Genet Resour Crop Evol
DOI 10.1007/s10722-007-9303-9

RESEARCH ARTICLE

Genic SSRs for European and North American hop (*Humulus lupulus* L.)

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Received: 17 October 2007 / Accepted: 18 December 2007

Abstract Eight genic SSR loci were evaluated for genetic diversity assessment and genotype identification in *Humulus lupulus* L. from Europe and North America. Genetic diversity, as measured by three diversity indices, was significantly lower in European cultivars than in North American wild accessions. Neighbor Joining cluster analysis separated the hop genotypes into European and North American groups. These eight SSRs were useful in uniquely identifying each accession with the exception of two sets of European landraces and a pair of Japanese cultivars, 'Shinshuwase' and 'Kirin II'. An accession from Manitoba grouped with the European (EU) cluster reflecting the group's genetic similarity to older Manitoba germplasm used to develop 'Brewer's Gold' and the gene pool arising from this cultivar. Cultivars grouped closely with one of their immediate parents. 'Perle' grouped with its parent 'Northern Brewer and 'Willamette' grouped with its parent 'Fuggle H'. Wild American accessions were divided into two subgroups: a North Central group containing mostly *H. lupulus* var. *lupuloides* and a Southwestern group containing *H. lupulus* var. *neomexicanus* accessions. These eight SSRs will be valuable for genotype identification in European and wild American germplasm and may potentially prove useful for marker-assisted selection in hop. PCR products from four previously reported primer pairs that amplify the same intronic SSR regions as do the genic SSRs in this study were compared in eight common cultivars. Different primer pairs generated robust markers at the *chs*2 and *chi* loci. However, only the HLC-004B and HLC-006 primer pairs amplified successfully at the *chs*3 and *chs*4 loci.

Keywords Functional SSRs · *Humulus lupulus* · Microsatellite · Sequence tagged Sites (STS) · Simple sequence repeats

Electronic supplementary material The online version of this article (doi:10.1007/s10722-007-9303-9) contains supplementary material, which is available to authorized users.

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Introduction

Humulus L., commonly known as hop, is one of two genera in the Cannabinaceae. Hop is indigenous to the Northern Hemisphere in Europe, Asia, and North America. Most hop cultivars are selections of the European native hop, *Humulus lupulus* L. var. *lupulus*, a diploid ($2n = 2x = 20$) perennial vine.

Variety *lupulus* was introduced from Europe to North America, Japan, and elsewhere, starting with European migration in the 18th and 19th century. This variety is botanically and morphologically distinct based on sparse glands and pubescence in leaves relative to North American varieties.

Small (1978) separated *H. lupulus* into five botanical varieties based primarily on leaf characteristics and geographical distribution. These varieties are the European *H. l.* var. *lupulus*; the Japanese *H. l.* var. *cordifolius* (Miq.) Maxim.; and three North American varieties (*H. l.* var. *neomexicanus* A. Nelson et Cockerell, *H. l.* var. *pubescens* E. Small, and *H. l.* var. *lupuloides* E. Small). Small's (1978) varietal concepts of *H. lupulus* were based primarily on a numerical analysis of leaf shape and pubescence. These morphological characters, however, can be highly variable even within a single population and may not be biologically meaningful. In particular, variety *lupuloides*, represents populations that cannot be classified into one of the other varieties. Novel genetic markers may provide important information for understanding *H. lupulus* phylogeography.

Much of the current range of *H. lupulus* in Eurasia and North America was either glaciated or cold mixed conifer forest as recently as 10,000 years ago (Delcourt and Delcourt 1993; Hewitt 2000). This implies that *H. lupulus* throughout the Northern Hemisphere has undergone a rapid range expansion emanating from southern populations during the Holocene Epoch and is most likely continuing to expand its range (Delcourt and Delcourt 1993).

In the early 1900's, Salmon (1934) crossed a selection of *H. l.* var. *lupuloides* from Manitoba, Canada that was resistant to powdery mildew (caused by *Podosphaera macularis* Braun et Takamatsu) with European selections and released 'Brewer's Gold', 'Northern Brewer' and 'Bullion'. 'Brewer's Gold' is included in the pedigree of many new varieties released from breeding programs around the world. As hop powdery mildew spreads throughout the world, interest in North American *H. lupulus* is increasing for its potential disease and insect resistance (Hummer 2005; Smith et al. 2006).

The US Department of Agriculture (USDA), Agricultural Research Service (ARS), National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon, manages a world hop collection of 1,444 plant and seed accessions originating from 24 countries. Clonal accessions are vegetatively propagated and can be easily misidentified based on phenotypic traits. Molecular markers offer a viable method to document the genetic identity of each accession in this collection.

DNA-based molecular markers are valuable tools for genotype identification, parentage analysis, diversity assessments, linkage mapping, marker-assisted selection and phylogenetic and evolutionary studies. Microsatellites or Simple Sequence Repeats (SSRs) are 1–6 bp tandem repeats that were first discovered in humans but have been found in the genomes of most organisms (Li et al. 2002). Microsatellite markers are particularly valued for their high degree of polymorphism, codominant expression, reproducibility, potential for automation and ease of transfer among labs (Morgante et al. 2002). Sequence tagged site (STS) markers are synonymous with microsatellite markers in hop (Brady et al. 1996; Patzak et al. 2007), and we will refer to them as microsatellite or SSR markers in this paper.

