Guan et al., 2008). To develop SSR markers in *Bupleurum* will be valuable for studies on genetic diversity and phylogenetic relationship of the genus *Bupleurum*. It will also be of significance to authenticate and promote the proper use of medicinal *Bupleurum* species.

As wild resources sharply shrinking, several *Bupleurum* species have been cultivated since 1970's in China. Nowadays about one third of *R. bupleuri* circulating in Chinese medicinal materials markets was derived from cultivation. *B. chinense* is the most universal species being cultivated in China. In Taiwan, the species of *B. kaoi*, *B. falcatum* and *B. chinense* and in Japan and Korea, the species of *B. falcatum* are commonly cultivated and pharmacologically studied as the source of *R. bupleuri*, respectively (Lin et al., 2008; Iida, 2006; Choi et al., 1995). *Bupleurum* cultivation methods have been researched in details (Minami et al., 1995; Shon et al., 1998; Liu et al., 1991; Kim et al., 1997; Kubo et al., 1993). It is obvious that plants with consistent agronomic traits and high saikosaponins content are the basis of standardization and modernization of the cultivation. To select and breed new cultivars with favorable agronomy traits and active components is a good way. There were some reports about the conventional breeding practices on *Bupleurum* cultivars. *B. falcatum* L. cv. Tainung No.1 was a breeding line developed through mass selection from three sources of *B. fatcatum* in Taiwan (Liu et al., 1989). In Japan, new *Bupleurum* plants were intended to acquire by crossing between the Japa-nese source plants and the Korean plants (Ohta et al., 2006). To improve cultivated *Bupleurum* in China, *B. chinense* DC. cv. Zhongchai No. 1 was mass-selected recently in our laboratory. Molecular marker-assisted selection (MAS) is an important method in modern breed-ing technologies. To develop SSR markers in *Bupleurum* species will be helpful for the identification of desired *Bupleurum* germplasm, the analysis of genetic structure of the cultivated population and the bred lines, the genetic map construction and quantitative trait loci (QTL) mapping.

Generally, two methods were used to develop SSR markers. For those organisms with plenty of genomic sequences or ESTs data, primer pairs of genomic and EST-SSR markers usually were developed by sequence database mining. For those organisms with little information of DNA sequences or ESTs, other methods for SSR markers isolation have been exploited. Inter-simple sequence repeat (ISSR)-suppression PCR, reported by Lian et al. (2001) for the first time, was a time and cost saving method for genomic SSR markers isolation and has been used in several plant species and fungi (Tamura et al., 2005; Lian et al., 2003). No SSR markers have been developed in the genus *Bupleurum*. We previously *in silico* analyzed and obtained 86 potential EST-SSR loci in 1650 uniESTs from a *B. chinense* root cDNA library (Sui et al., 2010, in press). Their primer pairs and validities to amplify were reported here. A set of nineteen genomic SSR markers were also developed using ISSR-suppression PCR method.

MATERIALS AND METHODS

Plant materials

B. chinense DC. cv. Zhongchai No. 1, which is a mass-selected cultivar of *B. chinense*, was used to isolate genomic SSR markers and analyze the validity of the EST-SSRs. Twenty two wild individual plants of *B. chinense* DC. f. *octoradiatum* (2n = 12), collected from Baihua Mountain, Beijing, were used to analyze variation of the genomic SSR markers. At least two plants of each of seven *Bupleurum* species collected from Shaanxi, Shanxi, Hebei, Sichuan, Qinghai, Hei Longjiang and Anhui provinces (Table 1) were used to test the species transferability of the genomic SSR markers. Plant materials used in this experiment were identified by Chunsheng Liu, Professor of Beijing University of Chinese Medicines and their specimens were deposited in the Specimen Museum of IMPLAD, CAMS and PUMC.

