Development of cleaved amplified polymorphic sequence (CAPS) and high-resolution melting (HRM) markers from the chloroplast genome of *Glycyrrhiza* species

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Abstract

Licorice (*Glycyrrhiza glabra*) is an important medicinal crop often used as health foods or medicine worldwide. The molecular genetics of licorice is under scarce owing to lack of molecular markers. Here, we have developed cleaved amplified polymorphic sequence (CAPS) and high-resolution melting (HRM) markers based on single nucleotide polymorphisms (SNP) by comparing the chloroplast genomes of two *Glycyrrhiza* species (*G. glabra* and *G. lepidota*). The CAPS and HRM markers were tested for diversity analysis with 24 *Glycyrrhiza* accessions. The restriction profiles generated with CAPS markers classified the accessions (2–4 genotypes) and melting curves (2–3) were obtained from the HRM markers. The number of alleles and major allele frequency were 2–6 and 0.31–0.92, respectively. The genetic distance and polymorphism information content values were 0.16–0.76 and 0.15–0.72, respectively. The phylogenetic relationships among the 24 accessions were estimated using a dendrogram, which classified them into four clades. Except clade III, the remaining three clades included the same species, confirming interspecies genetic correlation. These 18 CAPS and HRM markers might be helpful for genetic diversity assessment and rapid identification of licorice species.

Keywords Licorice · Genetic diversity · SNP · CAPS · HRM

Abbreviations

SNP	Single nucleotide polymorphism
CAPS	Cleaved amplified polymorphic sequence
HRM	High-resolution melting
INDEL	Insertion and deletion
GD	Genetic distance
PIC	Polymorphism information content
N_A	Number of alleles

M_{AF}	Major allele frequency
SSR	Simple sequence repeats

Introduction

Licorice is a perennial plant belonging to the subfamily Faboideae of the family Leguminosae; it has long been used in oriental medicine (Fenwick et al. 1990). About 20 known plant species belonging to the genus *Glycyrrhiza* exist

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globally and they are mostly grown or cultivated in Asia, Europe, Australia, and the US (Kim et al. 2011). Among the *Glycyrrhiza* species, *G. glabra* (European licorice), *G. echinata* (Russian licorice), and *G. uralensis* (Chinese licorice) were cultivated mostly in Europe, Russia, and Far East regions as medicinal herb, respectively (Carmen et al. 2012). Glycyrrhizin, a triterpenoid saponin, is the most important active ingredient in licorice, 3–5% of which is found in the roots; it has various bioactive functions such as anti-allergic, antioxidant, anti-ulcer, antiviral, and anti-cancer activities (Montoro et al. 2011). Recently, the demand for licorice as an ingredient in functional products such as foods and cosmetics has increased, and hence has the size of its market (Kim et al. 2013).

Contents of glycyrrhizin, a marker component of licorice, and other bioactive substances showed differences in species (Kondo et al. 2007), and glycyrrhizin above a certain level (225 mg/day) caused electrolyte disturbance by increasing the cortisol level in the body (Kageyama et al. 1992). Moreover, excessive intake might cause temporary visual impairment (Fraunfelder 2005). Hence, it is necessary to determine the glycyrrhizin contents accurately in different species (Liao et al. 2012). However, most commercial licorice are sliced or powdered, which could lead to its misuse or mixed use. Therefore, there is an urgent need for an easy and accurate identification of licorice species. At present, studies on the identification of licorice species have relied on comparisons of morphological characteristics of roots (Lee and Jo 2004) and content variations in glycyrrhizin (Yang et al. 2010; Choi et al. 2011). However, such methods have limitations in accurate identification since variations might appear depending on the growth environment.