The small number of genomic microsatellite markers available in hop prior to 2005 was limited to 25 primer pairs: 4 by Brady et al. (1996); 11 by Jakše et al. (2002); and 10 by Hadonou et al. (2004). These SSRs were used for cultivar identification (Brady et al. 1996; Cerenak et al. 2004), genetic diversity evaluation among wild and cultivated hop (Jakše et al. 2004; Murakami et al. 2006b), and for genetic mapping (Čerenak et al. 2006). Sixty primer pairs were recently developed from six hop genomic-enriched libraries (Stajner et al. 2005). Development of SSR markers from microsatellite-enriched libraries is time-consuming and expensive. An alternative approach is to mine sequences deposited in GenBank for SSRs in the genus of interest and then search for SSRs based on Expressed Sequence Tags (ESTs). These EST-SSRs or genic SSRs represent functional molecular markers indicating presence in genes of known or putative (homology-based) function. They tend to be more conserved than are genomic SSRs and consequently exhibit higher cross-species transference. Genic SSRs have been isolated in many plants and were reviewed by Varshney et al. (2005).

The hop collection at the USDA-NCGR in Corvallis, Oregon is highly diverse and contains landraces and cultivars of European hop as well as a large collection of wild native North American

accessions. Therefore, a set of SSR markers that can identify each accession and evaluate the diversity in such a diverse collection must exhibit high cross-transference. Since genic SSRs are highly conserved as opposed to genomic SSRs, we mined GenBank for microsatellite-containing hop sequences in 2005 and identified ten polymorphic simple sequence repeats (SSRs) in seven hop genes (Bassil et al. 2005). Patzak et al. (2007) reported the development of microsatellite markers from six of these same hop genes and from an additional sequence encoding a DNA binding protein. The presence of nine cultivars in common with the Patzak et al. (2007) study allowed us to compare the resulting allelic composition at five identical genic regions. The main objective of this study, however, was to evaluate eight of these genic SSRs for their ability to identify and determine the genetic diversity of 46 genotypes representing European landraces, cultivars, and North American native hops in the Corvallis Genebank collection.

Materials and methods

Plant material and DNA extraction

Forty-six clonal hop accessions maintained in screenhouses at the USDA-NCGR, in Corvallis, Oregon, were evaluated in this study (Table 1). This material was divided into a European (EU) and a wild North American (NA) group. The European group of *H. l.* var. *lupulus* contained 10 landraces (L) and 14 cultivars (CV). All cultivars used in this study contain some native North American material in their pedigree and should be considered intervarietal hybrids. We chose to divide the North American accessions into two groups based on taxonomic variety and geography (Fig. 1). These groups were: North Central (NC) for individuals of *H. l.* var. *lupuloides* (nine accessions) and *H. l.* var. *pubescens* (three accessions); and Southwestern (SW) for individuals of *H. l.* var. *neomexicanus* (nine accessions).

DNA was extracted from actively growing young leaves in the spring, by using a modified Puregene (Gentra Systems Inc., Minneapolis, MN) extraction protocol. Proteinase K and RNase A digestion steps were included in the extraction and protein precipitation was repeated twice.

PCR and SSR analysis

SSR mining from hop GenBank sequences and primer testing and design were previously described (Bassil et al. 2005). Ten of 15 primer pairs designed in different regions of seven hop genes generated polymorphic PCR amplicons and were evaluated further by fluorescent capillary electrophoresis. Of these 10 primer pairs, HLC-002C did not generate a PCR product in 4 of the 46 genotypes, and HLC-004A generated more than two PCR amplicons in three wild American accessions. Results from these two SSR loci are not included in this study, which reports data from the remaining 8 SSR loci (Table S1, supplementary data).

Table 1 List of 24 European (EU) accessions and 22 wild North American (NA) genotypes used in this study

Accession name	PI number	Taxon	Origin
European group, Cultivars			
Brewer's Gold	302781	*H. lupulus* var. *lupulus*	UK
Bullion 10A	558668	*H. lupulus* var. *lupulus*	UK
Canterbury Golding	617391	*H. lupulus* var. *lupulus*	UK?
Fuggle H	558664	*H. lupulus* var. *lupulus*	Oregon
Galena	617287	*H. lupulus* var. *lupulus*	Idaho
Huller (Hueller Bitterer)	558946	*H. lupulus* var. *lupulus*	Germany
Kirin II	558695	*H. lupulus* var. *lupulus*	Japan
Late Cluster seedling	558559	*H. lupulus* var. *lupulus*	Oregon
Newport	632858	*H. lupulus* var. *lupulus*	Oregon
Northern Brewer	558710	*H. lupulus* var. *lupulus*	England
Nugget	558948	*H. lupulus* var. *lupulus*	Oregon