Development of genomic SSR primer pairs

The ISSR-suppression PCR technique established by Lian et al. (2001) was used to develop genomic SSR markers. A total of 100 different ISSR-PCR amplified fragments were cloned and sequenced to determine one flanking region for each SSR locus. SSR Hunter 1.3 software (Li and Wan, 2005) was used to find SSR loci on the inner sequence of clones and then forward and reverse primers to clone these SSR loci were designed. For SSR loci on the terminal of ISSR-PCR amplified fragments, the other unknown region flanking each locus was determined by the following steps: Genomic DNA was separately digested with one of the restriction enzymes: *Eco*RV, *Hae*III, *Rsa*I, *Ssp*I and *Alu*I (New England Biolabs, Beijing). After digestion, fragments were precipitated by alcohol with 1/10 volume of 3 M NaOAc (pH 5.2) and ligated with an adaptor (consisting of two single-stranded oligonucleotides: 5′-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGG GCTGGT-3′ and 5′-ACCAGCCC-NH_2-3′) using DNA Ligase (TaKaRa). The ligation products were diluted 10 times with ddH_2O and used as the template of the first PCR. For the second PCR, primers IP1 and its corresponding nested primer IP2 based on the sequence between IP1 and the SSR, were designed from the determined sequences. An adaptor-primers AP1 (5′-CCATCGTAATA CGACTCACTATAGGGC-3′) and the nested primer AP2 (5′-CTATAGGGCACGCGTGGT-3′) were also prepared. The first PCR reaction was conducted using primers IP1 and AP1 with the diluted ligation product. The second PCR reaction was conducted using primers IP2 and AP2 with a 100-fold dilution of the first PCR products. Single-banded fragments were amplified after second PCR and were then cloned and sequenced. Finally a primer (IP3) was designed with the opposite flanking sequence of SSR locus to primers IP1 and IP2. The primer pair, IP1/IP3 or IP2/IP3 was examined as a potential SSR marker. All primers were designed by Primer Premier 5.0 software (Premier Biosoft International). Primer pairs that yielded expected size fragment were then subjected to loci polymorphism analysis.

Polymorphism evaluation of genomic SSR loci

Total DNA was separately isolated from leaves. PCR amplification conditions were: 25 µl reaction mixture containing 1× PCR buffer, 100 ng template DNA, 1.0 U Taq DNA polymerase (TaKaRa), 1.5 mmol/l $MgCl_2$, 0.2 mmol/l of each dNTP, 0.4 µmol/l reverse primer

Table 1. Origin of plant materials used in the present study, *B. chinense* DC. cv. Zhongchai No. 1 used to isolate genomic SSR markers, *B. chinense* DC. f. *octoradiatum* (Bunge) Shan et Sheh used to analyze polymorphism of SSR loci and the others to test species transferability of the SSR markers.

Specimens no.	Species	Origin	Above sea level (m)	Latitude and longitude
Wild				
06Y07-CH19	*B. longiradiatum* Turcz. var. *porphyranthum* Shan et Y. Li	Mei County, Shaanxi	1806	E107°48´808´´, N34°00´808´´
06Y07-CH20	*B. yinchowense* Shan et Y. Li	Xinjiang, Shanxi	660 - 670	E111°04´622´´, N35°44´098´´
06Y07-CH04	*B. sibiricum* Vest var. *jeholense* (Nakai) Chu	Xinglong, Hebei	1750 - 1760	E117°28´862´´, N40°35´347´´
06Y07-CH33	*B. wenchuanense* Shan et Y. Li	Erlang Mountain, Sichuan	-	-
06Y07-CH30	*B. smithii* Wolff var. *parvifolium* Shan et Y. Li	Ping'an, Qinghai	3194 - 3310	-
06Y07-CH16	*B. chinense* DC. f. *octoradiatum* (Bunge) Shan et Sheh	Baihua Mountain, Beijing	1803, 1925	E115°34´534´´, N39°48´889´´
Cultivated				
06Y07-CH25	*B. scorzonerifolium* Wilid.	Mingshui, Hei Longjiang	-	-
06Y07-CH31	*B. falcatum* L.	Bozhou, Anhui	-	-
06Y07-CH27	*B. chinense* DC. cv. Zhongchai No. 1	IMPLAD, Beijing	-	-

