

Genbank-derived Microsatellite Markers in Hop

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Abstract

A method was developed to identify microsatellite-containing sequences from existing *Humulus lupulus* L. entries in the Nucleotide GenBank database. We screened 45 genomic, mRNA and expressed sequence tag (EST) *Humulus* nuclear sequences and identified 7 that contained simple sequence repeats (SSRs). Primer pairs were designed for 15 sequences and the optimum annealing temperature was determined by gradient PCR. SSR primers were screened for polymorphism in 24 wild accessions of hops. Ten primer pairs generated repeatable polymorphic bands and seemed to amplify single loci. These 10 polymorphic loci will be useful for genotype identification and for estimation of genetic diversity in the hop collection maintained at the U.S. Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository, Corvallis, Oregon.

INTRODUCTION

Hop (*Humulus lupulus* L.) is a member of the Cannabinaceae family. The genus *Humulus* contains three species: *H. lupulus*, *H. japonicus* Siebold & Zucc. and *H. yunnanensis* Hu (Small, 1978). All commercial hops are of the species *Humulus lupulus*, a dioecious perennial diploid with a chromosome complement $2n=2x=20$. Small (1978), using numerical analysis, separated *H. lupulus* into five taxonomic varieties based primarily on morphological characteristics and geographical location: *H. lupulus* var. *cordifolius*; *H. lupulus* var. *lupuloides*; *H. lupulus* var. *lupulus*; *H. lupulus* var. *neomexicanus*; and *H. lupulus* var. *pubescens*. *H. lupulus* var. *lupulus* originated in Europe but has been naturalized in North America and Japan by escaping cultivation. *H. lupulus* var. *cordifolius* is found in Asia and Japan. The remaining three subspecies are indigenous to North America.

The primary *Humulus* germplasm collection of 587 accessions is maintained in a field collection at the USDA-ARS NCGR in Corvallis, Oregon in collaboration with Oregon State University and John Henning, a USDA-ARS hop breeder and geneticist. A core collection of 68 virus-tested genotypes is maintained in vitro and in screenhouses. Molecular PCR-based markers are neutral valuable tools for genotype identification. They provide guidance in core accession designations and for estimating the genetic diversity of a collection.

Microsatellite or simple sequence repeat (SSR) have become the markers of choice for genetic fingerprinting and genetic diversity assessments because of their ease of transfer between laboratories, abundance, high degree of polymorphism, co-dominance and suitability for automation. In hop, four SSR markers were first developed (Brady et al., 1996). They were found to be more reproducible and polymorphic than random amplified polymorphic DNA (RAPD) markers for discriminating breeding lines. These four SSRs were used in genetic mapping (Seefelder et al., 2000), in fingerprinting and estimation of genetic diversity (Jakše et al., 2001). De novo isolation of microsatellite markers has been recently reported (Hadonou et al., 2004; Jakše et al., 2002) through the construction of microsatellite-enriched genomic libraries. GenBank has been thus far

untapped as a potential source of microsatellites in hops. The objective of the present study was to develop SSR markers from these public *Humulus* sequences and to evaluate their usefulness in identifying hop genotypes and in estimating genetic diversity in the hop collection.

MATERIALS AND METHODS

DNA was extracted from young actively-growing leaves using the Puregene kit (Gentra Systems Inc., Minneapolis, Minn.) according to the manufacturer's recommendations. Twenty four wild genotypes (eight European, 15 North American, one accession from Kazakhstan) (Table 1) were used in this study.

A total of 45 *Humulus* nuclear DNA sequences were obtained from GenBank. A microsatellite search tool, SSRIT, was used to detect microsatellites and is available at <http://brie2.cshl.org:8082/gramene/searches/ssrtool>. The genomic DNA sequence and the mRNA sequence were aligned in FASTA format using CLUSTALW, which enabled the identification of the SSR location with respect to the gene. For each sequence, the location of the microsatellite [5' untranslated region (5'UTR), intron, exon, or 3' untranslated region (3'UTR)] was predicted by aligning the coding or cDNA sequence to the genomic sequence using CLUSTALW. Fifteen primer pairs (Table 2) were designed for 7 sequences using Primer3 (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi). The locus name is 'HL' for *Humulus lupulus*, 'C' for Corvallis, followed by a three-digit number and alphabetical letters when more than one microsatellite was present in the same sequence.

PCR was performed in a total reaction volume of 15 µl containing: 1 x PCR buffer, 2 mM MgCl₂, 0.2 mM each dNTP, 10 µM of each primer, 0.05 U of Biolase enzyme (Bioline USA Inc., Randolph, Mass.) and 5 ng of DNA template. For each primer pair, a gradient PCR ranging from 45°C to 65°C was used to determine the optimum annealing temperature (T_m) in an Eppendorf Gradient thermocycler (Brinkmann Instruments, Inc., Westbury, New York) or an MJ Research Tetrad thermocycler (MJ Research, Inc., Watertown, Mass.). The PCR conditions consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 40 sec, annealing for 40 sec and extension at 72°C for 30 sec. A final extension was performed at 72°C for 30 min. Amplification products were separated by electrophoresis on 3% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator using a Bio-Rad GelDoc 2000 digital imaging system (Bio-Rad Laboratories, Hercules, Calif.).

