University of São Paulo
"Luiz de Queiroz" College of Agriculture

Application of genotype-by-sequencing for diversity and genetic marker identification of hop cultivars in Brazil

Ashley Noel Burricks

Dissertation presented to obtain the degree of Master in Science. Area: Genetics and Plant Breeding

Piracicaba
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BSc Life Sciences

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versão revisada de acordo com a Resolução CoPGr 6018 de 2011

Advisor:
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I would like to dedicate this dissertation to my nieces Andrea Dina and Lydia Williams

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CONTENTS

RESUMO

Aplicação de genotipagem por sequenciamento para estudo de diversidade e identificação de marcadores genéticos de cultivares de lúpulo no Brasil

Humulus lupulus é uma planta originalmente cultivada na Europa para produção de cones, que são utilizados na fabricação de cerveja. O Brasil tem uma grande indústria cervejeira, mas o cultivo do lúpulo tem sido limitado devido às condições climáticas inadequadas para a cultura. Um programa de melhoramento de lúpulo no Brasil é necessário para o desenvolvimento de cultivares adaptadas ao clima e rentáveis para o produtor rural. Para construir uma base sólida para os programas de melhoramento é necessária uma compreensão mais profunda do germoplasma disponível. Objetivou-se no estudo suprir essa necessidade utilizando o método de genotipagem por sequenciamento (GBS, em inglês) para ampliar a compreensão da diversidade genética de lúpulo disponível no Brasil, assim como desenvolver meios discernir com precisão a identificação de cultivares. Para isso, primeiramente foi identificado que o genoma de referência da cultivar Cascade e o chamador de variantes bcf tools como os mais adequados para os dados deste trabalho. Em seguida, 5971 SNPs de alta qualidade foram utilizados para distinguir duas populações.Foram encontrados quatro marcadores SSR, mas a análise não confirmou a eficiência do seu uso para identificação de cultivares. Tanto para a análise de marcadores quanto para o estudo de diversidade está claro que existiram erros de identificação de cultivares de lúpulo no Brasil, entretanto, não foi possível seu uso para identificação das cultivares nesse estudo.

Palavras-chave: *Humulus lupulus*, Genotipagem por sequenciamento, SSRs, SNPs, Estrutura genética, Genômica de populações

ABSTRACT

Application of genotype-by-sequencing for diversity and genetic marker identification of hop cultivars in Brazil

Humulus lupulus is a plant originally cultivated in Europe for their cones, which are used in the beer making process. Brazil has a large beer industry but its traditionally unsuitable climate has limited cultivation of the plant. A dedicated hop breeding program is necessary to produce unique Brazilian cultivars that are suited to the climate and are profitable for the growers. A deeper understanding of the available germplasm is necessary to build a strong foundation for breeding programs. This study seeks to meet those needs by using a genotype-by-sequencing approach; first to provide an understanding of the available genetic diversity of hops in Brazil, and second, to provide a means for discerning the accuracy of cultivar labeling. The study first isolated the Cascade reference genome as well as the bcftools variant caller as the most suitable for the current data set. The study then used 5971 high-quality single nucleotide polymorphisms to distinguish 2 distinct populations. Four SSR markers were found, but analysis has called into question the use of these SSR markers for cultivar identification. From both the marker analysis and the diversity analysis it is clear that there has been some mislabeling among the cultivars present in Brazil, however, it is not yet possible to use these markers for cultivar identification.

Keywords: *Humulus lupulus*, Genotype-by-sequencing, SSRs, SNPs, Genetic structure, Population genetics

LIST OF FIGURES & TABLES

List of Figures

List of Tables

1. INTRODUCTION

Hop (*Humulus lupulus* L.), from the *Cannabaceae* family, is a dioecious, herbaceous perennial climbing plant indigenous to the Northern Hemisphere. The plant can be divided into two parts, namely the perennial rootstock (underground) and the above-ground annual vegetative growth. The aerial vegetative growth is considered a bine, which is a modified stem that twists around a structure via spiral growth of the growing tip; in the case of *H. lupulus* the bine twists in a clockwise direction (Boutain, 2014; Edwardson, 1952). Most of the rootstock is underground; however, a section called the crown occurs above the ground. Multiple bines with leaves and other lateral bines sprout from the crown or rhizomes. The reproductive structures are inflorescences found as lateral growths from the bine — cymose panicles in males, and racemes of strobiles in females. Furthermore, female cones have overlapping bracts with rich oil deposits at the base (Boutain, 2014; Shephard et al., 2000).

Humulus. lupulus is primarily of interest to humans due to the enriched oils in the cones. Hop cones may contain more than 100 different types of essential oils in different ratios, resulting in a myriad of complex flavors ranging from fruity to spicy. Hop resins, commonly known as alpha and beta acids, are also important to beer flavor as they provide the classic bitterness of the beverage (Inui et al., 2013). These constituents are essential in that they provide the characteristic flavors associated with beer, but also help with preservation due to their antimicrobial properties. Outside of the brewing industry, hops are also used for flavoring in soft drinks and cooking, was named medicinal plant of the year in 2008, and is being researched for medical applications (Eyres & Dufour, 2008; Sakamoto & Konings, 2003).

The vast majority of hops is used for beer production, and a recent upward trend in craft beer production has had large impacts on demand for hops. In 2021, 128,068 metric tons of hops was needed for global beer demands (IHGC, 2021b). China, the United States of America (USA), and Brazil are currently the world's top three producers; however, only the USA and China have notable hop industries. Despite having one of the largest breweries in the world (ABinBev) and one of the top 10 worldwide craft beer industries, Brazil still imports 99,9% of its hops (Kirin Holdings Company, 2019; USDA FAS, 2020).

The Brazilian climate remains a major barrier to hops production. Traditionally, hops prefer long summer days and a dormancy period in cold winters. Although hops

have grown in Brazil for over 200 years, poor harvest predictions and low oil contents caused stagnation of the industry (da Silva Durello, 2019). Recently, renewed efforts have paved the way for improved hops production in Brazil, including the registration of hop cultivars and the formation of a hop society, Aprolúpulo (*Aprolúpulo*, 2018). These efforts have been accompanied by research into propagation, *terroir*, and plant breeding.

This study aims to assist current plant breeding efforts, with a primary goal of establishing a baseline for the genetic diversity of the registered hop cultivars in Brazil as well as specific landraces brought to Brazil by German and Polish immigrants. A pedigree constructed of known relationships between the different cultivars was used as a baseline to compare the genetic relationships as presented by the data. A secondary goal of the study was to evaluate genetic methods for cultivar identification, using the base assumptions that either single nucleotide polymorphism (SNP) or simple sequence repeat (SSR) markers are consistent between the same cultivars, and that a combination of at least five markers can be used to distinguish different cultivars. These markers will be used to assess the authenticity of the labeling of the available hops germplasm. A genotype-by-sequencing (GBS) approach using SNP markers was used along with draft genomes of the Cascade and Shinsuwase cultivars, sequenced and assembled at laboratories in the USA and Japan, respectively.

2. LITERATURE REVIEW

2.1 Hop Diversity

As one of only two members of the Cannabaceae *sensu stricto* family, *Humulus lupulus* is the closest known relative of *Cannabis*. Three species have been identified in the *Humulus* genus, namely *H. lupulus* from Europe and North America, and *H. japonicus* and *H. yunnanensis*, both from Asia (Boutain, 2014; Small, 1978; Tembrock et al., 2016). Small (1978) further classified *H. lupulus* into five botanical varieties based on eleven phenotypic traits: *H. lupulus* var. *cordifolius*, *H. lupulus* var. *neo-mexicanus*, *H. lupulus* var. *lupuloides*, *H. lupulus* var. *pubescens*, and *H. lupulus* var. *lupulus*. The origins of *H. lupulus* are thought to be in Asia, where all three species, including two of the five subspecies can be found, making it the most diverse region for *H. lupulus* (Boutain, 2014). It has been concluded that the plant migrated from Asia in two directions (Boutain, 2014; McCallum et al., 2019; A. Murakami et al., 2006). The European *H. lupulus* var. *lupulus* population split off from the Chinese population ca. 1.3 million years ago and spread through Russia to western Europe. Approximately one million years ago, eastward migration of the plant to Japan took place (*H. lupulus* var. *cordifolius*), as well as migration over the Bering Strait, resulting in diversification into three varieties in the Americas (*H. lupulus* vars. *pubescens, neomexicanus*, and *lupuloides*).

