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Determining Pollinizer Success Rates among Several Apple (*Malus domestica* L.) Cultivars Using Microsatellite Markers

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Abstract: In order to determine the pollinizer success rates between twelve apple cultivars in 2021 and 2022, 671 apple embryos were collected from 19 different orchards in Ullensvang (southwestern Norway) and Svelvik (southeastern Norway). Genomic DNA was extracted from the collected embryos and, afterward, a genetic characterization with 15 polymorphic microsatellite markers was conducted. An identical set of markers was also used on all twelve mother cultivars, as well as on six crabapple pollinizers, which were found in the investigated orchards. The obtained molecular data enabled paternity analyses to be performed with the objective of assigning a male parent to each embryo. The paternity analyses identified pollen donors for all, except for 3% of the embryos. In most cases, it was possible to identify the most successful pollinizers for each cultivar, with 'Aroma' and 'Discovery' being the most efficient pollen donors overall. Tree abundance seems to be a major factor in pollinizer success, while semi-cross-compatible characteristics represent a hindrance. Only 7% of the analyzed embryos were determined to have been fertilized by pollinizers outside the orchard, confirming the significance of pollinizer proximity for efficient pollination.

Keywords: paternity analyses; SSR; embryos; pollinizers; cross compatibility; tree abundance

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1. Introduction

Apple (*Malus domestica* Borkh.) is one of the most commercially significant temperate fruit crops in the world with an annual global production of approximately 87 million tons. China is the worldwide leading producer, accounting for 47% of the world's production [1]. Large gene pools, successful production in both northern and southern hemispheres, different appearances, pleasant aromas and tastes, low prices, good transportability, and year-round storage all make apples one of the most popular snack fruits (Fotirić Akšić et al., 2022) [2]. In Norway, the acreage of high-density apple orchards is about 1500 ha, with an annual output of over 12,000 tons. The fruit cultivation is located mostly around the fjords in southwestern Norway and around the lakes in southeastern Norway, representing the most northerly fruit tree-producing areas in the world [3]. The cultivation of this crop has a long tradition in Norway, as demonstrated by the country's rich apple germplasm [4,5]. High-intensity apple production, which is situated foremost in the Hardanger district in the Telemark and Oslofjord region, relies on high-yielding foreign cultivars and modern high-density plantings.

Commercial apple production is conducted via the usage of pollinizers [6], which usually consist of other apple cultivars or, more recently, of flowering crabapple trees. This

is due to the gametophytic self-incompatibility present in domesticated apples, as well as in their wild relatives (*Malus* spp.), which is controlled by the multi-allelic *S*-locus [7], and which has been mapped to linkage group 17 [8]. Consequently, when there is a match that is present between the *S* allele in the pollen and the one in the pistil, the pollen tube growth is inhibited within the style. Therefore, successful cross-pollination can only be accomplished by a cross-compatible pollinizer, either with both *S*-alleles differing from those present in the genotype it needs to fertilize (fully compatible) or when there is only one (semi-compatible). However, semi-compatibility has been revealed to cause significant reductions in apple fruit yield when environmental conditions for pollination are suboptimal [9]. This situation is further complicated by the existence of triploid cultivars, which, due to their lower ability to produce balanced gametes, have reduced fertility [10]. This makes them very poor pollinizers, which represents a significant consideration during orchard design. Furthermore, triploid cultivars have three *S* alleles, compared to two in diploids, which complicates the compatibility/semi-compatibility/incompatibility relationships between the pollinizer and main cultivar [11].

Although some apple cultivars display a genetic affinity toward parthenocarpy, cross-pollination in pears, a related fruit crop, has shown several benefits in terms of fruit size [12] and marketability [13] when compared to parthenocarpy. Additionally, treatments with hormones such as gibberellic acid can stimulate the development of parthenocarpic apple fruit; however, these treatments result in fruit with morphological deviations [14]. Even though the main commercial apple cultivation in Norway is conducted in the available environmentally suitable regions for large-scale fruit production, low temperatures and precipitation during spring pose significant challenges for pollinator activity, as well as for the overall pollination and fruit set in certain seasons. Additionally, Norway is located outside the climatic range of many wild bee species, which adversely affects the pollination service delivery [15]. In order to combat this, fruit growers need to rent beehives during the flowering period to secure pollination and fruit set. Wild bumble bees (*Bombus* sp.) and other early flying bees (from genera *Osmia*) operate at lower temperatures than honeybees (*A. mellifera*), and are also important pollinator insects [16].

Inadequate insect pollination and consequent seed and fruit set deficits do not only significantly impact the quantity, but also the quality of apples, influencing their classification for the market [17]. The negative effect of inadequate pollination and fertilization on specific fruit quality parameters, such as the symmetry of the apple fruit, has also been reported [18,19]. Although a low level of pollination deficit might benefit producers, as it reduces the necessity for fruit thinning, limiting pollination in the hope of reducing the labor associated with thinning is not a good strategy. It must be noted that apple flowers will set fruit with even minimal cross-pollination; however, this results in poor-quality fruit with low seed content [20]. Furthermore, a global meta-analysis identified a general presence of a 30% pollination deficit in European apple production [21], indicating that the current level of pollination deficit is substantial.

Aside from the cross-compatibility and the meteorological conditions, other factors such as flowering overlap [22] and pollinizer's diversity [23] have been identified as significant contributors to the seed and fruit set in apple. A diversity of pollinizers is usually present in Norwegian apple orchards, where it is common to interplant rows of at least two pollinizers with overlapping flowering times, and which are evenly distributed between the trees of the main cultivar in the orchard. Better insights into the success rate among these pollinizers and the most commercially significant apple cultivars grown in Norway would provide valuable information regarding the factors contributing to individual pollinizers' efficacy in specific field conditions of the main Norwegian fruit-producing regions.

