RESEARCH ARTICLE

# Genetic diversity of *Ustilago hordei* in Tibetan areas as revealed by RAPD and SSR

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**Abstract**

Covered smut, which is caused by *Ustilago hordei* (Pers.) Lagerh., is one of the most damaging diseases of highland barley (*Hordeum vulgare* Linn. var. nudum Hook. f) in Tibetan areas of China. To understand the molecular diversity of *U. hordei*, a total of 27 isolates, which were collected from highland barley plants from Tibet, Sichuan, Qinghai, and Gansu provinces/ autonomous region, were analyzed using random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers. Among the 100 RAPD primers used, 24 primers exhibited polymorphism. A total of 111 fragments were amplified, of which 103 were polymorphic with a polymorphic rate of 92.79%. The average observed number of alleles (*Na*), effective number of alleles (*Ne*), Nei's genetic diversity (*H*), Shannon's information index (*I*) and polymorphism information content (*PIC*) value in the RAPD markers were 1.9279, 1.5016, 0.2974, 0.4503, and 0.6428, respectively. For the SSR markers, 40 of the 111 primer pairs exhibited polymorphism and provided a total of 119 bands, of which 109 were polymorphic and accounted for 91.60% of the total bands. The *Na*, *Ne*, *H*, *I* and *PIC* values of the SSR markers were 1.9160, 1.4639, 0.2757, 0.4211, and 0.4340, respectively. The similarity coefficients ranged from 0.4957 to 0.9261 with an average of 0.7028 among all the 27 isolates used. The dendrogram, which was developed based on the RAPD and SSR combined marker dataset showed that the 27 *U. hordei* isolates were divided into 3 clusters at similarity coefficient of 0.7314. We determined that RAPD and SSR markers can be successfully used to assess the genetic variation among *U. hordei* isolates. The RAPD markers revealed higher levels of genetic polymorphism than did the SSR markers in this study. There existed moderate genetic difference among isolates. The molecular variation and differentiation was somewhat associated with geographical origin but not for all of the isolates.

**Keywords:** highland barley, *Ustilago hordei*, RAPD, SSR, genetic diversity

## 1. Introduction

The Qinghai-Tibet Plateau (containing all of the Tibet Autonomous Region and Qinghai Province, the west of Sichuan Province, south of the Xinjiang Uygur Autonomous Region, and part of Gansu and Yunnan provinces) is the largest plateau in China and is the highest in the world with an av-

erage elevation of 4 000–5 000 m.  The specific geographic condition causes a distinctive agricultural ecosystem and species diversity in the Qinghai-Tibet Plateau.  Highland barley (*Hordeum vulgare* Linn. var. nudum Hook.f), also called hulless barley, is one of the most important crops in Tibetan areas of China.  The cultivated area of highland barley accounts for 60% of the total grain acreage, and the yield accounts for more than 55% of the total grain output (Lou 2000).  The covered smut of barley (*H. vulgare* L.), which is caused by *Ustilago hordei* (Pers.) Lagerh., occurs everywhere that barley grows.  The occurrence of covered smut is 10% generally and can even reach 60%.  Economic loss is due to not only a decreased yield but also teliospore contamination of healthy seeds (Mathre 1997).  Hulless barley cultivars are more susceptible to covered smut than hulled barley (Grewal *et al.* 2006).  The most efficient method to control covered smut is planting resistant cultivars. However, the genetic variation in the pathogen population reduces the efficiency of host resistance during disease management.  Therefore, the knowledge of the variability of the pathogen population is significant for determining disease management strategies.

Variations in fungal pathogens have been traditionally characterized based on virulence, mating type, cultural characteristics, sporulation, morphology, host-parasite physiological and biochemical interaction and disease reaction on differential hosts.  The *U. hordei* population has a variation in virulence, and it has been reported that there was differentiation in various aspects of the *U. hordei* races.  Five races were first identified by Faris (1924a, b).  Afterwards, many researchers identified *U. hordei* races in Canada (Aamodt and Johnston 1935; Semeniuk 1940), America (Tapke 1937, 1945), and China (Yu 1940; Yu and Fang 1945).  Other differences among the isolates were also studied.  Gaudet and Kiesling (1991) studied the aggressiveness and disease severity of 13 *U. hordei* races and isolates on Hannchen, Nepal and Odessa barleys, and they found significant variations in the head sorus type and peduncle compaction rating but none in the plant dwarfing.  Hellmann and Christ (1991) examined 63 haploid *U. hordei* isolates from Ethiopia and North Dakota in the USA, for isozyme variation using starch gel electrophoresis.  The results revealed that the isozyme variation was inconsistent with the pathogenicity, and they demonstrated that isozymes and buffer systems could not be used solely to identify *U. hordei* races.  McCluskey and Mills (1990) used electrophoretic karyotypes to detect the variation of monosporidial strains that represented 14 *U. hordei* races.  Their results implied that the chromosome length polymorphism and electrophoretic pattern of the total soluble proteins could not be efficiently used to differentiate the strains of *U. hordei*.