Table 1 List of 24 European (EU) accessions and 22 wild North American (NA) genotypes used in this study

Accession name	PI number	Taxon	Origin
Perle	558667	*H. lupulus* var. *lupulus*	Germany
Shinshuwase	264597	H. lupulus var. lupulus	Japan
Willamette	558665	*H. lupulus* var. *lupulus*	Oregon
European group, Landraces			
Hallertauer mittelfrüher	558736	*H. lupulus* var. *lupulus*	Germany
Hersbrucker-8	558761	*H. lupulus* var. *lupulus*	Germany
KAZ-098	635262	*H. lupulus* var. *lupulus*	Kazakhstan
Nadwislanska (Nadwislanski)	558706	*H. lupulus* var. *lupulus*	Poland
Saazer 38	558784	*H. lupulus* var. *lupulus*	Czech Republic
Swiss Tettnanger	558678	*H. lupulus* var. *lupulus*	Germany
Tardif de Bourgogne	558677	*H. lupulus* var. *lupulus*	France
Tettnanger	558682	*H. lupulus* var. *lupulus*	Germany
USDA 21087M	558607	*H. lupulus* var. *lupulus*	Yugoslavia
USDA 21090M	558602	*H. lupulus* var. *lupulus*	Southern Yugoslavia
North American Group, North Central			
Manitoba-1000-SG1	635241	*H. lupulus* var. *lupuloides*	Manitoba
Montana-544	559234	*H. lupulus* var. *lupuloides*	Montana
NDakota-1001-SG16	635242	*H. lupulus* var. *lupuloides*	North Dakota
NDakota-1006-SG22	635247	*H. lupulus* var. *lupuloides*	North Dakota
NDakota-1018-SG7	635258	*H. lupulus* var. *lupuloides*	North Dakota
Saskatchewan-1008-SG19	635249	*H. lupulus* var. *lupuloides*	Saskatchewan
Saskatchewan-1011-SG14	635252	*H. lupulus* var. *lupuloides*	Saskatchewan
Iowa-758	558906	*H. lupulus* var. *lupuloides*	Iowa
Missouri-1020-SG1	635259	*H. lupulus* var. *pubescens*	Missouri
Missouri-1019-SG45	617471	*H. lupulus* var. *pubescens*	Missouri
Missouri-755	558903	*H. lupulus* var. *pubescens*	Missouri
Nebraska-492	559198	*H. lupulus* var. *lupuloides*	Nebraska
USDA21117M	558589	*H. lupulus* var. *lupuloides*	Wisconsin
North American Group, Southwestern			
Arizona-1437-FryeCanyon	635233	*H. lupulus* var. *neomexicanus*	Arizona
Arizona-1441-LookoutCanyon	635237	*H. lupulus* var. *neomexicanus*	Arizona
Colorado-1355-DeerGulch1	635450	*H. lupulus* var. *neomexicanus*	Colorado
Colorado-1367-WillowCreek	635462	*H. lupulus* var. *neomexicanus*	Colorado
Colorado-1401-PhantomCanyon2B	635490	*H. lupulus* var. *neomexicanus*	Colorado
NMexico-1386-Pecos2	635480	*H. lupulus* var. *neomexicanus*	New Mexico
NMexico-1433-WindyPoint	635231	*H. lupulus* var. *neomexicanus*	New Mexico
USDA60023M	558591	*H. lupulus* var. *neomexicanus*	Colorado
Utah-752	558900	*H. lupulus* var. *neomexicanus*	Utah

The EU group contained 14 cultivars (CV) and 10 landraces (L) while the NA group was subdivided by geographic location into 13 North Central (NC) and 9 Southwestern (SW) accessions. Plant Introduction (PI) numbers, taxon, and origin are included

