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Genetic Diversity Inferred from Microsatellites of Wild Hops in Galicia (Spain)

Molecular analysis of wild hops growing in the North-West of Spain (Galicia) show a high genetic diversity. Despite the highly clonal reproduction characteristic of hops, over 70 % of Galician accessions show a unique genotype. Oncor assignment genetic analysis using a representative hop reference collection of cultivars, wild European and wild American hop plants confirmed that Galician hops are highly similar to wild hops growing in other parts of Europe and, therefore, to most of the cultivated hops. Bayesian analysis using Structure showed the existence of only two different genetic groups in our dataset: (i) wild American hops, and (ii) wild European hops including cultivars. No genetic signature of wild American germoplasm was found, which is coincident with the area historical records and also allows us to reject the hypothesis of these hops being the result of escapes from the Spanish main hop growing area, León, sited southeast of Galicia.

Descriptors: genetic diversity, *Humulus lupulus* L., microsatellite DNA

1 Introduction

Spain is the eighth largest world hop (*Humulus lupulus*) producer both in acreage (Ha) and production (Tons) [1]. Nowadays Spanish hop production is mostly localized in the Órbigo riverbank (León), being Nugget the most prevalent cultivar (almost the 97 % of the total production). However, in XIX and early XX centuries hop was broadly cultivated in all the Northern Spanish regions [2]. Historical data confirm the existence of commercial hops in Galicia in the early years of the XX century, with production peaking in the area in the sixties. Galician hop was cultivated by local growers in small farms mostly specialized in English cultivars, such as those from the Golding family. These small production areas have been disappearing in the last decades while the bulk of the Spanish hop production was concentrated in the Northern Spanish plateau (S.A.E. Fomento del Lúpulo, León).

Driven by the presence of the company 'Hijos de Rivera Inversiones Corporativas S.L' manufacturer of 'Estrella Galicia' beers, there is increasing interest in restoring local hop production in Galicia. In recent years, promising production results have been obtained in experimental plots using well-known cultivars as Nugget, Perle, Magnum, Sladek, Taurus, Saazer, Hallertau Mitterfruer, Merkur, Columbus and Cascade. But despite their good results in lupulin content, the plants were affected by downy mildew caused by *Pseudoperonospora humuli*. At the same time that commercial hops are affected by local pathogens, hops growing spontaneously in Galician riverbanks show no symptoms of disease. There is little information on these hop plants which could be native wild plants, naturalized from old farms or the result of crosses between cultivars and local wild hops. Up to date, Galician hop's origin is unknown.

Wild hops are considered a promising source of new genetic variation to identify new resistance genes to the virulent pathogenic strains of powdery mildew [3]. Identification of plants resistant or tolerant to endemic pathogens of a given region is an initial step towards sustainable hop production, by the reduction of pesticide or by the usage of new cultivars resistant to locally adapted pests.

The aim of this work was to identify the origin of the wild hops growing spontaneously in Galicia (Spain) using molecular markers. Hop identification can be performed by GC-MS analysis of the essential oils contents in cones [4] or by using morphological characters. Differences found using these phenotypical characterizations among some cultivars can be very subtle and be affected by abiotic conditions. In order to allow varietal identification, mature plants able to produce cones are needed [5]. DNA-based identification methods provide information at the genotypic level so their results are not influenced by plants age or any of the environmental variations that determine the final phenotype of each individual. In the last decades, several polymorphic microsatellite loci have been developed for hops [6, 7, 8, 9, 10], and found to be extremely useful in varietal identification [11, 12, 13]. *Čerenak* et al. [14] proposed using at least five microsatellite loci (5–2, 11a59, 3a88, 7a82, HlGT4) for hop cultivar identification and reported a success rate of 70 % (for chromosomal mapping consult 15 and 16). In the present study we characterized Galician hops using microsatellite loci and comparing it to a reference collection of hop plants representative of genetic diversity found in cultivars, wild European and wild American plants.

The results of this study are key to evaluate their potential as starting breeding material to develop a local genetic improvement program.