Various diversity studies have been done on wild hops using different genetic (Hadonou et al., 2004; Jakse et al., 2004; Murakami et al., 2006; Patzak & Henychová, 2018), chemical, or morphological markers (Mafakheri et al., 2020; Ocvirk et al., 2018; Patzak et al., 2010). Studies on wild hops have shown clusters of Asian, European, and North American hops; high genetic diversity has been confirmed in North America in particular (Howard et al., 2011). Studies on various cultivated hops showed that most shared ancestry is with European hops. While there was some admixture with North American hops they seemed to be more closely related to European hops (Peredo et al., 2010; Small, 1980). Most studies have focused on European wild hops; consequently, there are limited data on hops growing further east than Russia. Wild hops in Japan have been incorporated into some breeding programs, but with only 17 commercial cultivars and limited pedigree records, it is difficult to know the relationship between these cultivars and their wild counterparts (IHGC, 2021a).

2.2 Hop Cultivation

2.2.1 Hop life cycle

According to various guides on hop cultivation (Dodds, 2017; Spósito et al., 2019), initial expenses for farmers can be quite high because of the required infrastructure. Hop yards are easily distinguished by growing structures called trellises that are up to 6 m in height. Hop rhizomes are purchased and planted but typically require two to three years to reach maturity. In the spring, bines that grow from the crown of the rhizome are trained on to ropes that are suspended from the trellis. During its growth phase the hops grow to the top wire, after which the secondary bines develop along with the cones (Spósito et al., 2019). Once the cones have attained their maximum oil content, hops are harvested. by cutting the rope from the trellis and hand-picking the cones or putting them into machines for extraction. Hop cones are usually dried and stored as compressed pellets, which are sold to as beer brewing ingredients. Occasionally, fresh hops are sold directly to nearby brewers (Dodds, 2017).

2.2.2 Hop Breeding History

Neve (1991) described many of the early tradition surrounding hops cultivation and breeding. The early stages of hop cultivation in Europe relied on transportation, which was often limited; therefore, brewing would happen near the hop yards and breeding efforts would have been very localized. As transport technology improved, different hop varieties could be shared between different countries. At that time, Mainland European countries tended to name their cultivars from the region it came from, such as the Czech Saaz (Zatec) or the German Hallertauer, while in the United Kingdom (UK) they tended to name them after their growers, e.g. Fuggle and Golding. Many of these landraces, e.g. the American Cluster, had different phenotypes, such as Early and Late Cluster, Early Green, and Semi-Early Saaz, named according to their flowering stages Typically, farmers would plant them in sequential blocks to maximize yields from their staggered harvest times (Neve, 1991; USDA, n.d.).

The earliest breeding effort in the West started as early as 1894 in Germany, while the first successful breeding program was started in 1906 by Salmon in the UK (Neve, 1991). As hops are asexually propagated, entire fields are often genetically identical, leaving them vulnerable to diseases such as verticillium wilt, powdery mildew, and blight, which wipes out entire crops. The development of disease resistance is

what instigated the establishment of hop breeding programs; however, other characteristics such as yield, alpha-acid content and flavor have become incorporated into the programs (Neve, 1991). The most notable of these programs was started at Wye College in the UK, where many important cultivars such as Northern Brewer, Bullion and Brewers Gold were developed (Gerard & Lemmens, 2013). These cultivars along with Fuggle were resistant to downy mildew and were used as parents for many subsequent cultivars (USDA, n.d.). Downy mildew disease prompted breeding programs in the USA to develop resistant cultivars suited to the USA climate such as Cascade and Crystal (Julian, 2021).

Another important trait explored in hop breeding has been maintaining the aroma characteristics. Asexually propagated hops are marked by relatively constant chemical profiles, whereas the volatile flavors of sexually propagated offspring can differ dramatically from that of the parent (Neve, 1991). Brewers had become used to this, were invested in maintaining consistent flavor profiles in their beers, and were unwilling to substitute hops without a good reason (Neve, 1991). This tradition most likely prompted breeding programs in Europe to use available materials rather than incorporating wild materials. However, some programs used breeding methods heavily reliant on wild resources such as those in the USA which produced Cluster, a descendant of European cultivars and American wild hops (Edwardson, 1952), or the UK breeding program which used a wide variety of wild resources. The increased production of craft beer has dramatically changed the market, as craft brewers are interested in experimenting with different aromas and seek out varied flavors in the hops they use (Garavaglia & Swinnen, 2017).

Despite its distinct aroma, Cluster was still popular with American breweries due to its high alpha-acid content as well as the storage stability of the oil's storage (Neve, 1991). Alpha-acids provide the classic bitter taste of beer, and at the time the 7% alpha-acid content of the Cluster variety was significantly higher than that of European hops at around 5% (Julian, 2021). Part of the success of Bullion, Brewer's Gold, and Northern Brewer, was due to their high-alpha-acid content. Current breeding programs differentiate between hops that are only used for bittering, those only used for aroma, and those that can be used for both (Dodds, 2017). After years of breeding some hops now have up to 20% alpha-acid content. USA breeding programs were also

responsible for some high-alpha content varieties with initial successes such as Galena and Comet and later successes such as Nugget (Neve, 1991).

While disease resistance, aroma and alpha-acid content were primary concerns, breeding programs also focused on some other traits. Yield in terms of cone size and number is a major consideration, and a focus on yield prompted some breeders to experiment with triploid hops. Using colchicine and crossing tetraploid and diploid parents, some triploid staples such as Crystal, Willamette and Triple Pearl from the USA (Roborgh, 1969; Skovested, 1938) were bred. Not only did triploids have a larger size, but also low possibility of seed fertilization, which made them easier to manage for farmers. Currently there are also some programs interested in shorter ‘dwarf’ hops that will eliminate the need for tall trellises, which are expensive and cumbersome (Nesvadba, 2016).

Outside of the USA, UK and Europe breeding programs are either newer or not much is known about them. Japan, for example, has a well-developed hop industry and their breeding programs have been producing Japanese hops for decades. Indeed, some notable cultivars such as Shinsuwase, Kirin II and Sorachi Ace came from this program (Kirin Holding Company, 2019). The only public data about these cultivars indicates some European parents mixed with some indigenous Japanese hops. At lower latitudes, breeding programs are well established in Argentina, Australia, New Zealand, and South Africa. New Zealand produced a variety of triploid cultivars, and the breeding programs in Australia have released some prominent cultivars (Beatson & Inglis, 1999). The South African breeding program is the one with the lowest longitude to have developed commercial cultivars. The Argentinian breeding program is newer but aims to drive local hop production, having already released two local cultivars (Trochine et al., 2020). The newest trend in hop breeding has seen breeding programs established in tropical climates, with — notable efforts being made in Florida (Buck, 2021) and in Brazil. See **Table 1** for a complete list of the hop cultivars relevant to this study as well as their origin and parentage.

Cultivar Name	Ploidy	Year Registered	Country	Female Parent	Male Parent (if known)
AlphAroma	3n	1970	N. Zealand	Smooth Cone 4n	
Brewer's Gold	2n	1919	UK	Unknown	
Bullion	2n	1910	UK	BB1	
Cascade	2n	1955	USA	BB1	
Centennial	2n	1974	USA	19124	63015M
Chinook	2n	1974	USA	OR6619-04	63012M
Cluster	2n	landrace	USA	Golding	USA Male
Columbus	2n	1995	USA	Unknown	
Comet	2n	1961	USA	Unknown	58006
Crystal	3n	1983	USA	Sunshine	21381M
East Kent Golding	2n	landrace	UK	Hallertauer Mittelfrüh 4n	
Fuggle	2n	1875	UK	Landrace	
Galena	2n	1968	USA	Unknown	
H7 Leones	2n	Unknown	Spain	Brewer's Gold	
Hallertauer Mittelfrüh	2n	1956	Germany	Northern Brewer	
Hersbrucker	2n	Unknown	Germany	Landrace	
Magnum	2n	1980	Germany	Unknown	75/5/3
Mapuche Tehueller	2n	1992	Argentina	Unknown	Q82M2/21
Neo	2n	Wild-type	USA	Galena	
Northern Brewer	2n	1934	UK	Cascade	OB21
Nugget	2n	1970	USA	Wild parents	63015M
Perle	2n	1978	Germany	Canterbury Golding	1963/5/27
Polaris	2n	2012	Germany	65009	
Saaz	2n	landrace	Czech	Northern Brewer	
Sorachi Ace	2n	1994	Japan	Unknown	Beikei No.2
Southern Cross	3n	1970	N. Zealand	Landrace	53-5-61
SuperAlpha	3n	1976	N. Zealand	70K-SH6	
Tahoma	2n	2013	USA	Smooth Cone 4n	
Teamaker	2n	2008	USA	Smooth Cone 4n	
Triple Pearl	2n	2013	USA	Glacier	
Willamette	3n	1967	USA	Unknown	Fuggle male
Yakima Gold	2n	2013	USA	Perle (4n)	Slovenian male
Zeus	2n	2013	USA	Fuggle (4n)	
Brazilinsk	2n	Unknown	Brazil	Early Cluster	
M3	2n	Unknown	Brazil	Unknown	

