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Pollen viability, self-incompatibility, and a very singular *S*-allele structure between the reasons for the limited potential productivity of traditional Basque cider apple varieties

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ABSTRACT

Cider regions conserve a broad diversity of traditional cultivars for which knowledge has hardly evolved. Key aspects of their reproductive biology are barely known, hindering improvement in orchard management and resulting in highly variable yields. In this study, we characterized key aspects of the reproductive biology of some traditional apple cultivars from the Basque-style cider-producing area in northern Spain (Basque Country and Navarre). We tested for pollen quality, self-compatibility, and cross-compatibility (*S*-genotyping). The pollen quality was good except for Urtebete, Errezila, Reineta Encarnada, and triploid varieties. Self-pollination results confirm the need for pollinators, as only Moko and Txalaka showed certain self-compatibility. Regarding *S*-genotyping, the population proved very singular, with an atypically high frequency of S_{26}, a frequent allele within crabapples, and the appearance of a novel unpublished allele (S_{60}). The knowledge generated for this variety pool will contribute to a better choice of suitable pollinators, preventing the use of popular crabapple varieties that are demonstrated to be partly incompatible with them, and will lead to an increase in potential yields in the region.

1. Introduction

Spain is one of the leading apple producers in the European Union, with a total production of around 585,000 tons for 2010–2020 (MAPA, 2021). According to its use, apple production comprehends two main categories: table (85%) and cider (15%). Cider apple production is concentrated in the North-western regions of Galicia, Asturias, Basque Country, and Navarre and majorly follows a traditional production model, with small semi-extensive or extensive orchards of mostly vase-trained trees. Another key differential aspect of cider apple production in this area is the plant material, as production is based on a diverse group of old traditional cultivars typical to each region. For example, the Appellation of Origin "*Sidra del País Vasco/Euskal Sagardoa*" (Cider of the Basque Country) allows the use of over 100 traditional cultivars, although the bulk of production is achieved with 22 of them (Fundación Hazi, 2016; Odriozola, 2016). This production model, including the great flexibility of the Appellation of Origin regarding apple cultivars, has encouraged the priceless preservation of ancestral genetic resources but also has resulted in difficulties in orchard management. Ultimately, those orchards planted with traditional cultivars tend to have smaller and more erratic productions. The reasons are certainly varied, but the scarce information on reproductive biology for most traditional cultivars may be relevant, as it might limit the suitability of cultivar selection when orchards are designed (Odriozola, 2016).

Apple (*Malus x domestica*) reproductive biology is known not to be simple since it is a self-incompatible species that, therefore, requires cross-pollination. The pollen donor cultivar should meet several requirements: (i) bloom synchronously with the receptor, (ii) produce pollen which reaches some quality standards, and (iii) be genetically compatible.

The blooming time for each cultivar, being an easily observable trait, is generally well known. Nevertheless, pollen quality and genetic compatibility remain unknown for many cultivars, including those used

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for cider production in the Basque Country and Navarre.

Pollen quality refers mainly to its viability and germinability, of which some standards are required to reach an average and stable apple production. Pollen viability is a term that describes the potential capacity of pollen to live, grow, germinate, and develop, whereas germination is the capacity to generate viable pollen tubes (Dafni and Firmage, 2000). Pollen quality is usually assessed by pollen viability tests, with techniques such as staining, and germination tests, that can be performed *in vitro* and *in vivo*. Pollen viability is a relevant trait since pollen fertility greatly varies between apple cultivars; the best-performing ones can reach almost 100% viability, while others may be below 10% (Ramirez and Davenport, 2013). Viability and germinability are generally much higher in diploid cultivars, most reaching at least >50% and very frequently >70%, while most triploid cultivars reach 10% viability/germinability or less (Ramírez and Davenport, 2013; Stott, 1972). Trying to improve orchard productivity, *Malus floribunda* or crabapple selections are often used as pollinators as they outperform commercial cultivars in terms of pollen production and quality (Kozma et al., 2003; Ramírez and Davenport, 2013).

As for genetic compatibility, apple tree sexual reproduction is regulated by a gametophytic self-incompatibility system governed by a multi-allelic *S* locus (Broothaerts et al., 2004; Nettancourt D. E., 1977; Sheick et al., 2018). In this system, pollen tube growth is inhibited when the self-incompatibility alleles (*S*-allele) of pollen match those present in the receptor cultivar (Dennis Jr, 2003). Consequently, apple cultivars can be compatible (when the *S*-genotype of the donor and the receptor are completely different), semi-compatible (if the *S*-genotype is partly shared between the donor and the receptor), or incompatible (when the *S*-genotype of the donor and the receptor is the same) (Schneider et al., 2005). To date, almost 60 *S*-alleles have been described in *Malus* (Sheick et al., 2020, 2018). However, in modern apple populations, the effective *S*-allelic diversity is rather low, as only ten alleles (S_1, S_2, S_3, S_5, S_7, S_9, S_{10}, S_{20}, S_{24}, and S_{28}) are part of approximately 80% of the non-redundant *S*-genotypes reported in the literature. That narrow diversity is due to the recurrent use of a few elite cultivars (*i.e.*, Red Delicious, Golden Delicious, Gala, Jonathan, Fuji, and Honeycrisp) in apple breeding programs (Sheick et al., 2020). In this globalized production model, some crabapples were selected to be widely used as compatible pollinators since they have more diverse ancestors and, therefore, carry *S*-alleles infrequent or absent in modern populations (Sheick et al., 2018). However, *S*-allele studies of local and traditional cultivars from different European regions have shown a greater *S*-allelic diversity than those of modern apples, with notable deviations in *S*-allele frequencies (Dreesen et al., 2010; Halász et al., 2011; Larsen et al., 2016; Nybom et al., 2008).

This work aims to determine to which extent the reproductive biology of traditional Basque apple cider varieties may explain their uneven and relatively scarce yield. The originality of this work resides in the combination of pollen characterization, pollination studies, and genetic compatibility depiction. The lack of studies of Spanish populations, including *S*-genotyping, enhances the importance of the provided results.

2. Material and methods

2.1. Plant material

Plant material from 54 apple cultivars was considered, including 42 traditional local cultivars from the Basque Country and Navarre, two crabapples and ten reference varieties (Table 1). Among the local cultivars, 36 were diploids and six triploids.22 of them (Bost Kantoi, Errezila, Gezamina (3n), Goikoetxea, Haritza, Ibarra (3n), Limoi, Manttoni, Mikatza, Moko, Mokote, Mozoloa (3n), Patzuloa (3n), Saltxipi, Txalaka, Txori Sagarra, Udare Marroi (3n), Urdin, Urtebete, Urtebi Haundi (3n), Urtebi Txiki, and Verde Agria) are the most cultivated cider cultivars among the allowed ones in the Appellation of Origin "*Sidra del Pais Vasco/Euskal Sagardoa*" (Fundación Hazi, 2016). The remaining local cultivars are also traditional varieties whose aptitude might be cider, table, or mixed. The crabapples Evereste and Golden Gem were included in the *S*-allele determination experiment due to their frequent use as pollinators in orchards in the region. In addition, ten cultivars were included as references for pollen viability tests and *S*-allele determination (Table 1).

