

Discrimination of Tsingtaodahua from Other Hop Cultivars and Its Quality Control by Molecular Analysis

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ABSTRACT

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Random amplified polymorphic DNA (RAPD) was used for hop varietal identification, primarily to distinguish Tsingtaodahua, a fine Chinese variety. Eleven typical varieties, including four aroma hops, five bitter hops, Tsingtaodahua and Cluster, were successfully identified on the basis of 28 polymorphic RAPD bands amplified by five random primers. UPGMA analysis of RAPD data showed genetic relationship among analyzed varieties consistent with traditional hop classification. Subsequently, one specific RAPD fragment was converted to a sequence tagged site (STS) marker which can detect as little as a 5% admixture of the variety Kirin 1 in Tsingtaodahua. The RAPD and STS markers can be successfully used for Tsingtaodahua identification and quality control.

Keywords: Discrimination, hop, *Humulus lupulus*, RAPD, STS marker, Tsingtaodahua, quality control.

INTRODUCTION

Hop, *Humulus lupulus* L., is a dioecious perennial climbing plant cultivated mainly for beer production. Its female flowers, or cones, have lupulin glands that contain chemicals giving beer unique aroma and bitter flavor. Hop varieties can be generally divided into two classes, bitter hops and aroma hops³. As their name implies, bitter hop varieties are those that impart bitter flavor to beer and have high α acid levels. Aroma hops, with low to medium α acid levels, mainly impart characteristic hop aromas to beer. Tsingtaodahua is an excellent hop grown in China and an important raw material in Tsingtao Beer. Although Kirin 1 has richer α acid than Tsingtaodahua, its α acid content decreases faster during storage of hop cones. Along with its α acid decreasing, Kirin 1 produces a rancid smell, changing the aroma and bitterness of beer. However, in order to increase α acid content, Kirin 1 has been mixed with Tsingtaodahua in beer production due to cost advantages. In order to ensure the flavor and quality

of Tsingtao Beer, it was necessary to discriminate Tsingtaodahua from other hop cultivars and determine its purity.

The identification of hop varieties was previously based on their constituent components, e.g. bitter components (α acid/ β acid components) and essential oil components (farnesene, caryophyllene). These chemical methods have limitations in being laborious, time consuming and the amounts of secondary metabolites are influenced by the environmental factors. DNA fingerprinting is an appropriate alternative method of hop variety identification since DNA polymorphisms are environmentally independent. Hop varieties are asexually propagating clones with identical genetic materials⁹. DNA fingerprinting distinguishes different varieties according to their DNA variations at a set of genetic loci. A powerful and convenient molecular marker system, randomly amplified polymorphism of DNA (RAPD)¹⁰ has been widely used for identification of individual, cultivars or species⁸, and for genetic diversity analysis¹¹. However, because of random primers' short length, RAPD markers may produce some artificial amplification products. Therefore, careful control of DNA quality and amplification conditions is necessary to ensure reproducible banding patterns⁷. Additionally, RAPD markers converted to sequence tagged site (STS) markers have proved to be successful^{1,2}.

The objective of this research was to develop a rapid and effective DNA marker assay to discriminate purity of Tsingtaodahua with respect to Kirin 1 contamination and thus provide an efficient method for purity determination.

MATERIALS AND METHODS

Plant materials and DNA extraction

The hop varieties used in this study are listed in Table I. In order to isolate high quality genomic DNA from hop leaves or cones, the CTAB (cetyltrimethylammonium bromide) protocol with some modifications was used to extract hop genomic DNA⁴. In brief, freeze-dried leaves or hop cones were ground into powder in liquid nitrogen. After grinding, about 70 mg powder was transferred into an Eppendorf tube, into which, 1 mL preheated (65°C) 6× CTAB extraction buffer [6% CTAB (w/v), 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 mol/L NaCl, 2% mercaptoethanol, 5% PVP] was added. The mixture was gently inverted a few times and incubated at 65°C for 1 h. After cooling to room temperature, 0.5 mL chloroform/isoamyl alcohol (24:1) was

