UNIVERSITÀ DEGLI STUDI DI PARMA
Dipartimento di Scienze degli Alimenti
Dottorato in Scienze e Tecnologie Alimentari
Ciclo XXVIII

Italian wild hop (*Humulus lupulus* L.): from biodiversity evaluation to varietal selection

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Index

Summary

Hop (*Humulus lupulus* L.) is a dioecius perennial plant. The cultivation is specific for female plants, used mainly for brewing and pharmacology. Female inflorescence, known as cone or strobili, contains bitter acids, essential oil and polyphenols.

Commercial hop cultivation provides better results in regions between 45 and 55 degrees north or south in latitude, an area that also includes the northern part of Italy, where hop is endemic. Despite several studies have been conducted on the characterization of wild hops biodiversity in the U.S.A. and Europe, a lack in literature concerning the description of Italian wild hops genetic variability is still present.

The increasing request of hop varieties improved in important traits, like diseases, resistance and valuable aroma profile, is bringing the hop industry. Moreover, Italian agricultural sector needs new impulse to be competitive in the market. In this view, Italian wild hop biodiversity is a resource, useful for the obtaining of Italian hop varieties, characterized by peculiar aromatic traits and more adaptable to Mediterranean climate, making their cultivation more sustainable.

Based on this consideration, the present Ph.D. thesis deals with the evaluation of the Italian hop biodiversity, through the characterization of the wild samples under different point of view.

The project started with the recovery of wild hop samples in different areas of north of Italy to consitue a collection field, where 11 commercial cultivars of US and European origin were grown, to have a complete vision of the hop panorama.

Ph.D. project followed different research lines, the results of each one contributed to completly characterize the northern Italian hop wild biodiversity:

- the morphological description showed a high phenological variability (Study 1);
- the genetic characterization confirmed the rich biodiversity of the Italian population and showed a significant genetic distance between Italian genotypes and the commercial cultivars, taken in consideration (Study 2);
- the need of an early sex discrimination method leads to an improvement of a genetic marker, developing a more efficient marker (Study 3);
- a complete morphologic, genetic and chemical analysis of plants gave results to select the most promising genotypes (Study 4);

- the comparison between the performance of wild hops and commercial cultivars in the same collection field indicated that some wild genotypes had a higher environment adaptability (Study 5);
- the evaluation of the terroir, obtained comparing commercial cultivars in the collection field and the same genotypes purchased in the market, showed the influence of the northern Italian environment on the aromatic profile (Study 5);
- a new analytical method for the revelation of bioactive metabolites and a simple extraction procedure were developed (Study 6).

In conclusion, the Ph.D. thesis, contains the first characterization of Italian wild hop, made under field condition. The present study: i) permits to obtain a complete and significative description of the genotypes; ii) allows the identification of the most promising wild Italian genotypes; iii) allows the identification of commercial cultivars more adaptable the northern Italian climate.

1. Introduction

1.1. Biodiversity: importance in agroecosystem

Biodiversity is "the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems (World Health Organization and Secretariat of the Convention on Biological Diversity, 2015 Connecting Global Priorities: Biodiversity and Human Health A State of Knowledge Review).

This definition reflects the different levels of biodiversity and the complex interaction between biotic and abiotic elements.

Research of biodiversity is a field in continuous expansion; Wilson (1988) reports that only a really small part of existing biodiversity (about 1,4 millions) as been described. Actually, the exact number of the species of earth is still not precisely known; however, it is possible to estimate the existence of 5 to 30 million of species, considering the still unexplored habitats such as coralline barriers, sea depths, tropical forests. The reason why biodiversity must be protected is in the uncountable function of microorganisms, plants and animals have in the ecosystem, ensuring resources indispensable for life as we know it (Holdren and Ehrilch, 1974; Ehrlich and Ehrlich, 1981). Thus, it is important to understand the great biologic diversity on earth and its essential and functional role for the equilibrium and for the maintenance of ecosystems. Moreover, it is necessary to consider that every species contains a great quantity of hereditary information and every single individual is virtually unique from the genetic point of view, due to the high polymorphism of the numerous *loci* present (Wilson, 1988).

In spite of biologic diversity is recognised as one of the most important resource of the planet, it is threatened by human beings. The great expansion of population and human activity, occurred in last centuries, has altered the global equilibrium, interfering with biogeochemical cycles, changing earth climate and intensifying organism disseminations. At the present, some consequences such as changing in plant flowering times and in the animal species migration and distribution are already visible. This alterations are causing modifications in the alimentary chain and in the ecosystem (Convention on Biological Diversity, 2010).

The use of fossil fuels, together with uncontrolled deforestation, has caused the increasing of CO_2 in the atmosphere; the rise of CO_2 is attested to be over 30%, and the amount of greenhouse gas is

doubled, with a strong increase in the last forty years. In the next century, it is expected a great change in climate conditions due to global warming, a change never seen on earth from the last glaciations, 18,000 years ago (Chapin et al., 2000).

Industrial agriculture and human activities have increased the presence of nitrogen in soil, with its consequent dispersion in soil and water ecosystems (Chapin et al., 2000).

Moreover, the transformation of natural ecosystem in cultivated fields is determining the loss of natural habitats and, consequently, the impoverishing of biodiversity (Convention on Biological Diversity, 2010). Furthermore, the globalization and easiness of transports are making very common the diffusion of plant and animal species in area different from origin ones (Chapin et al., 2000). This phenomenon is causing ecosystem disequilibrium due to food competition, diseases spreading and cross breeding with autochthon population (Convention on Biological Diversity, 2010).

The destruction of a species of insects causes the death of a culture dependent from it for pollination, or the loss of a natural enemy for harmful insects. Moreover, the possible disappearing of microorganisms connected with nitrogen assimilation, causes damages to agriculture, compromising soil fertility (Ehrlich, 1984; Ehrlich, 1988).

The crucial point is that every organism in the ecosystem has a fundamental role, functional for the surviving of the ecosystem themselves (Ehrlich, 1988).

1.2. Agrobiodiversity

Agricultural biodiversity (often referred to as agrobiodiversity) includes all the components of biological diversity of relevance to food and agriculture, and those that constitute the agroecosystem: the variety and variability of animals, plants and microorganisms at the genetic, species and ecosystem levels, which sustain the functions, structure and processes of the agroecosystem (FAO/PAR 2010). Agrobiodiversity is the result of natural selection processes and the careful selection and inventive developments of farmers, herders and fishers over millennia. Agrobiodiversity is a vital sub-set of biodiversity. Agrobiodiversity decline is an increasing phenomenon; the reason is the increasing request of food, and the request of uniformity and standardization from the global market that lead to the use of intensive monoculture, with consequent disappearing of local varieties (FAO, 2004).

Effectively, about 80% of the global food production derives from less than a dozen of species, plants and animal included (CEQ, 1981). This reliability/entrustment for human alimentation to a

restricted number of varieties of cereals, vegetables, fruit, nuts and legumes lead to the inevitable genetic erosion, as reported by a study conducted on 104 countries (FAO and PAR, 2010). The use of genetically uniform monoculture in agricultural systems makes it very vulnerable to extreme meteorological events, like drought or strong storms or climate changing and diseases (FAO and PAR, 2010).

To overcome the impoverishing of agrobiodiversity and make cultivated plants more adaptable to the aforementioned problems caused by climate change, it is necessary to exploit the existent biodiversity, through breeding programs. In this way, it will be possible to create diseases resistant variety and give yield stability in case of unpredictable or unfavorable events. (Rosenzweig and Perry, 1994; Chloupeck et al., 2004; Olesen et al., 2007; Seguin et al., 2007).

Knowledge of the morphological, ecological and agronomical characteristics of all available genotypes is indispensable for selection of the most promising individuals, best-suited to modern cultivation techniques. The genotype identification is the first step in order to classify and describe the existing germplasm and describe and isolate genotypes with valuable characteristics. Genotypic characterization use different types of markers, that could be of morphological, biochemical or molecular types. The conservation of the biodiversity is becoming a world priority. The *ex situ* germplasm conservation is one of the method mainly used and consists in collecting, characterizing and transferring in a collection field the plant material of interest, in order to create germplasm banks. Another valuable method of germplasm conservation exploits the potentialities of *in vitro* culture; with this option large number of plants can be stored in restricted space and diseases safe.

1.3. Hop biodiversity

Humulus comprise three species, *Humulus lupulus* L. (the common hop used especially in brewing) and *Humulus japonicus* Sieb. & Zucc. (the Japanese hop, used as ornamental plant) and *Humulus yannenensis* (Small, 1980) (grown at high altitude in southern China and only a little is known). The *Humulus lupulus* L. specie included the European variety *lupulus*, the western United States variety *neomexicanus* Nelson & Cockerel, the Midwest Unites States var. *pubescens* Small, the Northern Great Plains Eastward United States variety *lupuloides* Small and the Asian and Japan variety *cordifolius* Miguel. (Rydberg, 1917; Small, 1980; Mez, 1969; Neve, 1991; Murakami et al., 2006a, b). Hop is cultivated in areas comprised between 34 and 66° latitude, thus in Europe, North America, Japan, Asia, Australia and New Zeeland, Argentina and South Africa, The correct altitude for its growing is between 250 and 800 metres (Mez, 1969). Hop grow in banks of rivers, forests

and clearings and in places where it can find support for its stem and big amounts of water (Rybacek, 1991).

Hop cultivation is based on a relative small number of cultivars, characterized by either bitterness properties and peculiar aromatic traits. In England and Germany, countries with a long hop tradition, the last centuries trend was the cultivation of the few hop varieties and the extirpation of the wild hops, in order to avoid the seeds in the final product. These cultural choices have determined an important loss of the natural biodiversity. Nowadays, brewers and hop growers are interested in hop varieties characterized by new aromatic profiles, but also in manage a more sustainable hop cultivation, less needy in external inputs, such as pesticides and synthetic fertilizers. Therefore, hops biodiversity could be a new genetic resource for breeding. The crop genetic diversity has its bases in wild relative species and the developing was influenced by the processes of selection, cultivation and by the environmental conditions. The result of those processes is the conception of different landrace, that become the beginning material for further breeding (20 years of the National Programme on Conservation and Utilization of Plant Genetic Resources and Agrobiodiversity. Published by the Ministry of Agriculture of the Czech Republic Těšnov 17, 117 05 Praha 1 eagri.cz, info@mze.cz Prague 2013 ISBN 978-80-7434-138-0). In hop cultivation, the process of breeding began, in England, in the 18th century and the first varieties selected were Goldings, Bramligs, and Fuggle varieties (Burgess, 1964; Mez, 1969). The same process took place, in the same period, in Germany, in where, aromatic variety like Tettnanger were developed using as a start materials, wild hops.

Successively, in response to the demand of growers and brewers for new cultivars with different bitterness levels, high yielding capacities, and resistance to pest, hop-breeding programs started including American germplasm in the breeding crosses.

The genetic characterization of the hop varieties used nowadays, shows the interferences of genome of different origin, European and American.

Using molecular markers, Peredo and collaborators (2010) analysed 182 hop accessions, including wild European (68), wild American (48), and cultivars (66); they concluded that all the hop cultivars are closely related to the European group, indicating that most of them derived from European wild plants. Deep differences in chloroplast and nuclear DNA were detected among the American and European accessions using microsatellites: while hop cultivars are closely related to the European group, indicating that most of them derived from wild plants grown in several regions of Europe. The new cultivars, which include American genome in their pedigree, present slight differences to these traditional hops, although none of them was more similar to the American hops

than to the European, indicating a strong European influence in their germplasm (Peredo ert al., 2010).

Patzak et al. (2004), in a recent research, showed a phylogeny maps of 68 of the existent varieties (Fig. 1). In the figure, it is clearly shown, the complicated origin of the cultivars developed, and the wild ancestral precursors, like cv Manitoba (U.S.A), and as said before, the cvs Fuggle and Goldings for England.

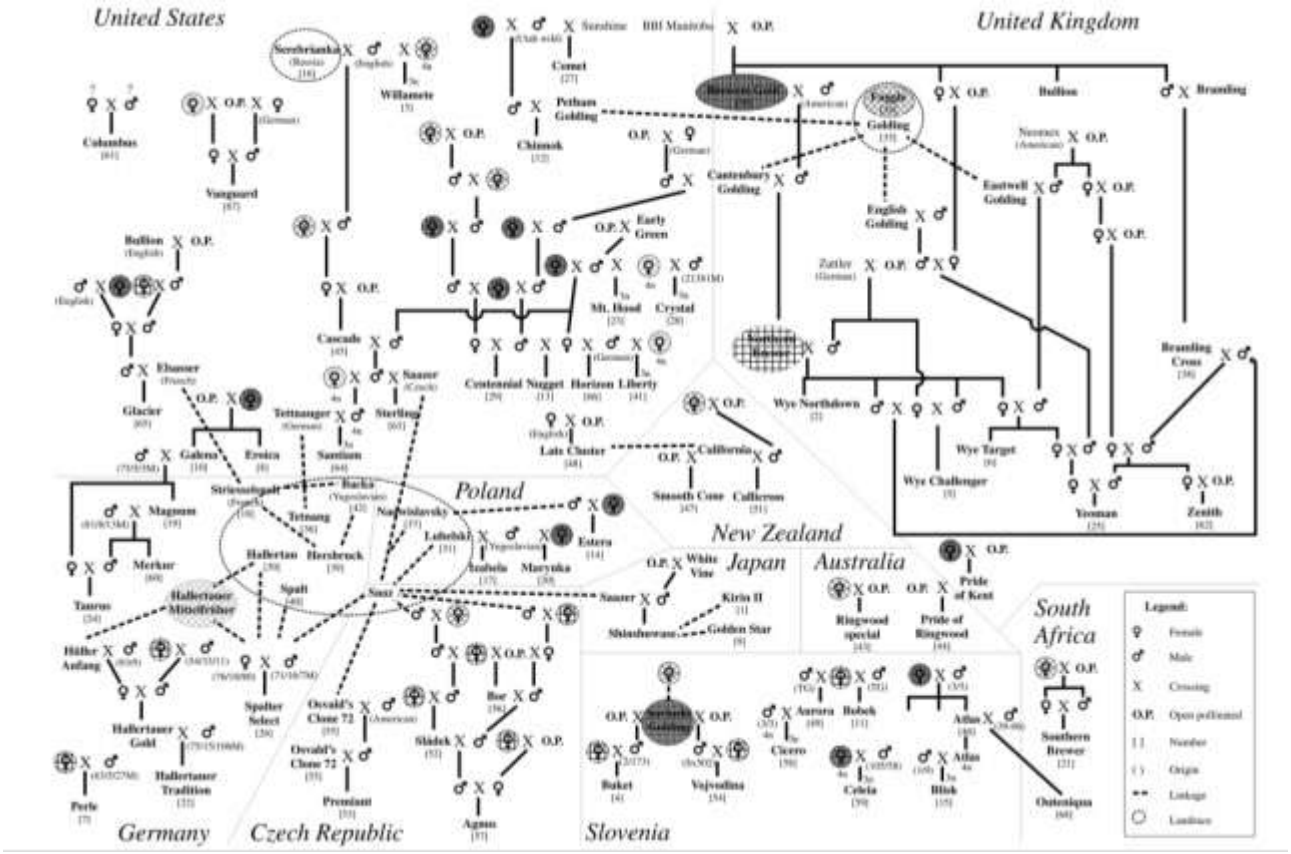


Figure 1 *Origins of 68 individual hop cultivar*

Nowadays more than 200 cultivars are grown worldwide (Patzak et al. 2007). An example of low exploitation the biodiversity is the fact that no breeding program includes Asiatic material at this moment in their crosses even though a high level of resistance to aphids was found in a wild Japanese male (Darby, 1999).

1.4. Hops in history

The hop (*Humulus lupulus* L.) is believed to be native of China, from where it diffused to Japan, America and Europe (Neve 1991; Murakami et al. 2003). Ever since the first century a.D. Pliny in his Natural History, cites hops as an ingredient for salads.

Hops were first used for dyeing tissues, making ropes and paper, and it was known as a medical source for liver, sleep and stomach disturbances by Arabic, Indian of America, Greeks and in the Ayurvedic medicine (Laws, 2010). The hop stabilizing properties were known by Egyptians and Sumerians who used fermented hops to produce beverages.

How hops become an essential ingredient for beer production is unknown; at the beginning, different aromatizing herbs were added during boiling to the beverage like rosemary, but this practise did not have success. Hop shows its stability property only after one hour and a half of boiling, and nobody knows how and who discovered this property. What it is documented from the beginning of IX century is that Swiss monks were enthusiastic brewers, so that in the design of their monastery were always expected the presence of spaces for beer production.

In 736 a.C., in a monastery in Weihensphan, near Munich (Germany), some documents described the first coupling of beer and hops; moreover, hops is cited by abbot Irminone, in his book "Polyptichus", in the first half of the IX century (Mez, 1969). Documented information about the utilization of hops, inherent to beer production, dated back 822 a.C., reports that a Benedictine monk from a monastery near Amiens (France) wrote some laws over the monastery life and hops were cited as a material to be stored for beer production, but it was not specified if hops were used as a stabilizing or aromatizing agent (Arnold, 2005).

An evidence of the utilization of hops as a stabilizing agent came from Rhineland (Germany), in the monastery of Rupertsberg, in 1150, where abbess Hildegard Von Bingen, philosopher and healer, published a book titled "Physica Sacra"; in this book, one chapter titled "De Hoppho" was dedicated to hops, and it is cited that the plant, as a consequence of its bitterness, contrasted the deterioration of bevereges, so it increases shelf life. Moreover she notice that hops has little use for humans, noting that it "increases melancholy in men." (Arnold 2005; Von Bingen, 2005). Beer production in monasteries was a tradition, and every monastery produced beers with distinct characteristics; notable of mention were and are the beers from Benelux and Holland, called Trappist beers. Hop gardens were practically of monastery ownership in France and Germany till the fourteenth century. In 1516, the Bavarian Reinheitsgebot (the German beer purity law) was put into effect declaring hops one of the three allowable beer ingredients, together with water and malt (yeast hadn't yet been discovered).

Hence, hop cultivation probably began in the 9th century in Germany and expanded in the 13th century, and testimonies of hop gardens are mentioned in state enactments of that time and Germany began to export hopped beer abroad.

The tradition of hop growing and processing in Czech Republic especially in the Saaz district, dates back to the Middle Ages (Rybacek, 1991) thanks to the introduction of beer from German population. Historic documentation reported information about hop gardens in Czech Republic, gone back to year 1348 (Mez, 1969). Žatec, an ancient Czech city, became the major beer production and developing place in the region till nowadays. In the 14th century, hop production and industries, had a drop down, and then again during the thirty-year war.

Hops diffused in the same period in Netherlands and only in the 16th century diffused in England, especially in Kent. The hop growing, was banned in England till 1500, then, it was allowed and the government engaged experts from the Netherland to teach English farmers the technique of hop growing. Books about English hop growing were published with instruction about cultural operations; one of them, written by Scot, in 1574, contains information still actual (Burgess, 1964). In Scot's book, also the instructions for hop drying are illustrated (Burgess, 1964). Before 1500, the hops used in English breweries were imported from France, Holland and Germany, with duty to be paid, so the hop cultivation were incentivized in England. England hop growing spread rapidly and arrived also in Wales, at the end of the 16th century.

In 1710, the English parliament banned the use of non-hop bittering agents, in part to prevent brewers from evading the new penny-per-pound hop tax. Thus, hops became the dominant bittering agent in beer, throughout the western world. Defoe, in 1724, reported that the growth of hops around Canterbury was exponential, and there were 6,000 acres of hop gardens in this district (Burgess, 1964). At the end of the 18th century, first steps on hop breeding were made, with the selection of cultivars Goldings and Bramligs, (Burgess, 1964); furthermore, in 1875, Richard Fuggle, a hop cultivator and researcher, selected the new variety Fuggle (Mez, 1969).

The cultivation of hops expanded around 1630, English and Dutch people arrived in America. Farmers, migrated to the new continent, began to cultivate hops around New York, California, Oregon and Washington. At the beginning, to produce beers, American brewers used firstly wild hops, then exploiting the advancement made in the old continent started to use also the European cultivars. From this moment, hops were developed greatly and new varieties were found, like the well-known aroma variety Cascade. In the New York district, in 1830, hops were the major crops cultivated.

Since the 1850, Western states of Washington, Oregon and California became one of the major pole for the beer production. The positive phase of hop production in the U.S.A. ended with the beginning of prohibition in 1919, and revived at the end of this period (around 1947). (Vang et al., 1996). Meanwhile, migration continued all over the world, and, during the 1800s, the far away islands of New Zealand became a destination for Europeans of many nationalities. British and mid-European settled in the new land, bringing with them also their hops (McLauchlan, 1994). New varieties were developed, crossing hops from Europe with wild hops and, by 1960, three resistant hop strains, known as Smoothcone, First Choice and Calicross were obtained. While other hop growing countries were approaching the problem of seeds in hop cones by eliminating male plants from their hop gardens to induce seedlessness, in New Zealand, researchers noted that in the botanical world, interest had surged in tetraploid and triploid plants that had successfully been produced in other plant varieties.

In parallel, a great flux of European migrants, reached the Australian coasts and in the 1930s, western Australia was the third state to establish hop growing on a commercial basis.

1.5. Hops in Italy.

It is commonly accepted that, also in Italy, like in all Europe, Benedictine monks began to produce beers, using before different herbs and spices and then hop cones.

Documented experience of hop cultivation in Italy is described by Gaetano Pasqui (Pasqui, 2010), Agronomist from the city of Forlì, in Emilia Romagna region, in the 19th century. The earliest documentation of beers produced by Pasqui dated back to 1847. Pasqui started to select, grow, and characterize plants of spontaneous hops (Pasqui, 2010), because of the high cost of the hops sold from Germany. In the selection, Pasqui considered as important morphological trait the colour of the stem: Pasqui (2010) supposed that hops with green stems were better than hops with red stems. After the selection program, Pasqui was able to launch the first handcrafted beer, using hop cones produced by Italian hop genotypes (Pasqui, 2010).

In 1876, in Marano sul Panaro (Modena), in the estate of Marquis Montecuccoli, Styrian and Bohemian hop varieties were used to start hop cultivation in that area. The results were comforting and the hops produced were publicly praised by numerous Italian and foreign beer brewers; the product, also, received a honourable mention at the international Hagenau (Alsace) exhibition, in 1860 (Mageira, 1875). There is a lecture of the "comizio agrario di Modena" in which Mageira explained practically the new unusual cultivation of hop; in his lecture, Mageira said: "it seems to

be possible to consider hop among the plants that take welfare in agriculture in Emilia Romagna". In this lecture, it was described the experience of Pasqui: in 1873, Pasqui's hopsyards were productive and allowed him to produce "good beer and a good quantity of money". Mageira asserted that, for a complete description of the cultivation, money are very important, so, he reported the prospect of Pasqui cultivation, inserting numbers of plants, yield, incomes, spending and profits. He repeated the good results and tried to communicate the advantages of cultivating hops in Italy, so that, Italian growers could become hop exporters and not only importers (Mageira, 1875). In Mageira's report, it is also cited Mr. Ottavi, owner of an experimental hop field in Bologna, with 450 hop plants, who said that hops were ten times more profitable than wheat. It is mentioned that Mr. Ottavi obtained 61.50 kg of dried hop cones, with an outcome really promising. Mageira reported, also, the Marquis Montecuccoli'experience who cultivated 220 hop plants: in the first two years of cultivation, the harvest was a little low, but always profitable, and from the third year, the harvest was better than in Rottembourg. This lecture represents the first evidence that hop cultivation in Italy was possible and took good result (Mageira, 1875).

After all the above reported experiences, other hop cultivation were carried out in Italy by Mr. Faina in 1908, by Luciani Brothers, in 1914, by Moretti in 1927, by Dandoni in 1959 (Caracausi 2006; Buiatti). Afterwards the interest in hop cultiavation declined, to increase again recently; in fact, the appeal of beer has rapidly grown and a great number of small breweries has flourished, spurring a renewed interest for local raw materials.

1.6. Hop production and sector analysis

In the last years, the hops cultivated areas has increased; the most important change reported by Barth and Haahs is the reconversion of the varietal panorama, substituting high alpha varieties with aroma variety; this reconversion started in 2006, when the area cultivated was 46.246 ha and carried out until the 2014, when the hectares were 47.766 (Figure 2). World hop production, in 2014, was estimated to be of 96,477 tonnes, value that has increased if compared to 2013 (83,232 t) (Barth and Haas Report, 2015); specifically,, between 2013 and 2014, high alpha acreage has been reduced in the world (-11%), while aroma hop acreage increased (+3%) (Figure 1).

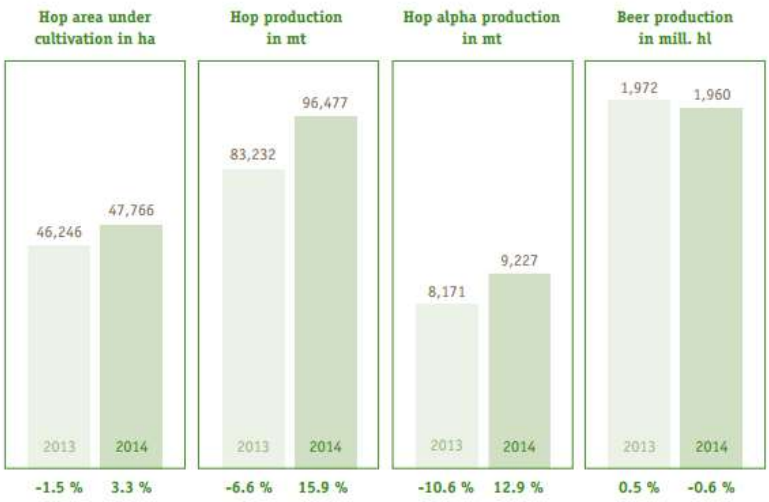


Figure 2 *World market key data*

The market analysis reported that, in 2014, aroma and flavour hops were sold out; the data confirm that consumer and beer producers are more interested in flavoured beers (Hintermeiner, 2014). In the last years, it was possible to observe, also, an increase in beer production that lead to an increase of hop request and, them in the hop production (Figure 3).

Figure 3 *Hop market development from 2003 to 2014*

The major world hop producer countries are China, USA and Germany. China showed a preponderance of bitter and high alpha hops cultivation. There is a decrease in the acreage dedicated to bitter varieties and a little increase in aroma hop areas. The yield for hectare was a little higher in

2014 than 2013, but the decrease of cultivation acreage has, as a result, the decrease of the total hop production (Table 1) (Bart and Haas Report, 2015).

Variety	Development of acreage Acreage ha			Development of production Ø Yield mt/ha		Production mt	
	2013	+/-	2014	2013	2014	2013	2014
Total Aroma	226	17	243	2.38	2.43	537.0	591.4
Total Bitter	2,081	-202	1,879	2.58	2.56	5,359.0	4,808.0
Total High Alpha	524	9	533	2.48	2.79	1,298.0	1,487.6
CHINA TOTAL	2,831	-176	2,655	2.54	2.59	7,194.0	6,887.0

Table 1 *China hop acreage and crop. Comparison between the years 2013 and 2014*

Regarding USA, the place in where the inversion of trend began, thanks to the development of craft brewers, the tendency remains the increase of aroma hop dedicated areas, versus the decrease of high alpha hop production areas. Strong demand from U.S. craft brewers continues to drive the market activity to aroma and flavour hops (Table 2) (Bart and Haas Report, 2015).

Variety	Development of acreage Acreage ha			Development of production Ø Yield mt/ha		Production mt	
	2013	+/-	2014	2013	2014	2013	2014
Total Aroma	7,062	1,954	9,016	1.77	1.77	12,499.2	15,923.2
Total High Alpha	7,192	-825	6,367	2.64	2.56	18,954.8	16,279.5
USA TOTAL	14,254	1,129	15,383	2.21	2.09	31,454.0	32,202.7

Table 2 *U.S.A. hop acreage and crop. Comparison between the years 2013 and 2014*

The first hop producer in Europe is Germany (Table 3) (Bart and Haas Report, 2015).

Area	Variety	Development of acreage Acreage ha			Development of production Ø Yield mt/ha		Production mt	
		2013	+/-	2014	2013	2014	2013	2014
Total Aroma		9,352	524	9,876	1.31	1.97	12,290.47	19,408.70
Total Bitter		299	-15	284	1.40	2.09	419.15	593.67
Total High Alpha		7,166	-47	7,119	2.07	2.59	14,813.44	18,465.65
Total Other		31	-3	28	1.00	1.13	31.08	31.75
GERMANY TOTAL		16,849	459	17,308	1.64	2.22	27,554.14	38,499.77

Table 3 *Germany hop acreage and crop. Comparison between the years 2013 and 2014*

In Germany, the production of aroma hops increased respect to the bitter ones. The decrease in the production of bitter hops signs a -47 ha,, and a increase of +524 ha in aroma hops; an important increase is registered both for the total production, from 27,554,14 t (2013) to 38,499,77 t (2014),

and for the yield of aroma hops from 12,290 mt (2013) to 19,408 mt (2014) (Table 3). This is a big change in German cultivation habits and represents the new tendency of the brewing industry, oriented in aromatic beer productions.

The same trend is visible also for the hop production in Czech republic (Table 4) (Bart and Haas Report, 2015), where the increase of acreage dedicated to aroma hop production is significant: from 2013 to 2014 there is a +150 ha, with an increasing in aroma hops production of about 1,000 mt in 2014. The increase of total hops production in 2014 is mainly due to the increase of aroma hops yield. The decreasing of hectares dedicated to bitter hops is lower than in Germany (Table 3-4)

Variety	Development of acreage Acreage ha			Development of production Ø Yield mt/ha		Development of production Production mt	
	2013	+/-	2014	2013	2014	2013	2014
Total Aroma	4,258	150	4,408	1.23	1.39	5,239.5	6,113.3
Total High Alpha	48	-6	42	1.72	1.93	82.6	81.0
Other	13	-3	10	0.59	0.77	7.7	7.7
CZECH REPUBLIC TOTAL	4,319	141	4,460	1.23	1.39	5,329.8	6,202.0

Table 4 *Czech Republic hop acreage and crop. Comparison between the years 2013 and 2014*

In Poland the trend is the same of the other European producing hop countries (Table 5), with a 10% increase in acreage dedicated to aroma hops respect to 2013, but the majority of hop production remain focused in bitter and high alpha hops (fTable 5) (Bart and Haas Report, 2015).

Variety	Development of acreage Acreage ha			Development of production Ø Yield mt/ha		Development of production Production mt	
	2013	+/-	2014	2013	2014	2013	2014
Total Aroma	532	54	586	1.49	1.34	793.1	784.2
Total Bitter/High Alpha	875	-51	824	1.86	1.56	1.627.6	1,288.1
POLAND TOTAL	1,407	3	1,410	1.72	1.47	2,420.7	2,072.3

Table 5. *Poland hop acreage and crop. Comparison between the years 2013 and 2014*

Regarding Slovenia's trend, a continue rising in the production of aroma hops is registered, in terms of hectares cultivated and total hop production (Table 6) (Bart and Haas Report, 2015).

Variety	Development of acreage Acreage ha			Development of production Ø Yield mt/ha		Development of production Production mt	
	2013	+/-	2014	2013	2014	2013	2014
Total Aroma	1,139	135	1,274	1.11	1.78	1,267.4	2,271.0
Total High Alpha	27	-5	22	1.09	2.14	29.8	47.8
SLOVENIA TOTAL	1,166	130	1,296	1.11	1.79	1,297.1	2,318.8

Table 6 *Slovenia hop acreage and crop. Comparison between the years 2013 and 2014*

England, one of the most ancient and traditional hop cultivation country is not in line with the other countries for the year 2014. Even if most of the English varieties are aromatic, and the total hop production was and is overall due to these varieties, the acreage destined to aromatic hop production decreased in 2014 (Table 7) (Bart and Haas Report, 2015).

Variety	Development of acreage Acreage ha			Development of production Ø Yield mt/ha		Production mt	
	2013	+/-	2014	2013	2014	2013	2014
Total Aroma	756	-51	705	1.21	1.57	915.7	1,105.1
Total High Alpha	229	-5	224	1.40	1.57	321.0	351.7
ENGLAND TOTAL	985	-56	929	1.26	1.57	1,236.7	1,456.8

Table 7 *England hop acreage and crop. Comparison between the years 2013 and 2014*

For 2015, hop acreage is expected to continue increasing, most of those, for the production of aroma varieties. World hop acreage in 2015 amounts approximately to 50,900 ha, with an increasing of 3,145 ha respect to 2014. In the last 26 years, only in 2008, such a development in hop areas has been registered. It is also expected a continuous decrease in the production of high alpha and bitter hops and an increase in the production of flavor and aroma varieties (Barth and Haas Report).

1.7. Element of hop botany

Domain	***Eukariota***
Kingdom	***Magnoliophyta***
Class	***Magnoliopsida***
Order	***Urticales***
Family	***Cannabaceae***
Genus	***Humulus***
Species	***H. lupulus L.***

Table 8. *Hop botanical classification.*

Figure 4 *Botanical table of* Humulus lupulus *L.*

Hop (*Humulus lupulus* L.) (Figure 4) is included in the Urticales order, family Cannabaceae (Table 8). This small family comprises only two genera: *Cannabis* and *Humulus*; *Cannanbis* is represented

only by Hemp (*Cannabis sativa* L.), used for textile industry and for the drug (hashish or marijuana) obtaining.

The name of the specie *H. lupulus* is originated by two terms: Humulus, derived from the latinization of the Slavic term for hops, chmele, and *lupulus* derived from lupus, the Latin word for wolf, based on the plant's habit of climbing on other plants as a wolf does on the sheeps. Its common name is derived from the Anglo-Saxon hoppan (to climb).

Hop is a herbaceous perennial climbing plant, the organs above soil die every year at the beginning of winter, but the underground organs and roots continue to survive; it is a rustic plant and can survive also with strength in the cold, at the dormant stage (Burgess, 1964). In Europe, the vegetative stage begins in spring, flowering stage is almost in July and August; August is also a critical month for the production of the cone resins, coincident with strobile formation.

Hop is a dioecius plant, with male and female flowers that grow on separate plants (Figure 5); sometimes, plants originated by seeds could be monoicous and often infertile (Burgess 1964).

a b

Figure 5 *Male (a) and female (b) hop inflorescences.*

In hops, there are four different organ systems that can be distinguished, two underground (roots and modify stems) and two above ground (vegetative and generative organs). The anatomy and morphology of the above soil stem and the underground roots have similarity, and differ only in proportions (Rybacek,1991).

Roots can reach 150 cm depth and 200-250 cm of radius length. Hops have two types of roots, differentiating each other depending on the direction, horizontal or vertical towards the surface of the soil (rhizome, generate new wood and above ground stems) The root system is different from the stem in the underground because it possessed no bud nodes. Depending on their maturity, two types of roots can be distinguished: skeleton roots (secondary thickened roots forming the skeleton of the whole system, are involved in the deposition of reserved substances) and terminal active rootlets (including the youngest rootlets that take up water and solution from the soil and participate in the primary metabolism of the plant) (Rybacek, 1991). An important part of the underground plant are the root tubers, secondary thickened roots, that are the principal organ for the storage of nutrients like starch and sugars. Root tubers are usually bottle shaped with an elastic cortex useful for the adaptation of the root to the soil (Rybacek, 1991).

The ratio between the two types of roots and the extension of the radical apparatus depends on the structure of the soil and on the variety (Pignatti, 1982). Radical apparatus is perennial and, in young stage, is white with a thin paper cortex. In adult stage, the cortex is thicker and begins to be fibrous and spongy, with brown-reddish coloration (Mez, 1969). The main function of the underground stem organs is giving the possibility to the plant to maintain the meristematic tissue during unfavourable cold period. In this way, hop plants continue to grow in the underground and ensure a rapid growth of the epigeic tissues, at the beginning of the vegetative season (Rybacek, 1991).

The epigeic parts of the plants are composed by vegetative organs (buds, stems and leaves) and generative organs (for the production of seeds). The stems are long, climbing, cave; in the youth stage, they are grassy and then they become woodier. The inner part of the stems is made by medullar parenchyma, hexagonal and branched, rough, with firm curved hairs. In autumn, it dries out and only the basal part remain alive; this part starts to swell and produces buds, before the beginning of dormancy, until spring. Stems grow upright, forming 5-6 internodes, then apex begins to rotate in a clockwise direction on pale or support forming helix at constant radius, consequently, the steepness at which the bine climbs, increases with decrease in diameter of its support (Mez, 1969, Bell, 1958).

The stems, when the weather become warmer, grow rapidly (up to 4 mt) until middle July/beginning of August; then the growth stops in October and the plant dries out in November. In the dormant stage, organic substances and mineral elements go from the stems to the roots. Stem colour varies, according to the variety, from reddish to light green or nearly white in albino varieties. Also the newly emerged shoots vary in colour from reddish to green (Mez, 1969). The hop stems carry on growing as long as they have a support, then, lateral branches start to develop.

The developing of lateral branches take place in the axil of the leaves on the main bine, in the lower part and in the upper part of the plants. In the upper part, lateral branches continue to elongate and form inflorescences. Stout hooked hair in the lateral bines are similar to those in the main steam, and directed towards the main bine (Burgess, 1964). Sometimes, an underground bud could from roots and develop shoot, known as "runner shoots" which, instead of growing vertically, grows horizontally through the soil as a rhizome, and emerges at some distance from the plant. Runner shoots must be removed in spring to prevent the plant from spreading and sometimes the mown rhizome is used for propagation (Burgess, 1964).

Leaves of the hops are opposite and grow from the nodes of the stem and lateral branches in pairs. They are thin, hairy and the margins are roughly serrated or palmate lobed, chordate at the basis, with three or five and occasionally seven lobes. The number of lobes in leaves is variety dependent, but sometimes, leaves with a different number of lobes grow on the same plant (Burgess, 1964, Mez, 1969). The hairy on the upper layer of the leaves are finer and softer than the underside, and the hairiness of the underside is a varietal characteristic (Davis, 1956). The leaves of stems and of shoots are different: the first developed earlier and are usually bigger with a hairy structure. Main veins protrude from the under surface of the leaves; instead in the surface, the venation is palmate and level. The upper surface is deeper green coloured than the under surface (Rybacek, 1991), except for the ornamental cultivar "Golden Hop", which have golden – yellow leaves. Variation in the depth of green is often due to varietal characteristic. In the reverse face of leaves, there are light coloured glands containing resins and essential oils. In different studies, a direct relationship between number of glands in leaves and resin content in hop cones is shown, and this relationship is useful in breeding for the selection of genotype with higher resin cones (Dark and Tachell, 1955; Srecec 2011). The length and width of the lamina of the leaves are approximately the same, they vary from different part of the plant and according to the state of growth (vigorousness). The petiole, fleshy in texture is about 3/5 of the lamina and has a shallow furrow on its upper side. It has hooked hairs pointing towards the stem (Burgess, 1964).

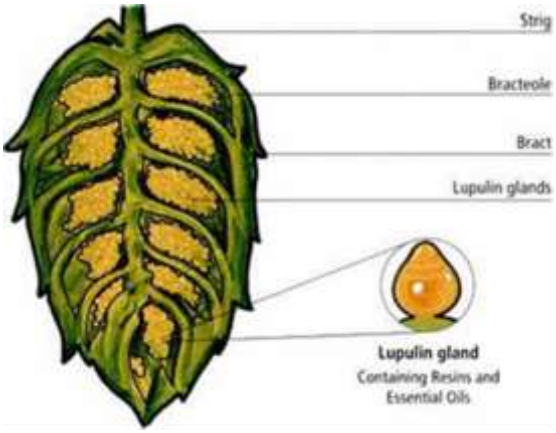
Male inflorescence is a richly branched cymose panicles, green-yellow coloured, with small individual flowers on short stalks, which grow from the axil of the leaves of lateral branches and the upper part of the main bine (Figure 5a) (Burgess, 1964). At blossom, flowers reach the dimension of 2 mm in length and 6 in width (Mez, 1969). The yellow-green sepaloid perianth is divided in five lobes, which at the bases, have five fine short filament bringing long anthers at their ends. Anthers, hang freely from flowers, present fine yellow pollen, that is carried by the wind to the stigma of

female inflorescence. The petals are also endowed with lupuline glands, but the number is smaller than in female inflorescence (Burgess 1964; Mez, 1969).

Pistillate flowers derived from the buds present in the axils of the leaves of lateral branches; the top of the main bine developed in short branches with round terminal buds. The round buds, with a short stalks terminating in female inflorescence called 'burrs'. Female inflorescences (5 mm of length and 6 mm of diameter) are green and with a short central axis, presenting alternate pairs of stipular bracts. These bracts are vestigial structures of a leaf, which has disappeared in the ancestral development of the plants; an exception is the hop variety "Northern Brewer", in which occasionally a leaf is formed between the stipular bracts (Burgess, 1964). In the axils of stipular bracts tiny branches arise and terminate in four small protuberances, each carrying minute bracteoles with a female flower enfolded at the base. Each individual flower has a minute green perianth which closely incorporates the bicarpelate ovary with one ovule (Rybacek, 1991). The ovary bears two filamentous stigmas, without styles. The stigmas are fixed near seed aperture, elsewhere they are free and on the surface there is a long papillae and the protruding of the stigmas give the inflorescences a brush like appearance which catch the pollen floating in the air (Burgess, 1964; Rybacek, 1991). If rainy period are followed by dry time during flowering, the plants that have already blossomed begin to blossom again producing a second inflorescence (Rybacek, 1991). Stigmas are white and atrophic, so very quickly they become brown and loose the pollen catching skill. At the end of their function, the whole inflorescence change to sincarpy. The translation into hop cone (strobile) (Figure 6) implicates different modifications for the inflorescence: the axis lengthens and thickens, stem is modified into rachis and bract and bracteoles enlarging, turn into covering bracteolets and true bracts. In bracts and bracteoles there are few fine hairs.

Figure 6 *Hop strobiles*

The most commercially important component in the hop cone is the lupuline, formed by multicellular lupulinic glands. Lupuline glands are present in rudimentary form in the flower bud, becoming cup-shaped or globular at maturity (Figuire 7 a-b). Quickly, they develop and increase the secretion of resins and essential oils, responsible of the golden yellow colour of lupuline (Burgess, 1964, Rybacek, 1991).



a

b

Figure 7 a) H*op strobile. b) Detail of the hop strobile: bracts with lupuline*

In over ripe hops, the cuticles sometimes go under rupture for the pressure exercised by the accumulation of lupuline. However, lupuline is fragilely connected with bracteoles and it is possible to lost some lupuline for wind effect or in picking and drying steps. The ovary contains single ovule which, when fertilized, develops into two coiled embryo, and the pericarps hardens and colour change to brown while the fruit ripens. Non fertilized flowers elongate stigmas and they eventually die off. The seedless cone is smaller than the fertilized cone and develops in delay, but produces more lupuline. Seeds or the residuals of the non fertilized flowers are covered, at the beginning, by the bracteole. The fruit of the hops is one seeded achene. The formation of seeds is dependent i) on the presence of male plant, flowering in the same period of the female flowers, ii) on the wind direction transporting pollen, iii) on the climate conditions and iv) on the flowering time. Seeds production is influenced by hops variety, but it is especially controlled by the fertilization of the female flowers. Commercially, seeds are often undesirable, and in commerce, hops must report in label the definition “seeded hops”, if seeds content exceeding 2% of their weight (Reg. No 1850/2006 EC). Female hop seedlings, generally, occurred more frequent than male, and this may be due to the production of parthenogenetic seeds. Moreover, female seed have the tendency to germinate earlier than male (Neve, 1991).

Specific attention is necessary for the particularity of the plant dormancy period. At the end of the growing season, as said above, hop plants and their seeds enter in a dormant phase, which stopped at the beginning of the growing season. This period lasts almost six months, from the second half of October to the beginning of April. Dormancy begins with a predormancy period, where the upper part of the plant begins to dry up and buds stop growing, upper bracteoles and buds thickens, giving

much protection against winter weather. A brown layer protection is made over the end of rootlets; then, the phase of deep dormancy begins; no changes in roots growth are detected and all the life processes are rested as much as possible, but clearly do not stop completely. This period usually ends in December. Subsequently, there is a period of post dormancy in where there is no growth, but important processes begin in the roots. Here, reserved stock, like polysaccharides, is transformed in monosaccharides, which become mobile; then they are transferred from root tubers to the rhyzome, reactivating the activity of roots absorption. Afterwards, the activation of the buds on the rhyzome begins, they grow differently depending on temperature and soil properties (Rybacek, 1991). Thanks to the dormancy, hops can survive in winter.

1.8. Chemical composition of hop cones

The term hops is used, also, to identify female inflorescence of hop plants (strobile), the most economically important part of the plants. The importance of female inflorescence of hop plants (the strobile) is due to the its richness in substances useful not only for brewing, but also for the presence of bioactive compounds, important in cosmetic and pharmacy. In table is reported the mean composition of hop strobile (cone) (Benitez et al., 1997) (Table 8):

Composition of air-dried hop cones (% m/m))	
α –acids	2-17
β – acids	2-10
Protein	15
Amino acids	0,1
Water	8-12
Ash	10
Polyphenols and Tannins	3-6
Monosaccharides	2
Pectin	2
Essential oils	0.3-3 (v/m)
Amino-acids	0,1
Cellulose, etc.	40-50

Table 8 *Composition of air dryed hop cones (Benitez et al., 1997)*

The most important and functional compounds present in hop cones are resins, tannins and essential oils. Resins are divided in soft and hard resin, depending on their solubility in organic solvent.

1.8.1. α-acids and β-acids

The bioactive and functional compounds present in the soft resin are the bitter acids, alicyclic phenolic acids, which are, respectively, di- or tri-prenylated phloroglucinol derivatives and their oxidation products known, as humulons (α-acids) and lupulons (β-acids); they represent the 5-20% of hop strobile weight (Chen and Lin 2004; Stevens and Page, 2004) (Figure 8). They are synthetized in glandular trichomes (lupuline glands), where they are accumulated during the cone ripening phase, inducing the lupuline glands increasing (De Keukeleire et al., 2003; Patzak et al., 2015). The hop acids, pale yellowish solids when extracted, are weak acids which dissolve poorly in water and exhibit almost no bitter taste (Keukeleire, 2000).

The level of bitter acids in hop cones depend on hop variety, on strobile maturation and on environmental factors (De Keuleleire et al., 2003; De Keuleleire et al., 2007).

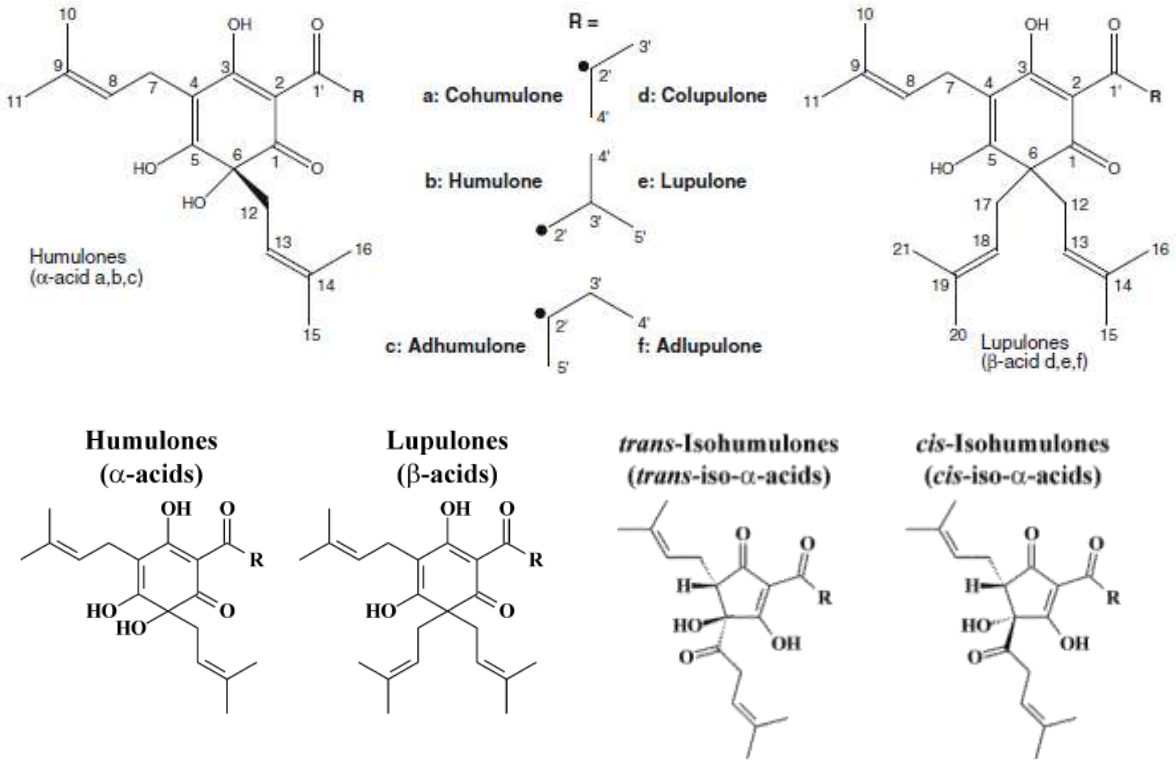


Figure 8 *Chemical structures of bitter acids and their isomers*

α- and β-Acids comprise three major analogues constituents, differing in the nature of the side chain: α-acids with three major analogous (cohumulone, humulone and adhumulone) and β-acids also with three major analogous (colupulone, lupulone and adlupulone) (Figure 8) (De Keuleleire et al., 2003). Hop bitter acids are very sensitive to oxidation thus, hops are rapidly dried after

harvesting, often pelleted and stored in airtight bags preferably at low temperature (Van Cleemput et al., 2011).