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Table 1 List of the Galician hop plants analysed with nuclear SSR

Accession	Locality	Sex	Seeds	Lupuline Glands
1	Porzomillos	F		yes
2	Sada	F	yes	yes
3	Limiñón	F		yes
4	Cos	?		
5	Cos	F	yes	yes
6	Cos	?		
7	Cos	?		
8	Cos	?		
9	Cos	F	yes	
10	Cos	F	yes	yes
11	Cos	F	yes	yes
12	Cos	F	yes	yes
13	Cos	F	yes	yes
14	Mabegondo	F	yes	yes
15	Mabegondo	F	yes	yes
16	Mabegondo	F		yes
17	Mabegondo	M		
18	Mabegondo	F	yes	yes
19	Mabegondo	?		
20	Mabegondo	F	yes	yes
21	Mabegondo	F	yes	yes
22	Sta. Marta de Babío	F		yes
23	Sta. Marta de Babío	M		
24	Sta. Marta de Babío	F	yes	yes
25	Rois	M		
26	Sta. Marta de Babío	F		yes
27	Piadela	F		
28	Piadela	F	yes	yes
29	Piadela	F	yes	yes
30	Piadela	?		
31	Piadela	F	yes	yes
32	Piadela	F		
33	Piadela	?		
34	Piadela	M		
35	Piadela	F	yes	yes
36	Paderne	F		yes
37	Paderne	F	yes	yes
38	Paderne	M		
39	Paderne	F	yes	yes
40	Betanzos	F		yes
41	Betanzos	F		yes
42	Betanzos	F		yes
43	Betanzos	F	yes	yes
44	Betanzos	F		
45	Betanzos	F		yes
46	Betanzos	F		yes
47*	Betanzos	F		yes
48	Betanzos	F		
49	Betanzos	F	yes	yes
50	Betanzos	F		yes
51	Betanzos	F	yes	yes
52	Betanzos	F	yes	yes
53	Betanzos	F	yes	yes
54	Betanzos	F	yes	yes
55*	Betanzos	F	yes	yes
56	Betanzos	F	yes	yes
57	Betanzos	F	yes	yes
58	Betanzos	?		
59	Souto	F	yes	yes
60	Souto	F	yes	yes
61	Paderne	?		
62	Paderne	F		yes
63	Paderne	F		yes
64	Paderne	F		yes
65	Paderne	F		yes
66	Paderne	F		yes
67	Paderne	F		yes
68	Cambre	F	yes	yes
69	Cambre	F	yes	yes
70	Cambre	M		
71	Betanzos	F		yes
72	Betanzos	F		yes
73	Betanzos	F		yes
74	Betanzos	F		yes
75	Chantada	F		yes
76	Oural	F	yes	yes
77*	Moaña	F	yes	yes
78*	Moaña	F		yes
79	Moaña	F	yes	yes
80	Ponte Ulla	F	yes	yes
81	Ponte Ulla	F		yes
82	Ponte Ulla	?		
83	Ponte Ulla	F	yes	yes
84	Ponte Ulla	F	yes	yes
85	Ponte Ulla	?		
86	Ponte Ulla	?		
87	Ponte Ulla	F	yes	yes
88	Ponte Ulla	F	yes	yes

* No quality DNA was extracted from the accessions in cursive

2 Material and methods

In the year 2010, leaves from hop plants grown in the region ‘As Mariñas’ (A Coruña), Galicia (Spain) (Fig. 1, see next page) were individually collected from the aerial part of 88 different individuals, each of them from an independent rhizome and sexually identified (Table 1). The genetic database obtained from the analysis of a reference collection is necessary to perform assignment tests and

Fig. 1 **Geographic location region As Mariñas (La Coruña) at Galicia (Spain). Numbers show the location of some of the sampled plants**

was generated using 194 hop accessions of known origin: 75 wild European, 46 wild American and 73 cultivars and breeding lines (see suplementary material in 13).

Plant tissue was stored at –80 °C until analyses. DNA extractions and PCR amplifications were carried out following *Peredo* et al. protocols [17, 13]. Four of the Galician samples failed in producing high quality DNA and were not included in the molecular analysis (see Table 1). Seven microsatellite loci, 11a59, 5–2, 3a88 [11], HIGA4, HIGT1, HIGT2 and HGT5 [6] were amplified in the dataset consisting of the unknown Galician samples and the reference collection (see Table 1 for list of accessions and genotypes). Fragment sizes were determined with an automatic sequencer ABI PRISM 3130 and Peak Scanner v.1.0 software (Applied Biosynthesis, CA, USA)].

Genetic variability and F_{ST} estimations (and their *P*-values) were calculated using Arlequin software v.3.5 [18]. Exploratory analysis of the data (frequencies, identity match, heterozygosity, genetic distance and PCoA) were performed using the add-in GenAlEx 6.5 [19] for windows Excel. Assignment tests of the plants to the reference collection were carried out with Oncor [18] and Structure v.2.3.4 [21]. This last program in combination with Structure Harvester [22] was used to define the number of genetic units present in our dataset, which included four *a priori* pools: (1) Galician plants of unknown origin, and known accessions representative of (2) wild European, (3) wild American and (4) cultivar/breeding lines hop plants as previously described above.