and 0.4 µmol/l forward primer (or 0.4 µmol/l reverse primer, 0.4 µmol/l forward primer with M13 (-21) tail at its 5′ end and 6-FAM labeled M13 (-21) primer (Schuelke, 2000)). Amplification was performed by a PCR thermal cycler (Biometra® T-Gradient 96) with the program of 5 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at the annealing temperature of each primer pair (Table 2) and 2 min at 72°C (additional 8 cycles of 30 s at 95°C, 30 s at 53°C and 2 min at 72°C for fluorescent dye labeled primer), 10 min at 72°C. PCR products with the GeneScan-500 ROX internal-lane size standard (Applied Biosystems) were electrophoresed on ABI 377 (Applied Biosystems). SSR alleles were resolved by GeneScan 3.7. GENEPOP version 3.4 (Raymond and Rousset, 1995) was employed to calculate H_O (Observed heterozygosity) and H_E (Expected heterozygosity) values and to test LD (Linkage disequilibrium) and HWE (Hardy-Weinberg equilibrium).

Development of EST-SSR markers

The primer pairs intended to amplify 86 potential SSR loci, *in silico* identified in 1650 uniESTs from a *B. chinense* root cDNA library (Sui et al., 2010, in press) recently, were designed by Primer Premier 5.0 software. PCR amplification was performed using DNA from leaves of two *B. chinense* DC. cv. Zhongchai No. 1 individual plants. Their conditions were the same to that of genomic SSR markers with annealing temperatures showed in Table 4.

RESULTS AND DISCUSSION

Nineteen primer pairs which amplified a clear PCR product with the expected size were obtained (Figure 1 and Table 2). All the loci comprised perfect (6), interrupted perfect (4) and compound (9) SSRs. Size of the amplified fragments ranged from 132 to 373 bp. A total of 126 alleles were produced, ranging from 3 to 13 alleles with an average of 6.63 per locus. The observed heterozygosity (H_O) value ranged from 0.23 to 1.00, while the expected heterozygosity (H_E) value varied from 0.29 to 0.92. Nine of the nineteen polymorphic loci (labeled in Table 2) departed significantly from the Hardy-Weinberg equilibrium (HWE) ($P < 0.05$) and eight pairs of loci, BC-ac001 and BC-tc005, BC-ag001 and BC-tc005, BC-tg001 and BC-tc004, BC-at001 and BC-tc004, BC-at006 and BC-ag001, BC-tc001 and BC-at006, BC-tc002 and BC-at006 and BC-ac001 and BC-tc001, showed significant Linkage disequilibrium (LD) (Fisher's exact test, $P < 0.01$). Sequences which harbored the nineteen genomic SSR markers were registered in GenBank (Accession Nos. EU596392-EU596410).

SSR markers developed in one species usually could be transferable to some other species of the same genus or closely related genera (Tang et al., 2006; Konishi et al., 2006). Therefore, to expand the use of these genomic SSR markers, the species transferability was tested in seven other *Bupleurum* species including wild and cultivated genotypes (Table 3). All loci were successfully amplified in *B. longiradiatum* var. *porphyranthum*, *B. yinchowense* and *B. smithii* var. *parvifolium*, eighteen in *B. falcatum*, seventeen in *B. scorzonerifolium* Willd., sixteen in *B. sibiricum* var. *jeholense* and eight in *B. wenchuanense*. These results showed most of the the genomic SSR markers had wide species transferability.

To obtain more useful SSR markers, primer pairs were designed and PCR amplifying verified based on our previous result of EST-SSR data mining in a *B. chinense*

Table 2. Characteristics of nineteen polymorphic genomic SSR markers, isolated from *B. chinense* DC. cv. Zhongchai No. 1, analyzed from 22 individual plants of *B. chinense* DC. f. *octoradiatum* (Bunge) Shan et Sheh