DNA barcodes are standard methods recommended by the convention on biodiversity for species identification (Hebert et al. 2003), and because they offer the advantage of using DNA sequences to assess and identify interspecies variations, they have been actively used in recent studies (Liu et al. 2011). The barcode used for plants is a core DNA barcode, which includes the internal transcribed spacer present in *matK*, *rbcL*, *trnH-psbA*, and 45S rDNA found mostly in plant chloroplast (Yang et al. 2012). Interspecies variations can be easily and rapidly identified using chloroplast genome than by using existing universal DNA barcode, and hence, the chloroplast genome has been used as the basis for developing markers for species identification and classification (Chen et al. 2010). Chloroplast-based single nucleotide polymorphism (SNP) markers have already been developed for the major medicinal crops, including ginseng, and they have been successfully used for species identification, phylogenetic analysis, and genetic diversity assessment (Lee et al. 2012; Kim et al. 2015; Zhao et al. 2015; Jo et al. 2016). Moreover, chloroplast-derived CAPS markers have also been reported to detect the intra- and interspecies variation

of different species (Kim et al. 2018). Similarly, the HRM analysis is suitable for genotype discrimination and has been applied to identify plant cultivars such as grapevine and olive (Mackay et al. 2008). Thus, this study aimed to investigate the differences in chloroplast sequences in licorice species and use the findings to develop CAPS and HRM markers. The findings of our study might provide a method for understanding the genetic relationships and structures among licorice species, as well as measures for preserving and managing genetic resources and avoid misuse and mixed use of medicinal herbs.

Materials and methods

Plant material and DNA extraction

The materials used in the present study which includes eight accessions of *G. uralensis* and *G. glabra* were obtained from the Ginseng Research Division at the National Institute of Horticultural and Herbal Science (NIHHS), South Korea and 16 accessions were obtained from the United States Department of Agriculture (USDA), including *G. uralensis*, *G. glabra*, *G. lepidota*, *G. echinata*, and *Glycyrrhiza* spp. (Table 1). Seeds were germinated and grown in a greenhouse. The genomic DNA extraction was performed by collecting leaves from 1-year-old plants; washed and grounded with liquid nitrogen into powder using a pestle-mortar. Subsequently, DNeasy Plant Mini Kit (QIAGEN, Germany) was used for DNA extraction, as per manufacturer's protocol.

CAPS and HRM marker conversion

Primers were developed by arranging chloroplast sequence data of *G. glabra* and *G. lepidota* by using ClustalW software to confirm SNPs. The chloroplast sequence data of *G. glabra* (accession no. NC_024038) were downloaded from NCBI database, whereas those of *G. lepidota* (accession no. NC_034229) were obtained from our previous study results (Raveendar et al. 2017). The dCAP FINDER 2.0 program was used to design the CAPS primer, where SNPs were designed to be included within the restriction enzyme recognition site (Table 2).

The ten sets of CAPS primers that had been prepared were used for performing PCR on the 24 licorice accessions. The mixture used in PCR contained 10 ng of genomic DNA, 20 pmol of primer, 2.5 mM of MgCl₂, 0.25 mM of dNTPs, and 0.5 U of Taq polymerase (Inclone, Deajeon, Korea), and the total volume of the reaction solution was 25 µL. The CFX96 PCR detection system (Bio-Rad Laboratories, Hercules, CA) was used for DNA amplification; the PCR included a total of 35 cycles with each cycle involving 5 min of pre-denaturation at 95 °C; 30 s of denaturation at 95 °C;

