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RESEARCH ARTICLE

Genetic diversity analysis of hulless barley from Shangri-la region revealed by SSR and AFLP markers

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Abstract For adding the hulless barley resources of Shangri-la region to the global barley resource library, a basic work was done by us to assess their genetic diversity of this region. The genetic diversity of 60 hulless barley samples collected from three counties in Shangri-la region of Yunnan Province, were studied using SSR (simple sequence repeats) and AFLP (amplified fragment length polymorphism) markers. A total of 70 alleles were detected for 19 pairs of SSR primers, and 525 band containing 464 polymorphic bands were revealed for 5 pairs of AFLP primers. The value of polymorphism information content (*PIC*) ranged from 0.03 to 0.86 for SSR primers. The total numbers of alleles were 51, 55, 43 in three populations and the polymorphic bands were 188, 205 and 141. The genetic distances and genetic identity among the three populations showed their close relationship. The gene diversity among populations relative to the total population diversity (*Gst*) was 0.13 for SSR markers and 0.02 for AFLP markers and indicated that just 13 and 2% variations were among populations, respectively. The UPGMA cluster analysis revealed that all of the samples grouped randomly rather than clustered into distinct groups corresponding to their populations, row types and spring/fall types. We concluded that there was high genetic diversity in the population of Shangri-la region and the formation of diversity was related to complex environment and inhabitants' traditional practices.

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Introduction

Domesticated barley (*Hordeum vulgare* L. subsp. *vulgare*) is classified into two major categories all over the world according to the grain type, namely, hulled barley and hulless barley (or naked barley). Taketa et al. (2004) concluded that hulless barley today can be traced back to a single, naked mutation and south-western Iran is the most likely site of it's origin. According to a survey by Takahashi (1955), hulless barley is distributed widely, but its frequency greatly differs among regions. Its distribution is skewed towards East Asia, occupying as much as 95% of domesticated barley in the highlands of Nepal, Bhutan, Korea, Japan and Tibet of China. China hulless barley genetic resources occupy 77% of the world's total hulless barley collection. Hulless barley in China is mainly distributed in the Qinghai-Tibet Plateau, covering Tibet, Qinghai, Gansu, Sichuan and Yunnan provinces. In Yunnan Province, hulless barley is mainly distributed in the Shangri-la region. Located in the southern part of the Qinghai-Tibet Plateau and the eastern Himalayas, Shangri-la is the junction of Yunnan, Tibet and Sichuan provinces.

With the increases of the altitude, the landform, soils, temperature, solar power and UV intensity changes correspondingly. The large range of altitudes in this area results complex ecological environments which build a foundation to create diverse barley cultivars. In addition, the majority population in Shangri-la is Tibetan (or Zang minority in Chinese) and they lived on the hulless barley from ancient till now. The hulless barley landraces in this region is closely associated with the traditional Tibetan culture, which emphasizes the value of traditional barley cultivars in agriculture, medicine, diet, nature worship, and religion (Walker et al. 1995). Traditional Tibetan practices play an important role in natural resource management and farming systems of hulless barley conservation (Pan 2005). Based on the complex ecological environments and the inhabitants' traditional practices, various hulless barley germplasm resources are grown in Shangri-la region (NeAn 2003). They should be made an elementary study in genetic background on molecular level for further deeper research.

SSRs, the simple sequence repeats, also called microsatellites, occur as highly repetitive elements in all eukaryotic genomes (Tautz 1989). SSRs are co-dominant, abundant and multiallelic, so SSRs is a popular marker system used in linkage mapping, germplasm surveys, phylogenetic studies (Liu et al. 1996) and the measurement of genetic diversity of breeding material and cultivars (Donini et al. 1998). AFLP have been also showed to be a powerful DNA fingerprinting technique (Vos et al. 1995). They are dominant markers but reveal a large number of loci, so it is efficient in diversity studies (Russell et al. 1997).

Based on the present situation that there are rare research on the hulless barley resource of Shangri-la region, a basic work was done by us to used SSR and AFLP markers to assess their genetic diversity. In addition, we try to explain the formation of the diversity with both natural and artificial reasons. The work should contribute not only to the gene pool of the global barley resource library, but also to the ecology and ethnobotany.