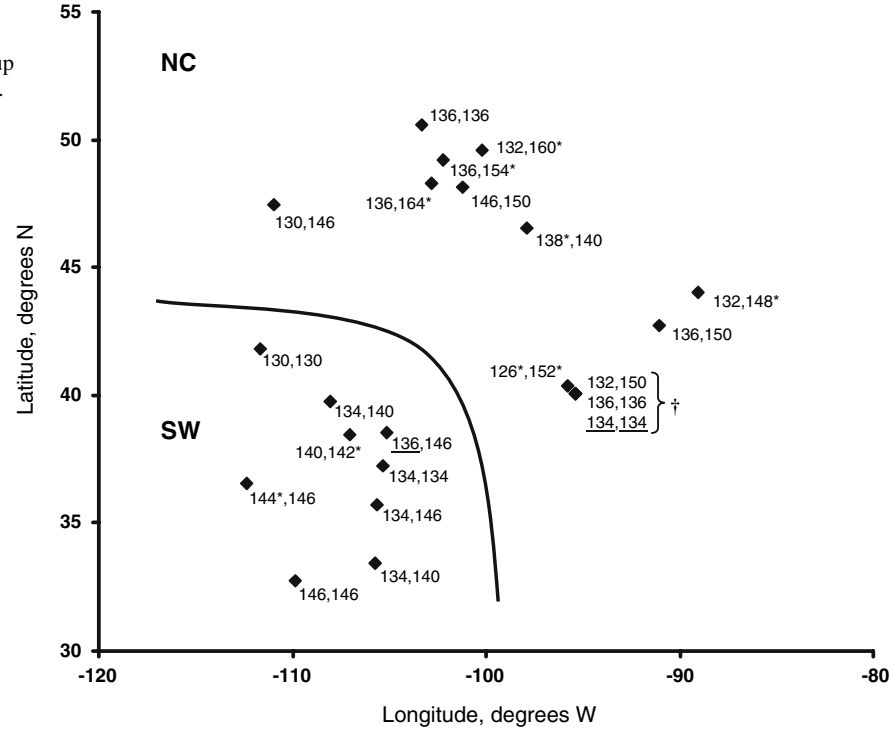

Fig. 1 Distribution of HLC-001A alleles in North American accessions. NC, North Central group (*H. l.* var. *lupuloides* and *H. l.* var. *pubescens* shown with †); SW, Southwestern group (*H. l.* var. *neomexicanus*). An asterisk indicates unique alleles; putative introgressed alleles are underlined

PCR were carried out separately for each primer pair by using a fluorescently labeled forward primer (FAM, HEX, or NED) and an unlabeled reverse primer. The reactions were performed in 10 µl volumes containing 1× reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of each primer, 0.25 units of Biolase *Taq* DNA polymerase (Bioline USA Inc., Randolph, MA), and 2.5 ng genomic DNA. After initial denaturation at 94°C for 3 min, DNA was amplified for 35 cycles in an Eppendorf Gradient thermocycler (Brinkmann Instruments Inc., Westbury, NY) or an MJ Research Tetrad thermocycler (MJ Research, Inc., Watertown, MA) programmed for 40 s at 94°C, 40 s annealing at the optimum annealing temperature of the primer pair, and 40 s extension at 72°C. Amplification was followed by a final extension at 72°C for 30 min. Up to three PCR products were included in each multiplex, and the final dilution factor ranged from 1:80 to 1:320. The size of the SSR amplicons was determined after separation on an ABI 3100 capillary (Applied Biosystems, Foster City, CA) sequencer at the Central Services Laboratory of Oregon State University (Corvallis, OR). GeneScan (version 2.1) was used for automated data collection. Genotyper (version 2.0) was used for computation of allele sizes and accurate visualization of these alleles.

Data analysis

PowerMarker (Version 3.25) (Liu and Muse 2005) was used to calculate the observed number of alleles (n_A), the observed heterozygosity (H_o), the expected heterozygosity (H_e) and polymorphic Information Index (PIC) for EU and wild NA accessions (Table 2).

H_e was calculated as

$$H = 1 - \sum_{i=1}^{n} p_i^2$$

and

$$\mathrm{PIC} = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 p_i^2 p_j^2$$

where p_i equals the frequency of the ith allele (Botstein et al. 1980).

Neighbor Joining (NJ) cluster analysis was based on the proportion of shared-allele distance (D_{sa})

$$D_{sa} = \frac{1}{m} \sum_{j=1}^{m} \sum_{i=1}^{a_j} \min(p_{ij}, q_{ij})$$

Table 2 Genetic diversity indices of eight genic SSRs evaluated using 24 European (EU) cultivars and selections (*H. l.* var. *lupulus*) and 22 wild North American (NA) accessions from the three taxonomic varieties, *lupuloides*, *neomexicanus* and *pubescens*

Marker	No. Alleles (n_A)		H_o		H_e		PIC	
Populations	Eu	NA	Eu	NA	Eu	NA	Eu	NA
HLC-001A	9	16	1	0.73	0.81	0.89	0.78	0.88
HLC-001D	6	11	0.67	0.68	0.62	0.8	0.59	0.78
HLC-002B	7	6	0.79	0.59	0.67	0.68	0.63	0.65
HLC-003A	5	7	0.83	0.77	0.72	0.75	0.68	0.72
HLC-004B	6	12	0.5	0.82	0.5	0.86	0.48	0.84
HLC-005B	3	9	0.21	0.64	0.19	0.75	0.18	0.71
HLC-006	4	3	0.63	0.27	0.51	0.3	0.48	0.27
HLC-007	3	7	0.08	0.5	0.43	0.81	0.37	0.78
Mean	5.38	8.88*	0.59	0.63	0.56	0.73*	0.52	0.70*

Number of alleles per locus (n_A), observed heterozygosities (H_o), expected heterozygosities (H_e) and Polymorphic Information Indices (PIC) were calculated for each population by using PowerMarker (Liu and Muse 2005)

* Indicates highly significant difference at 95% confidence level between mean n_A ($P = 0.008$), H_e ($P = 0.044$), and PIC ($P = 0.037$) of the WA group as opposed to the EU genotypes

where p_{ij} and q_{ij} are the frequencies of the ith allele at the jth locus, m is the number of loci examined, and a_j is the number of alleles at the jth locus.