Morphological, physiological, and biochemical studies are

time-consuming and can be affected by the environment; thus, they are not very precise.  However, a molecular marker, especially a DNA marker, can test the genetic variation of a material, which is more essential and accurate than other markers (Weising *et al.* 1995).  Random amplified polymorphic DNA (RAPD), which is based on PCR, is a molecular technique that is able to analyze the unknown sequence of the entire genome for polymorphism.  Because of its richness of polymorphisms and high individual specific and environmental stability, RAPD has been widely used to analyze the genetic diversity of various species (Fernández *et al.* 2002; Shang and Song 2005; Du *et al.* 2011; Hu *et al.* 2012).  Simple sequence repeat (SSR) has been used for fingerprinting, genetic diversity, genetic linkage mapping, and the quantitative trait locus (QTL) and population genetic structure studies because of its high number of available polymorphisms, good repeatability, co-dominance, and ubiquitous occurrence (Ellegren 2004).  Recently, SSR has been used for studying plant fungal pathogens, such as *Venturia inaequalis* (Hu *et al.* 2008), *Magnaporthe grisea* (Shen *et al.* 2004), *Phytophthora infestans* (Zhao *et al.* 2008), and *Rhynchosporium secalis* (Bouajila *et al.* 2007).  To our knowledge, *U. hordei* fingerprinting and genetic diversity studies that are based on DNA markers have not been conduct.  Thus, the present study was designed to study the genetic variation of the *U. hordei* isolates collected from the Tibetan areas of China.

## 2. Materials and methods

### 2.1. Isolates

A total of 27 *U. hordei* isolates were collected from different regions of Tibetan areas (Tibet, Sichuan, Qinghai and Gansu provinces/autonomous region) in China.  The geographic origins and host lines of the isolates are listed in Table 1, and the distribution of these areas is shown on Fig. 1.  Teliospores were collected from smutted ears of naturally-infected barley, suspended in sterile water, and smeared on PDA medium and incubated at 25°C for 3 d. Individual haploid isolates were obtained as described by McCluskey and Mills (1990).

### 2.2. DNA extraction

The method reported by Karwasra *et al.* (2002) was adapted for the DNA extraction.  The concentration of DNA was determined using a spectrophotometer.  The DNA was diluted to 100 ng $\mu L^{-1}$ and stored at –20°C.

### 2.3. RAPD-PCR amplification

A total of 100 decamer primers were obtained from Shang-

**Table 1** Geographic origin and host line of the *Ustilago hordei* isolates

| Isolate | Geographic origin | Host line | Isolate | Geographic origin | Host line |
|---|---|---|---|---|---|
| 1 | Ganzi, Sichuan | Aqing 5 | 15 | Lhunzhub, Tibet | Zangqing 690 |
| 2 | Danba, Sichuan | Aqing 4 | 16 | Damxung, Tibet | Zangqing 690 |
| 3 | Daofu, Sichuan | Aqing 5 | 17 | Nimu, Tibet | Zangqing 690 |
| 4 | Kangding, Sichuan | Kangqing 5 | 18 | Mozhugongka, Tibet | Zangqing 690 |
| 5 | Jiulong, Sichuan | Kangqing 5 | 19 | Qushui, Tibet | Zangqing 690 |
| 6 | Luhuo, Sichuan | Aqing 4 | 20 | Gyangze, Tibet | Ximala 22 |
| 7 | Xinlong, Sichuan | Aqing 4 | 21 | Shigatse, Tibet | Ximala 22 |
| 8 | Yajiang, Sichuan | Aqing 5 | 22 | Langkazi, Tibet | Ximala 19 |
| 9 | Qamdo, Tibet | Zangqing 320 | 23 | Gongbo'gyamda, Tibet | Zangqing 25 |
| 10 | Gonjo, Tibet | Zangqing 320 | 24 | Nyingchi, Tibet | Zangqing 25 |
| 11 | Jomda, Tibet | Zangqing 320 | 25 | Wulan, Qinghai | Chaiqing 1 |
| 12 | Luolong, Tibet | Zangqing 320 | 26 | Dulan, Qinghai | Chaiqing 1 |
| 13 | Markam, Tibet | Zangqing 320 | 27 | Xiahe, Gansu | Ganqing 1 |
| 14 | Baxoi, Tibet | Zangqing 320 | | | |



**Fig. 1** Map of sampling sites.

hai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China) and diluted to 2.5 ng µL⁻¹. RAPD-PCR was performed in 10 µL reaction volumes that contained 1 µL of 10× buffersolution, 0.8 µL of 25 mmol L⁻¹ MgCl₂, 0.2 µL of 10 mmol L⁻¹ dNTP, 0.1 µL of 100 ng µL⁻¹ template DNA, 4 µL of 2.5 ng µL⁻¹ primer, 0.1 µL of 5 U µL⁻¹ *Taq* polymerase, and 3.8 µL of ddH₂O.  Amplification was performed using an Eppendorf Mastercycler (Eppendorf Netheler-Hinz GMBH, Germany), that was programmed, for initial denaturation at 94°C for 5 min, 50 cycles of 94°C for 40 s, 48°C for 70 s, and 72°C for 2 min.  Amplification was completed with a 10-min final extension at 72°C.  The amplified DNA samples were separated on 1.0% agarose gel at 120 V using 1× TAE.  The gels were stained with ethidium bromide and photographed under UV light.