In a highly impactful review paper on apple pollination, Ramirez and Davenport [24] concluded that further research on successful pollination should include molecular markers, such as simple sequence repeats (SSRs) or new creative methodologies that can be used to determine the pollen parent. SSRs or microsatellites have previously been used for

diversity studies on fruit germplasm in Norway [4,5,25]. However, this marker system is also highly efficient in parent identification [26], parent–offspring analyses [27], as well as multi-generational pedigree network reconstruction [28]. More recently, microsatellite markers have been used to identify the pollen donors of the pear and plum seeds collected in commercial orchards in Norway in order to determine the success rate of individual pollinizers [29–31]. Regarding similar molecular studies on apples, allozyme and isoenzyme profiles of apple seeds have been employed to conduct paternity analyses and thus to gain insight into the pollen dispersal and pollination in apple orchards [22,32]. SSR markers, in addition to the leaf color and molecular typing of *S* alleles, have been analyzed in the seeds and progeny of the 'Fuji' cultivar in order to trace the pollen flow from 'Maypole' and 'Dolgo', as well as to investigate the effect of distance on the pollinizers on fruit set [33]. The mentioned study relied, however, on only two SSR markers, thus limiting the application of the approach in a setting with numerous main cultivars and pollinizers (which is a setting that characterizes commercial apple production in Norway).

In this study, embryos from the fruit of twelve apple cultivars—'Asfari', 'Aroma', 'Discovery', 'Eden', 'Elstar', 'Fryd', 'Gravenstein', 'Julyred', 'Katja', 'Rubinstep', 'Summered', and 'Vista Bella'—were collected during two growing seasons and analyzed using SSR markers. The aim of obtaining this molecular dataset was to identify the individual pollen donors for each embryo, as well as to assess the pollinizer success rate among the examined apple cultivars within the orchards located in Ullensvang (southwestern Norway) and Svelvik (southeastern Norway).

2. Materials and Methods

2.1. Plant Material and Experimental Design

During the 2021 and 2022 growing seasons, five random, fully mature and fully developed apple fruits, from every sampled orchard, were harvested from each of the twelve main cultivars at the beginning, middle, and end of the planting row. Fruits were collected from the following apple cultivars: 'Asfari', 'Aroma', 'Discovery', 'Eden', 'Elstar', 'Fryd', 'Gravenstein', 'Julyred', 'Katja', 'Rubinstep', 'Summered', and 'Vista Bella', which were all grown in 19 different orchards at 10 sites located in either Ullensvang (southwestern Norway) (UL1–UL5) or Svelvik (southeastern Norway) (SV1–SV5). Every cultivar was sampled in the two orchards for both years. Five fruits from each of the three trees (one tree at the beginning, one middle, and one at end of the row) per cultivar were sampled, resulting in a sample size of 30 fruits per cultivar for each growing season. The only exceptions, in terms of the number of sampling orchards, were the cultivars 'Asfari' and 'Katja', which were each harvested from a single orchard in 2021 and from two orchards in 2022, as well as 'Vista Bella', which was sampled from a single orchard in 2021 and 2022. The experimental design entailed that all the main cultivars were to be sampled from a single orchard in Ullensvang, as well as from another single orchard in the Svelvik municipalities. However, this was not possible for the cultivar 'Julyred', as it is cultivated exclusively in southeastern Norway, nor was it possible for 'Vista Bella', which is commercially present only in southwestern Norway. Out of all examined mother cultivars and pollinizers, not a single pair was cross-incompatible (i.e., possessing identical *S* allele profiles). However, 23 pairs of the analyzed main apple cultivars were semi-compatible (possessing a single, common *S* allele). The data on the *S* allele composition of the investigated genotypes were obtained from https://www.agr.nagoya-u.ac.jp/~hort/apple/ (accessed on the 25 January 2023) [34]. However, in the case of 'Eden' (*S*7 *S*24) and 'Fryd' (*S*2 *S*3), these were obtained through personal communication with the breeder (Fresh Forward Breeding & Marketing, The Netherlands).

Within the orchards included in this study, all the pollinizers were planted in single rows. With very few exceptions, pollinizers consisting of commercial cultivars and crabapple varieties constituted a minimum of 5–10% of all the fruit trees present in the examined orchards. The following six crabapples served as pollinizers in the sampled orchards: 'Dolgo'—*Malus baccata* (L.) Borkh.; 'Evereste'—*Malus* PERPETU®; 'Golden

Hornet'—*Malus* × *zumi* (Matsum.) Rehder; 'Kobenza'—*Malus* 'Kobenza', 'Professor Sprenger'—*Malus* × *zumi* (Matsum.) Rehder; and 'Red Sentinel'—*Malus* × *robusta* (Carrière) Rehder. The abundance of each apple cultivar and crabapple pollen donors within the sampled orchards is presented in Table 1. It is important to note that several of the sampled orchards are located in each other's vicinity, thus enabling a potential pollen flow between the orchards. Conversely, however, significant wild apple population was not registered in the instances of close proximity in the sampled orchards.

The sampled apple trees were all grafted on M9 rootstocks, planted 1 × 3.5–4 m apart, and were trained as uniform spindles; they were also pruned to a maximum height of 2.5–3 m and fully matured during the study. All sampled trees were uniform in terms of flower set, vigor, and health status. Orchard floor management consisted of grass in the interrow and a 1 m wide herbicide strip within the row, which is the industry standard for orchard management, together with frequent grass mowing between the rows. Pest management was carried out according to integrated protocols, where spraying against major pests (insects and diseases) was performed when needed. In the case of water deficits, trees were irrigated using the drip approach. All trees received the same amount of fertilizers, determined by the soil and leaf analysis. Hand thinning was carried out at the end of June in order to achieve optimum crop loads of good fruit quality (i.e., 15 cm in between the fruitlets).

Weather data before, throughout, and after the apple flowering during the 2021 and 2022 seasons were collected from the meteorological stations located in Ullensvang and Svelvik (Available online: https://lmt.nibio.no/agrometbase/getweatherdata_new.php (accessed on the 15 February 2023), and which are presented in Figure 1.

