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Evaluation of Genetic Variability within Actual Hop (*Humulus lupulus* L.) Cultivars by an Enlarged Set of Molecular Markers

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Abstract

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Traditional hop (*Humulus lupulus* L.) cultivars have been used in the brewing industry for a long time. Globally, about ten new breeding lines were released to the market in each decade from ~1970 to 1999. Since 2006, the rate of release of new cultivars has increased tenfold. It is, therefore, important to identify their genotype and origin. Molecular genetic methods based on DNA are the most appropriate technology for this purpose. Recently, we developed an efficient marker system for the authenticity control of hop genotypes based on expressed sequence tag-simple sequence repeats (EST-SSR). In the present study, we enlarged the previously established EST-SSR set with 27 new polymorphic markers and evaluated molecular genetic variability within 135 traditional and new world hop cultivars. Two sets of 10 markers effectively differentiated all used cultivars, with the exception of cultivars derived from the same original genotype such as Saaz, Spalt, Tettnang and Nadwislawsky. Results of molecular genetic variability analyses corresponded with the genealogical and geographical origin of the key cultivars.

Keywords: expressed sequence tag-simple sequence repeat (EST-SSR); cluster analysis; principal coordinate analysis

Current increased demand for the production of hops (*Humulus lupulus* L.), a key raw material for brewing industry, has been driven predominantly by development of craft breweries. With marketing often based on new flavours, there is a clear demand from the beer market for new hop cultivars, and therefore hop breeders have provided an expanded list of cultivars every year in all hop growing regions. The Barth-Haas Group (2013) produced a record of 125 hop cultivars in common use worldwide which was an increase of 17 cultivars since 2009. Thirty new hop cultivars were introduced in 2016 alone (Tim Kostelecky, John I. Haas, Yakima, personal communication). With this large number of new cultivars, it is not always easy to understand how new cultivars relate to existing established hop cultivars, especially when the origin of new cultivars is sometimes secret or poorly described. Every cultivar can be precisely described by its content of bitter acids, essential oils and polyphenols in hop cones, but there are overlaps among cultivars. The composition is influenced by growing season, growing technology and environmental conditions (Krofta & Patzak 2011), and is not necessarily indicative of the character of that cultivar in beer. Nowadays, molecular genetic methods based on DNA provide a reliable tool for the evaluation of individual cultivars and genotypes. Microsatellite SSR (simple sequence repeat) markers have become a standard DNA identification method for species and cultivars within different organisms. A recent technical advance in next generation sequencers (NGS) opened a way to obtain huge amounts of transcriptomes (Nagel *et al.* 2008; Clark *et al.* 2013; Xu *et al.* 2013) and whole genome sequence information (Natsume *et al.* 2015), which provided us with the possibility to

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seek new gene specific molecular markers. From this information, new types of molecular markers such as expressed sequence tag-simple sequence repeats (EST-SSR) (Jakše *et al.* 2011; Patzak & Matoušek 2011; Koelling *et al.* 2012; Singh *et al.* 2012) and single nucleotide polymorphisms (SNP) (Matthews *et al.* 2013; Yamauchi *et al.* 2014; Henning *et al.* 2015) were derived. Recently, we reported an efficient marker system for genotyping and authenticity control of Czech hop cultivars based on EST-SSR, which was implemented for the identification of hop genotypes and control of cultivar purity (Patzak & Matoušek 2013a, b). This marker system is successful and efficient not only for cultivar determination, but also for evaluation of molecular genetic variability with addition of highly polymorphic molecular markers (Patzak *et al.* 2007; Patzak & Matoušek 2011).

In the present study, we enlarged the set of EST-SSR markers with 27 new polymorphic markers, and evaluated molecular genetic variability within 135 traditional and new world hop cultivars (Table S1 in Electronic Supplementary Material (ESM)) by hierarchical clustering analysis and principal

Table 1. Gene region, amplification and polymorphism characteristics of 27 hop expressed sequence tag-simple sequence repeats (EST-SSR) loci