### 2.4. SSR-PCR amplification

The genomic sequences of *U. hordei* were downloaded

from Genbank (USA, http://www.ncbi.nlm.nih.gov/genbank) (Laurie *et al.* 2012).  SSR loci were screened using the MISA program (http://pgrc.ipk-gatersleben.de/misa/) with the default parameters.  Compound SSRs were defined as ≥2 SSRs interrupted by ≤100 bases (Sonah *et al.* 2011).  Then 111 randomly selected primer pairs were synthesized (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China).  SSR-PCR was performed in 10 µL reaction volumes that contained 1 µL of 10× buffersolution, 0.8 µL of 25 mmol L⁻¹ MgCl₂, 0.2 µL of 10 mmol L⁻¹ dNTP, 0.1 µL of 100 ng µL⁻¹ template DNA, 1 µL of 2.5 ng µL⁻¹ primers mix, 0.05 µL of 5 U µL⁻¹ *Taq* polymerase, and 6.85 µL of ddH₂O.  The amplification was conducted using an Eppendorf Mastercycler (Eppendorf Netheler-Hinz GMBH, Germany).  PCR amplification was adopted, including initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and a 10-min final extension at 72°C.  The PCR products were electrophoresed in a vertical 6% polyacrylamide gel with 1× TBE and stained using a 0.1% AgNO₃ solution.

### 2.5. Data analysis

Each fragment of the sampled *U. hordei* isolates that were obtained from the RAPD and SSR analyses was scored as a binary variable of 1 or 0 to indicate the presence or absence of the fragment, respectively.  Popgene 32 software was used to calculate the observed number of alleles ($Na$), effective number of alleles ($Ne$), Nei's genetic diversity ($H$), and Shannon's information index ($I$).  The polymorphism information content ($PIC$) value of the individual primer was calculated based on the formula: $PIC=2\times F(1-F)$ (Anderson *et al.* 1993), where $F$ is the frequency of marker fragments that were present, and $1-F$ is the frequency of marker fragments that were absent.  $PIC$ values greater than 0.5 indicate loci with a high polymorphism, values between 0.25

and 0.5 indicate loci with intermediate polymorphism, and values lower than 0.25 indicate loci with low polymorphism (Lamine and Mliki 2015). The genetic similarity coefficient was calculated using NTSYS 2.02 software. The correlation of similarity matrices, which was obtained from the RAPD and SSR profiles, was judged using two-way Mantel test (Mantel 1967) using MxComp Module of NTSYS 2.02. A dendrogram (phylogenetic tree) was constructed based on unweighted pair group method using arithmetic averages (UPGMA) using NTSYS 2.02 software. Bootstrap analysis with 1 000 simulated samples was conducted using MEGA 6.06. Principal coordinate analysis (PCoA) was also performed using NTSYS 2.02 software.

## 3. Results

### 3.1. RAPD analysis

100 random decamer oligonucleotide primers were used and 24 of them were polymorphic (Table 2). The number of amplified bands per primer ranged from 2 (S407) to 8 (S71 and S284) with an average of 4.6 bands per primer. A total of 111 bands amplified, of which 103 bands were polymorphic and the polymorphic rate was 92.79%. The fragments varied in size from 150 to 2 000 bp. The amplification profile of one of the primers is presented in Fig. 2. The average $Na$, $Ne$, $H$ and $I$ values were 1.9279, 1.5016, 0.2974, and 0.4503, respectively. The highest $PIC$ value (0.9409) was observed for primer S284 and the lowest $PIC$

**Table 2** Polymorphic profile and characteristics of the 24 random amplified polymorphic DNA (RAPD) primers

| Primer | Sequence (5´→3´) | Bands | Polymorphic bands | $PIC^{1)}$ |
|---|---|---|---|---|
| S24 | AATCGGGCTG | 4 | 3 | 0.2817 |
| S27 | GAAACGGGTG | 6 | 5 | 0.7217 |
| S28 | GTGACGTAGG | 7 | 7 | 0.9164 |
| S29 | GGGTAACGCC | 5 | 4 | 0.6356 |
| S30 | GTGATCGCAG | 3 | 2 | 0.2817 |
| S38 | AGGTGACCGT | 3 | 2 | 0.2407 |
| S39 | CAAACGTCGG | 4 | 4 | 0.7621 |
| S40 | GTTGCGATCC | 3 | 3 | 0.4942 |
| S62 | GTGAGGCGTC | 6 | 6 | 0.8581 |
| S65 | GATGACCGCC | 5 | 5 | 0.8622 |
| S68 | TGGACCGGTG | 4 | 4 | 0.8493 |
| S71 | AAAGCTGCGG | 8 | 8 | 0.9015 |
| S282 | CATCGCCGCA | 4 | 3 | 0.4708 |
| S284 | GGCTGCAATG | 8 | 8 | 0.9409 |
| S286 | AAGGCTCACC | 5 | 5 | 0.7693 |
| S287 | AGAGCCGTCA | 6 | 6 | 0.8378 |
| S288 | AGGCAGAGCA | 5 | 4 | 0.6815 |
| S289 | AGCAGCGCAC | 3 | 2 | 0.3681 |
| S293 | GGGTCTCGGT | 3 | 3 | 0.4544 |
| S294 | GGTCGATCTG | 4 | 4 | 0.6926 |
| S401 | GTTGGTGGCT | 3 | 3 | 0.5612 |
| S407 | CCGTGACTCA | 2 | 2 | 0.4175 |
| S413 | GGTGGTCAAG | 6 | 6 | 0.6735 |
| S414 | AGGGTCGTTC | 4 | 4 | 0.7550 |
| Mean | | 4.6250 | 4.2917 | 0.6428 |

