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Genetic Diversity and Distinctness of Wild Nebraska Hops and Hop Cultivars (Humulus lupulus L.)

Megan Franklin
University of Nebraska-Lincoln

GENETIC DIVERSITY AND DISTINCTNESS OF WILD NEBRASKA HOPS AND HOP CULTIVARS (*Humulus lupulus* L.*)*

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by
Megan Franklin, BS
Plant Biology
College of Agricultural Science and Natural Resources

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Faculty Mentors:
Keenan Amundsen, PhD, Bioinformatics and Computational Biology
Brian Waters, PhD, Agronomy and Nutritional Sciences

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Abstract

Background Commercial hop (*Humulus lupulus)* cultivars that are being grown in the Midwest are not performing as successfully as when they are grown in the Pacific Northwest, the region to which they are adapted. To increase adaptation to the Midwest environment, one strategy is to draw from the genetic pool of wild native Midwest hops, which have developed genes that allow them to grow successfully in this environment. Wild hop plants that are genetically distinct from commercial cultivars are likely to have more adaptations, such as pest/disease resistance and drought tolerance, which can be bred into commercial lines. The purpose of this study is to identify the genetic distinctness of two wild native Nebraska hop plants from 18 hop cultivars tested.

Methods DNA extractions and amplified fragment length polymorphism (AFLP) analysis was done on the 20 hop accessions to generate hop genetic marker data. These markers were used for further software analyses of genetic diversity. Structural, Cluster, and Principle Component analyses were used to assess diversity among the 20 hops accessions and generate a phylogenetic tree of hop accessions.

Results We found that, of the two native Nebraska hops tested, Wild-1 was genetically distinct from the commercial cultivars, while Wild-2 was genetically similar to the cultivars Cascade and Saaz.

Conclusion Native Nebraska hop Wild-1 demonstrates that there indeed are native Midwest hops that are genetically distinct from the commercial cultivars tested. Therefore, breeders have a genetic source of potential environmental adaptations that can be utilized for the breeding of more successfully grown hops.

Key Words: Genetics, Hops, Phylogeny, Plant Biology

Introduction

Hops (*Humulus lupulus* L.) are perennial climbing plants, typically reaching up to 6 meters tall. They are found naturally growing near rivers, streams, and well-drained regions between the 35° and 55° parallels. Hops can be utilized for pharmaceuticals, textiles, bread making (Hampton *et al.*, 2001), as well as antimicrobial agents (Arsene *et al.*, 2015). Their potential for treating anxiety and depression is also being investigated (Kyrou *et al.*, 2017). The most common use of hops is for making beer, being used as bittering and preservative agents, and for flavor and foam stability (Schonberger and Kostelecky, 2011).

Hops are predominantly grown in Europe and North America, but are also grown in countries such as Japan, China, and New Zealand**.** Hop plants are not native to North America, and were first brought over from Europe to be grown for beer brewing. Growing began on the east coast, but with time, hop production expanded across the United States. It is speculated that wild hops began growing in hedges and along fields and transportation routes as seeds were dropped during transportation. These seeds eventually produced genetically distinct lines from cultivated hops as they grew wild and adapted to different environments. Now, there are three separate genetic pools which include European, Wild North American, and Hybrids (Henning *et al.*, 2004). Breeders have used wild hop plants to incorporate desirable traits, such as disease resistance, into their breeding programs (Jakse *et al.*, 2001, Townsend and Henning, 2009). In fact, high alpha-acid content and disease resistance to *Verticillium* wilt characteristics in modern cultivars originated from wild hops(Neve, 1991).

Today in the United States, most hops are grown in the Pacific Northwest (Washington, Oregon, and Idaho). Meanwhile, the beer industry is continuing to grow, both throughout the United States as well as in Nebraska. From 2011 to 2016, the number of craft breweries in

Nebraska increased from 18 to 42 (National Brewers Association). Hop production is increasing to meet the demand. In Nebraska, crop acreage has increased by 50% from 2014-2016, and is expected to continue increasing (Hop Growers of America, 2017). Commercial hop cultivars from the Pacific Northwest are facing environmental stressors in the Midwest to which they are not adapted. Breeders are trying to improve the quality of hop flavors (such as alpha-acids for bitterness, and aroma), their yield, resistance to pests, diseases, and drought. To do this, breeders are looking to genetically diverse hop plants, which could contain traits that may be useful for hops production in the Midwest.