Golding	2n	landrace	UK	Landrace	
Hallertauer Tradition	2n	landrace	UK	Landrace	
Mount Hood	3n	1983	USA	Hallertauer 4n	19058M
Pacific Gem	3n	1970	N. Zealand	Smoothcone 4n	OP
Pocket Talisman	2n	1970	USA	Late Cluster	OP
Saaz	2n	landrace	Czech Rep.	California Cluster	OP
Smooth Cone	2n	1960	N. Zealand	Fuggle N	OP
Southern Brewer	2n	1970	South Africa		
Spalt Spalter	2n	landrace	Germany	Early Green	
SpalterSelect	2n		Germany		
Sterling	2n	1990	USA	76/18/80	71/16/07
SUNBEAM	2n	1990	USA	Saaz 38	21361M
Tettnanger	2n	landrace	Germany	Saaz 38	OP
USADA 19058m	2n	unknown	USA		

Table 1: List of all the cultivars in the study along with their known ploidy, date of origin (if registered as a cultivar) and country of origin. Parents are only named if there is a record of them; OP under male parent implies open pollination.

2.2.3 Hop Cultivars in Brazil

Hops have been around in Brazil for a long time; however, records are sparse. One of the first mentions of hops can be found in an 1885 article from 'Revista Agrícola do Imperial Instituto Fluminense de Agricultura', published in Rio de Janeiro (Caminhoa, 1885). Other evidence points to immigrants from Europe bringing hops with them for personal use on two separate occasions: in Paraná in 1869 and in Rio Grande do Sul in 1953 (Puccilini, 2020). Apart from personal growers and a few small farms, the hop industry has remained relatively stagnant until the formation of a Brazilian Hops Society, Aprolúpulo, in 2018, following a boom in the Brazilian craft beer industry that prompted a demand for locally sourced hops with a unique Brazilian flavor. This association has united various players in the hop growing industry, including growers, breeders and researches, in the hopes of improving the hop production sector (Aprolúpulo, 2018).

As a relatively untapped industry sector, the Brazilian hops industry has no channels to register new hop cultivars and landraces. This means that even though

there are claims of some local cultivars and landraces, there are no means to assess these claims and formalize the cultivars. There is evidence of one cultivar that originated from the south-west of Brazil called “Mantiqueira”. Allegedly the cultivar was discovered in a discard heap by accident after the breeder gave up on hop breeding efforts. At this stage, little is known about the cultivar apart from its existence (Globo Rural, 2016). The hop variety brought to Paraná by Polish immigrants is called ‘Brazilinsky’, which probably comes from the Polish for ‘Brazilian’. In Rio Grande do Sul the variety is called Crioula (Puccilini, 2020). The origins of both varieties are unknown. Hop cultivation in a tropical climate presents some challenges for hop breeders. Given the lack of any formal studies done in Brazil thus far, the actual problems that hop growers in Brazil encounter are mostly unknown, and only inferences can be drawn from available literature. Hops are traditionally grown at latitudes higher than 35 degrees. Historically, only South Africa was cultivating hops at lower longitudes of 34 degrees — and even at these longitudes extra lighting was necessary to increase the day length (Joseph, 2015). Hop flowering is triggered by size of the plant unless the day lengths are long, in which case the plant grows indefinitely (Thomas & Schwabe, 1969). Another potential problem is that winters are warmer in Brazil than in Europe. Until 2019 it was thought that hops will not grow well without a dormancy period; however, Baurele (2019b) disproved this hypothesis and showed that no dormancy period was required between harvests. Since Brazil has no native hop species it will also be difficult to breed cultivars that are well-suited to the climate as well as resistant to diseases and predators endemic to tropical climates. More studies are required to understand the breeding needs for cultivars in Brazil.

To date, five individuals and one organization have registered a total of 46 cultivars in Brazil (Brasil, M. da A.). The registered cultivars are mostly from the USA (47%), followed by Germany (20%), the UK (11%) and New Zealand (11 %), with one cultivar each from Argentina, Japan, South Africa, Spain and the Czech Republic. Among the registered cultivars there is only one male, which was registered by the United States Department of Agriculture.

It is worth noting that Saaz and Saazer are both acceptable names for the Czech cultivar and have been registered twice. A similar situation can be found with two registrations for the Golding landrace, which has many variations, with one (East Kent

Golding) being more specific, while the other is simply registered as a Golding. Furthermore, H7 Leones is the Spanish name for a clonal selection of Northern Brewer.

2.2.4 Hop Cultivar Identification

Hop plants cannot be easily distinguished because most look the same and defining characteristics such leaf lobe shape can vary significantly even on individual plants. Some plants have distinguishing features, e.g. cone shape and color, but these are usually not unique to the cultivar. Chemotaxonomic identification methods can also be used by analyzing the alpha-acids, the essential oils of the flavor profile (Olsovska et al., 2016).

The *terroir* or location of the hop yard can have a massive effect on the chemical composition. Studies focusing on the Cascade cultivar reported substantial variation in the flavor profile as well as the contents of alpha and beta acids (da Silva Durello, 2019; Van Holle et al., 2021). Considering that different seedlings are often visually indistinguishable, they can easily be mislabeled, either accidentally or intentionally. Therefore, genetic methods for identifying cultivars has become increasingly popular.

2.3 Hop Genome

2.3.1 Hop Cytology

Hop cytology can be a complicated topic. The first studies in the early 1900s proved that the hop chromosomes can be elusive, especially when considering the number of sex chromosomes as well as the identity of the sex chromosomes. Two researchers, Winge (1929) and Sinoto (1929), established two different identification models after studying wild hops. Winge identified 9 bivalents, and Sinoto identified 8 bivalents with one quadrivalent. Neve later confirmed the presence of quadrivalents in hops, but was uncertain about whether they were sex chromosomes (Neve, 1991). Apart from specially bred cultivars, the current understanding is that hops are diploid with ten chromosomes — two of which are sex chromosomes, i.e., a larger X and smaller Y chromosome (Divashuk et al., 2011; Shephard et al., 2000).

A cytological study (Easterling et al., 2018) which included wild North American hops proved that, even with more modern tools many questions about the hop genome remain. DNA content assays indicated that one 2C parent, Apollo, had 3C offspring; fluorescence peaks indicated the possibility of endoreduplication in this instance.

Additionally, DAPI stains demonstrated frequent occurrences of meiosis bridges. They found that there was indeed a presence of a normal bouquet during early prophase in both wild-type and cultivated hops. However, during mid-prophase they noticed a lack of consistency in fiber appearance.

It seems that during diakinesis there is considerable variation, with canonical bivalents as well as some other complexes. In fact, the average number of bivalents ranges from 2.2 to 6.0 depending on the variety. This meant that on average between 8 and 22.5 homologues were left over to form other complexes. Among the other complexes found were double rings, rings of four, NOR-linked plus X, as well as various multiple complexes (Easterling et al., 2018; Zhang et al., 2017). As a result of these complexes, anaphase bridges are highly prevalent at an expected frequency of approximately one in two. This is indicative of a breakage-fusion-bridge cycle as described by McClintok (1941).

Hop sex determination has been demonstrated to be controlled by a dosage system (Jacobsen, 1956) where sex is determined by the ratio of X to autosomal sites in the organism. Generally, a 0.5 X dosage indicates a male organism and 1.0 X dosage indicates a female organism; however, different variations depend on the number of X chromosomes and polyploidy (Neve, 1991). In polyploid species, variations in the expression of male and female phenotypes can be observed on the same plant. It seems that the Y chromosome is not essential for the male phenotype. However, it is presumed that in *H. lupulus* the lack of this chromosome will result in the inability of pollen to mature, and development will be halted in the tetrad stage (Parker & Clark, 1991). While hops are usually dioecious, several cases of monoecious individuals have been observed (Sirrine, 2017). This phenomenon has also been observed in diploid hops, with an unequal distribution of male and female reproductive organs (Skof, et al., 2012). While extremely rare, there have also been studies that have observed bisexual plants (Neve, 1991; Shephard et al., 2000). It remains unclear whether monoecious diploid hops are XY or XX individuals, and the mechanisms underlying these variations are still unknown.