α-Acids are considered the most important quality parameters of hops and are present in hop cones in amount between 2% and 17% of the dry weight, depending on variety and environment (Benitez et al., 1997; Bamforth, 2000) (Table 8). Moreover, α-acids value represents an important factor in crop predictions, stock estimation and contract market initiatives (Pavlovic, 2009). Humulone represent about the 15% of the total α-acids, adhumulone the 20-65% and cohumulone, is present in quantities varying between 35 and 70% (Van Cleemput, 2011; Kolpin, 2010). Other minor α-acids are prehumulone and posthumulone (Jaskula et al., 2007). In particular, the quantity of cohumulone is used as an important marker of quality for commerce, because it is the most bittering acid (Benitez et al., 1997; Kolpin, 2010).

The importance of the α-acids fraction is, mainly, due to the major contribution to the beer bitter taste. Effectively, during the brewing process, the water insoluble α-acids of the hop extract are converted into the more soluble iso-α-acid (Figure 8). Isomerization of α-acids generated cis/trans iso-α-acids. A remarkable instability of α-acids and trans-iso-α-acids during beer storage was found (Intelmann et al., 2009; Caballero et al., 2012). Iso-α-acids occur in beer in concentration up to 4 mg/l and they improve foam stability, thanks to tensioactive properties, suppress gushing and contribute to preserve the beer against microorganisms (De Keukeleire, 2000; Blanco et al., 2006). One example is the antibacterial effect against bacteria Grahm-positive and the antifungal effect against *Candida albican, Fusarium* and *Mucor* species (Zanoli and Zavatti, 2008).

At the same time iso-α-acids are responsible for the "lightstuck" flavour of beer, undesirable in the final product, due to the high vulnerability of these compounds to light (De Keukeleire, 2000; Schönberger and Kostelecky, 2011).

β-acids are less acidic than α-acids. β-acids differ from α-acids for the presence of one more prenyl in the lateral chain, and in this complex mix of bitter acids, the most present are lupulone (30-55%), colupulone (20-55%) and adluplone (10-15%) (Figure 8) (Van Cleemput, 2011). Prelupulone and postlupulone are present only in trace. β-acids, together with α-acids, are implied in the foam stability of beer (De Keukeleire, 2000; Van Cleemput, 2011).

Other than for the above reported properties, the importance of the bitter fraction rely on their the sedative activities; it is known since ancient time the use of hop against insomnia and anxiety; experiment on mice have shown a real antidepressant and sedative activity of this substances (Zanoli et al., 2005; Schiller et al., 2006; Negri et al. 2010).

Moreover bitter acids are studied as potential cancer preventive agents thanks to antioxidative (acting as radical quencher), anti-inflammatory and other biological activities, such as, antitumor-promoting effects on mice skin carcinogenesis (Gerhauser, 2005; Lee et al.,2007; Bohr et al.,2008; Van Cleemput et al., 2011).

Furthermore, bitter acids are effective against inflammatory and metabolic disorders, which makes them challenging candidates for the treatment of diabetes mellitus, cardiovascular diseases, and metabolic syndrome (Van Cleemput et al., 2011).

1.8.2. Polyphenols

Dry hop cones contain 4-14% of polyphenols, in which are present proanthocyanidins, also named condensed tannins (Li and Deinzer, 2006), phenolic acids (ferulic and chlorogenic acids) (Zanoli et al., 2007; Li and Deinzer, 2006; Callemien and Collin, 2008), flavonoid aglycones and glycosides, (Segawa et al., 2006; Arraez-Roman et al., 2006) and catechins (Magalhães et al. 2010). Proanthocyanidins exhibited a wide range of biological activities, among them as antioxidants, they offer protection against cardiovascular and neurodegenerative diseases and immune disorders (Garcia and Villalba, 2006). In the tannins fraction, the prenylated chalcones, xanthohumol, isoxanthohumol and desmotoxylxanthohumol (present in the hard resin, insoluble in exane), have the major interest from pharmaceutical industries, thanking their antioxidant activities. Xanthohumol, hydrophobic flavonoid specific for *Humulus lupulus*, is the major polyphenol of female hop inflorescences (Figure 9). Xanthohumol is a calchone and it is implied in the biosynthetic pathway of 8-prenilnaringenina, a potent phitoestrogen; studies show its efficiency is similar to estradiol (Figure 9) (Chadwick et al., 2006; Zanoli and Zavatti, 2008).

Flavonoids represent a substantial group of secondary plant metabolites that display several health-promoting effects. Xanthohumol has antibacteric properties, it is a strong antioxindant and for that implied in the prevention of cancer; moreover it has active action against bacteria Grahm-positive, viruses and malarial protozoa.

Yui et al. (2014) examined the effects of dietary xanthohumol-rich hop extracts in obese rats feeded with a high-fat diet. It is shown that xanthohumol-rich hop extracts may inhibit the increase of body weight, liver weight, and triacylglycerol in the plasma and liver, induced by feeding high-fat diet through the regulation of hepatic fatty acid metabolism and inhibition of intestinal fat absorption. Therefore, xanthohumol-rich hop extracts may exert preventive function on the increase of body weight and tissue triacylglycerol levels by overnutrition.

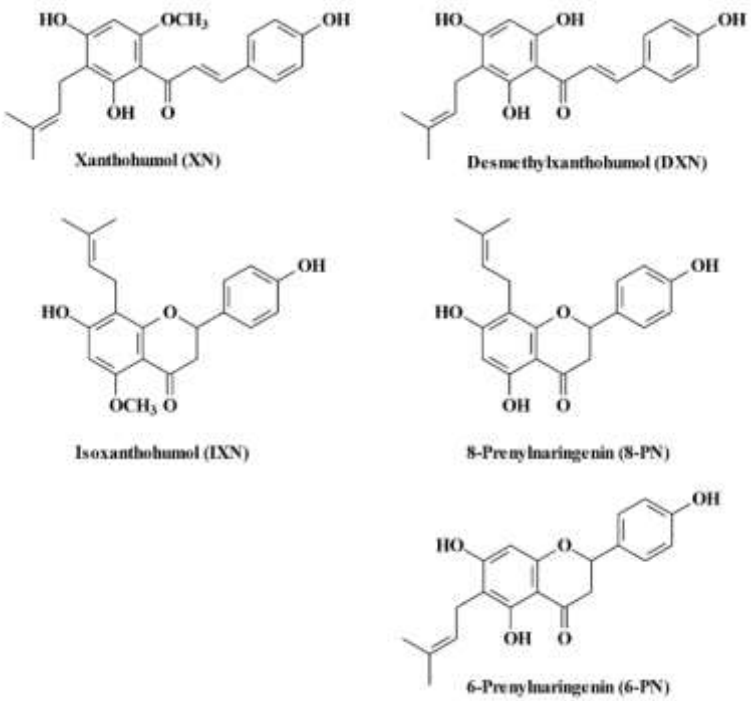


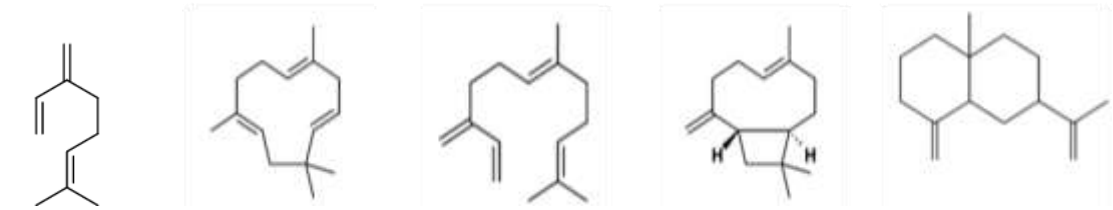
Figure 9 *Chemical structures of Xanthohumol and isoforms, and 6- and 8-Prenilnaringenin*

Other two prenylflavonoids present in hops and beer are 6-prenylnaringenin (6-PN) and 8-prenylnaringenin (8-PN): Busch et al. (2015) reported the results of the investigation of the possible anti-cancer potential, where it is demonstrated a strong dose-dependent reduction of cellular proliferation of human prostate cancer and renal carcinoma cells upon treatment.

1.8.3. Essential oils

As shown in Table 8, essential oil corresponded in an amount of 0.3-3% of the cone weight, but are very important constituent for brewing. They are responsible for the flavour of beers and the product characterization. They are produced by the secondary metabolism of the plants, in glandular trichomes (Wang et al., 2008), are genotype-dependent, and their biosynthesis is influenced by climate and soil. Most of the breeding programmes in recent years have been carried out to imply aroma compound in hops and to find out new varieties characterized by appreciated aromas. Hop oil is considered one of the most complex in nature: at present, more than 450 volatiles have already been identified and it has even been suggested that hop oil comprises over 1,000 different volatile

compounds (King and Dicknson, 2003; Roberts et al., 2004; Van Opstaele et al., 2013). Hop oil constituents are generally classified into three chemical groups: hydrocarbons (50−80%), oxygenated compounds (20−50%) and organosulfur compounds (<1%) (Sharpe, 1981). The therpenes fraction is the most present and the compounds myrcene, α-humulene and β-cayophillene, represent the 90% of the total aromas (Figure 10) (Eri et al., 2000, Wang et al., 2008). The sulfur fraction contains only trace of individual substances; however, due to their low flavor thresholds, they might, significantly, affect the taste and smell of beer, especially in a negative way. The oxygen fraction of essential oils is a mixture of alcohols, aldehydes, ketones, epoxides, esters and acids. Due to their higher solubility in aqueous solutions, these substances might influence the flavor of beer in a significant way. Linalool is one of the major components of this group (Štěrba et al., 2015).



Myrcene Humulene Farnesene β-Caryophyllene Selinene

Figure 10 *Chemical structure of the principal aroma compounds of hop essential oils*

Myrcene (Figure 10) is the most common monoterpene and comprises 10−72% of hop essential oil. Aroma compounds are important especially in brewing. Myrcene usually does not make a contribution to hop aroma in beer, because its concentration is often far below the sensory threshold level, due to its evaporation during worth boiling (Kishimoto et al., 2005). In the last years, there is a development of new brewing techniques, used to avoid or reduce the aroma loosing and better characterize beer with hops. The "dry hopping" is a hopping technique, in which hops are introduced in infusion at the end or at the last minutes of the boiling, in order to preserve its spicy and fruity aromas. Moreover, as monotherpenes are the last produced in ripening, myrcene could be used as markers for cone ripening (Briggs et al. 2004).

Among the hop essential oil, the most abundant sesquiterpenes are **α-humulene** (15−42% of hop essential oil) and **β-caryophyllene** (2.8−18.2%) (Nijssen et al., 1996) (Figure 10). The α-humulene and β-caryophyllene quantities recover in hops, are traditionally used as quality markers; specifically, a ratio value above 2.5 between α-humulene and β-caryophyllene is considered a quality index for aromatic hops; this value is higher in European aroma varieties than in the others

(Deinzer and Yang, 1994). The aroma is earthy and spicy (Patzak et al., 2010; Nance and Setzer, 2011). This characteristic is, indeed, typical of traditional European aroma varieties, that are also known with the name of "noble hops".

Farnesene isomers are sesquiterpenes very important that seem to be indicators of noble hops; they are characterized by woody, grassy and citrus aroma. Expecially the presence of trans-β-farnesene is used as index to determine if a hop has "noble" characteristics (Kofra et al., 2003).

Selinene, with its two isomers, belongsto the sesquiterpenes family and is typical of wild hops. A study on wild and cultivated hops in Europe, reported that the quantity of this compound is very high in wild hops, compared with North American hops (Patzak et al., 2010). Selinene is characterized by grassy aroma.

Sensory descriptor used in beer to express flavour originated from hop essential oil are: fruit, citrus, floral spicy, herbal, hoppy and woody principally. For the floral-fruity and citrus hoppy flavour, the principal responsible are mainly monotherpene alcohols, like geraniolo, linalool and citronellol, present in less quantities but not of minor importance; particularity, linalool gives, if alone, a hoppy scent, but together with geraniolo, takes to fruity and flower aromas (Peecoock et al 1981, Hanke, 2009). Regarding linalool, it is one of the most aromatic flavour components of hop essential oil and it has been considered as a primary substance for hoppy aromatic beers (Fritsch and Scieberle, 2005; Kaltner et al, 2003). It is a very flavourful terpene alcohol, with citrus- and bergamot-like odor. Linalool is contained in hop essential oil in amounts of up to 1.1% by weight (Moir, 1994).

Thioesters are mention as contributors to hops fruity–floral aroma, whereas the spicy, herbal hoppy scents, remain undefined. The spicy flavour has a special importance; it is associated with noble hops and is very complex. Noble aroma is a particularly desirable character in beers and it is usually associated with the use of hop aroma varieties from Europe, such as the cvs. Hallertauer, Hersbrucker and Saaz (Eyres et al., 2007; Graham et al., 2007); noble aroma seems to be associated to the oxygenated sesquiterpenoids fraction and to a complex of aromatic molecules, effective even at low concentration, due to synergic mechanisms (Moir, 1994, De Keukeleire, 2000; Goiris et al. 2002; Peacock and Deinzer, 1981; Peacock et al., 1981).

Eyres and collaborator (2007) studied aroma compounds and, in particular the spicy fraction of oils from four noble aroma hops. They reported that humulene epoxid II is the predominant constituent, but also oxygenated sesquiterpeni are mentioned.

Compared to the volatile profile of essential oils from dry cones, the aroma profile in beers, is very different and complex. First of all, the boiling step, during beer production, causes lots of reaction and volatilization of aromatic and volatile compounds; then, fermentation by yeasts, gives origin to

new compounds and changes the equilibrium of flavour substances. Lot of studies focussed on the characterization of the aromatic profile of hops in beers (Lemusieau et al. 2001, Fritsch and Schieberle, 2005; Haseleu et al. 2010, Nance and Setzer, 2011; Clark et al. 2011; Gonclaves. et al 2012: Van Opstaele et al. 2013; Masek et al 2014) and tried to explain the different reactions, like synergism and masking, taking place during brewing process; but it has to be still explained the way in which the reaction happens (Hanke et al. 2009); for this reason, the experience of the brewers plays a fundamental role in the production of a beer with the desired characteristics.
Moreover, essential oils have bioactivity against Grahm positive bacteria (Zanoli and Zavatti, 2008; Van Cleemput et al., 2011).

1.9. Hop biodiversity evaluation methods

In order to exploit the existing hop biodiversity and to manage successfully the breeding programs, it is necessary to characterize hop germplasm with a morphological, chemical, agronomical and genetic approach.
The study of hop biodiversity has been carried out by several authors in different countries, exploiting diverse approaches based on phytochemical fingerprinting (Henning et al. 2004; Stevens et al. 1997; Stevens et al. 2000), molecular methods, such as RAPD (Patzak et al. 1999), AFLP (Amplify Fragment Length Polymorphism) (Solberg et al. 2014), ISSR (Danilova et al. 2003), STS (Patzak et al. 2007) and SSR (Koelling et al. 2012) markers. They found that wild genotypes represent a source of interesting characters to be used in breeding programs. This results are encouraging for the exploiting of the wild hop germplasm, really wide and rich, in countries like Italy, where hop is not a traditional crop. Actually, in Italy, hop is mainly cultivated as an experimental crop, using commercial varieties bred in USA, New Zealand and east Europe. Deeping the knowledge on Italian hop biodiversity could represent an useful starting point for breeding programs, aimed at enriching the existent commercial varieties with interesting characters, carried by wild ecotypes. Wild germplasms have been used as donors for several important disease- and insect resistance genes and genes for adaptation to stressful environments (Acquaah 2007) and of peculiar phytochemical profiles (i.e., aroma and flavour).
Current breeding practices are aimed, primarily, at improving the disease resistance (*Verticillium* wilt, downy mildew and powdery mildew), at increasing the resin content, at increase and stabilize yield, at ameliorating agro-technical parameters and at combining traits by utilization of cultivars and breeding lines or wild hops with favourable properties (Stajner et al. 2008).

Another important goal of hop breeding is the selection of seedless plants since, from the point of view of the brewing industry, the presence of seeds in the cones is undesirable, as their fats and proteins adversely affect beer fermentation (Hildebrand et al. 1975).

Traditional hop breeding started with clonal selections from adapted wild hops, while modern hop cultivars are derived by hybridization, but even if made from carefully selected parents, the proportion of improved varieties obtained is extremely small. In addition, the hop breeder difficulty is further increased by the long period necessary for the evaluation of the new genotype (three to four years from germination). With advances in science and technology and the application of biotechnologies to plant breeding (e.g., gene transfer, somaclonal and gametoclonal variation), new sources of genetic variability have become available to accelerate the breeding process (Acquaah 2007).

1.9.1. Morphological marker

Hop cultivars are registered and characterized in every aspect, morphological, chemical and genetic. In the morphological approach that comprises the phenotypical manifestation of the individuals, several aspects are observed, like the characteristics of cones, leaves, plants and bines (Figure 11) (Cerenak et al., 2012); moreover, qualitative and quantitative physiological and agronomical aspects, like yield, are detected. A perfect morphological marker must be clearly distinguishing, that means, it must convey clear, uniform and constant differences in the population and in the future generations. Nevertheless, in reality, morphological markers are too much affected by biotic and abiotic factors and not sufficient discriminating (AA. VV. 2008). For hops, for instance, the shape of the leaf is useful to distinguish between *Humuls lupulus* and *Humulus japonicus*, but it does not differentiate European and American cultivars (Davis, 1957). UPOV (2006) proposed a specific descriptor list for hop (Annex 2). In that list, whole plant characters, shoots, leaves, inflorescences, strobiles and bracts were taken into consideration, together with the agronomical characteristics like ripening in early or late stage and yield (UPOV 2006; Rígr et Faberová, 2000). The aim was to standardize the methodology of data collection, and to indicate a prior list usable by all researchers who seek to describe hop cultivar.

1.9.1. Chemical attributes

The study of hop secondary metabolites (bitter acids, polyphenols, essential oils) is useful for quantitative description, but also because it can be used to differentiate among hop varieties.

Chemical profiles are, often, use to distinguish among varieties, and, for example, in hops several studies were made (De Cooman et al., 1998; Jelinek et al., 2010; Jelinek et al., 2011; Perpete et al., 1998; Li et al., 2006). De Cooman and collaborator (1998) stated that the analysis on hop acids and essential oils, or hop acids and flavonoids, are not suitable to obtain satisfactory results in term of discrimination. In the same study, they compared the results of all the analysis, finding out that bitter acids revelation is not enough to discriminate unambiguously among the cultivars taken in consideration; instead, with flavonoids and with essential oil analysis, a complete discrimination is possible. However, numerous studies focussed on the varietal discrimination of hops based on chemical composition, and especially on the aromatic profile of hops (Kovacevic and Kac, 2001; Eri et al., 2000; Sterba et al., 2015), due to the less dependence to external factor of the oil fraction. The α humulene/β cariophillene ratio is a varietal trait used as a marker, because it is independent to ripeness and storage, and it is characteristic of each varieties (Kralj et al., 1991). Eri and collaborators (2000) studied the complete oil profile fraction of four cultivars and find out oil component useful for the discrimination among the varieties; moreover, the complete aroma profile could be useful to create a database in which aroma profile's cultivar could be registered and used for variety identification.

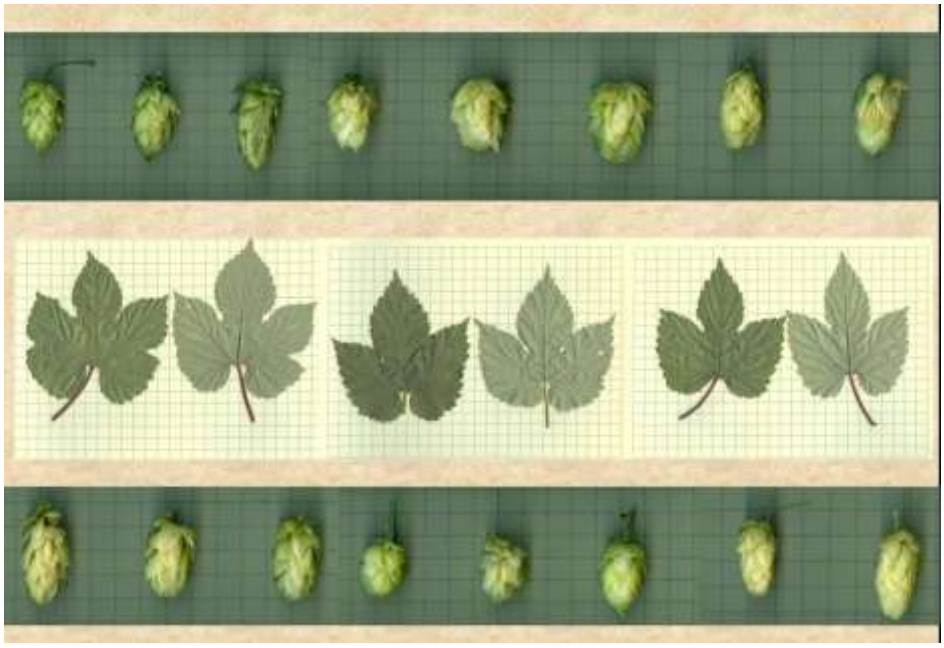


Figure 11 *Example of morphological biodiversity of hop cones and leaves*

Linalool, along with a wide spectrum of hop compounds, can be used to identify the hop cultivar (Sterba et al., 2015), but also some terpenoid and sulfur compound can significantly be used as distinguising factor among varieties (Gross, 2011).

On the contrary, Green an co-workers (1997) explained that oil composition, which is produced by the secondary metabolism of the plants, is influenced by biotic factors, so that the composition of the essential oil is not an unambiguous marker. In this study, it is described that the hops variety "Tettnanger", grown in Australia, U.S.A. and in Hallertaü district of Germany, has different chromatographic aroma profile. Moreover, Kovacevic and Kac (2001) explained that in the hop world production, there are numerous varieties characterized by minor aromatic differences and the distinction among all the varieties could not be possible using only aromatic profiles.

1.9.2. Discrimination by molecular markers

As described above, in the past, the varietal recognition was based on morphological or chemical methods. Those procedures are now overcome by DNA analysis that are able to reveal variations in genomic sequence with frequencies over 1% among individuals of the same species (Ganino et al., 2006). The advantage of the use of molecular analysis for varietal discrimination is the complete independence of the analysis from environmental factors. A molecular marker is a genetic *locus*, determined using specific primers and probes able to distinguish, unambiguously, the correspondent chromosome trait; it is useful to find out variability caused by mutations (insertion, deletion, translation, duplication) of DNA homologous region of different individuals of the same species. The perfect molecular marker must be stable and not influenced by pleiotropic or environmental effects, sex and age independent, polymorphic and ubiquitous, codominant in order to differentiate between homozygosis and heterozygosis, neutral and universally applicable (Ganino et al., 2006). Therefore, molecular markers are stable, cost-effective and easy to use, providing a handy tool for numerous applications, like genome mapping, gene tagging, genetic diversity, phylogenetic analysis and forensic investigations. A large number of molecular marker techniques have been developed in the last three decades. However, the techniques, namely RAPDs (Random Amplify Polymorphic DNA), AFLPs (Amplify Fragment Length Polymorphism), ISSRs (Inter Simple Sequence Repeated), SSRs (Simple Sequence Repeated) and SNPs (Single Nucleotide Polymorphic) have received the most attention. The choice of the marker will depend on the targeted use, microsatellites and SNPs largely fulfil most of the user requirements (Grover and Sharma, 2014). From the use of polymorphic molecular marker, it is possible to obtain a varietal fingerprinting; moreover it is possible to obtain a large number of information with high reproducibility. The only

limiting factors are the costs, sometimes high, and the practice; time and knowledge is often needed (Ganino et al., 2006).

In the study of hop population, RAPD (Brady et al. 1996; Pillay and Kenny 1996; Murakami 1998; Patzak et al. 1999), AFLP (Hartl and Seefelder 1998; Seefelder et al. 2000; Jakše et al. 2001; Solberg et al. 2014), ISSR (Zietkiewicz et al. 1994; Danilova et al. 2003) and SSR (Hadanou et al., 2004; Jakše et al. 2004; Patzak et al.2007; Nahla et al. 2008; Bassil et al. 2008; Patzak et al. 2010; Patzak and Matausek 2011; Koelling et al. 2012) markers were used. RAPDs are very useful for fingerprinting; they are fast and do not need high quantities of DNA, they are easy to use and have low execution cost. A deep knowledge of the genomic sequence is not needed because the primers tie in any case. RAPDs are useful for organisms not well known. The limits of the analysis is the low reproducibility due to the low strength of the amplification reaction (i.e.: low annealing temperature) (Yang et al., 1996). Moreover, they are dominant markers and it is not possible to differentiate between heterozygosis and homozygosis, giving relatively low throughput (Ganino et al., 2006). AFLP have found application in the characterization of germplasm (Hill et al., 1996), in the creation of genetic maps and in breeding programs (Becker et al., 1995; Van Eck et al., 1995). The advantage of AFLP is the high sensitivity and easy reproducibility but they need DNA in large quantities and of high quality. Moreover, those are complex and expensive techniques and the use of radioactive substances may be needed.

Solberg and coworkers (2014), utilizing AFLP markers, analyzed 62 Danish and 34 Norwegian hop plants, and found that a correlation exists between genetic variability, zone of origin, chemical composition and morphology.

In many studies on spontaneous and cultivated hop, polymorphic microsatellite sequences were isolated and characterized (Hadanou et al. 2004; Bassil et al. 2008; Jakše et al. 2011; Patzak e Matausek 2011; Koelling et al. 2012). SSR or microsatellites are characterized by a tandem repeat of only 1–6 bp in a sequence. Microsatellites are widespread and dispersed in the genomes of all plants and display an elevated level of hypervariability within each species. These features makes them excellent both for gene mapping and fingerprinting. Presently, the SSR technique has provided reliable markers to solve the problems of varietal identification and for genetic characterization of hop, because of their high transferability, elevated polymorphism and codominance; moreover, the advantages in the use of microsatellites are the high throughput and the tiny quantity of DNA required, that could be not extremely purified. The majority of the studies that use SSR marker to determine hops biodiversity come to the conclusion that there are two primary genetic groups: European (including wild and cultivated material) and North American (wild

material only) (Bassil et al. 2008; Henning et al. 2004; Jakse et al. 2004; Murakami et al. 2006a; Patzak et al. 2010; Peredo et al. 2010; Stajner et al. 2008). In detail, Jakše et al. (2004) and Murakami et al. (2006a), by utilizing 4 and 11 SSR loci, respectively, effectively evaluated the biodiversity and the existing relations among spontaneous and cultivated hops from Europe, North America and Asia. Patzak et al. (2010) conducted chemical (essential oils, polyphenols and bitter acids) and molecular studies to evaluate the genetic variability in native hops of Canada (62 plants) and of Caucasus (58 plants) in comparison with European (104 samples) and North American (27 samples) plants. The genetic analyses involved the utilization of 9 SSR loci and of 3 STS sites. The chemical analyses allowed the distinction between North American and European hops, while the genetic study also permitted the discrimination between Caucasian and European hops.

Until present, few studies on hops have used SNPs to discriminate varieties; these molecular markers permit to reveal differences of single nucleotides and the use of these markers can give a highly informative DNA assay (Ganino et al., 2006). The main problem of SNPs use is the large amount of work required to project them (Ganino et al., 2006). Henning et al. (2015a), in their work, identify 7 SNP markers that could be used to recognize hop varieties and accession and in the study they were able to differentiate among 116 genotypes.

While the use of the molecular markers discussed above has greatly expanded our understanding of genetic variation in hop, the cost of these marker technologies remains an obstacle to their utilization in breeding programmes

1.10. From biodiversity study to breeding

The biodiversity study is an essential step in order to proceed with breeding programs. In the history of hop cultivation, due to domestication and breeding, hop suffered from a shrinking of intraspecific agrobiodiversity. Breeding is the solution to create hop genotypes endowed with interesting phytochemical profiles, but also suitable for sustainable cultivation, considering the emergency of climate changes. It may improve disease resistances, increase resins content and confer good yield. The never studied wild hop patrimony is a huge biodiversity source that could enrich the existing world agrobiodiversity; wild hops can be used as donors of genes for several important disease and insect resistances, for adaptation to stressful environments (Acquaah 2007) and for peculiar phytochemical profiles (i.e., aroma and flavour). Current breeding practices are aimed to: (i) improve disease resistance (Verticillium wilt, downy mildew and powdery mildew), (ii) increase the resin content and (iii) confer high and stable yield and good agro-technical parameters (Stajner et al.

2008). Another important goal of hop breeding is the recovery of seedless plants: from the brewing industry point of view, the presence of seeds in the cones is undesirable, as their fats and proteins adversely affect beer fermentation (Hildebrand et al. 1975). Traditional hop breeding started with clonal selections from adapted wild hops, while modern hop cultivars are derived by hybridization; but even from carefully selected parents, the proportion of improved varieties obtained is extremely small. In addition, hop breeders activity is further complicated by the long period (three to four years from germination) which is necessary for the characters of interest to assume their mature form. However, for successful breeding programs a large variability of genotypes to start from is indispensable.

1.10.1. MAS-marker assisted selection

Hop breeding is a long process and for speeding breeding programs molecular approaches have been developed to support conventional programs. The discovery of markers for the individuation of characteristics of interest is important. The use of a molecular marker, associated to resins content, for example, could reduce the time needed to see if the new plant actually possesses the desired new characteristic (Ĉerenak et al., 2006; Ĉerenak et al., 2009). In hops, several studies have been made to individuate genetic sequences correlated with phytochemical and agronominc characters (Quantitative Trait Loci or QTL). Marker Assisted Selection can shorten lengthily hop breeding based on phenotypes selection. In 2008, a study made by Cerenak and coworkers applied AFLP and SSR markers to a segregation population to detect QTL linked with α-acids content and yield traits. They identified QTLs probably associated to the studied traits, and established an important improvement in MAS recovery, especially for α-acids. Henning and collaborators (2011) carried out a study for the discovery of genes related with susceptibility to powdery mildew. In this study, they found two AFLP markers, linked with QTL associated with disease susceptibility. In 2015 (b) Henning and co-workers, tried to identify QTL to select genotypes with downy mildew resistance in hops, based on SNP markers. Researchers reported a high-density genetic map of hop for the identification of SNP markers that can be potentially used to select for downy mildew resistance in hop.

Another need in hops breeding programmes is the selection of the female plants. As a rule, the phenotypic manifestation of hops sex occurs after one year and cytological analysis is not useful for all varieties (Haunold, 1991). Specific molecular markers for sex identification have been developed in different species like hemp (Sakamoto et al., 1995), pistachio (Hormanza et al., 1994), papaya (Parasnis et al., 2000), silene (Gill et al., 2000), asparagus (Reamon-Büttner et al., 1998), and kiwi (Zhang et al., 1998). Polley et al. (1997) developed a molecular marker (STS) located in

the Y chromosome of hops which allowed an early distinction between male and female plants. The advantage of the use of the STS markers is the achieving of a quick selection of female hops; this would allow DNA extraction to be made from the first leaves and there would be no need to wait for plant flowering, but it is not totally discriminating. McAdam et al. (2013) used, for sex identification in hop, a SSR marker named HlGA7 that showed a significant association with the male phenotype.

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2. Aim of the Ph.D. project

The Italian agricultural sector is now in decline and the introduction of a new crop, with unique characteristics, might represent a valuable alternative, also for the international hops market. The increase of beer consumption and moreover the rise of craft beer production in Italy, Europe and U.S.A. in particular, was also a motivation for the present project.

A selected Italian hop variety with aromatic peculiarities and with resistance to diseases will be able to better answer to the damages caused by climate changes, in view of a sustainable cultivation for use in the brewing industry, and eventually have a place in the world hops market.

This Ph.D. thesis is a part of a project began in 2012, funded by Marano sul Panaro Council (Modena, IT). Ph.D. project idea started from historic evidence of a hop cultivation in the Marano sul Panaro district, in the XIX century, and aimed at recreating, today, a hop industry in Italy and at selecting Italian hop varieties. During the Ph.D. project, the existing experimental field collection (42 native varieties and 11 cultivars) was expanded, implementing the number of accessions up to 80, with plants coming from almost all north Italy.

The general objective of this Ph.D. project is to study, expand and enrich agrobiodiversity, starting from the natural biodiversity present in wild hops, to find and create hop varieties with excellent aromatic characteristics, adaptable to Mediterranean climate.

To realize this aim, the following goals must be achieved:

- genetic evaluation of the Italian wild hop biodiversity compared with well-known cultivars;
- agronomical and morphological characterization of selected hop genotypes;
- chemical characterization of genotypes, in order to evaluate qualitative characteristics;
- obtention of suitable markers for an early sex recognition, useful for hop breeding programs;
- evaluation of the “terroir” effect on hops yield and quality;
- assessment of the adaptability of Italian wild genotypes and of commercial cultivars to Italian environment , in field conditions.

More than one university in Italy is engaged in the study of brewing process, from all raw materials to beer, but this is first project that aims at finding and selecting completely Italian varieties, seeing wild Italian hops as a resource.

3. List of the studies

3.1. Study 1 - Conservazione e caratterizzazione di luppolo italiano: studi preliminari

Rodolfi M., Silvanini A., Mongelli A., Beghè D., Fabbri A., Ganino T. (2014) **Conservazione e caratterizzazione di luppolo italiano: studi preliminari.** X Convegno Nazionale sulla Biodiversità – Roma 3-5 settembre 2014, p. 83.

Italian wild hops conservation and characterization: preliminary study.

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Abstract

Humulus lupulus L.is a dioecius plants of the Cannabaceae family and grows in the temperate zone of the globe. Only female plants are of commercial interest (in brewery and for pharmaceutical preparation); in the hop strobile (cone) is prodced the lupulin, a resinous substance in which α-acids, β-acids, xanthohumol and essential oils are contained. Several are the commercial cultivars developed in breeding programme overall in England, Germany, East Europe, America, Japan and Australia. Selection was made in order to obtain hops genotypes with the desired characteristics, useful in brewing (bitter, peculiar aromas), disease resistant and also with particular morphological characteristics. Cone morphology, for example, is a character considered in the genotype selection; effectively, depending on the cultivation area, it is advisable to select genotypes with open or closed bracts in cones. Nevertheless, in country like Italy, where hop cultivation has not been developed in the lasts centuries, a great biodiversity is preserved. Using UPOV paramethers as references, data reguarding 22 wild Italian hops and H. Magnum cultivar, cultivated in the field collection of Marano sul Panaro (MO-Italy), were registered and analyzed. Data collected reguarded: the growth behaviour, colour and shape of the leaf, anthocyanin coloration of the main shoots, shape and size of cones, shape of the plants, flowering period, yield per plants and tolerance or sensibility to biotic and abiotic stresses. The resuts showed a great phenotypic variability in the hops collected: differeces regarded not only leaf and cone shape and size, but also the plant shape (clavate, conic and fusiform) and diseas resistance, like powdery and downy meldew.

Key words: *Humulus lupulus* L, Morphological characteriszation, Phenotypic variability, Discriminant characters.

Introduction

Hops are dioecious, perennial climbing plants (Neve, 1991). It is a dicotyledon coripetala. The genus Humulus, included, principally, two species *H. lupulus* L. and *H. japonicus* (Sieb et Zucc). *Humulus lupulus* L. originated from the boreal hemisphere, belongs to Urticales order, Cannabaceae family. Male hop flowers are richly branched cimose panicles; instead, female inflorescences (strobile) are cone shaped, composed by bracts and rich in resin called lupulin. Hop resins contain α and β-acids (humulons and lupulon), polyphenols (flavan and xanthohumol) and essential oils, used in brewing to confer to the beer the characteristic flavour (De Keukeleire et al., 2003). Only female plants are of commercial interest for brewing and pharmaceutical propose, because cones have higher lupulin content than male flowers. This is the first time that morphological studies on wild Italian hop are illustrated; that means, a prior characterization of the studied plants, grown in the experimental field of Marano sul Panaro (MO-Italy). The objective of this study is the morphological characterization of wild hops, collected in the experimental field. Results will be used for a more complete characterization of Italian wild hops, for future breeding programs and to obtain valuable Italian cultivars.

Materials and methods

In this study, 23 Italian genotypes were considered, 22 of which have their origin in different Italian regions (Table 1), instead the cultivar H. Magnum is of German origin. In 2013, the observations began, using as a guide the indication of UPOV (2006) manual. Growth behaviour was observed measuring plants high in 4 different moments, beginning from the sprout, to the maturity of the plants. Tolerance to diseases (*Pseudoperonospora humuli* (Miyabe & Takah.) G.W. Wilson e *Podosphaera macularis* (Wallr.) U. Braun & S. Takam), was evaluated during the growing period in field.

Table 1*. Genotypes used in the study and its provenience.*

ID	PROVENIENZA	ID	PROVENIENZA
BIBBIANO	Emilia Romagna	MOGLIA	Lombardia
BUSSETO	Emilia Romagna	PASQUI 1	Emilia Romagna
CAMPUS CANNA	Emilia Romagna	PASQUI 2	Emilia Romagna
ET3	Emilia Romagna	CINGHIO	Emilia Romagna
ET4	Emilia Romagna	PIATTELLO	Emilia Romagna
ET7	Emilia Romagna	RIO GAMBERO	Emilia Romagna
ET7 2	Emilia Romagna	ROGGINO 2	Lombardia
ET8	Emilia Romagna	TANGE	Emilia Romagna
ET8 1	Emilia Romagna	TAVERNELLE	Emilia Romagna
FISICA	Emilia Romagna	TORRE	Toscana
GIANNI	Emilia Romagna		
LIVELLO	Emilia Romagna	H. MAGNUM	Europaen hop farms

Results

Hops morphologic characters are reported in table 2 (quantitative characters), table 3 (qualitative characters) and in figure 1. Differences in every character were observed. In order to evaluate the differences between the sampled genotypes and find the most significant characters, data were analyzed using PCA (Principal Component Analysis) methods (Figure 2). Considering all data, 6 were the variables (main shoot: anthocyanin coloration, leaf: shape, cone: intensity of green color, cone: shape, bract length, bract: length apex), that explain the cumulative variance.

Flowering periods was different between cultivars and Italian genotypes; H. Magnum began the blooming the last decade of July, whereas, wild genotypes flowered in the second decade of August. As concern susceptibility to *Pseudoperonospora humuli* (Miyabe & Takah.) G.W. Wilson, the observed genotypes showed different reactions; the genotypes Moglia, Roggino 2 e H. Magnum resulted tolerant to the fungi attack, instead ET3, ET4, Pasqui 2 e Torre, resulted the most susceptible genotypes. Regarding powdery mildew (*Podosphaera macularis* (Wallr.) U. Braun & S. Takam), the most intolerant genotypes were Cinghio, Pasqui 1 and Tange.

Tabella 2 *Mean±DS and ANOVA of the morphological traits belonging to the 23 hop genotypes (22 Italian genotypes and H. Magnum cultivar). Different letters are idex of statistic significant with a confidence interval of 0.95 (Tukey's test).*

Genotypes	Cone shape	Cone width (cm)	Cone length (cm)	Fresh cone weight (g)	Bract shape	Bract width (cm)	Bract length (cm)
Bibbiano	0,786±0,145^{a-e}	2,058±0,375^{a-f}	2,667±0,536^{b-f}	0,171±0,036^{de}	0,549±0,041^{ab}	0,810±0,128^{a-c}	1,470±0,176^{ab}
Busseto 13	0,728±0,079^{c-f}	2,080±0,396^{a-f}	2,900±0,741^{a-f}	0,375±0,157^{a-c}	0,601±0,059^{ab}	1,086±0,157^{ab}	1,829±0,349^{a}
Campus Canna	0,656±0,102^{c-f}	1,700±0,189^{d-f}	2,650±0,561^{b-f}	0,160±0,077^{de}	0,464±0,249^{b}	0,713±0,379^{c}	1,538±0,184^{ab}
Cinghio	0,735±0,095^{b-f}	2,200±0,254^{a-e}	3,000±0,212^{a-f}	0,120±0,022^{e}	0,543±0,137^{ab}	0,780±0,192^{a-c}	1,440±0,114^{ab}
Et3	0,871±0,125^{c-f}	2,500±0,545^{a}	2,875±0,465^{a-f}	0,314±0,067^{b-d}	0,592±0,209^{ab}	1,010±0,137^{a-c}	1,820±0,373^{a}
Et4	0,613±0,162^{d-f}	1,600±0,126^{d-f}	2,733±0,578^{a-f}	0,161±0,050^{de}	0,593±0,239^{ab}	0,956±0,441^{a-c}	1,622±0,281^{ab}
Et7	1,021±0,171^{a}	2,500±0,408^{a}	2,500±0,577^{c-f}	0,195±0,012^{c-e}	0,621±0,056^{ab}	0,975±0,095^{a-c}	1,575±0,170^{ab}
Et7 2	0,999±0,027^{ab}	2,500±0,200^{a}	2,500±0,158^{c-f}	0,157±0,069^{de}	0,580±0,161^{ab}	0,900±0,158^{a-c}	1,600±0,292^{ab}
Et8	0,725±0,102^{c-f}	2,625±0,478^{a}	3,625±0,478^{ab}	0,398±0,135^{ab}	0,600±0,189^{ab}	0,800±0,141^{a-c}	1,425±0,434^{ab}
Et8 1	0,769±0,098^{a-e}	2,080±0,293^{a-f}	2,720±0,335^{a-f}	0,209±0,033^{c-e}	0,540±0,110^{ab}	0,833±0,173^{a-c}	1,544±0,052^{ab}
Fisica	0,694±0,048^{c-f}	1,833±0,288^{b-f}	2,667±0,577^{b-f}	0,142±0,070^{de}	0,510±0,069^{ab}	0,822±0,109^{a-c}	1,622±0,172^{ab}
Gianni	0,590±0,065^{ef}	1,450±0,333^{f}	2,450±0,454^{c-f}	0,192±0,050^{c-e}	0,443±0,076^{b}	0,680±0,122^{c}	1,550±0,246^{ab}
Livello	0,672±0,062^{c-f}	1,817±0,194^{b-f}	2,733±0,436^{a-f}	0,285±0,071^{b-e}	0,548±0,043^{ab}	0,913±0,064^{a-c}	1,675±0,198^{ab}
Moglia	0,873±0,055^{a-d}	1,780±0,148^{c-f}	2,040±0,151^{f}	0,154±0,050^{de}	0,543±0,049^{ab}	0,809±0,094^{a-c}	1,491±0,137^{ab}
Pasqui 1	0,487±0,053^{f}	1,557±0,325^{ef}	3,186±0,508^{a-e}	0,259±0,133^{b-e}	0,438±0,072^{b}	0,733±0,173^{bc}	1,667±0,223^{ab}
Pasqui 2	0,619±0,077^{d-f}	2,070±0,149^{a-f}	3,380±0,393^{a-d}	0,239±0,065^{b-e}	0,509±0,057^{ab}	0,827±0,119^{a-c}	1,636±0,237^{ab}
Piattello	0,728±0,145^{c-f}	2,434±0,476^{a-c}	3,389±0,537^{a-c}	0,317±0,081^{b-d}	0,538±0,043^{ab}	0,956±0,088^{a-c}	1,789±0,236^{ab}
Rio Gambero	0,783±0,147^{a-e}	2,255±0,250^{a-d}	2,973±0,638^{a-f}	0,278±0,104^{b-e}	0,475±0,055^{b}	0,760±0,084^{bc}	1,610±0,166^{ab}
Roggino 2	0,650±0,058^{c-f}	1,520±0,109^{f}	2,360±0,336^{d-f}	0,135±0,061^{de}	0,460±0,067^{b}	0,690±0,099^{c}	1,510±0,191^{ab}
Tange	0,902±0,198^{a-c}	2,250±0,377^{a-d}	2,563±0,495^{c-f}	0,168±0,034^{de}	0,672±0,109^{ab}	0,917±0,116^{a-c}	1,383±0,213^{b}
Tavernelle	0,998±0,249^{ab}	2,380±0,329^{a-c}	2,510±0,678^{c-f}	0,133±0,055^{de}	0,722±0,227^{a}	1,150±0,414^{a}	1,580±0,139^{ab}
Torre	0,792±0,081^{a-e}	1,709±0,230^{d-f}	2,173±0,337^{ef}	0,184±0,044^{de}	0,459±0,065^{b}	0,700±0,124^{c}	1,520±0,113^{ab}
H. Magnum	0,665±0,036^{c-f}	2,471±0,138^{ab}	3,729±0,292^{a}	0,533±0,078^{a}	0,472±0,076^{b}	0,771±0,160^{bc}	1,629±0,170^{ab}

Tabella 3 *Morphological descriptors (shoots, leale, cones and bracts) of the 23 hop genotypes 22 Italian genotypes and H. Magnum cultivar.*

Genotypes	Main shoot: anthocyanin coloration	Leaf: shape	Cone: size	Cone: shape	Cone: intensity of green color	Cone: degree of opening bracts	Cone: productivity per one plant	Bract: ratio width/length	Bract: length apex	Bract: length
Bibbiano	Strong	Three-lobed	Medium	Medium ovate	Medium	Slightly open	Very low	Medium	Very short	Large
Busseto 13	Strong	Five-lobed	Medium	Broad ovate	Light	Slightly open	Very low	Large	Very short	Large
Campus Canna	Weak	Three-lobed	Medium	Cylindrical	Light	Slightly open	Very low	Medium	Short	Large
Cinghio	Medium	Three-lobed	Medium	Broad ovate	Light	Slightly open	Low	Medium	Very short	Large
Et3	Medium	Three-lobed	Medium	Broad ovate	Light	Slightly open	Very low	Medium	Very short	Large
Et4	Very strong	Five-lobed	Medium	Narrow ovate	Medium	Slightly open	Very low	Large	Very short	Large
Et7	Medium	Three-lobed	Medium	Broad ovate	Light	Clearly open	Very low	Medium	Very short	Large
Et7-2	Strong	Three-lobed	Medium	Broad ovate	Light	Clearly open	Very low	Medium	Very short	Large
Et8	Medium	Three-lobed	Large	Broad ovate	Medium	Slightly open	Very low	Medium	Medium	Large
Et8 1	Medium	Five-lobed	Medium	Broad ovate	Light	Closed	Very low	Medium	Very short	Large
Fisica	Strong	Three-lobed	Medium	Broad ovate	Medium	Clearly open	Very low	Medium	Very short	Large
Gianni	Medium	Five-lobed	Medium	Broad ovate	Medium	Slightly open	Very low	Small	Very short	Large
Livello	Medium	Three-lobed	Medium	Medium ovate	Light	Slightly open	Very low	Medium	Very short	Large
Moglia	Weak	Three-lobed	Small	Globose	Light	Slightly open	Low	Medium	Very short	Large
Pasqui 1	Strong	Five-lobed	Large	Narrow ovate	Medium	Slightly open	Very low	Small	Medium	Large
Pasqui 2	Medium	Three-lobed	Medium	Narrow ovate	Light	Closed	Very low	Medium	Very short	Large
Piattello	Strong	Three-lobed	Medium	Medium ovate	Medium	Slightly open	Very low	Small	Very short	Large
Rio Gambero	Strong	Five-lobed	Medium	Broad ovate	Medium	Slightly open	Intermediate	Medium	Very short	Large
Roggino 2	Strong	Three-lobed	Small	Broad ovate	Light	Closed	Very low	Medium	Very short	Large
Tange	Medium	Five-lobed	Medium	Globose	Medium	Clearly open	Very low	Large	Very short	Large
Tavernelle	Strong	Five-lobed	Medium	Broad ovate	Medium	Clearly open	Very low	Large	Very short	Large
Torre	Strong	Three-lobed	Small	Globose	Light	Slightly open	Very low	Medium	Very short	Large
H. Magnum	Absent	Three-lobed	Large	Cylindrical	Light	Closed	Very low	Medium	Very short	Large

Figura 1 *Examples of the morphological variabilità in Italian wild hops. (A) Five-lobed leaf (ET8); (B) Trilobed leaf (Torre); (C) Large cone (ET8) and (D) small cone (Gianni).*

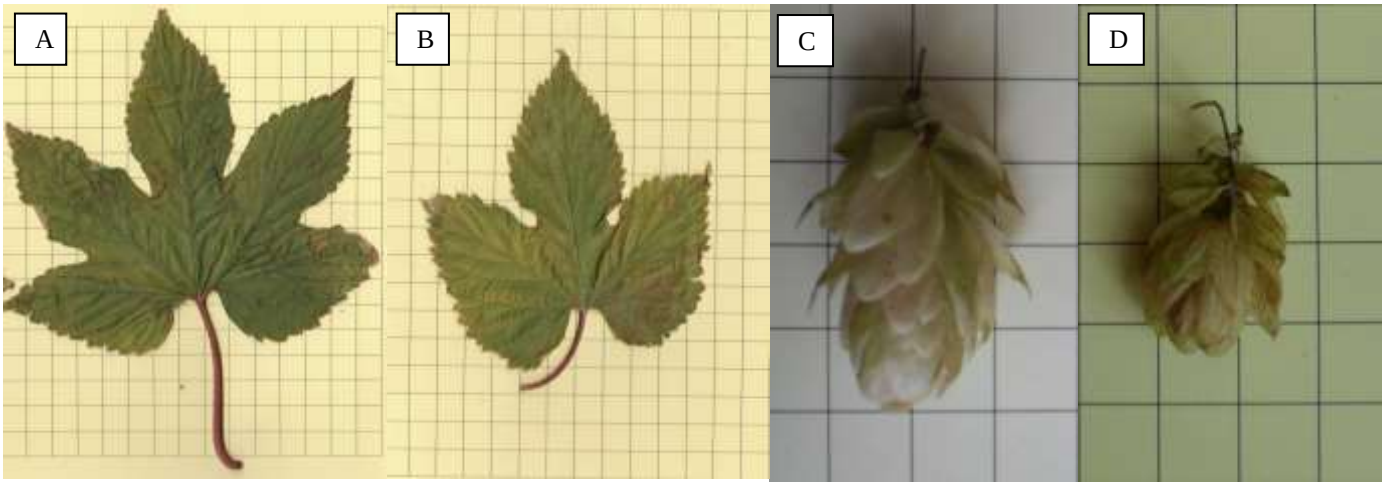
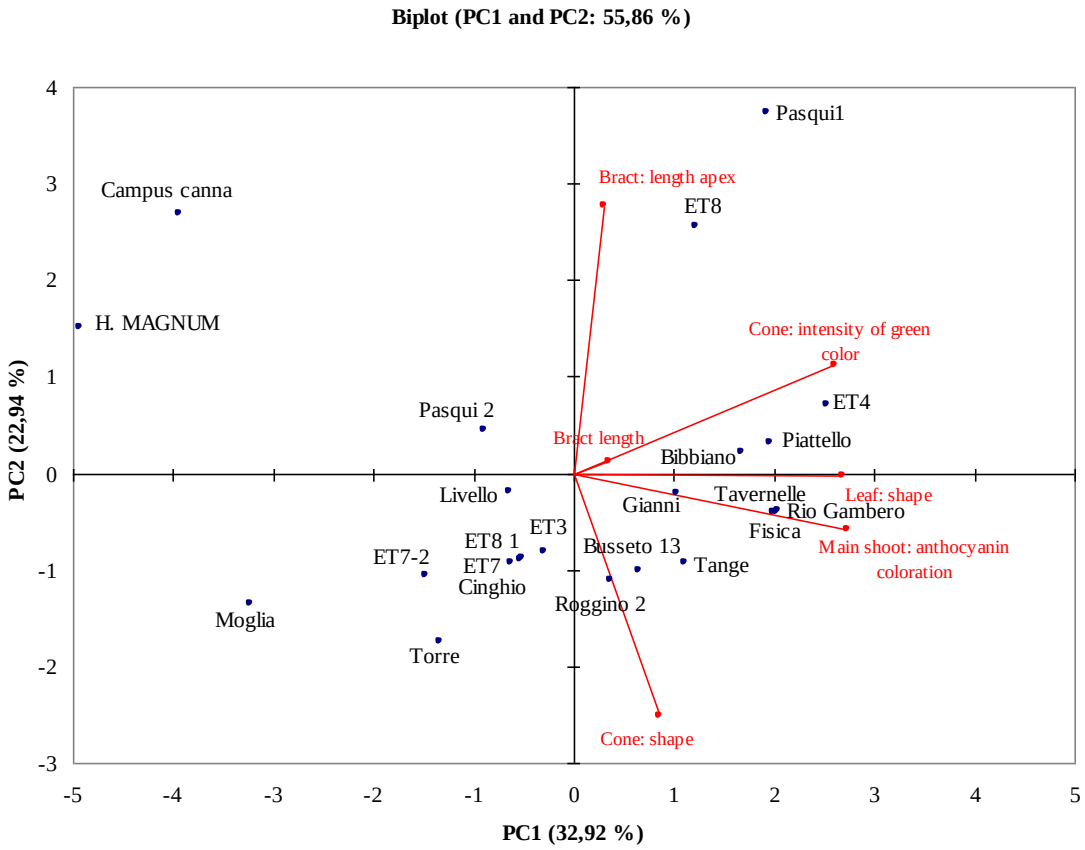


Figura 2 *Principal Component Analysis made by using the most discriminant variables.*



Conclusions

In conclusion, it is possible to assert that:

- In the Italian wild hops used in the study, there is a great biodiversity;
- Morphological markers revealed to be an useful tool for biodiversity evaluation in the species *H. lupulus* L.;
- Main shoot: anthocyanin coloration, Leaf: shape, Cone: intensity of green color, Cone: shape, Bract length, and Bract: length apex, are the characters that lead to the discrimination between the genotypes.
- The discovered biodiversity will allow the selection of new genotypes, useful for the production of Italian hop cones, that are essential for the Italian craft beers market characterization.