Table 1
Names of the traditional cultivars of N-W Spain, the modern cultivars used as references, and the crabapples included in this study. Triploid cultivars are indicated as (3n).

Type	Cultivar name
Traditional (42)	Agostera, Aranache-10, Arraiza-01, Baztan Sagarra, Bost Kantoi, Cinco Cantones, De Mine, Errezila, Frantzes Mikatza, Genaro Sagarra, Gezamina (3n), Goikoetxea, Gollano-03, Haritza, Hierro, Ibarra (3n), La Rica, Limoi, Manttoni, Manyaga, Manzana Enana, Manzana Tomate, Maxel Gorri, Mikatza, Moko, Mokote, Motela, Mozoloa (3n), Nano de Cascante, Orache, Patzuloa (3n), Pero, Roja, Saltxipi, Txalaka, Txori Sagarra, Udare Marroi (3n), Urdin, Urtebete, Urtebi Haundi (3n), Urtebi Txiki, and Verde Agria
Crabapples (2)	Evereste, and Golden Gem
Reference (10)	Fuji, Galaxy, Golden Delicious, Granny Smith, Jonagold (3n), Red Chief, Top Red, Starking Delicious, Reineta Encarnada, and King of the Pippins

The plant material was obtained in the 2021 growing season from different sources; Basque cultivars were obtained from trees conserved on the Otalarrea farm (Otalarrea s/n, Villabona, Gipuzkoa, Spain), belonging to the Provincial Council of Gipuzkoa, the references along with the Navarre cultivars were obtained from the public apple germplasm bank of the Public University of Navarra (El Sario, Arrosadia Campus s/n, Pamplona, Navarra, Spain), and the crabapples were purchased from commercial nurseries. Young leaves or twigs were sampled and stored at −80 °C until further use.

Before carrying out further experiments, varietal identification, including the 42 traditional local cultivars, was conducted to confirm the varietal identity of the samples and avoid duplicates. For DNA isolation, 100 mg of leaf material was ground to a fine powder using a microdismembrator (B. Braun Biotech International, Germany). Genomic DNA was isolated from this fine powder with Qiagen Dneasy Plant Mini kit (Qiagen, Germany) following the manufacturer's instructions. The DNA concentration of each sample was determined in an Omega plate reader coupled with its LVis plate (BMG Labtech, Germany). A set of 17 SSR markers (CH02D08, CH01f02, CH01h02, CH01h10, CH04E05, CH04c07, CH02c09, CH01h01, CH02c11, CH05f06, Hi02c07, CH02c06, NZ05g8, CH03d07, GD147, CH04f10 and GD12) were studied in three independent multiplex PCRs as described in Urrestarazu et al. (2012). The fragment analysis was performed with an ABI PRISM 3730 sequencer (Applied Biosystems, USA). Peak identification and fragment sizing were performed in Peak Scanner Software 3.1 (Applied Biosystems, USA).

2.2. Evaluation of pollen viability

To obtain the pollen, one hundred flowers were collected at the E_2 phenological stage according to the Fleckinger scale (Fleckinger, 1945) or BBCH59 according to the BBCH scale (Meier et al., 2009) (buds not opened but swollen). The flowers were dried *in silica* gel for approximately 48 h, and the pollen was extracted from the anthers 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. Pollen quality was tested in the most cultivated cultivars (Bost Kantoi, Errezilla, Gezamina (3n), Goikoetxea, Haritza, Manttoni, Mikatza, Moko, Mokote, Patzuloa (3n), Reineta Encarnada, Saltxipi, Txalaka, Txori Sagarra, Urtebete, Urtebi Txiki, Urdin, Urtebi Handi, Udare Marroi (3n), and Verde Agria). Eight reference cultivars were also included as comparing standards (Fuji, Galaxy, Golden Delicious, Granny Smith, Jonagold (3n),

Red Chief, Starking Delicious, and Top Red).

The pollen viability rate was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining. The staining mixture was prepared with 1% TTC, 60% sucrose, and ddH_2O, and stored in darkness (Sulusoglu and Cavusoglu, 2014). The pollen was spread on a microscope slide, and after two hours in the dark, five spots distributed along the coverslip were observed under the microscope (Axiolab 5 KMAT, Zeiss, Germany). Pollen grains stained in bright red or orange were considered viable, whereas those stained in brown, ochre, light orange or whitish were considered 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, 300 ppm $Ca(NO_3)_2$, and 0.02% $H_3(BO)_3$ at pH 6.5 was prepared and poured into Petri dishes. Pollen was hydrated in 20 mM of buffer Tris pH 6.5 and spread over three plates per cultivar (Calzoni et al., 1979). The plates were incubated for 48 h at 25 °C in the dark. After 48 h, were observed under the microscope in five spots distributed along each dish. Pollen grains were considered germinated when the pollen tube length was at least three times the diameter of the pollen grain. A minimum of 100 pollen grains per variety were counted to calculate the rate of germinated pollen (%) under these conditions.

Values for viability and germination rates were tested with ANOVA, and cultivars were grouped according to the post-hoc Scheffé test.

2.3. Self-compatibility evaluation

Self-pollinations were performed *in vitro* and *in vivo*. The modern cultivar Golden Delicious was included as a control due to its low but certain self-compatibility degree (Schneider et al., 2001).

In vitro self-compatibility was tested in those cultivars more widely used in cider orchards and, so, with higher economic importance (Bost Kantoi, Errezila, Gezamina (3n), Goikoetxea, Haritza, Manttoni, Mikatza, Moko, Mokote, Mozoloa (3n), Patzuloa (3n), Saltxipi, Txalaka, Txori Sagarra, Udare Marroi (3n), Urdin, Urtebete, Urtebi Haundi (3n), Urtebi Txiki, and Verde Agria). The reference variety Reineta Encarnada was also included due to its use for cider production in the region. Self-pollination tests in the laboratory were done in 50 flowers in E2 phenological stage. First, the flowers were emasculated and placed stiff in a water tray for 24 h to complete their maturation. The next day, flowers were self-pollinated. Germination took place for 72 h since after that pollen viability is strongly reduced, being unlikely for the pollen tube to reach the end of the style and the ovule. Then, pollinated flowers were placed for five days in FAA fixative solution and conserved at 4 °C in 70% ethanol until observation. Before flower observation, the samples were treated for 4 h with 8 N NaOH to soften the tissues and allow the stain to penetrate. The tissues were stained with 0.1% aniline blue solution diluted in 0.1 N K_3PO_4 for a minimum of 24 h, and without washing, they were mounted on a microscope slide with a few drops of the staining solution as a medium. The cover was gently pressed to crush the softened pistils. The preparations were observed under DAPI fluorescent light (adapted from Martin (1959)). A minimum of 20 pistils were observed per cultivar. For quantification, germinated pollen grains were counted, and the length of each pollen tube was measured and rated to the stigma's length.