2.2. SSR Genotyping

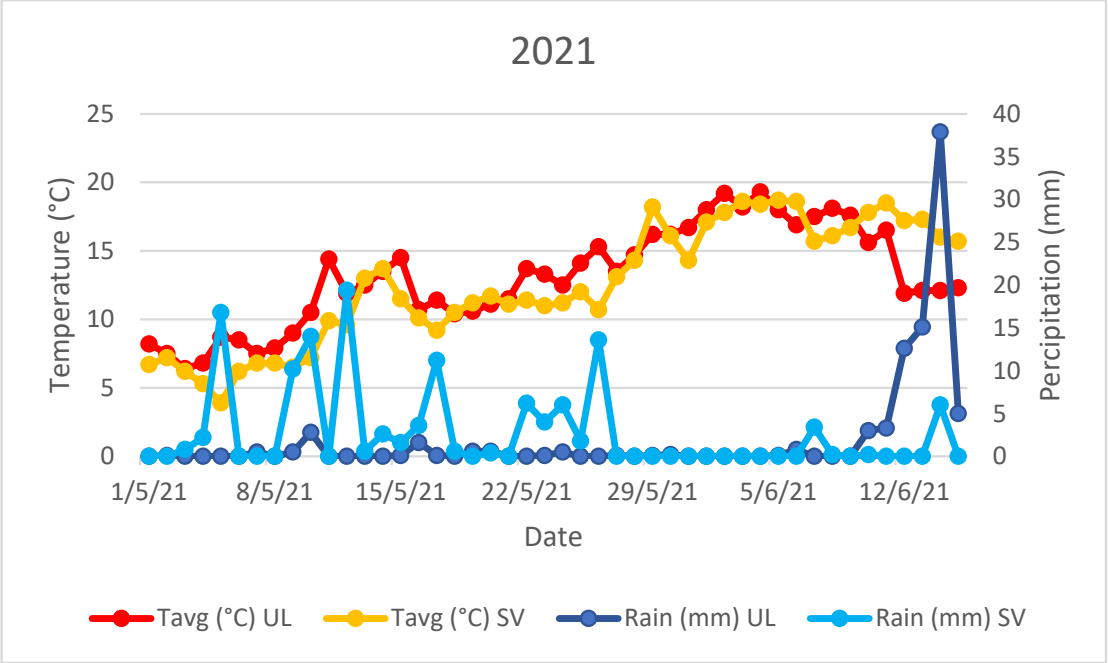
In order to quantify the pollinizer success rate among the analyzed apple cultivars, all the sampled apple fruits were cut open and their seeds were extracted. From the extracted seeds, a single embryo was randomly selected from each fruit, resulting in a sample size of 15 embryos per cultivar and orchard for each growing season. A notable exception was the fruit collected from the cultivar 'Gravenstein' in which, due to its triploid state, empty carpels were frequently found. Consequently, during the two-year trial, 45 'Gravenstein' embryos were genotyped. In instances where the extracted seeds were of a smaller size, additional embryos were included in the analyses. This was performed in case the DNA extraction/genotyping failed in these samples. During 2021 and 2022, a total of 671 apple embryos were analyzed.

Additionally, in the summer of 2020, tissue samples (young leaves) were collected from twelve main apple cultivars, as well as from six crabapples that were grown in the investigated orchards. The genomic DNA from the embryos and leaves was isolated via a commercially available NucleoSpin Plant II, which is a mini kit for obtaining DNA from plants (Macherey-Nagel, Dueren, Germany). The kit was utilized following the manufacturer's recommendations.

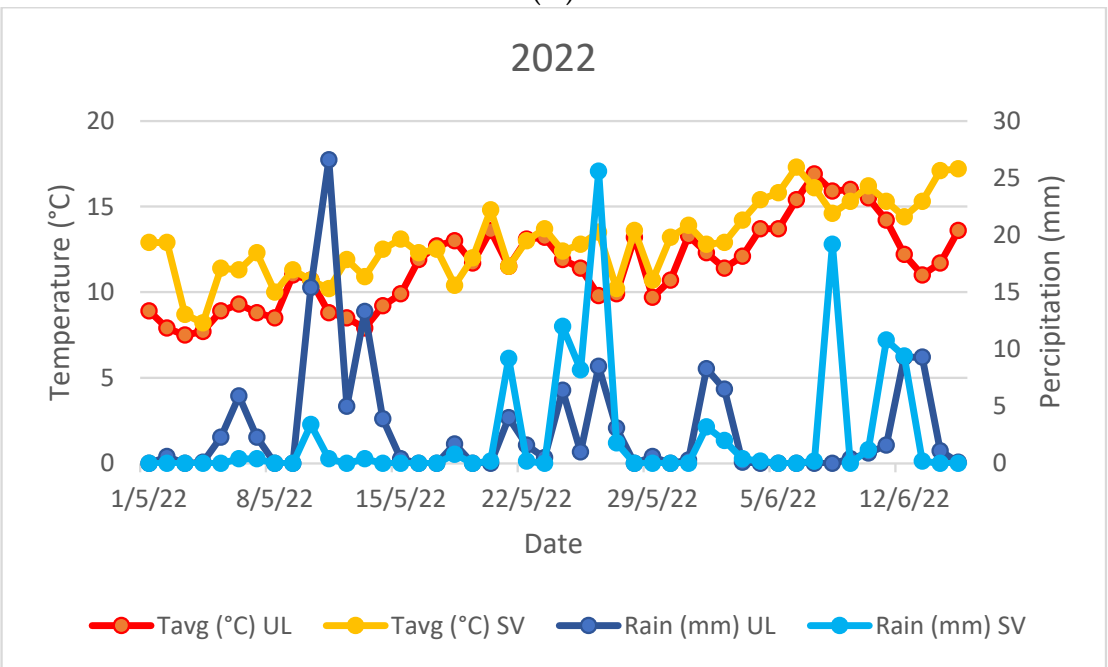
Fifteen SSR markers were chosen based on their polymorphism, as was reported in the previous study on apple germplasm in Norway [5]. Forward primers were fluorescently labeled with 6-FAM, HEX, or TAMRA dye. Based on a size range and annealing temperature, fifteen primer pairs were combined into five multiplex reactions (MIX1-CH02B10, CH05E03, CH02C02a; MIX2-CH03D12, CH02C11, CH01D03; MIX3-CH01F07a, CH04E03, CH01D09; MIX4-CH01H01, CH01H02, CH01H10; and MIX5-CH02C02b, CH04E02, CH02D08) (Table S1).

