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Assessment of genetic diversity by simple sequence repeat markers among forty elite varieties in the germplasm for malting barley breeding*

Jun-mei WANG, Jian-ming YANG†‡, Jing-huan ZHU, Qiao-jun JIA, Yue-zhi TAO†‡

(*Institute of Crop and Nuclear Technology Utilization, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China*)

†E-mail: jmyang@163.com; tao_yuezhi@hotmail.com

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Abstract: The genetic diversity and relationship among 40 elite barley varieties were analyzed based on simple sequence repeat (SSR) genotyping data. The amplified fragments from SSR primers were highly polymorphic in the barley accessions investigated. A total of 85 alleles were detected at 35 SSR loci, and allelic variations existed at 29 SSR loci. The allele number per locus ranged from 1 to 5 with an average of 2.4 alleles per locus detected from the 40 barley accessions. A cluster analysis based on the genetic similarity coefficients was conducted and the 40 varieties were classified into two groups. Seven malting barley varieties from China fell into the same subgroup. It was found that the genetic diversity within the Chinese malting barley varieties was narrower than that in other barley germplasm sources, suggesting the importance and feasibility of introducing elite genotypes from different origins for malting barley breeding in China.

Key words: Barley (*Hordeum vulgare* L.), Genetic similarity, Simple sequence repeat (SSR) marker, Cluster analysis, Genetic diversity

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1 Introduction

Barley (*Hordeum vulgare* L.) is the major raw material for malting and brewing. Therefore, barley grain and malting qualities are critical in their commercial use. Breeding of new malting barley varieties is a complex program that involves the improvement of at least 20 agronomic and malting characteristics (Rasmusson and Phillips, 1997). This program normally restricts the use of parents in improving a variety of traits, and barley breeders have had to work within narrow gene pools (Horsley *et al*., 1995). The modern malting barley cultivars are becoming more genetically homogeneous and more vulnerable to pathogens and adverse environments (Asins and Carbonell, 1989). This threat has stimulated the study for new genetic resources for barley breeding, and more researchers in many countries have placed emphasis on the necessity for the collection, conservation, and utilization of the landrace and cultivated varieties (Brown *et al*., 1990). It has been reported that there were 470470 barley accessions in the GenBank (FAO, 2009). Although a wide range of genetic resources is available in the GenBank, only a small part of these resources have been evaluated (Matus and Hayes, 2002).

Traditionally, morphological traits, cytological characters, biochemical tests, and pedigree information are used to assess genetic diversity and classify barley germplasm. However, these methods are always associated with various limitations and are insufficient to reveal the whole information within barley resources (Matus and Hayes, 2002). Many

‡ Corresponding authors

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types of molecular markers, including restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and inter-simple sequence repeat (ISSR), have been used to characterize crop resources (Liu *et al.*, 1996; Russell *et al.*, 1997; Pejic *et al.*, 1998; Shi *et al.*, 2004; Reddy *et al.*, 2009). SSR markers, an excellent molecular marker system with the advantages of being codominant, abundant, highly reproducible, highly polymorphic, and easy to assay, have been used in many types of genetic analyses such as the construction of linkage maps, diversity assessment of germplasm, and identification of molecular markers for marker-assisted selection (Matus and Hayes, 2002; Marcel *et al.*, 2007; Pushpendra *et al.*, 2007). Quantitative trait locus (QTL) analysis of malting quality has been performed in recent years on a number of crosses derived from different germplasm sources originating from North America, Europe, and Australia, and significant advances in knowledge have been achieved (Zale *et al.*, 2000; Hoffman and Dahleen, 2002; Li *et al.*, 2003; Emebiri *et al.*, 2004; Marcel *et al.*, 2007; Panozzo *et al.*, 2007; Varshney *et al.*, 2007).