Table 1 List of studied *Glycyrrhiza* accessions

Accession name	Species	Germplasm source	Geographic origin	Type of accession
CBG 1	<i>Glycyrrhiza uralensis</i>	NIHHS, RDA	CHN	Cultivated
CBG 2	<i>Glycyrrhiza uralensis</i>	NIHHS, RDA	CAN	Cultivated
CBG 3	<i>Glycyrrhiza uralensis</i>	NIHHS, RDA	CHN	Cultivated
CBG 4	<i>Glycyrrhiza uralensis</i>	NIHHS, RDA	RUS	Cultivated
CBG 5	<i>Glycyrrhiza uralensis</i>	NIHHS, RDA	CHN	Cultivated
CBG 7	<i>Glycyrrhiza glabra</i>	NIHHS, RDA	CAN	Cultivated
CBG 9	<i>Glycyrrhiza glabra</i>	NIHHS, RDA	UZB	Wild material
CBG 10	<i>Glycyrrhiza uralensis</i>	NIHHS, RDA	CAN	Cultivated
CBG 16	<i>Glycyrrhiza lepidota</i>	USDA, ARS	USA	Wild material
CBG 17	<i>Glycyrrhiza lepidota</i>	USDA, ARS	USA	Wild material
CBG 18	<i>Glycyrrhiza lepidota</i>	USDA, ARS	USA	Wild material
CBG 19	<i>Glycyrrhiza lepidota</i>	USDA, ARS	USA	Wild material
CBG 20	<i>Glycyrrhiza lepidota</i>	USDA, ARS	USA	Wild material
CBG 21	<i>Glycyrrhiza echinata</i>	USDA, ARS	YUG	Wild material
CBG 22	<i>Glycyrrhiza</i> spp.	USDA, ARS	RUS	Wild material
CBG 23	<i>Glycyrrhiza</i> spp.	USDA, ARS	KAZ	Wild material
CBG 24	<i>Glycyrrhiza echinata</i>	USDA, ARS	DEU	Wild material
CBG 25	<i>Glycyrrhiza uralensis</i>	USDA, ARS	RUS	Wild material
CBG 26	<i>Glycyrrhiza uralensis</i>	USDA, ARS	KAZ	Wild material
CBG 27	<i>Glycyrrhiza uralensis</i>	USDA, ARS	MNG	Wild material
CBG 28	<i>Glycyrrhiza uralensis</i>	USDA, ARS	KAZ	Wild material
CBG 30	<i>Glycyrrhiza glabra</i>	USDA, ARS	KAZ	Wild material
CBG 31	<i>Glycyrrhiza lepidota</i>	USDA, ARS	USA	Wild material
CBG 32	<i>Glycyrrhiza lepidota</i>	USDA, ARS	USA	Wild material

CAN Canada, CHN China, DEU Germany, KAZ Kazakhstan, MNG Mongolia, RUS Russia, USA United States of America, UZB Uzbekistan, YUG Yugoslavia

30 s of annealing at 55–60 °C; and 1 min of extension at 72 °C. After PCR amplification, PCR products were cleaved using restriction enzymes *HinfI* (G^ANTC), *MseI* (T^ATAA), *ApoI* (R^AAATY), *HpyCH4IV* (A^ACGT), and *BfaI* (C^ATAG; NEB; Ipswich, England). The PCR mixture included 0.05 U of restriction enzyme, 1× reaction buffer, and 5 µL of PCR products, and PCR was conducted for 3 h at 37 or 50 °C. The reaction products were used to check for diversity by assessing cleavage in 3% agarose gel. The size of the restriction fragment for each marker was scored by Image Lab Touch Software 2.2 software (Bio-Rad Laboratories, Hercules, CA).

For the eight loci that did not have restriction enzyme recognition site where SNP was located, HRM analysis was applied (Table 2). For the HRM analysis, 1 µL of template DNA, 5 µL of 2× SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA), and 2 µL of 10 µmol primer were mixed to a final volume to 10 µL; next, CFX96 Real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) was used to perform the analysis. PCR was performed for 40 cycles with each cycle involving 2 min of pre-denaturation at 95 °C, 20 s of annealing at 56–60 °C, and

20 s of extension at 72 °C. The melting curve of fragments amplified after PCR was analyzed by raising the temperature by 0.2 °C, from 65 to 95 °C, while measuring the intensity of fluorescence emitted during denaturation. Generated data were analyzed using Precision Melt Analysis software (Bio-Rad Laboratories, Hercules, CA). HRM analysis was performed to detect polymorphisms between the sequences in each sample based on specific SNPs.

Cloning and sequencing

We performed cloning to compare the base sequences of samples with confirmed variations in the HRM analysis. After 10 µL of PCR products was verified using 1% agarose gel, pGEM-T easy vector (Promega, Madison, MI, USA) was inserted, as per manufacturer's protocol, and transformed into *Escherichia coli* DH5α. Colonies that had formed were randomly selected for amplification of the cloned fragments; DNA clean kit (Bioneer, Deajeon, Korea) was used for purification, and the base sequences were analyzed using ABI3730 automatic sequencer.