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added, mixed gently and centrifuged at 12000 rpm for 10 min. The supernatant was mixed with 1/10 volume of 10× CTAB solution [10% CTAB (w/v), 0.7 mol/L NaCl] and held at room temperature for 10 min. The solution was extracted with equal volume chloroform/isoamyl alcohol and the DNA was precipitated with 1× CTAB buffer [1% CTAB (w/v), 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 1% mercaptoethanol] at 60°C for 30 min. DNA was dissolved in 0.5 mL high salt TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0, 1.0 M NaCl) and precipitated again with 0.6 volume of cold isopropyl alcohol. DNA was rinsed twice with 70% ethanol, air-dried and dissolved in 50 µL TE containing 10 mg/mL RNase. The concentration and purity of DNA were determined by agarose gel electrophoresis and ultraviolet light. With this technique, the absorbance ratio (A_{260}/A_{280}) of DNA obtained from fresh and/or dried hop leaves or cones was 1.6–1.8. The DNA was stored at –20°C. DNA was diluted to 100 ng/µL with deionized distilled water for PCR reaction.

RAPD analysis

PCR reactions for RAPD amplification were optimized as follows. Amplification reactions were carried out in a 25 µL reaction mix containing 120 µM of each dNTP, 1.5 mM Mg²⁺, 0.4 µM primer (Table II), 2.5 µL 10× PCR buffer [100 mM KCl, 80 mM (NH₄)₂SO₄, 100 mM Tris-HCl, pH 9.0, 0.5% NP-40], 20 ng genomic DNA, 1.0 U of Taq polymerase (Bioasia, China). The PCR reaction was performed with a thermal cycler PTC-200 (MJ Research, Inc., USA) with the following temperature profile: DNA denaturation at 94°C for 4 min, followed by 35 cycles at

94°C for 15 s, 37°C for 30 s and 72°C for 1.5 min. The reaction was terminated with a final step of 10 min at 72°C. The amplification products were separated by gel electrophoresis on 1.5% agarose gels containing ethidium bromide (10 mg/mL) in 0.5× TBE buffer. Bands were visualized by illumination with ultraviolet light and photographed with a gel imaging system (Syngene, USA). GeneTools™ software (Syngene, USA) was used to detect bands and to estimate their molecular weight.

Conversion of RAPD to STS (sequence tagged site)

Tsingtaodahua and Kirin 1 were amplified with random primer S1. Kirin 1 yielded a specific band approximately 490 bp in length (Fig. 1), which is absent in Tsingtaodahua. This band was cut out, purified using NucleoTrap Kit (Clontech, USA) and re-amplified using S1. The re-amplified fragment was ligated into pBluescript II KS+ T Vector (Stratagene, USA), transformed into competent *E. coli* DH5α cells, and sequenced with an ABI3730 DNA sequencer (Applied Biosystems, USA). Cloning and sequencing of this band, converted the marker into a STS.

STS were amplified using primers that were chosen from sequences located at positions 136 to 157 (STSF: 5'-GTA GCT TGT GCT AGC TAC ATG C-3') and 473 to 490 (STSR: 5'-CCG CTT GTC ATA AGT AG-3') (Fig. 2). The DNA obtained from the mixture of Tsingtaodahua and Kirin 1 raw hops cones, at various ratios (w/w) was tested using the STS procedure with STSF and STSR. STS amplification was carried out in the same manner as RAPD profiling except for thermo cycling condition and gel concentration. The initial denaturation was followed by 30 cycles of 94°C for 30 s, 48°C for 30 s and 72°C for 30 s. PCR product was separated on 0.8% agarose gel. The amplification fragment was 355 bp.

Table I. The list of hop varieties used in this study together with their pedigrees and origins.

No	Variety	Pedigree	Origin
1	Saaz	Selection from an old Czech land race	Czech
2	Willamette	Tetraploid Fuggle × Fuggle seedling	USA
3	Cascade	[Fuggle × (Serebrianka × Fuggle's seedling)] × OP	USA
4	SA-1	Female Czech Saaz and male Tettngang	China
5	Tsingtaodahua	US selection from late Cluster	China
6	Cluster	Old American variety	USA
7	Nugget	(Brewers Gold × 5-29-4) × [Brewers Gold × (Early Green × OP)]	USA
8	Galena	Brewers Gold × OP	USA
9	Magnum	Galena × 75/5/3	Germany
10	Marcopolo	Unknown	USA
11	Kirin1	Selection from Shinshuwase	Japan

OP: open pollinated

Table II. The list of arbitrary primers used and obtained data.