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3.2. Study 2 - First identification and characterization of wild Italian Humulus lupulus L. and comparison with European and American hop cultivars using nuclear microsatellite markers

Rodolfi M., Fabbri A., Bruni R., Ganino T. **First identification and characterization of wild Italian *Humulus lupulus* L. and comparison with European and American hop cultivars using nuclear microsatellite markers**. *Three Genetic and Genome*.
SUBMITTED

First identification and characterization of wild Italian *Humulus lupulus* L. and comparison with European and American hop cultivars using nuclear microsatellite markers

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Abstract

Nine genic SSR loci were used to evaluate the genetic diversity and identify accessions in wild Italian *Humulus lupulus* L., in comparison with European and American commercial cultivars. A collection of 80 wild hop samples from Italy and 43 hop cultivars from Europe and America, were characterized. Allelic frequency analysis revealed 65 distinct Italian genotypes and differentiated all the common commercial cultivar; moreover, specific alleles were observed for wild and cultivated hops. The number of alleles identified in the wild population were 104 and 123 within all the accessions. The average number of alleles per locus detected in the Italian population was 11,5 (ranging from 2 to 24) and 13,8 (ranging from 4 to 27) in the complete population . The maximum polymorphic information content was evidenced for locus HlGA23 in the Italian wild population and in the whole accessions (0,905 and 0,902 respectively). The dendrogram constructed from Euclidean distance with the UPGMA method showed two main clusters, one composed by American and European accessions and one most composed by wild Italian accessions. The study showed for the first time the great biodiversity present in Italy, and the remarkable differences with European and American hops; this genetic richness could be used in future breeding programs in order to develop new hop varieties carrying characteristics useful for brewers.

Keywords: genetic diversity, Italian hop germplasm, SSR markers, *Humulus lupulus* L., allele frequency.

1. Introduction

The genus Humulus belongs to the Cannabaceae family, and is composed by three species: *Humulus lupulus* L., *Humulus japonicus* Siebold&Zucc., *Humulus yunnanensis*Hu (Small 1978; Neve 1991); it is believed to be native of China, from where it diffused to Japan, America and Europe (Neve 1991; Murakami et al. 2003). *Humulus lupulus* L., commonly known as hop, is spontaneous in Central Europe and widely cultivated in the temperate zones of the planet, mainly to be utilized in the beer industry; the female flowers contain a resin called lupolin which, thanks to its composition in α-acids, β-acids and essential oils, contributes to the typical beer bitterness, and to its aromatic characteristics (Eri et al. 2000; Kornyšova et al. 2009). The use of hops in brewing, both as a protection against spoilage and for aromatic purposes, is subsequent to the first beer production and, is only documented as an ordinary practice starting from the sixteenth century. Italian hop in particular it has been evaluated only in the first half of the XIX century by the agronomist Gaetano Pasqui, who started to select, grow and characterize plants of spontaneous hop (Pasqui 2010). The earliest documentation of beer produced by Pasqui date back to1847. A similar experience took place in 1876 in Marano sul Panaro (Modena), in the estate of Marquis Montecuccoli. The results were comforting, and the hops produced were publicly praised by numerous Italian and foreign beer brewers; the product also received a honorable mention at the international Hagenau (Alsace) exhibition in 1860 (Magiera 1875). Experiments of hop cultivation were also conducted in 1908 by Faina, in 1914 by the Luciani Brothers, in 1927 by Moretti and in 1959 by Dandoni (Treccani 1938; Caracausi 2006/Buiatti personal communication). Hop cultivation and culture has since gradually decreased in Italy, until recently, when for the appeal of beer has rapidly grown and a great number of small breweries has flourished, spurring a renewed interest for local raw materials. Hops is native in the whole Italian peninsula, and since plant identity is not known, an evaluation of biodiversity of the countless ecotypes has become a necessity. Hop varietal analysis can be made by using a number of different markers (morphological, chemical and genetic). Morphological markers are useful for the distinction between *Humulus lupulus* and *Humulus japonicus*, since the two species exhibit a number of differences, such as the amount of lupolinic glands, but are not suited to discriminate the cultivars of European and American provenance (Davis 1957). To assess diversity among cultivars, some researchers have utilized the chemical profile (Stevens et al. 1997; Stevens et al. 2000; Hanning et al. 2004). Varietal differences were found regarding the content in chemical compounds, as for instance flavonoids; in particular, by evaluating the prenylated flavonoid fraction, hops of European

origin could be distinguished from those of Asian origin. However, to avoid variability in chemical and phenotypical features due to the environment, molecular methods appear more suitable. Among them are RAPD, (Random Amplify Polimorfic DNA) (Brady et al. 1996; Pillay and Kenny 1996; Murakami 1998; Patzak et al. 1999), AFLP (Amplify Fragment Length Polimorfism) (Hartl&Seefelder 1998; Seefelder et al. 2000; Jakše et al. 2001; Solberg et al. 2014), ISSR (Inter Simple Sequence Repeted) (Zietkiewicz et al. 1993; Danilova et al. 2003), STS (Sequence Tagged Site) (Patzak et al. 2007) e SSR (Simple Sequence Repeated) (Hadanou et al., 2004; Jakše et al. 2004; Patzak et al.2007; Nahla et al. 2008; Bassil et al. 2008; Patzak et al. 2010; Patzak and Matausek 2011; Koelling et al. 2012) markers. Patzak (2001) carried out a research in which the efficacy of some of these methods (RAPD, STS, ISSR and AFLP) was compared in identifying the biodiversity among ten hop cultivars. Solberg and coworkers (2014), utilizing AFLP markers, analyzed 62 Danish and 34 Norwegian hop plants, and found that a correlation exists between genetic variability, zone of origin, chemical composition and morphology. In many studies on spontaneous and cultivated hops, polymorphic microsatellite sequences were isolated and characterized (Hadanou et al. 2004; Bassil et al. 2008; Jakše et al. 2011; Patzak e Matausek 2011; Koelling et al. 2012). Jakše et al. (2004) and Murakami et al. (2006), by utilizing 4 and 11 SSR loci, respectively, effectively evaluated the biodiversity and the existing relations among spontaneous and cultivated hops from Europe, North America and Asia. Patzak et al. (2010) conducted chemical (essential oils, polyphenols and bitter acids) and molecular studies to evaluate genetic variability in native hops of Canada (62 plants) and of Caucasus (58 plants) in comparison with European (104 plants) and North American (27 plants). The genetic analyses involved the utilization of 9 SSR loci and of 3 STS sites. The chemical analyses allowed the distinction between North American and European hops, while the genetic study also permitted the discrimination between Caucasian and European hops. The main result was then that chemical analyses are not as distinctive as the genetic ones.

The present research aimed at evaluating the genetic variability of native hop accessions, never identified so far, from different provenances of Italy (Northern Italy), by utilizing 9 microsatellite markers, as compared with several known hop cultivars. A proper description of the *Humulus lupulus* L. biodiversity is an essential step to select the starting material needed to develop new hops varieties that will hopefully find a relevant place in the hops commerce.

2. **Materials and methods**

2.1. Plant material

Eighty wild hops accessions were collected in Northern Italy regions (Lombardy, Emilia Romagna, Tuscany, Veneto and Val D'Aosta) at altitudes ranging from 20 to 1200 mt ca., as reported in Table 1.

Other accessions had no name and were of unknown origin, and were tagged with an alpha-numerical code or with the name of the collected zone.

European and American hop varieties (Tab. 2), were purchased from commercial sources.

Table 1. *List of the 80 Italian accessions and their provenience. In parenthesis the number of accessions used are indicated.*

ID	Region	ID	Region
ET (15 accessions)	Emilia Romagna	SANTA CLARA (1 accessions)	Lombardy
FISICA (1 accession)	Emilia Romagna	ROGGINO (2 accessions)	Lombardy
CAMPUS CANNA (2 accessions)	Emilia Romagna	QUINTO (4 accessions)	Veneto
TANGE (1 accession)	Emilia Romagna	SANT'ILARIO (1 accession)	Emilia Romagna
CINGHIO (1 accession)	Emilia Romagna	VIA CASTIGLIONE (1 accession)	Emilia Romagna
BIBBIANO (1 accession)	Emilia Romagna	PASQUI (3 accessions)	Emilia Romagna
RIGO ROSSO (1 accession)	Emilia Romagna	ROCCAMALATINA (1 accession)	Emilia Romagna
RIBO B (1 accession)	Emilia Romagna	MOGLIA (1 accession)	Lombardy
SAN POLO (1 accession)	Emilia Romagna	MAURIZIANA (1 accession)	Emilia Romagna
MARON (1 accession)	Emilia Romagna	BOERO (1 accession)	Emilia Romagna
LIVELLO (1 accession)	Emilia Romagna	TAURO (1 accession)	Emilia Romagna
PIATTELLO (1 accession)	Emilia Romagna	PICO (1 accession)	Emilia Romagna
BUSSETO (15 accessions)	Emilia Romagna	PELOSA (2 accessions)	Emilia Romagna
BRENTO (1 accession)	Emilia Romagna	COMO (1 accession)	Lombardy
GIANNI (1 accession)	Emilia Romagna	TORRE (1 accession)	Tuscany
TAVERNELLE (1 accession)	Emilia Romagna	CORTINA (1 accession)	Emilia Romagna
RIO GAMBERO (1 accession)	Emilia Romagna	DOLCETTO (1 accession)	Emilia Romagna
MA (4 accessions)	Emilia Romagna	PESCIA (2 accessions)	Tuscany
PRISMI (2 accessions)	Lombardy	SIBOLLA (1 accession)	Tuscany
Valle d'aosta (1 accession)	Valle d'Aosta		

Table 2. *List and origin of the 43 European and American hop cultivars. In parenthesis the number of genotypes used are indicated.*

ID	Region	ID	Region
BRAMLING CROSS (1 genotype)	Kent, Sussex (UK)	MOSAIC (1 genotype)	United States
TETTNANGER (1 genotype)	Baden-Bitburg-Rheinpfalz (DE)	AURORA (1 genotype)	Herefordshire, Worcestershire (UK)
WILLAMETTE (1 genotype)	Oregon, Washington (USA)	CHALLENGER (1 genotype)	United Kingdom
SAAZ (3 genotypes)	Czech Republic	HALLERTAU MAGNUM (2 genotypes)	Germany
ADMIRAL (1 genotype)	United Kingdom	HERSBRÜCKER (1 genotype)	Germany
SLÁDEK (1 genotype)	Czech Republic	HALLERTAU HERCULES (1 genotype)	Germany
SUPERGALENA (1 genotype)	Stati Uniti	TRADITION (1 genotype)	Germany
PREMIANT (1 genotype)	Czech Republic	BREWER'S GOLD (1 genotype)	United Kingdom
SAPHIR (1 genotype)	Germany	CHINOOK (1 genotype)	United States
PERLE (1 genotype)	Germany	CENTENNIAL (2 genotypes)	United States
CASCADE (2 genotypes)	Washington (USA)	HALLERTAUER MITTELFRÜH (1 genotype)	Germany
GOLDINGS (1 genotype)	United Kingdom	COLUMBUS (1 genotype)	United States
EL DORADO (1 genotype)	United States	SPALTER SELECT (1 genotype)	Germany
PILGRIM (1 accessione)	United Kingdom	HALLERTAU TAURUS (1 genotype)	Germany
STYRIAN GOLDINGS (1 genotype)	Slovenia	SMARAGD (1 genotype)	Germany
EAST KENT GOLDINGS (1 genotype)	United Kingdom	NORTHERN BREWER (1 genotype)	United Kingdom
TARGET (2 genotypes)	Kent, Sussex (UK)	FUGGLE (1 genotype)	Herefordshire, Worcestershire (UK)
NUGGET (1 genotype)	Oregon, Washington (USA)	MARYNKA (1 genotype)	Poland

2.2. SSR analysis

Genome DNA of the 123 samples (Tables 1 and 2) was extracted from young leaves (L) collected from young Italian wild hops or from vacuum dried flowers (Fl) and pellets (P) (European and American accessions) or young leaves of hop cultivars grown in Italy, in the collection field (LC) or cultivated by Italian farmers (LF). The samples, after immersion in liquid nitrogen, were stored at

-80 °C until DNA extraction. Genomic DNA was extracted following the CTAB procedure (Doyle and Doyle 1987).

In order to evaluate the validity of analyses (PCR amplification and sequencer analysis) a control was set through the insertion in each analysis of a sample standard (the variety Cascade, Fuggle, Mgnum and Marynka were used) to allow an internal control at each analysis.

For DNA amplification 9 couples of SSR primers were used which had shown a high discriminating capacity (Stajner et al., 2005) (Tab. 3).

Table 3. *list of SSR loci, primer sequence and their respective annealing temperature (T °C) used in this study.*

Primer	For 5'→ 3'	Rev 5'→ 3'	Size (bp)	T (°C)
HlGA31	CY5-CAAACTTGGTGCTCTAAGATGAA	CGTTTTCCCAACACCTAGTTC	163	55
HlGT14	CY5-GGCATGGCTAACTCTATATGC	AAATAGAAGTGCCATAACTGA	165	54
HlGT16	CY5-CCGTGATACAAATCTACCCAAA	CTCCAGTCAGCAATCTCTTCAA	228	54
HlGT17	CY5-GGTCCTTAGTCACTTGCCAAT	GACTGTTCGAAGCACAATCAA	182	54
HlACA3	CY5-CAAGTTGTTGGTTGATTTCACAT	CTCCTTCCTGTGTTCACCAC	215	52
HlAGA6	CY5-GTTAGAATCTCGTTGGCAA	TCTGAAACTTCACTAATCATC	192	55
HlAGA7	CY5-ACAAGCAGTAATGATGAGGA	TCCAAGTCTCTCAATTAGGAA	180	54
HlAGA35	CY5-ATTATCTCAAACACTCAACCCC	AGCAGGCTATAAAAAGAAGTGC	203	55
HlGA23	CY5-AAGCACGAAAACTGACTTG	GTTGCCCAAAATCACTGTT	245	54

The PCR amplification was performed in a 25 µl volume containing: 1x Reaction Buffer (Biotools, B&M Labs, S.A., Madrid, SP), 1.5 mM $MgCl_2$ (Biotools, B&M Labs, S.A., Madrid, Spain), 0.2 mM dNTPs (Amersham Biosciences, Piscataway, USA), 0.2 µM primer (MWG Biotech, Ebersberg), 20 ng genomic DNA and 0.6 U of *Taq* polymerase (Biotools, B&M Labs, S.A., Madrid, SP). For primer HlACA3, HlGA31, HlGA35, HlGA23, $MgCl_2$ concentration was 2.5 mM, to obtain a better quality of amplification.

The PCR amplification was optimized in thermal cycler MJ PCT 100 Research (Watertown, Mass.), programming a first step at 95°C for 5 minutes followed by 30 cycles of 45" at 94°C, 30" at the specific annealing temperature for each couple of primers, and 90" at 72°C, for denaturation, annealing and primer extension, respectively; at the end of the cycles were allowed 8 minutes of incubation at 72°C.

The amplification products were separated with a CEQ 2000 Genetic Analysis System (Beckman Coulter, Inc.) sequencer on acrylamide gel CEQ Separation Gel LPA-1 (Beckman Coulter, Inc.). A marker CEQ DNA Size Standard kit 400 (Beckman Coulter, Inc.) was used to estimate the

approximate molecular weight of the amplified products. One of the two PCR primers in each reaction was end-labeled with a fluorescent dye (Cy5, MWG-Biotech, Ebersberg).

2.3. Data analysis

For a better comparison, the analysis were performed first evaluating the Italian population, then the entire totality of samples including commercial cultivars.

The fragments were sized by using a conservative binning approach (Kirby 1990) through the statistical R software (R Development Core Team 2005), which takes into account the type of replicate and compensates for the limits of fragment resolution.

In order to evade wrong estimations of allelic frequencies due to the presence of clones, individuals identical at all loci were removed from the data set using an Excel spreadsheet (© Microsoft Corporation).

The genotypes that showed only one amplified allele at locus were assumed to be homozygous at that same locus. The information content of the SSR markers were evaluated according to number of alleles per locus, allele frequency, observed (H_O) and expected (H_E) heterozygosities, presence of null alleles (f_{nu}), effective number of alleles (N_E) (Brookfield 1996), (Paetkau et al. 1995) and polymorphic information content (PIC) (Botstein et al., 1980). Such values were obtained by using the Cervus 3.0 software (Marshall et al. 1998; Kalinowski et al. 2007).

The level of similarity/dissimilarity among the examined Italian wild hops was obtained through the genetic similarity matrix utilizing Euclidean distance. Cluster analysis and construction of the dendrogram relative to genetic distances were obtained by using the unweighted pair-group method with arithmetic mean (UPGMA) algorithm, with XLSTAT 2009 software (Addinsoft™ 1995–2009).

3. Results

3.1. SSR markers polymorphism

The 9 primer pairs belonging to the series HlGT, HlGA, HlAGA e HlACA, were used to analyze the 80 local genotypes and the 43 commercial cultivars. According to the analysis of heterozygosity and of allelic frequencies, on the 80 Italian accessions the SSR markers allowed the identification of 104 alleles (Tab. 4).

Table 4. *Allele size (bp), allele frequency (f), number of alleles(N), observed (H_o) expected heterozygosity (H_e) null allele frequency (f_{nu}), effective number of alleles (N_E) and polymorphism information content (PIC)*

	Locus		**Locus**		**Locus**		**Locus**		**Locus**		**Locus**		**Locus**		**Locus**		**Locus**	
	HlGT14	**f**	**HlGT16**	**f**	**HlGT17**	**f**	**HlAGA6**	**f**	**HlAGA7**	**f**	**HlACA3**	**f**	**HlGA31**	**f**	**HlGA35**	**f**	**HlGA23**	**f**
a	154	0,006	209	0,013	165	0,006	165	0,006	163	0,043	205	0,056	152	0,006	193	0,988	243	0,063
b	159	0,056	211	0,163	169	0,006	168	0,006	166	0,013	208	0,069	158	0,113	223	0,013	244	0,131
c	161	0,100	213	0,006	173	0,013	171	0,156	169	0,163	211	0,444	160	0,369			247	0,006
d	162	0,006	225	0,156	175	0,013	174	0,019	172	0,006	220	0,150	162	0,056			251	0,013
e	163	0,156	227	0,181	177	0,281	177	0,013	181	0,081	223	0,563	164	0,263			255	0,162
f	165	0,263	229	0,206	179	0,156	180	0,025	184	0,094	226	0,019	166	0,050			257	0,050
g	167	0,344	231	0,113	181	0,131	183	0,250	187	0,325	229	0,106	168	0,006			265	0,013
h	169	0,050	233	0,106	183	0,006	186	0,138	190	0,244	232	0.019	182	0,006			267	0,006
i	173	0,006	235	0,031	185	0,057	189	0,281	193	0,031	235	0.056	184	0,006			271	0,006
j	175	0,006	237	0,025	187	0,281	192	0,088			238	0,013	186	0,031			273	0,006
k	177	0,006			189	0,013	195	0,013			241	0,013	188	0,088			275	0,006
l					193	0,025	201	0,006					200	0,006			277	0,438
m					195	0,013											279	0,031
n																	281	0,031
o																	283	0,044
p																	285	0,013
q																	287	0,031
r																	289	0,056
s																	291	0,019
t																	293	0,094
u																	295	0,013
v																	297	0,056
w																	299	0,025
x																	301	0,138
N	11		10		13		12		9		11		12		2		24	
H_E		0,772		0,848		0,796		0,806		0,790		0,754		0,768		0,025		0,911
H_O		0,650		0,688		0,813		0,700		0,613		0,788		0,600		0,025		0,938
f_{na}		0,069		0,087		-0,009		0,059		0,099		-0,019		0,095		-0,000		-0,014
N_E	3,59		6,57		4,90		5,15		4,76		4,06		4,31		1,02		11,23	
PIC	0,738		0,829		0,767		0,780		0,762		0,733		0,738		0,024		0,905	

of the Italian hop population.

The average expected heterozygosity (H_E) and observed heterozygosity (H_O) values are 0,718 e 0,646, respectively. The effective allele number (N_E) ranges from a value of 1,025 for marker HlGA35 to 11,23, relative to HlGA23, with an average of 5,070.

Among the analyzed accessions we can assess the most frequent alleles per locus as concerns Italian accessions in Table 4. The allelic frequency for the Italian population ranges between 0,006, with

unique alleles present in all loci except HlACA3 and HlGA35, and 0,988 in HlGA35 with allele 193, followed by HlACA3 with allele 223, for a frequency of 0,563. Expected heterozygosity (H_E) is always above 0,75, except for locus HIGA35, which has the value of 0,025; the values of observed heterozigosity (H_O) were all above 0,600 with the exception for locus HlGA35 (H_O value of 0,025). Observed heterozygosity was instead higher than expected in primers HlGT17 and HlACA3 (Tab. 4). To evaluate locus ability to discriminate among accessions PIC values (Polymorphism Information Content) were calculated, and ranged from 0,024 for locus HlGA35, to 0,905 for locus HlGA23, with a mean PIC value corresponding to 0,6973.

To achieve a more exhaustive view of the biodiversity of the Italian accessions, the analysis were then performed including the European and American cultivars.

From the total analysis of the 123 accessions, the SSR markers allowed the identification of 125 alleles (Tab. 5); the effective number of alleles (N_E) ranges from 1,088 for HlGA35 to 10,397 for HiGA23, with an average value of 5,510.

The average values of expected (H_E) and observed (H_O) heterozygosity for all analyzed accessions were 0,812 and 0,645, respectively. The most frequent alleles per locus are explain in table 5.

Table 5. *Allele size (bp), allele frequency (f), number of alleles(N), observed (H_O), expected heterozigosity (H_E) null allele frequency (f_{nu}), effective number of alleles (N_E) and polymorphism information content (PIC) of the of the entire hop population.*

	Locus		**Locus**		**Locus**		**Locus**		**Locus**		**Locus**		**Locus**		**Locus**		**Locus**	
	HlGT14	**f**	**HlGT16**	**f**	**HlGT17**	**f**	**HlAGA6**	**f**	**HlAGA7**	**f**	**HlACA3**	**f**	**HlGA31**	**f**	**HlGA35**	**f**	**HlGA23**	**f**
a	151	0,008	209	0,052	165	0,008	165	0,004	163	0,040	205	0,036	152	0,004	193	0,960	241	0,012
b	153	0,004	211	0,127	167	0,004	168	0,004	166	0,012	208	0,063	158	0,071	205	0,008	243	0,008
c	155	0,004	213	0,087	169	0,028	171	0,103	169	0,103	211	0,377	160	0,353	207	0,016	245	0,833
d	157	0,012	225	0,103	173	0,008	174	0,012	172	0,004	217	0,008	162	0,063	221	0,008	247	0,004
e	159	0,052	227	0,151	175	0,008	177	0,008	175	0,004	220	0,099	164	0,349	223	0,008	249	0,230
f	161	0,036	229	0,139	177	0,190	180	0,020	181	0,103	223	0,250	166	0,040			251	0,008
g	162	0,091	231	0,107	179	0,171	183	0,163	184	0,004	226	0,012	168	0,159			255	0,103
h	163	0,004	233	0,198	181	0,100	186	0,155	187	0,131	229	0,071	170	0,004			257	0,036
i	165	0,099	235	0,020	183	0,012	189	0,413	190	0,246	232	0,012	172	0,008			265	0,020
j	167	0,198	237	0,016	185	0,044	192	0,107	193	0,159	235	0,036	182	0,008			267	0,278
k	169	0,433			187	0,214	195	0,008	196	0,024	238	0,028	184	0,004			269	0,004
l	173	0,468			189	0,123	201	0,004	199	0,004	241	0,008	186	0,020			271	0,004
m	175	0,004			193	0,020			205	0,012			188	0,055			273	0,004
n	177	0,004			195	0,060			209	0,004			200	0,004			275	0,004
o		0,004			197	0,012			212	0,044							277	0,028
p									218	0,103							279	0,020
q										0,004							281	0,036
r																	283	0,028
s																	285	0,012
t																	287	0,278
u																	289	0,040
v																	291	0,012
w																	293	0,060
x																	295	0,008
y																	297	0,036
z																	299	0,048
aa																	301	0,099
N	14		10		15		12		16		12		14		5		27	
H_E		0,749		0,869		0,857		0,756		0,861		0,773		0,739		0,077		0,904
H_O		0,643		0,738		0,841		0,563		0,738		0,690		0,690		0,079		0,825
F_{na}		0,061		0,070		0,008		0,110		0,066		0,046		0,028		0,002		0,041
N_E	3,96		7,65		6,97		4,10		7,18		4,39		3,82		1,08		10,39	
PIC	0,727		0,856		0,841		0,729		0,845		0,747		0,704		0,078		0,902	

Allelic frequency of the whole population ranges from 0,004 (all primers except HlGT16, HlACA3 and HlGA35) to 0,960 for HlGA35 with allele 193, followed by HlGA23 with a frequency of 0,833 for allele 245 (Tab. 5). The effective number of alleles (N_E) ranges from 1,08 for HlGA35 to 10,39 for HiGA23, with an average value of 5,510. To evaluate locus ability to discriminate among accessions mean PIC value was calculated, as corresponding to 0,7144. PIC values ranged from 0,078 for locus HlGA35 to 0,902 for locus HlGA23 (Table. 5). From the comparison (Tab. 6) of the alleles present in the Italian population and the cultivars population, we can observe that the Italian hops have much more own alleles (47) than the cultivars population (18), and more unique alleles (15 alleles in Italian population and 6 alleles in cultivars population).

Table 6. *Indication of the own alleles of Italian genotypes (IT), European and American genotypes (EA) and the alleles present in both population (P). The bolded numbers are unique alleles.*

	HIGT14			HlGT16			HIGT17			HlAGA6			HlAGA7			HlACA3			HlGA31			HIGA35			HlGA23		
	IT	**EA**	**P**	**IT**	**EA**	**P**	**IT**	**EA**	**P**	**IT**	**EA**	**P**	**IT**	**EA**	**P**	**IT**	**EA**	**P**	**IT**	**EA**	**P**	**IT**	**EA**	**P**	**IT**	**EA**	**P**
a	159	151	155	235		209	173	**167**	165	**165**		171	169	**175**	163	205	217	208	**152**	**170**	160	223	205	193	245	241	243
b	163	153	161	237		211	175	197	169	**168**		180	**172**	**196**	166	226		211	158	172	162		207		**247**		257
c	**173**	157	165			213			177	174		183		199	181	232		220	**184**		164		221		249		265
d	**175**		167			225			179	177		186		**205**	184	235		223	186		166				251		267
e	**177**		169			227			181	195		189		212	187	241		229	188		168				255		281
f						229			183	**201**		192		**218**	190			238	**200**		182				**269**		287
g						231			185						193										**271**		289
h						233			187						209										**273**		299
i									189																**275**		301
j									193																277		
k									195																279		
l																									283		
m																									285		
no																									287		
op																									291		
p																									293		
q																									295		
r																									297		

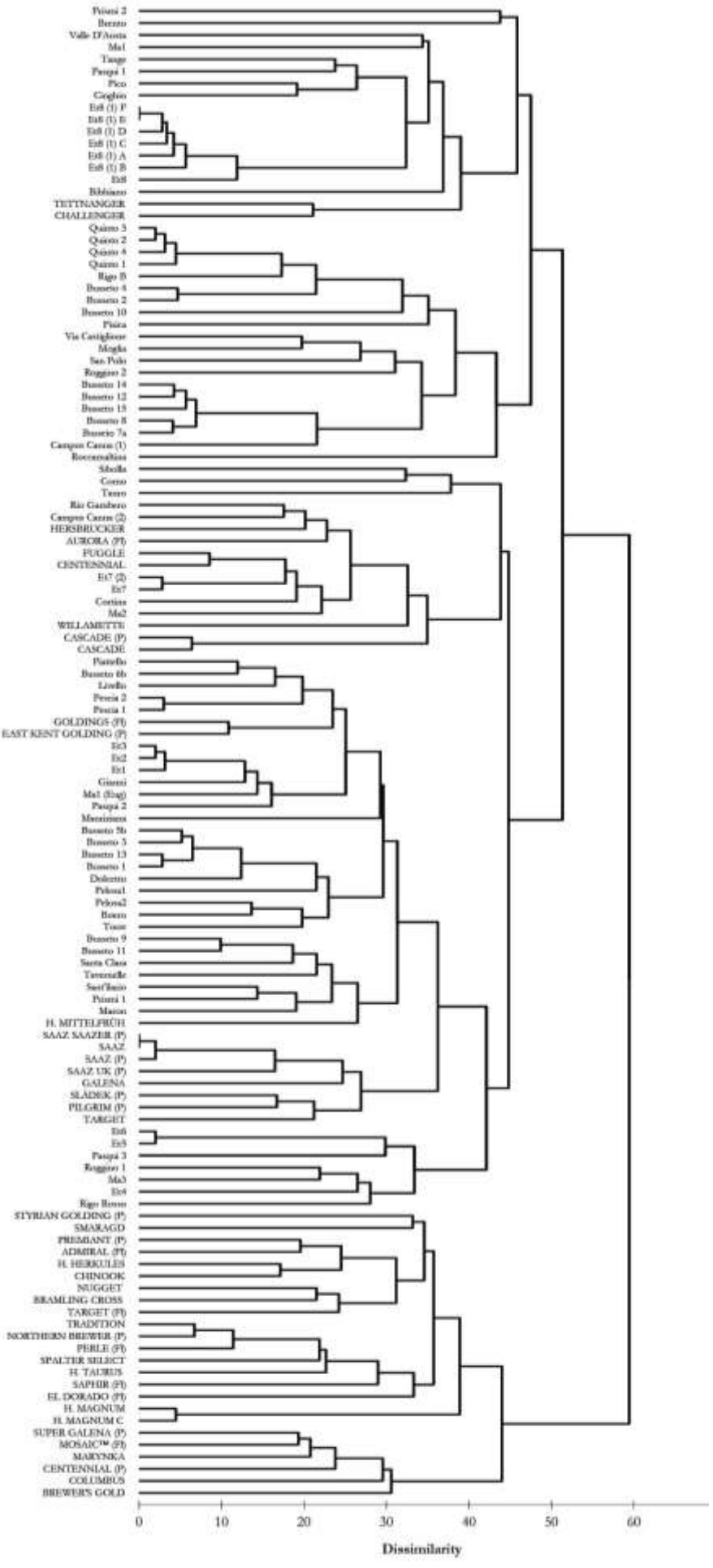
3.2. Identification of the examined population and internal relationships

Thanks to the utilization of the nine SSR primers, almost all 80 wild accessions could be identified. The relationships among all accessions were analyzed and observed by cluster analysis (UPGMA) at Euclidean distance, by which dissimilarity between studied wild hops and cultivars is highlighted (Fig.1). In the dendrogram (Fig. 1) differences can be observed between accessions with a dissimilarity index between 0 and 60% and the examined population is divided in two main clusters (I e II). Cluster I comprises the majority of analyzed genotypes, and splits into 2 subgroups. Subgroup A is almost exclusively composed by wild accessions, with the exception of cultivars Tettnanger and Challenger. This subgroup is then divided in 2 sets, set 1 including, besides the above mentioned cultivars, the whole Et8 group and other genotypes. Set 2 is instead exclusively composed of wild accessions, among which those named Busseto and Quinto. In subgroup B we find both cultivars and wild types; it is divided in two sets, the first including a smaller number of accessions, among which are cultivars Hersbrücker, Aurora, Fuggle and Centennial. In the second set is instead present a majority of wild type accessions, among which it worth remarking the similarity between Et3 and Et2 and between Et6 and Et5.

Cluster II is made of cultivars: it has two subgroups (A e B). Subgroup IIA is made of two sets, the first in which cultivar H. Magnum is differentiated, and the second in which we find most cultivars. The second subgroup (IIB) is instead represented by cultivars Super Galena, Mosaic, Marynka, Centennial, Columbus and Brewer's Gold, the latter being in a different set with respect with the rest of accessions. All accessions with a dissimilarity index below 10% and with a genic profile

differentiating by few alleles were reamplified and reanalyzed, and profiles were confirmed.

Figure 1. *UPGMA dendrogram based on Euclidean distance of Italian wild and European and American cultivated hops.*



4. Discussion

The nine primer pairs used in this study (HlGT14, HlGT16, HlGT17, HlAGA6, HlAGA7, HlACA3, HlGA31 HlGA35, HlGA23) (Table 3) were selected from a set of 60 microsatellites identified and developed by Stajner et al. (2005). Primers were selected by taking into account discriminatory ability, number of alleles amplified, segregation and genetic distance between locus; this last characteristic was observed in a mapping study made up by Jakše et al. (2010) in which these primers were used. For data analysis, two populations were considered: the first was composed of Italian accessions, and the second considered the whole population included cultivated European and American hops. Primer HlGA35 amplified an effective number of alleles of 1,02 (2 alleles), with an expected heterozygosity of 0,025 in the Italian population; it amplified 5 alleles (1,8 effective alleles) with an expected and observed heterozigosity lower than 0,08 in the complete population: this translates to a low variability and to a bad discrimination capacity between the accessions (Tables 4-5). As concerns the analysis of wild Italian hops, the most polymorphic primers were HlGA23 and HlGT17 individuating 24 and 13 alleles respectively (Tables 4 and 5). Instead, in the analysis among all the accessions the most discriminating primers were HlGT23 and HlAGA7 with 24 and 17 alleles detected respectively. The abundant number of alleles detected by these primers is in agreement with the study of Stejner et al. (2005). The microsatellites used made possible the study of genetic diversity in the 80 wild hops accessions, revealing 65 distinct genotypes and differentiating all the 43 cultivated cultivars. The analysis made on Italian hop genotypes and on the whole population (Italian, European and American hops), showed an high mean of observed heterozigosity (tables 4 and 5); this indicates an high degree of genetic biodiversity in the analyzed population. Allele analysis shows more alleles in the Italian wild hops accessions (104 alleles detected) than in the European and American cultivated hops (78 alleles detected), thus revealing a greater biodiversity in the Italian population (Tab. 6). Moreover Italian wild hops possessed more unique alleles than the cultivated hops (Tab. 6) and these data are in accordance with the study of Jakše et al. (2004) on wild and cultivated hops from America and Europe, in which the wild populations showed the highest number of unique alleles compared with cultivated hops. Unique alleles could be a useful tool for fingerprinting analysis and effective cultivar identifications (Cerenak et al. 2003, Jakše et al. 2004). The smaller number of alleles, and in particular of unique alleles in cultivated hops if compared to wild hops, may be due to the former being derived from breeding activities and selection within limited genetic resources; accordingly, the study of Li et al. (2008) on peach confirmed that after a long history of selection and cultivation,

the more primitive varieties still contain more allelic variations and rare allelic variations than the more evolved ones; in the long history of natural evolution and selection by human beings, although new variations have developed, the allelic variations in peach decrease, thus leading to a diminution of biodiversity.

The division of the study into two parts allowed us single out some characteristic alleles, specific or exclusive of the Italian population or of the European and American genotypes (Tab. 6); Table 6 shows the abundance of specific Italian alleles (47 alleles), if compared to the number of specific alleles of cultivated hops (18 alleles). The most interesting primers for allelic specificity were HlAGA7 with 6 alleles out of 16 exclusive for cultivars, and HlGA23 with 18 alleles out of 27 specific for wild Italian hops. The noted allelic differences, in spite of the relatively small number of cultivars sampled, are indicative of a significant separation existing between wild and cultivated hops.

The dendrogram (Fig. 1) shows that wild Italian and cultivated American and European hops belong to different clusters (cluster I and II), confirming the existence of remarkable genetic differences among the different populations examined. The presence of a significant genetic distance between wild hops and cultivated hops is consistent with previously reported data of different authors (Jakše et al. 2004; Murakami et al. 2006; Bassil et al. 2008; Patzak et al. 2010). The dendrogram does not show for a precise clustering according to geographical origin among Italian accessions. Within the Italian population some accessions only show one or two differences in the allelic profile (Supplementary Material, SM 1). The genotypes characterized by such small allelic differences were considered as having the same genetic identity; in detail, the accessions named Quinto3 and Quinto2, Et3 and Et2, Et6 and Et5 and 3 of the 5 accessions of ET8(I) were identified as the same genotype. The elevated genetic diversity present in the rest of the Italian population, strengthen the observations made in a previous study made by Murakami et al. (2006), in which with 11 SSR loci 133 hop samples coming from Asia, North America and Europe could be analyzed; among European hops, a limited pool of 4 accessions from different part of Italy were examined. Italian hop accessions had an elevated genetic difference among them, although being in the same cluster, together European germplasm. Even Figure 1 does not show distinct separation between European and American hops and some varieties are grouped in the first cluster with wild Italian genotypes. Therefore, some cultivated hops are genetically different from the cultivars present in the second group, exclusively made of cultivated hops. For instance, Tettnanger (Germany), Challenger (United Kingdom), Cascade (United States), Williamette (United States), Goldings (United Kingdom), East Kent Goldings (United Kingdom) are cultivated hops, but are

clustered in the first group with Italian wild accessions. As concerns Cascade and Willamette (American cultivars), this result is probably a consequence of breeding, because they both derive from Fuggle, an English hop variety that seems to have its origin from English wild hops. Jakše et al. (2003) have found differences in the group of wild and cultivated accessions: the majority of wild, European or American, were collocated in a separated clusters, showing significant differences from cultivated hops. Patzak et al. in 2010, in a biodiversity study, observed that Fuggle variety was grouped in the cluster of European wild hops; however, American an Asian cultivated hops were in a separate group, but close to European germplasm. This result is a confirmation of our results, in which Fuggle, Willamette and Cascade are clustered in group I with Italian wild genotypes (Figure 1). The lack of a geographical separation of the cultivated hops in the dendrogram (Fig. 1) may be due to incapacity of our set of primers to recognize origin, but also to breeding, since American cultivated hops are often a result of breeding with European genotypes. This last hypothesis is in accordance with Peredo et al. (2010), where the presence of an European genetic influence on American varieties was studied.

5. Conclusions

The results presented here describe for the first time the identification and characterization of Italian wild hops biodiversity, using molecular methods. Differences and similarities between wild and cultivated hops are representative of a remarkable genetic richness and show the need to study it in depth, researching new hops from different regions in the Mediterranean area; in this way it would be possible to obtain Italian biodiversity maps, useful as a starting point for further analyses, be they morphological, phytochemical or agronomical; a complete characterization to be used in order to select the best genotypes, to be used as such or for breeding programs. Moreover, the comparison of wild accessions with cultivated genotypes allows us to affirm that Italian hops are genetically different from most well-known cultivated hops. It was also found that within the population of north-central Italy a large genetic variability is present, ready to be studied and exploited; a great hops biodiversity which, after a careful work of selection, might lead to the development of hops endowed with unique and valuable characteristics.

Acknowledgements

This research was supported by fund from the town Marano sul Panaro (MO), so special thanks for the Major Emilia Muratori, that believe in this project from the first time.

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SUPPLEMENTARY MATERIAL

S.M.1. *Allele sizing of the 9 investigated loci. Accessions are grouped by genotype; different genotypes are separated by horizontal lines. The allele sizes of accessions differing from those typical of the group are in bold and underlined*

	HlACA3		**HlAGA6**		**HlAGA7**		**HlGA23**		**HlGA31**		**HlGT14**		**HlGT16**		**HlGT17**		**HlGA 35**	
Prismi 2	211	235	183	183	169	187	271	287	160	164	163	165	211	225	165	177	223	193
Brento	211	220	171	186	187	187	301	301	164	164	167	167	211	233	169	179	223	193
Valle D'Aosta	229	238	171	186	181	187	279	297	162	164	167	167	227	233	177	177	193	193
Ma1	211	235	183	189	169	181	297	301	162	162	161	161	225	225	173	173	193	193
Tange	211	220	183	189	187	187	293	301	164	164	159	163	227	233	177	179	193	193
Pasqui1	208	232	171	186	187	187	301	301	158	162	161	167	225	225	179	187	193	193
Pico	208	226	171	171	187	193	293	301	160	164	169	177	229	229	177	179	193	193
Cinghio	211	220	171	171	190	190	287	301	160	160	163	165	227	229	179	187	193	193
Et8 (1) f	205	229	171	192	181	187	283	289	158	166	159	165	211	231	177	187	193	193
Et8 (1) e	205	229	171	192	181	187	283	289	158	166	159	165	211	231	177	187	193	193
Et8 (1) d	205	229	171	192	181	187	283	***287***	158	166	159	***167***	211	231	177	187	193	193
Et8 (1) c	205	229	171	192	181	***190***	283	289	158	166	159	165	211	231	177	187	193	193
Et8 (1) a	205	229	171	192	181	187	***285***	***291***	158	166	159	165	211	***233***	177	187	193	193
Et8 (1) b	205	229	171	192	181	***190***	283	289	158	166	***154***	***163***	211	231	177	187	193	193
Et8	205	229	165	183	184	190	283	289	158	166	159	165	211	233	177	187	193	193
Bibbiano	220	229	183	183	190	190	293	293	166	186	163	165	211	227	185	185	193	193
TETTNANGER (L)	211	238	192	192	182	209	287	301	164	164	167	167	209	231	179	195	193	193
CHALLENGER (L)	211	238	192	192	166	196	287	299	164	164	165	165	209	231	177	193	193	193
Quinto 3	211	220	189	189	169	187	279	301	160	186	167	167	227	233	181	187	193	193
Quinto 2	211	220	189	189	169	187	279	301	160	***188***	167	167	227	233	181	187	193	193
Quinto 4	211	220	189	189	169	187	279	301	***162***	***188***	***165***	167	227	233	181	187	193	193
Quinto 1	211	220	189	189	169	187	279	301	160	***188***	167	167	***229***	***235***	***179***	***185***	193	193

Rigo B	220	229	183	186	169	184	277	301	164	186	163	165	225	237	177	183	193	193
Busseto 4	211	220	186	186	169	187	277	293	164	186	165	167	211	227	177	187	193	193
Busseto 2	211	220	186	***189***	169	***190***	277	293	164	***188***	165	167	211	227	177	187	193	193
Busseto 10	211	211	186	189	169	190	255	299	160	200	167	167	229	229	179	181	193	193
Fisica	211	235	168	171	163	193	281	297	158	184	165	167	227	231	177	187	193	193
Via Castiglione	211	235	171	183	187	190	265	297	164	188	167	175	227	227	177	181	193	193
Moglia	220	241	171	189	181	187	267	301	164	188	163	165	225	233	177	177	193	193
San Polo	211	235	183	183	187	187	247	301	160	188	165	165	231	231	177	179	193	193
Roggino 2	229	235	192	192	187	187	273	301	160	188	167	167	229	229	179	179	193	193
Busseto 14	211	220	183	186	187	187	255	293	160	188	167	167	211	225	177	181	193	193
Busseto 12	211	220	183	186	***190***	***190***	255	293	160	188	167	167	211	225	177	181	193	193
Busseto 15	211	220	183	186	***190***	***190***	255	293	160	188	***163***	***165***	***209***	225	177	181	193	193
Busseto 8	211	220	183	186	187	187	255	293	160	188	***161***	***163***	211	225	177	181	193	193
Busseto 7	211	220	183	186	***184***	187	255	293	160	188	***163***	***165***	211	225	177	181	193	193
Campus Canna (1)	220	223	183	189	193	193	245	281	160	182	165	167	211	225	177	177	193	193
Roccamaltina	235	235	183	192	181	187	245	301	168	186	161	161	233	233	179	187	193	193
Sibolla	211	235	171	189	169	184	255	281	160	188	165	165	231	231	177	189	193	193
Como	211	238	171	183	169	187	245	265	160	164	167	169	231	237	177	187	193	193
Tauro	223	241	171	183	169	190	255	293	164	164	169	169	213	235	179	195	193	193
Rio Gambero	211	220	180	183	181	187	245	257	160	164	163	163	211	237	175	177	193	193
Campus Canna (2)	211	223	183	192	187	190	245	255	164	164	162	165	211	231	177	187	193	193
HERSBRUCKER (L)	223	223	186	186	184	184	249	249	160	164	161	167	213	233	179	189	193	193
AURORA (FI)	211	223	189	189	184	184	249	267	162	164	165	169	213	233	187	189	193	193
FUGGLE (L)	208	223	189	192	181	190	249	265	160	166	167	167	227	231	179	189	193	193
FUGGLE (P)	211	211	189	189	184	212	249	267	164	172	157	167	213	233	169	179	207	193
Et7 (2)	211	226	183	189	184	190	257	257	158	158	163	165	229	229	179	187	193	193
Et7	211	226	183	189	184	190	257	257	***160***	***160***	163	165	229	229	179	187	193	193
Cortina	211	220	180	183	181	184	243	257	160	164	167	169	227	227	179	187	193	193
Ma2	211	220	189	189	169	181	245	255	152	164	165	167	229	229	177	185	193	193

WILLAMETTE (L)	211	223	189	189	175	184	241	249	164	182	161	165	209	231	187	189	193	193
CASCADE (P)	211	211	186	189	163	187	241	267	160	164	157	169	233	233	177	187	205	193
CASCADE (L)	211	211	189	189	163	187	241	267	164	166	155	169	231	231	177	187	205	193
Piattello	208	229	183	183	190	190	255	295	158	162	161	163	211	225	179	187	193	193
Busseto 6b	211	220	183	186	190	190	255	293	158	162	161	163	211	225	177	181	193	193
Livello	211	223	189	189	187	190	245	297	164	164	163	165	211	225	177	187	193	193
Pescia 2	211	232	183	183	187	187	255	281	160	164	161	161	211	227	181	187	193	193
Pescia 1	208	232	183	183	187	187	255	281	160	164	161	161	211	227	181	187	193	193
GOLDINGS (Fl)	223	223	189	189	181	184	249	285	160	164	165	167	213	233	181	189	193	193
EAST KENT GOLDINGS (P)	223	223	180	186	181	184	249	289	160	164	167	167	213	233	179	187	193	193
Et3	211	229	189	189	184	190	245	291	164	164	165	165	227	231	177	187	193	193
Et2	211	229	189	189	184	190	245	***293***	164	164	165	165	227	231	177	187	193	193
Et1	211	229	189	189	184	190	245	***293***	164	164	***167***	***167***	227	231	177	187	193	193
Gianni	211	229	183	195	190	190	245	291	164	164	161	163	227	227	179	185	193	193
Ma4	211	220	192	192	184	187	245	287	160	164	167	167	225	233	177	187	193	193
Pasqui 2	205	223	189	189	187	187	245	301	160	160	167	167	229	229	177	187	193	193
Mauriziana	208	211	189	189	169	190	245	289	160	164	167	167	211	227	179	187	193	193
Busseto 5b	211	211	183	186	190	190	255	277	158	158	165	167	225	229	181	193	193	193
Busseto 3	***208***	***208***	***180***	186	190	190	255	277	158	158	165	167	225	229	181	193	193	193
Busseto 13	211	211	183	186	187	187	255	277	160	160	165	167	225	229	181	193	193	193
Busseto 1	211	211	183	186	187	187	255	277	160	160	***163***	***165***	225	229	181	193	193	193
Dolcetto	208	211	189	192	184	187	255	275	160	160	163	167	227	227	181	189	193	193
Pelosa 1	211	211	189	189	190	190	245	289	160	164	167	169	229	233	187	187	193	193
Pelosa 2	211	211	171	189	187	187	245	289	160	164	163	169	229	233	187	187	193	193
Boero	211	211	174	186	187	187	245	287	160	160	163	167	229	237	179	195	193	193
Torre	211	211	171	189	184	190	257	289	160	160	167	169	229	235	179	179	193	193
Busseto 9	211	211	180	183	169	190	255	299	160	160	167	167	229	229	179	181	193	193
Busseto 11	208	208	186	189	169	190	255	299	162	162	167	167	229	229	179	181	193	193
Santa Clara	211	223	189	189	169	190	255	297	160	164	165	173	227	227	175	185	193	193

Tavernelle	205	208	183	186	184	187	255	301	160	160	163	165	225	225	177	177	193	193
Sant'ilario	211	220	171	189	169	184	245	297	164	164	161	167	227	233	177	181	193	193
Prismi 1	211	223	171	189	169	187	255	297	160	160	165	167	231	231	179	185	193	193
Maron	211	211	171	189	169	193	255	297	160	164	163	165	233	233	187	187	193	193
H.MITTELFRÜH (L)	217	223	186	189	181	187	265	299	162	164	167	167	229	233	185	189	193	193
SAAZ SAAZER (P)	211	223	189	189	181	209	249	299	162	164	161	167	227	233	189	195	193	193
SAAZ (L)	211	223	189	189	181	209	249	299	162	164	161	167	227	233	189	195	193	193
SAAZ (P)	211	223	189	189	181	209	249	299	162	164	161	167	227	233	189	***197***	193	193
GALENA (L)	211	211	189	189	163	209	249	299	164	164	161	167	227	233	187	189	193	193
SLÁDEK (P)	223	223	186	186	181	212	249	299	160	160	167	167	213	233	179	195	193	193
PILGRIM (P)	220	223	189	189	181	212	249	299	160	164	157	167	213	227	189	195	193	193
TARGET (L)	223	238	183	189	187	212	249	249	164	168	157	167	213	213	181	189	193	193
Et6	211	211	174	177	163	163	251	285	160	160	159	165	229	229	187	187	193	193
Et5	211	211	174	177	163	163	251	***283***	160	160	159	165	229	229	187	187	193	193
Pasqui 3	211	229	171	183	166	166	245	301	164	164	161	167	227	235	177	187	193	193
Roggino 1	211	223	186	189	169	169	245	299	160	160	167	167	225	225	185	185	193	193
Ma3	211	223	189	201	169	169	245	301	158	160	161	163	209	227	187	187	193	193
Et4	211	211	183	189	163	163	257	295	160	160	167	167	211	229	177	187	193	193
Rigo Rosso	211	211	192	195	169	172	255	301	160	160	165	165	231	235	177	179	193	193
STYRIAN GOLDINGS (P)	211	223	171	189	184	212	249	267	160	164	165	169	213	233	187	189	193	193
SMARAGD (L)	211	223	186	189	187	212	243	249	162	164	165	167	233	233	195	195	193	193
PREMIANT (P)	223	223	186	189	184	212	249	249	160	164	167	167	213	233	189	195	193	193
ADMIRAL (FI)	223	238	189	189	181	212	249	249	160	164	157	167	213	233	183	197	193	193
H. HERCULES (L)	223	223	189	189	184	199	249	249	160	168	157	167	213	233	179	179	193	193
CHINOOK (L)	223	223	186	189	184	212	249	249	160	164	167	167	213	233	179	179	193	193
NUGGET (L)	208	223	192	192	187	212	249	249	160	164	153	167	209	209	189	189	193	193
BRAMLING CROSS (L)	223	223	189	189	181	218	249	249	160	164	165	167	209	209	187	189	193	193
TARGET (FI)	223	238	183	189	187	212	249	249	164	168	157	167	213	213	181	189	193	193
TRADITION (L)	223	223	189	189	209	212	249	249	160	164	167	167	211	233	179	195	193	193

NORTHERN BREWER (P)	223	223	186	189	209	212	249	249	160	164	167	167	211	233	179	189	193	193
PERLE (FI)	223	223	189	189	209	212	249	249	160	164	157	167	213	233	181	189	193	193
SPALTER SELECT (L)	211	223	186	192	205	209	249	249	160	164	167	167	227	233	181	195	193	193
H. TAURUS (L)	211	223	186	189	199	212	249	249	160	164	157	167	213	233	167	189	193	193
SAPHIR (FI)	223	229	189	189	209	212	249	269	160	160	167	167	227	233	183	187	193	193
EL DORADO (FI)	223	223	186	186	199	212	249	257	164	168	157	167	213	213	169	189	207	193
H. MAGNUM (L)	223	223	189	189	184	212	249	249	160	164	157	167	213	233	189	195	221	193
H. MAGNUM (P)	223	223	189	189	184	212	249	249	160	164	157	167	***209***	***231***	189	195	221	193
SUPER GALENA (P)	208	211	192	192	187	212	249	281	160	164	155	167	213	233	169	189	193	193
MOSAIC ™ (FI)	208	223	189	189	187	212	249	267	160	164	155	167	213	233	165	189	193	193
MARYNKA (L)	211	223	189	189	187	212	249	281	164	164	157	167	209	231	169	189	207	193
CENTENNIAL (P)	211	211	189	189	184	212	249	267	164	172	157	167	213	233	169	179	207	193
COLUMBUS (L)	223	223	189	189	184	212	249	281	160	170	151	151	209	231	169	179	193	193
BREWER'S GOLD (L)	211	211	189	189	184	212	249	281	164	172	157	167	211	211	169	179	207	193

3.3. Study 3 - Optimization of PCR marker for early sex identification in Hop

Rodolfi M., Mongelli A., Marieschi M., Ganino T., Bruni R., Torelli A. (2015) **Optimization of PCR marker for early sex identification in Hop**. EBC-European Brewering Convention, 24-28 may 2015, Porto.