In vivo self-pollination tests, which were carried out in the field, were performed in Errezila, Gezamina (3n), Goikoetxea, Manttoni, Mikatza, Moko, Mozoloa (3n), Patzuloa (3n), Saltxipi, Txalaka, Urdin, Urtebi Haundi (3n), Urtebi Txiki, and Verde Agria. The test could not be performed in other varieties due to the scarce availability of flowers. A minimum of 5 corymbs with flowers in the E_2 stage were covered with a net to prevent cross-pollination by insects. Then, the fruits set were counted, and, to quantify self-pollination capacity, the rate of fruits set to the total number of flowers was calculated.

Values for self-pollination rates were tested with the analysis of variance (ANOVA), and cultivars were grouped according to the post-hoc Scheffé test.

2.4. S-allele characterization

In the first phase, *S*-alleles were amplified with degenerate primers (PycomC1fa / PycomC5ra) and identified by their sequence following the indications in De Franceschi et al. (2016) and Sheick et al. (2018). In detail, 100 ng of genomic DNA were amplified following the manufacturer's instructions using Ex Taq DNA polymerase (Ref RR001C, Takara Bio, Japan) and PycomC1fa / PycomC5ra primers with the following amplification conditions: an initial denaturation, 95 °C - 3 min; 30 cycles (95 °C – 20 s; 58 °C – 30 s; 72 °C – 1 min); a final extension 72 °C – 8 s (Sanzol and Robbins, 2008). The amplified fragments were then separated in a 3% agarose gel stained with SYBR Safe (Fisher Scientific AG, Switzerland). The gel was run at 120 V for 1.5 h, and the amplified *S*-allele fragments were observed with a blue light transilluminator (Fisher Scientific AG, Suisse). The DNA bands were cut individually, and the DNA was extracted from the gel using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany) and sequenced with Sanger (3730xl DNA Analyzer, Thermo Fisher, USA). Finally, for preliminary *S*-allele identification, the sequences were compared against the NCBI genomic database (https://www.ncbi.nlm.nih.gov/genome/).

In the second phase, *S*-allele identity was confirmed by the amplification with specific primers. PCR was prepared with Ex Taq DNA polymerase Kit (Takara Bio, Japan) and run according to published conditions (Broothaerts, 2003; Long et al., 2010). The PCR products were separated in agarose gels (1%). The gels were stained with SYBR Safe DNA Gel Stain (Thermo Fisher, USA) and visualized under a blue light transilluminator (Fisher Scientific AG, Switzerland).

3. Results

3.1. Cultivar identification

Cultivar identification results showed that two out of the 17 SSR markers used, NZ05g8 and CH04f10, were problematic in terms of insufficient fluorescence signal or complex scoring pattern and, as a consequence, were not considered. The multi-locus SSR profiles obtained for the remaining 15 SSR markers revealed the presence of 39 genotypes within the 42 different accessions tested (Suppl. Table 1). The three duplicated accessions were: Mozoloa (3n), which matched with Gezamina (3n), Goikoetxea with Frantzes Sagarra, and Manzana Enana with Nano de Cascante. Each of the other 36 cultivars represented a unique genotype.

3.2. Evaluation of pollen quality

3.2.1. Pollen viability

Pollen viability differed significantly among cultivars, ranging from a mere 2.5% in Gezamina (3n) to 95.5% in Verde Agria (Fig. 1A-C). Most diploid traditional cider cultivars had a viability above 60% and were grouped with the references (Table 2). In special, Verde Agria (95.5%), Goikoetxea (94.9%), and Bost Kantoi (92.9%) stand out due to their significantly higher viability. Three exceptions appeared within traditional diploids: Reineta Encarnada (34.7%), Errezila (17.7%), and Urtebete (16.8%). As expected, for the triploids, viability was low (<10%) and similar to that of Jonagold (3n), except for Udare Marroi (3n) (53.1%) and Urtebi Haundi (3n) (70.5%), whose viabilities were very close to that of the diploid group.

3.2.2. Pollen germination

The germination rate also differed significantly among cultivars ($p<0.001$), ranging from 1.2% (Gezamina (3n)) to 84.8% (Mikatza) within the cider cultivars (Fig. 1 D-F). Among the diploids, cider cultivars with germination rates below 50% significantly differed from the references (Table 2). In this group, it must be highlighted the low performance obtained by Urdin (42.1%), Reineta Encarnada (39.9%), Verde Agria (37.4%), and Urtebi Txiki (26.1%). Although these rates

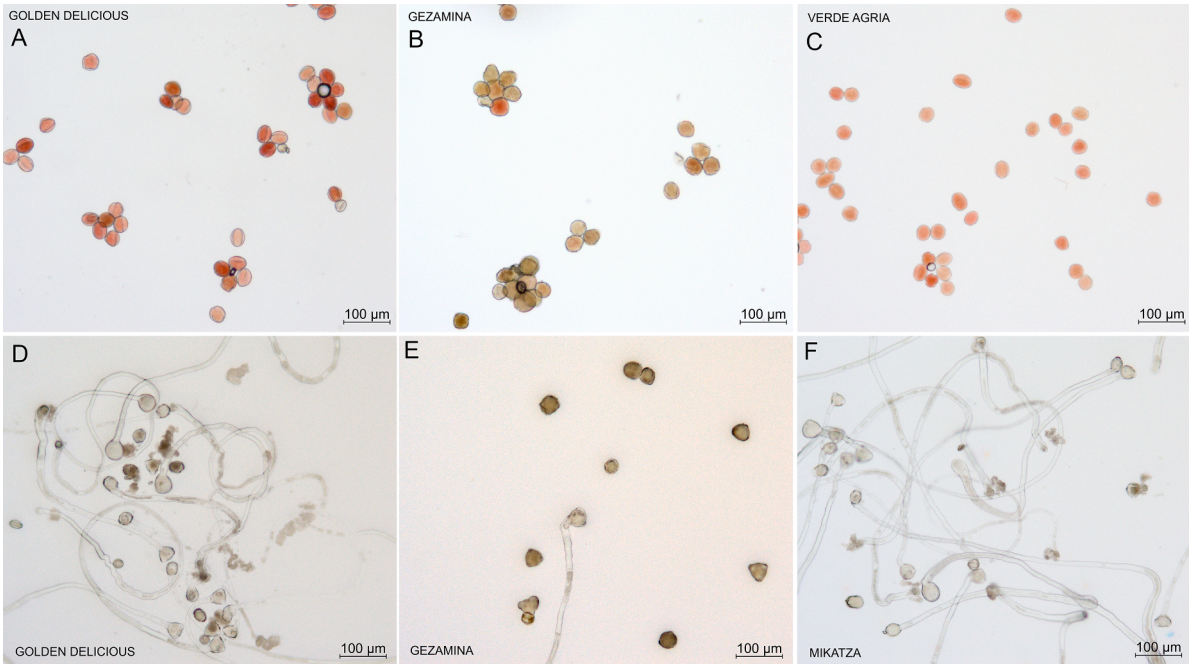

Fig. 1. Microscope images of the pollen viability (A-C) and germination tests (D-F). Golden Delicious was used as a control for both tests (A, D). Gezamina (3n) (B, E) represents a cultivar with a low pollen quality as it has a low viability rate and a low germination rate. Contrary, Verde Agria showed a high viability rate (C) and Mikatza a high germination rate (F).