[1] $PIC$, polymorphism information content.

value (0.2407) was recorded for primer S38. The average $PIC$ value was 0.6428.
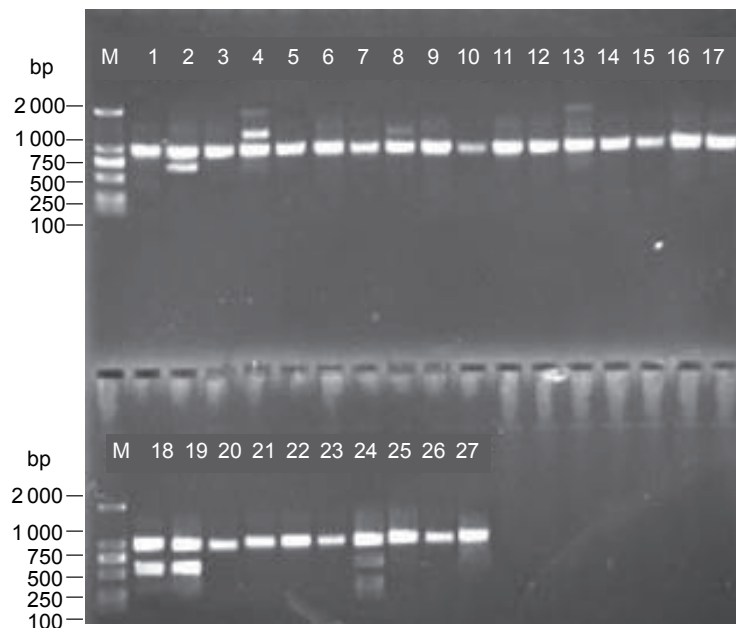


**Fig. 2** Random amplified polymorphic DNA (RAPD) profiling of *U. hordei* isolates with primer S38 (M, DL 2000 DNA marker; lanes 1–27, *U. hordei* isolates).

## 3.2. SSR analysis

In this analysis, 111 pairs of primers were used to amplify all of the genotypes, of which 40 provided reproducible and well-resolved bands (Table 3). The highest number of SSR bands was recorded for primers U47 and U108 (6 bands), followed by U30 and U88 (5 bands), whereas most of the other primers scored low (2 bands). There were 2.975 average bands amplified per primer. These primers produced 119 bands, of which 109 were polymorphic bands, which accounted for 91.60% of the total bands. The amplification profile of one of the primers is presented in Fig. 3. The average $Na$, $Ne$, $H$, and $I$ values were 1.9160, 1.4639, 0.2757, and 0.4211, respectively. Primers U88 exhibited the highest $PIC$ value (0.7502), and primers U15, U18, U27, and U93 exhibited the lowest $PIC$ value (0.0688). The average $PIC$ was 0.4340.

## 3.3. Cluster analysis

RAPD and SSR data sets were combined together to determine the genetic relationship between the different isolates. The correlation between the RAPD and SSR similarity coefficient matrices was 0.9249. The similarity coefficients, which were calculated based on the combined RAPD and SSR data, ranged from 0.4957 to 0.9261 with an average of 0.7028 among all of the 27 isolates. The nearest genetic distance occurred between isolates 22 and 21, which were collected from Langkazi and Shigatse, Tibet, and between isolates 22 and 15, which were collected from Langkazi and Lhunzhub, Tibet. The largest genetic distance occurred between isolates from Baxoi and Gyangze, Tibet. All above indicated that there was a moderate genetic difference among isolates.

The genetic relationship between the isolates is shown in the dendrogram which was obtained using UPGMA. The correlation coefficient between the dendrogram and its similarity matrix was 0.9355 indicating that the dendrogram may be a good representation of the genetic relationship. The dendrogram showed that all the isolates were divided into 3 clusters at the similarity coefficient of 0.7314 (Fig. 4). Cluster I consisted of 10 isolates, of which 2 isolates were collected from Sichuan Province and 8 isolates were collected from Tibet. Cluster II included 11 isolates, of which 5 isolates were collected from Sichuan Province, 3 isolates were from Tibet, 2 isolates were from Qinghai Province, and 1 isolate was from Gansu Province. There were 6 isolates grouped into cluster III, with 1 isolate from Sichuan Province and 5 from Tibet.

The result of the PCoA was consistent with the UPGMA

**Table 3** Sequences of the simple sequence repeat (SSR) primers that were used in this study with the repeat motif, product size, bands, polymorphic bands and $PIC$