Results and discussion

Genic SSRs

Eight SSR primer pairs that are located in known hop genes and that amplified PCR products in the expected size range in all European and North American hop accessions were tested. Some of these hop genes are key enzymes in biochemical pathways that produce secondary metabolites responsible for the aroma and bitterness sought for beer production. Hop cones (the female infructescences) contain large numbers of 'lupulin glands' where resins and essential oils accumulate and where some of these genes are expressed. One of these genes encodes farnesyl pyrophosphate (FPP) synthase (fpps) (SSR = HLC-001A, HLC-001D), which catalyzes the synthesis of geranyl pyrophosphate (GPP) and FPP, the precursors of terpenoids such as essential oils (Okada et al. 2001). Five of these genes belong to the chalcone synthase family. Valerophenone synthase (VPS) (SSR = *vps*, HLC-005B) produces the polyketide precursors of the bitter acids in hop cones that play a major role in the taste of beer (Okada et al. 2004; Novak et al. 2006). *Chs*-H1 (SSR = HLC-003A) is believed to have true chalcone synthase (CHS) activity and to catalyze the formation of naringenin chalcone, the precursor of prenylflavonoids, while the function of the remaining three CHS-like genes, *chs*2 (SSR = HLC-007), *chs*3 (SSR = HLC-006) and *chs*4 (SSR = HLC-004B), is unknown. The last SSR-containing gene is an endochitinase gene (HCHI) (class I, Henning and Moore 1999) which might play a role in defense against plant pathogens. In this study, we did not evaluate the accessions studied for production of secondary metabolites encoded by any of these genes. These markers could be useful for marker-assisted selection (MAS) if an association was found between a desirable phenotype and a specific SSR allele. Such use in MAS awaits further evaluation by hop breeders and is not the scope of this study.

Genetic diversity in European and American hop

Our results agree with previous morphological (Small 1978), chemical (Hummer et al. 2005), and molecular studies (Pillay and Kenny 1996; Jakše et al. 2004; Murakami et al. 2006a, 2006b) that observed high genetic variability of American accessions compared to European genotypes. In this study, genetic diversity, as measured by n_A, H_e and PIC, was significantly lower in European landraces and cultivars than in wild North American accessions [n_A ($P = 0.008$); H_e

($P = 0.044$); and PIC ($P = 0.037$)] (Table 2). Number of alleles per locus ranged from 3 to 9 (averaging 5.38) in the EU group and from 3 to 16 (averaging 8.88) in the wild NA group. The eight SSRs generated 22 alleles in the 10 European landrace genotypes and 71 alleles in the 22 North American accessions. The lowest H_e and PIC were found at HLC-005B in the European accessions and at locus HLC-006 in the American genotypes. HLC-001A had the highest H_e and PIC in both groups. At every SSR locus, the most frequent allele(s) in the EU and the NA groups were different (Table S2, in bold). The largest number of unique alleles was also found in the NA accessions (three in Southwestern group and 15 in North Central group). In contrast, European landraces displayed no unique alleles and EU cultivars but one.

The large number of alleles from the NA group that were shared with the cultivar group demonstrate the significant contribution of North American *H. lupulus* in the development of modern hop cultivars (Table S2, Figs. 2, 3). The group of 14 cultivars generated 38 alleles. Of these 38 alleles, one was unique; eight were found in both EU and NA; nine were shared with the EU landraces (often as the most common allele), and 20 were shared with the NA genotypes (Table S2). This hybrid origin of modern cultivars was recently also documented with chemical and morphological characters (Stevens et al. 2000; Henning et al. 2004).

Clustering

Our NJ cluster analysis, based on the proportion of shared-allele distance, D_{sa}, separated the 46 accessions into distinct European and North American clusters (Figs. 2, 3), consistent with other molecular studies in hop (Jakše et al. 2004; Murakami et al. 2006a, 2006b). In a study of 124 hop genotypes based on four SSR primer pairs, Jakše et al. (2004), found that 14 wild NA accessions formed a separate cluster, distinct from EU landraces and cultivars, and had both longer alleles and the highest number of unique alleles when compared to EU landraces and cultivars. In our study, Kaz-098 from Kazakhstan and a wild accession from Southern Yugoslavia (USDA 21090M) appear separate from the Northern European landraces, which is consistent with previous