Locus name	Repeat motif	Primer sequence (5'-3')	T_A[a] (℃)	N_A[b]	Size range (bp)	H_O[c]	H_E[d]	GenBank Accession No.
BC-at001	$(AT)_3GTATGT(AT)_9$	F: TTCCTTCTAGTCTCCGTGCTG R: GAGGGTCTTCTCAAAGCCAAT	58	6	132-144	0.70	0.76	EU596392
BC-ttgaa001	$(TTGAA)_3+(GA)_3+(AG)_4+(AG)_3+(GA)_3$	F: GTTGCCCAGGTGAAGATAGC R: TGCAGCTCTAACTCTAGGTCTCA	60	4	313-336	0.23	0.29	EU596393
BC-at002	$(AT)_8$	F: GAAGAACAGCATCATTTGGGAGA R: GGGGTGTCTGAACATTAAAACAGT	59	8	196-224	0.47	0.59	EU596394
BC-ac001	$(AC)_5(GC)_2(AC)_3$	F: AGAAGAACAGGCACAAACTCAG R: TTTCCACTCCGATTGGTCGT	58	5	198-236	0.58	0.71	EU596395
BC-tg001	$(CT)_3+(TG)_3+(TA)_3+(TG)_6$	F: GTCTTCACGCTTGGTTCATC R: TTCTATGACCAGCAATACAACA	57	7	324-337	0.55	0.61	EU596396
BC-at003*	$(ACAT)_3C(AT)_9$	F: TTCTTTTACTTCCATCTCCCG R: GCATCTTTTCTACACTTGCTTTG	57	10	169-205	0.86	0.87	EU596397
BC-at004*	$(AT)_{10}+(AT)_8$	F: TTGAATGGGAGGCTCATGTAAC R: AAACCGTCGAATTTGATGATGT	56	5	242-262	0.43	0.73	EU596398
BC-at005	$(TA)_7+(GT)_5+(AT)_{10}$	F: TGTGCAGATGGGATTGATTGT R: CAAAACCACAGCCGACTCTTA	57	3	207-237	0.30	0.35	EU596399
BC-tc001*	$(TC)_{20}(AC)_{13}$	F: CATGATCTGAAGCCACATTCC R: GATGTGATCCACCATGTGAACTA	59	13	214-276	0.95	0.92	EU596400
BC-tc002	$(CT)_3+(TGTC)_3+(TA)_3$	F: TGGGCATTAGTCATATTCCTCTT R: CTGGATATGTATGCCACTTCGT	57	5	283-342	0.68	0.59	EU596401
BC-tga001*	$(TGA)_5$	F: AGACGCAGAGCAAGTTTGTGAAG R: CAGCAACCAATAAGCAACCCTAA	59	5	319-331	0.25	0.57	EU596402
BC-ac003	$(AC)_{10}$	F: TTCTGTCAGGGTCCTCTACTCAT R: GCAGATAAGTTAAAAGGAATGGAA	56	7	153-165	0.81	0.84	EU596403
BC-at006*	$(TA)_7$	F: CTGTGCGATTCTTGGTATGGA R: TCACATGCAACCACGATCACT	58	6	205-215	0.53	0.76	EU596404
BC-tc003*	$(CT)_{10}$	F: TCAAAGAACTAGGCTTAGGACATT R: TGATGATGAGTGAGATTGTAGGC	57	4	296-304	0.57	0.74	EU596405
BC-ag001*	$(AC)_6+(GA)_4T(AG)_4G(GA)_8T(AG)_{10}$	F: CATAATAGCCTCGGCCTGGAT R: GGTACGGTGAATGTCTTTGATGAT	59	9	196-205	0.48	0.85	EU596406
BC-tc004*	$(TC)_{11}(TA)_8$	F: CACTAATCCTCACTCTTCTGCTT R: AAGACTGGCTTTGTCCAACA	57	11	320-354	0.76	0.90	EU596407
BC-ag002*	$(AG)_3+(CTT)_3+(TGA)_3+(TC)_3$	F: GTGAGCATGTGAGGAGGGATG R: AAAGCCCAGAGGTAACTGTTGAT	59	5	336-361	1.00	0.70	EU596408
BC-at007	$(AT)_{11}$	F: TCTTGCTAGTGACCTCCATTG R: ATCGTCCGAAGATGTCAGTATA	56	7	347-373	0.55	0.74	EU596409
BC-tc005	$CTCA(CT)_8$	F: TCTTGTGGGATGTAATGATGGA R: TCGTAGCACTGAACCTGGACT	57	6	312-324	0.77	0.80	EU596410