Table 1. The abundance of each main cultivar and pollinizer ('Asfari'—AS; 'Aroma'—AR; 'Discovery'—DI; 'Dolgo'—DO; 'Eden'—ED; 'Elstar'—EL; 'Everest'—EV; 'Fryd'—FR; 'Golden Hornet'—GH; 'Gravenstein'—GR; 'Julyred'—JU; 'Katja'—KA; 'Ko Benza'—KB; 'Professor Sprenger'—PS; 'Red Sentinal'—RS; 'Rubinstep'—RU; 'Summered'—SU; and 'Vista Bella'—VB) within the 19 orchards that were sampled in the 10 sites (UL1–UL5 and SV1–SV5) located in two Norwegian municipalities (i.e., in Ullensvang, southwestern Norway and Svelvik, southeastern Norway), expressed in a percentage (%) of the overall number of trees. All cultivars were sampled within the individual orchards are stated.

Municipality	Site	Orchard	Cultivars Sampled	AS	AR	DI	DO	ED	EL	EV	FR	GH	GR	JU	KA	KB	PS	RS	RU	SU	VB
Ullensvang	UL1	UL1-A	AR, DI, EL, RU		33	34			17										16		
Ullensvang	UL1	UL1-B	RU		11	12	2		11	2		2				2	2	2	42	12	
Ullensvang	UL1	UL1-C	SU				9			2										89	
Ullensvang	UL2	UL2-A	AS	55		37									1	6	1				
Ullensvang	UL2	UL2-B	KA	30		62									1	6	1				
Ullensvang	UL3	UL3	ED, FR		3	2	2	35		2	50		2		2		2				
Ullensvang	UL4	UL4	GR						20				35			5	5			35	
Ullensvang	UL5	UL5	VB		5	10				5										30	50
Svelvik	SV1	SV1-A	AR, JU	1	50					1		1	1	45		1					
Svelvik	SV1	SV1-B	EL		1				96				1	1							1
Svelvik	SV2	SV2-A	DI		1	83	1			1			1	1	10				2		
Svelvik	SV2	SV2-B	GR		4	1	1			1			91	1	1						
Svelvik	SV2	SV2-C	JU		2	1								97							
Svelvik	SV2	SV2-D	KA		3	92	1			1			1	1	1						
Svelvik	SV3	SV3-A	ED		1			62	1		35									1	
Svelvik	SV3	SV3-B	FR		1			35	1		62									1	
Svelvik	SV3	SV3-C	SU							7										93	
Svelvik	SV4	SV4	AS	85		15															
Svelvik	SV5	SV5	RU		1	1				2		5							91		



(**A**)



(**B**)

Figure 1. Average temperature (Tavg) and rainfall in May and the first half of June 2021 (**A**), as well as in the same period in 2022 (**B**) at the sampling locations of Ullensvang (western Norway—UL) and Svelvik (Eastern Norway—SV).

All PCR reactions were conducted in a total volume of 15 µL, containing 1.5–2 mM of $MgCl_2$, 1 × PCR buffer, 0.2 mM of dNTPs, 0.05 U/µL of TaqNovaHS DNA Polymerase (Blirt, Gdansk, Poland), and 10–50 ng of template DNA. All the primer pairs were amplified, as was described in Gianfranceschi et al. [35], Liebhard et al. [36], and Gasi et al. [37], with minor modifications. Diluted PCR products were mixed with Hi-Di formamide (Applied Biosystems, Foster City, CA, USA) and a prepared size standard. An ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) was used for the electrophoretic separation of the PCR products. Alleles were sized relative to the internal

size standard LIZ 500 (Applied Biosystems). GeneMapper v. 5 (Applied Biosystems) was used for allele scoring.

The number of different alleles and gene diversities [38] within the embryos, as well as in the main cultivars, was calculated using a Powermarker v3.25 [39]. Due to its triploid nature, 'Gravenstein' was excluded from these analyses. Paternity analyses were estimated based on the allele frequencies and the number of candidate parents to be tested for each offspring. The objective was to assign a male parent to each offspring. In order to check the mothers' genotypes, a maternity analysis was also conducted, which was identical to the paternity analysis except with respect to the fact that the objective was to assign a female parent to the offspring. Calculations were conducted within PAPA 2.0 (Package for the Analysis of Parental Allocation) [40] and PASOS 1.0 (Parental Allocation of Singles in Open Systems) [41]. Again, due to 'Gravenstein' being a triploid, the described approach to paternity analyses was not feasible. Therefore, the obtained SSR data for the sampled cultivar (i.e., 'Gravenstein'), pollinizers, and for each analyzed embryo were searched in order to determine the most likely male parent for each individual progeny, which represents a slight modification of Decroocq et al. [42].

3. Results and Discussion

3.1. SSR Polymorphism

The fifteen primer pairs utilized in this study managed to amplify the 158 distinct alleles that were among the 11 main cultivars ('Gravenstein' is excluded due to its triploid nature), resulting in an average value of 10.53 alleles per locus (Table S2). Among the analyzed loci, the number of alleles ranged from 4 for locus CH02C02b to 17 for CH05E03. In order to put the calculated values into context, two recent molecular studies on apple germplasm in Norway, based on the same marker system, reported an average of 14.30 [5] and 11.53 alleles per loci [4]. The comparatively high number of alleles, which were detected among only 11 genotypes, is probably due to the diverse pedigree of the analyzed cultivars and due to the inclusion of crabapples. The mean calculated gene diversity [38] between the main cultivars ranged from 0.58 for CH02C02b to 0.91 for CH05E03, and had an average of 0.79. This is slightly higher than the values reported on the Norwegian apple germplasm by Meland et al. [5] (0.76) and Gasi et al. [4] (0.75). It is worth noting that the locus CH02C02b also displayed the lowest values for polymorphism measures in both of those studies, possibly due to the presence of null alleles.

