Assessing the causes of the low productivity of cider apple orchards in the Spanish region of Gipuzkoa

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Abstract

Apple cider-making has a long tradition in many northern Spanish regions, such as Gipuzkoa in the Basque Country. The production in this region is largely based on traditional local cider cultivars for which very little information is available on their pollination requirements, resulting in a generalized low productivity of the orchards. In this work the self-(in)compatibility, *S*-alleles and pollen quality have been studied for the main cider varieties grown in Gipuzkoa, which represent a wide genetic diversity. The *S-RNase* genotype of 25 cultivars is firstly reported here, which allows allocating the cultivars in their corresponding incompatibility group. All the *S*-alleles have been previously described, being S_{26}, S_3 and S_5 the most frequently found. Regarding pollen quality triploid cultivars, as expected, showed lower viability (<70%) and germination rates (<20%). Whereas most diploid cultivars showed good pollen viability (>80%) and germination (>30%) rates, it is noteworthy the low performance of 'Urtebete', very similar to that of triploids. Self-compatibility tests both in vitro and in situ showed low rates of fruit set and, together with an upper position of the stigmas related to stamens, reveal a low degree of self-compatibility for most cultivars. Surprisingly, two cultivars called 'Moko' and 'Txalaka', showed certain self-compatibility with around 7% fruit set. All in all, the results will enable to improve traditional varieties orchard design as well as the profitability of apple cider farms in the region.

Keywords: S-alleles, fruit set, pollen viability, incompatibility, self-compatibility

INTRODUCTION

Apple cider-making has a long tradition in many northern Spanish regions, such as Gipuzkoa in the Basque Country, where cider has been produced at least since the Middle Ages. The production in this region is largely based on traditional local cider cultivars (more than 100 cultivars are used), and it is recognized 2016 with an appellation of origin (Euskal sagardoa/Sidra natural del Pais Vasco). Despite their quality, the productivity of cider apple orchards in Gipuzkoa is generally insufficient to achieve an adequate profitability. This is due to several reasons, among which are the biennial nature of many cultivars, inadequate weather conditions during the flowering season, inadequate agronomic management and, especially, the lack of information about the reproductive biology of the varieties ((Diputación de Gipuzkoa, 2015)). Their blooming time is known, but the intercompatibility properties (*S*-genotypes), self-compatibility, and pollen quality are unknown.

The pollinizers planted in apple orchards should flower at a similar time and have abundant, cross-compatible, and active pollen. Gametophytic self-incompatibility is present in the reproductive process of several Malus species, including *Malus* × *domestica* Borkh. (Broothaerts, 2003). Control of self-incompatibility is performed by the multiallelic locus '*S*', located in the terminal portion of chromosome 17 (De Franceschi et al., 2016). The recognition between the allelic products of the *S*-locus determines whether further pollen-tube growth is arrested in the style. Moreover, not every apple cultivar is a good source of pollen, as pollen viability or germination percentage varies among cultivars and species (Jahed and Hirst, 2017; Kwon et al., 2017; Matsumoto, 2014). In this work, we determine the S-alleles and pollen viability for 25 traditional Basque cider cultivars.

This work tries to fill this gap, at least for the 25 main cultivars used in the appellation of origin, by determining their *S*-alleles and pollen viability.

MATERIALS AND METHODS

Plant material

Twenty four cultivars (19 diploid and 5 triploid) of traditional Basque cider, corresponding to those recommended by the appellation of origin (Fundación Hazi, 2016), were analyzed in this study (Table 1). Eight additional modern cultivars were also included as references. The trees of the selected varieties were planted in the Otalarrea farm, belonging to the Provincial Council of Gipuzkoa, or in the apple germplasm bank of the Public University of Navarra.

Table 1. List of traditional Basque cider cultivars and modern cultivars used in this study. Triploid cultivars are indicated as (3n).