Table 2 Primers used for the experimental verification of chloroplast DNA markers

Primer name	SNP loci	Base substitution		Marker type/restriction enzyme	Primer sequence (5' → 3')	
		<i>G. l</i>	<i>G. g</i>		Forward primer	Reverse primer
GCP 1	matK ~ trnK-UUU	G	C	CAPS/ <i>Hinf</i> I (G [^] ANTC)	CCCATCAATGGACTATCTCTCC	ATGGGGTAGCGAGAAATGTG
GCP 2	trnK-UUU ~ rbcL	T	C	CAPS/ <i>Mse</i> I (T [^] TAA)	AAGACCTGGTGTGCGTATATCT	AAACTCGGCCCAATCTTTTA
GCP 3	trnV-UAC ~ ndhC	G	T	CAPS/ <i>Mse</i> I (T [^] TAA)	AGTAGTAGTAGAAAGAATAAATAAAATTC	TGAATGTGGAAAAGATATCGAA
GCP 4	ndhJ ~ trnF-GAA	T	C	HRM assay	CCCCAGACATTCCTTGAATA	GGCTTGACTCATCCGAAATA
GCP 6	ycf3 ~ ycf3	T	G	HRM assay	CGAAACCCGTTCTTACCTCA	AAAAATCGTTGTTTTGGAAGA
GCP 8	petN ~ trnC-GCA	C	T	HRM assay	CCGAGACGAAAAGATTACTGC	GCAAATCCTTTTCCCGAATGT
GCP 9	trnC-GCA ~ rpoB	A	C	CAPS/ <i>Apo</i> I (R [^] AATY)	CCGGAGTTAGATCTATGTCATGT	GCACTTAACCTTTTCAATTGTTT
GCP 12	atpA	C	T	CAPS/ <i>Hpy</i> CH4IV (A [^] CGT)	CCTATTGACGGTCGAGGAGA	CCTGTTTGTCTGTCCCGAAT
GCP 13	atpA ~ trnR-UCU	A	T	CAPS/ <i>Mse</i> I (T [^] TAA)	TCTTTTAATATCTTTTCTTTGAATTTGA	CAATTTGTGTTTTGTCCCTCT
GCP 14	trnQ-UUG ~ accD	A	T	CAPS/ <i>Mse</i> I (T [^] TAA)	GATCTAGATTCAAAATAGACTCG	AACTGGACAATTCATTTCTAAAG
GCP 15	accD ~ psaI	A	G	CAPS/ <i>Bfa</i> I (C [^] TAG)	TGTTATCCATATATAGTCATTTAGC	AAAGGAACAAAAATGGAAGGT
GCP 16	cemA ~ petA	T	A	CAPS/ <i>Apo</i> I (R [^] AATY)	AAAAACGATCCACTGATCCAA	GCATCGTCCCTATCCATGA
GCP 17	petB ~ petD	G	A	HRM assay	GCCGATTTCATGTTAATGCAC	GCAATGAAATACTATTGCTCCTC
GCP 18	rpl36 ~ rps8	G	C	HRM assay	TCTCTCTCTTTTCACAAATATGG	GGGATGGGGATTGTAATTCT
GCP 23	rps12 ~ trnV-GAC	G	T	HRM assay	GTCAATTTTGCAGGGTCTCG	CCCGACTCGGTATTCTTCAA
GCP 24	trnI-GAU ~ trnI-GAU	C	T	HRM assay	ATAGATGGGGCGATTCAAGT	CCGAACCTAAACCTGTGCTC
GCP 25	trnR-ACG ~ trnN-GUU	G	C	HRM assay	CCCCGGACTGTAGAACAAAG	AACGGTTTGCTTTGACCAT
GCP 26	ndhA ~ ndhA	A	G	CAPS/ <i>Apo</i> I (R [^] AATY)	CCACAATTCGGATCCAGAGT	TTTGTGCTATGAAAAAGGGACT

G.l *Glycyrrhiza lepidota*, *G.g* *Glycyrrhiza glabra*

Data analysis

The analyses on the number of alleles (N_A), major allele frequency (M_{AF}), GD, and PIC were performed using PowerMarker (ver. 3.25). Phylogenetic analysis was performed using C.S. Chord 1967 distance (Cavalli-Sforza and Edwards 1967), included in PowerMarker, where phylogenetic trees were constructed using UPGMA method after analyzing the genetic distance of each licorice accession.