Primer	Sequence (5'–3')	Fragment size range (bp)	No. of scored fragments
S1	GACCGCTTGT	490–2126	14
S5	TGCGCCCTTC	486–1564	9
S8	GTCCACACGG	230–1473	6
S37	GTTCGCTCC	451–1920	14
S39	CAAACGTCGG	505–1814	7
Totals			50

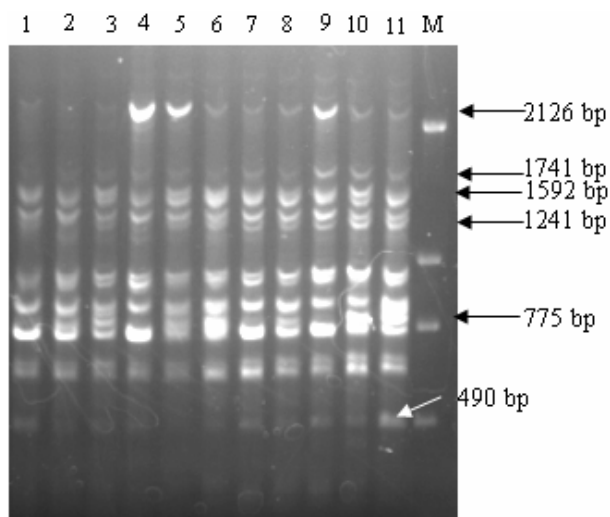


Fig. 1. RAPD fingerprinting amplified with S1 primer in different hop varieties. Lanes: 1. Saaz 2. Willamette 3. Cascade 4. SA-1 5. Tsingtaodahua 6. Cluster 7. Nugget 8. Galena 9. Magnum 10. Marcopolo 11. Kirin 1 Lane M: Molecular size marker DL-2000 (Takara Biotech Co., Ltd). The arrows indicate the polymorphic bands. The white arrow indicates the 490 bp band that was present in Kirin 1 but absent in Tsingtaodahua.

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1  GACCGCTTGT ATGAAAAGATA CAGGAAGCAA CAACTTCCTA CCTTTGAGGG TGGATCGGAT
   RAPD primer S1
61  CCACTGCGGG CAGAGCAGTG GATGAATATG GTTTCCTTGA TCTTAGACTT TATGAGGGTC

121 GAGGGAAGTG AAATGGTAGC TTGTGCTAGC TACATGCTTA GGGAGGATGC CCGTATCTAG
      STSF
181 TGGGATGTGG TGAGTCAGCG GAGGAATGTT GCAGTCATGA CATGGGAAGA ATTTAAGAGT

241 ACCTTTAATG AGAAATACTA TAGCATAGCA GTTCAAGATG TGAAGGTTGA TGAGTTTATC

301 AACCTGACCT AGAACAGGTT ATTAGTTACT GAGTACGCCT TGAGATTCTGA CAGACTGGCA

361 TAGTTTGCAC CAGACTTAGT GCCGACGGAT GCGGCCAGAA GAGATAGATT CGTACAGGGG

421 TTGAATGTGA TGATCGCCCG TGATGTGAAT ATTACGTTGA TTCCAGAGAC TACTACTTAT
      STSR
481 GCAACAAGCGG TC
   RAPD primer S1

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Fig. 2. DNA sequences of random amplified polymorphic DNA markers from Kirin 1 using random primer S1. RAPD primer S1 binding sequences are boxed. Sequences used to design the primers STSF and STSR for the STS specific amplification are underlined.

Data analysis

RAPD patterns of different varieties were scored as 1 or 0 for the presence or absence of a given band in a variety with Cross Checker™ Version 2.8 program. Genetic distances (GD) were calculated between any two varieties according to the formula $GD_{xy} = 1 - 2N_{xy}/N_x + N_y$, where N_{xy} is the number of bands shared by two varieties and N_x and N_y are the number of bands presented in x and y variety respectively⁵.

Relationships of hop varieties were also evaluated using the unweighted pair method for arithmetic averages (UPGMA)⁶. The dendrogram showing such relationships was plotted using MEGA 3™ program.