Table 2
Pollen viability and germination data of *in vitro* tests (n = 29). Data are shown as mean values with standard error (SE). Statistical differences were tested by ANOVA and post-hoc Scheffé test. Underlined letters indicate reference cultivars.

	Pollen viability (%) Mean ± SE	Germination (%) Mean ± SE
Starking Delicious	96.93 ± 0.90 ^{a}	88.43 ± 1.92 ^{a}
Granny Smith	96.75 ± 0.72 ^{a}	79.43 ± 3.07 ^{ab}
Verde Agria	95.54 ± 0.84 ^{a}	37.70 ± 4.40 ^{efg}
Red Chief	95.23 ± 2.12 ^{a}	84.11 ± 1.93 ^{ab}
Goikoetxea	94.85 ± 0.89 ^{a}	56.68 ± 3.29 ^{abcde}
Golden Delicious	94.11 ± 0.76 ^{a}	65.03 ± 5.23 ^{abcde}
Top Red	93.41 ± 1.12 ^{a}	77.93 ± 3.59 ^{ab}
Bost Kantoi	92.92 ± 1.95 ^{a}	42.17 ± 3.77 ^{cdef}
Manttoni	88.88 ± 1.32 ^{ab}	59.65 ± 3.28 ^{abcde}
Moko	87.94 ± 2.11 ^{ab}	76.92 ± 3.91 ^{ab}
Fuji	85.96 ± 2.37 ^{abc}	68.81 ± 3.17 ^{abcde}
Saltxipi	85.42 ± 2.32 ^{abc}	82.08 ± 4.07 ^{ab}
Mikatza	84.25 ± 1.15 ^{abc}	84.82 ± 2.84 ^{ab}
Urtebi Txiki	84.09 ± 1.28 ^{abc}	38.98 ± 5.60 ^{def}
Txalaka	83.94 ± 2.56 ^{abc}	71.09 ± 2.80 ^{abcd}
Galaxy	83.70 ± 1.74 ^{abc}	68.36 ± 3.29 ^{abcde}
Txori Sagarra	83.39 ± 0.91 ^{abc}	55.85 ± 3.61 ^{bcde}
Urdin	82.12 ± 1.79 ^{abc}	42.13 ± 2.77 ^{cdef}
Urtebi Haundi (3n)	70.51 ± 1.81 ^{abcd}	15.81 ± 5.77 ^{fgh}
Haritza	65.78 ± 2.28 ^{abcd}	74.27 ± 4.02 ^{abc}
Mokote	56.60 ± 10.11 ^{bcd}	68.50 ± 5.14 ^{abcde}
Udare Marroi (3n)	53.08 ± 7.52 ^{cd}	1.83 ± 0.88 ^{h}
Reineta Encarnada	45.36 ± 6.64 ^{de}	39.88 ± 4.75 ^{def}
Errezila	17.74 ± 7.52 ^{ef}	56.41 ± 4.13 ^{abcde}
Urtebete	16.79 ± 5.85 ^{ef}	2.10 ± 0.95 ^{h}
Patzuloa (3n)	8.85 ± 3.39 ^{f}	1.48 ± 1.15 ^{h}
Jonagold (3n)	8.59 ± 4.30 ^{f}	5.74 ± 2.25 ^{gh}
Mozoloa (3n)	7.84 ± 2.74 ^{f}	3.1 2 ± 1.15 ^{h}
Gezamina (3n)	2.45 ± 1.55 ^{f}	1.16 ± 0.79 ^{h}

cannot be considered poor, they indicate that a greater amount of their pollen would be needed to achieve results comparable to those of the better performing cultivars. In the case of Errezila, its pollen viability was low (<20%), but its germination level was moderate (56.4%). In the case of the triploid cultivars, as expected, the germination rates were very low (< 5%), except for Urtebi Haundi (3n). Around 15% of its pollen grains managed to germinate and, along with its high viability rate, it would perform as a poor pollinator, which is much better than expected in a triploid cultivar.

In general, the level of pollen germination was well related to their viability, so cultivars with high viability also had pollen with high germination power, and *vice versa*. The exceptions were Urtebi Haundi (3n), Udare Marroi (3n), Urdin, Verde Agria, and Urtebi txiki, for which their germination rates were much lower than for the viability, and Errezila, in which the reverse situation was observed.

3.3. Compatibility tests

3.3.1. Self-compatibility evaluation

For effective pollination, pollen should germinate, and the pollen tube must elongate till reaching the ovule, which is usually inhibited in self-pollination. In the *in vitro* self-pollination tests, these two aspects were quantified by the rate of flowers with germinated pollen and the length of the pollen tubes (Table 3). Regarding germination rates, the cultivars Moko (Fig. 2 E), Urdin (Fig 2G), Bost Kantoi, and Txori Sagarra outperformed Golden Delicious, having significantly higher rates (>88%). Saltxipi showed a level similar to Golden Delicious (~75%) (Fig. 2C-D). Other varieties, such as Manttoni, reached medium germination rates (Fig. 2F). Several cultivars displayed very low rates, equal or below 25%, as Verde Agria, Patzuloa (3n), Goikoetxea, Gezamina (3n), Reineta Encarnada, Udare Marroi (3n), Urtebete, and Mokote (Fig 2H) whereas, the remaining showed modest intermediate levels, between 25 and 75%. Regarding pollen tube development, some varieties showed pollen tubes reaching around ¾ of the stigma after 72 h (Moko, Bost Kantoi, Txori Sagarra, Saltxipi, and Mikatza) (Fig. 2E). Those, which also had high germination rates, demonstrated some degree of self-compatibility. Mikatza showed the highest pollen tube development, 83% of the stigma, but its germination rate was modest. On the contrary, Urdin behaved as Golden Delicious, which, despite having a high germination rate, was not able to develop enough pollen tubes confirming their low self-compatibility capacity (Table 3, Fig. 2C-D-G).

Table 3
Results of the *in vitro* and *in vivo* germination tests (n = 22). The *in vitro* test results were quantified as flowers with germinated pollen grains (germination%) and the length of the pollen tube, with maximum and mean values. *In vivo* germination was tested in fourteen of the cultivars and fertile flowers covered under the net were quantified. Underlined letters indicate reference cultivars, and - indicates missing values.