The native Nebraska (NE) hops, which grow wild, could be a key source of genetic material for breeding improvements. Using wild crop plants to breed in useful traits has been shown to be very profitable. Conferring increased yield traits from wild crops contributed about $115 million per year worldwide (Pimentel *et al.*, 1997). Although we know the pedigree of the commercial cultivars, we do not know the genetic relationships or diversity that wild NE hops have with the commercial hop cultivars. The purpose of this study is to determine how genetically distinct the wild NE hops are from commercial cultivars, and to compare genetic marker profiles to yield data to determine if there is an association between marker profiles and field performance. To do this, AFLP and cluster analyses were used to determine the genetic diversity of 20 lines. This will guide breeders in selecting the varieties to cross that will improve adaptation to the Midwest.

Materials and Methods

Plant Material

Twenty hop varieties were selected. Only female plants were used since female plants produce the hop cones used for brewing. These varieties included two wild native Nebraska hops, six seeded hops plants (S#), and twelve commercial cultivars (Table 1). The native hop samples, Wild-1 and Wild-2, were taken from Bennet, NE (40.7 N, -96.5 W) and Plattsmouth, NE (41.03 N, -95.9 W) respectively. The seeded hops were grown from seed, which originated from open pollinated hop plants in North Dakota. Therefore, their parentage is not known, and they are not grown commercially. These seeds were obtained from the National Plant Germplasm System, requested from the Germplasm Resources Information Network (GRIN). Seeds were planted and grown in University of Nebraska-Lincoln (UNL) greenhouses. Commercial cultivars were provided as plants by Midwest Hop Producers (Plattsmouth, NE), and were grown in greenhouses once they arrived at UNL.

Hop Cultivar	**Type**	**Location Trial Plants Received From**	**Region Cultivars Typically Grown**
Wild-1	Wild NE Hop	Bennet, NE	Midwest USA (wild)
Wild-2	Wild NE Hop	Plattsmouth, NE	Midwest USA (wild)
S1-Logan	Seeded	North Dakota	
S2-Burlington	Seeded	North Dakota	
S3-White Earth	Seeded	North Dakota	
S4-Enderlin	Seeded	North Dakota	
S5-Indian Head	Seeded	North Dakota	

S7-Oxbow	Seeded	North Dakota	
Cascade	Commercial	Plattsmouth, NE	Pacific Northwest USA
Centennial	Commercial	Plattsmouth, NE	Pacific Northwest USA
Chinook	Commercial	Plattsmouth, NE	Pacific Northwest USA
Columbus	Commercial	Plattsmouth, NE	Pacific Northwest USA
Galena	Commercial	Plattsmouth, NE	Pacific Northwest USA
Golding	Commercial	Plattsmouth, NE	Pacific Northwest USA
Mt. Hood	Commercial	Plattsmouth, NE	Pacific Northwest USA
Willamette	Commercial	Plattsmouth, NE	Pacific Northwest USA
Fuggle	Commercial	Plattsmouth, NE	United Kingdom
Saaz	Commercial	Plattsmouth, NE	Czech Republic

Table 1. Hop accession information

DNA Extraction

Hop tissue of newly emerging leaves was collected from hop plants and placed immediately in liquid nitrogen to prevent degradation and enzymatic reactions. Tissue was removed from liquid nitrogen and stored at -70°C until DNA extraction. DNeasy Plant Mini Kit (Qiagen, Inc, Valencia, CA) was used for DNA extraction, and the kit's protocol was followed with the following modifications: Frozen hop leaf tissue was disrupted using a mortar and pestle, using liquid nitrogen to keep tissue frozen. The step which involves adding 100 microliters Buffer AE, incubating the solution for 5 minutes at room temperature, and centrifugation for 1 minute at 6,000 x g was only performed once and not repeated. DNA was stored at -20°C until AFLP analysis.