Accession No.	Gene	Region	N_A	H_O	H_E
GAAW01049621	abscisic acid intensive 5	intron	7	0.696	0.783
AB543053	aromatic prenyltransferase HlPT-1	CDS	2	0.704	0.491
GAAW01059666	auxin-repressed protein	3'UTR	9	0.615	0.639
JQ063073	branched-chain aminotransferase 1 (BCAT1)	3'UTR	9	0.585	0.651
GAAW01078174	bZIP transcription factor 25	CDS	5	0.652	0.517
FJ617541	cinnamate 4-hydroxylase	CDS	4	0.430	0.439
AB290349	dihydroflavonol 4-reductase	CDS	3	0.711	0.575
LA679232	endoglucanase 6	3'UTR	10	0.793	0.780
GAAW01061092	flavanone 3-hydroxylase	5'UTR	5	0.489	0.669
GAAW01082226	geraniol 10-hydroxylase	CDS	4	0.548	0.641
LA438938	gibberellic acid 2 oxidase 2	promoter	7	0.563	0.736
		promoter	8	0.652	0.822
LA407469	gibberellic acid intensive gene 1	promoter	3	0.452	0.437
		promoter	4	0.674	0.603
AB292244	HlMYB1 transcription factor	promoter	2	0.489	0.494
		promoter	8	0.844	0.829
AB292245	HlMYB2 transcription factor	CDS	2	0.430	0.472
		CDS	4	0.526	0.564
GAAW01070905	MYB transcription factor 5	CDS	4	0.304	0.518
		3'UTR	3	0.741	0.526
HG983335	MYB transcription factor (Myb8)	CDS	4	0.674	0.644
LA458143	MYB transcription factor 46	3'UTR	4	0.474	0.465
GAAW01039204	MYB transcription factor 78	intron	5	0.430	0.517
GAAW01009048	rRNA 2'-O-methyltransferase fibrillarin	CDS	2	0.393	0.317
GAAW01049743	small auxin up RNA (SAUR) protein	5'UTR	4	0.252	0.625
GAAW01063497	WRKY transcription factor 9	CDS	3	0.407	0.352
LA429216	WRKY transcription factor 20	5'UTR	4	0.430	0.472

CDS – coding sequence; UTR – untranslated region; N_A – number of alleles; H_O – observed heterozygosity; H_E – expected heterozygosity

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coordinate analysis (PCoA) based on 276 amplified polymorphic markers. Six SSR (Jakše *et al.* 2002; Štajner *et al.* 2005), five STS (sequence-tagged sites) (Patzak *et al.* 2007) and previous ten EST-SSR (Patzak & Matoušek 2011) loci were amplified in PCR reactions (2 min at 94°C, 35 cycles (30 s at 94°C; 60 s at 54°C, 90 s at 72°C); 10 min at 72°C). Using the CTAB method according to Patzak (2001) DNA was isolated from the young leaves of samples from the hop garden containing the world hop collection of Hop Research Institute Co. Ltd., Žatec and from dried cones or pellets from samples obtained from hop merchants (Yakima Chief – Hopunion, LLC., Belgium; Simon H. Steiner, Hopfen, GmbH, Germany; John Barth & Sohn GmbH, Germany; Charles Faram & Co. Ltd., United Kingdom; Comptoir agricole, France; Slovenian Institute of Hop Research and Brewing, Slovenia). Amplification products were resolved via 5% denaturing (8 M urea) polyacrylamide gel vertical electrophoresis and visualized by silver-staining (Patzak 2001).

Within 27 newly together used EST-SSR markers, the number of alleles (N_A) per locus ranged from two to ten (Table 1) and was similar to our previous results (Patzak & Matoušek 2011). Jakše *et al.* (2011) reported also very similar results, where N_A ranged from two to seven and from four to twenty for multi-allelic loci, respectively. The observed (H_O) and expected (H_E) heterozygosities of new EST-SSR markers were calculated by GENEPOP version 4.2 (Raymond & Rousset 1995) and ranged from 0.252 to 0.848 and from 0.317 to 0.822, respectively. It was also similar to previous results (Jakše *et al.* 2011; Patzak & Matoušek 2011). Analysis by the Minimal Marker computer program (Fujii *et al.* 2013) was used for evaluation of the strength and efficiency of new EST-SSR markers. Two sets of 10 markers effectively differentiated all used cultivars, except for cultivars derived from the same original genotypes such as Saaz, Spalt, Tettnang and Nadwislawsky. Markers of flavanone 3-hydroxylase (F3H), abscisic acid intensive 5 (ABI5), auxin-repressed protein (ARP1), MYB transcription factor 1 (HlMYB1), MYB transcription factor 2 (HlMYB2), MYB transcription factor 46 (MYB46) and gibberellic acid 20 oxidase 2 (GA20oxy2) were included in these sets of new EST-SSR markers (Table 1). Previous EST-SSR markers (Patzak & Matoušek 2011) from our authenticity control system (Patzak & Matoušek 2013a, b), WRKY transcription factor 1 (WRKY1), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (CMPS),