The breeding of improved malting barley varieties to meet a stable, increasing demand of beer consumption is becoming a significant challenge for barley researchers and breeders in China. The genetic bases and relationships among different genotypes selected for malting barley breeding are, however, still unclear. In this study, we used 35 previously mapped SSR markers to characterize 40 barley accessions, including some parental lines of several mapping populations and elite genotypes of interest to our malting barley breeding program. The objectives of this study were: (1) to distinguish these genotypes, (2) to estimate the genetic diversity and relationship among these barley resources, and (3) to provide useful information for the conservation of genetic resources and the enhancement of malting barley breeding.

2 Materials and methods

2.1 Barley varieties

Forty barley varieties were chosen for this study (Table 1). Among these accessions, 12 are malting barley varieties released in recent years in China, and 28 are cultivated barley varieties collected from different countries, including the parental lines of several mapping populations and some commercial varieties imported to China as malting barley.

2.2 Genotyping of SSR

DNA was extracted from the leaf tissues of three-week-old seedlings (a single seedling per genotype), based on a modified cetyltrimethylammonium bromide (CTAB) method described by Stein *et al.* (2001). Thirty-five SSR markers, five from each of the seven linkage groups with known map locations, were selected and used in this study after searching the web site at http://www.genetics.org/cgi/content/full/156/4/1997/DC1. These SSR primer sets were developed and mapped by Ramsay *et al*. (2000). Detailed information about primer sequences and allele sizes is shown in Table 2.

Polymerase chain reactions (PCRs) were performed in a MyCycler™ thermocycler (Bio-Rad Laboratories, USA). The volume of PCR solution was 15 μl, containing 75 ng of template DNA, 1× PCR buffer (Mg^{2+} free), 0.375 U of *Taq* DNA polymerase, 300 μmol/L of deoxynucleotide triphosphates (dNTPs), 2.25 mmol/L of Mg^{2+}, and 0.75 μmol/L of forward and reverse primers. The optimized PCR amplifying conditions used are available at http://www.geneics.org/cgi/content/full/156/4/1997/DC1 (Ramsay *et al*., 2000). The amplified fragments were separated on 6% (w/v) native polyacrylamide gels. The electrophoreses were performed at 90 W for 2 h in 1× TBE [Tris-borate-ethylenediaminetetraacetic acid (EDTA)] buffer, and the gels were visualized with the silver stain method as described by Bassam *et al*. (1991). The sizes of all fragments were determined by comparing the most intense band with the NoLimits™ DNA sequence marker (Shanghai Sangon Biological Engineering Technology & Services Co., China).

2.3 Data analysis

The number of alleles detected by each SSR marker was estimated for each genotype and all SSR marker loci were scored as described by Struss and Plieske (1998). The resulting matrix was used to estimate genetic similarity (GS) among all varieties by Dice coefficient of similarity (Nei and Li, 1979):

$$GS=2N_{ij}/(N_i+N_j),$$

where N_{ij} is the number of allele types presented in both genotypes *i* and *j*, N_i is the number of allele types presented in genotype *i*, and N_j is the number of allele types presented in genotype *j*. Based on the similarity matrix, a dendrogram showing the genetic relationships between genotypes was constructed by the unweighted pair group method with arithmetic average (UPGMA) using the software NTSYS-pc (numerical taxonomy and multivariate systems) Version 2.01 (Rohlf, 1998).