Optimization of PCR marker for early sex identification in hops

Rodolfi M., Mongelli A., Bruni R., Marieschi M., Ganino T., Torelli A.

Summary:

Humulus lupulus L. is a dioecious perennial species and only the female plants are of commercial interest. Female inflorescence of hops, referred as cone, contain secondary metabolites which give bitterness, flavor and aroma to beer. Usually, for a definitive determination of the sex phenotype of hop progeny, two season growth are required. Moreover, to determine the sex of a wild preexisting plant is necessary to wait flowering season.

A molecular method to reduce time and costs of breeding programs is the Marker Assisted Selection (MAS). Previous researches reported the identification of male and female plants with markers associated with Y chromosome, in order to allow the plant screening from the first leaf.

The aim of this study is enhancing the STS DNA marker, previously selected by other researchers, and improving the robustness of the method. To achieve the maximum potential of molecular sex identification, new primers associated with Y chromosome, two reverse (R0 and R1) and two forward primers (F0 and F1), were designed. The PCR amplicon F0-R0, resulted to be accurate and sensitive for an early identification of male plants.

Introduction:

Humulus lupulus L. is a dioecious perennial species, commonly known as hop, but only female inflorescence are of commercial interest for the greater content in valuable substance, such as bitter acids, essential oils and antioxidants. Hop is diploid and has 2n=20 chromosomes of which nine pair are bivalent autosome and 2 are sexual chromosomes. The male gender and phenotype is expressed by an X autosome, but the Y chromosome is essential for the pollen fertility (Parker and Clark, 1991, Dallapetra and Calderon Urrea , 1993).

For breeding and selection of new hop hybrid genotypes, it would be highly desirable to have rapid sex identification method at early stage. The sex individuation in field is possible only after flowering, that means 1 or 2 years after sowing. Cytological analysis is another method for sex discrimination in hops, but it is not useful for every variety (Hounold, 1991).

For this reason, the use of MAS could be the solution, providing rapid and reliable identification of agricultural important traits. Molecular markers specific for sex identification were prior obtained for papaya, pistachio and asparagus. Polley et al. (1997) developed a specific PCR molecular marker associated to the Y chromosome of hop (STS marker).

In this study the efficacy of STS primers and other new four primers designed in our laboratory was evaluated.

PCR conditions

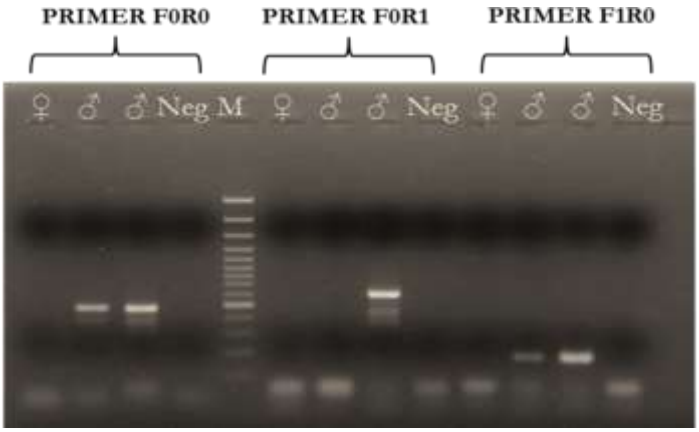
The PCR reaction was carried following Polley methodology (Polley et al. 1997). Annealing temperature were: 54°C for the STS primers, and 60°C for the others primers designed. PCR products were analyzed by electrophoresis on horizontal 2% agarose gel, stained by ethidium bromide and visualized under UV light.

Methodological approach

Preliminary test: primers combination

After the development of the new primers, every combinations (*F1R1 (fragment size 304bp), F0R0 (fragment size 486 bp), F0R1 (fragment size 607 bp), F1R0 (fragment size 182 bp)*, were tested on 3 sample, 1 female and 2 male hops DNA. (Figure 1). DNA extraction was made using CTAB method. We observed that the most discriminating pair of primers was F0R0 (Figure 1).

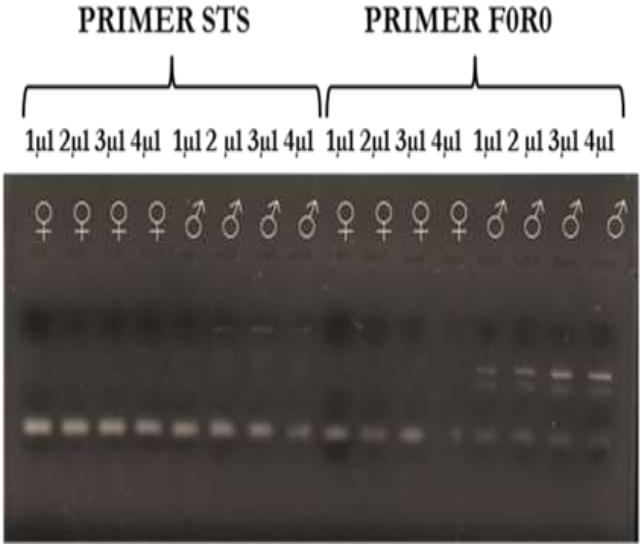
Figure 1 *New primers (F0R0, F1R1, F1R1) amplification capacity test*



Preliminary test: DNA quantity

We compared the efficacy F0R0 primers with STS primers (fragment size 1500 bp)(Polley et al 1997), using different DNA quantities of 4 females and 4 males: 20ng (1µl), 40ng (2µl) and 60 ng of DNA (3µl)) (Figure 2.). The DNA extraction was made using CTAB method and the quality of the DNA was low. The DNA quantity to use to obtain the best result was 40 ng.

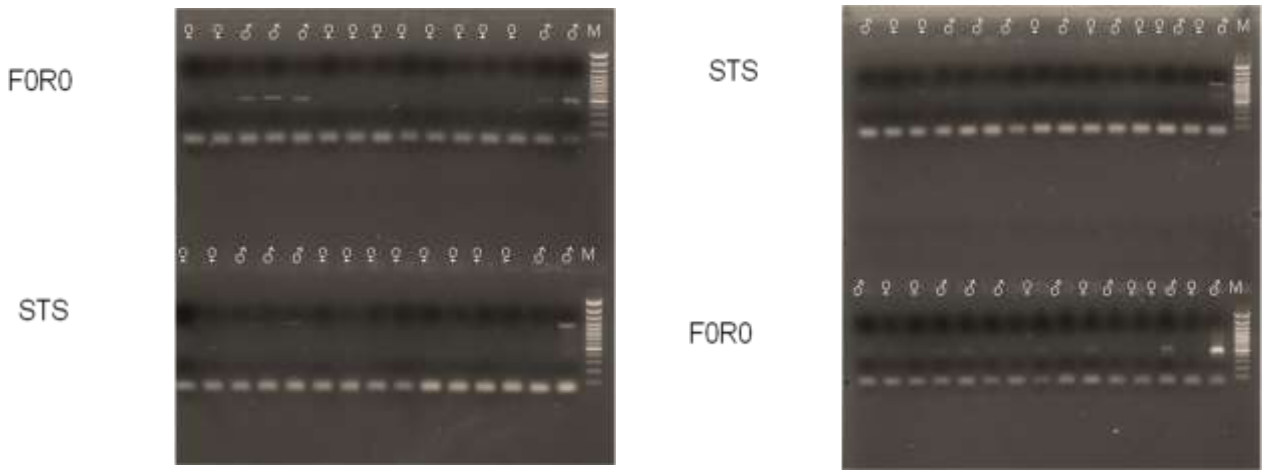
Figure 2 *Evaluation of the best DNA quantity for the two primers*



Test with low quality DNA

This test was made on 80 hops samples. After the extraction of low quality DNA, 20 ng of the nucleic acid were used in PCR for the amplifications of the fragments corresponding to STS and F0R0 primers.

Figure 3. *Example of STS and F0R0 accurancy test made on 80 male and female hop samples*



The results discriminated between the two primers and STS was almost worthless (Figure 3.), instead, F0R0 recognized all male samples. The reason is probably due to the shorter dimension of the fragment amplified by the new primers (F0R0, 486 bp) compared to STS fragment (1500 bp), thus easily amplifiable.

The DNA of 23 male samples was purified using Ammonium Acetate. In the PCR mix with both primers, 20 ng and 10 ng of purified DNA were used. Moreover was placed a female sample as control and a PCR control sample without DNA (-) (Figures 4. and 5.).

Figure 4.*STS (a) and F0R0 (b) amplification results obtained using 20 ng of DNA*

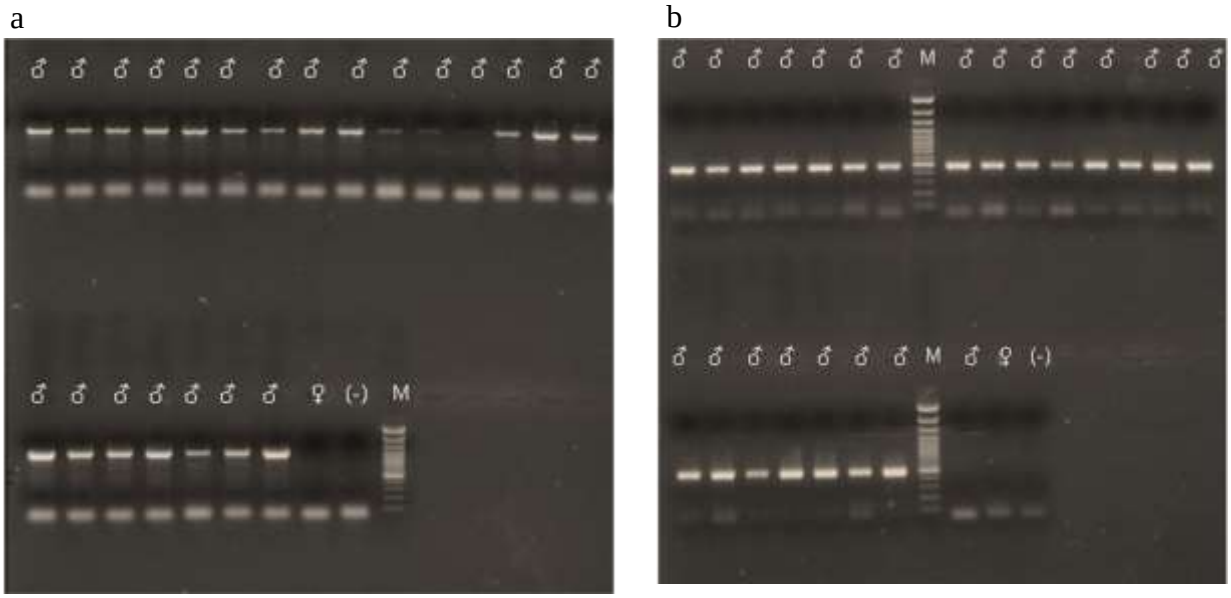
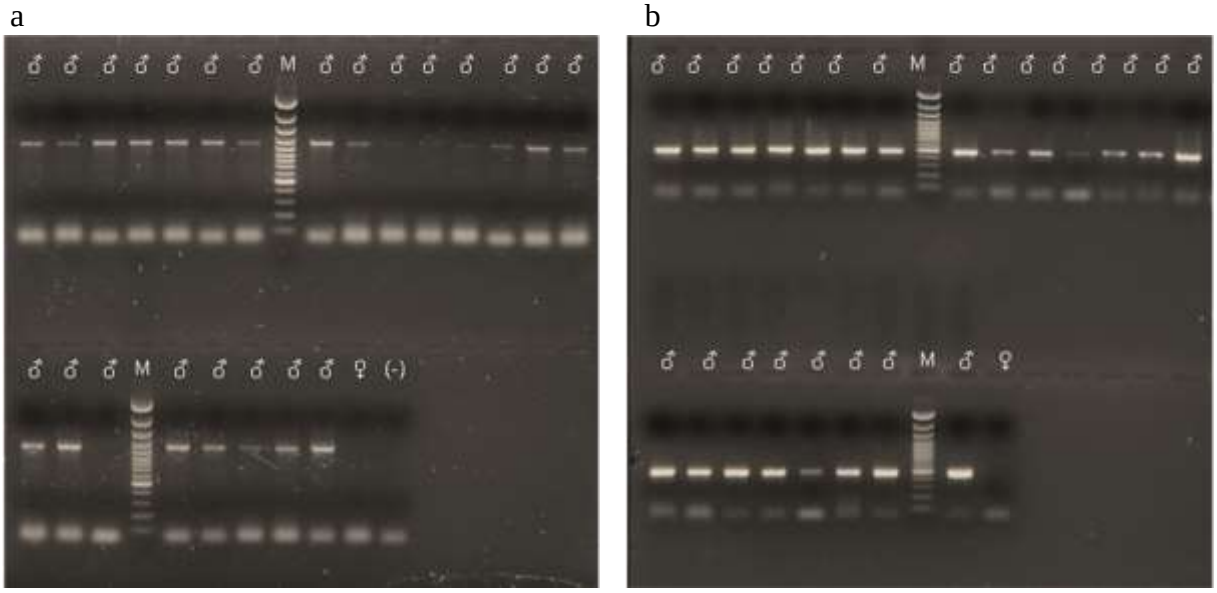
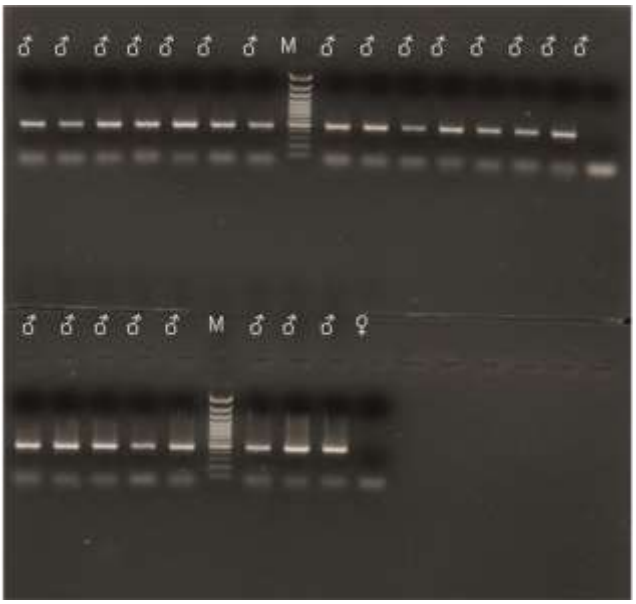


Figure 5 *STS (a) and F0R0 (b) amplification results obtained using 10 ng of DNA*



The use of purified DNA, changed the obtained results. The gel showed a better amplification for both primers, but, F0R0 showed better and clear fragments, especially uaing10 ng of DNA.
Moreover a PCR using 2,5 ng of DNA was made and the result was amazing (Figure 6.).

Figure 5 *F0R0 amplification results obtained using 2,5 ng of DNA*



Conclusions:

- Primer combinations: F0R0 showed the best discrimination power.
- DNA quantities: with low quality DNA the more useful quantity of DNA was 40 ng .
- DNA qualities: using low quality DNA, the only pair of primers usefull to discriminate between male and female were F0R0, instead, STS primers were not useful.
- Using purified DNA the amplification is better for both primers but the highest amplification resulted using F0R0 primers.
- The amplification continued to be of very good quality also with very small amount of DNA (2,5 ng) if the DNA is of high quality.
- F0R0 was more sensitive and the amplification was higher, showing better fragments than STS primers.
- In this study we showed the better efficacy, accuracy and sensitivity of the new marker F0R0 compared with the STS marker identified by Polley et al. (1997).

References:

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Polley A., Ganal M., Seigner E., 1997. Identification of sex in hop (*Humulus lupulus*) using molecular markers. Genome, 40(3): 357-361

3.4. Study 4 - Italian hop germplasm: Characterization of wild Humulus lupulus L. genotypes from Northern Italy by means of phytochemical, morphological traits and multivariate data analysis

Mongelli A., Rodolfi M., Ganino T., Marieschi M., Dall'Asta C., Bruni R. (2015) **Italian hop germplasm: Characterization of wild *Humulus lupulus* L. genotypes from Northern Italy by means of phytochemical, morphological traits and multivariate data analysis.** Industrial Crops and Products, vol. 70, p. 16-27, ISSN: 0926-6690, doi: 10.1016/j.indcrop.2015.02.036

Italian hop germplasm: characterization of wild *Humulus lupulus* L. genotypes from Northern Italy by means of phytochemical, morphological traits and multivariate data analysis.

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Abstract

To evaluate the intraspecific diversity of wild *Humulus lupulus* in Northern Italy, 22 hop accessions were collected from distinct populations. The selected germplasm was genetically probed with 9 SSR primers, grown in a collection field and compared with 5 commercial varieties (Columbus, Fuggle, H. Magnum, Tettnanger, Willamette) cultivated under the same conditions. Hops were evaluated for their aromatic profile, xanthohumol and bitter acids composition by means of GC-MS and HPLC-UV, and further analyzed by principal component and cluster analysis. A great phytochemical diversity was found and wild populations were classified in three distinct clusters in terms of volatile composition and in two separate groups in terms of α–, β–acids content. Multivariate data analyses revealed that selinenes, α-acids, trans-β-farnesene and α-caryophyllene/β-humulene ratio, both contribute the most to the classification of Italian genotypes. Xanthohumol and α-acids content were higher than previously reported for European wild hops. Total α– and β– acid content ranged between 2.98 g/100 g (Busseto 13) and 11.11 g/100 g (Rio Gambero). In the volatile fraction, selinene isomers were above 40% in the essential oils isolated from 6 samples; myrcene ranged between 0.5 (Pasqui 1) and 30% (ET8), while α–humulene/β–caryophyllene ratio was comprised between 0.26 (ET8) and 4.16 (Roggino 2). The phytochemical variability and the presence of traits valuable for brewing suggest the potential exploitation of some ecotypes for breeding and improvement of the cultivated varieties, in particular for plant material aimed to be used in the dry hopping stage of brewing. Some wild genotypes may be valuable as dual purpose or aromatic hops, with properties similar to Fuggle and Tettnanger. The results of the first comparative report of commercial *H. lupulus* cultivars in Italy are also reported.

Keywords: hop germplasm, clonal selection, essential oils, bitter substances, xantohumol.

1. Introduction

Hop (*Humulus lupulus* L., Cannabaceae) is a worldwide distributed crop, a dioecious perennial species whose female inflorescences are used to impart bitterness and fragrance to beer, to preserve its durability and to produce herbal and cosmetic products (Zanoli and Zavatti, 2008). Hop strobiles, and hop leaves to a very lesser extent, display a substantial density of hop glandular trichomes (lupulin), mostly located in the outer lower surface of bracteoles and in the whole surface of the female perianth (Kavalier et al., 2011 and Srecec et al., 2011). These glands store an unique and complex pool of secondary metabolites biosynthesized in the foliar mesophyll, comprising both prenylflavonoids (e.g., xanthohumol), prenylphloroglucinols (known as bitter α- and β-acids, prevailing in bitter hops) and a volatile terpenic fraction (prevailing in aromatic or finishing hops) that confers a characteristic fragrance, extremely valuable in the brewing process (Kishimoto et al., 2006, Wang et al., 2008, Zanoli and Zavatti, 2008 and Srecec et al., 2012).

Thanks to the easiness of its cultivation, a rustic habit and to an high degree of intraspecific genetic variability, hop adapted itself to different climatic and ecological conditions and is therefore recognized as an extremely polymorphic species (Patzak et al., 2010a, Patzak et al., 2010b, Patzak et al., 2010b, Murakami et al., 2006 and Patzak, 2001). Hop breeding, indeed, has been historically performed by hybridization or by development of European ecotypes and clonal selection of wild hops from different regions.

Besides a remarkable genetic heterogeneity, a great deal of variability in the volatile secondary metabolite spectrum of *H. lupulus* has been described among different populations and significant differences were noticed between American, European and Asiatic wild populations, further increased by the variability induced by the human intervention during selection for productive purposes (Eri et al., 2000, Seefelder et al., 2000, Henning et al., 2004, Murakami et al., 2006 and Patzak et al., 2010b). Similarly, different amounts and profile of α-, β-acids have been described in the literature for wild hops from Central Europe, British Islands, far East and Northern America, allowing the classification of wild and commercial hops in 4 categories such as fine aroma (α-acids 3–4.0% w/w, essential oils <1%), aroma (α-acids 3–7% w/w, essential oil 1–2%), bitter (α-acids <8.0% w/w, essential oils 1–2%) and high-alpha (α-acids <15.0% w/w, essential oils 1.5–3%), and subsequent separation the basis of α/β acid ratio, according to the needs of the brewing industry (Krofta, 2003). Despite the vast heritage of biodiversity, the increasing appreciation for particular flavors and tastes and the great diversification requested by the whole beer industry are pushing towards a larger diversification with strong local ties. This translates into a need for genotypes with original phytochemical traits, to be introduced directly in cultivation or to be included in tailored

breeding programmes. As the phytochemical profile is a key factor in defining both quality and value of hop strobiles, and being its composition vastly depending on heritable traits, breeding efforts to discover new *H. lupulus* genotypes rich in α, β-acids and/or specific aromatic compounds will be significant only if all the variables are taken into account (Kralj et al., 1991 and Kac and Kralj, 1998). At the same time, the availability of multivariate analysis tools allows the development of predictive models, correlating local genotypes with established commercial cultivars.

In order to select genetic materials useful to improve the commercial hop germplasm, European wild hops have been screened in the last decade (Patzak et al., 2010a, Patzak et al., 2010b and Patzak et al., 2010b). The presence of bitter secondary metabolites, and of course of essential oils, have been evaluated in wild populations of *H. lupulus*growing in the United States, in Chile and in countries of Central and Northern Europe (del Valle et al., 2003, Bernotienë et al., 2004, Jirovetz et al., 2006 and Patzak et al., 2010a). On this regard, it must be noticed that some geographical areas have not been properly scrutinized and the genetic pool at present screened do not include mediterranean accessions, despite the fact that *H. lupulus* can be easily found in the wild also in subalpine areas (Murakami et al., 2006 and Patzak et al., 2010b). Noticeably, wild*H. lupulus* thrives in the whole Italian peninsula in disturbed habitats like roadsides, grazing lands, pastures or at the border of forests. In some dedicated areas of Northern Italy hop was also cultivated until the beginning of the last century, but despite being widely used by small-scale brewers or to manufacture herbal products, the whole Italian production comes from foreign-grown hops (Prencipe et al., 2014).

Given the strong interest for the development of cultivars and varieties from ecotypes with unique aroma qualities and adequate yield, a first set of Italian wild hops has been screened for their phytochemical, genetic and morphological properties and compared to commercial cultivars. A further goal is to determine if local germplasm may offer a sound basis for the breeding of new varieties suitable for developing hop cultivation in Northern Italy. Therefore, by means of morphological measurements, GC–MS and HPLC-UV analysis, we provide an overview of the intraspecific chemodiversity on a selection of the Italian wild hop germplasm cultivated in a collection field in Northern Italy. Twenty-two different wild genotypes selected were compared with 5 commercial aromatic, bitter and dual-purpose varieties grown under the same conditions and classified by means of multivariate data analysis.

2. Materials and methods

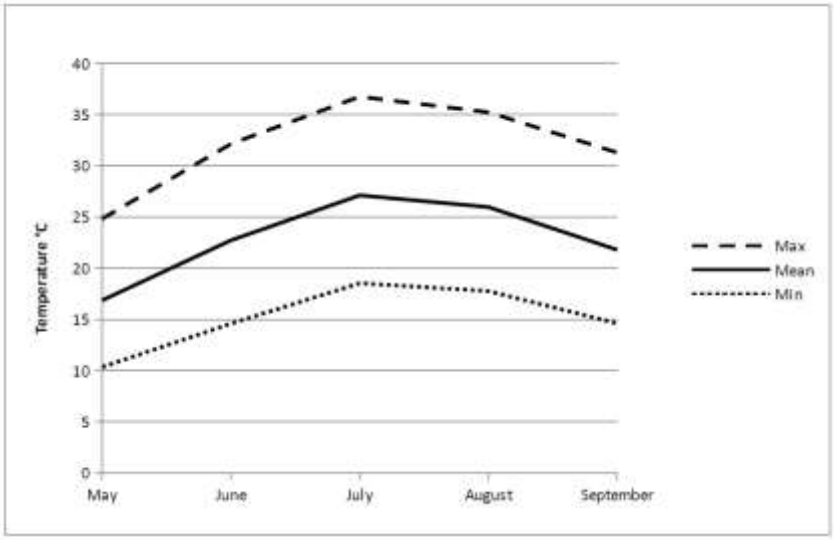
2.1 Chemicals and solvents

Dichloromethane and anhydrous sodium sulfate were purchased from Sigma-Aldrich (Milan, Italy). Toluene was purchased from Carlo Erba (Milan, Italy). Ultra-pure water was in house produced by using a Milli-Q-System (Millipore, Bedford, MA, USA). Standards of caryophyllene, myrcene, humulene, and (+)-linalool were purchased from Sigma-Aldrich (Milan, Italy). Methanol (HPLC grade) was purchased from Sigma-Aldrich (Milan, Italy). Bitter acids mixture standard (international calibration extract, ICE-3) was from Labor Veritas Co. (Zürich, Switzerland). The mixture standard contained α-acids with 13.88% of cohumulone and 30.76% of *n*-humulone + adhumulone, and β-acids with 13.44% of colupulone and 10.84% of *n*-lupulone + adlupulone. Xanthohumol from hop (*Humulus lupulus*) ≥ 96% HPLC grade standard was purchased from Sigma-Aldrich (Milan, Italy).

2.2 Field germplasm collection

The wild hop genotypes were retrieved in different provinces of northern Italy (Parma, Reggio Emilia, Forlì, Mantova and Pavia) at altitudes ranging from 20 to 100 m. a.s.l.. Hop accessions were grown in a field germplasm collection in Marano sul Panaro (MO, 44°27'00" N, 10°58'00" E), Emilia-Romagna, Italy. The collection germplasm field is 1600 m^2, with 4 drills of 80 m. length, spaced with 2.5 m. between drills and 1 m. between plants in the drills. The model of growth is based on the Y growth form and the height of the structure is 4.30 m. The plants are arranged in blocks of 4 plants per genotype. The means, minimum and maximum monthly temperatures of the growing period are reported in Figure 1.

Figure 1. *Means, minimum and maximum monthly temperatures in field collection in the 2013. Temperature data were collected in field by using a HOBO® Pendant Temperature Data Logger (Onset, Cape Cod, MA, USA).*



2.3 Plant material

In this study several Italian hop were considered, namely Bibbiano, Busseto 13, Campus Canna 1, Cinghio, Et3, Et4, Et7, Et7 2, Et8, Et8 1, Fisica, Gianni, Livello, Moglia, Pasqui 1, Pasqui 2, Piattello, Rio Gambero, Roggino 2, Tange, Tavernelle, Torre and five international cultivar (H. Magnum, Willamette, Fuggle, Columbus, Tettnanger) for a total of 27 genotypes. Each genotype was grown and collected in Marano sul Panaro (MO, Italy) under the same environmental conditions. Cone hop samples were harvested in September 2013. In order to emulate farm picking, drying and conservation, samples were dried in a thermostated oven (55°C for 12 hours) with forced ventilation until a relative humidity of 10% was reached. Samples were dried according to methods available in the literature and used to mimic industrial processing (Hoffmann et al., 2010; Nielsen et al., 2013) in order to obtain a residual moisture of approx. 10%, . Precise values for relative humidity were determined for each sample and data obtained were then used to calculate the actual content of bitter substances as g/100g DW for each ecotype or cultivar. Afterward, samples were vacuum-packed and stored at -20°C until analyses were performed.

2.4 Morphological traits

The morphological characteristics of Italian hops were evaluated using UPOV (2006) and Rígr and Faberová (2000) descriptors. The morphological data were compared with cv H. Magnum grown in Italy. Shape, width, length, fresh weight of cones and shape, width and length of bracts were measured. Moreover, other qualitative descriptor were considered (anthocyanin coloration of shoot, leaf shape, cone size, cone shape, cone intensity of green color, degree of opening bracts, production per one plant, bract ratio width/length, length apex of bract and bract length). For each genotype a total of 40 leaves, cones and bracts were collected, belonging to four plants per genotype.

2.5 DNA analysis

Total genomic DNA was extracted from young leaves collected from young, healthy and actively growing shoots. The material, after immersion in liquid nitrogen, was kept in a -80°C freezer. Genomic DNA was extracted following the procedure described by de la Rosa et al. (2002).

The samples were subjected to characterization with nSSR markers. For DNA amplification 9 couples of nSSR primers were used: five simple sequence repeats di-nucleotides, HlGT14, HlGT16, HlGT17, HlGA23 and HlGA31 (Stajner et al., 2005), and four simple sequence repeats tri-nucleotides, HlACA3, HlAGA6, HlAGA7 and HlAGA35 (Stajner et al., 2005) (Table 2).

Table 1. *List of SSR loci, source (*), primer sequence and their respective Size (bp) and annealing temperature (Ta, expressed in °C) used in this study.*

Primer	For 5'→ 3'	Rev 5'→ 3'	Size (bp)	Ta (°C)
HlAGA35*	CY5-ATTATCTCAAACACTCAACCCC	AGCAGGCTATAAAAAGAAGTGC	203	55
HlGT14*	CY5-GGCATGGCTAACTCTATATGC	AAATAGAAGTGCCATAACTGA	165	54
HlGT16*	CY5-CCGTGATACAAATCTACCCAAA	CTCCAGTCAGCAATCTCTTCAA	228	54
HlGT17*	CY5-GGTCCTTAGTCACTTGCCAAT	GACTGTTCGAAGCACAATCAA	182	54
HlGA23*	CY5-AAGCACGAAAACTGACTTG	GTTGCCCAAAATCACTGTT	245	54
HlGA31*	CY5-CAAACTTGGTGCTCTAAGATGAA	CGTTTTCCCAACACCTAGTTC	163	55
HlACA3*	CY5-CAAGTTGTTGGTTGATTTCACAT	CTCCTTCCTGTGTTCACCAC	215	52
HlAGA6*	CY5-GTTAGAATCTCGTTGGCAA	TCTGAAACTTCACTAATCATC	192	55
HlAGA7*	CY5-ACAAGCAGTAATGATGAGGA	TCCAAGTCTCTCAATTAGGAA	180	54

*Stajner et al., 2005

The decision to select markers from these four series was imposed by the necessity to exploit the positive features, and to compensate for the negative aspects of both. Di-nucleotides are more common in the genome and more polymorphic, but require greater care in data analysis (e.g., are often subject to polymerase slippage during amplification, which makes microsatellite profile analysis more complex); Tri-nucleotides on the contrary are less frequent in the genome but more easily interpreted, and therefore more reliable and superimposable to data produced in other laboratories.

PCR amplification was performed in a final volume of 20 μl containing: 1x Reaction Buffer (International PBI, Milan, IT), 1.5 mM $MgCl_2$ (International PBI, Milan, IT), 0.2 mM dNTPs (Amersham Biosciences, Piscataway, USA), 0.2 μM primer (MWG Biotech, Ebersberg), 20 ng genomic DNA e 0.6 U of *Taq* polymerase (International PBI, Milan, IT). PCR amplification was optimized in thermal cycler MJ PCT 100 Research (Watertown, Mass.), programming a first passage at 95°C for 5 min. followed by 30 cycles of 45 sec at 94°C, 30 sec at the specific annealing temperature for each couple of primers, and 1 min. at 72°C, for denaturation, annealing and primer extension, respectively; at the end of the cycles were allowed 8 min. of incubation at 72°C. One of the two PCR primers in each reaction was end-labelled with a fluorescent dye (CY5, MWG Biotech, Ebersberg, Germany). The amplification products were separated with a CEQ 2000

Genetic Analysis System (Beckman Coulter, Inc.) sequencer on acrylamide gel CEQ Separation Gel LPA-1 (Beckman Coulter, Inc.). A marker CEQ DNA Size Standard kit 400 (Beckman Coulter, Inc.) was used to estimate the approximate molecular weight of the amplified products. Two reference samples were used in all runs.

2.6 GC/MS analysis

Prior the analysis the samples were extracted with steam distillation for four hours to obtain essential oils for each accessions. The essential oil was diluted in CH_2Cl_2 (1:200 v/v) in a vial and added of a small amount of anhydrous sodium sulfate to eliminate possible water's trace. After addition of toluene (25 µl of a solution 4000 ppm in CH_2Cl_2) as internal standard, the diluted sample (1 µl) was analysed by GC/MS. All samples were analysed with a Thermo Scientific (San Jose, CA, USA) TRACE 1300 gas-chromatograph coupled to a Thermo Scientific ISQ™ Single Quadrupole mass spectrometer. The gas-chromatograph was equipped with Supelcowax 10 (30 m x 0.25 mm, f.t. 0.25 µm) (Supelco, Bellefonte, USA) capillary columns and helium was used as carrier gas (constant flow of 1 ml min^{-1}). GC/MS oven temperature gradient started from 50°C, this condition was maintained for 3 minutes, then the temperature was raised to 200°C (5°C/minute). The final temperature was maintained for 18 minutes. The injector was maintained at 230°C operating in split modality, ratio 1:20. The mass spectrometer was equipped with an electron impact source (EI, 70 eV) and the acquisition mode was full scan (from 40 m/z to 500 m/z). A solvent delay time of 4 minutes was applied. The main volatile compounds were identified on the basis of their mass spectra compared with the reference mass spectra libraries (WILEY275, NBS75K, Adams 2001) and of their calculated Retention Indexes through the application of the Kovats' formula (KI) compared with those reported in the literature. When it was not possible to find the KI in the literature, a tentative identification was obtained by matching with mass spectra libraries data: a match quality of 98% minimum was used as a criterion. In order to determinate the RI of the components, a mixture of alcanes (C8-C20) was injected in the GC-MS equipment and analysed under the same conditions described above. The gas-chromatographic signals were manually integrated and the resulting peak areas were compared with the total sum of area and expressed in percentage.

2.7 HPLC-UV analysis

Dried cones (0.5 g) were extracted with 20 ml of methanol in a flask and homogenized with Ultra Turrax mixer (IKA®, T18 Basic) for two 20 sec periods, to avoid sample overheating (Farag et al,

2012). Extracts were stirred for 3 hours at room temperature in the dark to prevent degradation and photo-oxidation. Subsequently the extracts were centrifuged at 4000 rpm at 20°C for 20 min. Supernatant was then transferred in a volumetric flask (50 ml). Exhausted matrix re-extracted with methanol (15 ml) centrifuged as above and then, the supernatant was pooled with the first extract; the volumetric flask was then filled to the final volume with methanol. After filtration through 0.45 µm PTFE filter, the sample (10 µl) was analysed by HPLC-UV. All samples were analysed by a 2695 Alliance separation system (Waters Co., Milford, MA, USA) equipped with a 2487 Dual λ Absorbance Detector (Waters Co., Milford, MA, USA). HPLC was equipped with Jupiter C18 column (3u, 300A, 150 x 2.00 mm) (Phenomenex® Castel Maggiore, Bologna, Italy). Solvent A (H_2O + 0.2 % CH_3COOH) and solvent B (CH_3CN + 0.1 % CH_3COOH) were used for mobile phase. Chromatographic conditions were set as follows: the flow rate was 0.2 ml/min; the column temperature was set at 30°C; the injection volume was 10 µl; gradient elution was performed using eluent A and eluent B: initial condition at 50% A, 0–5 min. isocratic step, 5–12 min. linear gradient to 70% B, 12–19 min. isocratic step at 70%B, 19–20 min. linear gradient to 85% B, 20-24 min. isocratic step at 85% B, 24-25 min. linear gradient to 90% B, 25-35 min. isocratic step at 90% B, 35-36 min. linear gradient to 50% B and requilibration step at 50% B for 14 min. (total analysis time: 50 min.). Chromatograms were acquired at 326 nm. Three injections were performed for each sample. For the quantification of α- and β- acids, a calibration curve was obtained from dilution of ICE-3 standard, according to the official method (Analytica-EBC, section 7, method 7.7).

2.8 HPLC method validation

Linearity was assessed in two diferent ranges for α– and β–acids, due to the different range of concentrations found in samples. Since ICE-3 contains a mixture of α- and β-acids, the concentrations of the standard solutions of each compound were calculated according to the percentages stated by the manufacturer. The amount of bitter acids in hop samples was determined by using these calibration curves and the same protocol was adopted for xanthohumol. For reference compounds, the limit of detection (LOD) and the limit of quantification (LOQ) were experimentally determined by HPLC analysis of serial dilutions of a standard solution to reach a signal-to-noise (S/N) ratio of 3 and 10, respectively. The accuracy of the analytical procedure was evaluated by adding a known quantity of standard compound to half the sample weight of grounded sample (Magnum) to reach 100% of the test concentration. The precision of the extraction technique was validated by repeating six times the extraction procedure on the same hop sample. The precision of the chromatographic system was tested by performing intra- and inter-day multiple

injections of the hop extract and then checking the %RSD of retention times and peak areas. Six injections were performed each day for three consecutive days.

2.9 Statistical analysis

The fragments were sized by using a conservative binning approach (Kirby, 1990) through the statistical R software (R Development Core Team, Vienna, Austria), which takes into account the type of replicate and compensates for the limits of fragment resolution. The genotypes showing a single allele in a given locus were indicated as homozygote. The level of similarity/dissimilarity among examined accessions was obtained through the genetic similarity matrix utilizing Euclidean distance. Cluster analysis and construction of the dendrogram relative to genetic distances were obtained by using the unweighted pair-group method with arithmetic mean (UPGMA) algorithm, with XLSTAT 2009 software (AddinsoftTM 1995-2009). The quantitative morphological characters were evaluated for their quantitative variables: mean and standard deviation (SD). The collected data were also analyzed by using univariate analysis of variance (ANOVA). One-way analysis of variance was carried out by applying Tukey's post hoc test ($p \leq 0.05$) using SPSS Statistics 21.0 software (SPSS Inc. Chicago, Illinois, 2003). Principal component analysis (PCA) has been used as an alternative way to represent inter-individual relationships using separately morphological and chemicals data. The PCA analysis was performed using XLSTAT 2009 software (Addinsoft^{TM} 1995-2009).

3. Results and Discussion

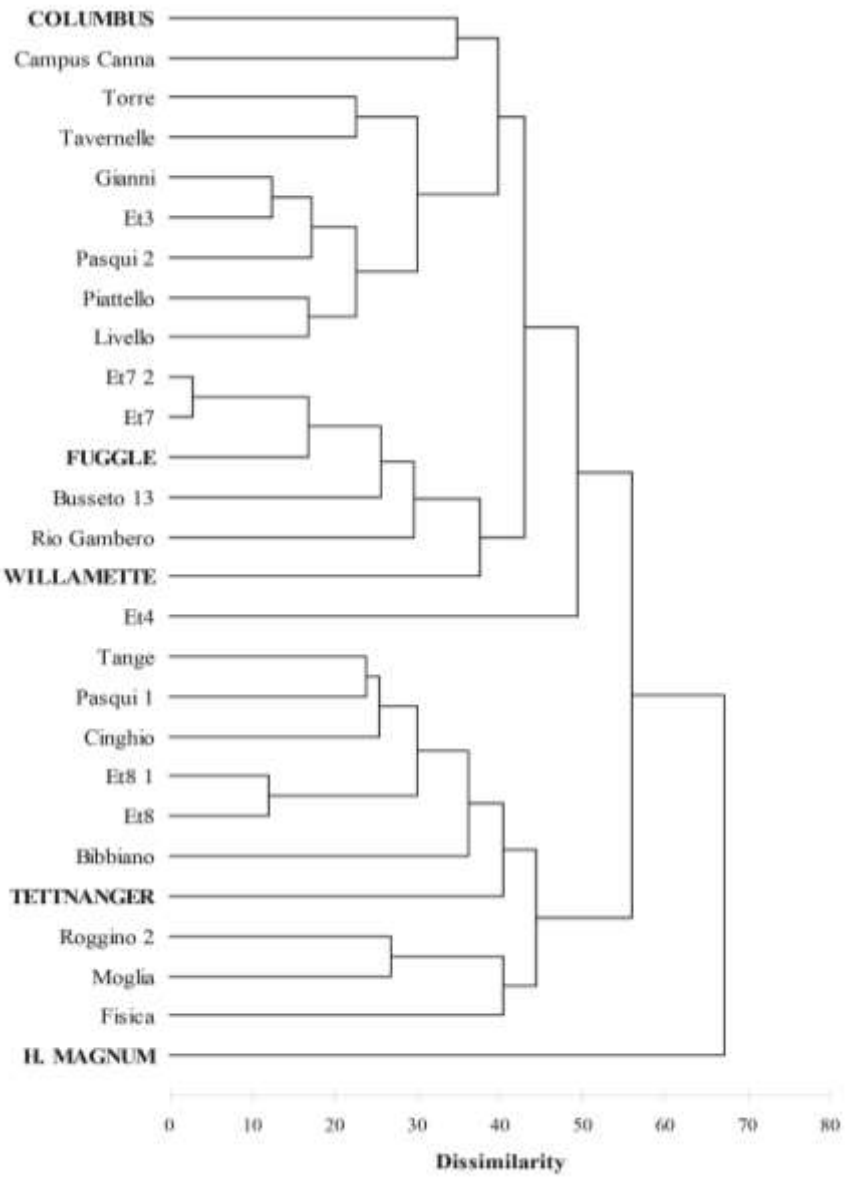
3.1 DNA analyses

General cluster analysis of 22 wild hop genotypes and 5 commercial cultivars were performed on data obtained from 9 SSR primers, revealing that the pool was composed by genetically different and heterogeneous material (Figure 2).

Some wild genotypes had a rather close genetic relationship with Fuggle (cultivar selected by European wild hops), Tettnanger (genotype daughter of cv. Saaz in turn selected by European wild hops) and Willamette (triploid genotype, daughter of Fuggle) cultivars, and this was somehow expected being those varieties derived from European ecotypes or hybrids with a strong European heritage (Patzak et al., 2006). On the other hand, the new cultivars developed to fulfil recent market requirements were produced by crosses of those old European hops with wild American hops (Peredo et al., 2010), as in the case of Columbus variety. On the contrary, their distance from H.

Magnum was relevant, and therefore these relationships were further evaluated through phytochemical evaluations.

Figure 2. *Dendrogram of the hierarchical cluster analysis among italian genotypes and commercial cultivars of Humulus lupulus L., as determined by genetic similarity using euclidean distance*



3.2 Morphological traits

The descriptive statistical analysis values for each quantitative trait is reported in Table 2. Differences were observed in the all traits of Italian hops. In the ANOVA analysis, the genotypes differed for all traits at a probability level $p \leq 0.05$.