Materials and methods

Site description

Shangri-la region, with a total area of 23,870 km^2, is located in the south of Qinghai-Tibet Plateau and the east of Himalayas (E98°37′–100°23′, N26°57′–29°12′) and the junction of Yunnan, Tibet and Sichuan provinces. The altitude in this region ranging from 1,480 to 6,740 m, together with its location provides a complex topography within high variation in climatic conditions. The altitude of this study ranges from 1,749 to 3,600 m, accordingly, the landforms, soils and temperature reveals differences at different altitudes. Three main types of landforms occupy the region: river-valleys (below 2,200 m), mountain areas (2,200–2,800 m), and the plateau (above 2,800 m). From the foot to the top of the attitude, the soils appear as red soils (below 2,600 m), yellow soils (below 2,900 m), brown soils (2,900–3,300 m) and dark brown soils (3,200–3,700 m). The region in our study is separated into three vertical climatic zones including the South Temperate Zone (below 2,300 m), the Warm Temperate Zone (2,300–2,800 m) and the

North Temperate Zone (2,800–4,200 m). The significant climates are among the river-valleys. Determined by the land relief and the blocking to air by the hills, the river-valleys has three types including dry and hot river-valleys, warm river-valleys, damp and muggy river-valleys (NeAn 2003).

Plant materials

A total of 60 hulless barley samples (Table 1) were collected from Diqing Tibetan Autonomous Prefecture of Shangri-la region which was divided into three parts including Shangri-la county, Deqin county and Weixi county. Among the 60 samples, 27 were collected from Shangri-la, 25 from Deqin and 8 from Weixi. The collection sites of the 60 hulless barley samples were located on the map (Figs. 1, 2). 51.7% of the 60 samples were 4-row barley; meanwhile 48.3% were 6-row barley and none of the samples were 2-row barley. 18.3% of the 60 samples were winter cultivars and 81.7% were spring cultivars.

DNA extraction

DNA was extracted with modified CTAB protocol from fresh leaf of 2–3 weeks after planting (Doyle and Doyle 1987).

Table 1 The information of 60 hulless barley samples

Code	Altitude (m)	Row type	Spring/fall type	Code	Altitude (m)	Row type	Spring/fall type
s1	3,233	4	Spring	d4	2,908	6	Spring
s2	3,296	4	Spring	d5	2,911	6	Spring
s3	3,320	6	Spring	d6	3,550	6	Spring
s4	3,036	4	Spring	d7	3,452	6	Spring
s5	3,135	4	Spring	d8	3,321	6	Spring
s6	3,171	4	Spring	d9	3,454	6	Spring
s7	3,230	4	Spring	d10	3,593	6	Spring
s8	3,230	4	Spring	d11	3,477	6	Spring
s9	2,686	6	Spring	d12	3,600	6	Spring
s10	2,845	6	Spring	d13	1,953	6	Spring
s11	2,845	6	Spring	d14	2,198	4	Spring
s12	2,330	6	Spring	d15	2,928	4	Spring
s13	2,330	6	Spring	d16	2,530	4	Spring
s14	2,350	4	Spring	d17	2,390	6	Spring
s15	3,397	4	Spring	d18	2,340	4	Spring
s16	2,914	4	Spring	d19	2,354	6	Spring
s17	3,294	4	Spring	d20	1,933	6	Spring
s18	3,378	4	Spring	d21	1,997	4	Spring
s19	2,841	6	Spring	d22	2,914	4	Spring
s20	2,841	6	Winter	d23	1,969	6	Spring
s21	2,650	6	Winter	d24	1,899	4	Spring
s22	2,650	4	Spring	d25	1,826	6	Spring
s23	2,583	4	Spring	w1	2,600	4	Winter
s24	2,774	6	Spring	w2	2,569	4	Winter
s25	2,995	6	Spring	w3	2,343	4	Winter
s26	2,438	6	Winter	w4	2,530	4	Winter
s27	2,560	4	Spring	w5	2,040	6	Winter
d1	3,172	4	Spring	w6	2,553	4	Winter
d2	3,272	4	Spring	w7	2,585	6	Winter
d3	3,255	6	Spring	w8	1,749	4	Winter