Accession	*In vitro*			*In vivo*
	Germination	*Rate* $\left(\frac{Length\ of\ the\ pollen\ tube}{Length\ of\ the\ stigma}\right)$		Fertile flowers
	%	Max (%)	Mean (%)	%
Moko	100.00a	62.86	30.99 a	8.00 a
Urdin	100.00a	28.34	19.32 abc	0.59 bc
Bost Kantoi	95.83a	73.16	30.83 a	–
Txori Sagarra	88.00a	74.89	21.02 abc	–
Golden Delicious	78.57ab	34.74	21.80 abc	–
Saltxipi	76.00 ab	77.36	29.23 ab	1.09 bc
Mozoloa (3n)	73.08 abc	20.62	7.90 abc	1.21 bc
Urtebi Haundi (3n)	72.00 abc	37.76	15.07 abc	0.00 c
Mikatza	72.00 abc	83.64	4.45 bc	0.00 c
Manttoni	68.00 abcd	55.78	12.09 abc	0.00 c
Urtebi Txiki	59.09 abcde	57.69	3.49 bc	0.95 bc
Txalaka	56.52 abcde	40.24	23.42 abc	6.23 ab
Haritza	44.00 abcde	28.8	20.65 abc	–
Errezila	40.00 abcde	33.35	15.37 abc	0.00 c
Verde Agria	25.00 bcde	10.19 a	16.18 abc	0.48 c
Patzuloa (3n)	12.00 cde	5.14 a	2.81 c	0.00 c
Goikoetxea	10.00 de	9.64	16.18 abc	0.83 bc
Gezamina (3n)	8.00 de	12.64	20.00 abc	3.35 abc
Reineta Encarnada	8.00 de	9.91	12.50 abc	–
Udare Marroi (3n)	4.00 e	2.99	2.99 c	–
Urtebete	0.00 e	–	–	–
Mokote	0.00 e	–	–	–

Table 3 shows also the results of *in vivo* tests. For most cultivars, the number of self-pollinated flowers were null or residual (<2%), which would confirm that are strictly self-incompatible and that require cross-pollination. Fruit set after self-pollination was only observed for three cultivars, Gezamina (3n), Moko, and Txalaka, showing some flowers set at the end of the trial (3 - 8%). These rates are far from what is achieved by cross-pollination and, in any case, insufficient to obtain an acceptable harvest.

From those cultivars with promising results in the *in vitro* self-pollination tests (Moko, Bost Kantoi, Txori Sagarra, Saltxipi, and Mikatza), Bost Kantoi and Txori Sagarra could not be tested *in vivo*, but a partial self-compatibility was confirmed for Moko. *In vivo* tests showed also a partial self-compatibility in Txalaka although *in vitro* germination tests showed discrete results.

3.3.2. Genetic inter-compatibility evaluation

For the *S*-allele study, the 39 traditional cultivars with unique genotype were included. Beside, two reference cultivars (King of the Pippins and Reineta Encarnada) and two pollinator cultivars (Evereste and Golden Gem) were also tested.

Thirty-six *S*-genotypes could be completely deciphered while one of the *S*-alleles remained unknown when *S*-genotyping three cultivars (Manyaga, Ibarra (3n), and Urtebi Haundi (3n)). Within the local population, a total of 14 previously described alleles were identified (S_1, S_2, S_3, S_4, S_5, S_7, S_{11}, S_{17}, S_{20}, S_{23}, S_{24}, S_{26}, S_{28}, and S_{34}). All the *S*-alleles identified from reference and pollinator cultivars were included within that list. Additionally, we found one novel *S*-allele in the cultivar 'Verde Agria', which has been designated as S_{60} (NCBI accession OP547868.1) following the nomenclature established by Kim et al. (2016) and continued in Sheick et al. (2018, 2020).

Regarding allele frequency, six alleles covered 0.77 of the total alleles of the traditional population: S_3 (0.21), S_{26} (0.16), S_2 (0.13), S_5 (0.11), S_1 (0.09), and S_{28} (0.07) (Fig. 3). As described in the introduction, among those *S*-alleles S_1, S_2 S_3, and S_5 are frequent alleles in modern cultivars nonetheless they also reach high rates among traditional cultivars. S_{26} and S_{28} raised as two frequent alleles in the population of the Basque Country and Navarre, but are very rare in the modern and traditional apple cultivars from other geographical origins studied to date (see references in Supplementary Table 2).

A total of 12 *S*-genotypes were unique (33.3%), meaning that those cultivars are compatible with all the others (Gezamina (3n), Gollano-03, Haritza, La Rica, Manttoni, Manzana Tomate, Maxel Gorri, Patzuloa (3n), Roja, Saltxipi, Udare Marroi (3n), Urdin, and Verde Agria) (Fig. 4). The rest belonged to one of the incompatibility groups identified: S_1S_3, S_2S_3, S_2S_5, S_2S_{26}, S_3S_5, S_3S_{11}, S_3S_{26}, S_3S_{28}, S_5S_7, S_5S_{28}, and $S_{26}S_{28}$. The cultivars that showed more incompatibilities in the study were Baztan Sagarra, Mikatza, Mokote, Ibarra (3n), and Pero (group S_3S_{26}), followed by Genaro Sagarra, Hierro, Nano de Cascante, and Patzuloa (3n) (group S_1S_3) and Errezila, Patzuloa (3n), Udare Marroi (3n), and Urtebete (group S_3S_5). The other incompatibility groups were composed of two or three cultivars. Regarding the other categories of cultivars included, the reference King of Pippins shared the *S*-genotype with the incompatibility group S_1S_3, while Reineta Encarnada had the same as Moko and Txalaka (S_2S_{26}), and, within the crabapples, while Evereste had a unique genotype, Golden Gem (S_1S_{26}) shared it with Limoi.

4. Discussion

4.1. Some traditional cultivars as Txalaka, Mikatza, Saltxipi, or Moko could be used as pollinators

Although pollen quality was high in most cultivars, the germination rate showed high variability throughout the population. In general terms, diploids frequently had germination rates over 98%, well above 70%, being the rate used to be classified as good (Ramírez and Davenport, 2013). Some exceptions appeared within diploids, such as Reineta Encarnada, Urtebete, and Errezila, which had very low pollen viability rates, similar to those in triploids (Fig. 5). Some traditional cultivars as Txalaka, Mikatza, Saltxipi, or Moko had outstanding values, and could therefore be used as pollinators.

Contrarily, triploid cultivars are known to reach normally less than 10% germination, which is confirmed here by most of the triploids within this population, such as Patzuloa (3n), Mozoloa (3n), and Gezamina (3n), which grouped with the reference triploid Jonagold (3n). However, the most remarkable result regarding the triploids was that some showed unexpectedly high viability values (*e.g.*, Udare Marroi (3n) and Urtebi Haundi (3n) >50% viability). In any case, in general terms, our results confirm the necessity of paying attention to pollination in the design of orchards where triploids will be cultivated.