Table 1 List of barley varieties investigated in this study

Barley variety	Origin	Row-type	Type	Pedigree
Baudin	Australia	2	Spring	Stirling/Franklin
Sloop	Australia	2	Spring	WI2468/Norbert//Golden Promise/WI2395/3/Schooner
Stirling	Australia	2	Spring	Dampier//(A-14)Prior/Ymer/3/Piroline
Schooner	Australia	2	Spring	Proctor/Prior-A//Proctor/Ci3576
Franklin	Australia	2	Spring	Shannon/Triumph
AC Legend	Canada	6	Spring	Chapais/CIMMYT-6
Harrington	Canada	2	Spring	Klages/3/Gazelle/Betzes//Centennial
Encore	Canada	6	Spring	Cadette/QB198.39
TR306	Canada	2	Spring	Unknown
AC Metcalfe	Canada	2	Spring	Oxbow/Manley
CDC Copeland	Canada	2	Spring	TR118/WM861-5
CDC Kendall	Canada	2	Spring	Manley/SM85221
Viviane	Canada	6	Spring	Unknown
OAC Kippen	Canada	6	Spring	York//Ci10853/Parkland/3/Perth
AC Kings	Canada	2	Spring	AB79-17/Iona
Island	Canada	2	Spring	TBR635-25/Symko
Grant	Canada	6	Spring	P885-4/P854-35
Yangnongpi 4	China	2	Winter	Sunong 91-7112/Tongying 1
Ci4196	China	2	Winter	Unknown
Zhedar 1	China	2	Winter	Unknown
ZJU 8	China	2	Winter	Shang 89-0917/92-18
Supi 3	China	2	Winter	Kinuyu Taka/Kanto Nijo 25//Hu 94-043
Daner	China	2	Winter	Naso Nijo/Sihuyu Taka
Kenpi 8	China	2	Spring	86-1/Gimpel//Hungary 92-25
Hua 30	China	2	Winter	Xiu 82-164/Xiumai l
Zhepi 8	China	2	Winter	Zhepi 2/93-125
Ganpi 4	China	2	Spring	Fawawit/Ba-nong-86259
Ganpi 3	China	2	Spring	S-3/Fawawit
Xiumai 3	China	2	Winter	Xiu 82-164/Xiumai l
Esterel	France	6	Winter	7761/Plaisant
Franka	Germany	6	Winter	Vogelsanger-Gold/Senta//Dura/Dea/3/Vogelsanger-Gold
Nudinka	Germany	2	Spring	Emir/Volla//Lbp-840-N/3/Goldthorpe/4/Dr. Baentsch-N
Triumph	Germany	2	Spring	Unknown
Amaji Nijo	Japan	2	Winter	Fuji Nijo/Seijo 17
Golden Promise	UK	2	Spring	(M)Maythorpe
Proctor	UK	2	Spring	Kenia/Plumage-Archer
Kym	UK	2	Spring	Georgie/Hanna
Excel	USA	6	Spring	Cree/Bonanza//Manker/Robust
Stander	USA	6	Spring	Excel//Robust/Bumper
Steptoe	USA	6	Spring	Washington-3564/Unitan

/: first cross; //: second cross; /3/: third cross; /4/: forth cross; M: mutation

Table 2 Primer sequences, fragment sizes, and repeat types of 35 barley SSR markers