The code with s means the samples came from Shangri-la County; with d from Deqin County; with w from Weixi County

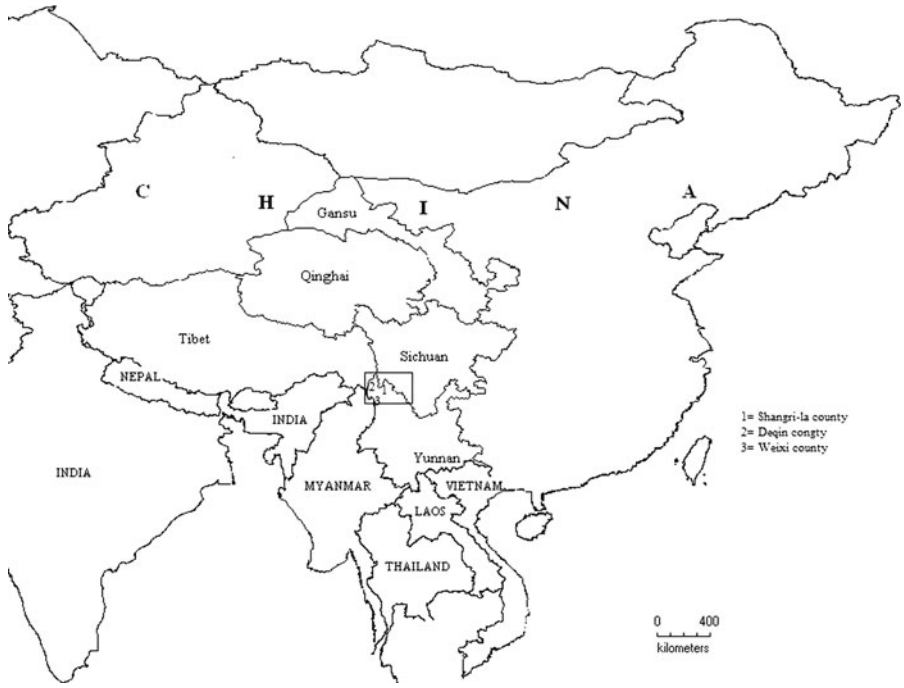

Fig. 1 A map of the origins of hulless barley samples marked with numbers

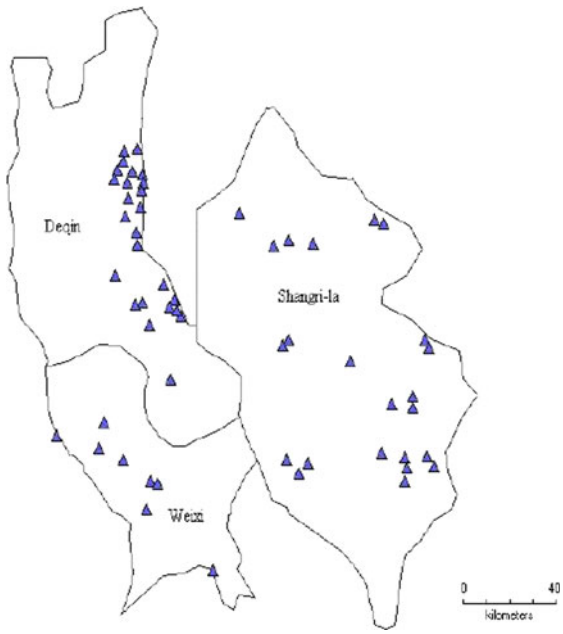

Fig. 2 The collection sites of 60 hulless barley landraces from Shangri-la region marked with triangle

SSR analysis

A total of 19 primers were selected from the SSR-based linkage map of barley (Ramsay et al. 1997). For polymerase chain reactions (PCR), each sample was prepared in a final volume of 15 μl containing 100 ng of template DNA, 1.5 μl of Taq polymerase buffer, 2.0 mM of $MgCl_2$, 0.2 mM dNTPs, 2.5 pmol of forward and reverse primers and 1 unit of Taq DNA polymerase (Tiangen). Information on primer sequences and PCR amplification conditions for each set of primers were available at the Genetics supplemental data site at http://www.genetics.org/supplemental/156/4/1997/DC1.