Results and discussion

SNP locus identification and application of CAPS marker

A total of 18 SNP loci were identified by comparing the chloroplast sequence data of *G. glabra* (accession no. NC_024038) and *G. lepidota* (accession no. NC_034229). The most common forms of base substitution were C → T and G → C, which were found in three loci each, followed

by A → T, A → G, G → T, and T → C in two loci each and A → C, G → A, T → G, and T → A in one locus each. Among these, ten chloroplast loci were converted to CAPS markers for detecting potential diversity in 24 *Glycyrrhiza* accessions (Table 2). Total of ten amplified chloroplast loci were analyzed with five restriction enzymes, *Hinf*I, *Mse*I, *Apo*I, *HpyCH4*IV, and *Bfa*I (NEB; Ipswich, England), in which SNP variations were observed with specific restriction fragment patterns (Fig. 1). The results confirmed that GCP 16 marker had the most restriction profiles (4 genotypes; Fig. 1i), followed by GCP 3, GCP 13, and GCP 14 with three genotypes (Fig. 1c, f, g) and GCP 1, GCP 2, GCP 9, GCP 12, GCP 15, and GCP 26 with two genotypes (Fig. 1a, b, d, e, h, j). All *G. lepidota* accessions showed species-specific restriction fragment patterns with GCP 3, GCP 13, GCP 14, and GCP 16 markers, which allowed their distinction from other accessions.

Among the tested markers, GCP 14 showed specific restriction profiles that allowed the identification of *G. lepidota* and *G. echinata* from other accessions (Fig. 1g). Within *Glycyrrhiza* species, various hybrids from natural hybridization have been reported (Ashurmetov 2009); further, interspecific hybrids have also been reported by artificial hybridization (*G. glabra* × *G. uralensis*). Thus, development of DNA markers that can be used to identify the species is

necessary. CAPS markers have been routinely used in plant breeding programs for fine mapping of important traits in crop plants (Arora et al. 2017; Babu et al. 2017). Recently, Kim et al. (2018) validated the chloroplast-derived CAPS markers to detect the intra- and interspecies variation of *Pinus* species. Similarly, the chloroplast-derived CAPS markers developed in the present study could be used for species identification of various licorice resources.

High-resolution melt assay and sequencing

HRM primers were produced for the remaining eight chloroplast loci that could not be converted to CAPS markers and used for diversity analysis of the 24 *Glycyrrhiza* accessions. In the HRM analysis, all eight loci showed a unique melting curve, and each accession was stratified into two (Group A and Group B) or three (Group A, Group B, and Group C) groups (Fig. 2). Among them, *G. lepidota*-specific melting curves were observed with GCP4, GCP 18, and GCP 24 markers, allowing its easy distinction from other species (Fig. 2a, e, g). When the segment amplified by GCP 4 (*ndhJ* ~ *trnF*-GAA), most base substitutions (4 SNPs, C → A, T → A, C → A, and C → T) were observed, and *G. lepidota* accession (ATCT) and *G. echinata* accession (CAAT) were distinguished from the other accessions

Fig. 1 Restriction profiles obtained after PCR products were digested with *Hinf* I (a GCP 1), *Mse* I (b GCP 2, c GCP 3, f GCP 13, g GCP 14), *Apo* I (d GCP 9, i GCP 16, j GCP 26), *HpyCH4*IV (e GCP 12)

and *Bfa* I (h GCP15), respectively. For 24 accession (lane 1–32) designations, refer to Table 1, M, 100 bp DNA ladder

Fig. 2 Sequence alignment and high-resolution melting (HRM) curve profiles of amplicons obtained using eight HRM markers (**a** GCP4, **b** GCP6, **c** GCP8, **d** GCP17, **e** GCP18, **f** GCP23, **g** GCP24, **h** GCP25). **a, b, e, h** Normalized plot showing three genotypes (group A, group B and group C) in 24 *Glycyrrhiza* accessions; **c, d, f, g** Normalized melting curves of the GCP7, GCP15, GCP19, and GCP20 polymorphisms. A typical plot showing the melting curves of the two polymorphisms; the two genotypes (group A and group B) for each SNP and INDEL are highlighted by color bar. The area enclosed by the boxes indicates SNP and INDEL sequence variations