Barley SSR marker	Chromosome	Motif	Primer sequence (5′→3′)	Annealing temperature (°C)	Expected product (bp)
Bmac0032	5(1H)	$(AC)_7T(CA)_{15}(AT)_9$	CCATCAAAGTCCGGCTAG GTCGGGCCTCATACTGAC	65	215
HVBDG	5(1H)	$(CT)_6$	GAGAGAGAAAGAGAATGGCAGG AAAAAACTGCACCCAATCACTT	60	145
EBmac0501	5(1H)	$(AC)_{13}$	ACTTAAGTGCCATGCAAAG AGGGACAAAAATGGCTAAG	58	151
Bmag0347	5(1H)	$(CT)_{28}$	CTGGGATTGGATCACTCTAA AAAACAAGTACTGAAAATAGGAGA	55	107
HVADH1	5(1H)	$(TGC)_7$	GAATTCTCATGAGGGATGCTTC CAACTGAAACTCATGGCCAT	60	206
HVM36	2(2H)	$(GA)_{13}$	TCCAGCCGACAATTTCTTG AGTACTCCGACACCACGTCC	55	114
Bmac0216	2(2H)	$(AC)_5$	GTACTATTCTTTGCTTGGGC ATACACATGTGCAAAACCATA	55	190
EBmag0793	2(2H)	$(GT)_{13}(AG)_{36}$	ATATATCAGCTCGGTCTCTCA AACATAGTAGAGGCGTAGGTG	55	177
Bmac0134	2(2H)	$(AC)_{28}$	CCAACTGAGTCGATCTCG CTTCGTTGCTTCTCTACCTT	55	148
HVBKASI	2(2H)	$(C)_{10}(A)_{11}$	ATTGGCGTGACCGATATTTATGTTCA CAAAACTGCAGCTAAGCAGGGGAACA	60	197
Bmac0209	3(3H)	$(AC)_{13}$	CTAGCAACTTCCCAACCGAC ATGCCTGTGTGTGGACCAT	58	176
Bmag0006	3(3H)	$(AG)_{17}$	TTAAACCCCCCCCCTCTAG TGCAGTTACTATCGCTGATTTAGC	58	174
Bmag0508A	3(3H)	$(AG)_{14}$	TCTCCGTATATTTAGGAAACG TATCTCCCCCTAGATAGAAGG	55	175
Bmag0905	3(3H)	$(TC)_{14}$	TTTATCTCCCCCTAGATAGAAG TCTCCGTATATTTAGGAAACG	55	177
Bmag0853	3(3H)	$(GA)_{15}$	ACAAGTATCCTGCAAACCTAA CGACCTTCTTAATGGTTAGTG	55	183
HVM40	4(4H)	$(GA)_6(GT)_4(GA)_7$	CGATTCCCCTTTTCCCAC ATTCTCCGCCGTCCACTC	55	160
HVRCABG	4(4H)	$(GA)_6$	TTTAAAAGAAAAGTGAATGGC TAATGAAGAATGAGGAGAAGC	55	123
EBmac0679	4(4H)	$(AC)_{22}$	ATTGGAGCGGATTAGGAT CCCTATGTCATGTAGGAGATG	55	148
Bmag0375	4(4H)	$(AG)_{19}$	CCCTAGCCTTCCTTGAAG TTACTCAGCAATGGCACTAG	58	135
Bmac0577	4(4H)	$(AC)_{12}$	TCATACAGAAGCCCACACAG TGCATGTTCATTCTAGACAGG	53	146
HVACL1	7(5H)	$(AT)_7$	TTTGGAATTATTCTGTGGGACC GGGATTCAATCAAGTATTCGGA	60	150
HVDHN7	7(5H)	$(AAC)_5$	TTAGGGCTACGGTTCAGATGTT ACGTTGTTCTTCGCTGCTG	58	177
HVLEU	7(5H)	$(ATTT)_4$	TTGGAAGTGTACAGCAATGGAG TGAAAGGCCCCACAAGATAG	60	166

(To be continued)

Table 2

Barley SSR marker	Chromosome	Motif	Primer sequence (5′→3′)	Annealing temperature (°C)	Expected product (bp)
Bmac0306	7(5H)	$(AC)_{10}$-$(AC)_5$	CCTTGTGTGAGTGTGTGTG ACATGCACATGAACTAATCAA	58	127
EBmatc0003	7(5H)	$(ATC)_4N_3(ATC)_3$	AATTTTGCAAAGCTGGAGG CATTATGGTGGGGTTCATGT	58	111
HVM34	6(6H)	$(GA)_{10}$	ACCATGTTGCGTGTTGCTT CGGTTCGAAATCGAGTGG	60	222
Bmac0127	6(6H)	$(AC)_{26}$	AACTATGTCCAGTCGTTTCC CTTGTCGTATCATCTTATTCAGA	58	118
Bmag0344	6(6H)	$(CT)_{10}GT(CT)_{16}$	GATCCAACTATATTAACAAAGCC TGAGGGTATGTACCACTAGCT	60	165
Bmag0103	6(6H)	$(AG)_{22}$	AAAATATTGGCATGAGCTTAG ATCAAAGATCACATCCTTCC	55	166
Bmag0807	6(6H)	$(TC)_{18}$	GGATATAAGGGTCCATAGCA AATTACATCAAATAGGCTCCA	55	111
HVCMA	1(7H)	$(AT)_9$	GCCTCGGTTTGGACATATAAAG GTAAAGCAAATGTTGAGCAACG	60	141
HVWAXYG	1(7H)	$(AT)_9$	TCCAATGGCATCTACAGGACGGCCAA GCAGGTTGAGCTGCGCAAAGTCGTCG	58	205
Bmag0135	1(7H)	$(AG)_{10}GG(AG)_{12}$	ACGAAAGAGTTACAACGGATA GTTTACCACAGATCTACAGGTG	58	161
Bmag0217	1(7H)	$(AG)_{19}$	AATGCTCAAATATCTATCATGAA GGGGCTGTCACAAGTATATAG	58	196
HVM04	1(7H)	$(AT)_9$	AGAGCAACTACCAGTCCAATGGCA GTCGAAGGAGAAGCGGCCCTGGTA	55	198