The value for the average number of alleles per locus, obtained through a genotyping of the sampled apple embryos in 2021, ranged from 3.20 for the 'Asfari' embryos to 7.13 for 'Rubinstep'. Regarding gene diversity, the lowest value was also obtained for the 'Asfari' embryos (0.58), while the highest was detected in the seeds extracted from the 'Summered' fruit (0.71). In 2022, the lowest values for both parameters were once more calculated for the 'Asfari' embryos (3.33 and 0.56), while the highest average number of alleles per locus (6.07), as well as the second highest gene diversity (0.67), were found in the embryos extracted from the 'Summered' fruit. The highest value for gene diversity in 2022 was calculated in the seeds extracted from the apple cultivar 'Aroma' (0.68).

When comparing the average number of alleles per locus and the gene diversity between the two seasons, it is notable that both parameters display higher values in 2021 (5.05 and 6.92) when compared to 2022 (4.55 and 6.73). The higher values calculated regarding the embryos collected in 2021 indicate a pollinizer success rate that was distributed across a more diverse set of pollen donors. Similar studies on plum [31] and pear [29], in a more diverse set of donors, have reported a clear correlation between the higher allele diversity among genotyped seeds and pollen contribution. Considering that, in most cases, the same orchards were sampled in both 2021 and 2022, changes in pollination patterns can usually be ascribed to variations in environmental factors. For instance, spring precipitation may have an indirect adverse effect on pollination and fertilization by limiting the activity of pollinators [43]. Additionally, the influence of temperature at bloom on pollination has also been well documented [44].

3.2. Climate and Flowering

Although this study relied on samples derived from two distinct fruit-producing regions in Norway (Ullensvang, southwestern Norway and Svelvik, southeastern Norway), there was very little difference in the average daily temperatures between the regions pre-, during, and after the flowering period, for both the 2021 and 2022 seasons (Figure 1). The differences in the amount of rainfall, during the same phenological periods, varied more between the seasons than between the different sampling regions; this can be seen in the fact that the lowest precipitation was registered in Ullensvang in 2021 (87.5 mm), and the highest was also observed in the same location, albeit in 2022 (141.1 mm). However, the distribution of this rainfall varied massively. The precipitation registered in Ullensvang during the actual flowering period in 2021 was 4 mm, compared to the 115.4 mm that was measured in the same region during the flowering period in 2022. A higher average temperature was registered during this period in 2021 (13.3 °C) when compared to 2022 (11.2 °C).

The earliest flowering genotype was 'Dolgo' (11 May), while the earliest flowering main cultivar in the first season was 'Summered' (13 May), with the latest being 'Asfari', which began to flower seven days later. In 2021, the flowering period lasted, on average, 12 days in Ullensvang, from the first bloom to petal fall (Table 2). In 2022, 'Dolgo' and 'Summered' were again the earliest bloomers, accompanied by 'Gravenstein', which started flowering on 6 May. The flowering period also lasted, on average, 12 days in Ullensvang.

Table 2. Dates of the first bloom (10% of flowers open), full bloom (80% of flowers open), and petal fall in May and June for the 12 main apple cultivars ('Asfari'—AS; 'Aroma'—AR; 'Discovery'—DI; 'Eden'—ED; 'Elstar'—EL; 'Fryd'—FR; 'Gravenstein'—GR; 'Julyred'—JU; 'Katja'—KA; 'Rubinstep'—RU; 'Summered'—SU; 'Vista Bella'—VB) and for the two most successful pollinizer crabapples ('Dolgo'—DO and 'Everest'—EV) in Ullensvang (western Norway) and Svelvik (eastern Norway) during the 2021 and 2022 seasons.

ULLENSVANG														
Year	**Flowering**	**AS**	**AR**	**DI**	**ED**	**EL**	**FR**	**GR**	**KA**	**RU**	**SU**	**VB**	**DO**	**EV**
2021	First Bloom	5/20	5/19	5/16	5/21	5/18	5/20	5/14	5/14	5/16	5/13	5/16	5/11	5/19
	Full Bloom	5/27	5/25	5/22	5/27	5/26	5/26	5/21	5/20	5/20	5/18	5/21	5/17	5/24
	Petal fall	5/31	5/28	5/31	5/31	5/31	5/30	5/30	5/27	5/27	5/26	5/27	5/22	5/29
2022	First Bloom	5/17	5/16	5/9	5/18	5/16	5/17	5/6	5/11	5/11	5/6	5/9	5/6	5/16
	Full Bloom	5/21	5/20	5/16	5/22	5/20	5/21	5/15	5/16	5/19	5/13	5/15	5/11	5/20
	Petal fall	5/26	5/25	5/22	6/5	5/24	6/2	5/21	5/20	5/22	5/21	5/20	5/16	5/25
SVELVIK														
Year	**Flowering**	**AS**	**AR**	**DI**	**ED**	**EL**	**FR**	**GR**	**JU**	**KA**	**RU**	**SU**	**DO**	**EV**
2021	First Bloom	5/22	5/21	5/17	5/18	5/21	5/18	5/17	5/22	5/16	5/21	5/17	5/18	5/20
	Full Bloom	5/25	5/23	5/19	5/22	5/25	5/22	5/20	5/25	5/20	5/25	5/19	5/25	5/25
	Petal fall	6/8	6/11	6/1	6/1	6/11	6/1	6/3	6/8	5/29	6/8	6/1	5/28	6/1
2022	First Bloom	5/18	5/15	5/14	5/16	5/18	5/14	5/10	5/12	5/12	5/16	5/7	5/9	5/14
	Full Bloom	5/22	5/20	5/18	5/22	5/24	5/18	5/16	5/16	5/16	5/22	5/14	5/16	5/19
	Petal fall	5/29	5/29	5/24	5/29	5/29	5/24	5/24	5/24	5/23	5/29	5/23	5/29	5/29

The seasonal differences in precipitation were smaller in Svelvik, where the rainfall during the actual flowering period in 2021 was 51.2 mm, compared to 62.6 mm in 2022. The differences in the average temperature between the two seasons were comparable to the one registered in Ullensvang (14.5 °C and 12.0 °C) but slightly warmer. The earliest flowering cultivar in the first season was 'Katja' (16 May), with the latest being 'Asfari' and 'Julyred', which began to flower six days later. In 2021, the flowering period lasted, on average, 16 days in Svelvik, from the first bloom to petal fall. The earliest flowering cultivar in the second season was 'Summered' (7 May), with the latest being 'Asfari' and 'Elstar', which began to flower 11 days later. In 2021, the flowering period lasted, on average, 12 days in Svelvik.