Amplified Fragment Length Polymorphism (AFLP) Analysis

Amplified Fragment Length Polymorphism (AFLP) markers were obtained by the following procedures as described in (Vos *et al.*, 1995) with the following modifications: The Invitrogen Core Reagent Kit (Thermo Fisher, Waltham, MA) and manufacturers protocol was used for restriction enzyme digestion of DNA with *Eco*R1 and *Mse*1 restriction enzymes provided by the kit. The Invitrogen Core Reagent Kit was also used for the ligation step of AFLP adapters. Pre-selective amplification was done using primers compatible to the Invitrogen adapters. Before samples were analyzed, they were run on a 1% agarose TAE (0.04M tris-acetate/EDTA buffer) diagnostic gel electrophoresis at 100 V for 30 minutes. This was to assess the quality of pre-selective amplification. Selective primers were chosen for amplification (Table 2).

Primer	**Sequence**
E-AAG	5’ GACTGCGTACCAATTCAAG 3’
E-ACG	5’ GACTGCGTACCAATTCACG 3’
E-AGC	5’ GACTGCGTACCAATTCAGC 3’
M-CTC	5’ GATGAGTCCTGAGTAACTC 3’
M-CAG	5’ GATGAGTCCTGAGTAACAG 3’
M-CTG	5’ GATGAGTCCTGAGTAACTG 3’

Table 2. Selective Primers
Primers used for amplification in AFLP analysis.

Selective primers were obtained from Integrated DNA Technologies (Coralville, IA). Selective *Eco*R1 primers were labeled with 6-FAM (6-carboxyfluorescein) to allow detection of amplified

products. Samples were sent to the University of Nebraska Medical Center Genomics Core Facility (Omaha, NE) and run on an Applied Biosystems 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA) to generate the AFLP markers used in analysis.

Data Analysis

Genetic markers were scored using the ABI GeneMapper software. A population structure analysis was done using Structure 2.3.4 to describe the probability of a genotype being assigned to a population based on allele frequencies. Default settings were used, with length of Burnin settings at 10,000 iterations and number of Markov Chain Monte Carlo (MCMC) replications at 10,000. The appropriate number of populations was confirmed using the *Evanno et al.*, 2005 analysis, testing (*K*) populations ranging from 2-7, with 10 replications of each population value.

Using NTSYSpc 2.2 (Rohlf, 2009) software, the SimQual Module was used to create similarity coefficients implementing the DICE (Lee R. Dice, 1945) similarity formula, which utilized the AFLP marker data. Genetic relationships were analyzed by the SAHN Module, which implemented the Unweighted Pair Group Mean Average (UPGMA) clustering method. Due to missing data points in Brewers Gold and Seeded-6 accessions, these varieties were removed from further analysis because missing data would lead to misleading groupings. A phylogenetic tree was created with NTSYSpc 2.2 software. Bootstrap values were calculated by doing 1000 resamplings using WinBoot and implementing the DICE coefficient. A Principle Component Analysis (PCA) was done by using the EIGEN module of NTSYSpc 2.2 software and a 3D plot of Eigen vectors created. The PCA was visualized using the Mod3D plot.

Hop yield data from 2nd year field grown commercial cultivars was provided by Midwest Hop Producers. Acceptable Yield Range per Plant was determined by setting a high and low yield within a 10% range of the average yield estimate for 2nd year plants provided by Midwest Hop Growers.

Results

Structure Analysis of Populations

To determine how many populations (K) the 18 studied hop cultivars and wild hops segregate into, 680 AFLP markers were first run through an Evanno Analysis (Figures 1 and 2), which determined the number of populations to be four. In Figure 1, there are two peaks, which suggest the most likely number of populations. The highest peak is found at six populations, while the second at four populations. In Figure 2, likeliness of a number of populations is based on the point before which the variability of estimated Ln probability becomes very high. The point at which variability is the highest is at five populations, thus four is more likely to be the most accurate estimate of the number of populations describing this set of accessions. Therefore, four populations were used for Structure Analysis (Figure 3).