| Primers | Sequence (5′→3′) | Repeat motif | Product size | Bands | Polymorphic bands | $PIC$ |
|---|---|---|---|---|---|---|
| U15 | F: CTATTGACCTGGTTGGAG | $(AT)_7$ | 203 | 2 | 2 | 0.0688 |
| | R: TACTGTTTGCTGCTGTTG | | | | | |
| U16 | F: GAGCAGCGGGAAAACATA | $(AC)_7$ | 129 | 2 | 1 | 0.2205 |
| | R: TGAGAAGAAAGCGAAACT | | | | | |
| U18 | F: AACAGCAGCAGCCACCAG | $(CA)_7$ | 111 | 2 | 1 | 0.0688 |
| | R: AAGGCAAGAAGAAACAAGTCAA | | | | | |
| U19 | F: GACAGTTAGAATGGGATGAG | $(AGG)_8$ | 281 | 3 | 3 | 0.5561 |
| | R: TGACCGTGCCACTTGAGA | | | | | |
| U20 | F: ACAGAACGCTGCCCAAGT | $(AG)_{18}$ | 154 | 2 | 2 | 0.3719 |
| | R: TGCTGCGACAAAGTGAAA | | | | | |
| U21 | F: GAGCACGACCATAGCCGAGGGA | $(GAG)_7$ | 187 | 3 | 3 | 0.4230 |
| | R: TGGGCAAAGCAACCACAACAGG | | | | | |
| U26 | F: GACCGCACGACAAGCAGA | $(TG)_6$ | 207 | 2 | 1 | 0.2205 |
| | R: CCTTCATCCATCAACCACC | | | | | |
| U27 | F: CTGGTGGAAGATGTTGCT | $(CA)_6$ | 105 | 2 | 1 | 0.0688 |
| | R: CTCTGACTTGCGTGTTGTA | | | | | |
| U29 | F: TTGAAGAGGGTGATAGGTG | $(CT)_{14}$ | 261 | 3 | 3 | 0.7437 |
| | R: TGGGTGGAGAATGAGTGA | | | | | |
| U30 | F: TGGTAGTTGGTGCTTGCC | $(TTG)_9$ | 181 | 5 | 5 | 0.5389 |
| | R: GCTGCTGATGGGAGTGATA | | | | | |
| U32 | F: GCCGTTGGTCTTGCGAATA | $(GCT)_8$ | 104 | 2 | 1 | 0.3747 |
| | R: GAGGCAGGTTGAGATGGGAT | | | | | |
| U33 | F: CCTGTAGGCGTCTGTCTCG | $(GAG)_{11}$ | 253 | 3 | 3 | 0.4986 |
| | R: AGCGTCTTCGCATTCTCG | | | | | |

(Continued on next page)