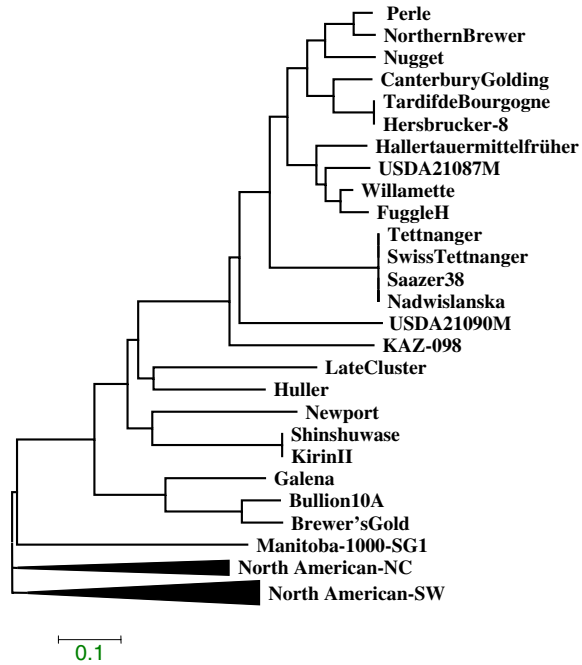

Fig. 2 Neighbor Joining cluster analysis of 24 European accessions and 22 wild North American (NA) genotypes based on the proportion of shared allele distance matrix using eight genic SSRs. Clustering among individual European accessions is shown in detail

studies that reported the distinctness of hop from the Caucasus (Jakše et al. 2004; Murakami et al. 2006b). These eight SSRs uniquely identified every accession with the exception of two sets of landraces ('Tardif de Bourgogne' and 'Hersbrucker-8'; 'Tettnanger', 'Swiss Tettnanger', 'Saazer 38' and 'Nadwislanska') and a pair of Japanese cultivars, 'Shinshuwase' and 'Kirin II' (Fig. 2), indicating low genetic variation in the European landraces as previously reported (Čerenak et al. 2004). Cultivars grouped closely with one of their immediate parents. 'Perle' grouped with its parent 'Northern Brewer' and 'Willamette' grouped with its parent 'Fuggle H'. Full sibs 'Bullion 10A' and 'Brewer's Gold' grouped together and with 'Galena', as all three have the same wild Manitoba accession as a parent. By comparing the NJ dendrograms of hop accessions with (Fig. 2) and without (Fig. 3) cultivars, the contribution of North American germplasm to the genetic diversity of modern cultivars was clearly evident.

The NA cluster separated into two geographic groups (Figs. 2, 3), split roughly at 40° N latitude and corresponding to our North-Central and Southwestern

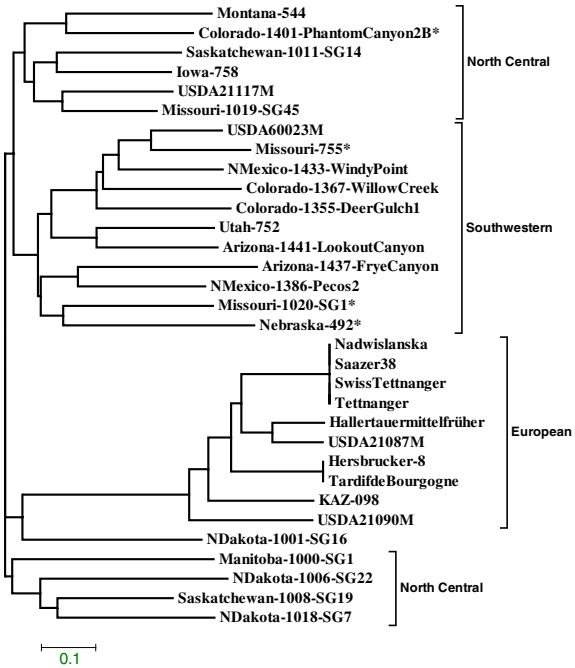

Fig. 3 Neighbor Joining cluster analysis of 10 European landraces and 22 wild North American (NA) genotypes based on the proportion of shared allele distance matrix using eight genic SSRs. Based on geographical location, the NA accessions were subdivided into two groups: North Central, and Southwestern

groups (Figs. 1–3). The geographic distribution of HLC-001A alleles illustrating these two groups in the North American accessions is shown in Fig. 1. Two main groups of NA accessions present in a distinct American cluster were also identified by Jakše et al. (2004). One group contained accessions collected from New Mexico, Arizona, and Colorado, which corresponds to the Southwestern group (*H. l.* var. *neomexicanus*) observed in this study (Figs. 1, 2). The second group contained accessions from Montana, Nebraska and Colorado and corresponds in part to the North Central group (*H. l.* var. *lupuloides*) (Figs. 1, 2). In the NJ dendrogram, the *H. l.* var. *pubescens* accessions did not group together. Such a small number of *H. l.* var. *pubescens* accessions does not allow us to draw any conclusions as to the molecular distinctness of this taxonomic variety. Clustering of an accession of *H. l.* var. *neomexicanus* collected at Phantom Canyon in Colorado (lacking xanthogalenol) with the NC group and the presence of alleles at several loci that are absent or rare in the SW group could indicate introgression. Observation of a characteristic allele from one group occurring in peripheral populations of a different group suggests that past introgression has occurred (Fig. 1). A PCR product of 136 bp at HLC-001A (Fig. 1) and a 238 bp (Table S2) allele at HLC-007 were widespread in the NC group but only present in the Phantom Canyon, Colorado accession of the SW group. At HLC-001D, the 246 bp allele was widespread in the NC group but only found in accessions from Phantom Canyon, Colorado and Lookout Canyon, Arizona. Conversely, a 134 bp allele amplified by HLC-001A that is widespread in the SW group was also present in a Missouri genotype (Missouri-755). At HLC-005B, the 190 bp allele was only observed in the Frye Canyon, Arizona (SW) and Missouri genotypes (NC). Two North Central accessions, Nebraska-492 and Missouri-1020-SG1, grouped together close to the Southwestern group because they share a unique allele, 429 at HLC-003A with accessions from the southern limit of distribution. Evidence for introgression between Southwestern and Eastern *H. lupulus* populations was previously presented by Hummer et al. (2005). In their study, samples of *H. l.* var. *neomexicanus* from the east side of the Rocky Mountains, lacked xanthogalenol, a prenylated flavonoid that is also absent in *H. l.* var. *lupuloides* and *H. l.* var. *pubescens* accessions. Further sampling and analysis of populations of *H. lupulus* along the southern and eastern limits of distribution is needed and could greatly resolve our understanding of phylogeography of *H. lupulus* in North America. The genetic diversity of these populations is threatened not only through loss of habitat from human development, but more importantly, introgression from naturalized *H. l.* var. *lupulus.*