The cone width ranged between 2.5 cm (Et8) and 1.52 cm (Roggino 2), while the cone length ranged from 3.73 cm to 2.04 cm; the highest values were observed in cv H. Magnum and the lowest values in Moglia genotype. The calculated ratio Cone width/Cone length (namely Cone shape) showed no marked differences between the accessions; the variation among the accessions ranged from 1.02 (Et7) to 0.49 (Pasqui 1). This ratio represents an index of cone shapes determined by means of a score scale described by UPOV (2006) and Rígr and Faberová (2000). The largest part of Italian hop cones was "broad ovate", only the cones of Campus canna and H. Magnum genotypes were "cylindrical", the other cones of Italian hops were "medium ovate", "narrow ovate" or "globose". The fresh cone weight ranged between 0.53 g (cv H. Magnum) and 0.12 g (Cinghio). The bract width ranged between 1.15 cm (Tavernelle) and 0.69 cm (Roggino 2), while the bract length ranged from 1.829 cm to 1.38 cm; the highest values were observed in Busseto 13 genotype and the lowest values in Tange genotype. The ratio Bract width/Bract length (Bract shape) showed few differences between the accessions; the variation among the accessions ranged from 0.72 (Tavernelle) to 0.44 (Pasqui 1). For the greatest part of Italian hop genotypes the score of bract ratio width/length was "medium" as only four genotypes (Busseto 13, Et4, Tange and Tavernelle) showed a "large" score and three genotypes (Gianni, Pasqui 1 and Piattello) provided a small bract ratio. The qualitative leaf shape of the hop accessions characterized in this study corresponded to those reported by Rígr and Faberová (2000) in characterizing a Czech hop collection. The leaf shapes of Italian wild hops evaluated were of two type: five-lobed (representing the largerst part of the evaluated germplasm) and three-lobed (Campus canna, Et7 2, Moglia and Torre). Under the climatic Italian conditions the productivity per plant ranged from "very low" (most Italian genotypes and H. Magnum) to "low" (Cinghio and Moglia) and "intermediate", a score obtained only for Rio Gambero genotype. In order to evaluate the morphological difference between Italian wild hops and identify the most significant variables in the data set, a PCA analysis was performed (Figure 3). Only 6 variables provided a good discrimination between the samples (Main shoot: anthocyanin coloration, Leaf: shape, Cone: intensity of green color, Cone: shape, Bract length, and Bract: length apex). The cumulative variance explained by the first two principal components (PCs)

in the model is 55.86%. (Figure 3), and the cumulative variance in the model is 77% if the first three PCs are taken into account. Depending on the variance explained by the first 3 PCs, it is possible to observe that the six variables are highly-discriminating traits, indeed the traits “Cone: intensity of green color”, “Bract length”, “Cone: shape”, and “Bract: length apex” offer an explained variance ranging from 43% to 69%. On the contrary, “Main shoot: anthocyanin coloration” and “Leaf: shape” are two poorly-discriminating traits, since they have an explained variance of less than 37%.

Table 2. *Mean, DS and ANOVA analysis of morphological traits of 23 genotyper (22 Italian hops and H. Magnum cltivar). Different letters in column indicate statistically significant differences mean at 0,95 confidence by Tukey’s test.*

Genotypes	Cone shape	Cone width (cm)	Cone length (cm)	Fresh cone weight (g)	Bract shape	Bract width (cm)	Bract length (cm)
Bibbiano	0.786 ± 0.145a–e	2.058 ± 0.375a–f	2.667 ± 0.536b–f	0.171 ± 0.036de	0.549 ± 0.041ab	0.810 ± 0.128a–c	1.470 ± 0.176ab
Busseto 13	0.728 ± 0.076c–f	2.080 ± 0.396a–f	2.900 ± 0.741a–f	0.275 ± 0.157a–c	0.601 ± 0.059ab	1.086 ± 0.157ab	1.829 ± 0.349a
Campus Canna	0.656 ± 0.102c–f	1.700 ± 0.189d–f	2.650 ± 0.561b–f	0.160 ± 0.077de	0.464 ± 0.249b	0.713 ± 0.179c	1.538 ± 0.184ab
Cinghio	0.735 ± 0.069b–f	2.200 ± 0.254a–e	3.000 ± 0.212a–f	0.120 ± 0.022e	0.543 ± 0.137ab	0.780 ± 0.102a–c	1.440 ± 0.114ab
Et3	0.871 ± 0.125c–f	2.500 ± 0.545a	2.875 ± 0.465a–f	0.314 ± 0.067b–d	0.592 ± 0.209ab	1.010 ± 0.137a–c	1.820 ± 0.373a
Et4	0.613 ± 0.162d–f	1.600 ± 0.126d–f	2.733 ± 0.578a–f	0.161 ± 0.050de	0.593 ± 0.239ab	0.956 ± 0.441a–c	1.622 ± 0.281ab
Et7	1.021 ± 0.171a	2.500 ± 0.408a	2.500 ± 0.577c–f	0.195 ± 0.012c–e	0.621 ± 0.056ab	0.975 ± 0.005a–c	1.575 ± 0.170ab
Et7 2	0.999 ± 0.027ab	2.500 ± 0.200a	2.500 ± 0.158c–f	0.157 ± 0.069de	0.580 ± 0.161ab	0.900 ± 0.158a–c	1.600 ± 0.292ab
Et8	0.725 ± 0.102c–f	2.625 ± 0.478a	3.625 ± 0.478ab	0.308 ± 0.135ab	0.600 ± 0.189ab	0.800 ± 0.141a–c	1.425 ± 0.434ab
Et8 1	0.769 ± 0.098a–e	2.080 ± 0.293a–f	2.720 ± 0.335a–f	0.209 ± 0.033c–e	0.540 ± 0.110ab	0.833 ± 0.173a–c	1.544 ± 0.052ab
Fisica	0.694 ± 0.048c–f	1.833 ± 0.288b–f	2.667 ± 0.577b–f	0.142 ± 0.070de	0.510 ± 0.069ab	0.822 ± 0.109a–c	1.622 ± 0.172ab
Gianni	0.590 ± 0.065ef	1.450 ± 0.333f	2.450 ± 0.454c–f	0.102 ± 0.050c–e	0.443 ± 0.076b	0.680 ± 0.122c	1.550 ± 0.246ab
Livello	0.672 ± 0.062c–f	1.817 ± 0.194b–f	2.733 ± 0.436a–f	0.285 ± 0.071b–e	0.548 ± 0.043ab	0.913 ± 0.064a–c	1.675 ± 0.198ab
Moglia	0.873 ± 0.055a–d	1.780 ± 0.148c–f	2.040 ± 0.151f	0.154 ± 0.050de	0.543 ± 0.040ab	0.800 ± 0.004a–c	1.401 ± 0.137ab
Pasqui 1	0.487 ± 0.053f	1.557 ± 0.325ef	3.186 ± 0.508a–e	0.259 ± 0.133b–e	0.438 ± 0.072b	0.733 ± 0.173bc	1.667 ± 0.223ab
Pasqui 2	0.619 ± 0.077d–f	2.070 ± 0.140a–f	3.380 ± 0.391a–d	0.239 ± 0.065b–e	0.509 ± 0.057ab	0.827 ± 0.119a–c	1.636 ± 0.237ab
Piattello	0.728 ± 0.145c–f	2.434 ± 0.476a–c	3.389 ± 0.537a–c	0.317 ± 0.081b–d	0.538 ± 0.043ab	0.956 ± 0.088a–c	1.789 ± 0.236ab
Rio Gambero	0.783 ± 0.147a–e	2.255 ± 0.250a–d	2.973 ± 0.638a–f	0.278 ± 0.104b–e	0.475 ± 0.055b	0.760 ± 0.084bc	1.610 ± 0.166ab
Roggino 2	0.650 ± 0.058c–f	1.520 ± 0.100f	2.360 ± 0.336d–f	0.135 ± 0.061de	0.460 ± 0.067b	0.690 ± 0.000c	1.510 ± 0.191ab
Tange	0.902 ± 0.198a–c	2.250 ± 0.377a–d	2.563 ± 0.495c–f	0.168 ± 0.034de	0.672 ± 0.106ab	0.917 ± 0.116a–c	1.383 ± 0.213b
Tavernelle	0.998 ± 0.240ab	2.380 ± 0.329a–c	2.510 ± 0.678c–f	0.133 ± 0.055de	0.722 ± 0.227a	1.150 ± 0.414a	1.580 ± 0.130ab
Torre	0.792 ± 0.081a–e	1.709 ± 0.230d–f	2.173 ± 0.337ef	0.184 ± 0.044de	0.459 ± 0.065b	0.700 ± 0.124c	1.520 ± 0.113ab
H. Magnum	0.665 ± 0.016c–f	2.471 ± 0.138ab	3.729 ± 0.292a	0.533 ± 0.078a	0.472 ± 0.076b	0.771 ± 0.160bc	1.629 ± 0.170ab

3.3 Phytochemical analysis

3.3.1 Chemical Composition of the Essential Oils.

The hydrodistilled oils of 22 Italian wild hop genotypes and 5 commercial varieties were analysed by GC/MS, taking into account only compounds of major relevance for breeding evaluation, due to geographical origin of the selected clones. Namely, myrcene, farnesene and selinene isomers, α–humulene and β–caryophyllene (Table 3, Table 4). Essential oil yields for Italian genotypes ranged between 0.20 and 0.6%, while values for commercial germplasm ranged between 0.17 and 2.30%. These results are in agreement with those regarding most common European aroma hops, which despite geographical and year-to-year fluctuations are usually characterized by a rather low content of essential oils, rarely exceeding 1.0% (Krofta, 2003). It must be noticed that, at present, a comprehensive and unequivocal evaluation of intraspecific diversity for European wild hop is lacking and data are scattered in different papers, in which analyses were performed on plant

material collected alternatively from wild and field grown plants, with obvious differences due to the changeable behaviour of hop under different geoclimatic conditions. Therefore, a rational and straightforward phenotypic comparison is often difficult to obtain.

Table 3. *Qualitative essential oil composition of Italian hop genotypes determined by GC–MS. Different letters in each column indicate statistically significant differences mean at 0.95 confidence by Tukey's test*

	Yield	Myrcene	β-Caryophyllene	*trans*-β-Farnesene	α-Humulene	(z,Z)-α-Farnesene	β-Selinene	α-Selinene	α-Humulene/ β-Caryophyllene ratio
					%				
Bibbiano	0.35	6.48	10.72	5.05	3.79	0.48	21.96	14.57	0.35
Busseto 1	0.35	3.88	5.81	2.99	3.77	1.15	21.14	18.24	0.65
Campus Canna	0.45	1.41	5.27	0.53	1.83	3.07	18.24	14.92	0.35
Cinghio	0.25	8.69	15.66	n.d.	25.01	0.88	9.25	7.62	1.6
ET7 2	0.45	12.73	13.18	1.28	30.32	1.25	8.43	9.72	2.3
ET3	0.35	7.79	13.21	1.11	3.83	0.94	22.59	24.3	0.29
ET4	0.45	0.59	5.06	0.21	2.77	0.44	25.18	23.7	0.55
ET7	0.4	10.2	11.88	0.18	19.96	1.56	8.59	10.16	1.68
ET8	0.25	29.68	5.34	8.56	1.39	1.15	12.16	10.13	0.26
ET8 1	0.45	11.73	7.45	10.22	1.05	1.13	28.93	10.63	0.14
Fisica	0.2	n.d.	3.44	n.d.	2.27	1.07	20.22	23.19	0.66
Gianni	0.4	8.26	5.17	n.d.	16.89	0.44	16.41	18.46	3.27
Livello	0.45	4.77	5.71	1.35	3.67	1.74	20.34	21.84	0.64
Moglia	0.25	7.22	3.9	1.96	2.82	3.21	14.08	15.44	0.72
Pasqui 1	0.45	0.59	0.1	n.d.	2.68	1.66	30.27	4.81	0.27
Pasqui 2	0.55	17.75	5.3	1.01	2.22	1.5	19.93	20.97	0.42
Piattello	0.3	7.58	4.63	10.29	1.74	4.51	6.86	8.09	0.36
Riogambero	0.6	20.78	11.73	7.01	2.82	1.54	17.39	19.99	0.24
Reggino	0.35	10.41	11.29	n.d.	46.91	n.d.	0.61	0.88	4.15
Tange	0.45	11.63	12.56	1.6	26.18	1.66	7.09	7.88	2.08
Tavernelle	0.25	13.95	7.41	5.76	26.83	1.18	8.68	11.06	3.62
Torre	0.5	19.28	5.99	12.33	2.65	0.39	13.88	15.32	0.44
Average	0.39	10.26	7.76	4.20	10.52	1.47	16.01	14.18	1.41

Table 4. *Qualitative essential oil composition of commercial hop cultivars cultivated in northern Italy, determined by GC-MS and compared with average commercial data.*

	Yield	Myrcene	Range in commerce	b-Caryophyllene	Range in commerce	trans-b-Farnesene	Range in commerce	a-Humulene	Range in commerce	(z,Z)-a-Farnesene	b-Selinene	a-Selinene	a-Humulene/b-Caryophyllene
Columbus	2,3	34,71	25-45	17,3	8--12	0,2	<1	19,11	15--25	0,23	2,5	1,51	1,1
Fuggle	0,6	0,91	40-50	23,14	6--12	n.d.	4--5	50,93	20--26	0,63	0,74	1,36	2,2
H.Magnum	1,45	21,45	30-35	14,27	8--12	n.d.	<1	50,64	34--40	0,33	0,86	1,31	3,5
Tettnager	0,17	0,33	36-45	10,04	6--7	0,83	5--8	26,58	18--23	1,24	1,95	2,64	2,64
Willamette	0,3	0,83	45-44	18,33	7--8	0,3	5--6	36,27	20--30	2,36	1,31	2,27	1,97

Myrcene content was extremely variable and ranging from less than 1 to 20-30% in the essential oils isolated from genotypes Rio Gambero, ET8 and Torre, whose values were comparable to the ones reported for Columbus and H. Magnum cultivars. Since the success of dry hopping technique in beer production, myrcene has gained an increasing relevance for its resiny, green to spicy notes and its presence is prized by brewers and consumers (Steinhaus and Schieberle, 2000; Marcelina and William, 2011; Whittock et al., 2012).This compound was previously reported to be in the 12-22% range in essential oils from wild samples, with the exception of hops collected in Lithuania (7.4%), while selected commercial hop varieties usually average more than 30% of myrcene in their essential oils. In particular, this is true for European traditional hops, which have a rather low myrcene content, while con the contrary American varieties may provide essential oils with more than 60% of this compound and are therefore particularly used in dry hopped beers. In our

screening only 10 essential oils exceeded the 10% limit with an average of 10.4%, lower than literature indication for essential oils from European wild samples. (Bernotienë et al., 2004; Patzak et al., 2010b). Regarding other constituents prominent from a brewing standpoint, 7 genotypes had an α–humulene content comprised between 17 and 46% and β–caryophyllene abundance exceeded 10% in the essential oils from 8 accessions. On average, these two sesquiterpenes, valuable for their warm woody-earthy note, were recorded at 11 and 8% respectively, with the highest amounts noticed in essential oils from ET7-2 for β–caryophyllene (13.2%) and Roggino 2 for α–humulene (46.9%). These values are very close to those obtained for essential oils isolated from Fuggle and H. Magnum cultivars grown under the same conditions (Table 4). Instead, Torre, ET7-2 and Tavernelle provided amounts comprised between 26 and 31%, comparable to Tettnanger and Willamette cultivar. It must be noticed that for α-humulene a dual range of concentrations was noticed, with essential oils from six genotypes above 25% and 15 below 4%. The pattern of its content is superimposable to the one reported from wild plants collected in Europe and grown under field conditions, with an average content of 11% and may represent an interesting starting point for the breeding of "noble hop aroma" varieties" (Patzak et al., 2010a; Patzak et al., 2010b). The α–humulene/β–caryophyllene ratio, whose greater value is considered as a proxy of quality for aromatic hops, ranged between 0.14 (ET8) and 4.15 (Roggino 2), with six accessions evidencing a ratio above 2. For aromatic purposes, most appreciated cultivars are characterized by an humulene/caryophyllene ratio exceeding 2.5 (Malizia et al., 1999; Krofta, 2003; Nance and Setzer, 2011).

Farnesene isomers, and *trans*-β-farnesene in particular, are prized in the so-called noble aroma hops, in which their abundance usually exceeds 15%, but are not biosynthesized by all aroma hop varieties (Krofta, 2003; Inui et al., 2013). The presence of *trans*-β-farnesene and z,z-α-farnesene was moderate in the essential oils obtained from Italian germplasm, with average values of 4.3 and 1.5 % respectively, in agreement with previous data from wild European hops and always higher than the amount scored by commercial cultivars grown in the same plot [Bernotiene et al., 2004; Patzak et al., 2010a; Cerenak et al.,2011]. On this regard, genotypes ET8, Piattello, ET8-1 and Torre were the best performers, with values for their essential oils in the 10-12 % range, making them a potential valuable starting point for the development of aromatic, finishing hops. α– and β–selinene were extremely abundant in the majority of the wild samples, as in the essential oils isolated from 6 genotypes their sum exceeded 40% and only in one accession (Roggino 2) it was below 2%, a value similar to most commercial cultivars grown under the same conditions. These

data are in agreement with previous observations, in which an high selinene content was deemed to be characteristic of European wild genotypes if compared to North American ones [Patzak et al., 2010]. However, it must be highlighted that Italian essential oils contained on average almost three times more selinene isomers than the average of European samples (30% versus 11%), while the content of commercial cultivars grown under the same conditions never exceeded 5% (Patzak et al., 2010a).

European wild hops are usually described by a low myrcene content, by the presence of farnesene, and by an high selinene content, allowing their diversification from non-European material but also somehow limiting their use in brewing. The results obtained from our screening suggest that, despite a total selinene content greater than usual, hop germplasm in northern Italy harbors some traits typical of European noble aroma hops. Some exceptions may indicate that few genotypes (e.g. Roggino 2) could retain characteristic similar to commercial cultivars or retain properties valuable for breeding (ET8, Torre). Torre, with a 19.8% myrcene, a 6% β-caryophyllene and a 12.3% *trans*-β-farnesene fits well within the parameters set for a promising candidate for noble aroma hop. At the same time, a higher content in α-selinene has been described as a trait potentially related to hop pathogens like *Podosphaera macularis* and, if confirmed and besides its limited value for brewing, may be valuable for resistance breeding (Cerenak et al., 2009). The large variability found in ecotypes in terms of volatile compounds seems to suggest the potential presence of different adaptations to the various climate and soil niches characterizing the Italian peninsula. The literature suggests also that the composition of the essential oils for a given ecotype offers limited variability from year to year, but may be characteristically different for different accessions. If confirmed by future investigations, the availability of a diversified wild germplasm pool in Italy may therefore represent an useful starting point for breeding aroma hops.

3.3.2 Quantification of bitter acids and prenylated chalcones.

Quali- and quantitative analysis of xanthohumol, α– and β– acids in female hop strobiles were performed by HPLC-UV and a significant variability in the content of these secondary metabolites was observed (Table 5, Table 6). Xanthohumol content was rather uniform in the evaluated pool, and some Italian genotypes (Campus Canna, Fisica, Rio Gambero and ET8 1) provided a maximum content equiparable to Fuggle, Willamette and H. Magnum commercial cultivars grown under the same conditions. Minimum values of xanthohumol was present in Busseto 13 and Pasqui 2 accessions (Table 5). An average content of 0.407 g/100g DW was obtained for Italian germplasm,

two time higher than the average elsewhere reported for European wild hops and within the range reported in the literature for northamerican wild hops (Patzak et al., 2010a; Patzak et al., 2010b, Hampton et al., 2002). Also the average α–acids content was higher in Italian germplasm if compared to previous determinations of European wild accessions, as the average obtained was 3.82 g/100g against a reported 2.38 g/100g DW (Patzak et al., 2010b).

Table 5. *Quantitative bitter acids composition of Italian hop germplasm determined by HPLC-UV. In column different letters indicate statistically significant differences mean at 0.95 confidence by Tukey's test*.

	Xanthohumol	Cohumulone	Humulone+adhumulone	Colupulone	Lupulone+adlupulone	Total α acids	COH % on total alpha acids	Total beta acids	COL% on total beta acids	α/β ratio
					g/100g DW					
Bibbiano	0.345±0.168a–c	1.060±0.082e–h	2.149±0.286g–i	1.300±0.318cd	1.767±0.401de	3.208±0.368g–i	33.101±0.873cd	3.067±0.719cd	42.331±0.388f	1.050
Busseto 13	0.206±0.003c	0.462±0.020k	1.273±0.022j	0.563±0.013j	0.677±0.013k	1.735±0.042j	26.640±0.344k	1.240±0.025j	45.432±0.065b	1.400
Campus Canna	0.583±0.021ab	1.450±0.044bc	3.634±0.034c	1.072±0.062de	1.405±0.054f–h	5.083±0.078c	28.514±0.298ij	2.477±0.115ef	43.275±0.334de	2.050
Cinghio	0.378±0.014a–c	1.117±0.028ef	2.082±0.047hi	0.583±0.022j	0.724±0.032jk	3.199±0.075g–i	34.907±0.036b	1.308±0.054ij	44.595±0.137bc	2.450
ET3	0.207±0.001c	0.466±0.023k	1.263±0.016j	0.568±0.003j	0.686±0.002k	1.729±0.038j	26.967±0.504jk	1.264±0.000j	44.912±0.137b	1.370
ET 7 2	0.424±0.011a–c	1.003±0.052g–i	2.281±0.102f–i	1.078±0.136de	1.730±0.191d–f	3.284±0.154f–i	30.538±0.375f–h	2.808±0.326c–e	38.368±0.278i	1.170
ET8	0.294±0.154bc	0.950±0.038hi	1.890±0.222i	0.960±0.193e–h	1.473±0.246e–g	2.858±0.250i	33.630±0.976bc	2.433±0.439ef	39.381±0.390h	1.170
ET4	0.445±0.012a–c	1.261±0.046d	1.940±0.112i	1.410±0.041bc	1.106±0.015hi	3.232±0.158g–i	39.077±0.098a	2.517±0.056d–f	56.037±0.265a	1.280
ET7	0.400±0.023a–c	1.040±0.048e–h	2.446±0.273f–h	0.983±0.042e–g	1.513±0.029e–g	3.486±0.321f–h	29.891±1.231g–i	2.497±0.071ef	39.374±0.591h	1.400
ET8 1	0.569±0.005ab	1.013±0.072f–i	2.674±0.100ef	1.618±0.042ab	2.287±0.029ab	3.686±0.172e–g	27.451±0.482jk	3.905±0.070a	41.421±0.229g	0.940
Fisica	0.631±0.015	1.004±0.040e–g	2.470±0.159f–h	1.050±0.045ef	1.498±0.045e–g	3.564±0.199e–h	30.710±0.413e–h	2.548±0.089d–f	41.192±0.237g	1.400
Gianni	0.302±0.233a–c	1.078±0.032e–g	3.034±0.189de	1.167±0.079de	2.101±0.118bc	4.112±0.221de	26.224±0.445k	3.268±0.197bc	35.702±0.196k	1.260
Livello	0.425±0.269a–c	1.541±0.028ab	4.343±0.024b	1.410±0.112bc	2.444±0.150a	5.883±0.052b	26.187±0.177k	3.854±0.262a	36.575±0.295j	1.530
Moglia	0.472±0.021a–c	0.853±0.009j	2.021±0.063hi	0.826±0.018f–i	1.015±0.046cd	2.874±0.072i	29.698±0.314hi	2.741±0.064c–e	30.143±0.025l	1.050
Pasqui 1	0.479±0.029a–c	1.124±0.026e	2.355±0.037f–i	0.979±0.005e–g	1.393±0.017gh	3.479±0.063f–h	32.314±0.120c–e	2.372±0.022e–g	41.267±0.130g	1.470
Pasqui 2	0.177±0.010c	1.253±0.018cd	2.952±0.029de	0.990±0.048e–g	1.215±0.080g–i	4.305±0.011d	31.430±0.347d–g	2.306±0.137e–g	42.064±0.340ef	1.870
Piattello	0.395±0.065a–c	1.125±0.000e	2.586±0.068e–g	0.669±0.047i–j	1.036±0.080ij	3.710±0.076e–g	30.314±0.274f–h	1.705±0.127h–j	39.254±0.121h	2.180
Rio Gambero	0.625±0.206ab	1.582±0.048a	5.736±0.725a	1.669±0.054a	2.126±0.091a–c	7.318±0.772a	21.706±1.161l	3.795±0.145ab	43.085±0.186cd	1.930
Ruggino 2	0.330±0.214a–c	1.063±0.011e–h	2.264±0.061f–i	0.739±0.002h–j	1.124±0.010hi	3.328±0.072f–i	31.954±0.262c–f	1.864±0.018g–i	39.672±0.326h	1.780
Tange	0.373±0.274a–c	0.930±0.039ij	2.074±0.121hi	1.113±0.119de	1.940±0.251cd	3.004±0.160hi	31.207±0.240e–h	3.062±0.370cd	36.367±0.364jk	0.980
Tavernelle	0.452±0.089a–c	1.119±0.084e	2.682±0.294ef	0.798±0.163g–j	1.226±0.263g–i	3.801±0.378d–f	29.478±0.509hi	2.023±0.426f–h	39.453±0.174h	1.880
Torre	0.359±0.193a–c	1.555±0.104ab	3.380±0.278cd	1.113±0.119de	1.468±0.150e–g	4.935±0.381c	31.521±0.237d–g	2.611±0.266de	42.623±0.113ef	1.890
Average	0.407±0.125	1.103±0.202	2.513±1.027	1.030±0.316	1.500±0.504	3.820±1.223	30.198±3.707	2.592±0.741	41.106±4.083	1.523

Table 6. *Quantitative bitter acids composition of commercial hop cultivars cultivated in northern Italy, determined by HPLC-UV and compared with average commercial data.*

	Xanthohumol	Cohumulone	Humulone + Adhumulone	Colupulone	Lupulone + Adlupulone	Total α acids	Range in Commerce	COH% in total α acids	Range in commerce	Total β acids	Range in commerce	COL% in total β acids	α/β ratio
					g/100g DW								
Columbus	2.240±0.159	5.788±0.410	13.823±1.082	3.977±0.273	3.195±0.157	19.611±1.492	14 – 16	29.520	30 – 35	7172±0.430	4 – 6	55.433	2.73
Fuggle	0.491±0.013	1.164±0.03	3.382±0.070	2.641±0.120	3.882±0.100	4.546±0.039	4 – 5	25.603	4 – 5	6.523±0.219	1 – 2	40.475	0.7
H.Magnum	0.648±0.420	3.287±0.171	13.694±0.998	2.987±0.282	7.197±0.537	16.981±1.168	12 –14	19.368	24 – 28	10.184±0.819	4 – 6	29.311	1.67
Tettnager	0.972±0.115	1.224±0.036	2.358±0.106	1.624±0.173	1.904±0.201	3.582±0.142	4 – 5	34.173	4 – 5	3.428±0.374	3 – 4	46.021	1.02
Willamette	0.538±0.002	0.980±0.004	1.948±0.054	0.888±0.018	1.217±0.006	2.928±0.058	4 – 6	33.468	30 – 35	2.106±0.024	3 – 4	42.183	1.39

Statistical differences are present in Rio Gambero, Livello, Campus Canna, Torre accessions (maximum values) and Busseto 13, ET3 accessions (minimum values). On the contrary, it must be noticed that the presence of β–acids was lower than the average known in the literature for wild European hops (2.59 versus 3.66 g/100g DW) (Patzak et al., 2010b). Table 5 show that ET8 1, Livello and Rio Gambero accessions (β–acids content ranged from 3.905 g/100g to 3.795 g/100g

DW) are statistically different from ET3 and Busseto 13, which are characterized by lower β–acids content. These observations translate into an high average for α/β acids ratio, a parameter set to define the quality for bitter hops, that was above 1.5 on average and close or above 2 in few genotypes (Piattello, Rio Gambero, Campus Canna, Cinghio). In particular, the highest amounts for bitter acids were found respectively in Rio Gambero for α–acids (7.3 g/100g DW, α/β ratio 1.93) and by ET8-1 (3.9 g/100g DW α/β ratio 0.94), Rio Gambero, (3.8 g/100g DW α/β ratio 1.93) and Livello (3.8 g/100g DW α/β ratio 1.53) for β-acids. Rio Gambero (11.11 g/100g DW) provided the highest amount of total acids in the screened pool. A further parameter used to grade commercial hops is the percentage of cohumulone within the total α-acids content. The average reported for wild Italian genotypes was above 30%, quite similar to values obtained for Columbus, Fuggle, Tettnager, Williamette. However, if compared to commercial cultivars, the bitter acids content was similar only to dual-purpose and aroma hops (Fuggle, Tettnager, Willamette), and much lower than high-α cultivars (Columbus, H. Magnum). This pattern should not come as a surprise, as wild accessions and genotypes usually score lower values on this regard.

3.3.3 Quality of commercial cultivars cultivated in Northern Italy.

As no detailed reports are available on the performance of commercial hop cultivars in subalpine areas, five commercial hop varieties with different quality traits were cultivated under uniform conditions. An aroma hop landrace and its triploid derivative (Fuggle and Willamette), a noble aroma variety (Tettnanger), and two high-alpha, bitter varieties (Columbus, H. Magnum) were chosen to cover a wide range of secondary metabolism specialization. The composition of the essential oils profile, the quantification of xanthohumol and bitter acids of their cones are reported in Table 3 and Table 5. The phytochemical evaluations showed some discrepancies if compared to data available in the literature and in commercial repositories (Directory of Hop data, Hopunion CBS, LLC). In terms of volatile compounds, Fuggle, Tettnanger and Willamette, despite being reported as high-myrcene producers (40-50% in essential oils), were almost devoid of this substance, which was below 1%, and a similar pattern was noticed for *trans*-β-farnesene (Table 6). On the contrary, cultivars grown in Northern Italy had essential oils with higher sesquiterpene content, with values for α–humulene and β–caryophyllene slightly above the ranges used to grade these cultivars at commercial level. Overall, on this regard solely Columbus provided results similar to those of a good commercial quality grade. Instead, the content in α– and β–acids and related parameters (α/β ratio, cohumulone and colupulone percentage in α– and β–acids) obtained for plants cultivated in Italy were similar to those used in commerce to define Fuggle, Willamette,

Tettnanger, H. Magnum and Columbus cultivars. Columbus was also the largest producer of xanthohumol, with a content 2-3 fold higher than other commercial varieties. In particular, those varieties with an higher content in α-acids, namely Columbus and H. Magnum, were slightly above such limit, thus leading to suggest that their cultivation under the Italian climate do not seem to alter the biosynthesis of bitter compounds. Similarly, the percentage of cohumulone in total α–acids was within, or very close to, the range suggested in the literature (Directory of Hop data; Stevens and Page, 2004; Zanoli and Zavatti, 2008). Further evaluations on a multi-year scale are under evaluation in order to confirm these preliminary indications.

3.3.4 Multivariate data analysis

A further objective of our work was to observe any patterns in phytochemical traits of wild and commercial hop specimens by means of multivariate chemometric analysis (Figures 3 and 4). PCA plots performed on GC/MS data allowed the definition of three well defined clusters for the Italian genotypes and a more careful definition of their relationship with commercial cultivars.

A first cluster, described mostly by warm, spicy sesquiterpenes α–humulene and β–caryophyllene, was related to fine aroma hops Tettnanger and Willamette, while the other two, described by trans-β-farnesene and selinene isomers respectively, were extremely distant from commercial cultivars (Figure 3). Noticeably, a wild genotype (Roggino 2) was closely related to Fuggle landrace in terms of aroma profile. On this regard the Italian wild population may be constituted by three main classes according to their aromatic profile. When the complete fingerprint was considered, the weight of the phloroglucinol profile became predominant, but nevertheless the genotype Roggino 2 confirmed its similarity to Fuggle and Tettnanger commercial varieties, mostly described by α–humulene/β–caryophyllene ratio (Figure 4). For what concerns the α– and β– acids profile, the Italian genotypes confirmed their relationship with Willamette and Tettnanger, while a large difference was noticed for Columbus, H. Magnum and, to a lower extent, Fuggle (Figure 5). In particular, Rio Gambero emerged as the most interesting genotype for potential breeding, given its rather close relationship with Fuggle and its different positioning on the plot respect to other Italian cultivars. Moreover, the overall patterns described by multivariate data analysis of phytochemical data were consistent with those provided by the dendrogram based on SSR data revealed in terms of phylogenetic relationships between genotypes and commercial cultivars.

Figure 3. *Score plots obtained from the PCA of morphological data of hop cones from italian genotypes and commercial cultivars of Humulus lupulus L.*

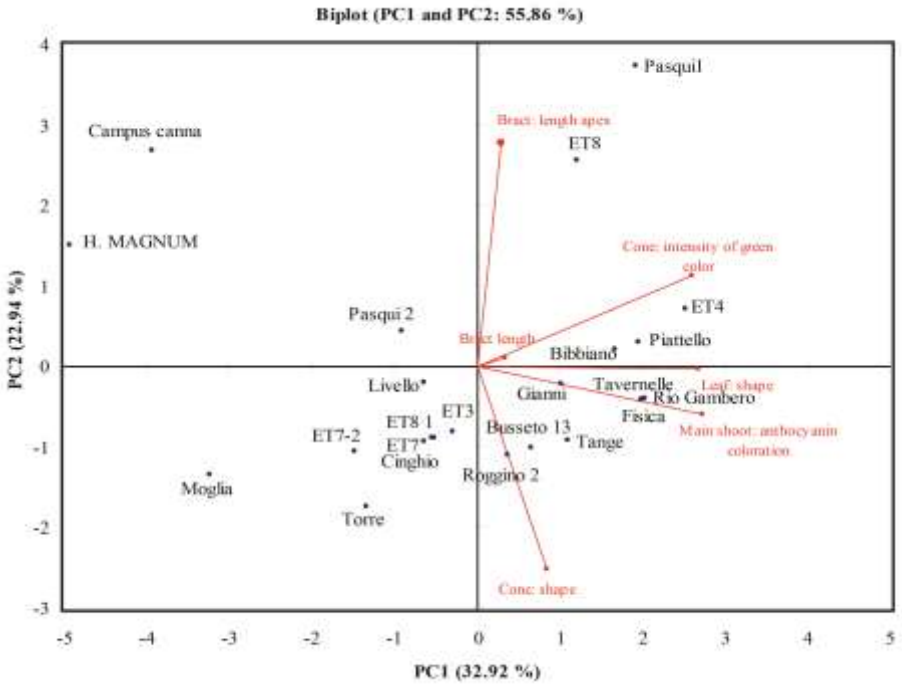


Figure 4. *Score plots obtained from the PCA of qualitative GC-MS data of essential oils extractd from italian genotypes and commercial cultivars of Humulus lupulus L.*

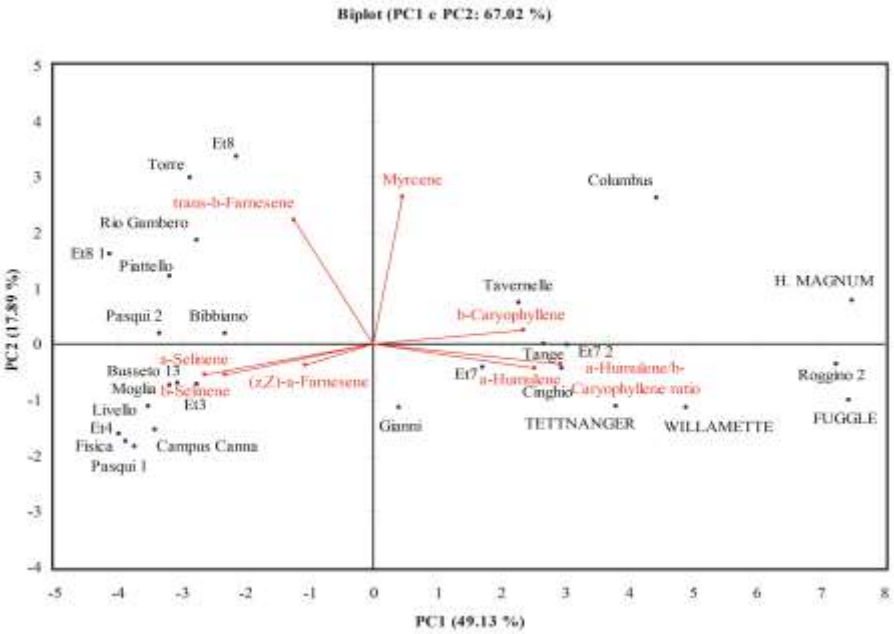
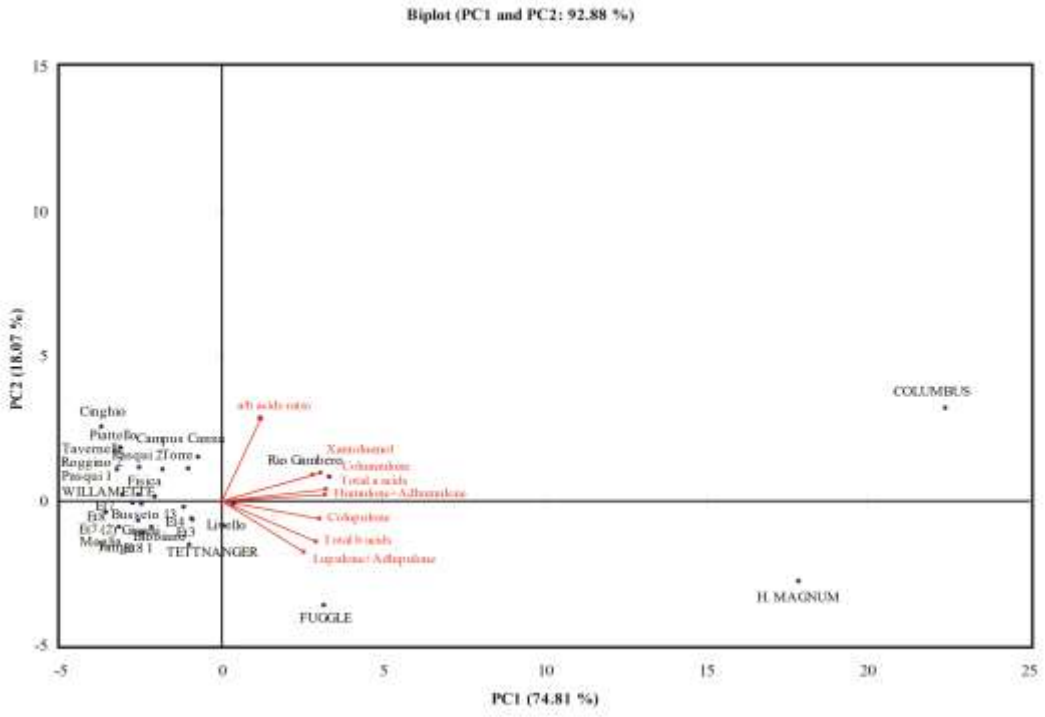


Figure 5. *Score plots obtained from the PCA of quantitative HPLC data of bitter acids and xanthohumol from italian genotypes and commercial cultivars of Humulus lupulus L.*



4. Conclusions.

Given the concurrent low essential oils content, the low bitter acid amount and the profile of its aromatic fraction, the Italian wild hop germplasm does not harbor candidates for high-alpha varieties but rather to dual-purpose or finishing hops. The phytochemical variability among the genetic pool screened and the presence of traits valuable for brewing, suggest the potential use of some Italian wild genotypes as sources for breeding and improvement of cultivated varieties. It must be however stated clearly that these preliminary results need confirmation after multi-year growing, also to estimate more properly the effects of local conditions for hop cultivation in Italy, but a fingerprint of the phytochemical profile in genetically different wild accessions represents a first, fundamental step to succeed in breeding improvement. (Mozny et al., 2009; Kučera and Krofta, 2009; Srečec et al., 2013; Pavlovič et al., 2013). Finally, our screening suggest that, from a phytochemical standpoint, the content of prenylflavonoids and phloroglucinols in commercial hop cultivars grown in Italy is in agreement with the percentages obtained in countries dedicated to hop cultivation, and therefore a commercial cultivation of hop could be suitable if these data are

confirmed. These preliminary evaluations confirm that careful, in-depth, screenings of wild hop genotypes in Italy may be warranted.

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Supplemetary material

Table. Linearity, sensitivity and recovery data for xanthohumol and bitter acids used as hop standards

Compound	Linearity range[a] (µg/ml)	r^2		Linearity range[a] (µg/ml)	r^2		LOD (µg/mL)	LOQ (µg/mL)	Mean recovery (%)	Intra-day repeatability (RSD%)	Inter-day repeatability (RSD%)
Xanthohumol	5 – 50	0.9996	y = 142138x - 115009				20	50	91.2 ± 1.3	1.48	0.90
Cohumulone	14 – 139	0.9943	y = 60801x + 73285	69.5 – 347	0.9960	y = 62843x + 452278	28	33.6	121.2 ± 2.5	0.76	2.70
N-Humulone/Adhumulone	30.8 – 308	0.9973	y = 60766x + 175025	154 – 370	0.9968	y = 62621x + 106	61.6	77	119.2 ± 1.9	1.04	2.20
Colupulone	13.4 – 134	0.9972	y = 58287x + 67827	67.2 - 336	0.9920	y = 58686x + 450778	26.8	33.5	97.6 ± 1.1	4.27	1.98
N-Lupulone/Adlupulone	10.8 – 108	0.9962	y = 57544x + 78458	54 – 270	0.9953	y = 59576x + 294876	21.6	27	97.1 ± 1.4	2.20	0.83

Legend: [a] Linearity was assessed in two diferent ranges for α– and β–acids, due to the different range of concentrations found in samples.

3.5. Study 5 - Are Humulus lupulus L. ecotypes and cultivars suitable for the cultivation of aromatic hop in Italy? A phytochemical approach

Mongelli A., Rodolfi M., Ganino T., Marieschi M., Cagiani A., Dall'Asta C., Bruni R. **Are *Humulus lupulus* L. ecotypes and cultivars suitable for the cultivation of aromatic hop in Italy? A phytochemical approach.** *Industrial Crops and Products.*
IN PRINT

Are *Humulus lupulus* L. ecotypes and cultivars suitable for the cultivation of aromatic hop in Italy? A phytochemical approach.

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Abstract.

Breeding programs are increasingly interested in the introduction of traits from wild hops and in the detection of the cultivars most suitable under different climatic conditions. Eleven *Humulus lupulus* ecotypes were selected from distinct populations in Northern Italy, grown in a collection field and evaluated for their yield and cone aromatic profiles after a three-year acclimatization. At the same time, the performance of 10 *H. lupulus* cultivars (Bramling Cross, Cascade, Challenger, Fuggle, Magnum, Marynka, Nugget, Target, Tettnanger, Willamette) grown under the same conditions was investigated by comparing them to commercially available material. Estimated gross yield for ecotypes ranged between 636 and 2491 kg/ha, largely exceeding hop production for commercial cultivars (277-1442 kg/ha), while essential oil accumulation was inverted. Essential oils obtained from mature cones were characterized by GC-MS. In the volatile fraction of most ecotypes, selinene isomers were extremely abundant and myrcene was low, while α–humulene/β–caryophyllene ratio was comprised between 0.15 (Pasqui 1) and 1.68 (Tavernelle and Roggino 2). The phytochemical variability among the genetic pool screened suggests its potential use for breeding and improvement of cultivated varieties, in particular for plant material aimed to be used in the dry hopping stage of brewing. Northern Italy seem to be particularly suitable for the cultivation of finishing, aromatic hops like Bramling Cross, Magnum, Marynka and Tettnanger.

Keywords: hop, wild germplasm, varietal selection, essential oils, biodiversity, selinene, humulene.

1. Introduction.

The genus *Humulus* comprises five species: *H. cordifolius* Miq., *H. neomexicanus* (A. Nelson & Cockerell) Rydb, *H. scandens* (Lour.) Merr, *H. yunnanensis* Hu and *H. lupulus* L., the latter being recognized as an extremely polymorphic species, described by five subspecies and characterized by a strong degree of intraspecific diversity (Pillay and Kenny, 1996; Sustar-Vozlic and Javornik, 1999; Jakse et al, 2004; Patzak et al., 2010a; Patzak et al., 2010b; Solberg et al., 2014). *Humulus lupulus* is a widely cultivated plant, whose female inflorescences (strobiles or cones) provide a key flavouring agent for the brewing industry and an appreciated

ingredient for herbal and cosmetic products (Zanoli and Zavatti, 2008). As a consequence of its commercial success, a plethora of hop cultivars has been developed worldwide, differing for gross biomass yield, climate adaptation, agronomic performance and secondary metabolite production (Kralj et al., 1991;Hampton et al., 2002; del Valle et al., 2003; Krofta, 2003; Murakami et al., 2006; Patzak et al., 2010a; Patzak et al., 2010b). Hop breeding has been traditionally focused on yield and strobile size or on the phytochemical profile of specific secondary metabolites, which are secreted in peculiar secretory glands (lupulin) and mostly present on hop bracts (Kavalier at al., 2011). An unique and complex pool of substances is biosynthesized in these structures, spanning from terpenophenolic polar metabolites to volatile terpenes, with the latter prevailing in aromatic or finishing hops used in specific stages of the brewing process (Kishimoto et al., 2006). As for other domesticated plants, a reduction of intraspecific diversity during the breeding process has been observed in *H. lupulus*, with a funnel effect on the genetic basis used for the development of new cultivars (Jarvis and Hodgkin, 1999). At the same time, the growing focus on local brewing and the widening of beer marketplaces increased the interest for hops endowed with peculiar phytochemical profiles and for new cultivation zones, in order to produce local hops with more peculiar bouquets. Both these constraints may be alleviated by the recourse to genetic material from wild accessions (to be introduced directly in cultivation or to be included in tailored breeding programmes), and by a better knowledge of the performance of established cultivars under different geoclimatic conditions. For instance, commercial hop cultivation provide better results in regions approximately comprised between 45 and 55 degrees north or south in latitude, an area that also includes the northern part of Italy, in which no cultivation is performed at present despite the presence of widespread populations of wild *H. lupulus* (del Valle et al., 2003; Prencipe et al., 2014). The interest for exhaustive screenings of wild hop germplasm from different parts of the world is testified by the literature on its chemodiversity, with particular reference for volatile constituents (Heywood, 2002; Figuereido et al., 2008). On this regard, a consistent deal of variability has been described within both commercial hop cultivars and wild populations from different countries and regions (Canada, Chile, United States, the Caucasus, some countries of Central and Northern Europe, among others) (Small, 1980; Kac and Kralj, 1998; Malizia at al., 1999; del Valle et al., 2003; Bernotiene et al., 2004; Jirovetz et al., 2006; Patzak et al., 2010a). However, it must be noticed that some geographical areas have not been properly scrutinized. For instance, wild *H. lupulus* ecotypes from subalpine areas of Europe may still be considered as an untapped source of genetic diversity, despite the common presence of the plant as a native

species in this region (Murakami et al., 2006; Patzak et al., 2010b; Solberg et al., 2014; Karlsson-Strese et al., 2014; Horreo et al., 2015). Furthermore, no report is available on the performance and behaviour of established hop cultivars under subalpine climatic conditions.

Therefore, this study aims at the screening of essential oil composition and yield of wild Italian *H. lupulus* ecotypes, grown under controlled agronomical conditions after three years of acclimatization. At the same time the performance of both ecotypes and some hop cultivars have been evaluated by comparing them to commercially available material. Overall, 11 different wild genotypes and 10 commercial varieties were acclimatized, analyzed and compared with samples from the marketplace.

2. Experimental

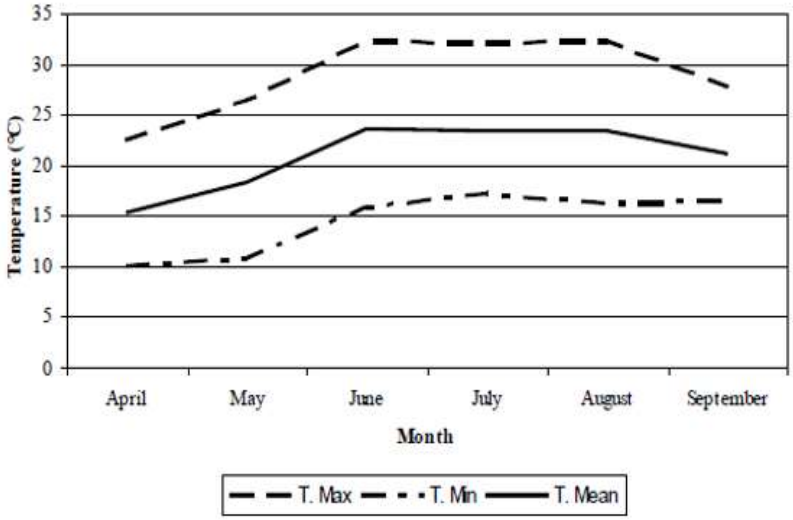
2.1 Chemicals and solvents

Dichloromethane and anhydrous sodium sulfate were purchased from Sigma-Aldrich (Milan, Italy). Toluene was purchased from Carlo Erba (Milan, Italy). Ultra-pure water was in house produced by using a Milli-Q-System (Millipore, Bedford, MA, USA). Standards of caryophyllene, myrcene, humulene, (+)-linalool and alkane solution C_8-C_{20} were purchased from Sigma-Aldrich (Milan, Italy). Methanol (HPLC grade) was purchased from Sigma-Aldrich (Milan, Italy).

2.2 Field germplasm collection

Rhizomes of commercial cultivars (Bramling Cross, Cascade, Challenger, Fuggle, Magnum, Marynka, Nugget, Target, Tettnanger and Willamette) were purchased from a European commercial nursery. Rhizomes from *H. lupulus* ecotypes were collected in Lombardia, Emilia Romagna, Tuscany and Veneto regions during 2011, verified for their genetic diversity and since then grown in a field germplasm collection in Marano sul Panaro (MO, 44°27'00" N, 10°58'00" E), Emilia-Romagna, Italy (Mongelli et al., 2015). The collection germplasm field is 1600 m^2, organized on 80 m long rows, spaced 2.5 m; the spacing on the row will be 1 m. The model of growth will be based on the Y growth form and the height of the structure will be 4.30 m. The plants will be arranged in blocks of 4 plants per ecotype. The means, minimum and maximum monthly temperatures of the growing period are reported in Figure 1. Temperature data were collected in field by using a HOBO® Pendant Temperature Data Logger (Onset, Cape Cod, MA, USA).

Figure 1. *Means, minimum and maximum monthly temperatures in field collection in the 2013. Temperature data were collected in field by using a HOBO® Pendant Temperature Data Logger (Onset, Cape Cod, MA, USA).*



2.3 Plant material

Each ecotype and commercial cultivar was grown under the same environmental conditions. Cone hop samples were harvested in September 2014. In order to emulate farm picking and industrial processing, samples were dried in a thermostated oven (55°C for 12 hours) with forced ventilation until a relative humidity of 10% was reached (Mongelli et al., 2015). Afterward, samples were vacuum-packed and stored at -20°C until analysis were performed. Hop cones of Marynka, (Poland), Willamette (USA), Target (United Kingdom), Challenger (UK) Bramling Cross, (UK), Magnum (Germany), Fuggles (UK), Tettnanger (Germany), Columbus (USA) were purchased in 2014 from Hopunion LLC, Yakima, WA, USA, while Nugget (USA) and Cascade (USA) were purchased from Rolling Beers, Vendargues, France.