**Table 3** (Continued from preceding page)

| Primers | Sequence (5′→3′) | Repeat motif | Product size | Bands | Polymorphic bands | *PIC* |
|---|---|---|---|---|---|---|
| U34 | F: TTTAGGGATGCGGATGCT | $(TG)_{12}$ | 209 | 2 | 2 | 0.4656 |
|  | R: CGAGGTATGCGATGTTGT |  |  |  |  |  |
| U37 | F: TCATCCCGCTGGAAACAT | $(ACC)_9$ | 261 | 2 | 2 | 0.5435 |
|  | R: GAGGTGGAAGAGGACTGG |  |  |  |  |  |
| U40 | F: CGAGGATGGGAGATGGTG | $(CT)_8$ | 154 | 2 | 2 | 0.2205 |
|  | R: GGCTCTGGCTCAGGAACA |  |  |  |  |  |
| U42 | F: GCCGTTGTTCCTGTTGCC | $(TGG)_{10}$ | 227 | 2 | 2 | 0.5898 |
|  | R: GGGATGGTGAGATGGGTAGTT |  |  |  |  |  |
| U44 | F: ATCAAGCAGCACAAGGAG | $(TC)_8$ | 162 | 2 | 1 | 0.1278 |
|  | R: GAGACATTGCGATTAGAAAA |  |  |  |  |  |
| U46 | F: CATCAATCTCGGCGTCTG | $(GGA)_6$ | 203 | 4 | 4 | 0.6815 |
|  | R: ATGCTGGGAATGAGGAAC |  |  |  |  |  |
| U47 | F: GCGATGGAGGATTGGACG | $(AC)_7$ | 183 | 6 | 6 | 0.5474 |
|  | R: GATGGAGCGATGGCAGAT |  |  |  |  |  |
| U51 | F: CTGCGTGAGTTGGTGCTT | $(TGT)_{16}$ | 266 | 2 | 2 | 0.5030 |
|  | R: GATTGTCGGCTCCTTCCT |  |  |  |  |  |
| U56 | F: TTGGGAGCAGAAGAAGGT | $(TCC)_6$ | 102 | 2 | 2 | 0.3301 |
|  | R: GATGTCGGAAGCAGGGTT |  |  |  |  |  |
| U57 | F: AAGACCCTTGCTTGAACT | $(CT)_{13}$ | 251 | 2 | 2 | 0.5227 |
|  | R: TGGTGGGATGGTAGAATA |  |  |  |  |  |
| U58 | F: GCGTCGCCATCCATTCCT | $(TCC)_8$ | 117 | 4 | 4 | 0.6124 |
|  | R: TCCGTGCGTAAGACCAAA |  |  |  |  |  |
| U60 | F: CGACAGGAACGCAAGTAA | $(TCC)_6$ | 185 | 3 | 3 | 0.4942 |
|  | R: CGTATCTCGCAGGATGGTAT |  |  |  |  |  |
| U65 | F: TCTCGTTTGTTTGCGGTTTC | $(CTC)_6$ | 181 | 4 | 4 | 0.6635 |
|  | R: AGCCCTGTTCTGGCGTTG |  |  |  |  |  |
| U75 | F: CGGCAATAAGCGAAAGAA | $(GGT)_9$ | 106 | 3 | 3 | 0.5513 |
|  | R: AATAGGAGCACCAGCATCAG |  |  |  |  |  |
| U80 | F: CAAAGCCGAAGCATAAGAA | $(GCA)_8$ | 207 | 4 | 4 | 0.6294 |
|  | R: GGCGGTAGGATTGTAGCA |  |  |  |  |  |
| U81 | F: CAAAGCCGAAGCATAAGAA | $(GCA)_8$ | 215 | 4 | 4 | 0.7233 |
|  | R: AGAAGGAAGGCGGTAGGA |  |  |  |  |  |
| U83 | F: GATGGAAGAGGGAAGGAA | $(GT)_8$ | 205 | 4 | 4 | 0.6961 |
|  | R: AGGCAGGTGACTGAAGAA |  |  |  |  |  |
| U85 | F: GAAGGTCGCACTCGGAAGG | $(TCA)_9$ | 257 | 3 | 2 | 0.1352 |
|  | R: TGGAGGCTCGCAAAGAAC |  |  |  |  |  |
| U88 | F: TCATTCACGCACAGCACA | $(TC)_8$ | 196 | 5 | 5 | 0.7502 |
|  | R: AGACAAGCAAACGCAACG |  |  |  |  |  |
| U89 | F: TGGACGACAATCAGAAGGG | $(CAA)_6$ | 224 | 3 | 3 | 0.2541 |
|  | R: GATGCGTGGTGGGAGGTA |  |  |  |  |  |
| U92 | F: GTTACTGCGGCGACTGGA | $(GTT)_7$ | 211 | 2 | 2 | 0.4247 |
|  | R: ATGATGGGCGGAATGGGT |  |  |  |  |  |
| U93 | F: CGTAGAGCAGCACAAAGAT | $(CTT)_6$ | 106 | 2 | 1 | 0.0688 |
|  | R: AACTAACCTAACCCTGGAAC |  |  |  |  |  |
| U95 | F: ACGCAGAGCACTGGTCAC | $(GA)_{13}$ | 304 | 2 | 1 | 0.2562 |
|  | R: TACGAGGGTTCCTTTGTC |  |  |  |  |  |
| U96 | F: GGATTGGCGATGGAGAAA | $(TCC)_6$ | 276 | 4 | 4 | 0.4008 |
|  | R: CTATGAGCGGGTGGTGGA |  |  |  |  |  |
| U101 | F: ACGCACATTCCAATCTCG | $(CT)_8$ | 241 | 3 | 3 | 0.4016 |
|  | R: GTTCAATACTCACCCTCCAT |  |  |  |  |  |
| U103 | F: GACGGGAAGAGGGATGAA | $(AG)_6$ | 285 | 3 | 3 | 0.5435 |
|  | R: CAGCAACTCCGAGACCAA |  |  |  |  |  |
| U108 | F: ACGAGGGTGGCACGATGT | $(CAC)_8$ | 118 | 6 | 6 | 0.4137 |
|  | R: AGCGAGGAAAGTGAGCAAAG |  |  |  |  |  |
| U110 | F: AGGTGGAAACAATGTGGAG | $(AGC)_8$ | 254 | 3 | 3 | 0.6535 |
|  | R: AGTGAACGAGTCGGATGAG |  |  |  |  |  |
| Mean |  |  |  | 2.975 | 2.75 | 0.4340 |
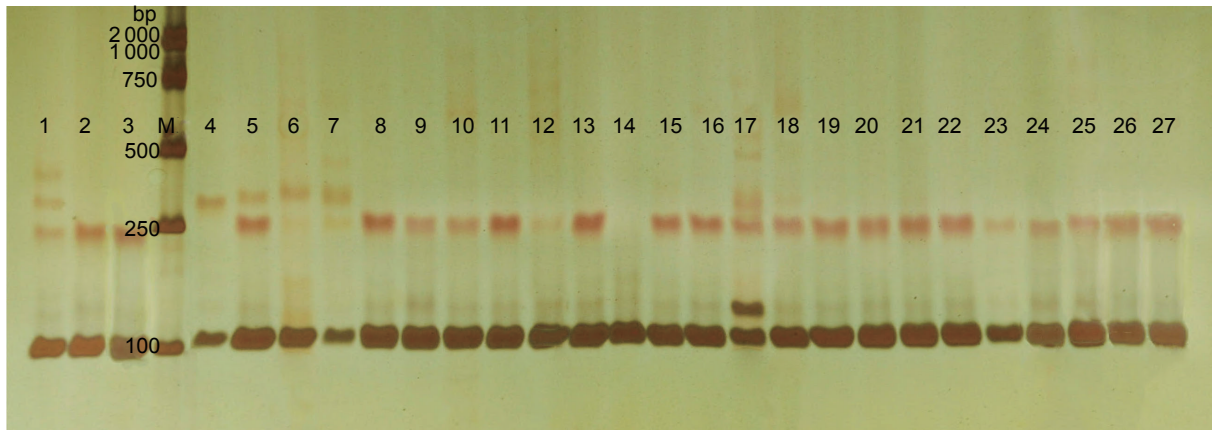
**Fig. 3**  Simple sequence repeat (SSR) profiling of *U. hordei* isolates with primer U8 (M, DL 2000 DNA marker; lanes 1–27, *U. hordei* isolates).
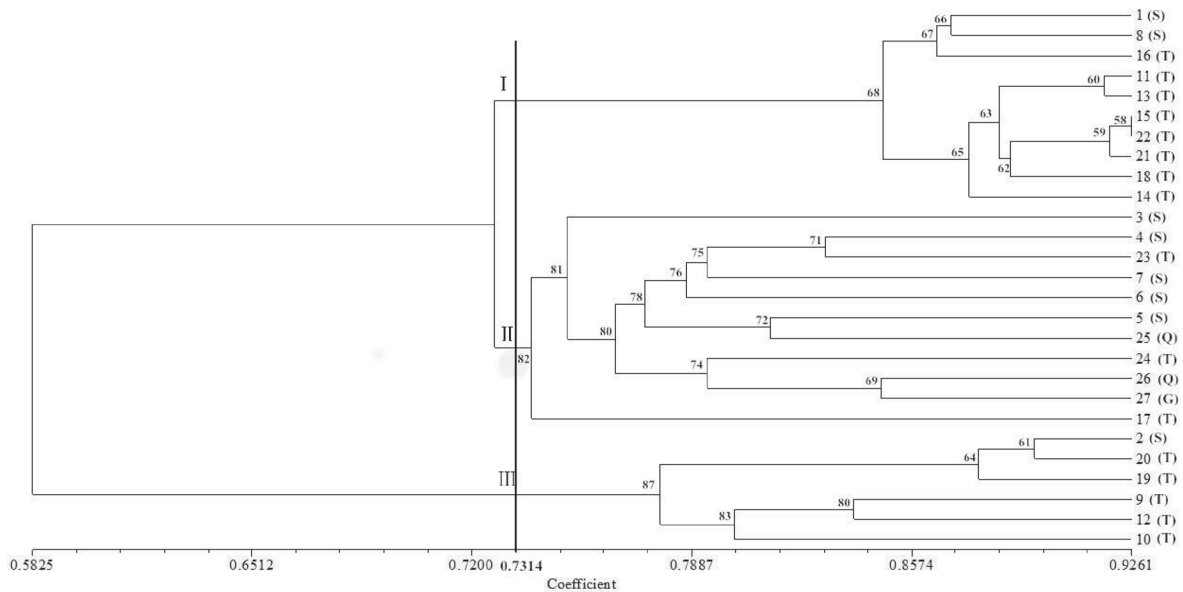