Type	Cultivar name
Traditional (24)	Aritza, Bost Kantoi, Errezila, Frantzes Mikatza, Gezamina (3n), Goikoetxe, Ibarra, Limoi, Manttoni, Mikatza, Moko, Mokote, Mozolua (3n), Patzolua (3n), Reineta Encarnada, Saltxipi, Txalaka, Txori Sagarra, Udare Marroi (3n), Urdin, Urtebete, Urtebi Haundi (3n), Urtebi Txiki, Verde Agria
Reference (8)	Fuji, Gala, Granny Smith, Jonagold (3n), Red Chief, King of the Pippins, Starking, Top Red Delicious

S-allele characterization

DNA was extracted from leaves with DNeasy Plant Kit (Qiagen) following the manufacturer's instructions. In a first phase, *S*-alleles were determined by sequencing. The *S*-alleles of the different varieties were amplified with degenerate primers (PycomC1fa / PycomC5ra) following the instructions of (De Franceschi et al., 2016, 2018). For this purpose, 100 ng of genomic DNA were amplified using Ex Taq DNA polymerase (Ref RR001C, Takara) following the manufacturer's instructions and PycomC1fa / PycomC5ra primers. Amplification conditions were initial denaturation, 95°C - 3′; 30x (denaturation 95°C - 20″; banding 58°C - 30″; extension 72°C - 1′); final extension 72°C - 8′. The samples were then run on an agarose gel. A 120ml 3% agarose gel was prepared using 1X TBE buffer and 1x SYBR Safe was added to label the DNA. Once solidified, 20µl of PCR was loaded per sample (along with 5µl of loading buffer) and the cuvette was filled with 1% TBE buffer. The gel was run at 120V for 1.5h and the amplified S-allele fragments were observed with a blue light source.

The fragments were cut individually, and the DNA was extracted from the gel. For this, 2% elution buffer was added to each fragment and the gel was diluted by incubating at 72°C in a thermoblock for 20' under agitation. After vortexing, the gel was centrifuged (30'', 10000g) removing the agarose and recovering the supernatant, where the amplified DNA is located. This mixture was purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and sent for Sanger sequencing. Once the sequences were received, they were run against the NCBI genomic database (https://www.ncbi.nlm.nih.gov/genome/) for identification.

Once the alleles were preliminarily identified, their identity was confirmed by amplification with specific primers (Broothaerts, 2003). For these PCRs, the same Ex Taq DNA polymerase amplification kit (Ref. RR001C, Takara) was used according to the manufacturer's instructions. Amplification conditions were initial denaturation, 94°C - 3′; 30x (denaturation 94°C - 15″; banding 60°C - 15″ (S_3, 57°C; S_{26}, 55°C); extension 72°C - 30′'); final extension 72°C - 2′.

Evaluation of pollen viability

To obtain the pollen, 100 flowers were collected at the E_2 stage (swollen buds, but without opening) in 2020 and 2021 seasons. For drying, they were placed in boxes in which silica-gel spheres had been previously placed, and on filter paper. The boxes were hermetically sealed with cellophane and placed in plastic bags to optimize the drying process. After approximately 48h, the pollen was extracted from the boxes using awls and tweezers. The pollen was stored in glass jars and kept at -80°C until further use to determine viability and germination rates.

The pollen viability rate was determined by staining with 2,3,5-triphenyltetrazolium chloride (TTC). It was prepared by mixing 1% 2,3,5-triphenyltetrazolium chloride (TTC), 60% sucrose and double distilled water. The solution degrades with light so it should be kept in the dark. It was then vortexed for 30'' or until the solution was homogenized. The pollen was spread on a microscope slide using a brush, 40µl of solution was added and covered with a coverslip. After 2h in the dark, the slides were observed under the microscope (x10 objective) and images of 5 spots distributed along the coverslip were taken. Pollen grains tinted in bright red, or orange were considered viable, those in light orange, transparent, ochre, brown or whitish non-viable. A minimum of 100 pollen grains were counted to calculate the rate of viable pollen under these conditions.

To evaluate the germination rate, a medium composed of 10% sucrose, 1% agar, 300ppm $Ca(NO_3)_2$ and 0.02% $H_3(BO)_3$ at pH 6.5 was prepared and poured into Petri dishes. Pollen was hydrated in 20Mm Tris pH6.5 buffer and spread over three plates per strain. The plates were incubated for 48h at 25°C in the dark. After 48h, the pollen plates were observed under the microscope (x10 objective) and images of 5 spots distributed along the plate were taken. Pollen grains were considered as germinated when their length was at least three times the length of the pollen grain. A minimum of 100 pollen grains were counted to calculate the rate of germinated pollen under these conditions.