3 Results

3.1 Allelic variation of SSR markers

Using DNA samples isolated from 40 barley accessions as templates, polymorphic DNA fragments were amplified from 29 among the 35 SSR primer pairs selected in this study, including 3 out of 5 SSRs (60%) located on chromosome 2H, 4 out of 5 (80%) on chromosomes 1H, 4H, 5H, and 6H, and all (100%) on chromosomes 3H and 7H, respectively (Table 3). The sizes of these fragments ranged from 100 to 300 bp. A total of 85 alleles with the average alleles per locus of 2.4 were detected at 35 loci. More than one allele was detected at 29 out of all 35 SSRs studied, with the polymorphic markers' ratio of 82.9% of polymorphic markers. The maximum of five alleles were observed at the loci of Bmac0134 on chromosome 2H and HVRCABG on chromosome 4H, respectively.

Table 3 Number of alleles detected and chromosome locations of 35 SSRs

Chromosome	Number of SSRs					Total allele number
	1 allele	2 alleles	3 alleles	4 alleles	5 alleles	
1H	1	1	2	1	0	13
2H	2	2	0	0	1	11
3H	0	1	4	0	0	14
4H	1	1	2	0	1	14
5H	1	3	1	0	0	10
6H	1	3	0	1	0	11
7H	0	3	2	0	0	12

3.2 Genetic similarity (GS)

Significant genetic variation was found among all barley accessions with the GS value ranging from 0.39 to 0.98. The GS value was ranged from 0.45 to 0.88 within the group of Chinese developed varieties, and from 0.39 to 0.98 within the group of introduced

varieties, indicating that there was a higher genetic diversity among introduced barley varieties.

3.3 Cluster analysis

All 40 barley accessions were discriminated successfully by SSR markers (Fig. 1). The accessions were classified into two groups (Groups 1 and 2) at the level of GS=0.57. Group 1 included 6 accessions, while Group 2 consisted of 34 accessions. At the level of GS=0.62, Group 2 was further divided into two subgroups (Subgroups 2a and 2b) containing 11 and 23 accessions, respectively.

Twelve Chinese malting barley varieties were clustered into different groups, two in Group 1, seven in Subgroup 2a and three in Subgroup 2b, respectively. All European barley varieties appeared in the same subgroup.

4 Discussion

In this study, 85 alleles were detected with 35 SSR loci, and allelic variations existed at 29 SSR loci. The average allele number per locus, ranging from 1 to 5 alleles detected from 40 barley accessions for each individual locus, was 2.4. This relatively small number is probably due to the limited accessions and relatively high GS within the investigated group of barley germplasm. However, since the SSR loci selected were evenly distributed along the barley genome, the genetic relationships revealed by this study within the investigated group of barley varieties are representative and meaningful.