**Fig. 4**  Clustering of 27 *U. hordei* isolates based on pooled RAPD and SSR markers.  Bootstrap values were obtained from 1 000 replications, and bootstrap values that were <50% were collapsed.  Capital letters (S, T, Q, and G) in parentheses represent Sichuan, Tibet, Qinghai, and Gansu provinces/autonomous region, respectively.

clustering analysis.  The grouping in the dendrogram was consistent with that shown in the 3D scatterplot (Fig. 5).  The PCoA also divided all of the isolates into 3 groups.  The first 3 principal coordinates accounted for 50.33% (30.85, 13.33 and 6.14% by 1st, 2nd, and 3rd principal coordinate, respectively) of the total variation.  Both the cluster analysis and PCoA indicated that there was some diversity among the *U. hordei* isolates in the Tibetan areas.

## 4. Discussion

This study revealed that RAPD and SSR markers may be successfully used to assess genetic variation among *U.*

*hordei* isolates.  Although RAPD was inefficient in detecting the genetic variation among the isolates of the loose smut fungus *Ustilago tritici* (Karwasra *et al.* 2002), it has been widely used for detecting the genetic variation among isolates of *Sporisorium reilianum* (Xu *et al.* 2003; He *et al.* 2007; Jiang *et al.* 2007; Ma *et al.* 2008).  Karaoglu *et al.* (2005) demonstrated that SSR could be useful for population genetics of fungal organisms.  In this study, RAPD markers revealed higher levels of genetic polymorphism than did the SSR markers.  The RAPD markers exhibited 92.79% polymorphic fragments compared with 91.60% polymorphism detected by the SSR markers.  The average observed number of alleles (*Na*), effective number of alleles
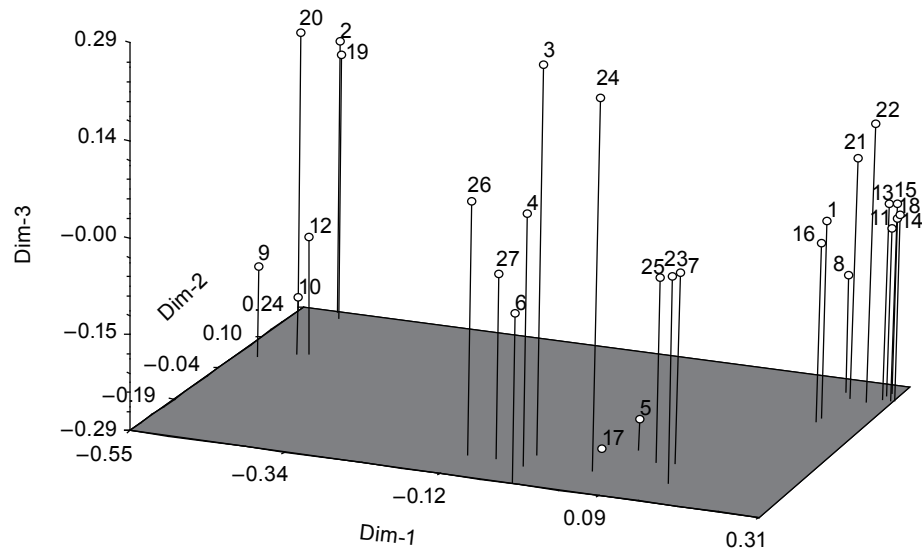
**Fig. 5** Three dimensional PCoA scaling of the 27 *U. hordei* isolates using RAPD and SSR markers.