*Loci with significant deviation from Hardy-Weinberg equilibrium (HWE) ($P < 0.05$), [a]T_A: Annealing temperature, [b]N_A: Number of alleles, [c]H_O: Observed heterozygosity, [d]H_E: Expected heterozygosity

root cDNA library (Sui et al., 2010, in press). Only 73 of 86 EST-SSR loci were suitable for primer pairs design. PCR products with expected sizes were amplified by 44 primer pairs (approximately 60.3%) (Table 4). The others primer pairs either had no clear PCR products or amplified bands without expected sizes. The uniESTs that harbored these EST-SSR markers were registered in GenBank (Accession Nos. FG341848, GR308099-EU308135).

In summary, we firstly provide genomic SSR and potential EST-SSR markers of *B. chinense* and also some species transferability genomic SSR markers of other

Table 3. Cross-amplification of nineteen SSR markers among seven *Bupleurum* species.

Species	Loci[a]																		
	at001	ttgaa001	at002	ac001	tg001	at003	at004	at005	tc001	tc002	tga001	ac003	at006	tc003	ag001	tc004	ag002	at007	tc005
Wild																			
B. longiradiatum var. *porphyranthum*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. yinchowense	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. sibiricum var. *jeholense*	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-
B. wenchuanense	+	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	-	+	-
B. smithii var. *parvifolium*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cultivated																			
B. scorzonerifolium Willd.	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-
B. falcatum	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+

[a] "BC-" in the names of loci was dispensed with; "+": Positive amplification yielding specific PCR products of expected size; "-": Negative amplification yielding PCR products of unexpected size or without PCR products.

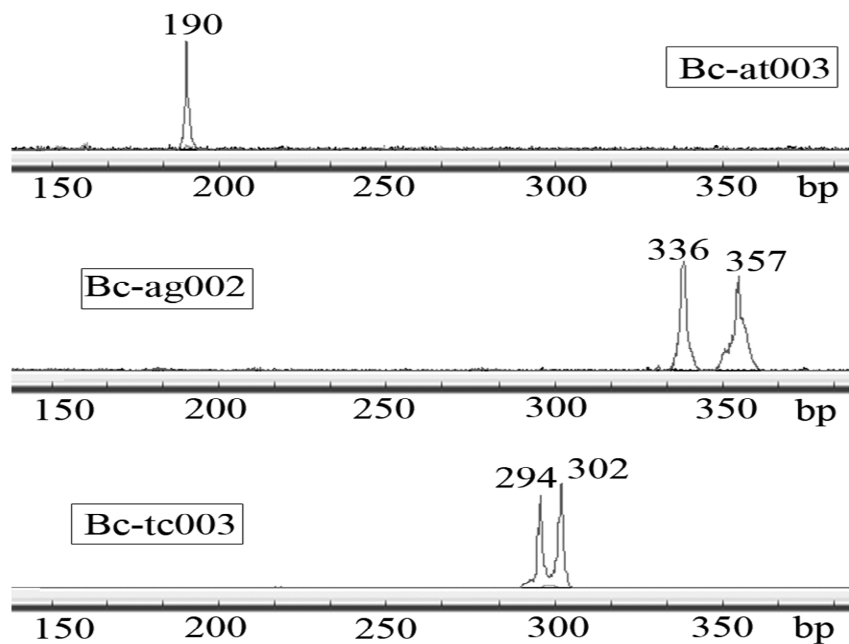

Figure 1. Capillary electropherograms of PCR products of genomic SSR markers, Bc-003, Bc-ag002 and Bc-tc003. Numbers indicate the size in base pairs (bp).