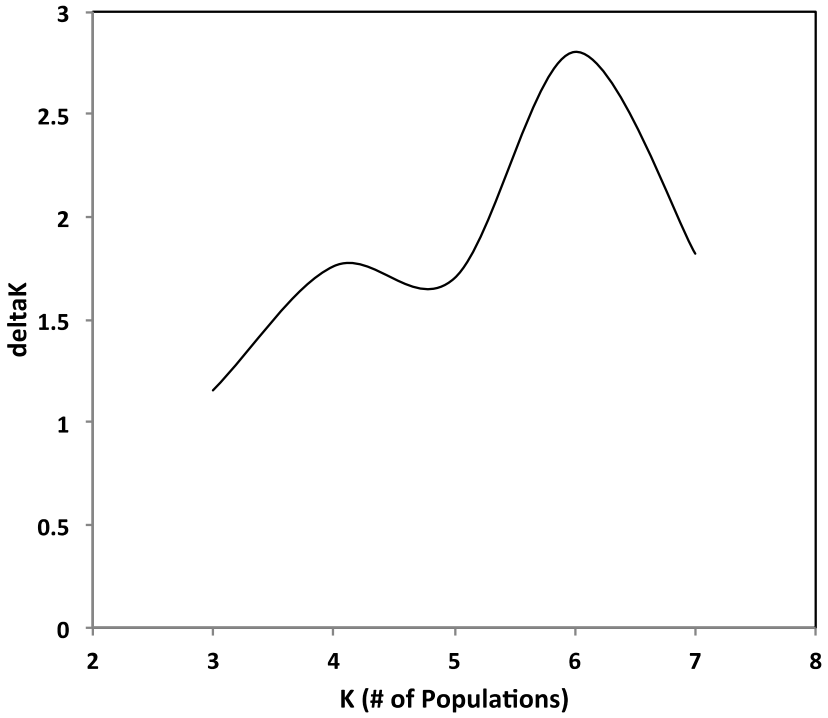

Figure 1. Evanno analysis to determine the number of populations (K). deltaK- m(1L"K1)/s[L(K)].

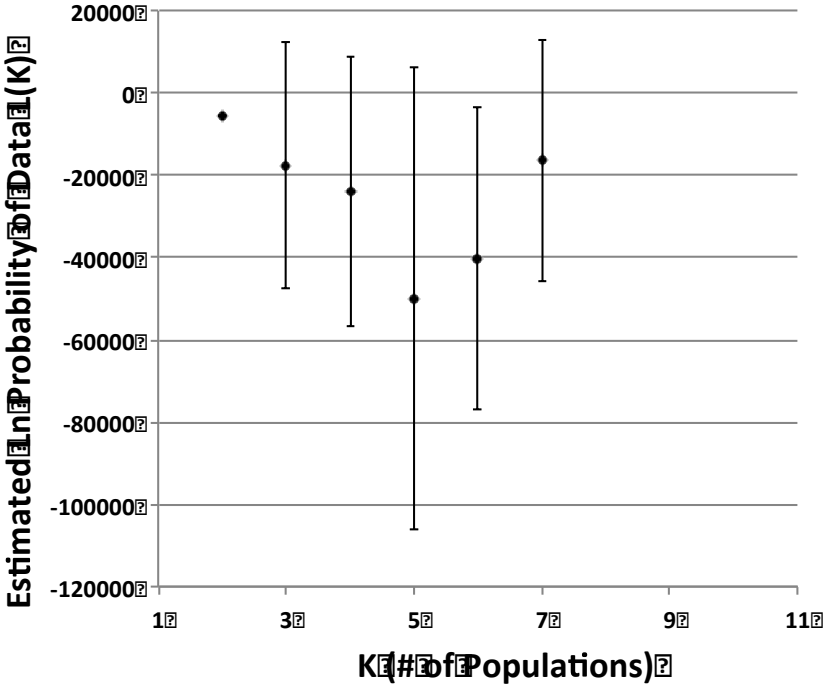

Figure 2. Estimated Ln probability. The more variability in K population, the less likely it is the representative number of populations in the accession set.

Individuals were then assigned by the Structure Analysis program into the four populations based on the genetic marker data (Figure 3). Seeded cultivars, as well as Fuggle, Mt-Hood, Golding, and Willamette associated mostly with Population 1. The native Wild-2 along with Cascade and Saaz, had the majority of markers associated with Population 2. Galena and Centennial associated mostly with Population 3. The native Wild-1 has the majority of its markers associated with Population 4, along with cultivars Chinook and Columbus.