After the amplification using PTC-200 Peltier Thermal Cycler (Bio-Rad), PCR products were separated by 8% polyacrylamide gels and visualized by silver staining. A 50 bp DNA ladder (Fermentas) was used as a standard for molecular size.

AFLP analysis

AFLP analysis was carried out as described by Vos et al. (1995) with minor modifications. Selective amplification was done with *Eco*RI and *Mse*I primers having three additional nucleotides. For fluorescent-based fragment analysis, *Eco*RI primers were labeled with TET (4, 7, 2′, 7′-Tetrachloro-6-carboxyl fluorescein) fluorescent dye. To prepare DNA fragments for separation by capillary electrophoresis, a sample loading solution was prepared with a 600-base-pair (bp) DNA size standard labeled with WellRED^{TM} dye

Table 2 Sequence of oligonucleotide adapters and primers used in pre-amplification and five selective AFLP primer combinations

Name	Sequence
*Eco*RI adapter	5′-AATTGGTACGCAGTCTAC-3′
	3′-CCATGCGTCAGATGCTC-5′
*Mse*I adapter	5′-GACGATGAGTCCTGAG-3′
	3′-TACTCAGGACTCAT-5′
*Eco*RI + 1 primer	5′-GACTGCGTACCAATTC A-3′
*Mse*I + 1 primer	5′-GATGAGTCCTGAGTAA C-3′
*Eco*RI + 3-AGG	5′-GACTGCGTACCAATTC AGG-3′
*Eco*RI + 3-AAC	5′-GACTGCGTACCAATTC AAC-3′
*Eco*RI + 3-ACC	5′-GACTGCGTACCAATTC ACC-3′
*Mse*I + 3-CTG	5′-GATGAGTCCTGAGTAA CTG-3′
*Mse*I + 3-CAT	5′-GATGAGTCCTGAGTAA CAT-3′
*Mse*I + 3-CTT	5′-GATGAGTCCTGAGTAA CTT-3′
*Mse*I + 3-CTA	5′-GATGAGTCCTGAGTAA CTA-3′

D1 (approximately 100:1; Beckman Coulter 608082 and 608098). This solution was thoroughly mixed by vortexing for a minimum of 2 min. A 20 µl aliquot of this cocktail was added to 3 µl of diluted (tenfold) selective amplification product. Each well was overlaid with a drop of Sigma olefin oil and samples were analyzed in the CEQ^{TM} 8000 Genetic Analysis Sequencer from Beckman Coulter, Inc (USA). Electrophoresis was done at 750 V for 1.5 h. Five primer combinations were selected based on the number of the polymorphic fragments. The sequences of selected primers and adaptors were listed in Table 2.

Statistical analysis

AFLP electrophenograms ranging in size from 50 to 600 bp were analyzed by CEQ 8000 Software (Beckman Coulter). Then the amplification fragments of SSR and AFLP were scored as either present (1) or absent (0), across all 60 hulless barley samples. The genetic diversity indexes were calculated with POPGENE (version 1.32) according to Nei's method (Nei 1973; Yeh et al. 1997): (1) *GD* (Nei's genetic distances) and I_N (Nei's genetic identity) (2) *Gst* (the coefficient of gene differentiation among population within species) using the formula $Gst = Dst/H_T$, $H_T = Hs + Dst$, where H_T was the mean allelic diversity, *Hs* was the genetic diversity within populations, and *Dst* was the genetic diversity among populations. The genetic similarity coefficient among the samples was estimated according to Dice (1945) by the software NTSYS-pc (version 2.11C) and similarity matrices were used to construct the unweighted pair group method with arithmetic average (UPGMA) dendrograms. To assess the informativeness of each SSR, the polymorphism information content (*PIC*), was calculated by the following formula: $PIC = 1 - \Sigma P_i^2$ and where P_i is the *i*th SSR allele frequency (Smith et al. 2000).

Results

Allelic variation of SSR markers

The number of alleles of each locus varied from two (Bmag0105, Bmag0692, Bmag0140, Bmac0209, Bmag0136, EBmac0701, EBmac0679, Bmag0223) to ten (Bmac0032) with an average of 3.68 per primer combination. There were altogether 70 alleles across 60 hulless barley samples. Considering the entire germplasm array, *PIC* values ranged from 0.03 (Bmag0105 and Bmag0136) to 0.86 (HVM33) with an average of 0.30 (Table 3).