2.3.2 Molecular Studies

A variety of studies on hops have used different genetic markers. RAPD (Araki et al., 1998; Polley et al., 1997), AFLP (Hartl & Seefelder, 1998), DArT (Howard et al., 2011; McAdam et al., 2013), and STS (Mafakheri et al., 2020) markers were used in

genetic diversity, dendrogram construction, quantitative trait loci, male identification and cultivar identification studies. SSR markers in particular seemed to be preferred by various researchers (Čerenak et al., 2004; Murakami et al., 2006; Patzak & Matoušek, 2011; Sivolap et al., 2010) as they were more transferable between different labs and studies. While the markers themselves were consistently present in the studied cultivars, the repeats often differed between two identification studies for the same cultivars (Brady et al., 1996; Čerenak et al., 2004). Five differences were confirmed between the two studies, while four differences remained ambiguous. More recent studies have been able to successfully use single nucleotide polymorphism (SNP) markers; however, there are limited studies using this kind of marker. Yamauchi (2014) did an identification study using SNPs at three loci, and Henning et al. (2004) constructed a dendrogram of 116 cultivars and was able to identify each cultivar using only seven SNPs. Matthews et al. (2013) conducted a study using genotype-by-sequencing (GBS) with the most SNPs (17,128), whereas a single digest was used with the ApeKI enzyme.

To date, the genome of three cultivars of hops have been sequenced. The first sequencing was done by Natsume et al. (2015) in Japan using Illumina technology and the genome of the Japanese Shinsuwase cultivar; it was subsequently released as a draft genome. The second sequencing was done by Hill (2017), although the paper was published in a database journal and the focus was on the development of Hopsbase, a database for *Humulus* genomics; therefore, the specifics of the sequencing and assembly are not clear. Finally, Padgett-Cobb et al. (2019) used PacBio technology in 2018 to sequence the Cascade genome, which was also released as a draft genome. A recent preprint (Padgitt-Cobb et al., 2022) used the same sequencing data along with Hi-C libraries by Dovetail Genomics for a better assembly with 10 primary contigs containing (93%) of the sequenced genome. The Cascade genome is the largest of the draft genomes; it also has the best N50 score. See **Table 2** for a summary of the hops genome assemblies to date.

Cultivar	Shinsuwase	Teamaker	Cascade (draft)	Cascade (dovetail)
Year Sequenced	2014	2017	2019	2022
Contig Number	292698	194438	37223	1533
Assembly Length	1.81 Gb	2.77Gb	4,31 Gb	3,7Gb
N50	11.13 Kb	39.33Kb	73.5 Kb	345,3 Mb

Table 2: Overview of the different hop cultivar genome sequences.

2.4 Sequencing

2.4.1 Next Generation Sequencing

Genetic sequencing has been one of the fastest developing sectors of technology. Currently, the speed, accuracy, and cost effectiveness of these methods are dramatically better than they were just a decade ago. The current generation of sequencing technology has been called Next Generation Sequencing and includes three front-runners, namely Illumina, PacBio and Nanopore. Briefly, Illumina makes use of flow cells to which multiple short reads are attached; all the reads are simultaneously sequenced with fluorescent-labeled nucleotides which emit light, and this light is recorded and interpreted into a sequence by the machine. While this method has limitations on sequence length (50–300 bp), it is highly accurate and allows for the use of multiplexing with barcodes, which also reduces costs (Illumina Inc., 2017). PacBio and Nanopore are less accurate but can perform longer reads than Illumina. Nanopore technology is compact and mobile, and can be used with any compatible computer (Nanopore Technologies, 2020). Different technologies are useful for various studies. Overall, the price and ability to multiplex make Illumina a preferred method for genetic diversity and marker studies (Liu et al., 2012).

2.4.2 Genotype-by-Sequencing

Genotype-by-Sequencing (GBS) is a versatile research tool which has gained popularity among research scientists. It is particularly used in plant and animal science as it is cheap and generates many SNP markers (Illumina, n.d.). The method was

developed by Elshire (2011) and makes use of restriction enzymes, barcodes and Illumina technology. The restriction enzymes serve a two-fold purpose: they help to reduce the size of the genome by targeting the same regions of the genome across multiple individuals, in addition to standardizing the ends of the target DNA to which barcodes can be ligated. The barcodes allow for multiple individuals to be placed in the same lane during the sequencing step, thereby substantially reducing costs associated with such studies.

3. MATERIALS AND METHODS

3.1 Plant Material

Fifty-eight different hop samples were collected from various nurseries throughout Brazil. The samples represent 33 of the 48 registered cultivars in Brazil as well as one Brazilian landrace and one Brazilian male. There were 30 samples from Viveiro Ninkasi (Ninkasi, 2020) in Rio de Janeiro, 10 from Viveiro Alto Tietê (Lúpulo Alto Tietê, 2020) in São Paulo, 8 from Viveiro Hops Brazil (Hops Brasil, n.d.) in São Paulo, six from Viveiro Lúpulo Gaúcho (Lúpulo Gaúcho, 2022) in Rio Grande do Sul, two from Viveiro Van de Bergen (VandeBergen, n.d.) in Minas Gerais, and one from Lúpulo Tropical (Lúpulo Tropical, n.d.) in São Paulo. Henceforth the nurseries will be referred to as Farms 1–6 in the order mentioned above. The samples from Farm 1, Farm 2 and Farm 4 were collected on location and immediately stored in bags with silica beads; the bags were packed in Styrofoam boxes and sent to the lab by courier on the same day that the samples were collected. Once in the lab the samples were stored in −80 °C freezers. The samples from Farm 3, Farm 5 and Farm 6 were provided as cuttings and grown at the university. Samples were collected and immediately stored in −80 °C freezers. See **Table 3** for a complete list of all cultivars provided by the farms.

3.2 DNA Extraction, Library Preparation & Sequencing

DNA extraction was done using a CTAB protocol (Doyle & Doyle, 1987). The plant material was macerated using the liquid nitrogen method. The library was prepared using a GBS approach and following the protocol proposed by Poland and Rife (2012). A double digest of MseI & NsiL was used. Sequencing was done with Illumina NextSeq 1000 & 2000.

Source	Number	Duplicates	Cultivar(Code)
Farm 1	25 + 5	Duplicate 2 (Dup_2) Duplicate 3 (Dup_3)	***Alph Aroma(Alp_1),*** *Brazilinsk(Bra), Brewer's Gold(Bre), Bullion(Bul), Cascade(Cas_3), Centennial(Cen), Chinook(Chi_2), Cluster(Clu_1), Comet(Com_3),* ***Crystal(Cry), Dr. Rudi(DrR)****, East Kent Golding(Eas), Fuggle(Fug), Galena(Gal), H7 Leones(H7L), Hallertaur Mittelfrüh(Hal_2), Magnum(Mag_2), Mapuche(Map), Neo-1(Neo), Nugget(Nug_2), Perle(Per), Polaris(Pol), Saaz(Saaz_1),* ***Southern Cross(SoCo_1)****, Sorachi Ace(SAce_2), Tahoma(Tah_1), Teamaker(Tea),* ***Willamette(Wil)****, Yakima Gold (Yak) ,Zeus(Zeus_2)*
Farm 2	7 + 3	Duplicate 4 (Dup_4)	***Triple Pearl(3Prl)****,* ***Alph Aroma(Alp_2)****, Cascade(Cas_4), Chinook(Chi_1), Columbus(Col_2), Comet (Com_4) , Magnum(Mag_3), Nugget(Nug* 1), ***Southern Cross(SoCo_2)****, Tahoma (Tah2)*
Farm 3	7 + 1	None	*Cascade (Cas_1), Cluster(Clu_2), Comet(Com_1), Nugget(Nug_3), Saaz(Saaz_2), Sorachi Ace(SAce_1),* ***Tahoma(Tah_3)****, Zeus(Zeus _1)*
Farm 4	6	Duplicate 1 (Dup_1)	*Cascade(Cas_2), Columbus(Col_1), Comet(Com_2), Hallertauer Mittelfrüh(Hal_1), Magnum(Mag_1), Saaz(Saaz_3)*
Farm 5	2	None	*Hersbrucker(Her), Northern Brewer(Nor)*
Farm 6	1	None	*Unknown Male (M3)*

Table 3: List of all the cultivars provided from each of the farms, with the code of each cultivar provided in the parenthesis after the name. The added numbers in bold represent the triploid cultivars that were not used in the analysis due to their ploidy, and one sample on Farm 3 that didn't sequence properly and was discarded from the study.