(CTCC) by SNP genotypes (Fig. 2a). GCP 18 (*rpl36~rps8*) could distinguish the *G. lepidota* accession (T/insertion) and *G. echinata* accession (CBG 24, C/deletion), collected from Deutschland, from other accessions (C/insertion) by one base substitution (C → T) and 10 bp INDEL (Fig. 2e). Lastly, two base substitutions (G → A and C → T) were confirmed in GCP 24 (*trnI-GAU~trnI-GAU*), and thus, the *G. lepidota* accession (AT) was distinguished from the remaining accessions (GC; Fig. 2g). In addition, specific melting curves that could identify *G. echinata* accession (CBG 21, YUG) and *G. uralensis* accession (CBG 28, KAZ) were found in GCP 6 (*ycf3~ycf3*) and GCP 25 (*trnR-ACG~trnN-GUU*) (Fig. 2b, h); in contrast, when GCP 8, GCP 17, and GCP 23 were used, each accession was stratified into two groups (Group A and Group B; Fig. 2c, d, f).

HRM analysis involves the process of checking for DNA amplification through electrophoresis and SNP exploration that does not require DNA sequencing; in recent times, it has been widely used as a molecular marker for distinguishing plant species or varieties (Ganopoulos et al. 2013). Moreover, the HMR markers were showed more effective in identifying plant species when considering the study of Jeong et al. (2010) rather than using other markers; thus the markers used in the present study might be used effectively for the identification of *Glycyrrhiza* species.

Genetic diversity of CAPS and HRM markers

Analysis on the diversity of CAPS and HRM markers for the 24 *Glycyrrhiza* accessions revealed a total of 60 alleles in the 18 markers. The number of alleles ranged from 2 (GCP8, GCP17, GCP23, and GCP24) to 6 (GCP14), with the mean number of alleles being 3.3. The range of M_{AF} that indicates the distribution of alleles was from 0.31 (GCP9, GCP14) to 0.92 (GCP1), with mean M_{AF} of 0.587. The genetic distance (GD) and polymorphism information content (PIC) values, which represent genetic diversity, showed the lowest value of 0.16 and 0.15 in GCP1 and GCP26, respectively; highest value of 0.76 and 0.72 in GCP 14; and mean value of 0.504 and 0.443, respectively (Table 3).

These genetic parameters were higher than those reported by Erayman et al. (2014), who analyzed European *Glycyrrhiza* species by using SSR markers (mean GD 0.375 and

mean PIC 0.305); however, the values were lower than those reported by Um et al. (2016), who analyzed *G. glabra* and *G. uralensis* in Asia, including China, Mongolia, and Korea, by using SSR markers (mean GD 0.60 and mean PIC 0.56). Therefore, the genetic diversity of *Glycyrrhiza* species in Asia, including China, is thought to be considerably more diverse than that in other regions. SSR markers are widely used in genetic diversity and evolution studies; however, considerable time and effort are needed in developing the primers, and it cannot be used in some cases involving genetically distant species owing to low transferability (Zhang et al. 2014). Moreover, the chloroplast genome-based CAPS and HMR markers can be employed to identify different varieties and hybrids without the requirement of the post-PCR procedures. In the present study, CAPS and HMR markers analysis showed genetic diversity at a level similar to those reported in previous studies. Therefore, the findings of this study might provide useful information for understanding the genetic variability and phylogenetic relationships of *Glycyrrhiza* species.