AFLP polymorphism

The number of bands that were amplified by each primer combination ranged from 64 to 142 (Table 3), in sizes from 50 to 600 bp. A total of 525 bands were generated across all 60 samples. Among these, 464 bands were polymorphic with a mean polymorphism rate of 88.4% ranging from 87.5 to 89.2%. The highest polymorphism rate (89.2%) was observed with primer combination E-AGG/M-CAT, whereas the least polymorphism rate (87.5%) was obtained with primer combination E-AGG/M-CTT.

Comparison of genetic diversity among the barley of Shangri-la, Deqin and Weixi

The numbers of alleles for SSR and polymorphic bands for AFLP in three populations were listed in Table 4. Deqin had the most alleles and polymorphic bands among three populations. The genetic distance (*GD*) and genetic identity (I_N) among the three populations showed that the genetic distance was low and the genetic identity was high, especially in AFLP analysis (Table 5), indicting that there were close relationships among the three barley populations.

Table 3 Alleles of each SSR primers and bands generated by five primer combinations for AFLP for 60 samples

SSR primer	Number of alleles	*PIC*	AFLP primer combination	Number of loci	Number of polymorphic bands	Polymorphism rate (%)
Bmag0211	4	0.34	E-AGG/M-CAT	129	115	89.15
Bmag0105	2	0.03	E-AGG/M-CTG	82	72	87.80
Bmac0032	10	0.85	E-AGG/M-CTT	64	56	87.50
Bmac0213	7	0.76	E-ACC/M-CTA	108	95	87.96
Bmac0134	5	0.77	E-AAC/M-CAT	142	126	88.73
Bmag0692	2	0.10	Mean	105	92.8	88.38
Bmag0140	2	0.15	Total	525	464	
HVM33	9	0.86				
Bmac0209	2	0.08				
Bmag0013	4	0.62				
Bmag0136	2	0.03				
Bmag0225	3	0.13				
HVM40	3	0.13				
EBmac0701	2	0.06				
EBmac0679	2	0.06				
Bmag0353	3	0.10				
EBmac0684	3	0.37				
Bmag0223	2	0.06				
HVCMA	3	0.26				
Mean	3.6842	0.30				
Total	70					

The gene diversity among populations relative to the total population diversity, namely the *Gst*, was 0.13 for SSR and 0.02 for AFLP. It revealed that the variations among populations were just 13 or 2%, while the variation within populations (87 or 98%) was the major component explaining the diversity.

Cluster analysis

Two dendrograms were constructed (Figs. 3, 4) to depict the genetic diversity among all 60 samples based on the SSR markers and AFLP markers. In Fig. 3, the 60 samples were divided into 4 groups

Table 4 Number of alleles for SSR and polymorphic bands for AFLP in three populations

SSR primers	Number of alleles			AFLP primer combinations	Number of polymorphic bands		
	Shangri-la	Deqin	Weixi		Shangri-la	Deqin	Weixi
Total	51	55	43	Total	188	205	141
Mean	2.68	2.89	2.26	Mean	37.6	41	28.2

Table 5 Nei's genetic identity (above diagonal) and genetic distance (below diagonal) among the three populations

SSR				AFLP			
	S	D	W		S	D	W
S	****	0.9736	0.9421	S	****	0.9982	0.9953
D	0.0267	****	0.9823	D	0.0018	****	0.9954
W	0.0597	0.0179	****	W	0.0047	0.0046	****