Temperature, especially in spring, is clearly the main determinant of tree phenology [45], while precipitation has either positive or negative influences on the phenology, depending

on the background climate conditions in a region [46]. The overall longest flowering period was recorded in 2022 in the Ullensvang orchards. Specifically, the first bloom started on 6 May ('Summered') and the last date for petal fall was recorded on 5 June ('Eden'). This prolonged flowering can be attributed to the low temperature during the flowering season (11.2 °C), as well as due to the very high precipitation registered for this region in 2022. This is in accordance with Heide et al. [47], who determined that, at 12 °C, flowering seems to be limited by low temperature depression of growth and leaf production. This is in addition to Grab and Craparo [48], who proved that extended rainy and heavily cloudy conditions can delay or stop tree flowering in apple trees in South Africa.

The flowering order between the different cultivars was similar across the years and sites. Although a prolonged flowering period might provide more opportunities for pollinator activities, albeit one that is encumbered by rainfall, the main factor influencing pollination patterns is the overlap in flowering periods among the main cultivars and pollinizers. However, it is interesting to note that the consistently early bloomer 'Summered' provided the embryos with the highest values for allele polymorphism parameters, indicating a more diverse set of pollen donors. In contrast, the embryos collected from the notable late bloomer 'Asfari' displayed the lowest values for the average number of alleles and gene diversity. Considering that the average flowering period for the 'Summered' trees was only slightly longer when compared to 'Asfari', the earlier blooming might have provided more opportunities for a broader range of pollen donors.

3.3. Identifying Most Successful Pollinizers

The apple cultivars 'Aroma' and 'Discovery' proved to be the most prolific pollinizers, regardless of the season and sampling site (Table 3). A more broadly distributed pollinizer success was noted in the apple embryos that were collected from the Ullensvang orchards when compared to Svelvik, with the sole exception being the seeds extracted from the 'Gravenstein' fruit. In addition, among the cultivars sampled in Ullensvang orchards during both seasons, the pollinizer success rate was, markedly, more evenly distributed across the different pollinizers in 2021 when compared to 2022. The sole exception was again 'Gravenstein', with 'Eden' and 'Katja' showing a similar distribution in both seasons. A possible explanation for the fact that more apple genotypes succeeded as pollinizers in 2021, compared to 2022, could be the lower precipitation registered in Ullensvang during the actual flowering period in 2021 when compared to 2022, as well as due to the somewhat higher average temperature (Figure 1). In addition, the mentioned meteorological factors may have also proved to be more conducive for pollination in 2021.

Although there was some seasonal variation in the pollinizer efficacy among the individual genotypes noted, as well as variations between the orchards sampled in both western and eastern Norway, in most cases it was possible to identify the most successful pollinizers for each of the twelve main cultivars. Namely, 'Discovery' proved to be the dominant pollinizer of 'Asfari' in both 2021 and 2022, independent of the sampling region. The two cultivars were found to be fully cross-compatible and displayed a flowering overlap ranging from 5 to 10 days during the two seasons (Table 2). In addition, 'Discovery' was, by far, the most abundant pollinizer in the orchards where 'Asfari' embryos were collected (Table 1). Interestingly, the lowest pollinizer success rate for 'Discovery' was detected in orchard UL2-A during the 2022 season, which coincided with the smallest flowering overlap (five days) between these two cultivars. The cultivar 'Discovery' being such a dominant pollinizer of 'Asfari' is also reflected in the fact that the 'Asfari' embryos possessed the lowest values for the average number of alleles per loci and gene diversity across all the analyzed samples, whereby the lack of pollinizer diversity directly affects the diversity between the progenies [31].

Table 3. The success rate, expressed in percentage, of the 14 pollinizers ('Asfari'—AS; 'Aroma'—AR; 'Discovery'—DI; 'Dolgo'—DO; 'Eden'—ED; 'Elstar'—EL; 'Everest'—EV; 'Fryd'—FR; 'Julyred'—JU; 'Katja'—KA; 'Ko Benza'—KB; 'Rubinstep'—RU; 'Summered'—SU; 'Vista Bella'—VB) among the 12 apple cultivars (calculated during two seasons in 19 orchards located in Ullensvang (UL) and Svelvik (SV) Norway, sampled in 2021 and 2022). Only the pollinizers that were positively identified in at least one sample are included in the table. "X" marks the absence of a sampled orchard. The data for calculation were obtained through genotyping the 671 apple embryos, which was achieved by using the 15 SSR markers and consequent parentage analyses. In cases where none of the 17 pollinizers included in this study were determined to be the father genotype, the male parentage of the sample was designated as unknown (UKN). Embryos with paternal genotypes located outside the orchard in which they were collected are displayed in cursive.

CULTIVARS	Year	Site	Orchard	AS	AR	DI	DO	ED	EL	EV	FR	JU	KA	KB	RU	SU	VB	UNK
'Asfari'	21	X	X															
		SV4	SV4			100												
	22	UL	UL2-A			87			*6*				*7*					
		SV4	SV4			100												
'Aroma'	21	UL1	UL1-A			13			27				*7*		53			
		SV1	SV1-A									100						
	22	UL1	UL1-A			87							*13*					
		SV1	SV1-A									100						
'Discovery'	21	UL1	UL1-A	6	20				20				*7*		20			27
		SV2	SV2-A		87										13			
	22	UL1	UL1-A		73				*7*						20			
		SV2	SV2-A		100													
'Eden'	21	UL3	UL3		13					6	44		37					
		SV3	SV3-A						13		87							
	22	UL3	UL3		27				*27*		40							6
		SV3	SV3-A		*7*				13		80							
'Elstar'	21	UL1	UL1-A		60	40												
		SV1	SV1-B		87												13	
	22	UL1	UL1-A		87	13												
		SV1	SV1-B		93												*7*	
'Fryd'	21	UL3	UL3		25			40					35					
		SV3	SV3-A					77	15							8		
	22	UL3	UL3		60	20			*7*				7					6
		SV3	SV3-A		*7*			73	20									
'Gravenstein'	21	UL4	UL4						10							90		
		SV2	SV2-B		75	9	9											7
	22	UL4	UL4			*11*			11							78		
		SV2	SV2-B		43	7	29			7						*7*		7
'Julyred'	21	SV2	SV2-C		46	40	*7*											7
		SV1	SV1-A		100													
	22	SV2	SV2-C		93	7												
		SV1	SV1-A		100													
'Katja'	21	UL2	UL2-B		*7*	93												