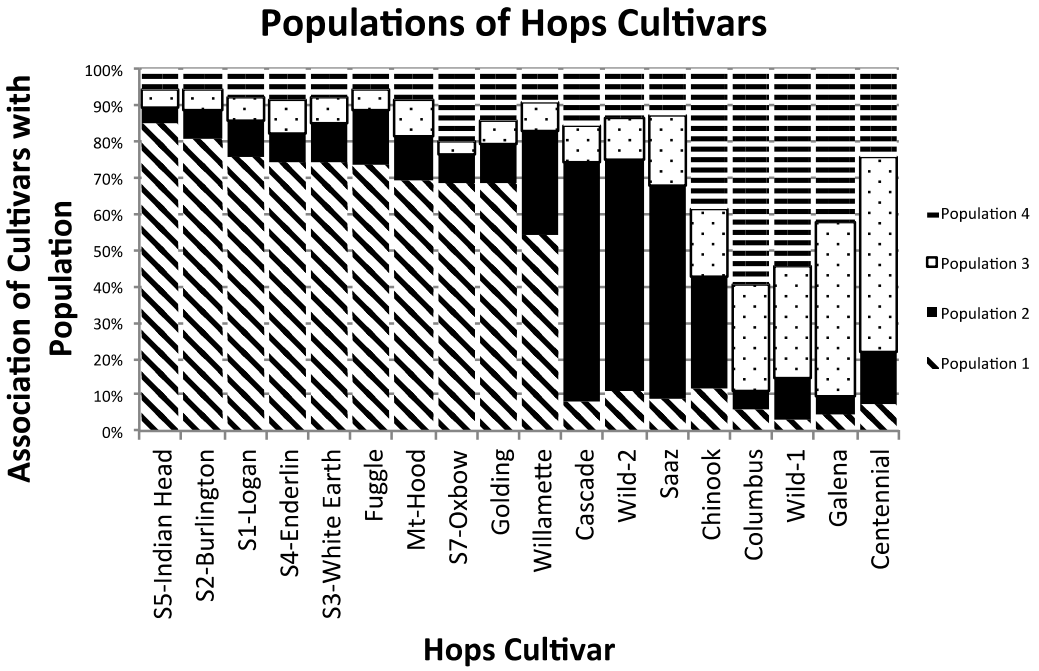

Figure 3. Structure analysis of 680 genetic markers to determine the number of segregating populations. Length of patterned bars represents the probability of an accession being grouped into four populations.

Phylogenetic Analysis

The genetic relationships of the 18 hop cultivars (Figure 4) was examined using cluster analysis to portray relationships based on a similarity coefficient in a phylogenetic tree. The non-grouping cultivars are Wild-1, Columbus, and Galena which all form their own clades. Two main groups form from the remaining cultivars, with a similarity coefficient of approximately .75. Group I includes the native cultivar Wild-2, and commercial cultivars Saaz, Cascade, Centennial, and Chinook. Group II includes all seeded cultivars, and commercial cultivars Willamette, Mt-Hood, Golding, and Fuggle. Seeded cultivars from 1 to 5 all cluster together, while Seeded-7 is outside of that grouping.