($Ne$), Nei's genetic diversity ($H$), and Shannon's information index ($I$) of the RAPD markers were all higher than those of the SSR markers. *PIC* as a feature of a primer is an ideal index to measure polymorphism. The *PIC* value for the RAPD markers (0.6428) was also higher compared with the SSR primer pairs (0.4340), indicating a higher polymorphism of RAPD loci than in the SSR loci. The different resolutions of the two markers may be due to different portions of the genome that these two techniques targeted. The RAPD markers distribute randomly throughout the entire genome to reveal the diversity of the entire genome, whereas SSR markers, which were designed on the basis of sequences of some *U. hordei* genes (Laurie *et al.* 2012), were only amplified at some particular region on the genome.

Measures of the genetic diversity in fungi, especially ascomycetes and basidiomycetes producing spores with potential for long-distance dispersal, often show a low level of differentiation (Hamelin *et al.* 1995). Zhou *et al.* (2008) determined a low level of genetic variation in 110 *Ustilaginoidea virens* isolates that were collected from North China. Amplified fragment length polymorphism (ALFP), which was used by Braithwaite *et al.* (2004), revealed a low level of genetic variation among 31 *Ustilago scitaminea* isolates. Additionally, Singh *et al.* (2005) demonstrated a limited diversity amongst geographically separate strains of *U. scitaminea*. Hellmann and Christ (1991) examined the isozyme variation of 63 *U. hordei* haploid isolates and observed a low allelic diversity of 0.189. In this study, the average similarity coefficient was 0.7028, indicating a moderate genetic variation among all of the 27 isolates. This may be attributed to the frequent exchange of highland barley varieties in these areas in recent years, which is important

because covered smut is a type of seed-borne disease. The clustering patterns generated by RAPD and SSR markers were different from each other. To best interpret the genetic relationships of the isolates, the genotyping data obtained using the RAPD and SSR markers were combined and used to construct the dendrogram. Cluster and PCoA analyses of the combined data showed the genetic relationship among the isolates. The isolates collected from Tibet were primarily clustered into cluster I and cluster III. The isolates collected from Sichuan Province were mainly included in cluster II. This result suggested that the molecular variation and differentiation was associated somewhat with geographical origin but not for all of the isolates. *U. hordei*, a heterothallic basidiomycete fungus, favors a high level of inbreeding, and only rarely is there opportunity for out-crossing (Fischer and Holton 1957). Therefore, the exchange of seed in these areas only caused a restricted gene exchange. Xu *et al.* (2004) also observed the phenomenon of different geographical origins with small dissimilarity coefficients in some *U. scitaminea* isolates.

Planting seeds that possessing a genetic resistance is the most effective and economical measure to control covered smut. However, symptoms of the covered smut are not visible until heading and natural or artificial inoculation occurs that typically results in inconsistent infection, even in highly susceptible lines. Thus, breeding for resistance to covered smut is time-consuming and difficult. Additionally, the existence of physiologic races in *U. hordei* also increases the difficulty of resistance breeding. Therefore, knowledge of the physiologic specialization in *U. hordei* is particularly important for predicting which resistance genes will likely be effective and stable once incorporated into new host

varieties. However, virulence is not a "neutral" trait because it is strongly influenced by host selection, and the genes involved represent only a small portion of the pathogen's genome (Caten 1987). The information obtained may not reflect the total genetic variability of the pathogen population. Therefore, it is necessary to combine molecular variation and virulence variability in the pathogen isolates. Meyer *et al.* (2005) examined patterns of genetic variation for virulence and for AFLP markers in four *Ustilago bullata* populations of *Bromus tectorum* in northern Utah and southern Nevada. Popovic and Menzies (2006) measured the genetic diversity of *U. tritici* isolates using virulence data and AFLP, and their AFLP analysis indicated a higher degree of genetic variability than did the virulence data. In this study, the *U. hordei* isolates were wild samples that were collected from the smutted head of infected highland barley plants. We only observed natural infection in open fields where *U. hordei* isolates were collected. The virulence of each isolate was unknown and must be investigate by using differential host lines. Thus, the next step is to evaluate *U. hordei* isolates for race designation and combine virulence data with the molecular analysis.

## 5. Conclusion

This study illustrated the combined effectiveness of the RAPD and SSR techniques for discriminating the *U. hordei* genotypes that were analyzed. The RAPD and SSR data separated the isolates into three major genetic similarity groups. However, the isolates that were collected from Qinghai and Gansu Provinces were so small that the information on the genetic diversity of *U. hordei* in these provinces was insufficient and unreliable. Thus, it is necessary to collect more isolates and combine the pathogenicity analysis to determine the relationship between genetic variation and pathotypes. Regardless, this investigation is the first report to assess the genetic variation of *U. hordei* from China using RAPD and SSR markers. These findings provide valuable information for barley resistance breeding and plant resistance mechanisms.

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