Evaluation of the amount of genetic variation in barley germplasm is the essential study for barley breeding. Outcomes of the assessments provide a

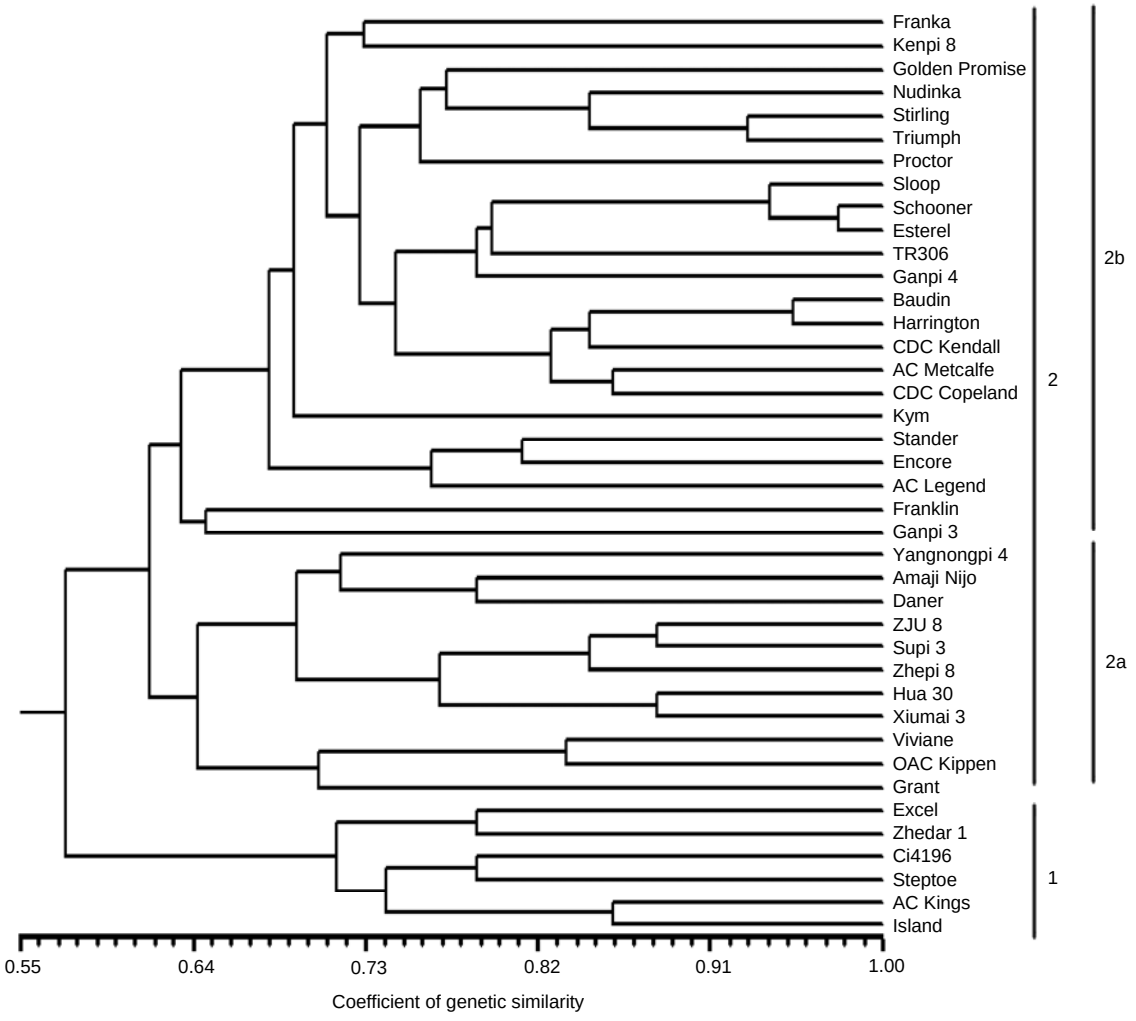

Fig. 1 Dendrogram of 40 barley varieties based on the UPGMA method

general guide for choosing parental lines to make suitable cross combinations for particular breeding purposes. The evaluation of genetic variation among barley resources from different countries has been reported (Russell *et al*., 1997; Pejic *et al*., 1998; Struss and Plieske, 1998; Ivandic *et al*., 2002; Matus and Hayes, 2002; Feng *et al*., 2003; Shi *et al*., 2004; Hou *et al*., 2005). As one of the genetic diversity centers of barley, China is rich in both landrace and cultivated barley (Feng *et al*., 2003). Several studies were conducted in China to evaluate the genetic relationships among different barley populations (Feng *et al*., 2003; Shi *et al*., 2004; Hou *et al*., 2005). Pejic *et al*. (1998) reported that the information of polymorphism would be sufficient if more than 70 alleles were detected. Shi *et al*. (2004) suggested that more than two SSRs from each of seven linkage groups should be selected to ensure the efficiency and representation of the genetic information among accessions. Saghai Maroof *et al*. (1994) reported that 71 alleles were detected in the 207 samples of wild and cultivated barley with four SSR primer pairs. The largest number of alleles found at locus HVM4 was 37. Other researchers have shown smaller numbers of alleles per locus in barley accessions, with a range of 1 to 16 (Becker and Heun, 1995; Struss and Plieske, 1998; Davila *et al*., 1999; Matus and Hayes, 2002; Feng *et al*., 2003).

Evaluation of the extent and the nature of genetic variation in barley germplasm provides valuable information for the conservation of germplasm. Genetic relationships were found to be very close among the Chinese malting barley varieties analyzed in this study. The fact that seven malting barley varieties developed in China were clustered into the same subgroup gave the strong indications of the narrow genetic background in Chinese malting barley germplasm. The introduced foreign genotypes investigated in this study, on the other hand, showed a broader genetic diversity. To avoid the potential risks associated with too little genetic diversity, the adoption of elite genotypes from different origins used as parental lines is highly recommended for malting barley breeding in China. Barley breeding organizations should stress the necessity for the collection, conservation, and utilization of the cultivated varieties and the landraces.