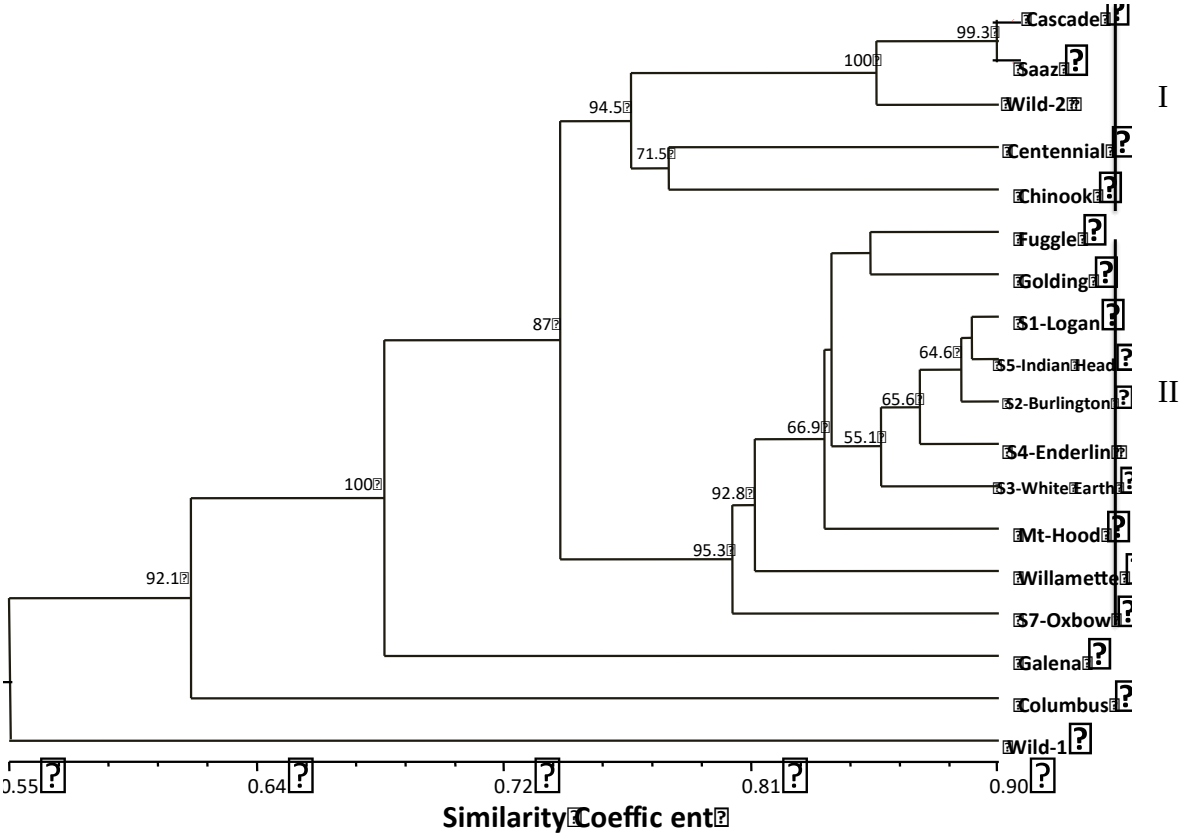

Figure 4. Cluster analysis of 680 hop genetic markers show the genetic relationship of hop cultivars in a phylogenetic tree. Bootstrap values show conservation of branching.

Yield Comparison

The 2016 yield data on commercial cultivar performance (Table 3) provided by Midwest Hop Producers (Plattsmouth, NE) showed Galena, Saaz, and Cascade cultivars produced more than satisfactory yields. Cultivars Columbus and Chinook produced at satisfactory levels. Centennial, Willamette, Mt. Hood, Golding, and Fuggle did not produce acceptable levels. Golding and Fuggle produced less than 1% of the minimum acceptable yield levels.

Table 3. Yield of Commercial Cultivars

Cultivar	Total Dry Yield (lbs)	Yield Per Plant (lbs)	Acceptable Yield Range Per Plant (lbs)	Yield Relationship to Acceptable Range
Galena	136.00	1.77	0.81-0.99	Above
Saaz	99.26	1.50	0.36-0.44	Above
Cascade	101.31	1.31	0.86-1.05	Above
Chinook	82.26	1.25	0.95-1.16	Within
Columbus	79.63	1.21	1.22-1.49	Within
Centennial	21.20	0.28	0.72-0.88	Below
Willamette	11.12	0.14	0.88-1.08	Below
***Mt. Hood**	9.84	0.13	0.72-0.88	Below
Golding	0.75	0.01	0.59-0.72	Below
Fuggle	0.09	0.001	0.52-0.64	Below

* Yield likely decreased by leafhoppers

Table 3. Hop yield data in 2016, second year of growth (provided by Midwest Hop Producers, Plattsmouth, NE) compared to acceptable yield based on literature.