RESULTS AND DISCUSSION

S-allele characterization

In total, we detected 10 different *S-RNase* genomic sequences (*S*-alleles) in our dataset, which were identified in the NCBI database as S_1, S_2, S_3, S_5, S_7, S_{11}, S_{20}, S_{23}, S_{26} and S_{28}. One additional *S-RNase* sequence could not be identified in the database (although the sequence was identified as located in the chromosome 17) and it could constitute a novel, previously undescribed *S*-allele. Two *S-RNase* alleles, present in the triploids 'Urtebi Haundi' and 'Mozolua' were coded as null-allele as they could not be identified by the methods used. Finding new alleles was not unexpected given that, to date, *S*-allele studies have focused mainly on modern varieties (Broothaerts et al., 2004; Dreesen et al., 2010; De Franceschi et al., 2018), and when traditional material has been analyzed (Broothaerts, 2003; Dreesen et al., 2010) previously undescribed alleles have been described. The *S*-allelic frequency found in the sample used in this study was also noticeably different to previous reports (Broothaerts et al., 2004; Dreesen et al., 2010; De Franceschi et al., 2018; Halász et al., 2011) made on large number of cultivars, both modern and traditional. S_3, S_5, S_{26} and S_{28} were common (>10% of the *S*-alleles) or very common (>15%) in our samples (Figure 1). It is particularly relevant the high frequency of S_{26}, found in eight of our cultivars. This *S*-allele had been described as extremely rare among *M.* × *domestica* but more frequent in *M. sylvestris* and *M. floribunda* (Broothaerts, 2003; Dreesen et al., 2010), and could have been introgressed in domestic apples. Other *S*-alleles, like S_9 and S_{10}, relatively common in both modern and old cultivars (Dreesen et al., 2010) were not found in our samples. Together, these frequencies would be an additional indicator of the existence of an apple Iberian genepool, which had already been detected by genetic structure analysis (Pereira-Lorenzo et al., 2017).

Apple cultivars were classified in 17 genotypes (Table 2), out of which 12 corresponded to diploid cultivars. Each triploid cultivar had its own *S*-genotype, but in the case of the diploids, in five cases there were two or three varieties sharing the genotype.

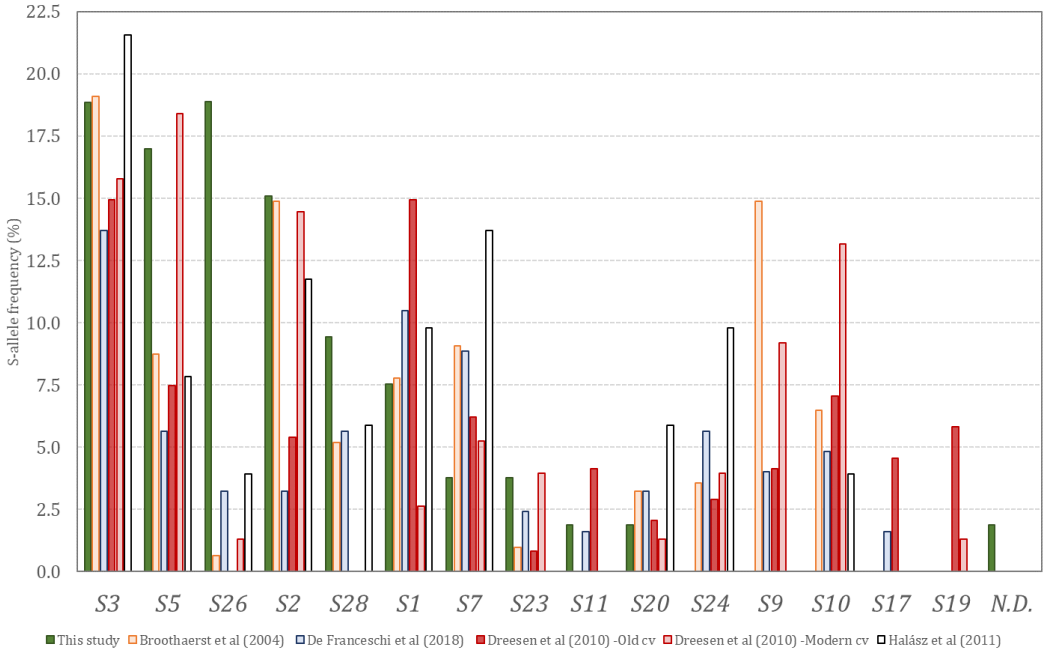

Figure 1. *S*-allele frequencies found in this study compared to previous reports. N.D. correspond to the non-identified allele found in this study.