Table 4. Repeat mortifs, primer sequences, expected PCR amplified fragment sizes and PCR annealing temperatures of 44 potential EST-SSR markers

Locus name	Repeat motif	Primer sequence (5'-3')	Expected size (bp)	T_A^a (℃)	GenBank Accession No.
CH001C12	$(TA)_6TT(TA)_5$	F: TTCTCCGTTCTGGGTTTATGA R: GAGTTTCCGCTCAAAGATGC	258	56	FG341848
CH002F09	$(TCC)_5$	F: GCCTTCAGACCACCTAAAAATG R: TGGGGAATAGAACAGAGATTGG	334	58	GR308099
CH004A09	$(AT)_{10}$	F: CGTCACTTAGCCTTGTATGGAAC R: TCGTTATCATTTTTCATGGCTTT	236	54	GR308100
CH005E04	$(AT)_{10}$	F: CTGAGACACCCCGTTCTGTT R: GCAATTCCCACCTTGTTGAT	395	56	GR308101
CH005G04	$(AAT)_6$	F: TGGAAACTCGACAGTCCCTTAT R: GGAACAAGCAAACAATGTACCA	232	56	GR308102
CH007D09	$(CA)_6CT(CA)_6$	F: AGCAGCAGCAAGGTCAAATAA R: CCCTGAAAACCTCTTCGACA	178	56	GR308103
CH008C06	$(ACA)_5$	F: GTTATCCCCGGATTCATGTG R: AACCATCAACCATCATCATCA	377	55	GR308104
CH009C07	$(ACT)_5$	F: GGGAGAAAGGTGAGGAAGAAG R: TAGGAAGGGATATGTGACTGTTG	362	59	GR308105
CH011H09	$(AT)_9$	F: AAGAAAGTATTGACGCAGAGGC R: CAAATCAGTGCCAAGATAAGGTA	198	57	GR308106
CH012E11	$(TA)_{10}$	F: GCGAAGACTGAGTGGCTTGATG R: AGAAACCATAACCATGCCTGCTA	249	59	GR308107
CH015C07	$(TAT)_6$	F: CAATGGCTGTTTCATCTGTTCTG R: GCAACTCCCAATCCCCAAATGA	343	59	GR308108
CH016E08-1	$(TC)_6+(CA)_8$	F: GAGAAGAAAGCGGCTGGTGGT R: AAGGCGATGAGATGACAAGGGT	155	60	GR308109
CH016E08-2	$(GAA)_2GAC(GAA)_5$	F: AAAACACCTCTGCCACCTCCA R: CACCATCGGAAGGGAAACCA	140	60	GR308109
CH016F08	$(GGT)_3+(TGG)_5$	F: TGGTGGACGAAAGATGGGTG R: TGTGGTGAATGTCCAGAGCC	306	60	GR308110
CH018E10	$(GCA)_5$	F: AAAATATCCCTGCTCCTTCTG R: ATTCTCGGTGGCTCGCTTAG	392	56	GR308111
CH018H12	$(AT)_{11}$	F: CAGCACCTAAAGTCTCAACG R: AGTTTCTCATCTCGGCTTGTT	472	56	GR308112
CH020D05	$(AT)_9$	F: CCCCAGAACAAGGGAAAGCAGC R: CAGCACCAACAATGTCATATCTCC	177	60	GR308113
CH022H03	$(GGA)_6$	F: GCTTCATCGCTTACCTCTATCC R: CTCCATTATTGTTGCCGTTTCC	275	58	GR308114
CH023D11	$(AC)_9$	F: CTCTTTCTCAAAACCCACCATC R: CATCTCCACCTTGTAATCACTC	142	58	GR308115
CH023E08	$(TA)_{10}$	F: CAGGACGATAAGGCTGATGTT R: CACATAAAATCTCCACCAAAATA	343	54	GR308116
CH025A01	$(AAG)_6$	F: TTCTACATTTTGGGCGTTTTAC R: ACCTGCTTGAGATGATTTTGA	155	54	GR308117
CH025G05-1	$(AAG)_5$	F: CTCCATTCCTCCTTTGTTAGTC R: TGAACCGAATCTATTGGGTGAAA	166	57	GR308118
CH025G05-2	$(ATC)_5$	F: CAATAGATTCGGTTCAAGTTCAG R: ATCAAAGCAAAGGTGGCAAAT	318	54	GR308118
CH026D06	$(AAG)_5+(AAG)_6$	F: TTGGGCATGACAATCACAGAA R: GAAAGTTATTAGGGTTTGAAGGGT	220	56	GR308119

Table 4. contd.