4.2. Moko and Txalaka show a certain self-compatibility degree

The self-pollination tests performed for the 20 most cultivated traditional apple cultivars confirmed the requirement of cross-pollination. Apple is majorly self-incompatible, and only a few cultivars are found to be self-pollinated. Some Japanese cultivars may reach a reasonable fruit set, such as Megumi, with rates up to 50% (Matsumoto et al., 1999). More frequently, cultivars show low self-compatibility, such as those reported for Elstar, Golden Delicious, or Fuji, which reach values under 10% (De Witte et al., 1995; Saito, 2007). In our work, the performance of Moko and Txalaka, with levels around 10% (Table 3), was unexpected. But, in any case, that level of self-compatibility is low, and not enough for sustainable apple production. So, pollinators should still be included, but that self-compatibility level can be considered a supplementary pollen source.

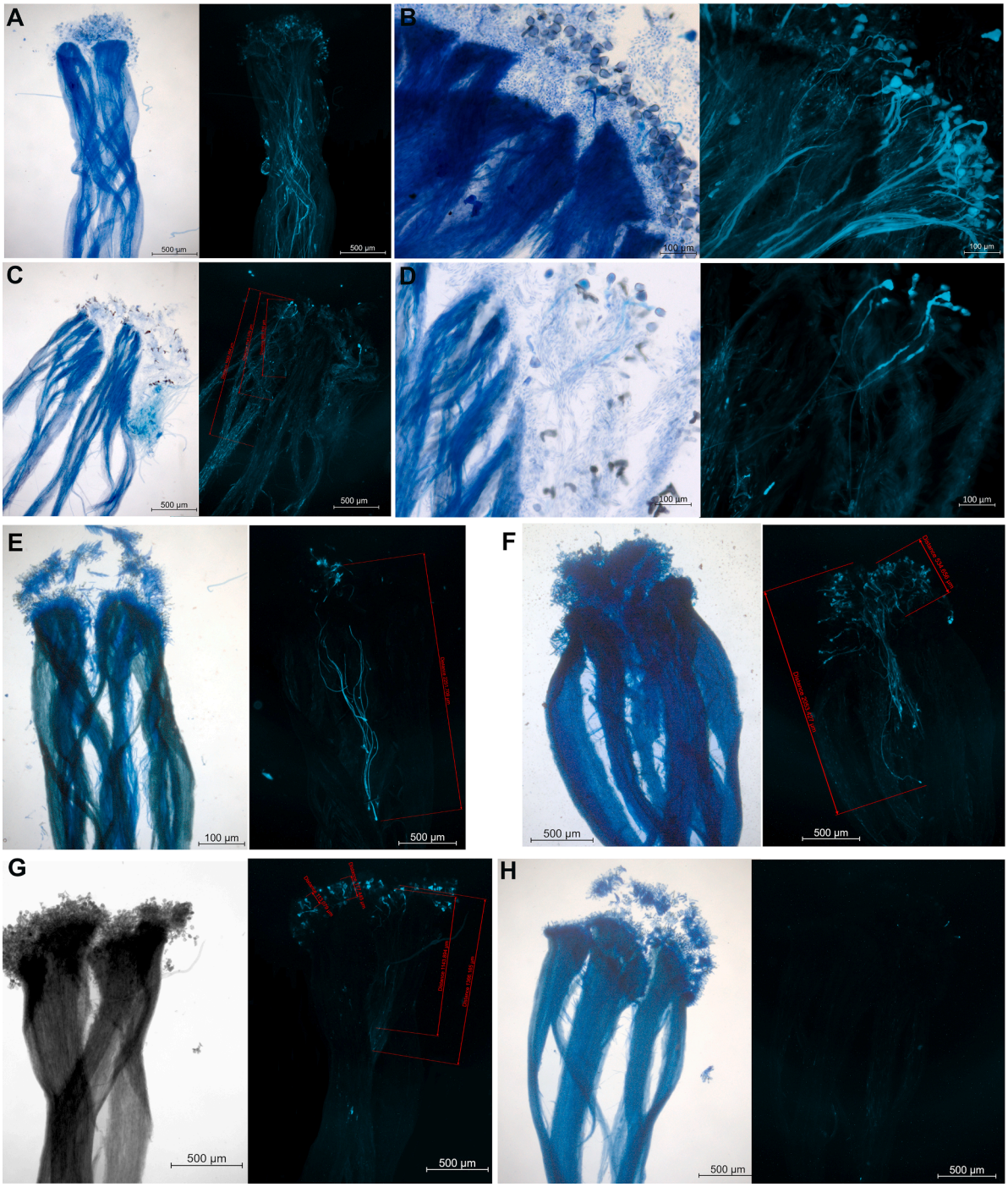

Fig. 2. Microscope images of the elongation of pollen tubes in the *in vitro* self-pollination tests. Pistils were stained with aniline blue and observed with DAPI fluorescence. (A-B) Naturally pollinated pistils were used as a positive control. Many germinated pollen grains and long pollen tubes can be observed. (C-D) Artificially self-pollinated Golden Delicious pistils were used as a control for artificial pollination. A few pollen grains germinated, and the pollen tubes did not develop well. (E-H) Some examples of traditional cultivars tested. (E) Moko showed self-compatibility, as some pollen grains germinated and pollen tubes elongated. (F) Manttoni showed a high germination rate, but the elongation of pollen tubes was scarce. (G) Urdin had a high germination rate but almost no pollen tube elongation. (H) Urtebete was fully-incompatible, as no germinated grains were found. Red lines indicate the length of the pollen tubes.

4.3. Specific allele composition and frequency in a particular gene pool population

Alleles S_1, S_2, S_3, S_4, S_5, S_7, S_{11}, S_{17}, S_{20}, S_{23}, S_{24}, S_{26}, S_{28}, and S_{34} conformed the *S*-genotype diversity of the population. The allele composition, along with their frequency, was compared with a compilation of *S*-allele studies (Fig. 3, Suppl. Table 2), concluding that the allele composition of this population differs from other modern, traditional, or wild/crab populations described until now.

On the one hand, alleles S_4 and S_{11} are infrequent in modern cultivars but mainly present in traditional and wild populations in Eastern Europe (Dreesen et al., 2010; Larsen et al., 2016). On the other hand, alleles S_1, S_2, S_3, and S_5, are uncommon in most traditional and wild ones but were also frequent in two Eastern Europe traditional populations (Dreesen et al., 2010; Larsen et al., 2016). So, although rare, the combination of those alleles (S_4, S_{11}, S_1, S_2, S_3, and S_5), a mixture of typically traditional