Table 2. Summary of the *S*-genotypes determined for 24 traditional Basque cider apple cultivars

***S*-genotypes[1]**		**Cultivars**		
Diploid cvs.	S_1S_3	King of the Pippins		
	S_1 S_{26}	Limoi		
	S_1 S_*	Verde Agria		
	S_2 S_5	Bost Kantoi	Urtebi Txiki	
	S_2 S_{20}	Haritza		
	S_2 S_{23}	Saltxipi		
	S_2 S_{26}	Reineta Encarnada	Moko	Txalaka
	S_3 S_5	Urtebete	Errezila	
	S_3 S_{26}	Mikatza	Mokote	Ibarra
	S_3 S_{28}	Txori Sagarra		
	S_5 S_{28}	Frantzes Mikatza	Goikoetxe	
	S_{23} S_{26}	Manttoni		
Triploid cvs.	S_1 S_3 S_5	Patzolua		
	S_2 S_3 $S_?$	Urtebi Haundi		
	S_3 S_5 S_{11}	Udare Marroi		
	S_5 S_7 S_{28}	Gezamina		
	S_7 S_{28} $S_?$	Mozolua		

[1]S_*: non-described *S*-allele, $S_?$: null allele

Evaluation of pollen viability

Pollen viability by TTC staining ranged from 96% ('Granny Smith' and 'Starking') to 2% ('Gezamina'). The reference varieties included in the study showed viabilities in agreement with existing knowledge. For example, 'Granny Smith' was included in the study because it is a variety well known for the high quality of its pollen and is used as a supplementary pollinator in many table apple orchards precisely because of this characteristic. An example of the opposite would be Jonagold, a triploid, which was included as a reference for the normal level expected in this type of variety, and which in our analysis had a viability of 10%.

The diploid cider varieties mostly had fertility rates comparable to those of the reference varieties and which, moreover, can be classified as good or very good, since in almost all cases they exceeded 70%. 'Haritza' and 'Mokote' showed somewhat lower viability, but in any case, they can also be considered good, exceeding 60%. However, the poor performance of 'Reineta Encarnada' (35%) and especially 'Urtebete' (26%) and 'Errezila' (18%), whose viabilities were low and like those of the triploids, should be noted. In the case of triploid varieties, viability was low in 'Patzolua', 'Mozolua' and 'Gezamina'. This result was expected, but, on the contrary, the viability of 'Urtebi Haundi' and 'Udare Maroi', higher than 60%, was surprisingly comparable to that of diploid varieties with moderate viability.

The germination rate ranged from 88% ('Starking Delicious') to 1.5% ('Gezamina'). In general, the level of pollen germination was well related to that of viability, so that varieties with good or very good viability also had pollen with high germination power. Most cider varieties had germination rates comparable to those of good quality reference varieties. Among the diploids, the discrete results obtained by the varieties 'Urdin', 'Reineta Encarnada', 'Verde Agria' and 'Urtebi Txiki' should be highlighted, with germinability around 40%, which implies that, as pollinators, they are somewhat worse than the others. Although they cannot be considered as bad, their results would indicate that it would be necessary to have a greater amount of their own pollen transported to the styles to achieve results comparable to the best performing varieties.

The germination results would confirm 'Urtebete' as unsuitable as a pollinator, despite being diploid. With a viability rate of around 20% and a germination rate of 2.5%, even in situations where there was a large amount of pollen of this variety, it would be unlikely to have satisfactory fruit set. In the case of 'Errezila', its viability was lower (<20%) even than that of 'Urtebete', but its germination level is moderate (55%). Overall, it shows that it is a very poor pollinator of other varieties, and that only in cases where there was a very large amount of pollen, its moderate germinability could partly compensate and obtain moderate fruit set.

In the case of the triploid varieties, as expected, germinations were very low, less than 5%, except for 'Urtebi Haundi', in which, around 20% of the pollen grains managed to germinate and, together with its high viability rate, it would behave as a mediocre pollinator, which is much better than expected in a variety of this type.

CONCLUSIONS

Eleven *S*-alleles and 18 *S*-genotypes were identified within the most relevant local cider apple cultivars grown in Gipuzkoa. One *S*-allele was not previously described. Allele frequencies in this cider apple germplasm show a different pattern to other European local and modern germplasm and constitute a further indication of the existence of an Iberian genepool previously revealed by genetic structure analyses. Pollen viability is generally good in diploid cultivars and poor in triploids. However, 'Urtebi Haundi' (3n) has shown a very good viability, and 'Urtebete' (2n) and 'Errezila' (2n), a very low one. The results will enable to improve traditional varieties orchard design as well as the profitability of apple cider farms in the region.

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