3.3 Demultiplexing, Alignment and SNP calling

Demultiplexing was done using the process_radtags function of Stacks (Catchen et al., 2013). The demultiplexed samples were then aligned to both the Shinsuwase and Cascade reference genomes with Bowtie2 (Langmead & Salzberg 2012). SNP calling was done using stacks and bcftools (Li, 2011). For the bcftools pipeline the aligned reads were sorted using samtools, then summary information was collected with the mpileup function in bcftools, and variants were called using the bcftools call function. For the Stacks pipeline the aligned reads were analyzed using the stacks ref_map.pl wrapper. SNPs were filtered using vcfutils varFilter (Danecek et al., 2011) and vcftools. Polyploid varieties were filtered out for the analysis, the filtered cultivars were Alph Aroma, Crystal, Dr. Rudi Southern Cross, Triple Pearl and Willamette.

3.4 SSR Markers

The output from bcftools included various kinds of variants, microsatellites were found by looking for loci with more than one allele and then visually confirming those with repetitive patterns, repetitions of one nucleotide were not considered. The remaining microsatellites were checked to make sure they did not occur on either end of the read. The positions of the remaining microsatellites were checked and used to determine the alleles of the successfully sequenced microsatellites.

3.5 Population Structure

Population structure was analyzed using adegenet (Jombart, 2008) package in R. Population number and clustering was determined using the **find.clusters** command all (52) Principal Component were used for K-means clustering. Six populations were chosen based on the BIC value. A STRUCTURE analysis was done using two, three and four populations. Two populations were chosen based on the BIC value and comparison with STRUCTURE plots.

3.6 Trees and Scatter Plot

A population tree was made using the poppr (Kamvar et al., 2014) and ape (Paradis et al., 2004) packages in R. A bootstrapped dendrogram was also constructed using Nei’s distance with 1000 iterations. The tree was visualized using the ggtree

package (Yu, 2020). Principal component analyses were performed on the same data. 1Missing data were imputed using the k-Nearest Neighbor Imputation found in the VIM package (Kowarik & Templ, 2016).

4. RESULTS AND DISCUSSION

4.1 Sequencing

In total, 266.8 million reads of length 111 bp each were sequenced, with an average of 4.4 million sequences per sample with a low of 1.4 million sequences on sample 54 to a high of 9.5 million sequences. The majority of the reads had a Phred score above 28, peaking around 33, while the average for each sample was consistently between 32 and 34. With a usual cut-off Phred score of 20, the quality of these reads are considerably high, with a possible error rate of 0.01% (Illumina, 2022). One sample, Tah_3, was discarded from the study as the data did not work with the aligner.

4.2 Alignment and SNP calling

In order to assess the best genome and variant caller, the alignment was done with all of the three available reference genomes as well as with two different variant callers. See **Table 4** for a summary of this step. Approximately 115 million reads aligned to the Cascade reference, 99 million reads aligned to the Teamaker reference and 103 million reads aligned to the Shinsuwase reference. Since the Cascade reference was phased and assembled using Pacbio data, the assumption was that it would perform the best. However, after filtering the SNPs for no missing data, 999 Phred quality and at least a minor allele count of one, the Teamaker reference had seven times more SNPs than the Cascade assembly. The Teamaker reference has the least background information of all the hops references, some basic information like DNA extraction methods and the kinds of sequencing technology used is missing. Further analysis showed that the Teamaker reference was 35% Ns, this alongside the fact that less reads were aligned to the Teamaker reference indicate a possibility of multiple different reads aligning to the same location on the Teamaker reference. The Cascade reference, on the other hand, was assembled from a PacBio read which has less accuracy with longer read lengths; a single read can have as much as 13–15% error (Ardui et al., 2018), whereas a smaller error rate of 0.19–0.22% was found in the Cascade draft (Padgitt-Cobb et al., 2019). While low, this error rate is 20× higher than the Illumina error rate and with short reads (111 bps) alignment each error can have a large impact on alignment. The Shinsuwase reference did not provide enough useful SNPs after filtering and was not considered.

Different Stage of SNP filtering	Reference Assembly		
	Shinsuwase	**Cascade**	**Teamaker**
All Variants Called	1561413	2065686	1991865
Only SNPs	1490019	1838364	1865618
No missing data and filtered for Phred quality	4472	4488	6822
No missing data and filtered for depth	5	261	1984

Table 4: Alignment comparison of the three hops reference genomes, numbers represent SNPs at different stages of quality filtering.

When comparing the bcftools variant caller and the Stacks variant caller, bcftools outperformed Stacks by a large margin, with up to 750% more SNPs came from the bcftools pipeline. The bcftools pipeline was also more flexible as it was also able to identify indels while Stacks was only able to identify SNPs. The bcftools pipeline included the option of including tags with extra information regarding the SNPs allowing for more control during the filtering process. A separate study comparing Stacks, Samtools and GATK variant callers found that Samtools performed the best when there was missing data (Wright et al., 2019). Considering between 98–99% of loci had missing data; these results are in line with this study. See **Table 5** for a comparison of the variant callers.

	bcftools		**Stacks**	
	Shinsuwase	**Cascade**	**Shinsuwase**	**Cascade**
Raw SNP reads	1490431	1217097	411990	529898
Filtered SNPs	9509	4715	1270	916

Table 5: Variant Caller comparisons on two hop reference genomes. Numbers represent total SNPs called and total quality SNPs after filtering for missing data and Phred Quality.

Approximately half of the SNPs sequenced had a read depth lower than 2, indicating a low read depth in comparison with a high call rate. The only other published study of this kind on hops found a similar trend with their SNPs even though they used a single digest with a different restriction enzyme, ApeKI (Matthews et al., 2013). This study had a total of 17,128 SNPs, using an average read depth cut-off of 0.84 and an average call rate of 82%. Herein, we found 48,368 SNPs with a minimum read depth of 2 and a minimum call rate of 90%. The difference in number and quality is most likely because Matthew et al. had 178 samples in comparison with 61 in the present study. For example, in this data set when only considering 30 individuals there were 60% extra SNPs due to missing data.

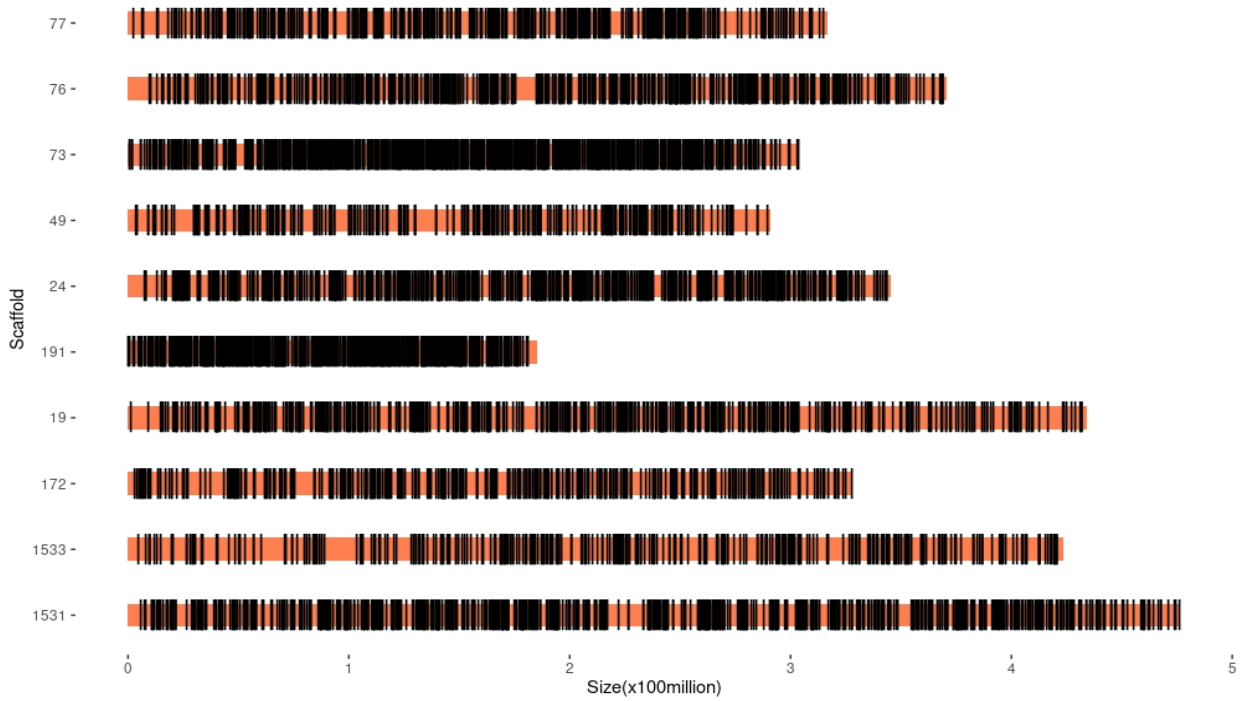

Figure 1: Distribution of SNPs on each scaffold from the Cascade reference. Scaffolds were named according to the names provided with the assembly and are not connected to any particular chromosome and are shown in red.