2.4 Essential oil extraction and sample preparation for GC-MS analysis

Prior the analysis, essential oils were extracted by steam distillation with a Clevenger apparatus for four hours. The essential oils were diluted in CH_2Cl_2 (1:200 v/v) in a vial and added of a small amount of anhydrous sodium sulfate.

2.5 GC/MS analysis

All samples were analysed with a Thermo Scientific (San Jose, CA, USA) TRACE 1300 gas-chromatograph coupled to a Thermo Scientific ISQ™ Single Quadrupole mass spectrometer. The gas-chromatograph was equipped with Supelcowax 10 (30 m x 0.25 mm, f.t. 0.25 µm) (Supelco, Bellefonte, PA, USA capillary columns and helium was used as carrier gas (1 ml min^{-1}). Oven temperature gradient started from 50°C, this condition was maintained for 3 minutes, then the temperature was raised to 200°C (5°C/minute). The final temperature was maintained for 18 minutes. The injector was maintained at 230°C operating in split modality, ratio 1:20. The mass spectrometer was equipped with an electron impact source (EI, 70 eV) and the acquisition mode was full scan (from 40 m/z to 500 m/z). A solvent delay time of 4 minutes was applied. The main volatile compounds were identified on the basis of their mass spectra compared with the reference mass spectra libraries (WILEY275, NBS75K, Adams 2001) and of their calculated Retention Indexes through the application of the Kovats' formula (KI) compared with those reported in the literature. When it was not possible to find the KI in the literature, a tentative identification was obtained by matching with mass spectra libraries data: a match quality of 98% minimum was used as a criterion. In order to determinate the RI of the components, a mixture of alkanes (C8-C20) was injected in the GC-MS equipment and analysed under the same conditions described above. The gas-chromatographic signals were manually integrated and the resulting peak areas were compared with the total sum of area and expressed in percentage.

2.6 Statistical analysis

The data obtained from chemical analysis were evaluated for mean and standard deviation (SD). The collected data were also analysed by using univariate analysis of variance (ANOVA). One-way analysis of variance was carried out by applying Tukey's post hoc test ($p \leq 0.05$) using SPSS Statistics 21.0 software (SPSS Inc. Chicago, Illinois, 2003).

3. Results and Discussion.

3.1 Hop production and essential oil yield.

Gross hop yields were calculated in order to estimate the potential production of local ecotypes and commercial cultivars, after their full acclimatization in the climatic conditions of Northern Italy. From the literature, the performances of wild hops from controlled environments are not usually available and the yields reported come from plants collected directly in the wild and therefore affected by local environmental and climatic variability (Small, 1980; Kac and Kralj, 1998; Malizia at al., 1999;del Valle et al., 2003; Bernotiene et al., 2004; Jirovetz et al., 2006;

Patzak et al., 2010a). The data obtained (Table 1) show a consistent hop biomass production by ecotypes, with promising outlooks (exceeding 1000 kg/ha DW) for 8 accessions and in particular for Cinghio (>2400 kg/ha DW). On the contrary, results for hop cultivars ranged from 277 kg/ha (Challenger) to 1442 kg/ha (Marynka), which are less encouraging if compared with the same cultivars grown elsewhere (Barth-Haas Hops Companion, 2009; Variety Manual USAHops, 2011). This behaviour may be due to a different adaptability to the local environment by genotypes developed to perform at their optimum at higher latitudes and in cooler climates. In terms of essential oil biosynthesis, ecotypes showed instead a less efficient performance, with an average yield halved if compared with commercial cultivars and ranging between 0.17 (Piattello) and 1.68% (Roggino 2 and Tavernelle), while values for commercial samples averaged between 0.96 (Tettnanger) and 2.13% (Magnum). These results are on average better than those regarding most common European aroma hops, which despite geographical and year-to-year fluctuations are usually characterized by an essential oil content rarely exceeding 1.0% (Krofta, 2003). The limited accumulation of essential oils in Italian ecotypes is however compensated by the larger production of hops and the estimated total essential oil production projected per hectare is conspicuous in some occasions. Tange (28.02 L/ha), Cinghio (27.65 L/ha) and Tavernelle (21.37 L/ha) were the best performers in terms of potential essential oil productivity, while only a single commercial cultivar (Marynka, 29.42 L/ha) provided similar values. If compared with samples from the same cultivars acquired from the marketplace, hop cultivars cultivated in Italy provided always a percentage of essential oil always close or slightly above to the values expected by hop traders (Variety Manual USAHops, 2011; Inui et al., 2013).

Table 1. *Gross and essential oil yield of hops cultivated in a Subalpine climate and estimation of their essential oil productivity*

	Yield	E.O. extraction yield	Potential E.O. production
Ecotypes	kg/ha, DW	%	L/ha
Bibbiano	1204	0,62	7,46
Cinghio	2491	1,11	27,65
ET8	1004	0,11	1,1
ET8 I	1081	0,22	2,38
Livello	1498	0,41	6,14
Pasqui 1	1580	0,15	2,37
Pasqui 2	636	0,26	1,65
Piattello	784	0,17	1,33
Roggino 2	984	1,67	16,44
Tange	1796	1,56	28,02
Tavernelle	1272	1,68	21,37
Average	1303	0,72	10,54
Hop cultivars			
Brangling Cross	400	1,3	5,2
Cascade	952	1,19	11,33
Challenger	277	1,42	3,94
Fuggle	526	1,63	8,57
Magnum	762	2,13	16,24
Marynka	1442	2,04	29,42
Nugget	292	0,98	2,86
Target	280	1,53	4,28
Tettnanger	304	0,96	2,92
Willamette	447	1,45	6,48
Average	568	1,46	9,12

3.2. Comparing wild germplasm to commercial cultivars grown under the same conditions.

More glaring differences emerged when the evaluation was extended to the essential oil profile of Italian ecotypes (Table 2) and of cultivars grown under the same conditions (Table 3).

Table 2. *Phytochemical composition of essential oils isolated from Italian H. lupulus ecotypes grown in collection field*

Compound	KI	RT	Bibbiano	Cinghio	ET 8_1	ET 8	Livello	Pasqui 1	Pasqui 2	Piattello	Roggino 2	Tange	Tavernelle
								%					
α-Pinene	1096	6,29	n.d.	n.d.	0.04±0.01hi	0.04±0.01hi	0.02±0.02hi	n.d.	n.d.	n.d.	n.d.	n.d.	0.03±0.01hi
β-Pinene	1105	6,52	0.11±0.00k-m	0.24±0.02i-m	0.25±0.03i-m	0.32±0.02h-l	0.06±0.05lm	0.11±0.05k-m	0.20±0.04j-m	0.09±0.01k-m	0.22±0.11i-m	0.05±0.04lm	0.04±0.00m
Myrcene	1155	8,12	9.56±0.17i-k	13.27±0.83hi	12.97±1.87hi	11.14±0.34ij	3.50±2.19n	6.57±2.35k-n	10.31±1.03i-k	4.95±0.22l-n	8.95±2.09i-l	5.10±0.14l-n	4.25±0.12mn
Limonene	1187	8,99	0.04±0.00jk	0.09±0.00i-k	0.26±0.01d-g	0.26±0.02d-g	0.10±0.05i-k	0.07±0.01jk	0.07±0.00jk	0.04±0.00jk	0.07±0.03jk	0.08±0.07i-k	0.08±0.01jk
Butyl isobutyrate	1197	9,24	0.04±0.01i	0.08±0.01i	0.05±0.04i	0.04±0.01i	0.02±0.02i	0.02±0.01i	0.03±0.02i	0.02±0.00i	0.01±0.01i	0.01±0.01i	0.01±0.01i
Heptanoic, Methyl ester	1278	11,48	0.17±0.01i-k	0.21±0.02h-k	0.94±0.24a-c	0.99±0.10a-c	0.09±0.05jk	0.11±0.04jk	0.02±0.01k	0.12±0.00jk	0.23±0.05h-k	0.09±0.06jk	0.17±0.01i-k
Methyl-4-ethyl-4 pentanoate	1326	12,77	0.15±0.00g-j	0.35±0.02c-h	0.26±0.00e-i	0.31±0.02c-i	0.37±0.32b-g	0.22±0.06f-j	0.31±0.04c-i	0.20±0.02f-j	0.20±0.04f-j	0.11±0.09h-j	0.09±0.00h-j
Methyl-6-methyl eptanoate	1332	12,95	0.08±0.00lm	0.19±0.01h-l	0.11±0.08k-m	0.03±0.01m	0.16±0.05i-m	0.20±0.04h-l	0.14±0.01j-m	0.04±0.01m	0.12±0.02j-m	0.08±0.07lm	0.10±0.01k-m
2-Nonanone	1380	14,25	0.36±0.01d-f	0.41±0.03b-d	0.26±0.02h	0.32±0.01f-h	0.33±0.06e-g	0.45±0.05bc	0.46±0.04b	0.27±0.02gh	0.39±0.05c-e	1.02±0.04a	0.46±0.01b
Nonanal	1384	14,38	1.26±0.02c	2.06±0.06a	0.76±0.05de	0.77±0.01de	0.57±0.21ef	1.74±0.22b	1.90±0.01ab	1.84±0.04ab	0.87±0.18d	1.69±0.35b	1.26±0.01c
5,9-Tetradecadiene	1477	16,63	0.05±0.00l-o	0.14±0.01f-h	n.d.	0.08±0.01i-m	0.02±0.02no	0.03±0.00m-o	n.d.	0.18±0.00c-g	0.23±0.01bc	0.12±0.02h-k	0.08±0.01j-n
Copaene	1492	16,84	0.18±0.02l-n	0.49±0.03f-h	0.07±0.00no	0.11±0.01no	0.16±0.02mn	0.15±0.00n	n.d.	0.18±0.00l-n	0.86±0.02b	0.39±0.06g-k	0.29±0.01k-m
2-Decanone	1497	16,97	0.31±0.01bc	0.22±0.03e-g	0.26±0.03c-f	0.27±0.01c-e	0.06±0.02j-l	0.06±0.02j-l	0.12±0.01h-k	0.08±0.00i-l	0.29±0.02b-e	0.27±0.10c-e	0.23±0.03d-g
2-nonanol	1503	17,12	0.05±0.01d-f	0.08±0.01d	0.03±0.02fg	0.07±0.01de	0.02±0.02fg	0.07±0.00de	0.07±0.01de	0.07±0.01de	n.d.	0.05±0.02ef	0.20±0.01b
Linalool	1535	18,29	0.26±0.01k-n	0.43±0.03i-n	0.36±0.01j-n	0.47±0.02i-m	0.12±0.01mn	0.24±0.00k-n	0.37±0.01j-n	0.17±0.01mn	0.22±0.02l-n	0.09±0.06n	0.17±0.01mn
β-Farnesene	1539	18,4	0.48±0.01d	n.d.	1.67±0.02b	1.89±0.02a	0.16±0.01hi	0.10±0.01jk	1.22±0.01c	0.45±0.01de	0.04±0.00l	0.08±0.01kl	0.35±0.00f
10,12-Octadecadiynoic acid	1570	19,22	0.35±0.00f-h	0.56±0.04c	0.06±0.05kl	0.12±0.02jk	0.14±0.01jk	0.27±0.01hi	0.12±0.01jk	0.14±0.01jk	0.92±0.03b	0.41±0.05d-f	0.38±0.01e-g
β-Caryophyllene	1577	19,37	14.28±0.46a-c	14.79±1.25a-c	7.02±0.04o-q	7.65±0.30m-p	9.33±0.35j-m	13.88±0.51b-d	10.97±0.06h-j	5.02±0.04r	14.84±0.9ab	11.33±1.53f-i	8.99±0.31k-n
2-Dodecanone	1584	19,54	0.21±0.00c-e	0.30±0.03b	0.17±0.04e	0.22±0.00cd	0.18±0.03e	0.21±0.00c-e	0.25±0.02c	0.17±0.00e	0.36±0.03a	0.24±0.03c	0.18±0.02de
2-Undecanone	1587	19,6	n.d.	n.d.	0.98±0.02ef	0.66±0.12fg	0.59±0.51f-h	n.d.	n.d.	0.92±0.05ef	0.03±0.01i	1.45±0.46d	1.42±0.07d
Longifolene	1591	19,74	n.d.	n.d.	n.d.	n.d.	n.d.	0.03±0.01c	n.d.	5.39±0.06a	n.d.	0.77±0.07b	n.d.
Decenoic acid, methyl ester	1613	20,22	0.27±0.01j-l	0.46±0.07h-k	0.46±0.04h-k	0.37±0.12i-l	0.70±0.13f-i	0.60±0.01g-j	0.50±0.02h-j	1.45±0.04c	0.46±0.03h-k	0.77±0.07f-h	1.62±0.22bc
Trans-β-Farnesene	1645	21,01	0.05±0.00j	0.23±0.03ij	10.81±0.45b	9.04±0.40c	0.11±0.10j	0.05±0.01j	0.43±0.06h-j	5.09±0.00fg	1.21±0.13h-j	1.72±0.18hi	8.08±0.79cd
α-Humulene	1656	21,18	8.91±0.17n	16.35±1.29h-l	1.56±0.03o	1.70±0.01o	3.81±0.44o	2.08±0.15o	2.86±0.06o	0.84±0.03o	24.81±1.39ab	17.25±0.56g-k	14.98±2.71j-m
cis,α-Bisabolene	1665	21,26	1.89±0.06a	n.d.	n.d.	0.39±0.01c	1.41±0.62b	0.50±0.04c	0.26±0.02cd	n.d.	n.d.	n.d.	n.d.
Germacrene D	1673	21,39	4.33±0.11b	1.79±0.05f-h	n.d.	n.d.	3.04±0.41c	6.10±0.26a	3.16±0.02c	1.18±0.04i-l	4.32±0.10b	1.70±0.09g-j	1.66±0.08g-j
(z,Z)-α-Farnesene	1678	21,51	1.00±0.09g-i	0.43±0.02kl	0.28±0.05kl	0.39±0.01kl	0.74±0.16h-k	1.64±0.02b-e	0.65±0.05i-l	0.26±0.02kl	1.28±0.06d-g	0.38±0.02kl	0.36±0.02kl
Methyl geranate	1681	21,69	0.73±0.04d-j	2.03±0.14b	0.28±0.12i-o	0.42±0.06h-o	0.69±0.14d-k	n.d.	0.27±0.02i-o	0.93±0.01c-g	0.14±0.01l-o	2.09±0.13b	1.35±0.06c
2-Dodecanone	1689	21,79	0.70±0.04d	1.17±0.19c	1.54±0.3b	2.26±0.22a	1.60±0.26b	0.57±0.07de	0.32±0.00ef	n.d.	0.88±0.07cd	1.70±0.23b	2.2±0.12a

β-Selinene	1703	22,44	14.00±0.33ab	8.67±0.37c	15.09±1.08ab	12.64±0.32b	14.74±4.37ab	15.54±0.85a	15.64±0.03a	6.94±0.10c	1.24±0.10de	8.81±0.55c	8.98±0.49c
α-Selinene	1708	22,56	14.41±0.38ab	9.03±0.40cd	10.69±0.09c	8.55±0.33cd	16.72±5.98a	15.79±0.97a	10.96±0.27bc	7.55±0.10c-e	2.24±0.17fg	9.45±0.60cd	9.40±0.96cd
α-Farnesene	1752	23,09	0.65±0.05bc	0.06±0.05hi	0.66±0.11bc	0.78±0.04ab	0.31±0.06e-g	0.15±0.02g-i	0.16±0.02g-i	0.53±0.01cd	n.d.	0.10±0.01hi	0.24±0.03f-h
τ-Cadinene	1761	23,29	1.40±0.14i-k	3.44±0.04b-d	0.95±0.17k	1.09±0.08jk	1.11±0.27jk	1.23±0.01jk	1.09±0.05jk	1.06±0.04jk	6.25±0.36a	4.24±0.71b	2.44±0.38d-i
δ-Cadinene	1763	23,33	0.72±0.09h-k	1.81±0.09b-d	n.d.	0.31±0.05kl	0.46±0.10j-l	0.74±0.02g-k	0.39±0.06j-l	0.39±0.02j-l	3.11±0.41a	1.14±0.36d-i	1.22±0.25c-i
Ar-Curcumene	1766	23,41	0.64±0.06b	0.28±0.01d	0.26±0.06d	0.29±0.00d	0.46±0.11c	1.24±0.04a	0.56±0.03b	0.12±0.01e	n.d.	0.22±0.02d	0.25±0.04d
α-Curcumene	1779	23,72	n.d.	n.d.	5.82±0.76a	5.23±0.53b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Eudesma-3,7(11)-diene	1785	23,83	0.07±0.02fg	0.27±0.02f	4.26±0.09d	4.61±0.04c	8.42±0.36b	0.04±0.03g	0.03±0.00g	9.39±0.11a	0.67±0.04e	0.19±0.02fg	0.17±0.00fg
Geranyl butyrate	1792	23,99	n.d.	n.d.	n.d.	n.d.	n.d.	0.09±0.08f	n.d.	0.06±0.02fg	n.d.	0.30±0.00bc	0.21±0.02de
2-Tridecanone	1813	24,43	1.16±0.11d-h	1.13±0.23e-h	0.51±0.24i-k	0.88±0.03g-j	1.23±0.32d-h	0.99±0.02f-i	1.66±0.02c-e	0.83±0.00h-j	1.51±0.06c-f	2.54±0.10b	1.86±0.23c
Geranoyl formate	1821	24,62	n.d.	n.d.	n.d.	n.d.	0.13±0.03d	n.d.	0.04±0.00e	n.d.	n.d.	n.d.	n.d.
Selina 3,7 (11) diene	1830	24,82	0.17±0.03cd	0.16±0.08de	n.d.	n.d.	0.28±0.03b	0.08±0.00f	0.22±0.01bc	0.10±0.00ef	n.d.	0.36±0.03a	0.22±0.05c
Calamenene	1837	24,96	0.12±0.02de	0.22±0.03de	3.35±0.24a	0.10±0.09de	2.00±0.17b	0.10±0.01de	n.d.	1.57±0.24c	n.d.	0.31±0.04d	0.18±0.02de
Methyl-3,6-dodecadienoate	1877	25,82	n.d.	n.d.	0.02±0.02b	3.38±0.30a	0.01±0.01b	n.d.	n.d.	n.d.	n.d.	n.d.	0.05±0.01b
2-Nonadecanone	1898	26,27	n.d.	n.d.	0.07±0.06c	n.d.	0.13±0.03b	0.11±0.01bc	n.d.	0.06±0.03cd	n.d.	0.11±0.07bc	0.26±0.01a
1,4-Undecadiene	1914	26,59	n.d.	n.d.	n.d.	0.10±0.01h	0.26±0.05d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
cis-3-Decen-1-ol	1935	27,02	0.59±0.03b	0.33±0.11de	0.43±0.16cd	0.62±0.03b	0.44±0.14cd	0.22±0.00e	0.82±0.04a	0.64±0.02b	n.d.	0.51±0.03bc	0.43±0.03cd
Caryophyllene oxide	1970	27,94	2.86±0.04j-l	1.78±0.02l	0.47±0.17h-j	0.57±0.02kl	0.61±0.07j-l	2.79±0.05kl	0.31±0.00l	0.58±0.02kl	1.65±0.07f-l	3.6±0.60b-d	1.03±0.02l
2-Dodecadiene	1999	29,13	1.60±0.16e-i	1.77±0.49d-g	1.15±0.47h-k	1.66±0.11e-h	1.06±0.32i-l	1.43±0.05f-i	2.99±0.05ab	0.62±0.02k-p	2.05±0.08c-e	2.59±0.10bc	1.31±0.01g-j
Humulene epoxyde II	2016	29,27	0.25±0.03e	2.15±0.02bc	0.01±0.01e	0.08±0.03e	0.02±0.02e	0.02±0.02e	n.d.	0.06±0.00e	2.88±0.24bc	4.93±1.77a	3.10±0.88b
Caryophyllenylalcohol	2054	29,58	0.44±0.04d-f	0.26±0.01e-j	0.06±0.01i-k	0.05±0.01jk	0.16±0.01g-k	0.51±0.01cd	0.11±0.02h-k	0.73±0.03bc	n.d.	n.d.	0.28±0.18e-j
Cubenol	2066	29,71	0.08±0.01d-h	0.18±0.02b-e	0.04±0.01gh	n.d.	0.05±0.04f-h	0.08±0.01e-h	0.05±0.02f-h	0.09±0.01d-h	0.30±0.03ab	0.16±0.01c-g	0.12±0.01c-h
17-Octadecen-14-yn-1-ol	2080	29,99	2.16±0.15d-g	1.88±0.24fg	1.09±0.41h-j	1.65±0.09gh	2.57±0.74c-e	1.56±0.01g-i	2.51±0.10c-f	2.13±0.11d-g	3.27±0.15b	4.29±0.25a	3.05±0.09bc
Globulol		30,7	n.d.	n.d.	0.09±0.03hi	0.13±0.02hi	0.11±0.03hi	0.18±0.01hi	0.06±0.02i	1.98±0.03bc	0.77±0.06e-g	0.11±0.01hi	n.d.
Germacrene-D-4-ol		32,85	0.46±0.01d-i	0.46±0.03d-i	0.57±0.31d-f	0.08±0.01i	1.70±0.53b	0.51±0.00d-h	0.14±0.01hi	2.29±0.01a	0.72±0.05d	0.54±0.05d-g	0.33±0.03e-i
Humule-1,6-dien-3-ol		33,26	4.64±0.1b	2.13±0.13d	0.03±0.02f	2.12±0.09d	3.34±0.94c	6.85±0.19a	4.13±0.08b	1.45±0.05e	n.d.	2.05±0.11d	1.91±0.05de
E,E Farnesol		35,27	0.07±0.03e	n.d.	0.11±0.10e	0.22±0.03e	0.09±0.08e	0.11±0.09e	n.d.	n.d.	n.d.	0.71±0.17de	0.13±0.01e
Longipinocarvone		35,81	0.25±0.03cd	0.27±0.07c	n.d.	0.09±0.01fg	0.11±0.10ef	0.67±0.08a	0.15±0.05d-f	0.30±0.05c	0.21±0.01c-e	n.d.	0.41±0.07b
Total			92,43	92,53	81,13	80,18	84,15	86,89	76,78	69,45	88,73	96,11	86,48
α-Humulene/β-Caryophyllene			0,62±0,12	1,11±0,01	0,22±0,00	0,22±0,01	0,41±0,09	0,15±0,02	0,26±0,01	0,17±0,01	1,67±0,01	1,56±0,37	1,68±0,51

Table 3. *Phytochemical composition of H. lupulus cultivars grown in collection field.*

Compound	KI	RT	Bramling Cross	Cascade	Challenger	Fuggle	Magnum	Marynka	Nugget	Target	Tettnanger	Williamette
						%						
α-Pinene	1096	6,29	0.51±0.02b	0.16±0.01e-i	0.06±0.05f-i	n.d.	0.33±0.28cd	0.26±0.00c-e	n.d.	0.15±0.02e-i	0.03±0.03hi	0.05±0.01g-i
β-Pinene	1110	6,59	0.90±0.06b-d	1.41±0.14a	0.88±0.12b-d	0.92±0.11b-d	1.06±0.22bc	1.09±0.15bc	0.53±0.10f-h	0.82±0.16c-e	0.87±0.13b-d	0.90±0.17b-d
Myrcene	1155	7,82	26.10±2.56cd	20.75±0.27ef	21.40±1.55ef	19.89±1.93e-g	27.98±0.76bc	23.05±3.59de	15.62±0.64gh	19.38±1.74e-g	21.67±1.37ef	18.18±0.27fg
Commercial range			**20-38**	**45-60**		**24-28**	**30-45**	**28-31**	**27-42**	**44-55**	**20-35**	**30-40**
Limonene	1187	8,64	0.37±0.03cd	0.54±0.01ab	0.31±0.06d-f	0.16±0.07g-j	0.37±0.03cd	0.68±0.06a	0.25±0.02d-h	0.17±0.03g-j	0.27±0.01d-g	0.27±0.05d-g
Butyl isobutyrate	1197	8,92	0.41±0.04g-i	0.28±0.01hi	0.28±0.03hi	n.d.	0.25±0.12hi	0.39±0.05g-i	0.30±0.01hi	0.74±0.56fg	0.14±0.01i	0.59±0.09gh
Heptanoic acid, methylester	1290	11,46	0.82±0.09b-d	0.39±0.01f-i	1.06±0.13ab	0.50±0.06e-g	0.61±0.12d-f	0.63±0.08d-f	0.33±0.02g-j	0.77±0.15cd	0.95±0.16a-c	0.46±0.08e-h
Methyl-4-ethyl-4 pentanoate	1326	12,44	0.55±0.05a-d	n.d.	0.56±0.08a-c	n.d.	0.45±0.06b-f	0.07±0.01ij	0.30±0.01d-i	0.24±0.23f-j	0.56±0.07a-c	0.19±0.03g-j
Methyl-6-methyl eptanoate	1332	12,60	0.51±0.06bc	0.30±0.03e-h	0.36±0.04d-f	0.28±0.03e-i	0.39±0.05c-e	0.17±0.02h-l	0.24±0.00f-j	0.46±0.08b-d	0.35±0.06d-g	0.11±0.02k-m
Ethylhexanol	1439	15,50	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.94±0.81a
5,9-Tetradecadiene	1477	16,45	0.14±0.01f-i	0.14±0.01f-i	0.12±0.02h-k	0.24±0.04b	0.13±0.01g-j	0.11±0.01h-l	0.20±0.00b-e	0.13±0.02f-j	0.15±0.04e-h	0.19±0.05b-f
Copaene	1492	16,84	0.53±0.06e-g	0.47±0.02f-h	0.44±0.04f-j	n.d.	0.47±0.00f-h	0.32±0.06i-l	0.70±0.01c	0.48±0.07f-h	0.54±0.15d-f	0.67±0.13cd
Linalool	1535	17,92	1.77±0.16b	0.89±0.05e-h	1.07±0.12d-f	1.12±0.14de	1.20±0.42de	0.52±0.08i-l	0.99±0.05ef	1.06±0.18d-f	1.12±0.22de	0.96±0.20e-g
10,12-Octadecadynoic acid	1577	18,96	0.12±0.01jk	n.d.	0.48±0.04cd	n.d.	n.d.	0.30±0.07gh	0.37±0.02fg	0.19±0.03ij	n.d.	0.41±0.10d-f
β-Caryophyllene	1584	19,14	13.4±0.61b-e	9.55±0.05i-l	12.23±0.88d-h	15.88±0.84a	13.14±0.81b-f	10.64±0.02h-k	13.09±0.5b-f	9.65±0.30i-l	11.18±0.86g-i	12.35±0.75d-h
Commercial range			**10 – 15**	**3 – 6**		**9 – 13**	**8 – 12**	**11 – 12**	**7 – 10**	**8 – 12**	**6 – 11**	**7 –8**
Decenoic acid, methyl ester	1613	19,84	1.90±0.21ab	1.02±0.09d-f	1.40±0.17cd	n.d.	1.99±0.29ab	1.90±0.30ab	2.06±0.10a	1.32±0.28c-e	n.d.	n.d.
trans-β-Farnesene	1645	20,60	n.d.	7.58±0.14cd	0.96±0.01h-j	n.d.	n.d.	15.95±1.48a	1.22±0.76h-j	0.03±0.03j	5.28±1.59e-g	6.75±0.64de
Commercial range			**1,00**	**3 – 7**		**5 – 7**	**1,00**	**1 – 3**	**1,00**	**1,00**	**16 – 24**	**5 – 6**
α-Humulene	1656	20,87	16.49±1.74h-l	13.86±0.03k-m	17.44±1.59g-k	22.68±1.50b-d	20.25±1.99c-h	12.8±1.49l-n	18.11±0.93e-j	18.52±3.09e-j	17.78±1.35f-k	18.46±0.67e-j

Commercial range			**20 – 38**	**8 – 13**		**33 – 38**	**30 – 45**	**26 – 33**	**16 – 19**	**17 – 22**	**22 – 32**	**20 – 27**
Germacrene D	1673	21,39	1.76±0.14gh	1.73±0.10g-i	1.81±0.22f-h	3.78±0.47b	1.64±0.01g-j	1.38±0.32h-k	2.40±0.23de	1.48±0.19g-k	1.89±0.40e-h	2.36±0.12d-e
(z,Z)-α-Farnesene	1678	21,46	1.27±0.11d-g	1.42±0.04c-g	1.73±0.22a-d	2.17±0.44a	1.22±0.06e-h	1.80±0.42a-c	2.1±0.24ab	1.28±0.12d-g	1.74±0.37a-d	1.18±0.08e-h
Methyl geranate	1681	21,97	0.36±0.04h-o	0.71±0.06d-k	0.45±0.05h-o	0.77±0.21d-h	0.59±0.26d-l	2.89±0.63a	0.34±0.02h-o	0.74±0.07d-i	0.47±0.17g-n	0.57±0.03e-m
β-Selinene	1703	22,09	1.70±0.12de	2.08±0.01de	7.23±0.14c	1.02±0.23de	1.53±0.05de	1.33±0.32de	3.5±0.25d	7.31±0.5c	6.67±0.7c	1.20±0.09de
α-Selinene	1708	23,09	2.38±0.21fg	3.14±0.02fg	8.05±0.46c-e	2.01±0.30fg	2.09±0.08fg	2.19±0.55fg	4.75±0.28ef	8.83±0.44cd	7.76±0.67c-e	2.78±0.17fg
α-Farnesene	1752	23,24	0.17±0.02g-i	0.36±0.05d-f	0.43±0.07de	0.16±0.03g-i	0.12±0.04hi	n.d.	0.40±0.05d-f	0.91±0.16a	0.44±0.13de	0.67±0.10bc
τ-Cadinene	1761	23,29	2.42±0.64d-i	5.62±0.61a	3.36±0.29b-d	5.88±0.36a	2.79±0.04d-g	2.43±0.69d-i	4.06±0.28bc	2.61±0.41d-h	3.07±0.67c-e	4.31±0.35b
δ-Cadinene	1763	23,99	1.22±0.28c-i	1.67±0.41b-e	1.38±0.16b-g	2.98±0.46a	1.36±0.05b-h	1.01±0.26e-j	1.88±0.04bc	1.21±0.12d-i	1.50±0.35b-e	1.91±0.25b
2-tridecanone	1813	24,04	0.90±0.07g-j	2.55±0.07b	0.98±0.14f-i	0.38±0.07jk	1.40±0.57c-g	n.d.	1.69±0.06c-e	0.53±0.10i-k	1.01±0.25f-i	0.24±0.05k
1,4-undecadiene	1914	26,59	n.d.	n.d.	n.d.	n.d.	0.17±0.05fg	n.d.	n.d.	n.d.	n.d.	n.d.
Caryophyllene oxide	1970	27,74	1.80±0.24c-e	1.37±0.31d-g	0.21±0.02kl	2.67±0.42b	1.08±0.50e-j	0.53±0.32g-l	0.82±0.48f-l	0.98±0.11e-k	1.22±0.16d-h	1.72±0.22c-e
2-dodecadiene	1999	28,68	n.d.	0.55±0.07l-p	0.22±0.03n-q	n.d.	1.89±0.06d-f	0.08±0.07pq	0.47±0.13m-q	0.54±0.01l-q	0.70±0.08k-n	3.22±0.44a
Humulene epoxyde II	2016	29,30	2.17±0.22bc	2.48±0.56bc	0.48±0.05de	4.86±0.71a	n.d.	n.d.	1.89±0.56bc	2.45±0.18bc	2.78±0.21bc	n.d.
17-Octadecen-14-yn-1-ol	2080	30,01	0.36±0.02d-g	0.25±0.03e-j	0.12±0.03h-k	0.31±0.10d-h	0.37±0.03d-g	0.16±0.04g-k	0.75±0.07b	0.11±0.10h-k	0.14±0.03g-k	0.28±0.11e-i
Globulol		30,70	1.84±0.12c	2.33±0.03b	1.07±0.16de	0.61±0.15fg	1.36±0.38d	0.23±0.06hi	3.88±0.09a	0.59±0.14fg	1.30±0.34d	0.22±0.08hi
Germacrene-D-4-ol		32,85	0.3±0.01e-i	0.32±0.01e-i	0.31±0.04e-i	0.63±0.14de	0.25±0.04e-i	0.18±0.04f-i	0.4±0.03d-i	0.31±0.10e-i	0.39±0.12d-i	0.45±0.10d-i
E,E Farnesol		34,68	n.d.	0.80±0.07de	0.57±0.04de	0.84±0.32de	n.d.	4.33±1.18a	0.18±0.10e	2.01±0.17bc	n.d.	2.70±0.83b
Total			82,34	84,63	87,54	90,75	86,57	86,64	83,85	86,00	91,89	87,54
α-Humulene/β-Caryophyllene			1,23	1,45	1,43	1,43	1,54	1,20	1,38	1,92	1,59	1,50

Likely as a consequence of agronomic selection on the plant secondary metabolism, few substances (myrcene, β-caryophyllene, α-humulene and δ-cadinene) were largely representative in the cultivars, while many different substances were present in trace amounts in plants of wild origin. For instance, 2-dodecanone, 2-decanone, nonanal, copaene, selina 3,7 (11) diene, *cis*-bisabolene, α-curcumene, *Ar*-curcumene and calamenene were produced only by wild hops. Overall, if compared with commercial cultivars grown under the same conditions, Italian hops produced lower averages of myrcene (8.2 versus 21.4%) and α-humulene (7.6 versus 17.7%) and higher amounts of α- and β-selinene (21.2 versus 7.8%). Myrcene has gained an increasing relevance for its green to spicy notes prized in beer-making, in particular for dry-hopping. In our screening, however, myrcene content ranged from less than 5 to 13%, with values slightly above 10% recorded only for ET8, ET8-1, Cinghio and Pasqui 2. These data are hardly comparable to commercial cultivars, in which the myrcene content of essential oils was at least two-folds greater. This compound was previously reported to be in the 12-22% range in wild accessions, with the exception of hops collected in Lithuania (7.4%), while selected commercial hop varieties usually average more than 30% (Bernotiene et al., 2004; Patzak et al., 2010b). At the same time, it must be noticed that most data regarding wild hops comes from plant material collected directly in the wild and not under controlled agronomic conditions, a factor that may alter some metabolic pathways while providing most reliable, reproducible and production-oriented data. Regarding other constituents relevant from a brewing standpoint, Italian ecotypes had a β−caryophyllene content comprised between 5 and 14.8%, with limited variability among accessions and values slightly lower or comparable to commercial cultivars. On the contrary, α−humulene abundance was extremely variable and ranged between 0.8% (Piattello) and 24.8% (Roggino 2), allowing the definition of two clearly distinct clusters: low and high α-humulene producers, the latter being composed by Cinghio, Tavernelle, Roggino 2 and Tange, whose content was higher or at least comparable to commercial cultivars grown in the same field. Noticeably, these ecotypes were also the best performers in terms of essential oil yield and forecasted production (Table 1). This behaviour confirms after a full acclimatization some preliminary evaluations obtained from the same genetic pool (Mongelli et al., 2015). The pattern for these sesquiterpenes is superimposable to the one reported from wild plants collected in Europe and grown under field conditions (Patzak et al., 2010a; Patzak et al., 2010b). The α−humulene vs. β−caryophyllene ratio, whose greater value is considered as a proxy of quality for aromatic hops, ranged between 0.15 and 1.68, with the best ratios provided once again by

Tavernelle, Roggino 2 and Tange. The most striking difference between cultivars and ecotypes lies within the different abundance of selinene isomers, with the sole exception of Roggino 2, for which the total selinene content was similar to commercial cultivars. Ecotypes Livello and Bibbiano in particular had a total content of compounds of the selinene group exceeding 36%. These data are in agreement with previous observations, in which an high selinene content was deemed to be characteristic of European wild genotypes if compared with North American ones (Patzak et al., 2010b). However, it must be highlighted that Italian samples contained on average almost two times more selinene isomers than the average of European samples (21% versus 11%) (Patzak et al., 2010b). Such trend represents a limitation to the potential exploitation of wild Italian hops for breeding purposes, at least concerning their aromatic quality. On the contrary, selinenes were rarely above the 10% limit (Challenge, Target, Tettnanger) in commercial cultivars grown under the same conditions. Farnesene isomers, and trans-β-farnesene in particular, are prized in the so-called noble aroma hops, in which their abundance usually exceeds 15%, but are not biosynthesized by all aroma hop varieties (Barth-Haas Hops Companion, 2009; Variety Manual USAHops, 2011). The presence of *trans*-β-farnesene, cis-β-farnesene and *z,z*-α-farnesene was fair but irregular in the Italian germplasm, with values in agreement with previous data from wild European hops and in some cases comparable with the amount scored by commercial cultivars grown in the same plot. On this regard, genotypes ET8, ET8-1 and Tavernelle were the best performers, making them a potential starting point for the development of aromatic, finishing hops.

3.3. Comparing cultivars grown in Northern Italy with commercial samples

The material produced by hop cultivars under the Italian climate was in agreement with commercial specifications, with some exceptions (Table 3). Target, Nugget, Willamette and Cascade, in particular, had a myrcene content almost halved if compared with the average accepted by the marketplace for high-quality hops of the same cultivars, while only for Bramling Cross, Magnum, Tettnager results were close to the suggested range (Variety Manual USAHops, 2011; Inui et al., 2013). All the cultivars provided amounts of α-humulene and of β-caryophyllene respectively lower and higher than the expected; in particular Cascade, Fuggle, Magnum, Nugget and Willamette had a β-caryophyllene content above the range usually reported for these cultivars. Selinene isomers were present in all the evaluated samples, with values ranging between 3% (Fuggle) and 16% (Target). With the sole exception of Challenger, the values reported by commercial cultivars cultivated in Northern Italy were higher than those

obtained from hops of the same cultivars collected elsewhere (Table 4), leading to suggest that an increased biosynthesis of these substances may be related to an adaptation to the subalpine climate or to the exposure to local biotic stress. On average, samples from Marano sul Panaro (MO, IT) had an average content of selinene isomers of 7.7% compared with 3.6% produced by commercial samples. Remarkably, Marynka presented a profile particularly rich in β-farnesene (>15%), a trait that might be valuable for dry-hopping and for the production of more floral bouquets in beers (Huell-Lutz et al., 2011; Nance et al., 2011). If compared with samples actually available on the marketplace, however, the percentage of myrcene in cultivars grown in Italy was higher in many occasions. This evidence contradicts common knowledge and may be related to harsher drying conditions or to non uniform storage of commercial material. In some occasions the quality of the samples cultivated in northern Italy was better than the commercial material, as for Magnum, Marynka and Tettnanger. Taking into account all the parameters evaluated, Cv Brangling Cross, Magnum, Marynka and Tettnanger seem to be the most viable for cultivation in Northern Italy in terms of aromatic quality, with Marynka being also by large the best performer in quantitative terms.

Further investigations may be warranted to confirm if the ecological and climatic conditions of subalpine zones may be the most suitable for a regular production of finishing hops.

Table 4. *Phytochemical composition of H. lupulus cultivars acquired from the marketplace*

Compound	KI	RT	Brangling Cross	Cascade	Challenger	Fuggle	Magnum	Marinka	Nugget	Target	Tettnanger	Williamette
						%						
α-Pinene	1096	6,29	0.21±0.00d-g	0.11±0.01e-i	0.01±0.01i	0.23±0.01d-f	0.84±0.02a	0.18±0.01d-h	0.35±0.03b-d	0.41±0.04bc	n.d.	n.d.
β-Pinene	1110	6,59	0.43±0.04g-j	1.13±0.01b	0.12±0.01k-m	0.22±0.01i-m	0.87±0.06b-d	0.76±0.02d-f	0.48±0.02f-i	0.36±0.04h-k	0.58±0.07e-h	0.69±0.01d-g
Myrcene	1155	8,03	18.78±0.98e-g	47.13±1.76a	3.33±0.08n	8.02±0.01j-m	30.31±0.31bc	18.77±0.98e-g	31.27±0.53b	5.01±0.0l-n	13.35±0.62hi	20.42±0.89ef
Commercial range			**20-38**	**45-60**		**24-28**	**30-45**	**28-31**	**27-42**	**44-55**	**20-35**	**30-40**
Limonene	1187	8,72	0.10±0.09i-k	0.15±0.02g-j	0.22±0.14e-i	0.51±0.08bc	0.34±0.01de	0.29±0.03d-g	0.11±0.02h-k	0.29±0.02d-g	0.17±0.03f-j	n.d.
Butyl isobutyrate	1197	8,92	2.35±0.21d	1.06±0.04f	0.57±0.16gh	2.92±0.04c	6.20±0.18a	2.85±0.24c	1.89±0.03e	4.12±0.11b	0.30±0.06hi	0.62±0.03gh
Heptanoic acid, methylester	1290	11,46	0.14±0.01i-k	0.20±0.01i-k	0.06±0.05k	0.08±0.07jk	1.18±0.08a	0.19±0.02i-k	0.14±0.01i-k	0.22±0.03h-k	0.65±0.05de	0.11±0.02jk
Methyl-4-ethyl-4 pentanoate	1326	12,75	0.62±0.03ab	0.08±0.01ij	0.31±0.05c-i	0.27±0.03e-i	0.51±0.01a-e	0.30±0.01d-i	0.22±0.01f-j	0.71±0.02a	0.45±0.06b-f	0.13±0.01g-j
Methyl-6-methyl eptanoate	1332	12,93	0.51±0.00bc	0.13±0.00j-m	0.16±0.02i-m	0.22±0.11g-k	1.14±0.04a	0.41±0.03c-e	0.35±0.00d-g	0.54±0.01b	0.40±0.04c-e	0.11±0.01k-m
2-Nonanone	1380	13,90	0.35±0.00d-f	n.d.	0.03±0.02jk	n.d.	0.09±0.03ij	0.12±0.01i	n.d.	n.d.	0.08±0.01ij	0.04±0.01jk
Nonanal	1398	14,35	0.22±0.03gh	0.09±0.01gh	0.08±0.01gh	0.23±0.01gh	0.71±0.01de	0.05±0.00gh	0.22±0.01gh	0.10±0.01gh	0.34±0.03fg	0.05±0.01gh
Ethylhexanol	1439	15,50	0.05±0.01b	n.d.	0.02±0.02b	0.12±0.01b	0.10±0.01b	0.07±0.01b	0.09±0.01b	0.2±0.15b	n.d.	0.01±0.01b
5,9-Tetradecadiene	1477	16,45	0.14±0.00f-h	0.07±0.01k-n	n.d.	0.2±0.01b-e	0.11±0.02h-l	0.16±0.01d-h	0.12±0.01g-k	0.36±0.03a	0.15±0.00e-h	0.21±0.01b-d
Copaene	1492	16,84	0.50±0.01f-h	0.21±0.01l-n	0.09±0.06no	0.65±0.01c-e	0.31±0.01j-l	0.55±0.02d-f	0.36±0.00h-k	1.18±0.01a	0.46±0.01f-i	0.72±0.02bc
2-Decanone	1497	16,95	0.07±0.03j-l	0.08±0.02i-l	0.18±0.03f-h	0.31±0.03b-d	0.28±0.03c-e	0.13±0.02h-j	0.16±0.02g-i	0.43±0.05a	0.37±0.02ab	0.04±0.03kl
2-Nonanol	1503	17,52	n.d.	n.d.	0.01±0.01g	0.12±0.01c	0.29±0.03a	n.d.	n.d.	0.07±0.01de	0.19±0.00b	0.02±0.02fg
Linalool	1535	18,27	1.68±0.03bc	0.59±0.01h-k	0.63±0.05g-j	0.94±0.02e-h	0.94±0.02e-g	0.73±0.06f-i	0.77±0.02f-i	2.17±0.01a	0.98±0.01e-g	1.36±0.05cd
cis–β-Farnesene	1575	18,91	0.26±0.01g	0.17±0.01hi	0.13±0.03ij	0.36±0.03f	0.16±0.01hi	0.25±0.01g	0.13±0.00ij	0.42±0.05e	0.16±0.01hi	0.18±0.02h
10,12-Octadecadiynoic acid	1577	18,96	n.d.	n.d.	0.07±0.01kl	n.d.	0.11±0.01jk	0.48±0.03c-e	0.26±0.00hi	n.d.	1.21±0.04a	0.51±0.05cd
β-Caryophyllene	1584	19,48	9.63±0.07i-l	5.95±0.23p-r	7.19±0.06n-q	7.63±0.20m-q	6.09±0.04p-r	8.73±0.06l-o	12.99±0.13c-g	4.61±0.09r	5.83±0.01qr	11.79±0.34e-h
Commercial range			**10–15**	**3–6**		**9–13**	**8–12**	**11–12**	**7–10**	**8–12**	**6–11**	**7–8**
2-undecanone	1601	19,56	2.30±0.04c	0.19±0.01hi	3.46±0.02b	2.50±0.09c	1.25±0.04de	0.90±0.03ef	0.27±0.03g-i	4.39±0.04a	2.16±0.09c	0.23±0.01hi
Decenoic acid, methyl ester	1613	20,28	0.22±0.01j-l	0.51±0.02h-j	0.99±0.12e-g	0.29±0.03j-l	0.24±0.03j-l	0.22±0.03j-l	1.63±0.02bc	0.37±0.04i-l	0.59±0.06h-j	0.08±0.01kl
trans-β-Farnesene	1645	21,17	n.d.	4.51±0.16g	0.58±0.02h-j	1.94±0.82h	n.d.	3.74±0.40g	0.22±0.04ij	0.05±0.05j	6.54±0.02d-f	4.97±0.14fg
Commercial range			**1,00**	**3–7**		**5–7**	**1,00**	**1–3**	**1,00**	**1,00**	**16–24**	**5–6**
α-Humulene	1656	21,23	21.63±0.54b-f	15.83±0.56i-l	19.25±3.18d-i	21.94±0.33b-e	21.15±0.02b-g	23.74±0.83bc	28.46±0.28a	11.08±0.23mn	20.01±0.32c-h	24.57±1.15ab
Commercial range			**20–38**	**8–13**		**33–38**	**30–45**	**26–33**	**16–19**	**17–22**	**22–32**	**20–27**
Germacrene D	1673	21,67	1.64±0.09g-j	0.69±0.07l	1.62±0.12g-j	1.96±0.08e-g	1.03±0.01kl	1.43±0.05g-k	1.15±0.01j-l	2.89±0.05cd	1.60±0.09g-j	1.74±0.07g-i
(z,Z)-α-Farnesene	1678	21,77	0.97±0.05g-j	0.50±0.03j-l	0.45±0.07kl	1.21±0.06e-h	1.55±0.08c-f	0.65±0.04i-l	1.23±0.02e-h	0.96±0.10g-j	1.14±0.02f-h	0.24±0.00l
Methyl geranate	1681	21,87	0.21±0.00l-o	0.38±0.00h-o	0.27±0.12j-o	0.25±0.03k-o	1.00±0.04c-e	1.06±0.07cd	0.1±0.01no	0.96±0.05c-f	0.11±0.09m-o	0.51±0.02f-n
β-Selinene	1703	22,38	1.33±0.06de	1.15±0.01de	6.45±0.39c	0.51±0.15e	0.57±0.13e	0.57±0.02e	1.87±0.03de	1.38±0.06de	0.36±0.03e	0.86±0.06de
α-Selinene	1708	22,50	1.84±0.05fg	1.56±0.02fg	6.90±0.21de	1.15±0.11fg	0.86±0.19g	1.00±0.04g	1.87±0.01fg	1.99±0.06fg	1.00±0.04g	1.95±0.06fg
α-Farnesene	1751	23,05	0.03±0.02i	0.10±0.00hi	0.09±0.08hi	n.d.	n.d.	n.d.	0.11±0.01hi	n.d.	n.d.	0.43±0.06de
τ-Cadinene	1761	23,24	2.03±0.15e-j	2.88±0.18d-f	1.85±0.05f-k	1.79±0.17g-k	1.07±0.05jk	1.75±0.14g-k	1.66±0.06h-k	2.44±0.30d-i	1.44±0.06i-k	2.65±0.04d-h

δ-Cadinene	1763	23,29	1.14±0.07d-i	n.d.	1.51±0.31b-e	1.60±0.13b-e	0.69±0.07i-k	1.13±0.08e-i	0.84±0.05f-k	2.71±0.19a	1.19±0.06d-i	1.46±0.12b-f
Geranyl butyrate	1792	23,99	0.23±0.01de	0.06±0.02fg	0.31±0.07b	n.d.	n.d.	0.24±0.02cd	0.17±0.02e	0.57±0.00a	0.21±0.05de	0.36±0.02b
2-Tridecanone	1795	24,42	1.54±0.01c-f	1.43±0.11c-g	3.18±0.44a	1.71±0.11cd	0.50±0.01i-k	1.69±0.01c-e	0.49±0.02i-k	3.55±0.02a	1.42±0.11c-g	0.21±0.00k
Geranoyl formate	1802	24,62	0.2±0.03c	0.73±0.06a	0.24±0.04c	0.21±0.01c	n.d.	0.35±0.02b	0.10±0.03d	0.23±0.01c	0.13±0.01d	0.03±0.03e
1,4-Undecadiene	1914	27,00	0.43±0.01b	0.32±0.02c	0.32±0.06c	0.21±0.03d-f	0.18±0.02e-g	0.23±0.00de	0.13±0.01gh	0.88±0.01a	0.37±0.02c	0.08±0.01h
Caryophyllene oxide	1970	28,13	3.86±0.09bc	0.80±0.17h-l	2.95±0.71b-d	7.44±0.36a	0.74±0.10i-l	2.22±0.27d-i	0.61±0.05i-l	3.60±0.04d-f	4.49±0.33bc	2.25±0.13c-f
2-Dodecadiene	1999	29,10	1.64±0.05e-h	0.15±0.00o-q	1.41±0.16f-i	0.78±0.10j-m	0.22±0.02n-q	0.49±0.04m-q	0.4±0.01m-q	2.24±0.03cd	0.65±0.01k-o	0.14±0.01o-q
Humulene epoxyde II	2016	29,29	n.d.	n.d.	n.d.	n.d.	1.65±0.33cd	n.d.	n.d.	4.71±0.28a	n.d.	n.d.
Caryophyllene oxide II	2044	29,30	5.83±0.32cd	2.08±0.17e	6.80±2.34c	12.69±0.91a	0.25±0.02f	4.28±0.68d	0.92±0.05ef	1.20±0.05ef	9.03±0.98b	4.28±0.15d
Caryophyllenylalcohol	2054	29,47	0.24±0.02f-j	0.14±0.01g-k	0.48±0.26de	1.39±0.09a	0.18±0.03g-k	0.18±0.03g-k	0.18±0.04g-k	n.d.	0.05±0.05i-k	0.22±0.02f-k
Cubenol	2066	29,71	0.17±0.02c-f	0.07±0.00e-h	0.17±0.14c-f	0.23±0.06a-c	n.d.	0.21±0.03a-d	0.11±0.03c-h	0.32±0.08a	0.11±0.10c-h	0.23±0.06a-c
17-Octadecen-14-yn-1-ol	2080	29,99	2.20±0.12d-g	1.13±0.04h-j	2.40±0.51c-f	2.07±0.21e-g	0.97±0.07i-k	1.61±0.24g-i	0.86±0.04jk	2.99±0.19bc	2.79±0.02b-d	0.38±0.07kl
Globulol		31,12	0.10±0.09hi	0.10±0.01hi	0.44±0.10gh	0.72±0.11e-g	0.08±0.03i	0.10±0.01hi	0.14±0.01hi	n.d.	0.87±0.01ef	0.19±0.03hi
Germacrene-D-4-ol		32,85	0.35±0.03d-i	0.17±0.04g-i	0.45±0.06d-i	0.47±0.05d-h	0.17±0.02g-i	2.17±0.00a	0.16±0.01g-i	1.21±0.05c	0.26±0.01e-i	0.52±0.03d-h
E,E Farnesol		35,27	0.60±0.05de	0.25±0.01e	0.18±0.01e	0.22±0.02e	n.d.	0.62±0.01de	0.29±0.02e	1.22±0.03cd	0.17±0.14e	1.94±0.11bc
Total			86,19	91,49	77,06	86,27	84,83	85,49	94,88	73,06	82,49	87,57
α-Humulene/β-Caryophyllene			2,25	2,66	2,68	2,88	3,47	2,72	2,19	2,40	3,43	2,08

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3.6. Study 6 - Development of a new high-performance liquid chromatography method with diode array and electrospray ionization-mass spectrometry detection for the metabolite fingerprinting of bioactive compounds in Humulus lupulus L.