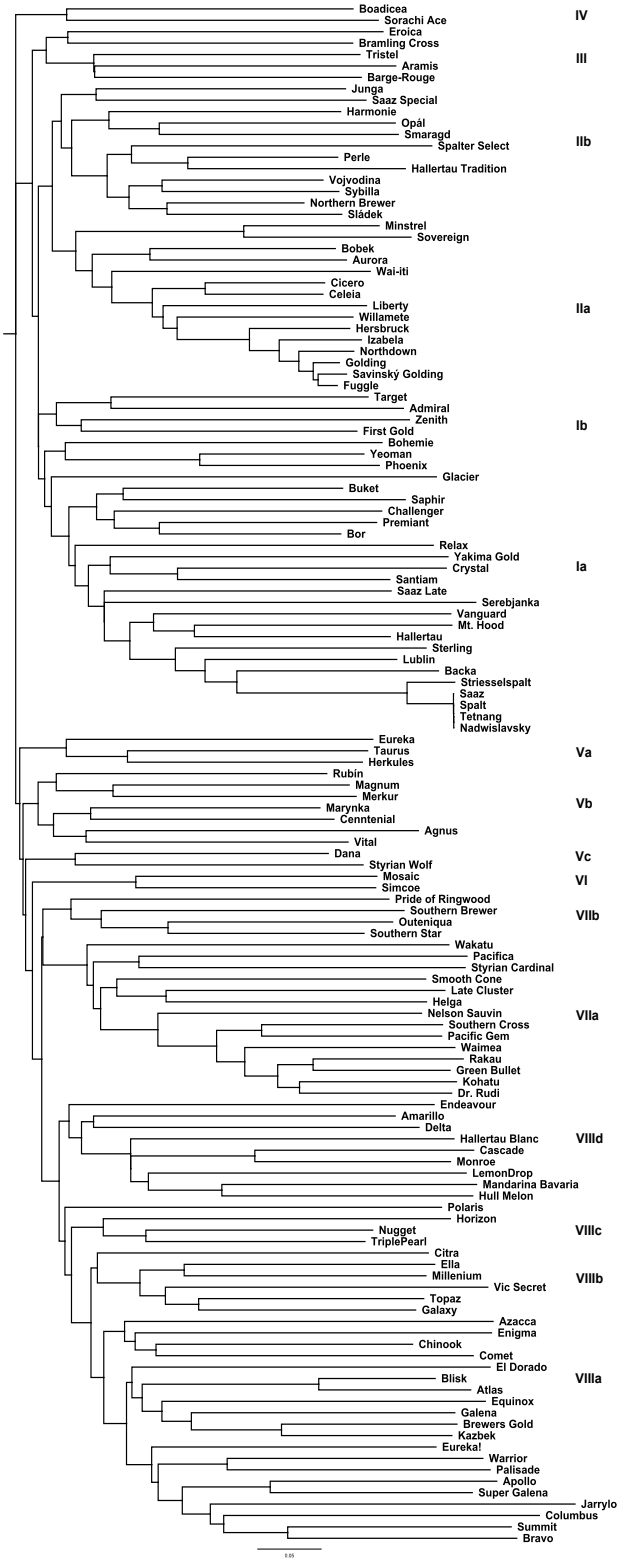

Figure 1. Dendrogram of genetic distances of 135 world hop cultivars revealed by unweighted pair group method with arithmetic means (UPGMA) and Neighbour-Joining (NJ) clustering based on the Jaccard similarity coefficient determined by 276 polymorphic molecular markers

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leucoanthocyanidin reductase 1 (LAR1) and calcium-binding EF hand family protein (CaEFh) were also included. Sets were complemented by previous STS markers (Patzak *et al.* 2007) of chalcone synthase 1 (CHS1), endochitinase 1 (HCH1) and nucleotide DNA-binding protein (NDBP) and by HlGA3 SSR marker (Jakše *et al.* 2002). F3H, GA20oxy2, HCH1, NDBP and HlGA3 were included in both sets. Henning *et al.* (2015) published similar results for SNP markers where seven markers differentiated 116 hop cultivars.