RESULTS AND DISCUSSION

Polymorphism of the varieties tested

Fifty arbitrary 10-mer primers (Sunbio, China) were screened with three hop varieties, i.e. Saaz, Tsingtaodahua and Nugget. Five primers S1, S5, S8, S37 and S39 yielded clear, consistent and reproducible bands. The number of bands yielded by different primers varied from 6 to 14 (Table II), with an average of 10 bands per primer and 5.6 polymorphic bands per primer. Totally, 50 bands were scored in 11 varieties, with their sizes ranged from 230 to 2126 bp (Table II), of which 28 were polymorphic, amounting to 56.0% average RAPD polymorphisms.

Identification and distinguishing of hop varieties using RAPD

RAPD fingerprints for primer S1 are shown in Fig. 1. An intense band 2126 bp for SA-1 distinguished it from the other three aroma hops, i.e. Saaz, Willamette and Cascade. Cascade had two polymorphic bands 1592 bp and 775 bp, which were absent in the other three aroma hops. The band 1241 bp was absent in all of the aroma hops, but present in all of the bitter hops, Tsingtaodahua and Clus-

ter. The fingerprints of Tsingtaodahua and Cluster were almost identical except the 2126 bp band was more intense in Tsingtaodahua.

Among high α acid varieties, there were three polymorphic bands 1592 bp, 775 bp and 490 bp. Marcopolo had a unique band 1592 bp. Nugget and Magnum did not have the 775 bp band which was present in Galena, Marcopolo and Kirin I. Among all of the 11 hop varieties, the 490 bp was only present in Kirin I. Analysis of polymorphic bands generated by primer S1 for all of the varieties are shown in Table III. The RAPD patterns of the selected five primers S1, S5, S8, S37 and S39 in combination could discriminate all of the 11 hop varieties. (RAPD profiles of S5, S8, S37 and S39 primers not shown).

Among the five primers, four generated polymorphism differences for Tsingtaodahua and Kirin I (Table IV). To detect contamination caused by mixing hop varieties, only the polymorphic band that was absent in Tsingtaodahua but present in Kirin I could be used as a specific marker. The band 490 bp yielded by primer S1 accorded with this qualification. When a mixture of DNA samples was used as a template, amplification products using random prim-

Table III. RAPD polymorphic bands generated by primer S1 for all of the varieties.

Variety	Molecular weight (bp)					
	2126	1741	1592	1241	775	490
Saaz	-	-	-	-	-	-
Willamette	-	-	-	-	-	-
Cascade	-	-	+	-	+	-
SA-1	+	-	-	-	-	-
Tsingtaodahua	+	+	+	+	+	-
Cluster	+	+	+	+	+	-
Nugget	+	+	-	+	-	-
Galena	+	+	-	+	+	-
Magnum	+	+	-	+	-	-
Marcopolo	+	+	+	+	+	-
Kirin I	+	+	-	+	+	+

(+) band present and (-) band absent

ers were not always consistent with those of the pure samples. This may be due to preferential annealing of primers to specific sites in the DNA mixture. Therefore, RAPD is probably not suited to detect mixtures of hop cultivars. Thus, RAPD was converted to STS in the following trials.

Purity determination using STS

STS amplification¹ was carried out to determine whether the PCR assay could detect contamination caused by mixing hop varieties. It was found that STS amplification could detect as little as a 5% contamination of Kirin 1 in Tsingtaodahua. Though this detection limit is not very sensitive compared to chemical methods, the PCR-based

Table IV. RAPD polymorphic bands generated by four primers for Tsingtaodahua and Kirin 1.

Cultivar	Primer (bands in base pairs)				
	S1 1592	S1 490 bp	S5 854 bp	S8 875 bp	S39 668 bp
Tsingtaodahua	+	-	+	+	+
Kirin 1	-	+	-	-	-

(+) band present and (-) band absent

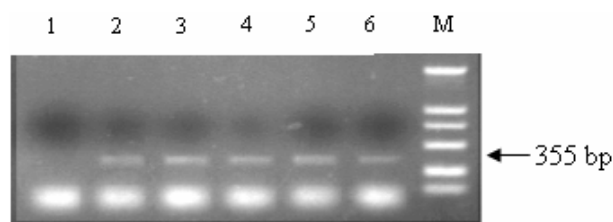


Fig. 3. The pallets of Tsingtaodahua and Kirin1 were mixed at various ratios (w/w), and the DNA was extracted and amplified with STS primers. The arrow indicates the band (355 bp) used to detect contamination. Lanes 1–6 show the specific amplifications of the DNA mixture of Tsingtaodahua (TD) and Kirin 1 (K) at different ratios: 1. TD:K 100:0, 2. TD:K 0:100, 3. TD:K 20:80, 4. TD:K 50:50, 5. TD:K 80:20, 6. TD:K 95:5. Lane M shows the molecular weight marker DL-2000 (Takara Biotech Co., Ltd).

technique is much easier and faster. The detection of the DNA mixture extracted from Tsingtaodahua and Kirin 1 is shown in Fig. 3.