Table 3. *Cont.*

CULTIVARS	Year	Site	Orchard	AS	AR	DI	DO	ED	EL	EV	FR	JU	KA	KB	RU	SU	VB	UNK
		X	X															
	22	UL2	UL2-B		*7*	93												
		SV2	SV2-D		*7*	87												6
'Rubinstep'	21	UL1	UL1-B		20	20	13		*7*	33						*7*		
		SV5	SV5		33		*7*			*7*				*13*		27		13
	22	UL1	UL1-A		46	*7*	*13*		27	*7*								
		SV5	SV5		60	13				20								*7*
'Summered'	21	UL1	UL1-C		*27*	*6*	*7*		*27*	33								
		SV3	SV3-C							53								47
	22	UL1	UL1-C		*7*	*33*			*20*	40								
		SV3	SV3-C		*27*		6			*67*								
'VistaBella'	21	UL5	UL5		20	40									*7*	33		
		UL5	UL5		14	53										33		
	22	UL5	UL5		20	27									*13*	40		
		X	X															

'Julyred' and 'Rubinstep' proved to be the dominant pollinizers of 'Aroma' in 2021, while in 2022, 'Julyred' and 'Discovery' were identified as the most efficient pollinizers of 'Aroma'. It is important to note that 'Julyred' was exclusively present in orchards that were located in Svelvik, which is where this cultivar had a 100% pollinizer efficacy in the analyzed 'Aroma' embryos. 'Aroma' shared a full cross-compatibility with these two apple cultivars and a flowering overlap that ranged from six to 17 days. Furthermore, 'Julyred' was the most abundant pollinizer in the Svelvik orchards where 'Aroma' fruit were collected, while 'Discovery' and 'Rubinstep' were the most abundant pollinizers in the Ullensvang orchards.

The pollinizer success that 'Discovery' possessed in the 'Aroma' embryos was reciprocal as 'Aroma' proved to be the overall dominant pollinizer of 'Discovery' in both 2021 and 2022. The single exception was registered in the UL1-A orchard during the 2021 season, where the pollinizer success rate was evenly split between 'Aroma', 'Elstar', and 'Rubinstep', with all cultivars being fully cross-compatible with 'Discovery'. The lower pollinizer efficacy of 'Aroma' in this instance could not be attributed to a smaller floral overlap.

The cultivars 'Eden' and 'Fryd' were each other's most successful pollinizers, which could be, at least, partly attributed to the fact that these cultivars are fully cross-compatible and that they share almost identical flowering dates. Additionally, these two cultivars were, by far, the most abundant in the orchards from which 'Eden' and 'Fryd' embryos were sampled (Table 1). In addition to 'Eden', the apple cultivar 'Aroma' also proved to be an efficient pollinizer of 'Fryd'. On the other hand, 'Aroma' was a much less efficient pollinizer of 'Eden', even though both 'Eden' and 'Fryd' embryos were sampled from the same or adjacent orchards and with the same abundance of 'Aroma' trees. This could, however, be due to the fact that while 'Aroma' and 'Fryd' are fully cross-compatible, 'Eden' shares a common *S* allele with 'Aroma'. The lower pollinizer success rate among the semi-compatible apple cultivars, compared to the fully cross-compatible ones, was reported by Schneider et al. (2005) [9], resulting in a recommendation that, under less-than-optimal conditions for pollination, the orchard design should rely on full compatibility.

In a rare instance where a semi cross-compatible cultivar served as the most efficient pollinizer in this study, 'Aroma' was identified as the dominant pollinizer of the apple cultivar 'Elstar'. This is most likely because these two cultivars share the *S*5 allele. While 'Aroma' trees were abundant in the Ullensvang orchards, from which the 'Elstar' embryos were collected, they were much less present in the Svelvik orchards, where 'Aroma' also displayed the highest pollinizer success. The pollinizer success of 'Aroma' could be attributed to very similar flowering periods between these two genotypes. With respect to this, a study by [22], which was similarly based on the paternity analyses of apple embryos, reported flowering overlaps as a more significant cause of siring success in apples when compared to cross-compatibility. However, it is important to note that the mentioned study was carried out in a climate that was more conducive to pollination. Considering the previously mentioned results on the pollination of 'Eden' and 'Fryd' by 'Aroma', as well as the fact that cases of semi-compatible genotypes serving as significant pollinizers in this study were few and far apart, full cross-compatibility can still be highlighted as a major factor for pollinizer success.

The very high pollinizer efficacy of 'Summered' in the 'Gravenstein' embryos collected from the Ullensvang orchard (UL4) during both seasons can explain the lower pollinizer diversity noted for this cultivar in Ullensvang. The high success rate of 'Summered' could also be attributed to its comparatively early dates for first bloom in both of these cultivars, as well as to the fact that 'Summered' was, by far, the most abundant pollinizer. Among the 'Gravenstein' seeds collected in Svelvik, 'Aroma'—the most abundant pollinizer in the sampled orchard—was the dominant pollinizer in both 2021 and 2022. All three cultivars were fully cross-compatible. 'Aroma' also proved dominant or, in some cases, the exclusive pollinizer of 'Julyred' in both seasons. In orchards where it was the exclusive pollinizer, 'Aroma' trees slightly outnumbered even the 'Julyred' trees.

The other rare instance of a semi cross-compatible cultivar serving as the most efficient pollinizer was the cultivar 'Discovery', which was identified as the most dominant pollinizer

of 'Katja'. Although semi-compatible (i.e., sharing *S*24), the two cultivars shared a flowering overlap that ranged from 9 to 11 days. The pollinizer success of the semi-compatible 'Discovery' can be explained by the fact that, in the sampled orchard, the abundance of 'Discovery' trees ranged from 62 to 92% (Table 1).