RESULTS AND DISCUSSION

Optimum annealing temperature for each primer pair was determined (Table 2) by gradient PCR. The optimum T_m was used to amplify DNA from 24 wild accessions of *H. lupulus*. HLC-001B and HLC-002A resulted in non-specific amplification of multiple DNA fragments. HLC-001C primers pairs failed to amplify in the 24 hop accessions while HLC-005A failed to amplify in 6 of the wild North American accessions. While HLC-005C appeared monomorphic, the remaining 10 loci amplified polymorphic DNA around the expected size range and will be evaluated further (Table 2).

Mining GenBank sequences for microsatellites succeeded in identifying ten potential SSR markers for hops. Our lab is investigating their usefulness for cultivar identification, genetic diversity and parentage assessments, and the ability to amplify fragments in other taxonomic varieties by capillary electrophoresis using fluorescent forward primers.

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Tables

Table 1. List of 24 wild hop accessions used for SSR screening, their accession number, name, taxon and origin.

Accession	Name	Origin
<i>Humulus lupulus</i> L. var. <i>lupuloides</i> E. Small		
CHUM1000	WA-Souris-E2 SG#1	Canada, Manitoba
CHUM1001	WA-Logan-N SG#16	United States, North Dakota
CHUM1008	WA-Oxbow-S SG#19	United States, North Dakota
PI558906	WA-Iowa 5	United States, Iowa
PI559234	BC-02	United States, Montana
<i>Humulus lupulus</i> L. var. <i>lupulus</i>		
PI558589	USDA 21117M	United States, Wisconsin
PI558591	USDA 60023M	United States, Colorado
PI558602	USDA 21090M	Yugoslavia
PI558607	USDA 21087M	Yugoslavia
PI558661	USDA 60015	United States, Arizona
PI558677	Tardif de Bourgogne	France
PI558678	Swiss Tettninger	Germany
PI558706	Nadwislanska	Unknown, Poland?
PI558736	Hallertauer mittelfruher	Germany
PI558784	Saazer 38	Czech Republic
CHUM1025	K-098	Kazakhstan
<i>Humulus lupulus</i> L. var. <i>neomexicanus</i> A. Nelson & Cockerell		
CHUM1355	WA-Deer Gulch #1	United States, Colorado
CHUM1385	WA-Pecos #1	United States, New Mexico
CHUM1386	WA-Pecos #2	United States, New Mexico
CHUM1401	WA-Phantom Canyon #2B	United States, Colorado
CHUM1441	WA-Lookout Canyon	United States, Arizona
PI558900	USDA 21599	United States, Utah
<i>Humulus lupulus</i> L. var. <i>pubescens</i> E. Small		
PI558903	WA-Missouri 3	United States, Missouri
PI559198	Wild American (WA)-7	United States, Nebraska

Table 2. The microsatellite loci for which primers were developed, their accession number, gene designation, motif, location in the gene, expected amplicon size, optimum annealing temperature, amplicon size range in 24 wild hop accessions where available and optimum annealing temperature (T_m). Highlighted SSRs will be evaluated further for usefulness in genotype identification and evaluation of genetic diversity.

Name	Accession Number	Gene	Motif	Motif Location	Expected Size	Observed Size Range	Optimum T _m (°C)
HLC-001A	AB053486	Farnesyl pyrophosphate synthase, (fpps)	(TA) ₇	5'UTR	132	129-168	60
HLC-001B			(TTA) ₂ T(TA) ₅ T(TA) ₃	5'UTR	444	NS ¹	45
HLC-001C			(AT) ₈	5'UTR	180	NA ²	
HLC-001D			(CT) ₆ , (TA) ₆	5'UTR	245	253-295	60
HLC-002A	AF147497	Endochitinase precursor, (HCH1)	(AT) ₉	INTRON1	243	NS	45
HLC-002B			(TA) ₂ C(AT) ₆	INTRON2	191	180-220	62
HLC-002C			(TAGTTA) ₅	3'UTR	249		52
HLC-003	AJ304877	Chalcone synthase, (chs_H1)	(CTA) ₆	5'UTR	434	433-469	62
HLC-004A	AJ430353	CHS-like protein 4, (chs4)	(TA) ₁₃ , (CA) ₇	5'UTR	176	148-170	50
HLC-004B			(TA) ₈ , (TA) ₃	Intron1	222	203-300	62
HLC-005A	AB047593	Valerophenone synthase, (vps)	(AT) ₃ , (AT) ₄ , (AT) ₅	5'UTR	247	nulls	56
HLC-005B			(TA) ₅ , (TA) ₅	5'UTR	182	180-210	64
HLC-005C			(TA) ₂ (TC) ₄ , (CT) ₃	Intron1	236	NP ³	62
HLC-006	AB061022	CHS-like protein 3, (chs3)	(TA) ₄ , (TA) ₄ , (TA) ₆	Intron1	287	280-305	62
HLC-007	AB061020	CHS-like protein 2, (chs2)	(CT) ₁₀ CC(AT) ₃ AC(AT) ₆	Intron1	238	219-287	62

¹NS indicates non-specific amplification

²NA refers to no amplification

³NP indicates refers to no polymorphism