UPGMA analysis

Fig. 4 shows Nei's unbiased measures of genetic distances between any two of 11 hop varieties. The overall genetic similarity between varieties was high. The highest genetic distance was found between Saaz and Marcopolo (GD = 0.3143).

The UPGMA dendrogram (Fig. 4) shows that the genetic relationships of varieties tested was roughly consistent with the traditional classification of hops into aroma and bitter types.

Varieties were divided into two clusters. Cluster 1 included most high alpha varieties, Galena, Magnum, Marcopolo and Kirin I. Tsingtaodahua and Cluster were also included in Cluster 1. Cluster 2 was mainly composed of European and US aroma landrace varieties, Saaz, Cascade and Willamette. Cascade and Willamette are Fuggle-related varieties. SA-1 gives beer an aroma similar to those brewed by Fuggle-related varieties. Although Nugget has Brewers Gold in the male parent's pedigree and contains high α acid content, it is different from Cluster 1 to which most high alpha varieties belong. This may be explained by predominant aroma type germplasm.

CONCLUSIONS

The present study confirmed the ability of RAPD analysis for Tsingtaodahua identification. Additionally, it was shown that the STS marker was a highly specific assay capable of detecting a minimum 5% of Kirin 1 in a Tsingtaodahua mixture. Genomic DNA extraction and RAPD or STS analysis could be carried out in only one day. Therefore, a rapid and effective DNA marker assay to identify Tsingtaodahua and detect Kirin I contamination of Tsingtaodahua has been developed. Similar DNA assays may be developed to detect purity in other mixtures.

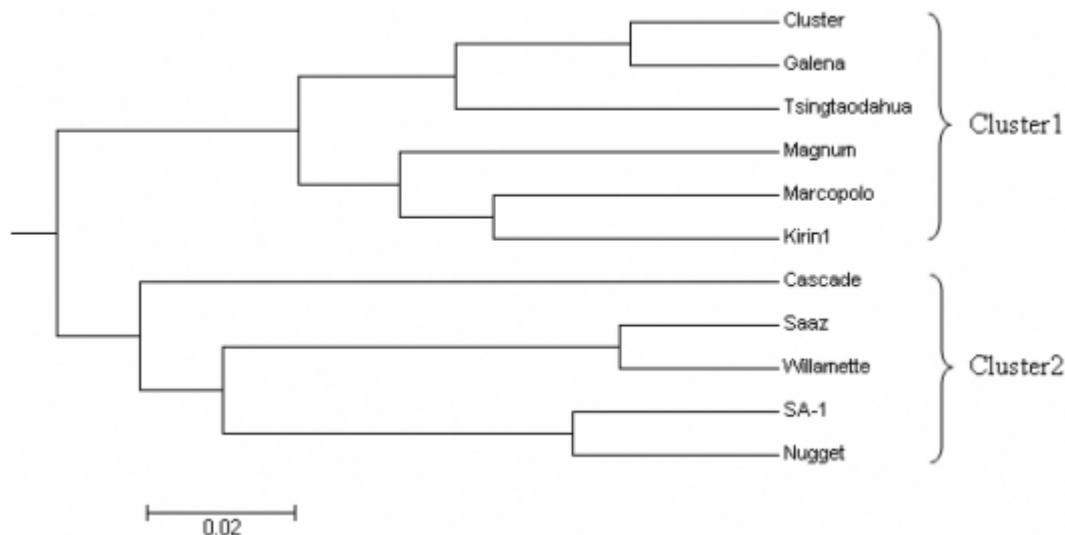


Fig. 4. UPGMA-derived clusters illustrating the relationship among hop cultivars determined by RAPD analysis.

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