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Development of a new high-performance liquid chromatography method with diode array and electrospray ionization-mass spectrometry detection for the metabolite fingerprinting of bioactive compounds in Humulus lupulus L.

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Keywords: Humulus lupulus; Hops; Prenylflavonoids; Bitter acids; Fused-core; HPLC.

Abstract

The study was aimed at developing a new analytical method for the metabolite fingerprinting of bioactive compounds in Humulus lupulus L. (hop), together with a simple extraction procedure. Different extraction techniques, including maceration, heat reflux extraction (HRE), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE), were compared in order to obtain a high yield of the target analytes. Dynamic maceration for 30 min with MeOH–HCOOH (99:1, v/v) as the extraction solvent provided the best result in terms of recovery of secondary metabolites. The analysis of hop constituents, including prenylflavonoids and prenylphloroglucinols (bitter acids), was carried out by means of HPLC-UV/DAD, HPLC-ESI-MS and MS^2, using an ion trap mass analyzer. An Ascentis Express C column (150 mm × 3.0 mm I.D., 2.7 m) was used for the HPLC analysis, with a mobile phase composed of 0.25% formic acid in both water and acetonitrile, under gradient elution. The method validation was performed to show compliance with ICH guidelines. The validated technique was successfully applied to the phytochemical analysis of ten commercial cultivars and twenty-three wild Italian hop genotypes, thus demonstrating to be a reliable and useful tool for the comprehensive multi-component analysis of hop secondary metabolites.

1. Introduction

Female strobiles (cones) of Humulus lupulus L. (hop) enjoy a wide spread use that encompasses pharmaceuticals, nutraceuticals, food and cosmetics. This plant material, in fact, represents both a key flavoring and bitter agent in brewing and a phytotherapic ingredient in sedative or phytoestrogenic products [1,2]. Its presence in beer is also considered as the determinant of some nutritional benefits ascribed to such beverage [3,4]. Besides the brewing industry, extracts obtained from hops are nowadays applied in herbal medicines, food supplements, functional beverages and cosmetics, spurring a steady interest for their thorough fingerprinting and for the development of hop cultivars provided with peculiar phytochemical profiles or for the evaluation of large germplasm accessions [5].

Hop cones are characterized by an unique and complex pool of secondary metabolites, comprising both prenylflavonoids and prenylphloroglucinols [2,6]. Three classes of compounds are of particular relevance in relation to bitterness intensity, sensorial properties and health benefits: prenylchalcones (xanthohumol, XN; desmethylxanthohumol, DMX), prenylflavanones (isoxanthohumol, IXN; 6-prenylnaringenin, 6-PN; 8-prenylnaringenin, 8-PN)and prenylphloroglucinols, also known as bitter acids or hop acids(including both α and β) (Fig. 1) [2,6].

While the main role of bitter acids is to provide a bitter flavor, to increase beer conservation and to stabilize its foam, prenylflavonoids are relevant for their phytoestrogenic and chemo-preventive activities [3]. The prenylated fraction of hops, due to its weak estrogenic activity, has also been suggested to induce a protective effect against some hormone-dependent diseases [7]. The compounds responsible for the sedative action of hop extracts, which is more valuable from a therapeutic point of view, have not been fully understood, even though bitter acids seem to be involved in this activity [8,9].

However, all these secondary metabolites are known to be quite variable in the plant material, according to intra-specific chemodiversity, different collection stage or cultivation zone and to post-harvest handling [10,11]. The presence and abundance of IXN, 6-PN and 8-PN, in particular, is related to a series of rearrangements that occur via thermal or enzymatic activity from XN and DMX and, notwithstanding, by gut microflora and yeasts during beer manufacturing [3,6]. These phytoestrogens can be found as minor constituents also in hop resin, due to the effects of pre and post-harvest management [12]. Their quantification, together with prenylchalcones and bitter acids, may be of relevance when hops are used not just as a brewing material, but as a raw ingredient in food supplements or cosmetic products. Since the overall quality and properties of H. lupulus

derives from the synergistic and simultaneous intervention of the whole phytocomplex [6], a comprehensive and accurate fingerprinting is valuable to properly evaluate hops for both commercial (quality grading), technological (evaluation of their presence during industrial processing), agronomic (breeding of enhanced cultivars) and ecological reasons (evaluation of wild germplasm).

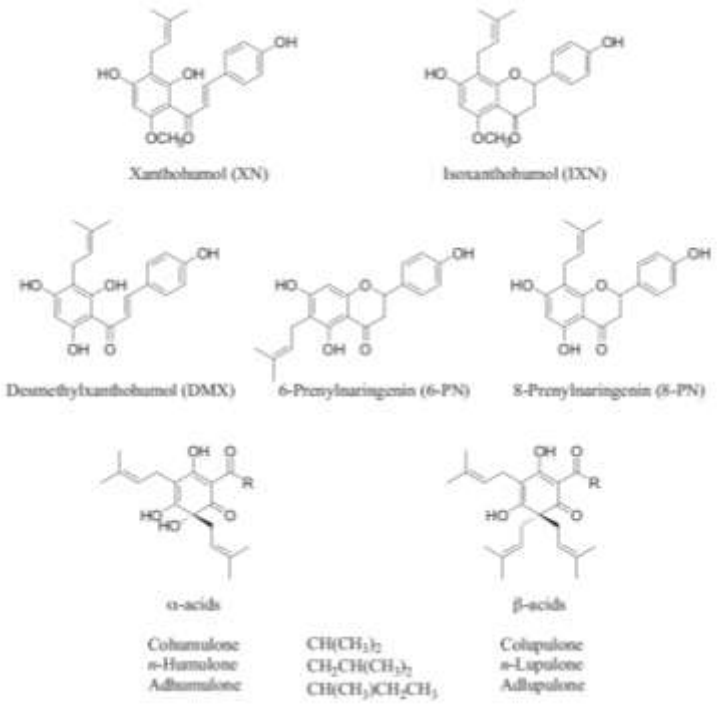

Figure 1. *Chemical structures of hop prenylflavonoids and bitter acids*

Several methods have been described in the literature to quantify prenylflavonoids and bitter acids in hop plant material, hop pellets, beer or beer by-products, and different approaches have been exploited [5]. Available protocols range from GC–MS with derivatization to HPLC-UV and HPLC-MS/MS, but upon closer attention many of these methods are focused on beer, brewing waste and biological fluids or are not properly validated [13–21]. In some cases, the literature involves techniques whose limitations in terms of sensitivity, easiness of use, sample pre-treatment or reduced availability of proper laboratory devices hinder a routine use [22–25]. Moreover, many of the existing methods applied to hop plant material are focused on a single class of compounds (bitter acids or prenylchalcones, and/or single prenylflavanones) and do not provide at the same time a rapid, fully validated and omni-comprehensive fingerprinting method for crude plant material [13,18,25–31].

Another critical step in the phytochemical analysis of H. lupulus plant material is represented by the sample preparation, both for its influence on the overall analytical throughput and for the relative instability of some hop constituents [5]. While IXN as well as 6-PN and 8-PN are usually stable under common extractive and analytical conditions, their common precursors XN and DMX may isomerize if proper conditions of pH, temperature and aqueous media occur [5,6]. Indeed, XN can isomerize into its corresponding prenylflavanone IXN, while DMX isomerizes to generate a mixture of 6-PN and 8-PN, due to the presence of two free phenolic groups [5,6].

Therefore, the management of samples must be carefully evaluated and a thorough comparison between different extraction protocols must be performed, in particular when different classes of phytochemicals are involved. On this regard, while ultrasoundassisted extraction (UAE) with methanol (MeOH) or ethanol (EtOH) is considered a good approach for prenylflavonoids [5], other techniques, such as microwave-assisted extraction (MAE), have not been evaluated so far. As for bitter acids, supercritical carbon dioxide (CO_2) extraction (SFE) is usually recommended [6,17], since it is particularly suited to dissolve non-polar compounds in a rather selective way, but it does not extract polar constituents, such as prenylflavonoids, or only traces of them [6,17].

In the light of all the above, this study was aimed at optimizing and validating a simple and comprehensive extraction procedure of hop secondary metabolites and at the development of a new and fully validated HPLC method with UV/DAD and ESI-MS^n detection, using a fused-core stationary phase, for the fingerprinting of both prenylflavonoids and bitter acids in hop extracts. To the best of our knowledge, this is the first validated method based on the fused core technology for the simultaneous analysis of all the bioactive constituents of hops. The method was successfully applied to the characterization of ten commercial cultivars and twenty-three different wild Italian hop genotypes. The fields in which this method can be applied range from routine quality control and standardization of hop extracts, to germplasm evaluation and breeding of new hop cultivars with tailored, high content of bioactive and aromatic secondary metabolites, up to the metabolite fingerprinting of the plant material to be used in pharmaceutical and nutritional investigations, in particular when the effects of crude extracts are evaluated.

2. Materials and methods

2.1. Chemicals and solvents

XN, IXN, 6-PN and 8-PN were purchased from Sigma–Aldrich (Milan, Italy). All reference compounds were of chromatographic grade. Bitter acids mixture standard (international

calibrationextract, ICE-3) was from Labor Veritas Co. (Zürich, Switzerland).The mixture standard contained α-acids with 13.88% of cohumulone and 30.76% of n-humulone + adhumulone, and β-acids with 13.44% of colupulone and 10.84% of n-lupulone + adlupulone. Formic acid (HCOOH), HPLC-grade MeOH and acetonitrile (ACN) were from Sigma–Aldrich (Milan, Italy). Water (H2O) was purified using a Milli-Q Plus185 system from Millipore (Milford, MA, USA).

2.2. Plant material

Thirty-three samples of hop female strobiles were considered in this study, including ten cultivars (indicated in the text asCV1–CV10) and twenty-three wild Italian genotypes (indicated in the text as GEN1–GEN23). The information about collected commercial samples and their origin is described in Table 1.An additional commercial hop sample (Saaz from Czech Republic, labeled as CV11), purchased from a Czech Republic farm, was used for the optimization of the extraction and chromatographic methods, because it was available in higher amount. The wild Italian hop genotypes were grown in a field germplasm collection in Marano sul Panaro (Modena), Emilia-Romagna region, Italy. All samples were harvested in 2013 during the hop season (August–September), dried at 55°C with air flow for 12 h and stored(vacuum-packed) at -20°C until chemical analysis. The samples were ground on an IKA M20 grinder (Staufen, Germany) before the extraction.

Table 1 *Commercial hop cultivars considered in this study*.

Cultivar	Origin	Code
Target	England	CV1
Columbus	USA	CV2
Cascade	USA	CV3
Willamette	USA	CV4
Bramling cross	England	CV5
Tettnanger	Germany	CV6
Magnum	Germany	CV7
Challenger	England	CV8
Fuggle	England	CV9
Marynka	Poland	CV10

2.3. Extraction of hop secondary metabolites

Four extraction techniques were compared in order to obtain a high yield of both prenylflavonoids and bitter acids from hop samples. The applied sample-to-solvent ratio (w/v) and the extraction

solvent (MeOH–HCOOH (99:1, v/v)) were the same for all method supplied in this study. All extraction procedures were carried out induplicate. Maceration extraction was performed on 0.5 g of sample with10 mL of solvent at room temperature for 30 min under magnetic stirring. Heat-reflux extraction (HRE) was carried out by refluxing 0.5 g of sample with 20 mL of solvent at 75◦C for 1 h under stirring, using a water bath. UAE was performed on 0.5 g of sample with 10 mL of solvent at room temperature for 30 min, using an ultrasonic bath (SonorexRK-100H, Bandelin, Berlin, Germany). Regarding MAE, a weighed amount of sample (0.1 g) was extracted with 2 mL of solvent in a 10 mL glass vessel at 50, 75 and 100°C for 20 min under stirring, using a monomode focused microwave apparatus with a closed-vessel system (Discover instrument, CEM, Metthews, NC, USA). Microwave power, with a maximum of 300 W and a magnetron frequency of 2450 MHz, and pressure were dynamically adjusted by temperature and power feedback control. All mixtures obtained from the extraction procedures described above were centrifuged at 4000 rpm for 5 min and the supernatant solution was filtered under vacuum in a volumetric flask. For HRE, the extract was directly diluted to volume (25 mL) with the same solvent. For maceration, UAE and MAE, the residue of the first extraction was re-extracted as previously described. Finally, the filtrates of the two extractions were combined and brought to 25 ml (for maceration and UAE) or 5 mL (for MAE) in a volumetric flask. The extracts were then filtered using a 0.45 µm PTFE filter into a HPLC vial and injected into the HPLC system.

2.4. HPLC-UV/DAD analysis

HPLC analyses were performed on an Agilent Technologies (Waldbronn, Germany) modular model 1100 system, consisting of a vacuum degasser, a quaternary pump, an auto sampler, a thermostated column compartment and a diode array detector (DAD).The chromatograms were recorded using an Agilent Chemstation for LC and LC–MS systems (Rev. B.01.03). An Ascentis Express C18 column (150 mm × 3.0 mm I.D., 2.7 µm, Supelco, Bellefonte, PA, USA) was used, with a mobile phase composed of 0.25% HCOOH in both (A) H2O and (B) ACN. The gradient elution was modified as follows: 0–20 min from 35% to 75% B, 20–40 min from 75% to 100% B, which was held for 5 min. The post-running time was 10 min. The flow-rate was 0.5 mL/min. The column temperature was set at 30°C. The sample injection volume was 3 µL. The UV/DAD acquisitions were carried out in the range 190–500 nm and chromatograms were acquired at 290 nm (for prenylflavanones), 330 nm (for bitter acids) and 370 nm (for prenylchalcones). Three injections were performed for each sample.

2.5. HPLC-ESI-MS and MS2analysis

HPLC-ESI-MS and MS2analyses were performed using an Agilent Technologies modular 1200 system, equipped with a vacuum degasser, a binary pump, a thermostated autosampler, a thermostated column compartment and a 6310A ion trap mass analyser with an ESI ion source. The HPLC column and the applied chromatographic conditions were the same as reported for the HPLC-UV/DAD system. The flow rate was split 3:1 before the ESI source. The HPLC-ESI-MS system was operated both in the positive and in the negative ion mode. For the positive ion mode, the experimental parameters were set as follows: the capillary voltage was 3.5 kV, the nebulizer (N2) pressure was 35 psi, the drying gas temperature was 350°C, the drying gas flow was 11 L/min and the skimmer volt-age was 40 V. For the negative ion mode, the MS conditions were the same as described above, with the exception of the capillary voltage which was set at 4.0 kV. Data were acquired by Agilent 6300 Series Ion Trap LC/MS sys-tem software (version 6.2). The mass spectrometer was operated in the full-scan mode in the m/z range 100–1000. MS^2 spectra were automatically performed with helium as the collision gas in the m/z range 50–1000, using the SmartFrag function.

2.6. HPLC-UV/DAD method validation

The validation of the HPLC-UV/DAD method was performed in agreement with international guidelines for analytical techniques for the quality control of pharmaceuticals (ICH guidelines) [32]. Regarding linearity, the stock standard solution of each com-pound (XN, IXN, 6-PN, 8-PN and ICE-3) was prepared as follows: an accurately weighed amount of reference standard was placed into a volumetric flask (0.8–1.8 mg in 10 mL for prenylflavonoids, 27.7 mg in 10 mL for ICE-3); MeOH was added and the solution was diluted to volume. The external standard calibration curve was generated using six data points. Three µL aliquots of each standard solution were used for HPLC analysis. Injections were per-formed in triplicate for each concentration level. The calibration curve was obtained by plotting the peak area of the compound at each level versus the concentration of the sample. Since ICE-3 contains a mixture of α- and β-acids, the concentrations of the standard solutions of each compound were calculated according to the percentages stated by the manufacturer. The amount of prenylflavonoids and bitter acids in hop samples was determined by using these calibration curves. As regards DMX, which has the same chromophore as XN, it was quantified using XN calibration curve and its amount was corrected by using the molecular weight ratio. For reference compounds, the limit of detection (LOD) and the limit of quantification (LOQ) were experimentally determined by HPLC analysis of serial dilutions of a standard solution to reach

a signal-to-noise (S/N) ratio of 3 and 10, respectively. The accuracy of the analytical procedure was evaluated using the recovery test. This involved the addition of a known quantity of standard compound to half the sample weight of grounded sample (CV11) to reach 100% of the test concentration. The fortified samples were then extracted and analyzed with the proposed method. The precision of the extraction technique was validated by repeating six times the extraction procedure on the same hop sample (CV11). An aliquot of each extract was then injected and quantified. The precision of the chromatographic system was tested by performing intra- and inter-day multiple injections of a hop extract (from sample CV11) and then checking the %RSD of retention times and peak areas. Six injections were performed each day for three consecutive days. The stability was tested using extracts from sample CV11 stored in amber glass flasks at 4◦C and at room temperature (about 25°C) and analyzed every 12 h for 72 h.

2.7. Statistical analysis

In order to have an overview of quantitative results obtained by HPLC-UV/DAD analyses, all thirty-three hop samples were submitted to multivariate statistical analysis by means of principal component analysis (PCA), using R-based chemometric software developed by the Group of Chemometrics of the Division of Analytical Chemistry of the Italian Chemical Society (freely downloadable from http://gruppochemiometria.it/gruppo-lavoro-r-in-chemiometria.html).

3. Results and discussion

3.1. Optimization of chromatographic conditions

The aim of this work was to develop a new analytical method for the metabolite fingerprinting of hop secondary metabolites, together with a simple extraction procedure. Regarding the HPLC method, even if there are other studies where prenylflavonoids and bitter acids have been analyzed simultaneously, albeit from different material and matrices [18,21], the complete separation of hop acids represents a challenging issue [18,21,33]. To do this, Česlová et al. [18] have used a C8column for the analysis of hop extracts and beer, but the method has not been completely validated. Kao and Wu [21] have used a C18column at high flow-rate to have an acceptable analysis time for beer lee extracts. In this study, the chromatographic performance of four columns, including two fully porous (Ascentis C18, 250 mm × 4.6 mm I.D., 5 µm, Supelco and Zorbax SB-C18, 150 mm × 4.6 mm I.D., 5 µm,Agilent Technologies) and two fused-core (Ascentis Express C18, 150 mm × 3.0 mm I.D., 2.7 µm, Supelco and Ascentis Express C18,150 mm × 4.6 mm I.D., 2.7 µm, Supelco), was evaluated. It is note-worthy that the fused-core technology was applied to the analysis of hop extracts for the first time in this work. An acidified mobile phase composed of

0.25% HCOOH in both H2O and ACN, under a suitable gradient elution, was used. The presence of HCOOH in the mobile phase is highly recommended, because a low pH value provides a better peak shape [5] and improves the resolution, especially for bitter acids [18]. The best separation for all hop components was achieved in only 35 min using the Ascentis Express C18column (150 mm × 3.0 mm I.D., 2.7 µm, Supelco). Compared with the fully porous stationary phases (Fig. 2), this column allowed to obtain a better chromatographic performance (in terms of both res-olution and sensitivity), a shorter analysis time and a considerable saving of solvent consumed, working at a flowrate of 0.5 mL/min instead of 1.0 mL/min; therefore, it was finally selected for use in this study.

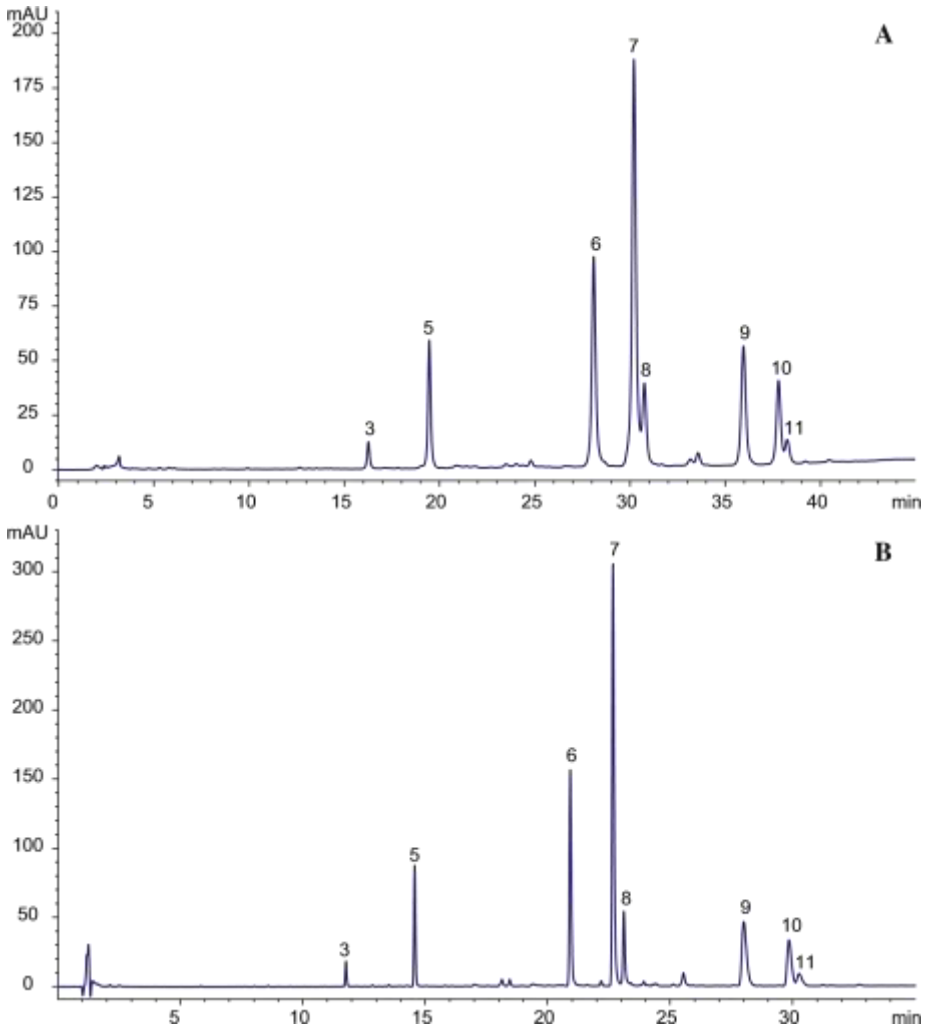

Figure 2. *Chromatograms obtained by HPLC-UV/DAD analysis of a hop extract (sample CV2) at 330 nm. Columns: (A) Ascentis C_{18} (250 mm × 4.6 mm I.D., 5 m, Supelco). (B) Ascentis Express C_{18} (150 m × 3.0 m I.D., 2.7 m, Supelco). For column B, experimental conditions as in Section 2.4. For peak identification, see Table 2.*

3.2. Optimization of extraction conditions

As regards the extraction optimization, several techniques were considered in this study, including maceration, HRE, UAE and MAE, which were evaluated in order to obtain a high yield of both prenylflavonoids and bitter acids. Maceration is an extraction pro-cess of plant material based on the use of a solvent with several daily shakings or stirrings at room temperature, while HRE, UAE and MAE are procedures that use heating, sound waves or microwaves to accelerate the extraction and increase the yield of secondary metabolites [34]. To make a reliable comparison of these techniques, all the experimental parameters (sample-to-solvent ratio, solvent and time) were kept constant. The solvent selection, which is typically influenced by the polarity of the secondary metabolites in the plant material, is a crucial part of the extraction method development [34]. Owing to the different polarity of prenylflavonoids and bitter acids, alcohols, such as MeOH and EtOH, were considered to be the best compromise for their extraction, in agreement with the literature [5,18,21]. It has also been suggested that the presence of HCOOH in the extraction solvent decreases the degradation and isomerization processes of hop prenylchalcones [26]. After several trials under different conditions, MeOH–HCOOH 99:1 (v/v) was finally selected as the best extraction solvent for hop secondary metabolites in this work. To obtain a better recovery, two equal volumes of solvent were preferred rather than all the volume in a single step [17]. By comparing the total amount of prenylflavonoids and bitter acids extracted from hops (sample CV11), dynamic maceration for30 min with MeOH–HCOOH 99:1 (v/v) repeated twice resulted to provide the higher yield (Fig. 3). It should be pointed out that maceration is an easy extraction technique and it does not require an expensive apparatus [34]. Therefore, it can also be easily transferred to routine analysis of this plant material and to quality control. The application of heating, ultrasound or microwave did not provide advantages in terms of extraction time and recovery for hop samples. This is probably due to the poor stability of hop secondary metabolites, suggesting the importance of mild conditions for their extraction. In any case, degradation products were not detected in hop extracts after the extraction techniques applied in this study.

Table 2 *Prenylflavonoids and bitter acids identified in hop extracts by HPLC-UV/DAD, HPLC-ESI-MS and MS2.*

Peak number	Compound	t_R (min)	UV λ_{max} (nm)	$[M+H]^+$ (m/z)	MS^2 (m/z)		$[M-H]^-$ (m/z)	MS^2 (m/z)	
					Base peak	Secondary peaks		Base peak	Secondary peaks
1	Isoxanthohumol[a]	5.9	290, 325	355	299	179 (64), 235 (24)	353	233	-
2	8-Prenylnaringenin[b]	9.5	294, 337	341	285	165 (41)	339	219	-
3	Desmethylxanthohumol	11.9	370	341	285	323 (21), 255 (21)	-	-	-
4	6-Prenylnaringenin[b]	12.9	292, 337	341	285	-	339	245	219 (81)
5	Xanthohumol[b]	14.7	370	355	299	179 (41)	353	233	339 (36), 119 (24)
6	Cohumulone[b]	21.2	237, 283, 323, 360sh	349	225	223 (94), 293 (81), 281 (47)	347	278	-
7	n-Humulone[b]	22.9	237, 285, 323, 360sh	363	223	307 (85), 239 (37), 295 (25)	361	292	249 (11)
8	Adhumulone[b]	23.3	235, 288, 323, 359sh	363	223	307 (90), 239 (44), 295 (25)	361	292	249 (12)
9	Colupulone[b]	28.3	230, 275, 332	401	275	277 (82), 345 (78), 219 (38)	399	287	330 (46), 356 (18)
10	n-Lupulone[b]	30.1	230, 275, 331	415	275	359 (82), 291 (70), 219 (24)	413	301	344 (21)
11	Adlupulone[b]	30.5	231, 273, 331	415	359	275 (92), 291 (47), 219 (30)	413	301	344 (74), 289 (29), 276 (25)

[a] Experimental conditions as in Sections 2.4 and 2.5.
[b] Confirmed with standard compound.

3.3. Identification of secondary metabolites in hop extracts

In this study, the complete characterization of prenylflavonoids and bitter acids in hop samples was carried out on the basis of their UV/Vis spectra, together with MS and MS2data, which where compared with those of reference standards and with the literature[5,17,21,27,35-37]. The compounds identified are shown in Table 2.Even though the negative ion mode has been considered as the most suitable one by some authors [5, 37], under the applied conditions hop secondary metabolites ionized well both in the positive and in the negative ion mode.

In the ambit of prenylflavonoids, XN and IXN showed the same MS and MS2behavior, as well as DMX, 6-PN and 8-PN. As reported in the literature [36], the thermal isomerization in the ion source of prenylchalcones to corresponding prenylflavanones make them not distinguishable by MS. In this context, UV/Vis data are very useful to identify both flavonoid classes, because prenylchalcones have a λ_{max} at 370 nm, whereas that of prenylflavanones is in the range 290–294 nm. As regards MS^2 data of prenylflavonoids in the positive ion mode (Table 2), XN and IXN showed an intense product ion at m/z 299, while DMX, 6-PN and 8-PN generated a strong fragment at m/z285, which can be attributed to the loss of the prenyl group. The other product ions observed in the MS^2 spectra of these compounds originated from retro-Diels–Alder (RDA) reactions [5]. The $[^{1,3}A]^+$ ion at m/z 235 was observed for IXN only, favored by its chemical structure [17]. In the negative ion mode (Table 2), the RDA cleavages of prenylflavonoids were the dominant fragmentation pathways, with the $[^{1,3}A]^-$ ion occurring at m/z 233 for XN and IXN and atm/z 219

for 8-PN and 6-PN, respectively. As regards prenylphloroglucinols, α-acids can be easily distinguished from β-acids on the basis of their retention time and UV spectrum. Within the same chemical class, the elution order observed in this study was co-, n- and ad-homologues, as previously reported in the literature for C18columns [21, 37]. As for MS and MS^2, bitter acids provided a good degree of fragmentation both in the positive and in the negative ion mode. Regarding MS^2 data of hop acids in the positive ion mode (Table 2), abundant product ions were obtained. The main fragments were generated by the loss of C_4H_8 (−56 u), corresponding to the cleavage of a prenyl group and by the loss of C_5H_8 (−68 u), corresponding to the total removal of a prenyl group, as described by Zhang et al. [37]. The loss of 56 u is preferentially followed by the loss of the acyl group, originating the product ions at m/z 223 for α-acids and 275 for β-acids [37]. The fragmentation mechanisms of bitter acids in the negative ion mode (Table 2) were similar to those previously described. For α-acids, the main product ions were attributed to the loss of C_5H_9 (−69 u), corresponding to a complete removal of a prenyl group. As for β-acids, the number of product ions generated in the MS^2 experiments was higher, due to the presence of an additional prenyl group; for these compounds, the main fragmentation pathway was based on the loss of 112 u, which originated from a consecutive loss of C_5H_9 (−69 u) and C_3H_7 (−43 u) [35]. For both α- and β-acids, other product ions were attributed to molecular rearrangements after the removal of one or more prenyl groups, as described by Hofte et al. [35]. In addition to the compounds described above, a series of minor constituents were found to be present in hop extracts, including cohulupone, humulinone, 4-deoxycohumulone, 4-deoxyhumulone, postlupulone, prehumulone, prelupulone andadlupone [38]. However, all these compounds were detected in very small amount in HPLC-ESI-MS chromatograms only and, therefore, they were not considered for quantification.

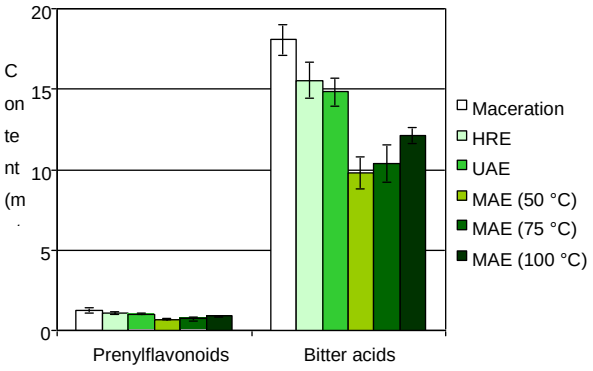

Figure 3. *Comparison of the extraction yield (mg/g) of hop components (sample CV11), using different extraction techniques.*

3.4. Method validation

HPLC-UV/DAD was chosen for quantitative analysis of the secondary metabolites in hop samples, because of the wider availability and use of this equipment in the phytochemical analysis and quality control of natural products [39, 40]. Over the concentration range tested, good linearity (r^2> 0.998) was observed for reference standard compounds used in this study (Table 3). The LOD value was in the range 0.3–1.0 µg/mL for prenylflavonoids and 2.8–5.8 µg/mL for bitter acids (Table 3). The LOQ value was in the range 1.3–3.8 µg/mL for prenylflavonoids and 8.1–21.4 µg/mL for bitter acids (Table 3). These values indicated that the proposed HPLC-UV/DAD method has a suitable sensitivity for the analysis of hop secondary metabolites. The accuracy of the analytical procedure was evaluated using the recovery test. The percentage recovery values, obtained by com-paring the results from samples and fortified samples, were found to be in the range 76–107%, in agreement with the literature [21], and can be considered satisfactory (see Table 4). The low intra- and inter-day SD values for content (≤0.1 mg/g for prenylflavonoids and ≤0.8 mg/g for bitter acids) (Table 5), and %RSD for retention times (≤0.1) and peak areas (≤2.9) (Table 6) indicated the high precision of both the extraction procedure and the chromatographic system. Stability was tested with hop extracts stored in amber glass flasks at 4°C and at room temperature (about 25°C) and analysed every 12 h. The analytes in solution did not show any appreciable change in the chromatographic profile over 72h. Degradation products were not detected as well. The validation data highlighted the suitability of the proposed method for the quali- and quantitative analysis of prenylflavonoids and bitter acids in hop extracts.

Table 3 *Linearity and sensitivity data for prenylflavonoids and bitter acids used as hop standards.*[a]

Compound	Linearity range (µg/mL)	Slope (*a*)	Intercept (*b*)	r^2	LOD (µg/mL)	LOQ (µg/mL)
Isoxanthohumol	2.1–166.0	14.24 (±0.11)	17.71 (±7.93)	0.9989	0.8	2.1
8-Prenylnaringenin	3.8–152.0	11.92 (±0.15)	−18.99 (±10.98)	0.9984	1.0	3.8
6-Prenylnaringenin	2.2–177.0	16.21 (±0.04)	1.76 (±2.86)	0.9999	0.8	2.2
Xanthohumol	1.3–81.0	36.00 (±0.14)	5.03 (±4.96)	0.9997	0.3	1.3
Cohumulone	9.6–383.9	8.49 (±0.08)	−46.40 (±13.85)	0.9987	2.8	9.6
n-Humulone	8.1–649.7	8.38 (±0.07)	−58.60 (±19.00)	0.9988	3.0	8.1
Adhumulone	9.9–198.6	8.34 (±0.06)	−16.49 (±5.76)	0.9994	3.1	9.9
Colupulone	18.3–365.9	8.41 (±0.06)	−42.32 (±11.01)	0.9994	5.8	18.3
n-Lupulone	21.4–214.1	8.62 (±0.09)	−39.91 (±10.97)	0.9989	5.3	21.4
Adhupulone	18.8–94.0	8.59 (±0.08)	−11.44 (±4.40)	0.9995	5.7	18.8

Experimental conditions as in Section 2.4.

[a] For each curve the equation is $y = ax + b$, where y is the peak area, x is the concentration of the analyte (µg/mL), a is the slope, b is the intercept and r^2 is the correlation coefficient. Standard error (SE) values are given in parenthesis. The p value was <0.0001 for all calibration curves.

Table 4 *Percentage recovery data of prenylflavonoids and bitter acids used as hop standards (sample CV11).*

Compound	Original amount (mg)	Spiked amount (mg)	Mean determined amount (mg)	Mean recovery (%) ±RSD (%) (n = 3)
Isoxanthohumol	0.01	0.03	0.04	79.9 ± 3.2
8-Prenylnaringenin	0.01	0.01	0.02	103.2 ± 1.1
6-Prenylnaringenin	0.01	0.01	0.02	91.1 ± 0.8
Xanthohumol	0.16	0.04	0.21	106.6 ± 7.3
Cohumulone	0.36	0.46	0.76	89.0 ± 2.4
n-Humulone	1.06	1.11	2.02	86.5 ± 4.4
Adhumulone	0.19	0.33	0.50	94.7 ± 1.7
Colupulone	0.36	0.82	1.01	78.6 ± 3.0
n-Lupulone	0.42	1.12	1.32	79.5 ± 2.1
Adlupulone	0.11	0.25	0.30	75.9 ± 0.2

Experimental condition as in Section 2.4.

Table 5 *Intra- and inter-day precision data for the extraction of prenylflavonoids and bitter acids from hops (sample CV11).*

Compound	Intra-day precision (n = 6, mean)			Inter-day precision (n = 18, mean) (mg/g ± SD)
	Day 1 (mg/g ± SD)	Day 2 (mg/g ± SD)	Day 3 (mg/g ± SD)	
Desmethylxanthohumol	0.1[a]	0.1[a]	0.1[a]	0.1[a]
Xanthohumol	1.1 ± 0.1	1.3 ± 0.1	1.1[a]	1.2 ± 0.1
Cohumulone	2.1 ± 0.2	2.4 ± 0.1	1.9[a]	2.1 ± 0.2
n-Humulone	7.0 ± 0.5	8.1 ± 0.4	6.4 ± 0.2	7.2 ± 0.8
Adhumulone	1.2 ± 0.1	1.4[a]	1.1[a]	1.2 ± 0.1
Colupulone	3.3 ± 0.2	3.4 ± 0.1	2.5[a]	3.1 ± 0.4
n-Lupulone	4.2 ± 0.3	4.5 ± 0.1	3.4[a]	4.0 ± 0.5
Adlupulone	1.0[a]	1.0[a]	<LOQ	1.0[a]

Experimental conditions as in Section 2.4.
[a] SD < 0.05.

Table 6 *Intra- and inter-day precision data for retention time (tR) and peak area of prenylflavonoids and bitter acids from hops (sample CV11).*

Compound	Intra-day precision (n = 6, mean)						Inter-day precision (n = 18, mean)	
	Day 1		Day 2		Day 3			
	t_R (min) ±RSD (%)	Area (mAU × s) ±RSD (%)	t_R (min) ±RSD (%)	Area (mAU × s) ±RSD (%)	t_R (min) ±RSD (%)	Area (mAU × s) ±RSD (%)	t_R (min) ±RSD (%)	Area (mAU × s) ±RSD (%)
Desmethylxanthohumol	11.9 ± 0.1	70 ± 0.7	11.9 ± 0.1	70 ± 1.0	11.9 ± 0.1	72 ± 2.9	11.9 ± 0.1	71 ± 2.1
Xanthohumol	14.7[a]	923 ± 1.1	14.7 ± 0.1	933 ± 1.0	14.7 ± 0.1	953 ± 1.4	14.7 ± 0.1	937 ± 1.8
Cohumulone	21.1 ± 0.1	318 ± 0.5	21.2 ± 0.1	314 ± 1.3	21.1 ± 0.1	312 ± 0.7	21.2 ± 0.1	315 ± 1.2
n-Humulone	22.9 ± 0.1	1145 ± 1.1	22.9 ± 0.1	1132 ± 1.8	22.9 ± 0.1	1146 ± 0.9	22.9 ± 0.1	1141 ± 1.4
Adhumulone	23.3 ± 0.1	187 ± 1.3	23.4 ± 0.1	186 ± 1.3	23.3 ± 0.1	185 ± 0.9	23.3 ± 0.1	186 ± 1.2
Colupulone	28.3[a]	421 ± 2.0	28.3 ± 0.1	402 ± 0.8	28.3 ± 0.1	401 ± 1.7	28.3 ± 0.1	408 ± 2.8
n-Lupulone	30.1[a]	591 ± 1.0	30.1 ± 0.1	561 ± 1.5	30.1 ± 0.1	561 ± 0.7	30.1 ± 0.1	571 ± 2.7
Adlupulone	30.5[a]	129 ± 2.2	30.5 ± 0.1	123 ± 2.5	30.5 ± 0.1	124 ± 1.1	30.5 ± 0.1	125 ± 2.9

Experimental conditions as in Section 2.4.
[a] RSD < 0.05.

3.5. Quantitative analysis of hop samples

The method developed in this study was applied to the quali- and quantitative analysis of secondary metabolites in female strobiles of ten commercial cultivars (Table 7) and twenty-three wild Italian hop genotypes (Table 8). Quantitative data were expressed as mg/g dry weight. A significant

variability in the content of secondary metabolites, including prenylflavonoids and bitter acids, was observed among commercial cultivars (Table 7). It is well-known that the amount of individual constituents strongly depends on the hop variety and, fora given one, on the conditions of growing, processing and storage [10,11]. In general, XN is considered the main prenylflavonoid of hops (0.1–1.0%, dry weight) [3], followed by DMX; bitters acids are usually the most abundant components (around 5–20%, dry weight) [6]. As shown in Table 7, the content of prenylflavonoids (1.0–5.9 mg/g) and bitter acids (13.8–128.5 mg/g) in commercial hop cultivars is in agreement with the described percentages [3, 6]. It is important to highlight that prenylflavanones were detected at very low amount in the cultivars analyzed, because these com-pounds are not biosynthesized in hops but obtained during storage or by microbial biotransformation [41]. This demonstrates the reliability of the method proposed, which avoids the isomerization of parent prenylchalcones during the extraction procedure and HPLC analysis. Commercial hop cultivars have been previously classified in four groups, such as fine aroma (α-acids 3.5–4.0%, w/w), aroma (α-acids 3.5–6.5%, w/w), bitter (α-acids <8.0%, w/w) and high-alpha (α-acids<15.0%, w/w), according to the needs of the brewing industry [42]. These groups were mainly distinguished on the basis of total α-acids and α/β acid ratio [42]. In view of this classification method, cultivars CV3, CV4, CV6, CV9 and CV11 can be attributed to the fine aroma and aroma groups, while CV2 and CV7 to the high-alpha group [43]. Regarding wild Italian hop genotypes (Table 8), total prenylflavonoids were in the range 1.0–3.2 mg/g, while total bitter acids were in the range 18.8–65.9 mg/g. The quantitative data obtained by HPLC were further processed by PCA, using an R-based software. Since the content of prenylflavanones was marginal in all hop samples, these compounds were not considered in the model. The first two principal components (PCs), explaining up to 90% of total variance, were used in this study. The score plot (Fig. 4A) shows the distribution of the samples along the PCs. The majority of hop samples is distributed in the center of this plot, highlighting a certain homogeneity among them. The loading plot (Fig. 4B) shows the contribution of each variable to the PCs, which is known to be influenced by the angle between them [44]. If the angle of the variable with a PC is closer to 0, the contribution of the variable to this component is strong [44]; this means that the content of prenylchalcones and α-acids influences mainly the first principal component (Fig. 4B), and the samples on the right of the score plot can be considered as the richest ones of these constituents (Fig. 4A). In particular, samples CV2, CV7 and GEN19 resulted to be the best performers in terms of both prenylchalcones and α-acids. As for prenylchalcones, the total value for samples CV2, CV7 and GEN19 was of 5.9±0.2, 3.1±0.1 and 3.2±0.3 mg/g (Tables 7 and 8), respectively, with XN representing the main constituent (>80%). In relation to α-acids, the total

value in samples CV2, CV7 and GEN19 was of 91.3±0.7, Fig. 4. Principal component analysis applied to quantitative data of all hop samples: (A) score plot and (B) loading plot for the first two principal components.79.9 ± 1.3 and 42.2 ± 3.9 mg/g (Tables 7 and 8), respectively. According to the loading plot (Fig. 4B), β-acids affect mainly the second principal component; in this ambit, samples CV9 and GEN3were found to be particularly rich of these compounds (36.7 ± 0.8and 32.2 ± 5.0 mg/g, respectively). Among wild Italian hop genotypes, the results of this study suggest GEN19 as a potential good source of secondary metabolites to be used in the pharmaceutical and nutraceutical fields. As regards the brewing industry, the high content of β-acids in sample GEN3 makes it a potential new flavoring agent for beer production. Both genotypes can represent a good starting point, at least from the phytochemical standpoint, for the breeding of new Italian hop cultivars.

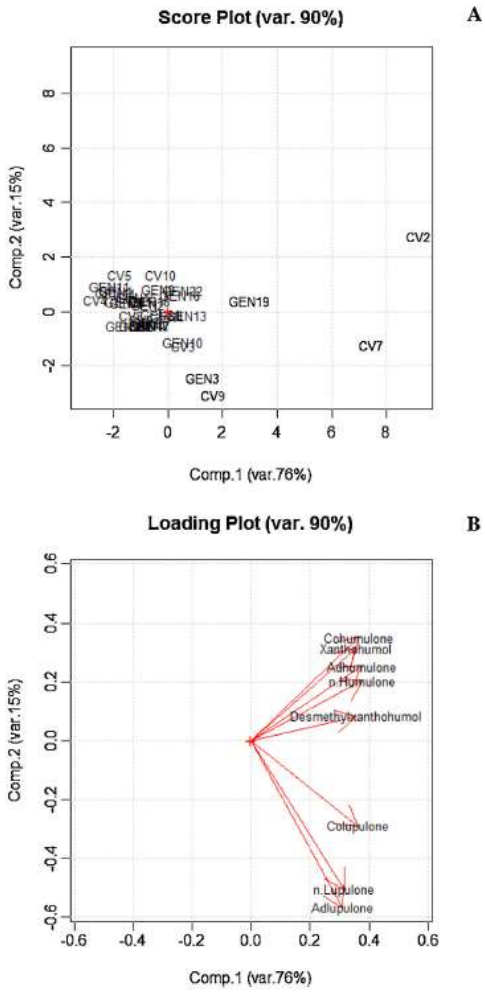

Figure 4. *Principal component analysis applied to quantitative data of all hop samples: (A) score plot and (B) loading plot for the first two principal components.*

Table 7 *Content of prenylflavonoids and bitter acids in commercial hop cultivars (CV1–CV10) by HPLC-UV/DAD (data are expressed as mg/g).*[a]

Compound	CV1	CV2	CV3	CV4	CV5	CV6	CV7	CV8	CV9	CV10
Isoxanthohumol	<LOQ	<LOD	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOQ	<LOD	<LOQ
8-Prenylnaringenin	<LOQ	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOQ
Desmethylxanthohumol	0.2[b]	0.9[b]	0.2[b]	<LOQ	<LOQ	0.1[b]	0.6[b]	0.3[b]	0.3[b]	0.1[b]
6-Prenylnaringenin	0.1[b]	<LOQ	0.1[b]	<LOD	<LOD	<LOQ	<LOQ	0.1[b]	<LOQ	<LOQ
Xanthohumol	0.8 ± 0.1	5.0 ± 0.1	1.1[b]	1.0[b]	1.1[b]	1.8 ± 0.3	2.5[b]	0.9[b]	1.2[b]	2.0 ± 0.1
Total prenylflavonoids	1.1 ± 0.1	5.9 ± 0.2	1.5[b]	1.0[b]	1.1[b]	1.9 ± 0.4	3.1 ± 0.1	1.3[b]	1.5[b]	2.1 ± 0.1
Cohumulone	5.3 ± 0.5	26.9 ± 0.3	6.2[b]	1.4[b]	5.3 ± 0.1	2.6 ± 0.4	15.7 ± 0.3	2.8[b]	3.6 ± 0.1	7.2 ± 0.3
n-Humulone	12.6 ± 1.1	54.2 ± 0.8	16.3 ± 0.2	3.4 ± 0.1	12.2 ± 0.2	5.9 ± 0.9	54.2 ± 0.8	4.0[b]	13.8 ± 0.3	21.4 ± 1.0
Adhumulone	3.0 ± 0.3	10.1 ± 0.2	2.4[b]	1.1[b]	2.9 ± 0.1	2.0 ± 0.3	10.0 ± 0.2	1.9[b]	2.6 ± 0.1	3.4 ± 0.2
Colupulone	5.8 ± 0.5	19.9 ± 0.4	12.2 ± 0.2	3.5 ± 0.1	3.6 ± 0.1	7.1 ± 1.2	15.7 ± 0.3	7.0 ± 0.1	14.2 ± 0.3	5.8 ± 0.2
n-Lupulone	5.9 ± 0.5	12.8 ± 0.3	14.1 ± 0.3	3.0 ± 0.1	2.7[b]	5.8 ± 0.9	27.3 ± 0.4	3.7 ± 0.1	17.4 ± 0.4	5.6 ± 0.2
Adlupulone	1.9 ± 0.2	4.1 ± 0.2	2.8 ± 0.1	1.4[b]	1.1[b]	2.9 ± 0.5	5.6 ± 0.1	2.4 ± 0.1	5.1 ± 0.1	1.6[b]
Total bitter acids	34.5 ± 3.8	128.1 ± 1.4	54.0 ± 0.8	13.8 ± 0.4	27.9 ± 0.2	26.3 ± 5.4	128.5 ± 1.6	21.9 ± 0.1	56.8 ± 1.2	45.1 ± 2.4
α/β acid ratio	1.53	2.48	0.86	0.75	2.75	0.66	1.64	0.67	0.55	2.45

[a] Data are expressed as mean ($n = 6$) ± SD.
[b] SD < 0.05.