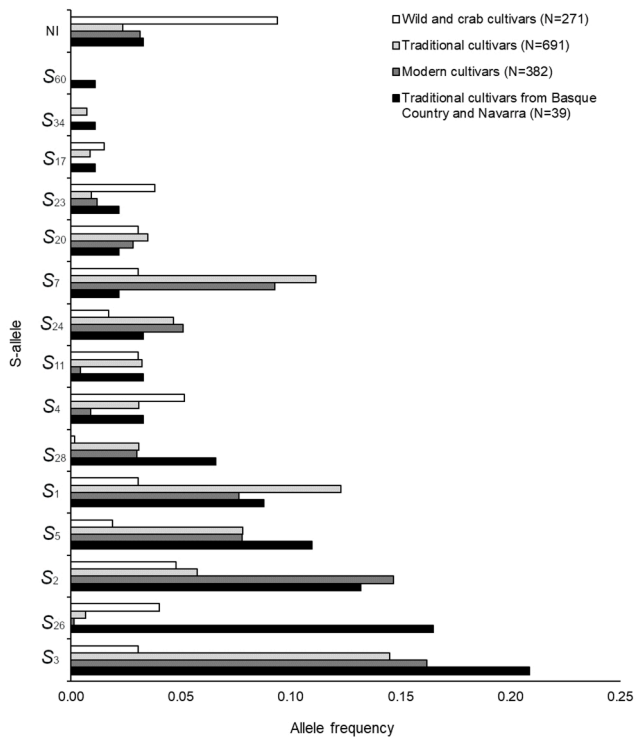

Fig. 3. *S*-allele composition of the population and their frequencies. The frequencies per *S*-allele of this population were compared with the compilation of the *S*-genotypes of modern cultivars, traditional cultivars, and wild/crab cultivars published in other studies. *S*-allele frequencies for other studies were compiled through a bibliographic review performed in the scope of this work and detailed in Supplementary Table 2. *Abbreviations*: NI: Not identified.

and typically modern *S*-alleles, has been described previously in other European apple traditional populations (Dreesen et al., 2010; Larsen et al., 2016).

The alleles S_{28} and, especially, S_{26}, are the rarest *S*-alleles found in our study. Regarding S_{28}, this allele has been found in several traditional populations, being an allele discreetly found in germplasm from Western Europe (Broothaerts et al., 2004; De Franceschi et al., 2018; Garkava--Gustavsson et al., 2008; Halász et al., 2011; Larsen et al., 2016; Nybom et al., 2008) while it has reached a higher frequency in a single group from Denmark (Larsen et al., 2016). However, it seems to have spread in southern Europe as, for example, has been reported herein. Beside, it also has been found in modern cultivars such as the widely cultivated Red Delicious (Broothaerts et al., 2004; López-Girona et al., 2021; Sheick et al., 2018). Concerning S_{26}, this allele has been reported as 'extremely rare' (Broothaerts, 2003), but within the population considered in our research, it has shown an exceptionally high frequency (≈15%). This frequency has not been previously described in any modern, traditional, or wild population until now and differentiates the Basque Country and Navarre population from all others studied so far.

In addition, the population presented an unpublished allele. The newly described allele, S_{60}, showed the highest amino acid similarity to that of the allele S_{26} (NCBI accession AB428432.1) (Kim et al., 2009), reaching an 85.1% identity. Other *S-RNases* sequences have shown similarities varying between 68 and 92% (Long et al., 2010). The high similarity between S_{60} and S_{26} may suggest a short evolutionary distance between both homologues.

The unique *S*-allele composition found in the germplasm evaluated in this study, mainly characterized by the high frequency of S_{26} and the appearance of a novel *S*-allele (S_{60}), indicates a singularity of the Basque Country and Navarre traditional apple population. Previously, compared their SSR markers with other populations in Europe and even Northern Spain, the population represented its genetic pool (Urrestarazu et al., 2012, 2016). The results of the *S*-allele study carried out here constitute evidence highlighting the singularity of the apple germplasm from this geographical area.

4.4. The high frequency of S_{26} may bring incompatibilities with crabapple pollinators

S_{26} is a frequent allele in crabapples, such as Evereste, Indian Summer, or Baskatong (Dreesen et al., 2010; Matsumoto et al., 2007; Sheick et al., 2020, 2018). In this work, we confirmed the already published *S*-genotype of Evereste ($S_{20}S_{26}$) and added Golden Gem (S_1S_{26}) to this list. This allele has also been found in *S*-genotypes of *Malus sylvestris* (Broothaerts et al., 2004; Dreesen et al., 2010; Li et al., 2012; Long et al., 2010). Regarding traditional cultivars, S_{26} has appeared in Hungary, Denmark, Italy, Greece, Russia, the United Kingdom, and Iran, although with discrete frequencies (De Franceschi et al., 2018; Halász et al., 2011; Larsen et al., 2016; Long et al., 2010). Beside, until now, only two modern cultivars carried S_{26} in its *S*-genotype (Long et al., 2010): Yoshkee ($S_{11}S_{26}$), a Canadian seedling of McIntosh, and Prime Gold ($S_{19}S_{26}$), found in the U.S in 1965, with parent-offspring relationships with Morgenduft and Red Delicious (Baric et al., 2020). All these facts support the hypothesis that the presence of the allele S_{26} in cultivated apples might be an introgression from *Malus floribunda* and *Malus sylvestris*, as suggested by Brooathers et al. (2003). This agrees with a recent genomic study suggesting that cider apples may derive from an ancestor *Malus sylvestris*, as they seem to have a closer relation than dessert apples (Migicovsky et al., 2021).

The *S*-allele composition of the population should be considered in orchard design, as it has been shown that it may not be rare that a traditional cultivar and a pollinator crabapple share totally or partially their *S*-genotype. The population of Navarre and the Basque Country is an example, and the high frequency of the allele S_{26} could explain, at least partially, the erratic harvests obtained. In this territory, placing crabapples as pollinators in the orchards is relatively frequent, based on the belief that including them will guarantee a successful pollination based on an alleged full compatibility and high pollen quality of crabapples. It is known that fully compatible pollinators are preferred to semi-compatible pollinators as semi-compatibilities produce reduced fruit set and harvest (Schneider et al., 2005). All traditional varieties carrying S_{26} in their *S*-genotypes have this situation with the recurrent crabapples Evereste or Golden Gem. In addition, the higher probability of complete incompatibilities with crabapples in a population with a high frequency of S_{26} cannot be dismissed, as described herein between Limoi and Golden Gem. These facts highlight the importance of describing the *S*-genotypes of not only main cultivars but also those traditional and crabapples and ensuring an efficient orchard design.

5. Conclusions

Apple cultivation in the Basque Country and Navarre (Spain) is mainly based on several traditional local cultivars. Yield is erratic between years, and it is difficult to know the causes to explain it due to the lack of information regarding the reproductive biology of these cultivars. Here, we report a multi-approach study to dig into their reproductive biology that included pollen quality determination, self-fertility, and *S*-genotypes of the most cultivated varieties to decipher the possible reasons for that fluctuation. In general pollen quality was good, and even some cultivars might be used as pollinators. That was not the case for Urtebete, Errezila, Reineta Encarnada and, as expected, the triploids. Regarding self-compatibility, surprisingly Moko and Txalaka showed a certain degree of self-compatibility, which certainly does not imply that pollinators are not needed. As for the *S*-genotypes, the *S*-allele composition of the population was exceptional, based on the outstandingly high frequency of S_{26} and the presence of S_{60}, an unpublished allele. S_{26} is frequent only within crabapples, and it might have introgressed into this population. Sharing S_{26} with widely used crabapples advocates