3 Results and discussion

All the seven microsatellite loci amplified successfully in the dataset showing high variability, with number of alleles detected per locus ranging from 7 (locus HGT2) to 14 (locus 5_2). The average number of alleles across loci in each population was 8, 8,75, 9. and 10,625 in the wild Sylvester (Galicia), wild European, cultivars and wild American pools, respectively (Table 2). These data confirm the utility of these microsatellite loci for population studies and genotyping, as previous reported [14, 12] and, even in analysis on small distribution areas as the analysed in the present study.

We want to stress the high diversity found among these accessions. Despite the highly clonal reproductive frequency characteristic of hops, over 70 % of the Galician samples had a unique genotype. Out of 84 accessions, only eight shared an identical genotype that could not be attributed to any of the 73 cultivars and breeding lines included in the present study. Accession 44, presented an identical genotype to that found in Tettnager, Saazer and other hops with 'Saazer genetic background'. Also, nine accessions of those collected in the wild were identical to Fuggle. There are historical records describing the cultivation of English hops in this area, so the presence of some naturalized individuals is not unexpected. However, further tests using molecular markers such as AFLP or SNP are necessary to unequivocally confirm the presence of Fuggle and Saazer-like hops in the Galician region.

Genetic differentiation (F_{ST} estimations) was always statistically significant (*P*-value <0.01; Table 3) among sample groups of the reference collection (cultivars, wild American and wild European). They are therefore genetically different and show that the microsatellite loci selected to perform the genetic characterization in this work are suitable to discriminate among hop populations of diverse origin. This is a requirement for the Oncor analyses [20] allowing unequivocal assignation of 84 samples of unknown origin growing in Galicia. Nearly half of the analysed accessions were assigned to the wild European pool (40 individuals; 47.6 %) while the other half was identified as members of the cultivar pool (44 individuals; 52.4 %). No genetic signature of wild American accessions was found among the Galician samples. These assignments were highly robust, being the assignment percentage of the wild European 95.7 % to themselves (standard deviation of 8.7 %) and 92.8 % (s.d. 10.1 %) to hop cultivars.

The F_{ST} analysis allowed to statistically discriminate among the *a priori* determined groups: cultivated, wild European, and wild American. The Bayesian approach in Structure is used to determine

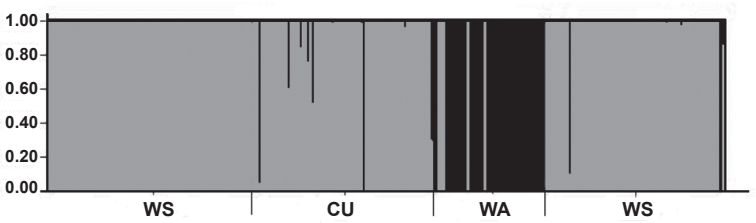

Fig. 2 **Graphical Structure output for the two genetic units (*K* = 2, black represents the wild American cluster and grey the European cluster) estimated by the software. Each vertical bar contains the membership proportion of each individual for the two genetic units. Acronyms: WS-Wild Sylvester (Galicia), CU-Cultivar, WA-Wild American, WE-Wild European**

Table 2 Genetic variability of the studied hop samples measured as the number of alleles per locus (Na). Acronyms: WS-Wild Sylvester (Galicia), CU-Cultivar, WA-Wild American, WE-Wild European, SD-Standard Deviation

Locus	WS	CU	WA	WE	Mean	SD	Total
5_2	7	12	20	11	12.5	4.219005	21
11a59	14	11	17	10	13	2.44949	27
HIGA4	7	12	9	9	9.25	1.596872	16
3a88	8	9	15	7	9.75	2.783882	23
HIGT1	11	10	6	16	10.75	3.185906	22
HIGT2	n.a.	7	7	7	7	0	9
HIGT5	n.a.	10	11	9	10	0.707107	14
Mean	8	9.000	10.625	8.750	9.333	1.28	16.625
SD	1.78	1.282	2.306	1.497	1.059	2.08	3.006

the probability of belonging of each sample to each differentiated group. Structure Harvester showed that the Structure software had defined two clearly different genetic units in the global dataset ($K = 2$): one of them included the wild American individuals in exclusively while the second group included hop cultivars, wild European accessions and Galician individuals (Fig. 2), all of them together. The average proportion of membership of each American accession to their own genetic unit was 0.891 while in the case of the 'European cluster', the calculated membership were 1.000 (Galician), 0.946 (wild European) and 0.973 (cultivars). Our results confirm that the wild American pool is highly distinct from the European pool, as previously reported in Peredo et al. [13] and elsewhere (e. g. [23, 24]). Hop cultivars could not be distinguished from their wild European relatives and were identified as a single pool. Cultivars and wild European hops share the same genetic background as many of the nowadays cultivars have their origin on selection over local European. More recently developed cultivars are the result of controlled crosses or open pollination crosses, in which American germoplasm was used to provide genetic variation that lead to an increase alpha acids yield and pest resistance. Molecular studies show high heterozygosity among hop cultivars, presenting genetic variability rates similar to that found in their wild relatives [25]. Cultivars are maintained as clonal lines through asexual reproduction, avoiding genetic erosion. This high variability makes it difficult to statistically differentiate the cultivated accessions from their wild European counterparts. So it was expected that all wild Galician plants were identified as members of the combined European and cultivated pools (Fig. 3, Supplementary Fig. 1).