Allele sizes at HLC-003A were significantly positively correlated ($r^2 = 0.407$ $P < 0.001$) with latitude in the North American group (Fig. 4). Our small sampling of NA genotypes along the southern limit of *H. l.* var. *lupuloides* and *H. l.* var. *pubescens* suggests that it is a region of high diversity. Only southern areas of the current distribution of *H. l.* var. *neomexicanus* are in unglaciated regions with suitable habitat during the Pleistocene (Small 1997; Delcourt and Delcourt 1993). North-Central populations are most likely derived from southern refugia east of the Rocky Mountains. As an impact of rapid range expansion, adaptive and physiological traits may vary

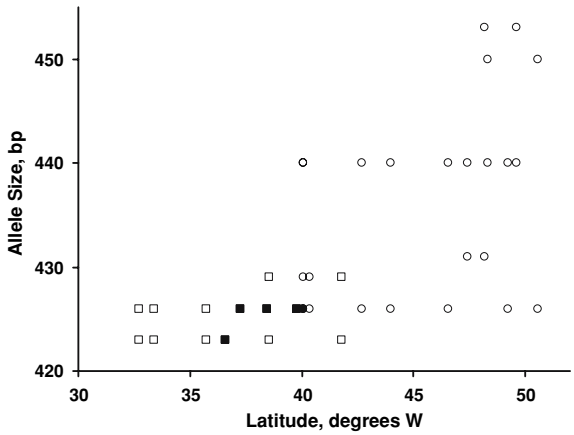

Fig. 4 Latitudinal distribution of allele sizes at HLC-003A in 22 wild North American accessions. □ = SW group; ○ = NC group. A solid symbol indicates individuals homozygous at this locus

clinally from south to north (Cwynar and MacDonald 1987). The significant positive correlation of allele sizes at HLC-003A with latitude observed in the North American group (Fig. 4) could result from a clinal selection of a linked adaptive gene.

Allelic composition using different primers and separation techniques

Patzak et al. (2007) used different primer pairs at T_a of 55°C to amplify the same intronic regions in five of the eight genic SSRs that we evaluated, including Intron 1 regions in *vps* (HLC-005C, Bassil et al. 2005), *chs*4 (HLC-004B), *chs*2 (HLC-007) and *chs*3 (HLC-006); and the intron 2 region of *chi* (HLC-002B). In this study, primers were always designed in conserved exon sequences flanking the SSR and a high annealing temperature (T_a) was used (60–64°C, Table S1) to amplify specific markers. While the *vps* intron1 region was monomorphic in both studies (Bassil et al. 2005; Patzak et al. 2007), the *chs*4 intron 1 primers did not generate polymorphic products in the Patzak study but amplified six PCR products in EU accessions and up to 12 products in the NA genotypes (Table 2 and S2). In both studies, polymorphism was found in intronic regions of the remaining three genes (*chs*2, *chs*3, *chi*), and the resulting PCR products were compared for eight cultivars common to both studies (Table 3). PCR products obtained from 'Brewer's Gold', 'Galena' and 'Kirin II' from these three intronic gene regions, *chs*2, *chs*3 and *chi*, are shown in Fig. S1 of the supplementary data.