Principle Component Analysis (PCA)

The 3-dimensional genetic relationship of the 18 hop cultivars and two wild hops (Figure 5) incorporated yield data from Table 3. to show any emerging patterns of clustered commercial cultivars with similar yields. The two clustered groups correlated with Groups I and II of the phylogram (Figure 4), with the exception of Galena. Of the two clustered groups, Group I had more of the commercial cultivars with yields above the acceptable range (Saaz, Cascade, and Galena), one cultivar within range, and one below. Wild-2 was also within Group I, but there is no yield data for that native NE hop. Group II had only cultivars with yields below acceptable range. The seeded cultivars were also in Group II, but have no yield data. Columbus and Wild-1 did not fall within either of these clusters.

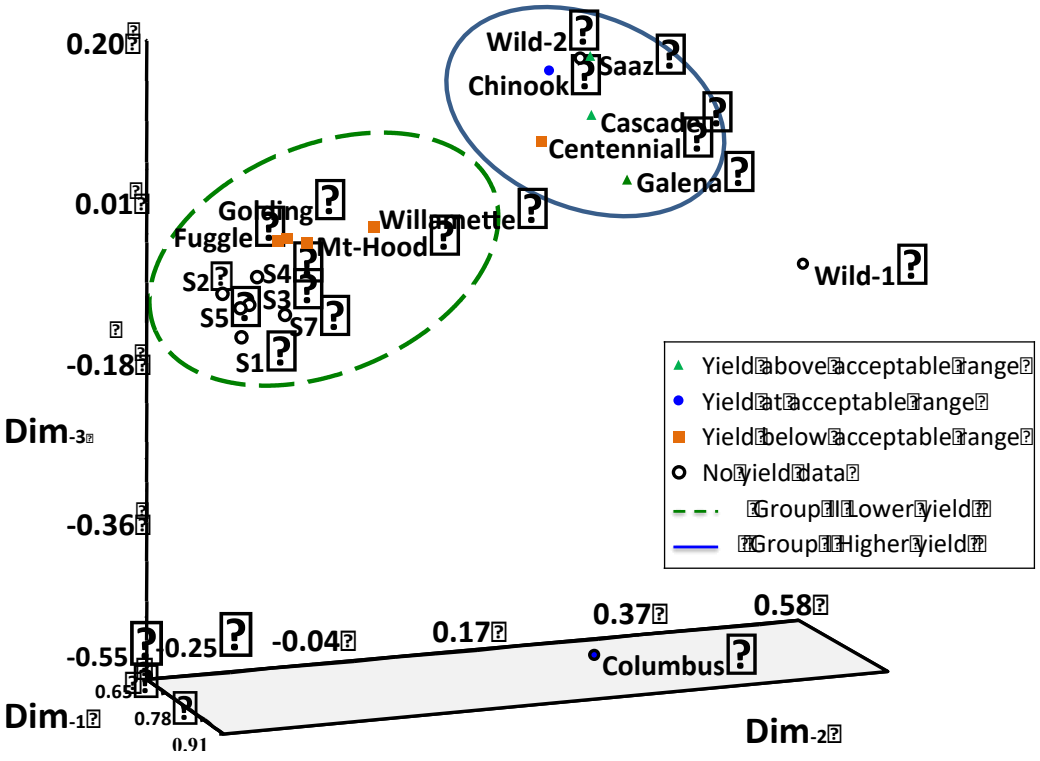

Figure 5. Principal Component Analysis(PCA) of hops commercial, seeded, and native varieties. S#= Seeded variety. Closed triangle represents variety with above expected yield; closed circle represents acceptable yield; closed square shows below expected yield; open circle represents no yield data. Blue solid line shows higher yield cluster (Group I).Green dashed line shows lower yield cluster (Group II).

Summary of Analyses

The similar results shown between the Structure Analysis (Fig. 3), Cluster Analysis (Fig. 4), and PCA (Fig. 5) confer high confidence in the assumptions being made about the distinctness of the NE hop Wild-1, and the relatedness to commercial cultivars of Wild-2.