A hierarchical cluster analysis was used for evaluation of molecular genetic variability within hop cultivars. It was based on the Jaccard similarity coefficient and Neighbour-Joining (NJ) clustering by unweighted pair group method with arithmetic means (UPGMA) in DARwin v. 5.0.155 (Dissimilarity Analysis and Representation for Windows, http://darwin.cirad.fr/darwin). The resulting dendrogram (Figure 1) was visualised by Geneious Pro 4.8.2 (Biomatters Ltd., Auckland, New Zealand) and corresponded with the combination of genealogical, geobotanical and analytical characteristics of individual cultivars. Hop germplasm has been shown by different methods of molecular analysis to be broadly divided into European and North American

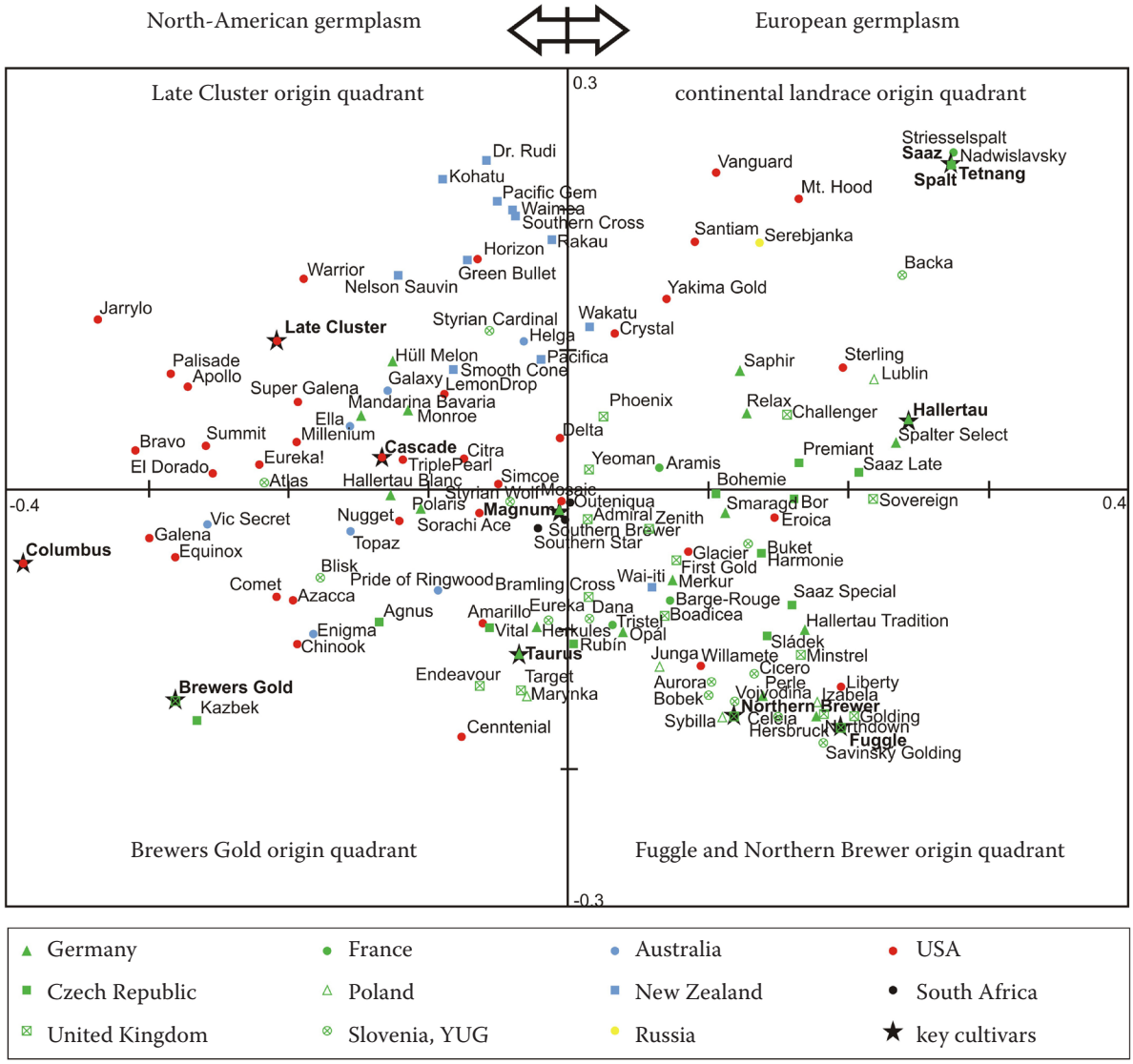

Figure 2. Principal coordinate analysis of 135 hop cultivars with country of origin revealed by DARwin v. 5.0.155 (Dissimilarity Analysis and Representation for Windows, http://darwin.cirad.fr/darwin) based on 276 polymorphic molecular markers

x, *y* – the first and the second principal coordinate, respectively; colours and signs represent the country of cultivar origin

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material (Seefelder *et al.* 2000; Štajner *et al.* 2008; Howard *et al.* 2011; Henning *et al.* 2015). A lot of cultivars have been developed through progressive hybridisation of European landrace germplasm with germplasm with its origin in wild populations of North America (Bassil *et al.* 2008; Štajner *et al.* 2008; Patzak *et al.* 2010; Howard *et al.* 2011). This was shown in cluster analysis when clusters I, II and III belong to European germplasm, VI, VII and VIII to North American germplasm and DNA information of cultivars in clusters IV and V showed patterns of variation from both North American and European origin (Figure 1). Genomes of aroma cultivars in cluster Ia were derived from continental European landrace genotypes which were used by US breeders in the development of these cultivars (Barth-Haas Group 2013; Lemmens 2014). Cultivars in cluster IIa originated from Fuggle and Golding, which were selected in the United Kingdom. Cultivars in cluster IIb originated from Northern Brewer (UK) which was also used for breeding of cultivars in cluster