Table 8 *Content of prenylflavonoids and bitter acids in wild Italian hop genotypes (GEN1–GEN23) by HPLC-UV/DAD (data are expressed as mg/g).*[a]

Compound	GEN1	GEN2	GEN3	GEN4	GEN5	GEN6	GEN7	GEN8	GEN9	GEN10	GEN11	GEN12
Isoxanthohumol	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
8-Prenylnaringenin	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Desmethylxanthohumol	0.2[b]	0.3[b]	0.4[b]	0.4[b]	0.3[b]	0.2[b]	0.3[b]	0.2[b]	0.3[b]	0.4[b]	0.2[b]	0.3[b]
6-Prenylnaringenin	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOQ
Xanthohumol	0.8[b]	1.2 ± 0.1	1.4 ± 0.2	1.4 ± 0.1	1.1[b]	1.1[b]	1.2[b]	1.2 ± 0.2	1.4 ± 0.1	1.6 ± 0.1	0.9[b]	1.2 ± 0.1
Total prenylflavonoids	1.0 ± 0.1	1.4 ± 0.1	1.8 ± 0.3	1.8 ± 0.1	1.4[b]	1.3[b]	1.5[b]	1.4 ± 0.2	1.7 ± 0.1	2.1 ± 0.2	1.1[b]	1.5 ± 0.1
Cohumulone	3.1 ± 0.2	4.8 ± 0.2	3.4 ± 0.3	3.5 ± 0.1	2.8[b]	2.2[b]	2.8[b]	2.5 ± 0.3	5.0 ± 0.2	2.8 ± 0.2	3.1 ± 0.1	2.6 ± 0.1
n-Humulone	9.0 ± 0.5	6.3 ± 0.3	8.0 ± 0.9	11.8 ± 0.4	9.3 ± 0.2	7.1 ± 0.2	9.2 ± 0.1	7.5 ± 0.8	15.0 ± 0.6	9.6 ± 0.6	6.4 ± 0.1	6.6 ± 0.3
Adhumulone	2.5 ± 0.1	2.6 ± 0.1	2.1 ± 0.2	2.9 ± 0.1	2.2[b]	1.9[b]	2.2[b]	2.3 ± 0.3	3.7 ± 0.1	2.9 ± 0.2	2.2[b]	2.2 ± 0.1
Colupulone	3.5 ± 0.2	8.3 ± 0.5	14.7 ± 1.7	7.0 ± 0.3	6.7 ± 0.1	6.5 ± 0.1	7.5 ± 0.1	4.6 ± 0.5	5.7 ± 0.2	8.2 ± 0.5	3.2 ± 0.1	7.3 ± 0.4
n-Lupulone	3.5 ± 0.2	4.5 ± 0.3	13.0 ± 1.6	7.9 ± 0.3	7.8 ± 0.1	7.1 ± 0.2	7.9 ± 0.1	4.5 ± 0.5	5.2 ± 0.2	9.8 ± 0.6	2.7 ± 0.1	6.8 ± 0.4
Adlupulone	1.4 ± 0.1	2.1 ± 0.1	4.6 ± 0.5	2.7 ± 0.1	2.6[b]	2.6 ± 0.1	2.7[b]	2.0 ± 0.2	2.0 ± 0.1	4.0 ± 0.2	1.2[b]	2.8 ± 0.1
Total bitter acids	23.2 ± 1.6	28.6 ± 2.0	45.7 ± 6.9	35.7 ± 1.7	31.4 ± 0.3	27.2 ± 0.6	32.2 ± 0.2	23.3 ± 3.4	36.6 ± 1.6	37.2 ± 3.0	18.8 ± 0.3	28.4 ± 1.9
α/β acid ratio	1.74	0.93	0.42	1.03	0.84	0.69	0.78	1.11	1.83	0.69	1.64	0.67

Compound	GEN13	GEN14	GEN15	GEN16	GEN17	GEN18	GEN19	GEN20	GEN21	GEN22	GEN23
Isoxanthohumol	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
8-Prenylnaringenin	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Desmethylxanthohumol	0.5[b]	0.2[b]	0.3[b]	0.4[b]	0.2[b]	0.3[b]	0.6[b]	0.2[b]	0.3[b]	0.4[b]	0.2[b]
6-Prenylnaringenin	<LOQ	<LOD	<LOD	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOQ	<LOD
Xanthohumol	1.4[b]	0.9 ± 0.1	1.0[b]	1.5[b]	1.3[b]	1.3[b]	2.6 ± 0.2	1.2[b]	1.4[b]	1.6 ± 0.1	1.0[b]
Total prenylflavonoids	1.9[b]	1.1 ± 0.1	1.3[b]	1.8 ± 0.1	1.4[b]	1.6[b]	3.2 ± 0.3	1.3 ± 0.1	1.6 ± 0.1	2.0 ± 0.1	1.3 ± 0.1
Cohumulone	4.3[b]	2.8 ± 0.2	3.8 ± 0.1	6.0 ± 0.1	2.7 ± 0.1	3.8[b]	6.8 ± 0.5	2.6 ± 0.1	3.9 ± 0.1	5.9 ± 0.3	1.3 ± 0.1
n-Humulone	14.4 ± 0.2	8.4 ± 0.7	9.4 ± 0.3	15.4 ± 0.3	11.2 ± 0.3	12.6 ± 0.2	29.9 ± 2.1	7.0 ± 0.3	9.4 ± 0.3	18.6 ± 1.0	6.1 ± 0.3
Adhumulone	3.9 ± 0.1	2.2 ± 0.2	2.6 ± 0.1	4.4 ± 0.1	2.4[b]	2.8[b]	5.5 ± 0.4	2.2 ± 0.1	3.3 ± 0.1	4.5 ± 0.2	1.6 ± 0.1
Colupulone	7.0 ± 0.1	3.7 ± 0.3	5.6 ± 0.2	6.6 ± 0.2	6.4 ± 0.1	5.6 ± 0.1	10.3 ± 0.8	4.5 ± 0.2	6.8 ± 0.2	6.3 ± 0.4	4.4 ± 0.2
n-Lupulone	8.9 ± 0.1	3.9 ± 0.3	4.6 ± 0.2	6.3 ± 0.2	8.7 ± 0.2	6.0 ± 0.1	9.9 ± 0.7	4.6 ± 0.2	6.4 ± 0.2	6.3 ± 0.4	7.5 ± 0.4
Adlupulone	3.0[b]	1.4 ± 0.1	1.7 ± 0.1	2.5 ± 0.1	2.7 ± 0.1	2.1[b]	3.6 ± 0.3	1.9 ± 0.1	2.9 ± 0.1	2.4 ± 0.1	2.5 ± 0.1
Total bitter acids	41.6 ± 0.3	22.4 ± 2.3	27.7 ± 1.1	41.2 ± 1.1	34.0 ± 0.7	33.0 ± 0.5	65.9 ± 6.2	22.8 ± 1.1	32.6 ± 1.1	44.0 ± 3.0	23.5 ± 1.5
α/β acid ratio	1.20	1.49	1.32	1.68	0.92	1.40	1.78	1.07	1.03	1.94	0.62

[a] Data are expressed as mean ($n = 6$) ± SD.
[b] SD < 0.05.

4. Conclusions

A new method was developed in this study for the comprehensive multi-component analysis of hop prenylflavonoids and bitter acids, aimed at the selection of varieties with high content of bioactive secondary metabolites. As regards the sample preparation, dynamic maceration with MeOH–HCOOH (99:1, v/v) gave the best results in terms of recovery of hop components, providing a

simple extraction technique. The analysis of hop secondary metabolites was performed by HPLC-UV/DAD, HPLC-ESI-MS and MS^2 analyses. The application of the fused-core column technology allowed an improvement of the HPLC performance in comparison with that of conventional particulate stationary phases, enabling a complete separation of all constituents in a shorter time and with low solvent usage. The proposed method was fully validated and then successfully applied to the characterization of commercial hop cultivars and wild Italian hop genotypes, thus demonstrating to be an efficient tool for the fingerprinting of this plant material. Furthermore, the easy extraction technique and the simple HPLC procedure developed in this study make this method easily applicable to hop quality control.

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4. Conclusions

The varietal selection carried out, in the recent past, by traditional hop growers country, like England and Germany, have impoverished the natural hop biodiversity; effectively, the modern hop agricultural system is based on a relative small number of varieties. Luckily, in several countries the hop biodiversity has been protected and it is used in breeding programs. In Italy, where hop is endemic, the evaluation and valorization of the wild hop biodiversity: i) is very important to enrich the genetic diversity in Europe; ii) is the first step for the development of breeding programs; iii) is the approach carried out to find Italian varieties with reliable characteristics.

In the present thesis, thanking a multidisciplinary approach, it was possible to increase the knowledge on hop Italian germplasm and on the adaptability of commercial cultivars to the north Italian environment.

The study of the Italian wild hop biodiversity has shown a great variability among the analyzed accessions. The morphological analysis allowed the individuation of associated phenological distinctive characters useful to discriminate between the different genotypes. The PCA analysis, performed and explained in the Study 2, allowed the extrapolation of the highly discriminating traits, correspondent to the intensity of the green colour of the cones, bract length, cone shape and length of bract apex. This prior results confirmed the richness of Italian biodiversity of put the basis for its deeper evaluation. Further studies were carried out with the objective of finding markers not influenced by environmental factors, like the phenologic markers. Genetic characterization of the accessions, through the utilization of molecular makers, seemed to have a higher discrimination power. Molecular characterization using the right molecular markers (endowed by the right discrimination capacity, reproducibility and robustness) is the most robust and accurate analysis for genotypes identification. The Study 3 presents the genetic characterization of the hop accessions using 9 SSR; the hierarchical analysis showed a great variability among the Italian accessions and highlighted an interesting genetic distance between Italian wild hops and the most known commercial hop varieties. Observing the allelic composition, it was possible to determine the efficiency of the utilised markers and to observe the high degree of heterozygosis owned by the hop population. The great hops biodiversity found in the Italian wild population is a richness and it is of fundamental importance in order to establish the most valuable ecotypes.

In order to facilitate and shorten breeding time, a Marker Assisted Selection for the early identification of sex in hops was developed. In the results, explained in Study 4, it is shown the

development of a new efficient marker F0R0 and the comparison with the already existing primer Short Tagged Sites. The results are very exhaustive and allowed a prompt sex identification with high efficiency, even with low quality DNA.

Subsequently, the chemical characterization (alpha and beta acids, aroma profile and essential oil content) of the accessions was carried out (Study 5), to evaluate positive and negative characteristics of the Italian hop plants. The quantification of alfa and beta acids revealed that Italian wild hops are characterized by a low bitter acid content (almost all below 5%); this characteristic makes the Italian genotypes unsuitable as bitter hops. This result showed the possibility to find aroma variety, rather dual-purpose or finishing hops (for dry hopping process) more reliable. Regarding aroma profile, genotypes ET8, Piattello, ET8-1 and Torre were particularly interesting for their high content farnesene, the aromatic molecules found in traditional "noble hops". Moreover, other genotypes contained elevated quantities of β-cariophillene and α-humulene, two terpenes responsible for the spicy and woody aroma of hops. The presence of those valuable traits are the evidence of a possible use of selected wild hops in brewing or for breeding programmes, to enrich known varieties with precious aromatic attributes.

An agronomic and chemical approach was used to compare the environment adaptability of Italian wild hops and commercial varieties, both cultivated in the hop field in Marano sul Panaro (IT); moreover, to evaluate the influence of terroir, chemical characteristics of commercial cultivars in the collection field and the same genotypes purchased in the market, were compared. In Study 6, different parameters were considered to evaluate the productive performances of genotypes. The highest yield was recorded in the Italian genotypes Cinghio; while, in the collection field, the yield of commercial cultivars was low, if compared to literature data. The essential oil analysis highlighted the variability of Italian genotypes. Among the Italian accessions, the genotypes Tange, Cinghio and Tavernelle had the highest essential oil production. The percentage of essential oil production of Italian genotypes was lower than commercial varieties; but, if the total oil production per hectare is considered, the oil production of Italian wild genotypes is similar to European cultivated varieties. As concerns the cultivars grown in Italy, they showed a higher oil content than the same cultivars purchased in the market. Considering the aromatic profile, a high selinene isomer content was individuated, not only in all the Italian accessions, but, also, in the commercial cultivars grown in Italy. The effect of *terroir* determined a more complex aromatic profile or the synthesis of different levels of single aromatic molecules; for example, cvs. Brangling Cross, Magnum, Marynka and Tettnanger develop an interesting aromatic profile, if cultivated in Italy.

Finally, a new analytical method for the analysis of hop prenylflavonoids and bitter acids was developed, with the aim of i) making routine analysis much more efficient and faster and ii) to select select varieties with a high content of bioactive secondary metabolites. The application of the fused-core column technology allowed an improvement of the HPLC performance in comparison with the conventional particulate stationary phases, enabling a complete separation of all constituents in a shorter time and with low solvent usage. (Study 1).

During the three years of research, a database with the genetic profile of all the studied varieties and accessions was created, proving the richness in biodiversity of the Italian wild hops; furthernore, more than one characterized Italian genotype seems to be suitable for the registration as commercial varieties. Countless are the characteristics that make the selected Italian genotypes unique. Nevertheless, the achievements obtained in this project constitue a scientific solid base to further research, targeted to the implementation of the genetic databank, the development of breeding programs and the technology transfer to the brewing industry.

Remain the hope of a future not too far, in where Italian genotypes and commercial hop varieties might be produced in Italy as a profitable crops, appreciated for its peculiar characteristics by the brewers all over the world.

ANNEX 1 - Agronomical aspects

Planting

Hop is a perennial plant that can occupy an area for 10-20 years. For this reason, a particular care must be devoted to the area selection, in terms of geographical location and soil texture, and to the agronomical practices. Management practices are very important during hop growing and soil must be enriched every year. At the beginning, for the obtainment of the right structure and nutrient enrichment of the soil, is advised manure together with potassium and phosphate fertilizer agent then nitrogenous dressing are necessary around plants. Very important for hop growing is water, so the land near river valleys are advantaged, for the easy irrigation and maybe for the presence of groundwater achievable. Land should be levelled out or only gently sloped, and protected by wind, maybe by a forest nearby or a high cultivation. A proper drainage is also necessary.

Plants grow in field, following the sun direction, East-West. The distances between plants are variable, too low space in rows between plants can affected the yield but also too much distance between plants, results maybe in more yield per plant but insufficient to compensate the reduction in plants population.

The distance is dependent not only by yield but also by the dimension of tractor used. Tractor have to pass between the raw without damaging the plants. Studied value suitable spacing for varieties is dependent on the system of growth imply: if shapes become bigger, more place is necessary between plants, and vary from 1,5 and 2 mt distance. As hops are climbing plants, for growing they require supports. Hop garden are equipped of systems of poles, for the supporting of wires, on which hops climb twisting. System of training are different and every country have its habits; generally the differences can be:

- poles height and plant height, from 3 mt to 6 m or little more;
-number of poles necessary;
-number of supports for bines, and number of bines growing
-inclination of bine support.

Umbrella system is used in Kent and consists in planting hops equidistant in rows and between the rows. The poles are higher in the outside than the poles in the inside and it is used a pole every six or nine hill in the inside. Wire are positioned at the top of the poles and pass across the garden at right angle (bearing wire). Other wires (parallel wires), two for each rows, are fixed to bared wires, parallel with rows and equidistant between the rows. Four strings pass from the hill to the top,

attached to “S” hooks positioned on the wires, distanced between one-another at the half of the space between plants. Anchorage is very important for the stability of the system, and anchor rods are made of galvanized iron, positioned 4 feet deep. Advantages are that cultivation can be made in both direction, there is a good exposure to light and air, thanks to the spaces throughout the garden, no wires interfere with machinery for picking.

In ***Worchester system*** poles are placed at intervals along the rows of hops, with stronger poles at each ends. Wires, two parallel horizontal, are fixed near the ends of the cross pieces and from this wires strings to the hop plants are taken. With this system plants are closer to each other, but the number of string per acre is smaller, only two strings for each plants is taken. The angle of slope of the string can vary depending on the position of hooks in the wires: the greater the angle of slope, the less will be the tendency for hop to forma a dense head, but the more the string deviate from the vertical position, the more difficult is to train the bine on the string and damages by wind are more likely. This method is advantageous when hops are growing in a less suitable soil, thanks to taking two bines per plants. Moreover, plants are easier to be treated than in the umbrella system.

In ***Butcher system*** poles are placed in each row of hops with two or three plants between neighbouring poles. Poles are connected by stout cross wires fixed with hooks a little below the top of the poles. Three or four string per plant are taken according to the spacing between plants. String should be arranged to slope away from the prevailing wind, preventing damages. This system is one of the best to minimize wind damages, the plants are well expose to air and light, and plants are easily sprayed thanks to the positioning of the strings in one plane. Some problems there are when lateral branches of adjacent strings become interlaced, because that cause problem when the picking is made by machines. This can be avoided, altering the stringing so that string come to the top wire alternatively (Burgess, 1964).

Propagation

Hop, as a dioecius plant, produce seeds in which characteristics are not note. Plants germinated from seeds do not have uniform characteristics and also the sex is unknown; thus to ensure the obtaining the same plant, agamic propagation is adopted. A type of propagation is propagation by cutting, in which it is necessary to do a cut near the rootstock of the parent plant, usually during the dormant season or in early spring. This technique is known as strap cutting. The straps are prepared from the thicker basal part of the new wood and if this is long enough, more straps can be obtained.

The bines too are useful for propagation: bines arising from buds in the lower part of the plants, growing horizontally under the soil, are covered of adventitious roots and they have to be removed

in spring and can be a material of propagation when they contain at least one node. (Burgess, 1964; Rybacek, 1991).

Water and nutrients requirements

After the dormancy, hops use for growing the reserves in the tubers and stock roots, but then plants need further supply, that must be absorbed from the soil. Nutrient deficiency in the period of bine and foliage growth can affect the final production. Water is very important for hops: several studies demonstrated the need of high level of water: from April to June, a hop plant consumes 482 mm water, more than 100 mm only in July and August (Rybacek, 1967) and 500-600 mm during the whole growth season (Pavlovic et al., 2010). Different studies reported irrigation have positive effects on yield and does not decrease the α-acids content; hops bitter acids are more temperature susceptible than water susceptible (Kučera et al., 2009; Krofta et al., 2013; Potop et al., 2014). The effect of water supply, especially the effect of drought stress, was studied by Ceh et al. (2007) to determine the modification in secondary metabolites production. Effectively, in various plants, stress caused an increased production of secondary metabolites, as a defence; in this case, the experiment showed that polyphenols content is more influenced by cultivar than by drought stress. Before July, if the amount of nutrient in the soil is not sufficient for a good developing of the plants, symptoms of nutrient deficiency begin to be visible (Burrges, 1964). The requirements of micro and macronutrient for hop is high and change in different growing periods (they begin to increase in spring and culminates during inflorescence and cone formation), but, also, they differ from variety to variety. Apart the main elements involved in the photosynthesis (carbon, oxygen and hydrogen), there are other components essential to the wellness of the plant. First of all, ***nitrogen***, present in various organic complexes in plant, like amino acids and chlorophyll, promotes the vigorousness of the hop, as for other plants. Moreover, hop plants have a rapid growing so they need big amounts of this micronutrient. Nitrogen deficiency is visible observing leaves, that assume a yellowish coloration and become thinner. In case of a severe deficiency of nitrogen, plants are dwarf in size, with light green and yellow leaves and are shed early. In case of excess of nitrogen, the plant is luxuriant, with great vigorousness, the leaves are strongly green and large, but cones are present in small amounts and oversized; moreover, the cones contain less lupuline, so they have a poor scent, the plants are more susceptible to diseases and there is a prolongation of the growing period. Abundance of nitrogen gives a limitation in the radical elongation, providing a strong root system in young hop plants (Rybacek, 1991).

Another macroelement necessary for a well growing plant is ***phosphorus***, essential in many organic tissues in the plant cells, participate in biochemical processes like the transferrin of energy. Phosphorous promotes the development of generative organs and its effect is opposite to nitrogen, causing an increase in the number of inflorescence. Deficiency of phosphorus inhibits roots growing and also the above soil part of the plant, limiting the transformation of sugar to organic acids. Moreover, there is a decrease in the number of inflorescence and cones, that developed badly.

Sulphur is another element necessary for hop growing, constituent of amino acids and protein, enzymes and vitamins, it is also implied in the production of essential oils in the cones. Symptoms of sulphur deficiency are similar to those of nitrogen deficiency (yellowish leaves) (Rybacek, 1991).

Metallic macro elements like ***potassium, calcium*** and ***magnesium*** are important for the metabolism of hop plants, participating in different processes in cells, tissues and organs. Potassium increases the strength against diseases and has a positive effect in the ripening of cone, production of lupuline and essential oils. Potassium deficiency is visible in leaves, which becomes pale and gradually yellow. Moreover, there is an increase in the production of lateral branches in plants, which grow more than the principal branch. Excess of potassium, causes damage to the cones and a decrease in lupuline and oil content (Rybacek, 1991).

Calcium forms salts with mineral and organic acids, and it is an important constituent for many part of the cells. In case of deficiency, apical leaves remain underdeveloped and then they are subjected to abscission. Abundance of calcium compromises the absorption of the other cations, causing chlorosis and also cones become yellow prematurely (Rybacek, 1991).

Magnesium is present in plant cells, in chlorophyll and is necessary for the synthesis of proteins and fats. Furthermore it influences the development of reproductive organs, quality and quantity of hop cones and the production of lupuline and essential oils. Deficiency of magnesium cause chlorotic coloration of leaves that fall down prematurely.

Other microelements necessary for hop plants growing are **iron, manganese, zinc, copper, molybdenum** and **boron**. All those microelements are involved in physiological and biochemical process in plant tissue. They are necessary and active in small amount but if there is a deficiency, the normal life cycle of plants is disturbed (Rybacek et al., 1991).

Diseases

Hops grown under cultivation is subjected to different biotic factors, like virus, bacteria parasites and fungus. It is important in a field situation the prompt individuation of diseases from the very

beginning, through a continuous control of the state of wellness of the plants, in this way, irreparable damage can be avoided. Fungal diseases and pests may induce stresses by reducing the green leaf area, the sap flow, or by altering plant metabolism (Baret et al., 2007). Diseases are very dependent on climate; for example, high humidity and temperature above ten degrees, are the perfect condition for fungi proliferation; high temperature without rain is the perfect condition for red spider mite.

The most common diseases are:

Downy mildew *(Pseudoperonospora humuli* L.*)*: is the most important fungal disease of hop. It can cause high losses in yield and quality of harvested hops. Incidence of infection are climate dependent and in particular, there is strong correlation between high rain and downy mildew infections. First symptoms of infestation appear on young shoots in early spring. Leaves are of green-yellow colour, buds are underdeveloped with contorted leaves. The under leaves presented grey violet cover and are visible sporangiums, commonly call spikes. Spikes are the main source for further spreading of the disease in the course of hop maturation (Vostřel, 2010). The upper leave present as symptom of yellow-green spots. An important regulation factor of downy mildew occurrence in the phase of flowering and cone forming is the application of copper fungicides. (Krofta et al., 2012).

Powdery mildew *(Podosphaera macularis*) of hops, may cause economic loss due to reductions in cone yield and quality. Infection on leaves and bines does not compromise final yield, but if the infestation arrive to cones or flowers, serious damage can be caused. Field studies in Washington State evaluated cone yield, bittering acid content and quality factors when fungicide applications were ceased at different stages of cone development. The incidence of cones with powdery mildew was linearly correlated with yield of cones, bittering acids and accelerated cone maturation (Gent et al, 2014).

Verticillium wilt, caused by *Verticilllium* spp., is a dangerous disease in hop. The fungus causes considerable economic crop failure (OEPP/EPPO 2007). *V. alboatrum* Reinke & Berthold is the most widespread Verticillium species in hops, whereas *V. dahliae* Klebahn is rarely found. These species belong to the group of soilborne pathogens. *Verticillium* can survive several years in the soil by producing resting structures (OEPP/EPPO 2007). After the infection of the roots by the fungus

the vascular system of the plant is colonized (Engelhard, 1957). *Verticillium* species also form resilient resting structures, making them difficult to eradicate from infested soil, which means that planting resistant cultivars is the most viable strategy to sustain production in affected areas (Radišek et al., 2006).

Line pattern viruses is known in the country where hops are cultivated, is characterized by the presence of little yellow-green spots, irregularly disposed on leaves usually in June. Usually the infection begins in the lower part of the plants. Another type of infection is extended to all the leaves, that gradually came necrotic. This disease stops in the dormant stage. Usually this virosis does not compromise the plant and yield, but in the necrotic type infection, affect quality and quantity of cones produced by the plants. In some cases, plants affected by necrotic viroses, reduced the productivity of the hop plants at about 50%.

Hop mosaic viruses, caused by Humulus virus 1 Salmon; it was described for the first time in England in 1923 by Salmon. It is found in every country were hop is cultivated.
Their symptomatology in infected plants. In many tolerant hop cultivars, some virus strains, fail to induce conspicuous symptoms. However, sensitive Golding-type cultivars infected with HpMV show severe symptoms, such as chlorotic vein-banding, leaf distortion and stunting, and poor yield; infected plants usually die prematurely. (Barbara and Adams., 1983; Yu and Liu, 1987).

Parasites that can infest hops are numerous, the more frequent are:

Damson-hop aphid, *Phodon humuli* Schrank, is the only aphid infesting hop plants. The infestation is relevant in the temperate-tropical zone of the globe. Plants are secondary hosts for the aphids, were, female aphids, go on the young leaves in the middle of May. In favorable condition (hot temperature and dry climate) they can reach ten generations in one seasons growth. Damage caused by aphids are the subtraction of nutrients, the developing of saprophyte fungi like the genera *Capnodium* and *Cladosporium* on the aphid faeces, that make difficult the leaves aeration and assimilation and in some serious cases, they can be vector of viruses. In some cases, plants stop growing, reducing yield and the quality of cones (Biancardi and Tone, 1989).

Spider mites is a pest threatening many crops all over the world. It grows under hot and dry weather conditions. Because of their great reproductive capacity, they are able to destroy plants within a

short space of time. Population density of spider mites depends on the temperature and relative humidity. A rather short development time ensures six to a maximum of nine generations during a season, which increases the danger of resistance to phytosanitary products (Malais and Ravensberg 1992).

Severe infestation can cause complete defoliation. Most economic damage is associated with cones. (Vostrel, 2010). Cones damaged by spider mites tend to shatter so that both quality and quantity of yield is reduced. Oxidation of damaged cones is accelerated and storability is reduced (Mahaffee et al., 2009).

Others pests present on hops, are less specific and in normal condition does not cause damages (I.e. *Psyllosydes* attenuate*, Tetranichus urticae Koch*, *Fusarium spp*)(Biancardi and Tone, 1989).

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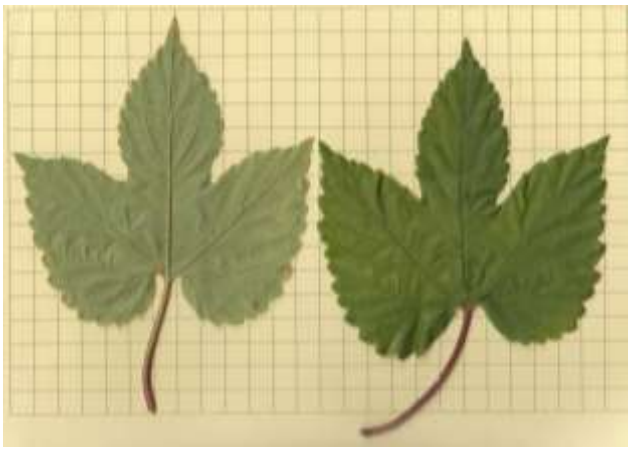
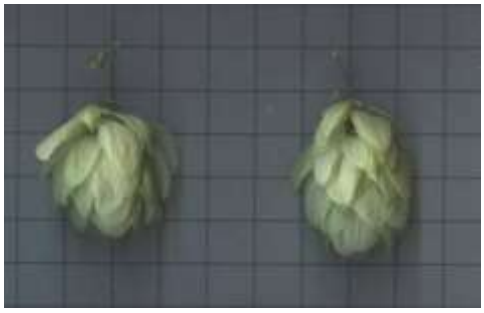
ANNEX 2 – UPOV Descriptors

www.upov.int/edocs/tgdocs/en/tg227.pdf

ANNEX 3 - Morphological cards

Genus: ***Humulus*** **Species:** ***H. Lupulus*** **L.** **Genotype: ET8**





DATA OF 3 YEARS RECORDING

MAIN SHOOT:	
ANTOCYANIN COLORATION	Medium
LEAF	
SHAPE	Three-lobed
SIZE OF BLADE	Medium
COLOUR OF THE UPPER SIDE BLADE	Green
INTENSITY OF THE GREEN COLOUR	Medium
PLANT	
GROWTH	Normal
SHAPE	From cylindrical to clavate
VOLUME OF THE HEAD	Medium
SIDE SHOOT	
MIDDLE THIRD OF THE PLANT LENGTH	Long
FROM THE UPPER THIRD OF THE PLANT LENGTH	Long
DESCRIPTION OF MIDDLE THIRD OF THE PLANT	
DENSITY OF FOLIAGE	Dense
TIME OF PICKING MATURITY	Late
CONE	
SIZE	Large
SHAPE	Broad ovate
DEGREE OF OPENING OF BRACTS	Slightly open
INTENSITY OF THE GREEN COLOUR	Medium
BRACT	
SIZE	Medium
RATIO WIDTH/LENGTH	Medium
LENGTH OF APEX	Short

MEAN BIOMETRICAL DATA	
LEAF	
WIDTH (cm):	12,5 ±1,94
LENGTH (cm):	11,0 ±1,46
CONE	
WEIGHT (g)	0,157±0,069
LENGTH (cm)	3,625±0,478
WIDTH (cm)	2,625±0,478
BRACT	
LENGTH (cm)	1,600±0,292
WIDTH (cm)	0,900±0,158

ET8 DOES NOT BELONG TO GMO
MOLECOLAR CHARACTERIZATION

Molecolar markers: SSR

ET8: HlGA31:158-166, HlGT14: 159-165, HlGT16: 211-233, HlGT17:177-187, HlACA3: 205-229, HlAGA6: 165-183, HlAGA7: 184-190, HlAGA35:193-193, HlGA23: 283-289

References:

- Prencipe F.P., Brighenti V., Rodolfi M., Mongelli A., Dall'Asta C., Ganino T., Bruni R., Pellati F. (2014) Development of a new high-performance liquid chromatography method with diode array and electrospray ionization-mass spectrometry detection for the metabolite fingerprinting of bioactive compounds in *Humulus lupulus* L. Journal of Chromatography A, vol. 1349, p. 50-59, ISSN: 0021-9673, doi: 10.1016/j.chroma.2014.04.097

- Mongelli A., Rodolfi M., Ganino T., Marieschi M., Dall'Asta C., Bruni R. (2015) Italian hop germplasm: Characterization of wild *Humulus lupulus* L. genotypes from Northern Italy by means of phytochemical, morphological traits and multivariate data analysis. Industrial Crops and Products, vol. 70, p. 16-27, ISSN: 0926-6690, doi: 10.1016/j.indcrop.2015.02.036

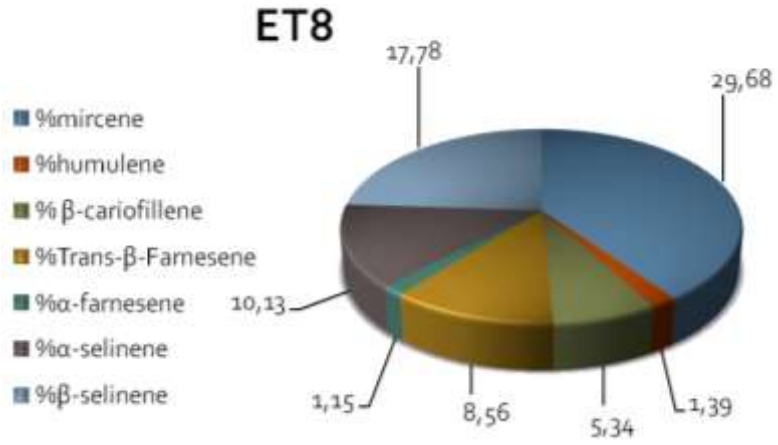
MORPHOLOGICAL CHARACTERIZATION MADE BY USING UPOV STANDARDS

CONSERVATION OF THE ACCESSION: Experimental field in Marano sul Panaro (Modena, IT)

Chemical characterization:

α-ACID CONTENT	**1,32%**
β- ACID CONTENT	**1,97%**
XANTHOUMOL	**0,18%**
ESSENTIAL OIL YIELD	**0,44%**

Essential oil composition: sensory characteristics

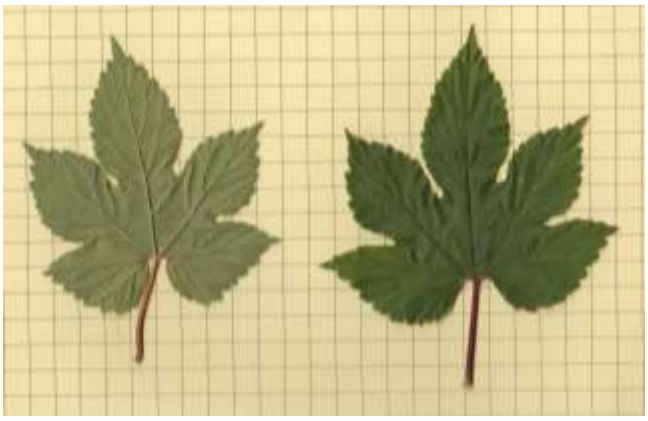


The peculiar sensorial characteristics of the genotype ET8, are due to the presence of high quantities of Farnesene, the molecule present in the most important European aroma hops; Farnesene, seems to be responsible for the characteristic “noble aroma” in hop.

Agronomical characteristics

This genotype came from Parma province, the principal characteristic of this accession is the resistance to Powderly Mildew; in 2014, low temperature and high humidity in spring and most of all in summer, have produced an great developing of the disease in the field, but ET8, was healthy and vigorous, without symptoms. Moreover, it demonstrated tolerance to Downy Mildew in 2013. The yield of fresh hop per hectare for ET8 hop is in mean 1840 tones.

Geneus: *Humulus* Species: *H. Lupulus* L. Genotype: TANGE



DATA OF 3 YEARS RECORDING

MAIN SHOOT:	
ANTOCYANIN COLORATION	Medium
LEAF	
SHAPE	Five lobed
SIZE OF BLADE	Medium
COLOUR OF THE UPPER SIDE BLADE	Green
INTENSITY OF THE GREEN COLOUR	Dark
PLANT	
GROWTH	Normal
SHAPE	From cylindrical to clavic
VOLUME OF THE HEAD	High
SIDE SHOOT	
MIDDLE THIRD OF THE PLANT LENGTH	Long
FROM THE UPPER THIRD OF THE PLANT LENGTH	Long
DESCRIPTION OF MIDDLE THIRD OF THE PLANT	
DENSITY OF FOLIAGE	Dense
TIME OF PICKING MATURITY	Late
CONE	
SIZE	Medium
SHAPE	Globose
DEGREE OF OPENING OF BRACTS	Slightly open
INTENSITY OF THE GREEN COLOUR	Medium
BRACT	
SIZE	Medium
RATIO WIDTH/LENGTH	Large
LENGTH OF APEX	Very short

MEAN BIOMETRICAL DATA	
LEAF	
WIDTH (cm):	12,0 ±1,94
LENGTH (cm):	11,9 ±1,46
CONE	
WEIGHT (g)	0,168±0,034
LENGTH (cm)	2,563±0,495
WIDTH (cm)	2,250±0,377
BRACT	
LENGTH (cm)	1,383±0,213
WIDTH (cm)	0,917±0,116

TANGE DOES NOT BELONG TO GMO
MOLECOLAR CHARACTERIZATION

Molecolar markers: SSR

TANGE: HlGA31:164-164, HlGT14: 159-163, HlGT16: 227-233, HlGT17:177-179, HlACA3: 211-220, HlAGA6: 183-189, HlAGA7: 187-187, HlAGA35:193-193, HlGA23: 293-301

References:

- Prencipe F.P., Brighenti V., Rodolfi M., Mongelli A., Dall'Asta C., Ganino T., Bruni R., Pellati F. (2014) Development of a new high-performance liquid chromatography method with diode array and electrospray ionization-mass spectrometry detection for the metabolite fingerprinting of bioactive compounds in *Humulus lupulus* L. Journal of Chromatography A, vol. 1349, p. 50-59, ISSN: 0021-9673, doi: 10.1016/j.chroma.2014.04.097

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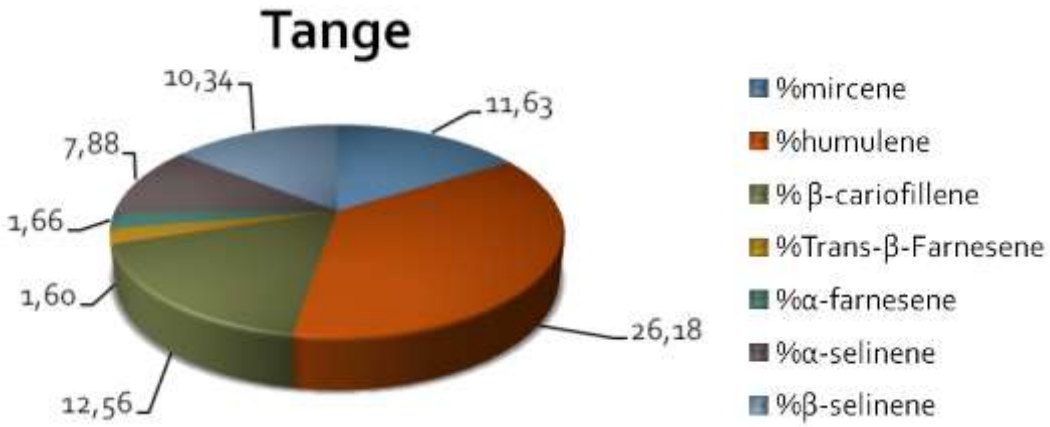
MORPHOLOGICAL CHARACTERIZATION MADE BY USING UPOV STANDARDS

CONSERVATION OF THE ACCESSION: experimental field in Marano sul Panaro (Modena, IT)

Chemical characterization:

α-ACID CONTENT	**1,08%**
β- ACID CONTENT	**2,56%**
XANTHOUMOL	**0,18%**
ESSENTIAL OIL YIELD	**0,51%**

Essential oil composition: sensory characteristics

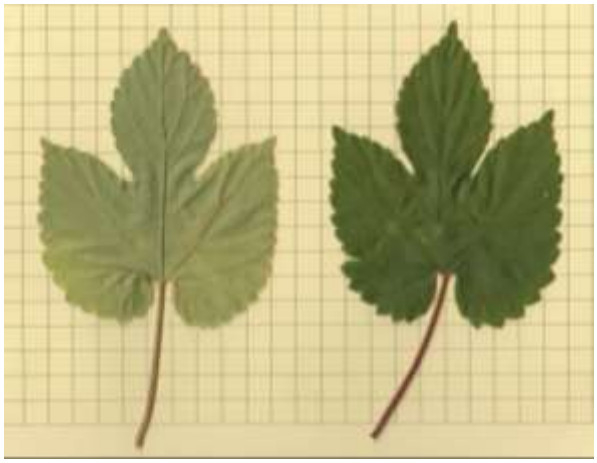


The peculiar sensorial characteristics of the genotype Tange, is due to the presence of high quantities of Humulene, the seems to be responsible of the characteristic spicy aroma in hop, .attribute very demanded in brewing sector.

Agronomical characteristics

This genotype came from Parma province, it is susceptible to powderly mildew and it demonstrated tolerance to downy mildew in 2013. The yield of fresh hop per hectare for Tange hop is in mean 961 tones.

Genus: *Humulus* **Species: *H. Lupulus* L.** **Genotype: CINGHIO**



DATA OF 3 YEARS RECORDING

MAIN SHOOT:	
ANTOCYANIN COLORATION	Medium
LEAF	
SHAPE	Three lobed
SIZE OF BLADE	Medium
COLOUR OF THE UPPER SIDE BLADE	Green
INTENSITY OF THE GREEN COLOUR	Dark
PLANT	
GROWTH	Normal
SHAPE	From cylindrical to clavated
VOLUME OF THE HEAD	High
SIDE SHOOT	
MIDDLE THIRD OF THE PLANT LENGTH	Long
FROM THE UPPER THIRD OF THE PLANT LENGTH	Long
DESCRIPTION OF MIDDLE THIRD OF THE PLANT	
DENSITY OF FOLIAGE	Dense
SHOOT FROM THE UPPER THIRD:	
TOTAL NUMBER OF CONE	
TIME OF PICKING MATURITY	Late
CONE	
SIZE	Medium
SHAPE	Broad ovate
DEGREE OF OPENING OF BRACTS	Slightly open
INTENSITY OF THE GREEN COLOUR	Light
BRACT	
SIZE	Medium
RATIO WIDTH/LENGTH	Medium
LENGTH OF APEX	Very short

MEAN BIOMETRICAL DATA	
LEAF	
WIDTH (cm)	9,6 ±1,28
LENGTH (cm)	9,9 ±1,05
CONE	
WEIGHT (g)	0,120±0,022
LENGTH (cm)	3,000±0,212
WIDTH (cm)	2,200 ±0,254
BRACT	
LENGTH (cm)	1,144±0,114
WIDTH (cm)	0,780±0,192

CINGHIO DOES NOT BELONG TO GMO
MOLECOLAR CHARACTERIZATION

Molecolar markers: SSR

CINGHIO: HlGA31:160-160, HlGT14: 163-165, HlGT16: 227-229, HlGT17:179-187, HlACA3: 211-220, HlAGA6: 171-171, HlAGA7: 190-190, HlAGA35:193-193, HlGA23: 287-301

References:

- Prencipe F.P., Brighenti V., Rodolfi M., Mongelli A., Dall'Asta C., Ganino T., Bruni R., Pellati F. (2014) Development of a new high-performance liquid chromatography method with diode array and electrospray ionization-mass spectrometry detection for the metabolite fingerprinting of bioactive compounds in *Humulus lupulus* L. Journal of Chromatography A, vol. 1349, p. 50-59, ISSN: 0021-9673, doi: 10.1016/j.chroma.2014.04.097

- Mongelli A., Rodolfi M., Ganino T., Marieschi M., Dall'Asta C., Bruni R. (2015) Italian hop germplasm: Characterization of wild *Humulus lupulus* L. genotypes from Northern Italy by means of phytochemical, morphological traits and multivariate data analysis. Industrial Crops and Products, vol. 70, p. 16-27, ISSN: 0926-6690, doi: 10.1016/j.indcrop.2015.02.036

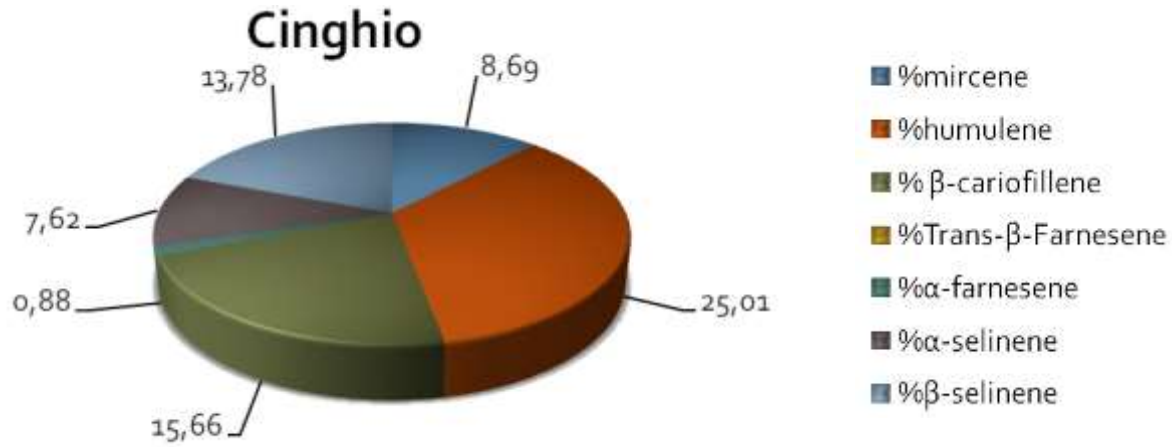
MORPHOLOGICAL CHARACTERIZATION MADE BY USING UPOV STANDARDS

CONSERVATION OF THE ACCESSION: experimental field in Marano sul Panaro (Modena)

Chemical characterization:

α-ACID CONTENT	**2,27%**
β- ACID CONTENT	**2,02%**
XANTHOUMOL	**0,25%**
ESSENTIAL OIL YIELD	**0,35%**

Essential oil composition: sensory characteristics



The sensorial profile of the genotype Cinghio, is characterized by the presence of high quantities of Humulene, that seems to be responsible of the characteristic spicy aroma in hop, attribute very demanded in brewing sector.

Agronomical characteristics

This genotype came from Parma province, it is tolerant to Powderly Mildew and it demonstrated tolerance to Downy Mildew in 2013. The greater value of this accession is in particular the yield of fresh hop per hectare, that is estimated to be 2,882.67 tones. Cinghio demonstrate to be the most vigorous and productive plants of the experimental field.

ANNEX 4 – Collection field management

Scheme of collection field

The collection germplasm field is 1600 m^2, organized on 80 m long rows, spaced 2.5 m; the spacing on the row will be 1 m (Figure 1). The model of growth will be based on the Y growth form (Figure 2) and the height of the structure will be 4.30 m. The plants will be arranged in blocks of 4 plants per ecotype (Figure 2).

Figure 1 *Scheme of system used in Marano sul Panaro field collection: poles distance, pole height, plant distance (green rows).*

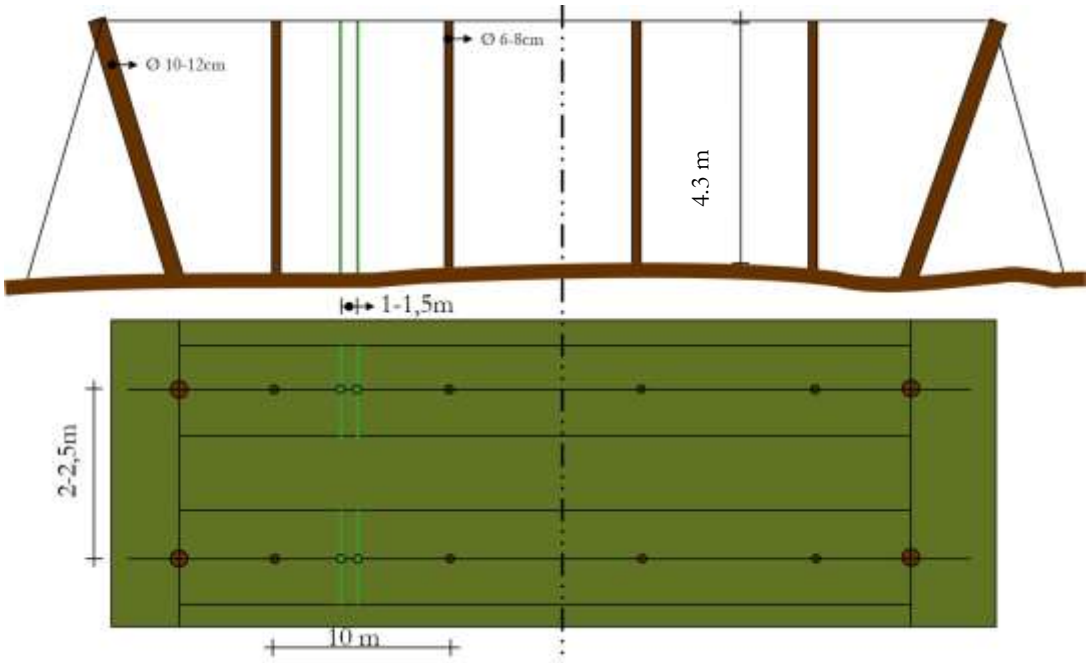
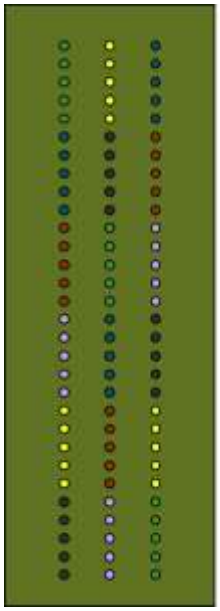
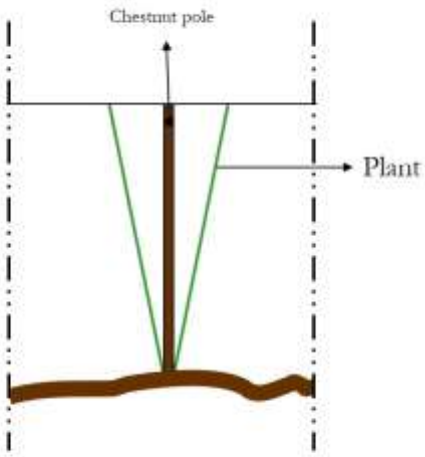


Figure 2 *Plant disposition (different colours indicate different ecotypes) and Y growth form.*





Collection field during vegetative cycle

Below the field arrangement and the vegetative cycle of hop plants is illustrated, indicating the plant development per each month, from April to October (Figures 3- 8).

Figures 3 *Setting up of the field and positioning of the cords (April). "Double Y" cord system (upper figures) and hop climbing sprouts (below figures)*

Figure 4 *Hop plants 3 weeks after sprouting (May): Italian genotypes (left) and commercial cultivars (right)*

Figure 5 *Hop plants in June (up) an in July (below). Hop reached maximum height and lateral shoots are developed.*

Figure 6 *Hop cones (August – September)*

Figure 7 *"Tettnanger" pollinized with Tauro (selected Italian male genotype) pollen*

Figure 8 *From picking to dried hop cones (September – October)*