Note: the significance of **** is the population's value with itself

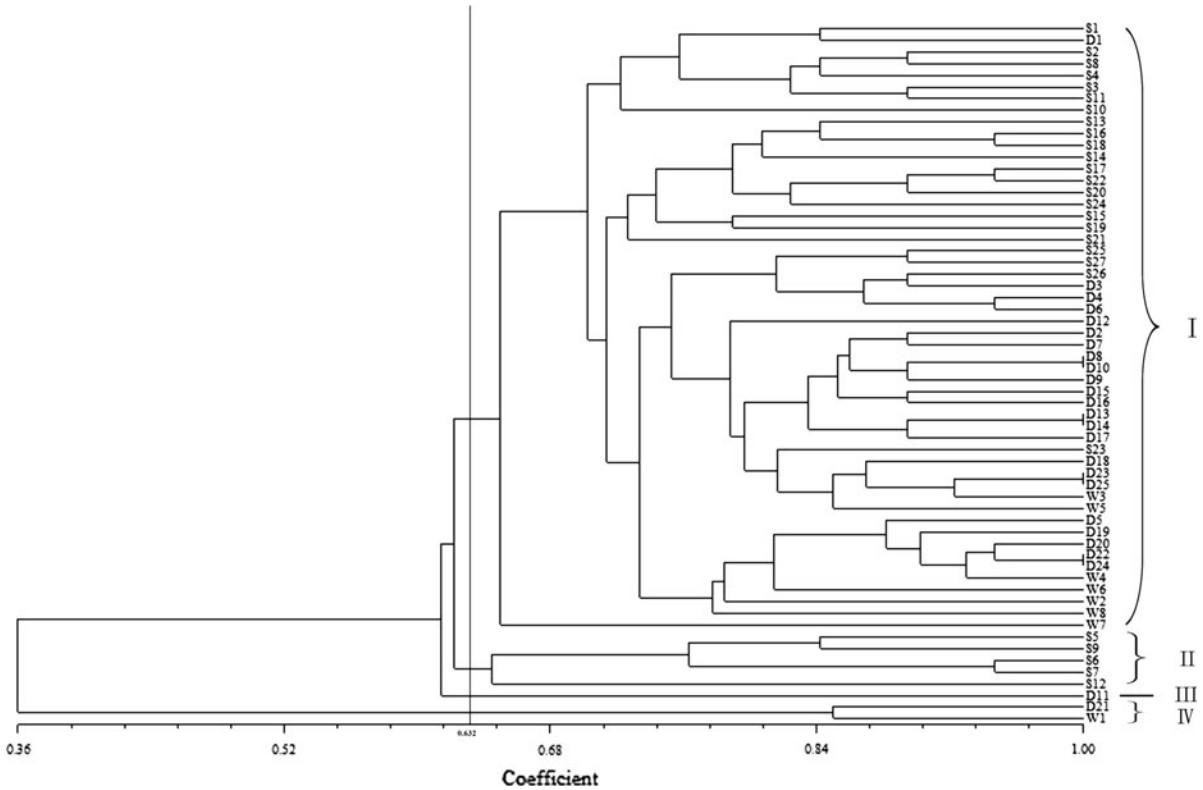

Fig. 3 UPGMA clustering dendrogram of 60 hulless barley genotypes based on the genetic similarity coefficients for SSR

where the genetic similarity coefficient was 0.632. The group I included 52 samples and they were random in origin populations, row types and spring/fall types. In Fig. 4, the 60 samples were divided into 3 groups where the genetic similarity coefficient was 0.84. The group I included 48 samples and they were also random in origin populations, row types and spring/fall types. The UPGMA cluster analysis resolved the genetic relationship among 60 genotypes and revealed that most of the samples grouped randomly rather than clustered into distinct groups corresponding to their origin populations, row types and spring/fall types. The genetic similarity coefficient varied from 0.36 to 1.00 for SSR and 0.75–0.95 for AFLP.

Discussion

SSR analysis revealed that there were 70 alleles and the genetic similarity coefficient ranged from a low of 0.36 to a high of 1.00 within the 60 hulless barley samples. The AFLP analysis showed that a total of 525 bands were generated across all 60 samples. The genetic similarity coefficient and its UPGMA cluster analysis revealed that there just 2 pairs of barley were completely similar for SSR and none of the collections were completely similar for AFLP in 1.00 similarity coefficient level. These facts indicated that the Shangri-la region which was only 23,870 km^2 had a large number of heterogeneous hulless barley recourses.