Using this information, the Cascade reference was used for further population structure and genetic marker analysis. In total 5971 high quality SNPs were found filtered for 80% maximum missing data, a minimum read depth of 4, a minimum genotype quality of 50, a minimum minor allele count of 1 and thinned to 1000 base

pairs. The SNPs were evenly distributed over the chromosomes (**Figure 1)** with the lower SNP to scaffold size ratio on scaffold 1533 with about 1 SNP to every 1,18 million base pairs and the highest SNP to scaffold size rations on scaffold 191 with about 1 SNP to every 271,000 base pairs. There were at least 300 SNPs found on each scaffold.

4.3 Population Structure

A K-means analysis was done on the SNPs, the BIC values indicated five clusters, which was too high for this small sample size. A STRUCTURE analysis done using K = 3 showed that one of the populations derived from the K-means analysis was a group of individuals that had 70% likelihood of being part of one population and 30% likelihood of belonging to another **(Supplementary Figure 1)**. K = 2 was chosen as the population number for this data set as both STRUCTURE and the K-means clustering showed the same populations (**Figure 2)**. The first population consisted of

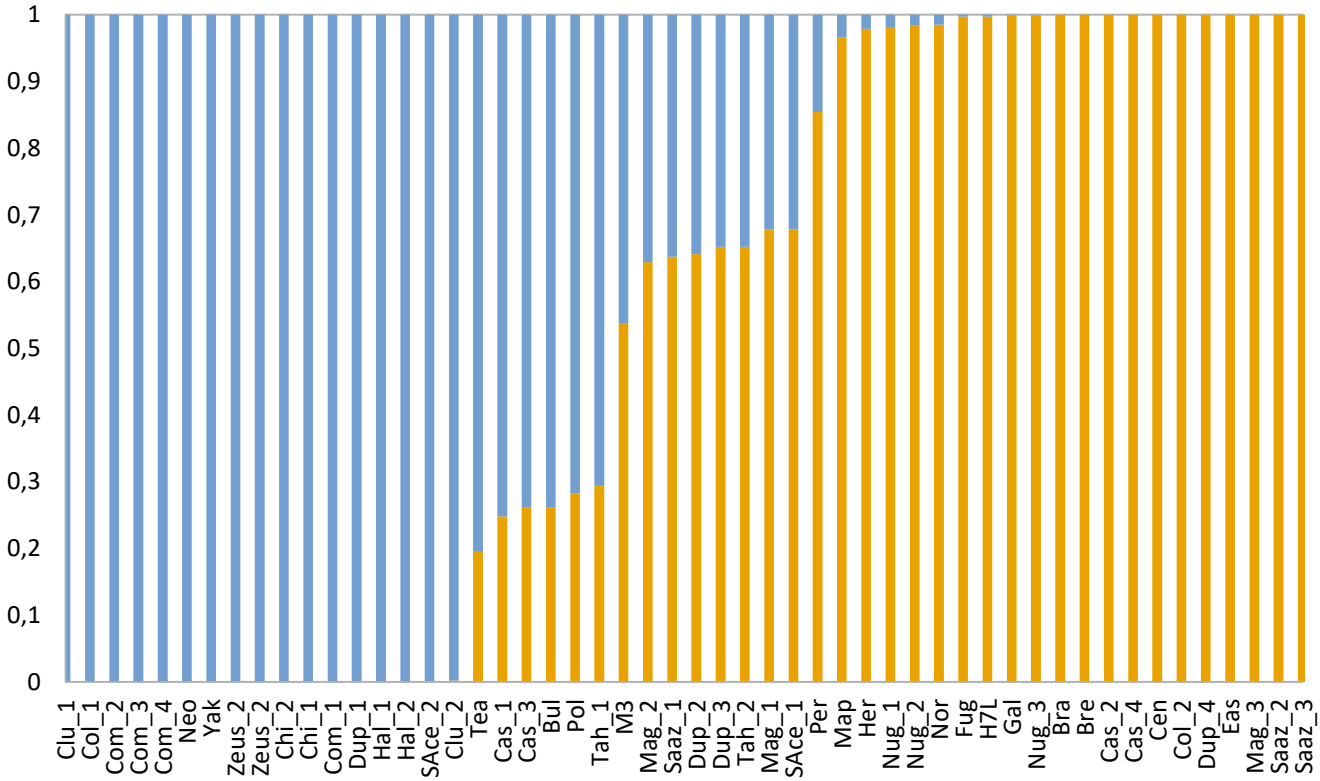

Figure 2: Membership Probability according to STRUCTURE. Population 1 = Blue; Population 2 = Orange.

23 individuals and 14 cultivars. Of the 14 cultivars, 10 have known origins in the US (Cascade, Chinook, Cluster, Columbus, Comet, Neo, Tahoma, Teamaker, Yakima

Gold, Zeus), 2 are from German breeding programs (Hallertauer Mittelfrüh, Polaris), and one each from the UK (Bullion) and Japan (Sorachi Ace). It is interesting to note that none of the US cultivars in this population have any known connection to Brewer's Gold. As Bullion is the daughter of BB1, a wild *H. lupulus var. lupuloides*, and many of the cultivars in this population are closely related to American wild types, it makes sense to see it in this population; however, it is surprising as Brewer's Gold is also a daughter of BB1. Seventeen individuals have more than a 99% probability of belonging to this population and six have more than a 70% probability of belonging to this population. Based on the high number of US cultivars represented in this population, it can be considered a US population.

The second population consists 29 individuals and 19 cultivars. Of the 19 cultivars, 6 are from the US (Cascade, Centennial, Columbus, Galena, Nugget, Tahoma), 4 are from the UK (Brewer's Gold, East Kent Golding, Fuggle, Northern Brewer), 3 are from Germany (Hersbrucker, Magnum, Perle), 3 are from South America (Brazilinsk, Mapuche, M3), and 1 each from the Czech Republic (Saaz), Japan (Sorachi Ace) and Spain (H7 Leones). Those individuals with more than 98% probability of belonging to this population are all descendants of cultivars from the UK (Brewer's Gold, Fuggle, East Kent Golding). Four of the cultivars from the US (Cascade, Centennial, Galena, Nugget) are either daughters or granddaughters of one of these cultivars. Five cultivars had a probability of between 60% and 70% of belonging to this population, two where present in both populations, and the other three were from Germany and the Czech Republic. Therefore, this population can be considered a European population centered around cultivars from the UK. The one male in the study (M3) is weakly part of this population with a 56% probability of belonging to it. A pedigree of the sequenced cultivars and their relation to each other are depicted in **Figure 3**.