CH027D09	$(TGT)_5$	F: CATAAAACAAAAGGCAAATCG R: GTCGGTAATGAATCCAAATGAG	337	53	GR308120
CH028D08	$(GT)_5$+$(AC)_5$	F: GAGACAGGGAATAAGAAAGTG R: TGAAGAAAGAGGCGAGAAC	161	55	GR308121
CH028G10	$(AC)_{10}$	F: TTGTTCAAGGATTTCGAGGCT R: ATCGGTGTTCAGCAGTAGCG	200	56	GR308122
CH028H08-1	$(TA)_9$	F: GAAGATAAGCAAGTTGAAGA R: TACCTGTTGAAACCGAATA	205	51	GR308123
CH028H08-2	$(TG)_6(TA)_8$	F: GTATTCGGTTTCAACAGGTA R: AGGAAGTCGGAAATAGTCAT	349	54	GR308123
CH032D05	$(TA)_{10}$	F: CTGGAAATGGAGTTAGAAGACA R: AACGAACTCAGACCCCTAAT	231	56	GR308124
CH032F06	$(CA)_{11}(TA)_7$	F: TCTAACTTAACACCACCATTTCA R: CCAACAATAGCAGTTACCCAT	272	55	GR308125
CH035A10	$(AAC)_5$	F: GTAATGATGATGCCTCCACAG R: CGGCGACAATACAAGACCAA	270	58	GR308126
CH035F03	$(TA)_9$	F: CTTAGATTCTTGCCCTTGC R: GTCAAATGTAGCCTTCAATCA	222	54	GR308127
CH036B04	$(GAA)_5$	F: AGAGGAGGATATGGGAACTGA R: TTGATGGAGCCTCTACCTTTC	167	58	GR308128
CH036C06	$(CTA)_7$	F: GCCATAGAAGTTGGTTCACG R: GCTGGTCCGAGTCATCATAG	190	58	GR308129
CH037E10-1	$(AAGTAG)_5$	F: GCAAGTAATGGGTAGTTGTATG R: AATGTTTCCAGGCTCAAGTT	298	54	GR308130
CH037E10-2	$(GCA)_8A(ATG)_5$	F: AGAAAGAGTTACGGTGGGACA R: AGTGGGCATAGGGATTTGTT	386	56	GR308130
CH037E10-3	$(GCA)_6$	F: ACAAATCCCTATGCCCACT R: TGTTAGTACCACCTGTTTCTTT	147	55	GR308130
CH038C11-1	$(TGA)_6$	F: ACCACAGTGATAACGAGGACA R: ATCTTTGGCTCAGAAACTAGAAC	142	57	GR308131
CH038C11-2	$(AAG)_5$	F: CCTCGTGACTTTAGGAGATGC R: GAGATTCTGCGACCCTGTTC	173	60	GR308131
CH038D02	$(GGT)_5$	F: ACCAAACCACCTATGTCACTAC R: CTCAAGGAGGCTGGAAACTG	131	58	GR308132
CH039A04	$(AT)_6$+$(AT)_6$	F: GACTCACCACGCCTAATAAACA R: TCTTCACCAGCAAGCCATTC	141	58	GR308133
CH040C04	$(GGA)_8$	F: ATGATGAACTGGGAAGAGGGT R: GCTTTGAGGACCTGGTTGTTA	197	58	GR308134
CH040D07	$(TG)_{11}$	F: GTTCAGCAAAGGAGAAGACGA R: TTTGTCCTGGTGCGTGGTG	301	58	GR308135

[a]T_A: Annealing temperature

Bupleurum species. These not only can be applied as a useful molecular tool for analysis of genetic variations, population structure in *Bupleurum* species and phylogenetic relationship of the genus *Bupleurum*, but may contribute to the promotion of the genetic linkage map construction, the development of DNA markers linked to the agronomic and medicinal characters in cultivated *Bupleurum*.

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