Ib and Ia. New French cultivars were grouped in cluster III. Very interesting was cluster IV where Boadicea (UK) and Sorachi Ace (Japan/USA) were grouped, which were both bred from male hops originated from Japan (Barth-Haas Group 2013). Cluster V grouped high alpha acid cultivars bred from Magnum and Taurus (Germany) which incorporate North American germplasm from Galena (USA) and Brewers Gold (UK) and European germplasm from Hallertau (Germany) (Lemmens 2014; Dresel *et al.* 2016). New Slovenian cultivars Eureka (cluster Va), Dana and Styrian Wolf (cluster Vc) were also bred from Taurus or Magnum, respectively. In North American germplasm, there are two major clusters VII and VIII, and cluster VI with cultivars Simcoe and its daughter Mosaic (USA) (Barth-Haas Group 2013; Dresel *et al.* 2016). New Zealand cultivars were grouped in cluster VIIa. They were mainly bred from Smooth Cone (New Zealand) which was bred from Late Cluster (USA) (Dresel *et al.* 2016). There are mainly aroma hops similarly like Slovenian cultivar Styrian Cardinal and Australian cultivar Helga, which were also included in this cluster. South African cultivars were grouped in cluster VIIb with cultivar Pride of Ringwood (Australia), which originated from Brewers Gold (UK) (Dresel *et al.* 2016). Brewers Gold influence also went through clusters VIIIa, VIIIb and VIIIc. Columbus (USA) breeding origin (cluster VIIIa) and Nugget (USA) breeding origin were also distinguishable (Figure 1). Cultivars made from Cascade (USA) were grouped in cluster VIIId (Dresel *et al.* 2016).

The principal coordinate analysis (PCoA) was also used for estimation of genetic diversity structure. PCoA was conducted by DARwin software based on a genetic similarity/dissimilarity matrix. The first principal coordinate (PCo) represented 12.65% of variation and the second PCo represented 6.85% of variation. PCoA corresponded with a previous dendrogram (Figure 1) when it divided cultivars into four quadrants: continental landrace origin, Fuggle and Northern Brewer origin, Brewers Gold origin and Late Cluster origin (Figure 2). There were also noticeable breeding influences of other key cultivars (Cascade, Magnum, Columbus) on new hop cultivars.

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