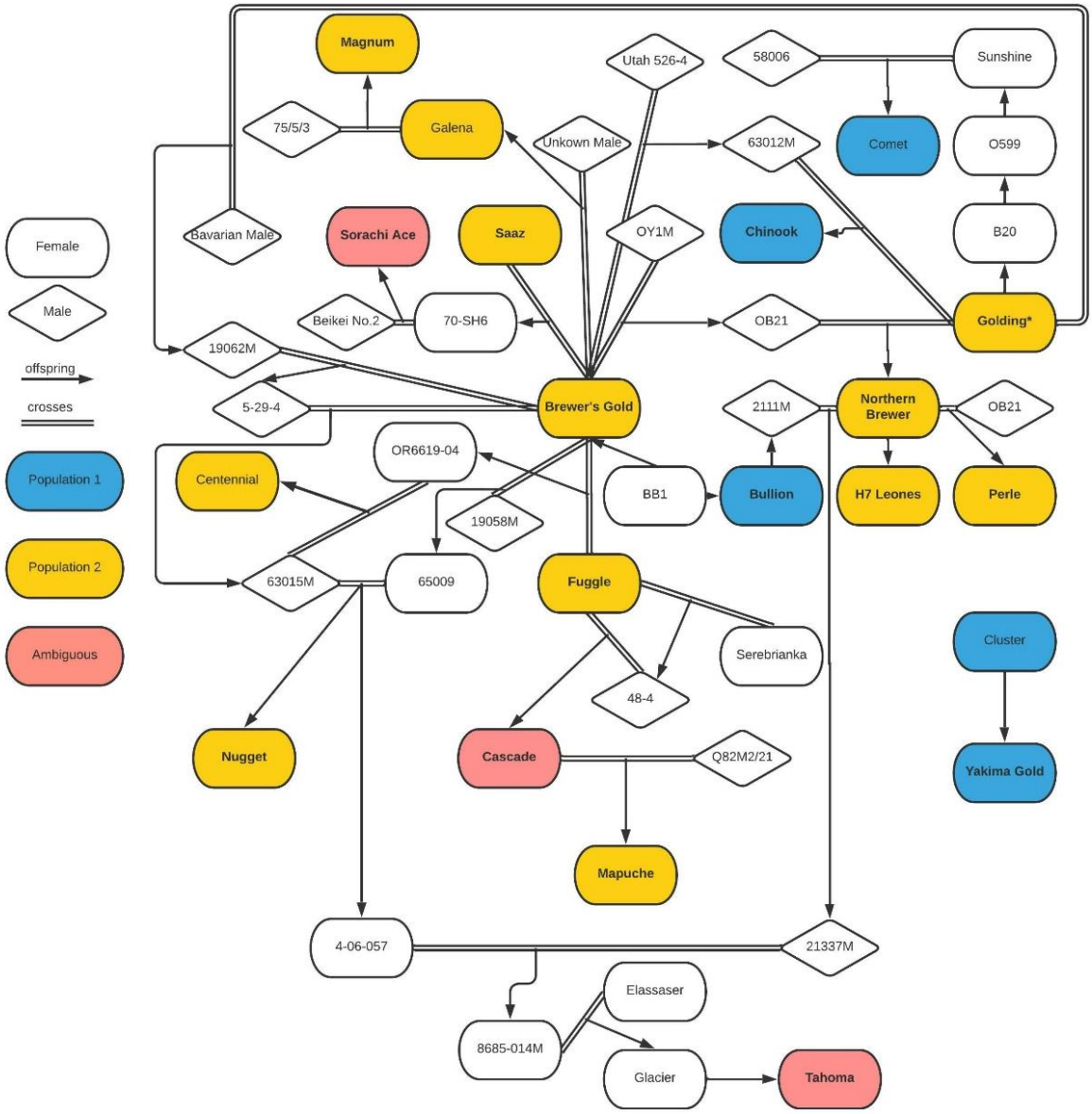

Figure 3: Hop Pedigrees from known and readily available resources. Boxes indicate genetic individuals, whether registered cultivars or breeding material. Diamond boxes and rounded boxes indicate males and females, respectively. Blue colored boxes indicate population 1 and yellow indicates population 2. Red boxes indicate those pedigrees that were present in both populations. Six of the cultivars in this study (Brazilinsk, Columbus, M3, Polaris, Teamaker, Zeus) have no known pedigree records.

4.4 Phylogenetic Tree and Scatter Plot

An unrooted neighbor-joining tree was generated along with a PCA scatter plot in order to get a better understanding of the tree and the relationship between the cultivars under investigation (**Figure 4**). The samples of Cascade, Columbus, Sorachi Ace and Tahoma were in different populations, indicating that these individuals are most likely not part of the same cultivar and have been misidentified. Some sources claim that there are at least two kinds of Cascade, namely the USA Cascade and the Argentinian Cascade, with clearly distinguishable differences in their alpha-acid and flavor profiles (Julian, 2021). However, it is unclear whether the differences are due to *terroir* or whether these cultivars are genetically distinct. Studies on *terroir* have found that Cascade from different areas can have a wide variety of flavors and alpha-acid content (Van Holle et al. 2021). Based on the known pedigree, the Cascade cultivars in population 2 are more likely the original due to their relationship to Fuggle and the presence of all other Fuggle-related cultivars in that population. Considering that Sorachi Ace is a descendant of Saaz and Brewer's Gold, it seems likely that the individual in the European population is the original as both of its ancestors are also in the same population. Without a pedigree it is not possible to make any assumption of the correctly labeled Tahoma or Columbus individual without further study. The male sample seems to be located in a strange position on this tree, as it is in the middle of another population, however, when looking at the population structure it is clear that this male has almost a 50% probability of belonging to either population.

The Chinook, Comet, Cluster, Hallertauer, Zeus and Nugget cultivars were all found in close proximity to each other on the neighbor joining tree. Based on these data, it is highly likely that all the samples of these cultivars are labeled correctly and are the same cultivar. The different Magnum and Saaz samples were all in the same population but were not close to each other. Saaz is a landrace, and due to its age, it is possible that many different genetic variants were labeled as Saaz in the past. Other notable deviations from expectation is that H7 individual, which should be very closely related to Brewer's Gold as a clonal selection of the cultivar, seems to be more closely related to Nugget. As siblings, Brewer's Gold and Bullion should be in the same population, and this was not found in this data set.

The duplicated samples were only considered against samples from the same location, even though the distance between them and another sample may have been

smaller. There were two duplicates from farm 1, the distance between them was very small and they appeared to be the same sample. They were most similar to the Saaz sample from the same location. The duplicate from farm 4 was identified as Columbus. The duplicate from farm 2 was closely joined to two other cultivars from the same farm, namely Cascade and

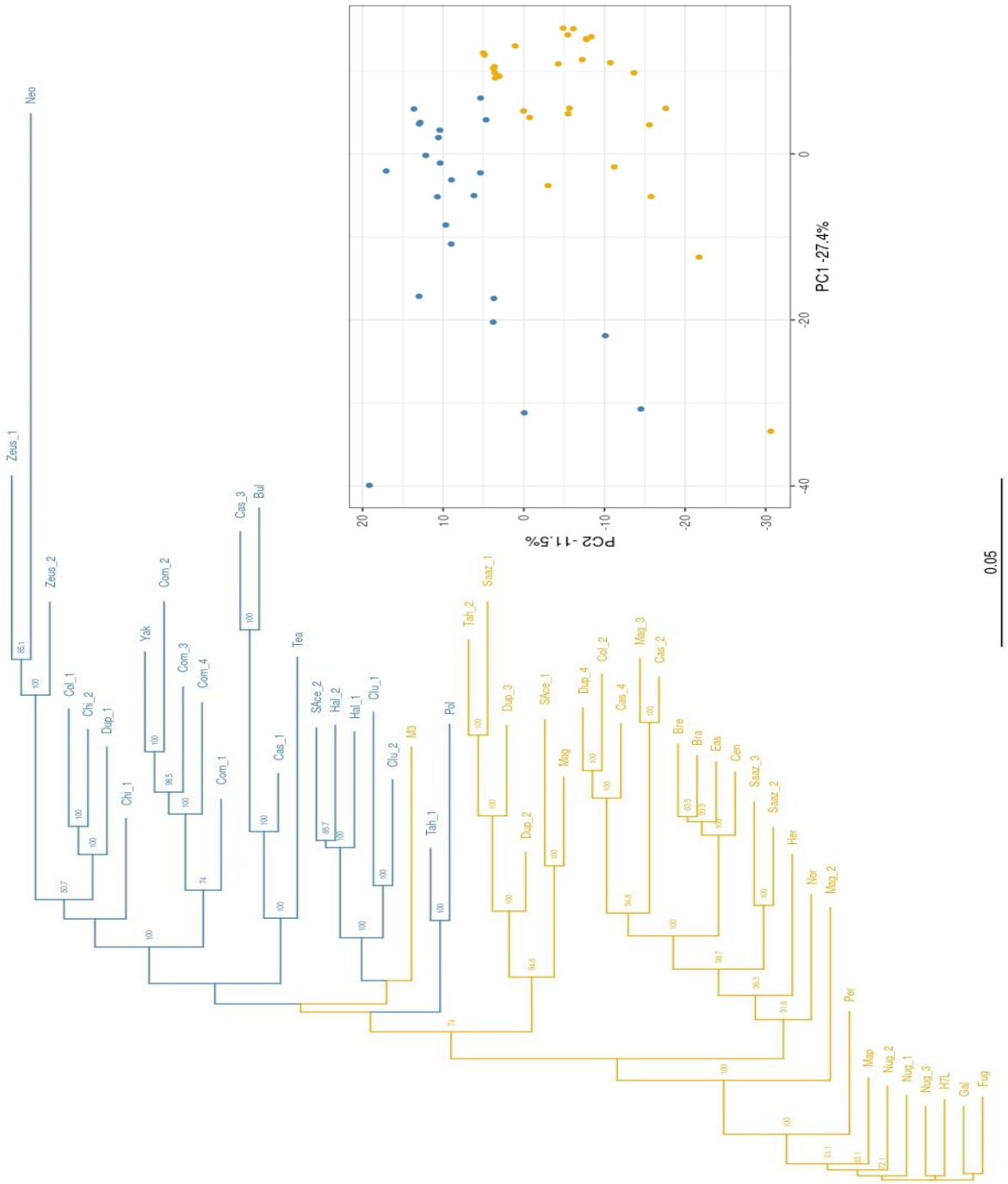

Figure 4: Unrooted neighbor joining tree and PCA scatter plot of all viable samples in the present study.