Table 3 F_{ST} values (below diagonal) and their correspondent P-values (above diagonal; **p-value < 0.01). Acronyms: CU-Cultivar, WA-Wild American, WE-Wild European

FST/P-value	CU	WA	WE
CU	–	**	**
WA	0.162	–	**
WE	0.112	0.146	–

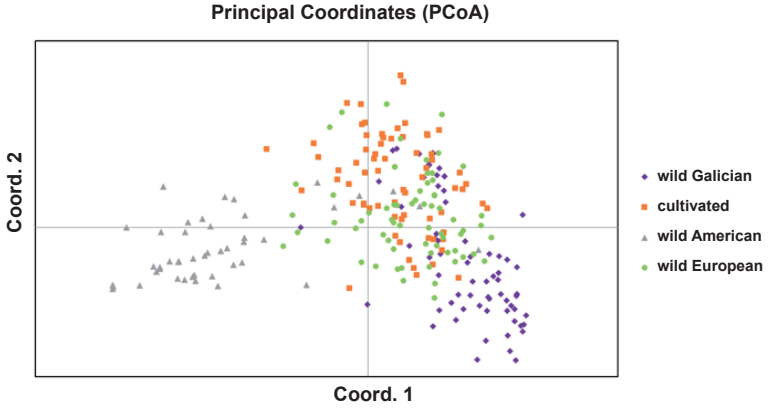

Fig. 3 PCoA illustrating the genetic distance among hop accessions included in this study. The three first axis account for nearly 40 % of the variance. Graphic represents axis 1 and 2 which account for 20.93 and 8.85 % of the variation respectively. Coordinate 1 differentiates Wild American hops (grey triangles) from all the other groups. Galician samples (purple diamonds) group with the European accessions (green circles) and cultivars (orange squares)

We can confirm that the Galician populations are not escapees from the commercially cultivated bitter hops nowadays in the Leon area, sited 300 km away, as the analysed samples are easily distinguishable from the most prevalent cultivar in Spain, Nugget. In addition, as no traces of American germplasm were found among the 84 Galician samples analysed in the present study, we can discard introgression of any of the bitter cultivars cultivated in Spain into the wild populations in northwest Spain. The lack of American germoplasm it is also coincident with the information available on the farming practises in the area. Our data confirms the historical records that only indicate the presence of English cultivars, mainly form the Golding family, in this area during the XX century. The Golding and Fuggle cultivars derive from European hops and their origin can be traced back to selection on local English landraces.

Recent microsatellite analysis of Swedish wild hops [25] concluded that the wild hops found in Sweden are different from that cultivated in the 19th century or the Nordic varieties bred in the early 20th century. The authors argue that the situation of the introduced material, in arable land, or the unsuitable flowering time of the introduced plants to that needed in those Northern latitudes might be the reason for lack of footprint of the former cultivars in the nowadays wild Swedish hop. The permissive climate and Southern latitude of the Northwest area of the Iberian Peninsula would have not act as a selective factor against any cultivated hops in case that bitter cultivars had been previously cultivated in the region or introduced from the Leon area.

4 Conclusion

Wild hops growing in the North-West of Spain (Galicia) have been analysed using molecular markers (seven nuclear loci microsatellites) and the results in alleles sizes compared with the ones of a genetic reference collection including cultivars, wild European and wild American hop

plants [13], with the aim of identifying their origin. Despite the highly clonal reproducction characteristic of hops, a high diversity was found among the wild Sylvester Galician accessions. Half of these accessions were assigned to the wild European pool and the other half to the pool of cultivars, and no genotype belonging to the wild American pool was found. The Bayesian test Structure showed the existence of two different genetic groups: (i) the wild American hops, and (ii) the wild European hops together with the cultivars. The results present here are a promising initial characterization of the diversity of wild hops in Spain, however future research will require the use of more powerful markers. The recent develop of pipelines that allow the use of next generation sequencing to obtain SNP has allowed a dramatic increase of resolution in the genetic fingerprinting [26] and it will be an inestimable tool to more precisely determine the origin of the wild or naturalized populations growing in Spain and across Europe.

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