Matus and Hayes (2002) found that germplasm classifications generally coincided with geographic origin and end-use quality. Pillen *et al*. (2002) also found that German barley varieties could be easily classified by SSR markers. On the other hand, Plaschke *et al*. (1995) and Russell *et al*. (1997) suggested that SSR markers could not differentiate genetic resources of related pedigrees. Our results demonstrate that the data generated from a set of 35 SSR markers were highly informative. The 40 varieties were distinguished successfully. Three Chinese spring varieties were clustered into Subgroup 2b with almost all spring barley varieties from other sources. Seven Chinese winter varieties were clustered into Subgroup 2a. Another two Chinese winter varieties 'Zhedar 1' and 'Ci4196' were classified into Group 1 with four North America (including USA and Canadian) varieties. It is interesting to note that the varieties in the same group always share one or more common breeding ancestors according to pedigree information. For example, the Chinese varieties 'Ganpi 3', 'Ganpi 4', and 'Kenpi 8' were present in Subgroup 2b. This group included many European varieties. Hungarian barley resources have been used as the parental lines over the time to develop these varieties (Wang *et al*., 2003; Li *et al*., 2006). Similar cases could be found in Group 1, in which two Chinese varieties 'Zhedar 1' and 'Ci4196' were adopted by the Canadian and American researchers in the 1990's (Evans *et al*., 2000; Urrea *et al*., 2002).

It was reported that the SSR marker EBmac0501 on chromosome 1H was associated with malt extract (Zale *et al*., 2000; Hoffman and Dahleen, 2002; Emebiri *et al*., 2004; Panozzo *et al*., 2007). In our study, the results generated from genotyping of EBmac0501 indicated that eight malting barley varieties 'Yangnongpi 4', 'Amaji Nijo', 'Daner', 'ZJU 8', 'Supi 3', 'Zhepi 8', 'Hua 30', and 'Xiumai 3' showed the same allele. Thus, it appears that the SSR marker EBmac0501 is also very likely to be associated with malt extract in Chinese malting barley. However, further validation is needed to confirm the candidate regions of key QTL for the characteristics of malting quality in Chinese barley.

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