Even though 'Aroma' proved to be the most efficient pollinizer of 'Rubinstep' in both 2021 and 2022, several other cultivars (mostly 'Everest' and 'Discovery') were also identified as successful pollinizers. The high average number of alleles detected in the 'Rubinstep' embryos, collected in 2021, reflected the fact that this cultivar had seven different identified successful pollinizers in 2021. A high pollinizer diversity, therefore, positively affected the genetic diversity that was noted across the progenies. In 2021, the 'Rubinstep' embryos were sampled from an Ullensvang orchard with ten different pollinizers and the more evenly distributed tree abundance across these genotypes. This highlights the influence of pollinizer abundance and distribution on the pollination patterns within apple orchards.

It is important to note that 'Everest' had almost identical flowering periods as 'Aroma' did throughout the trial, which may explain why it was the overall most successful pollinizer for crabapples. Furthermore, 'Everest' proved to be the dominant pollinizer of 'Summered' in both 2021 and 2022, with significant contributions from 'Aroma', 'Discovery', and 'Elstar'. The pollinizer success of 'Everest' in 'Summered' orchards can also be attributed to a larger abundance of this crabapple when compared to other orchards. The apple embryos with the highest values of gene diversity calculated in this study belonged to 'Summered'. This distinction is due to the diverse set of successful pollinizers that were detected in the 'Summered' seeds, including those from crabapples.

Although its pollinizers were very heterogeneous, 'Discovery', 'Summered', and 'Aroma' proved to be the most efficient pollinizers of 'Vista Bella' both in 2021 and 2022. This was also the third and final case of semi-compatible cultivars serving as one of significant pollinizers. Namely, 'Discovery' and 'Vista Bella' share a single, common *S* allele (*S*24). However, the success rate of 'Discovery' was, in most cases, below 50%. Again, the abundance of trees might have played a crucial role in the success of a semi-compatible pollinizer. Specifically, 'Discovery' was the second most abundant pollinizer in the 'Vista Bella' orchards.

In cases where paternity analyses failed to identify the male parent of the apple genotypes in the orchard, additional paternity analyses were carried out using the SSR profiles of all the 12 main apple cultivars and the 6 crabapples (excluding the mother genotype). Overall, 7% of the analyzed embryos were determined to have been fertilized by pollinizers that were genotyped in this study, but which were planted outside the sampled orchard (Table 3). However, this approach did not manage to help identify the male parent in approximately 3% of the 671 apple embryos that were analyzed in this study. Therefore, these samples were classified as having unknown paternity. The overwhelmingly highest percentage of these cases were determined in those embryos that were extracted from the cultivar 'Summered' in the orchard LI1 during the 2021 season—which is where almost half of the embryos were designated as having unknown paternity. This might serve as an additional explanation for why 'Summered' embryos displayed such high levels of genetic diversity, since it is impossible to assert how many different genotypes served as pollen donors in those cases. The overall low number of embryos with unknown paternity was somewhat surprising considering the large number of diverse and old cultivars cultivated in Norway [4,5], as well as due to the presence of *Malus sylvestris* populations in the country, which could have served as pollinizers. Feurtey et al. [49], who used microsatellites to investigate gene flow between *M. domestica* and *M. sylvestris*, reported that pollen dispersal could occur up to 4 km. The low presence of successful pollen donors outside the orchards could be attributed to the adjacent pollinizers' spatial advantage. Namely, the distance between the pollinizer and the pollinated cultivar was shown to represent a significant factor in pollinizer success [22,32,33,50–52]. In addition, the high tree density surrounding the maternal apple trees, which was present in the sampled high-density orchards was shown to guard against external pollen flow [50,53].

Similar studies, relying on the genotyping of embryos with SSRs, reported the importance of proximity on pollinizer efficacy within macadamia [54] and chestnut [55] orchards. Conversely, the SSR genotyping of olive seeds and consequent paternity analyses indicated cross-compatibility and flower overlap as the more significant drivers in pollinizer efficacy when compared to tree abundance and to the proximity of the pollinizers [56].

4. Conclusions

The fifteen SSR markers used in this study positively identified the individual pollen donors for all except for 3% of the 671 analyzed apple embryos that were collected from the fruit of twelve different cultivars. In most cases, it was possible to identify the most successful pollinizer for each cultivar, with 'Aroma' and 'Discovery' being the most efficient pollen donors across the board. The very rare instances of an efficient pollinizer sharing an *S* allele with the cultivar it was pollinating indicated that full compatibility should be an important consideration in Norwegian apple orchard design. The success of the few semi-compatible pollinizers that were detected in this study can be attributed to a very high tree abundance of said pollinizers within the orchards. Tree abundance seems to be a major factor in pollinizer success as most of the investigated cultivars and pollinizers possessed good flowering overlap. Relatively few cases of successful pollen donors from outside the sampled orchards confirmed the significance of pollinizer proximity for efficient pollination. Embryo genotyping and successive paternity analyses are arguably the most effective approach for future research on pollinizer efficacy in apple and other such important crops.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13041106/s1, Table S1. The microsatellite (simple sequence repeats—SSR) code, DNA sequences, annealing temperatures, fluorescence labels, and references for the 15 primer pairs that were used for the genotyping of 671 apple embryos, the mother cultivars, and potential pollinizers. Table S2: The number of alleles per locus (Na) and gene diversity (He) (Nei, 1978) for the 11 main apple cultivars ('Asfari', 'Aroma', 'Discovery', 'Eden', 'Elstar', 'Fryd', 'Julyred', 'Katja', 'Rubinstep', 'Summered', and 'Vista Bella') and crabapples ('Dolgo', 'Evereste', 'Golden Hornet', 'Ko Benza', 'Professor Sprengler', and 'Red Sentinel'), as well as for the embryos that were collected from the main cultivars in 2021 and 2022, based on 15 SSR (simple sequence repeat) loci. The cultivar 'Gravenstein' was excluded due to its triploid state.

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