Columbus. Based on Nei's distance the closest is Columbus; however, it is possible that it is a duplicate of the Cascade **(Supplementary Figure 2)**. It is worth nothing that the Columbus from farm 2 and the Columbus from farm 4 are most likely not the same cultivar.

4.5 Markers for cultivar identification

Microsatellites have been commonly used in hop studies for cultivar identification. Most commonly used are four markers identified by Jakse et al. (2001) and known by their loci, namely 11a59, 7a82, 3a88, and 5-2. Two independent studies (Brady et al., 1996; Patzak et al., 2007) published data about the numbers of SSR repeats found in some common cultivars. Microsatellites are typically good genetic markers because of low intergenerational variation; however, this rate of change can vary depending on the microsatellite (Li et al., 2004). Markers found in non-coding regions tend to be more variable, as well as markers that consists of triplets as they do not change the reading frame; it is important to validate the quality of microsatellite markers.

Marker	**191_190**	**73_194**	**77_140**	**76_779**
Scaffold	191	73	77	76
Position	1901184	194122362	140350153	77902117
SSR (Reference)	TCC x 3	TG x 5	GT x 6	AAC x 3
SSR (Variant)	TCC x 2	TG x 2	GT x 4	AAC x 2

Table 6: Position and sequence of four SSR markers located in the study

While GBS studies are not ideal for studying microsatellites, the indels from the Cascade alignment data were examined and four microsatellites were found (**Table 6**). Two dinucleotide (repetitions of two bases) and two trinucleotide (repetitions of three bases) markers were considered. These markers were present in

all the duplicated cultivars in the study as well as in the unknown duplicates. For quality, only markers with a depth of three or more were investigated. Six of the cultivars (Alph Aroma, Chinook, Cluster, Columbus, Tahoma, and Zeus) had identical markers on each individual. Four of the cultivars (Nugget, Comet, Hallertauer Mittelfrüh, and Southern Cross) had one mismatched marker. Two cultivars (Saaz and Sorachi Ace) had two mismatches, and Magnum had three mismatches, while Cascade had four mismatches. On Cascade, the mismatches were on two of the four individuals, with two matching perfectly. No cultivar with more than three individuals matched across all the markers.

The microsatellites were analyzed for matches to previously identified markers, but none were found. None of the sequenced regions included any of the previously defined microsatellites. Further inspection of the microsatellites used in the identification studies (Brady et al., 1996; Patzak et al., 2007) found that four out of nine cultivars used in both studies did not match in 10 loci; two of the cultivars had three mismatches, one cultivar had two mismatches and two had one mismatch. To further investigate the markers, SSR and SNP markers for identification were compared to the Cascade reference genome. The SSR markers (Čerenak et al., 2004) were all located, but comparisons to the present study were complicated because the bp lengths were estimated from electrophoresis. The SNP markers (Yamauchi et al., 2014) were also located; however, they did not match the SNPs from the Cascade sample used in the study. **Table 7** presents the results of the marker identification on select cultivars in this study.

Cultivar		191_190	73_194	77_140	76_779
Sorachi Ace	1	0	0	1	0
	2	0	0,5	1	0,5
Southern Cross	1	0	0,5	1	0,5
	2	0	0,5	1	0
Hallertauer Mittelfrüh	1	0	0,5	1	0,5
	2	0	0	1	0,5
Magnum	1	0,5	0	1	N/A
	2	0	0,5	0	1
	3	0	0	0,5	1
Nugget	1	0	1	0,5	0,5
	2	0	1	0,5	0,5
	3	0	1	0	0,5
Saaz	1	0	0	N/A	N/A
	2	1	0	0,5	N/A
	3	1	0,5	0,5	N/A
Cascade	1	0	0	0	N/A
	2	0	0	0	1
	3	0	0	0	0
	4	1	1	1	0
Comet	1	0,5	N/A	1	N/A
	2	0	0,5	1	N/A
	3	0,5	0,5	1	N/A
	4	0,5	0,5	1	N/A

Table 7: Distribution of SSR markers along eight selected cultivars. Markers were found at all locations but only those with a read depth greater than three were counted.

5. CONCLUSION

This study revealed two aspects of hop breeding that need to be addressed by Brazilian hop breeding programs, namely genetic material and genetic markers. Picking the right parents for a breeding program can be essential to its success, as genetically distinct parents will produce a wider variety of offspring to choose from. Herein, we showed that the hops available in Brazil can generally be classified in two populations — one with genetic material that is more European and the other with genetic material that is more American. The American cultivars Neo and Zeus were the most genetically different from the rest of the cultivars in this study, and can provide more genetic diversity to hops in Brazil.

Many of the cultivars with the same name did not occur in the same population, indicating a potential for mislabeling among Brazilian hops cultivars. While microsatellites were identified, they were also unable to answer any questions of similarity between cultivars. The study also calls into question the transferability of SSR markers from other studies and their usefulness in helping to resolve hop identification issues in Brazil. To definitively categorize hops in Brazil, the material needs to be further analyzed for markers that prove consistently reliable.

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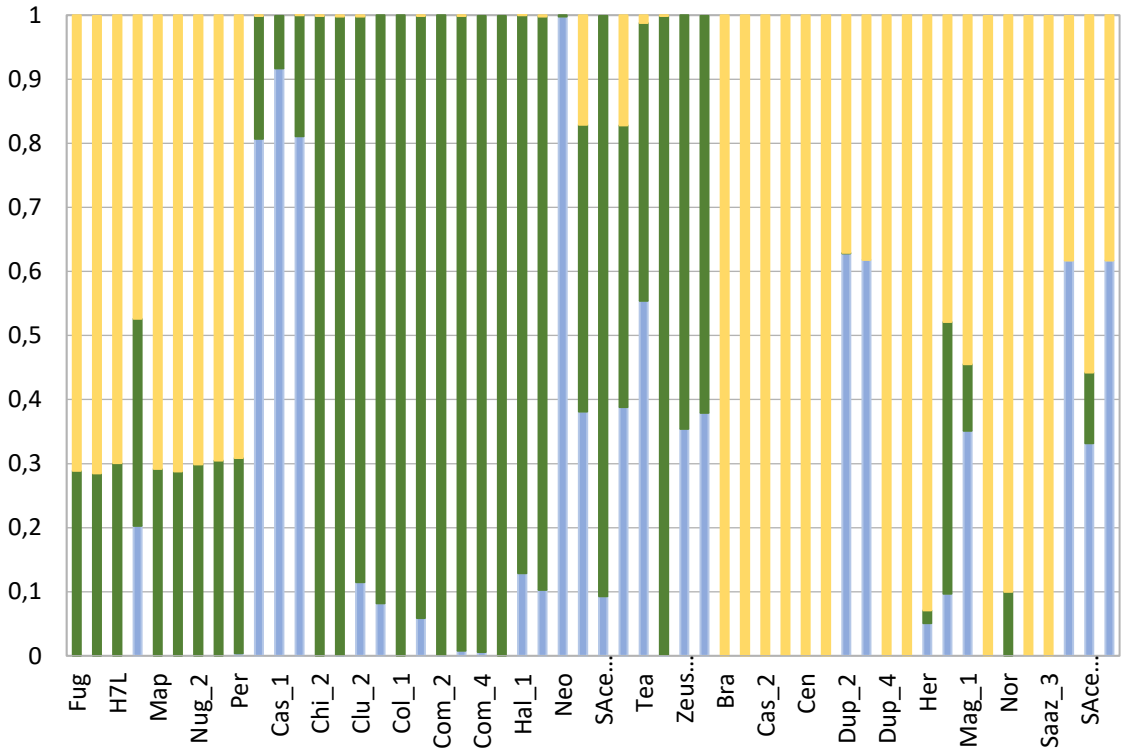
SUPPLEMENTARY MATERIAL

Supplementary Table 1: Structure Plot of K = 3 comparison between K-means and STRUCTURE.



Supplementary Table 2: Distance of the non-triploid individuals in the study