In the Patzak study, four markers were amplified at *chs*2 in 68 European hop cultivars in: A = 240 bp, B = 260 bp, C = 460 bp and D = 480 bp. From the *chs*2 gene, our HLC-007 primers amplified three PCR products in the 24 EU accessions (218, 232 and 236) and seven products in the NA germplasm (218, 232, 234, 236, 238, 240 and 242 bp). The 218 bp allele appears to be equivalent to Patzak's A (240 bp) variant since it was the most frequent product in European accessions in the two studies. The 236 bp allele could correspond to the B (260 bp) variant even though the difference between the A and B variants is only 18 bp as opposed to the 20 bp observed in the Patzak study. High molecular-weight products equivalent to the C and D gene variants were occasionally observed

Table 3 PCR product obtained to the same three gene regions using different PCR primers in eight common cultivars

Gene region	*chs*2, Intron 1						*chs*3, Intron 1				Endochitinase precursor, Intron 2					
Cultivar	HLC-007		*chs2*				HLC-006			*chs3*	HLC-002B			*chi*		
Brewer's Gold		236	240		460			284	290	220		188		198	350	360
Fuggle	218		240						290	–	178	188			340	350
Galena		236		260		480	282	284		220				198		360
Kirin II		236	240		460		282		290	220	178		192		340	350
Northern Brewer	218		240						290	–	178	188			340	350
Nugget	218		240		460				290	220	178	188			340	350
Perle	218		240		460				290	–	178	188			340	350
Willamette	218		240						290	–	178	188			340	350

Primers were designed to amplify SSR motifs in intron 1 of *chs*2 (HLC-007, *chs*2) and *chs*3 (HLC-006) and in intron 2 (HLC-002B, *chi*) of the endochitinase precursor gene, HCH1

(Fig. S2) but were not scored. The higher annealing temperature (62°C as opposed to 54°C) used to amplify the *chs*2 locus could have resulted in lack of robust molecular variants C and D in this study. Six of eight cultivars contained the same A and B variants at the *chs*2 intron locus in both studies. In 'Kirin II' and 'Brewer's Gold', however, variant A was amplified in the Patzak study while variant B was generated in this study.

At the *chs*3 locus, primers CHS23F and CHS23R amplified a 220 bp fragment in 21 out of 68 EU cultivars (Patzak et al. 2007) while our primers, HLC-006F and HLA-006R, generated four PCR fragments in the 24 EU cultivars (282, 284, 288 and 290 bp) and three in NA genotypes (282, 284 and 286 bp). The forward primer, HLC-006F, was designed in exon 1, and the reverse primer, HLC-006R, was designed to start in exon 2 and end in intron 1 (Bassil et al. 2005). These primer sequences were conserved in EU and NA germplasm and generated products ranging from 282 to 290 bp in every accession tested. In the Patzak study, the smaller amplicon size (220 bp as opposed to ≥282 bp) implies that at least one of the primers is in the intron sequence which is less conserved than exon sequences used in designing the HLC-006 primers. The absence of this 220 bp product in 65 of the 86 accessions could result from polymorphism in primer sequences which prevented annealing and product amplification. Therefore, we recommend the HLC-006 primers to amplify the *chs*3 SSR sequence.

Despite the differences in primers, PCR and instruments used to separate PCR amplicons, a 10 bp difference was obtained between variants A, B and C of the *chi* gene in both studies. The three gene variants, A (340 bp), B (350 bp) and C (360 bp), amplified by CHIF and CHIR (Patzak et al. 2007) appear to correspond to three (178, 188 and 198, respectively) of seven alleles observed in the European accessions evaluated in this study. Genotype composition at the *chi* locus was similar in both studies except in 'Kirin II' where we observed two PCR amplicons (178 and 192 bp) instead of the single 188 bp allele identified by Patzak et al. (2007). The most frequent allele in the European cultivars was also the 178 bp (or variant A, 340 bp), and variant C was detected alone in 'Galena' and with variant B in 'Brewer's Gold'.

Ease of allele scoring, use of different algorithms in fragment analysis and high resolution of capillary elecrophoresis used in this study as opposed to 5% polyacrylamide gel elecrophoresis used by Patzak et al. (2007) could account for the additional markers observed in this study at *chs*2 and *chi*. A 232 bp fragment was observed in addition to the 236 bp (variant B, 260 bp) and 218 bp (variant A, 240 bp) fragment at *chs*2. Four new markers (176, 192, 196 and 202 bp) were also generated at the *chi* gene in a smaller number of European cultivars (24 as opposed to 68) in addition to the three markers observed in both studies. Differences of 2–6 bp existed between these additional four markers and the three common markers (178, 188, 198 bp). Such small differences in size might not be easily scored in polyacrylamide gels.

Conclusions

Comparison of the results of the Patzak study to this study identified robust markers at the *chs*2 and *chi* loci irrespective of the primers used. However, only the HLC-004B and HLC-006 amplify successfully at the *chs*3 and *chs*4 loci. Differences in allelic composition of 'Kirin II' at *chs*2 and *chi* and of 'Brewer's Gold' at *chs*2 should be further evaluated to determine if one of these cultivars was misidentified or if an error in genotyping occurred. Also, differentiation between alleles that vary by 2–6 bp appears easier when using capillary electrophoresis than when using polyacrylamide gels for PCR fragment separation.

The genic SSRs we present in this study amplify robust markers in EU and NA hop. Their presence in genes that encode economically important traits might prove valuable for marker assisted selection which awaits further evaluation. Some of these loci can also be used as part of a fingerprinting set for cultivar identification.

Acknowledgements We thank laboratory technicians April Nyberg and Isabela Mackey for data entry and PCR. This research was supported by USDA-ARS CRIS 5358-150-033-00D.

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