The formation of diversity could be partly explained by its ecological environment variations. Pandey et al. (2006) pointed out that the diversity of the hulless barley in Himalayan were largely related to the altitude. The collected elevation in this study ranged from 1,749 to 3,600 m determined different ecological environments in landform, soils, temperature, solar power and UV intensity. Genetic diversity is the result of the interactions of genotypes and their environment, with the system developing over the long-term. The rich genetic diversity of hulless barley of Shangri-la region at different altitudes can be explained by the species' need to adapt to the differentiation of environmental conditions and other

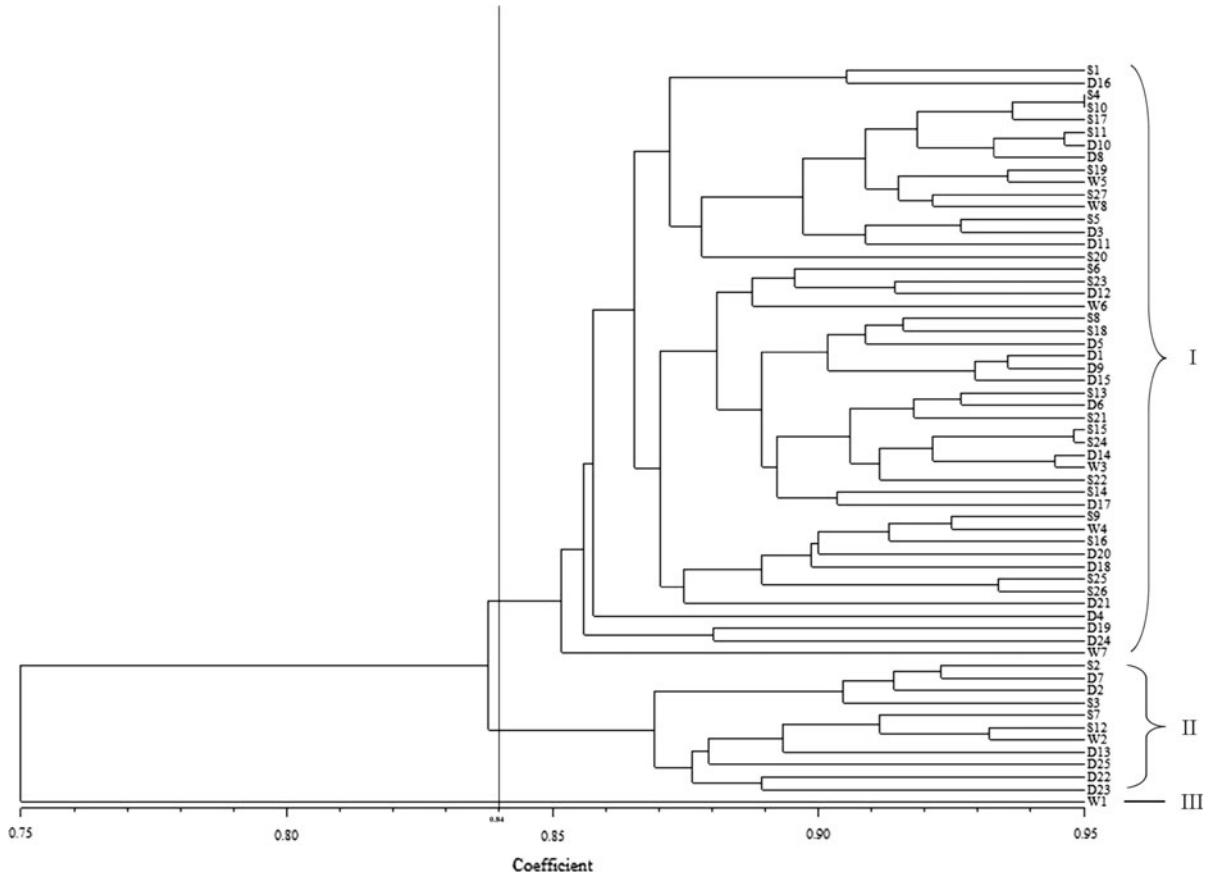

Fig. 4 UPGMA clustering dendrogram of 60 hulless barley genotypes based on the genetic similarity coefficients for AFLP

factors. This can be proved by the numbers of alleles and polymorphic bands in Shangri-la and Deqin barley populations. The samples numbers collected from the two county were nearly the same while the collection altitude ranges of the two populations were different. Shangri-la ranged from 2,330 to 3,397 m; Deqin ranged from 1,826 to 3,600 m. With the wider range of collection altitudes, Deqin population had more alleles and polymorphic bands than Shangri-la populations. This indicated that the diversity was really related to altitude.