Phylogenetic relationship

By using SNP-based CAPS and HRM markers, we further analyzed the genetic diversity between the 24 *Glycyrrhiza* accessions. The phylogenetic analysis based on UPGMA cluster analysis revealed that the genotypes of *Glycyrrhiza* species were clearly distinguished, which allowed their grouping into four major clusters (Fig. 3). In Clade I, ten *G. lepidota* accessions collected from the US were grouped together, which allowed their distinction from the other three species (*G. glabra*, *G. uralensis*, and *G. echinata*). *G. lepidota* is the only known species among *Glycyrrhiza* species that can grow in the wild in North America (Hayashi et al. 2000). Therefore, the independent grouping of Clade I comprising ten *G. lepidota* accessions might be attributed to geographical isolation. Clades II and IV included *G. glabra* accessions (CBG7 CAN and CBG9 UZB) and *G. echinata* accessions (CBG21 YUG, CBG24 DEU), respectively, and were distinguished from the other species. However, Clade III included ten *G. uralensis* accessions collected from Kazakhstan, Mongolia, China, Russia, and Canada; two *Glycyrrhiza* spp. accessions collected from Kazakhstan and Russia; and one *G. glabra* accession (CBG30 KAZ). Two *Glycyrrhiza* spp. within the same group were suspected of being *G. uralensis* considering their natural habitats and genotypes. However, *G. glabra* (CBG30) collected from Kazakhstan was found to have a closer phylogenetic relationship with *G. uralensis* of Clade III than with *G. glabra* (CBG7 CAN and CBG9 UZB) of Clade II. This finding was similar to those of other studies that analyzed phylogenetic relationships of licorice based on chloroplast sequence variations (Hayashi et al. 2000; Lim et al. 2012), while also being

Table 3 Characteristics of the 18 set of CAP and HRM markers in *Glycyrrhiza* spp.

Primer name	M_{AF}	N_A	H_O	GD	PIC
GCP 1	0.92	3	0.08	0.16	0.15
GCP 2	0.50	3	1.00	0.62	0.55
GCP 3	0.35	5	1.00	0.72	0.67
GCP 4	0.63	3	0.00	0.52	0.44
GCP 6	0.88	3	0.04	0.22	0.21
GCP 8	0.63	2	0.00	0.47	0.36
GCP 9	0.31	4	1.00	0.73	0.69
GCP 12	0.50	3	1.00	0.62	0.55
GCP 13	0.35	5	1.00	0.73	0.69
GCP 14	0.31	6	1.00	0.76	0.72
GCP 15	0.44	3	0.88	0.60	0.52
GCP 16	0.33	5	0.71	0.70	0.65
GCP 17	0.63	2	0.00	0.47	0.36
GCP 18	0.69	3	0.04	0.44	0.36
GCP 23	0.63	2	0.00	0.47	0.36
GCP 24	0.71	2	0.00	0.41	0.33
GCP 25	0.85	3	0.04	0.25	0.23
GCP 26	0.92	3	0.08	0.16	0.15
Mean	0.587	3.3	0.438	0.504	0.443

M_{AF} major allele frequency, N_A number of alleles, H_O observed heterozygosity, GD genetic diversity, PIC polymorphic information content

consistent with the random amplified polymorphic DNA and restriction fragment length polymorphism analysis results reported by Yamazaki et al. (1994). Therefore, additional studies are needed to compare barcode sequences, such as 45S rDNA, for the accurate identification of *Glycyrrhiza* species.

Further, although the *G. glabra* accession (CBG7 CAN and CBG9 UZB) in Clade II and *G. glabra* (CBG30 KAZ) in Clade III were the same species, they were classified into different clades. Such results suggest the possibility of genetic variation of licorice species under different ecological growth environments (Linhart and Grant 1996). Although these results were derived from a limited sample size, we believe they can be useful in studies on genetic variations based on geographical locations of *G. glabra*. With recent increase in the use of licorice, accurate species identification has become essential for their safe use. However, because licorice is processed in sliced or powder forms, it is hard to identify it at the species level. Moreover, considering that glycyrrhizin above a certain level can cause adverse effects in the body and its content shows interspecies differences (Kondo et al. 2007; Kageyama et al. 1992), the results derived in the present study might allow the easy and accurate identification of *Glycyrrhiza* species, indicating that it might be used for preventing misuse and mixed use of licorice and ensuring product quality.

Fig. 3 Phylogenetic tree for the 24 *Glycyrrhiza* accessions generated based on 10 CAPS and 8 HRM markers. At a C.S. chord genetic distance of 0.18, 24 *Glycyrrhiza* accessions were clustered in four groups

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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