The Structure Analysis (Fig. 3) supports the outcome of the Cluster Analysis (Fig. 4) since it showed Wild-2 segregating into Population 2 with Saaz and Cascade. This close relationship between these cultivars was also shown on the Cluster analysis (Fig. 4). With the exception of Centennial and Chinook, which formed a clade in Group I of the phylogenetic tree (Fig. 4), all other populations in the Structure Analysis (Fig. 3) followed the outline of the phylogenetic tree. This is understandable, as the bootstrapping value was only 71.5% for the Centennial and Chinook branch, which suggests lower confidence of Chinook and Centennial placement in the tree.

Cluster analysis (Fig. 4) is supported by PCA (Fig. 5). All cultivars were assigned to the same groups, or were segregated as outliers just as they were in the Cluster Analysis, with the exception of Galena. In PCA, Galena was put into Group I while Cluster Analysis shows it without a group. However, Wild-1 was not assigned to a specific group in either analysis, and Wild-2 is more closely related to Saaz, Cascade, Chinook, and Centennial. These similarities allow us to be confident in our assertions of the distinctness of the wild NE hops.

Discussion

Genetically Distinct

Of the two native Nebraska (NE) hops, Wild-1 is the most genetically distinct from the commercial hop cultivars. This was demonstrated through the Cluster (Fig. 4) and PCA (Fig. 5) which did not assign Wild-1 to one of the other clusters or groups. Therefore, Wild-1 is not closely related to the commercial cultivars tested. Wild-2 was shown within Group I of both the Cluster (Fig. 4) and PCA (Fig. 5), and is more closely related to Saaz and Cascade cultivars. Because Wild-1 is more genetically distinct, it is more likely to have unique environmental adaptation traits than Wild-2, due to the fact that when breeding for yield and physiological/morphological adaptations, it is best to pair unrelated cultivars (Henning *et al.*, 2010). A similar study on hops genetic relatedness that used some of the same cultivars, (Galena, Fuggle, Cascade, and seeded varieties) saw similar segregation and groupings of these cultivars, in which Galena was distinct from other commercial cultivars, Cascade and Fuggle were in separate groups, and the seeded cultivars formed a group together (Townsend and Henning, 2009).

Although there are limited studies on the success of breeding wild hops with commercial cultivars, there are numerous examples of using the wild accessions of other crops to incorporate adaptive genes into commercial crop cultivars. For example, the wild non-edible banana *Musa acuminata* was used to provide resistance to black Sigatoka, a disease that was seriously impacting banana production (Escalant *et al.*, 2002). The genes of the wild relatives of tomatoes, *Lycopersicon pennellii* and *Lycium chilense*, were used to increase drought and salinity tolerance in cultivated tomatoes (Rick and Chetelat, 1995). Since the wild NE hops are genetically distinct from cultivated hops, they too have the potential to serve as a genetic resource for adaptive traits.

Yield

Patterns between yield performance and clustering groups emerged. The Principle Component Analysis (Fig. 5) showed that commercial cultivars that clustered together in Group I had higher yields compared to the commercial cultivars that clustered together in Group II. This suggests that cultivars may share markers that will allow genetic screening to predict yield performance. Further testing must be done due to the limited amount of yield data and hop lines used in this study, and to determine what those markers may be.

Conclusion

The findings that the native NE hop, Wild-1, is genetically distinct from commercial cultivars, and that we see a correspondence between yield success and the groupings of related cultivars, can aid in the search for hop plants with adaptive traits to the Midwest. Our results imply that breeders can further investigate what adaptive traits Wild-1 may have. Going forward, more wild NE hops should be assessed for genetic diversity. Markers should then be identified and used to detect any adaptive traits within wild hops that could be utilized for breeding. Focus should be on traits such as abiotic stress and disease resistance, since traits for increased yield are typically not selected for in the wild, and are not likely to be traits found in wild hops. However, the relationship between yield and the different genetic groupings of commercial hops should be investigated to see if there are any recurring markers in one group that would allow us to predict yield success of cultivars and breeding lines in the future. Increasing the knowledge about the diversity of native hop cultivars can aid breeders in choosing varieties that may lead to increased success in the growing of hops in the Midwest. Additional studies are necessary to continue the search for distinct wild hops that confer useful adaptive traits.

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