Many studies had shown that most crop germplasm had been conserved by indigenous societies with their traditional culture and agricultural mode (Altieri and Merrick 1987; Plucknett et al. 1983; Pei and Xu 1997). Information from ethnobotanical surveys, the most common approach to the collection of indigenous knowledge about the uses and biodiversity associated with plants, can be a highly valuable source of information for biodiversity assessments (Martin 1995). In Shangri-la region, hulless barley may be eaten three times a day. The most popular food made from hulless barley is *Zangba*, a powder with fragrant smell, and *Qingkejiu*, a favorite alcoholic drink brewed from hulless barley. The pliable straw of hulless barley was good fodder for animals including yaks, cattle, and sheep. Some farmers also use hulless barley grains to feed poultry. The ash of the straw is also used for cooking. Due to its functional importance in daily life, many rare germplasm resources of hulless barley have been conserved. In all the communities surveyed, locals present certain cultivars of hulless barley, wheat, rice and corn as ceremonial sacrifice to pray for a great harvest, flourishing livestock and health. Many Tibetans associate the practice of conserving different hulless barley landraces with respecting their ancestors as these resources were reserved by their ancestors from generation to generation, and thus should not be discarded. Crop varietal diversity has been shown to help to increase

productivity by regulating diseases in agroecosystems (Martin 2000; Zhu et al. 2000). In most fields visited, two or three or more landraces were directly mixed and sown together to control pests and diseases to improve the yield and quality. These traditional culture and agricultural mode were other reasons to explain the barley diversity in Shangri-la region.

Local community members usually exchange landrace seeds between families or villages. Cultivars are either inherited from parents and conserve with great care or obtained from friends or from local markets. Annually, a certain quantity of seeds are determined per variety and retained on farm from the new harvest to serve for the next sowing. The sowing and harvest time varies among producing areas and depends on the altitude. In the north area of Shangri-la region where the altitude is below 2,800 m, the types of hulless barley cultivated are winter cultivars. The sowing time here is in November and the harvest time is in next May. In southern area of Shangri-la region where the altitude is above 2,800 m, the types of hulless barley cultivated are spring cultivars. The sowing time here is in March and the harvest time is in October. The different altitudes and the accompanying differences in climates have resulted in differences in time of maturation. Winter cultivars are early maturing and have a low yield in the area with a high altitude. On the contrary, spring cultivars are late maturing and have low yield in the area with an altitude below 2,800 m. Since different cultivars adapt to climates of different altitudes, the diversity of hulless barley landraces was necessary to meet the local Tibetan's consumption. *Gst* analysis showed that the degree of diversity was greater within than among populations. The genetic distance and genetic identity analyses also showed there were close relationships among the three barley populations. Zhang et al. (2009) analyzed the relatedness of hulless barley which was originated from six areas of Yunnan province. The *Gst* based on SSR analysis was 0.1917 and showed that the majority of the variation was within the populations. The introducing of the barley among the different areas may explain the greater variation within the populations. In this study, all of the barley collected from Weixi was winter cultivars and most of barley collected from Shangri-la and Deqin was spring cultivars, but the population of Weixi had close genetic relationships with the other two populations rather than significant differences with them. The practices of exchanging seeds and seeds trades in the markets maintained a high seeds flow systems among the three counties. So it can explain their close genetic relationships and the high diversity within populations. This also indicated the barley cultivars in Shangri-la region had a flexible adaptability to the temperature.

We concluded that there was high genetic diversity of the barley resource in Shangri-la region and the formation of diversity was related to complex environment and inhabitants' traditional practices. However, the evaluation of genetic diversity on molecular level was just a basic work. The DNA marker polymorphism and variation of agromorphological traits considered together would indisputably help the breeder to diversify the sources of germplasm and optimise the choice of parents to be used in crossing programs. For the utilization of the resources in breeding, the agronomic examination of this materials is in.

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