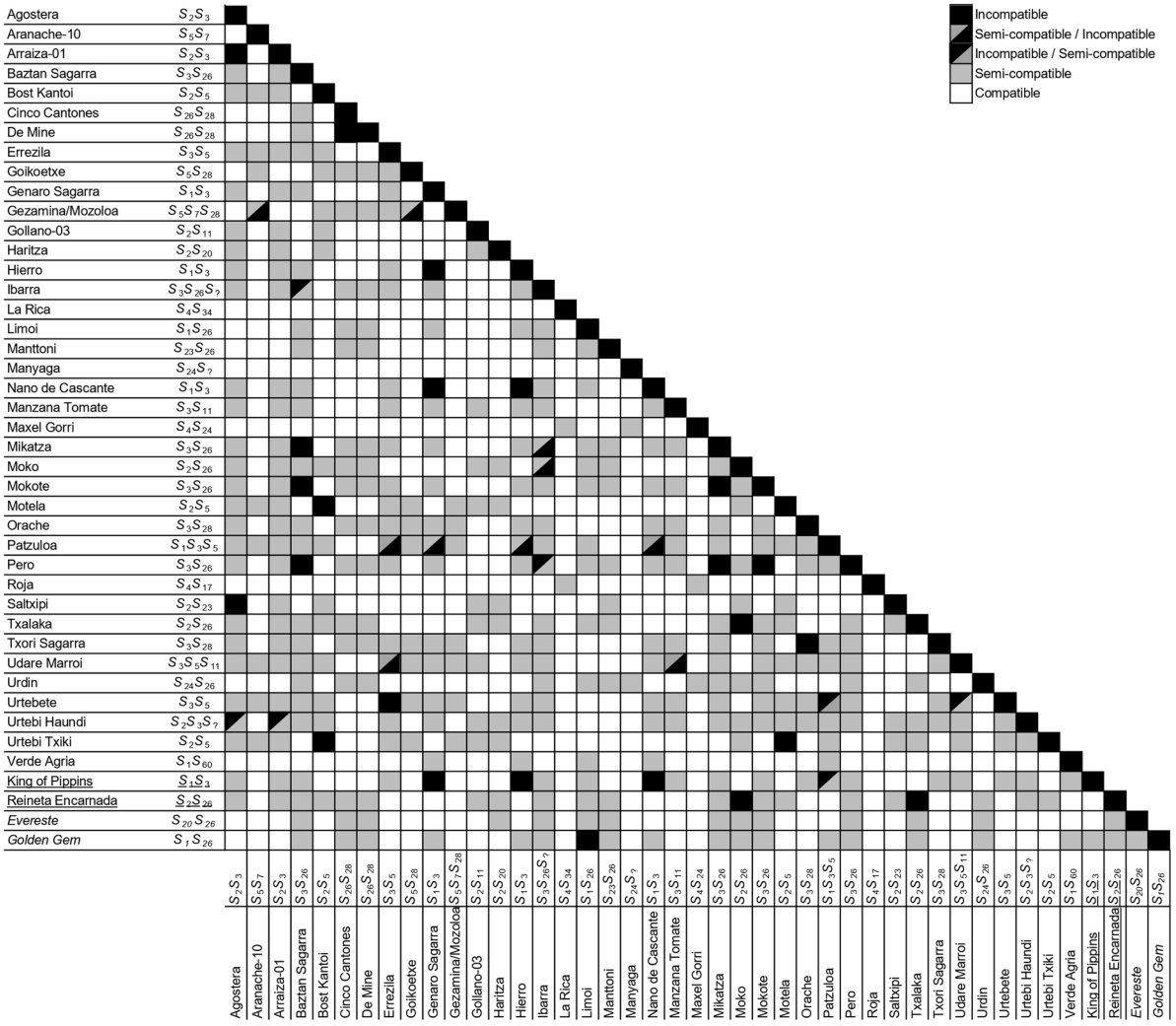

Fig. 4. *S*-allele compatibility chart displayed in a correlation matrix. Cultivars included were the traditional apple cultivars of the Basque Country and Navarre, the two reference cultivars (underlined letters), and the two crabapple cultivars (*italics*) . Varieties were classified into 3 categories: incompatible (when sharing the complete *S*-genotype), semi-compatible (when sharing part of the *S*-genotype), or compatible (when not sharing any *S*-allele).

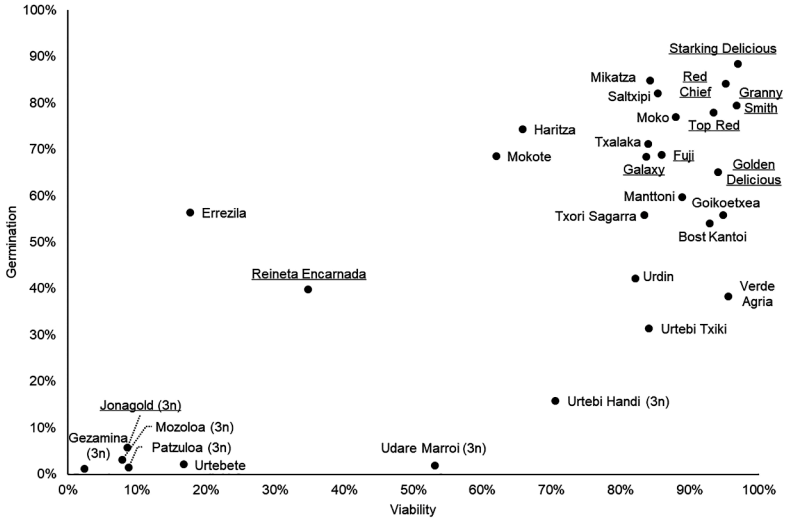

Fig. 5. Viability (%) and germination (%) of pollen of different traditional cider cultivars. Cultivars used as references are marked in italics. Pollen viability (% viable grains) according to TTC staining and pollen germination rate (%) of the cultivars under study. Pollen viability results range between 96.32% for Granny Smith and Starking to 2.72% for Gezamina (3n). Reference cultivars are marked in italics. The ploidy of the cultivar is specified between brackets, diploid (2n) or triploid (3n). Reference cultivars are indicated in underlined letters.

conscious pollinator choice to avoid incompatibilities and semi-compatibilities that, to date, have been overseen and may explain erratic yields. The significance of this study goes well beyond the population under study since it points out the importance of *S*-genotyping of all the varieties cultivated to provide an effective orchard planning with an appropriate pollinator selection.

CRediT authorship contribution statement

S. Crespo-Martínez: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **O. Oneka:** Data curation, Formal analysis, Investigation, Methodology. **M.J. Laquidáin:** Conceptualization, Methodology, Supervision, Writing – review & editing. **J. Urrestarazu:** Conceptualization, Methodology, Writing – review & editing. **L.G. Santesteban:** Conceptualization, Writing – original draft, Writing – review & editing. **C. Miranda:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Carlos Miranda reports financial support was provided by Ministry of Science and Innovation. Carlos Miranda reports financial support was provided by Diputación Foral de Gipuzkoa.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2023.112395.

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Further reading

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