S-Genotyping and Seed Paternity Testing of the Pear Cultivar 'Celina'

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Abstract: The diploid Celina/QTee® ('Colorée de Juillet' × 'Williams'), one of the most promising pear cultivars developed by the Norwegian breeding program Graminor, was launched in 2010. In Norway, the flowering is medium to late, while the fruits ripen in the beginning of September. The fruits are attractive with an intense red blush (50%) on a green background. Although, 'Celina' is cultivated in the most climatically suitable regions for fruit cultivation, present in Norway, unfavorable environmental conditions for pear pollination can have a very negative effect on fruit set and consequent yield. The aim of this study was to determine the *S*-alleles of 'Celina', as well as its frequently used pollinizers, and, through paternity testing of 'Celina' seeds, give a recommendation regarding the most important pollinizers of this pear cultivar. In order to accomplish this, 'Celina' and its potential pollinizers were all *S*-genotyped. After harvest, seeds collected from 'Celina' fruit in 2017 and 2018 were genotyped using eleven microsatellite markers. Genomic DNA was also extracted from leaf material collected from 'Celina', as well as from five pear cultivars used as pollinizers in the three examined orchards, and analyzed using the same marker set. Subsequently a simple sequence repeat (SSR) database was constructed and used for gene assignment analyses with the aim of quantifying pollen donor contribution from individual pollinizers. The obtained results indicate that 'Anna', the only examined pollinizer that was fully cross-compatible with 'Celina', together with 'Fritjof', the genotype which had the highest flowering overlap with 'Celina', proved to be the most successful pollinizers across all seasons and orchards. Although both cultivars were ubiquitous in the examined orchards, either as planted trees or as branches introduced during the flowering period, they were the most abundant pollinizers in only one orchard each. It is therefore possible to conclude that pollinizer abundance has a secondary significance in pollinizer success within investigated 'Celina' orchards.

Keywords: *Pyrus communis* L.; pear seeds; gene assignment analyses; *S*-alleles; cross-pollination

1. Introduction

Celina/QTee® ('Colorée de Juillet' x 'Williams'), one of the most promising pear cultivars developed by the Norwegian breeding program Graminor, was released in 2010 [1]. In Norwegian orchards, this cultivar flowers medium to late, usually in mid-May and produces attractive fruits with red blush and good fruit quality, storability and shelf life, which ripen in the beginning of September. There are

currently significant areas planted with 'Celina' cultivars in other countries, mainly Europe but also in South Africa.

Although 'Celina' is cultivated in some of the most suitable environmental conditions for fruit growing in Norway, generally unfavorable environmental conditions for pear pollination, during the Nordic spring, can have a very negative effect on yield quantity in pear orchards [2]. Adverse weather conditions and a low attractiveness of pear flowers to insect pollinators [3] often strongly hamper cross-pollination [4] and consequently the fertilization of pear ovules. Adequate fertilization represents a prerequisite for seed development, which is in turn essential for growth and development of high-quality fruits. It is considered most favorable for the production of high-quality fruit that majority of ovules of the flowers are fertilized [5]. In case only a sub-fraction of the ovules is fertilized, resulting fruits can be small [6,7].

The majority of the commercially grown European pear (*Pyrus communis* L.) cultivars are predominantly self-sterile [8]. Self-fertilization in pear, similar to other fruit species of the *Rosaceae* family, is prevented by gametophytic self-incompatibility [9]. Due to the self-incompatibility mechanism, fruit-set and overall commercial pear production is conditioned upon successful cross-pollination and fertilization [5]. Consequently, inter-planting of suitable pollinizer genotypes in pear orchards ensures fertilization of the pear ovules, which in itself leads to a successful set of an optimum crop load [10]. Successful cross-pollination can be accomplished by a cross-compatible pollinizer, in which either both S-alleles differ from those present in the genotype it needs to fertilize, or if they differ in only one S-allele. Although pollen from both semi- and fully-compatible pollinizers can successfully fertilize ovules in pear, a high and significant increase in seed and fruit set by fully- compared to semi-compatible pollinizers has been noted in this fruit crop [11]. It is also important to mention that semi-compatibility has been revealed to cause significant reductions in fruit yield when environmental conditions for pollination have been suboptimal in apple, a closely related crop [12]. Insight into the S-allele composition of various pear cultivars is therefore very important for orchard design [13].

In order to guarantee fruit set, commercial pear orchards need to contain at least two cross-compatible cultivars [5], which is the practice in Norwegian orchards. In case of shortage of pollinizers, branches in bloom, collected from additional pear cultivars are oftentimes introduced into the orchards. But this job can be time consuming and cannot be a permanent solution.

In addition of being semi- or fully-compatible, a further criterion which potential pollinizers need to meet is an adequate overlapping of flowering period with the main cultivar. This criterion is essential for effective cross-fertilization and seed set which in its turn stimulates fruit development [14]. Aside from cross-compatibility and coincidental flowering time, the abundance of pollinizer trees, planted within orchards, has been reported to affect the pollinizer efficacy among pear cultivars in Nordic conditions [2].

Identifying cross-compatible pear cultivars is traditionally accomplished with testcrosses. Additional information, such as the level of gametophytic cross-compatibility (fully or semi compatibility), can be obtained using polymerase chain reaction (PCR) based S-genotyping [15–17]. The procedure of S-genotyping involves the use of consensus and allele specific primers, which through polymerase chain reaction amplify targeted sections of the self-incompatibility ribonucleases (S-RNase) gene. The amplified sequences, either through their length polymorphism or through amplification success/failure indicate a presence of specific S alleles within a genotype. Furthermore, identifying the most efficient cross-compatible pollinizer in specific field conditions can also be accomplished using PCR based genotyping. Early efforts to investigate the gene flow within pear orchards have been conducted using RAPD (Random Amplification of Polymorphic DNA) markers [18,19]. Recent studies on pear [2] and plum [20] have used codominant DNA markers to genotype embryos and subsequently conduct gene assignment analyses in order to identify the pollinizer successes rate among several different genotypes used as a source of compatible pollen. Both of the mentioned studies relied on microsatellite markers or SSRs (simple sequence repeats) usage, which have previously been proven efficient in parent-offspring analyses on plum [21] and pear [22]. The combination of S-genotyping and

SSR genotyping of embryos can also provide an insight into the effect of various factors on pollinizer success in open pollination setting.

The aim of this study was to determine *S*-alleles of Celina, as well as its frequently used pollinizers, and, through paternity testing of 'Celina' seeds, give a recommendation regarding the most important pollinizers of this pear cultivar. Furthermore, this study aimed to determine if the level of gametophytic cross-compatibility, between 'Celina' and its recommended pollinizers, influenced the pollinizer efficacy within commercial orchards in Norway. Additionally, the impact of flowering overlap and pollinizers abundance in individual orchards was also examined.

2. Materials and Methods

2.1. Plant Material

In total, three pear orchards were included in this study (Table 1), all of which are located in Ullensvang, a municipality of the Hardanger district in Norway. GPS positions are: Orchard 1–60.359699, 6.669818, Orchard 2–60.362620, 6.671651, and Orchard 3–60.318669, 6.654565. Selected pear trees located within these orchards were used as a source of plant material for the *S* and SSR genotyping. Additionally, fruits harvested from the mentioned orchards were used for the extraction of seeds, which were genotyped using microsatellite makers. Within the investigated orchards, the cultivar composition and abundance varied significantly. 'Kristina' was only present in one of the three investigated orchards as a bordering cultivar of Orchard 1.

Table 1. Pear cultivar compositions and the abundance of each cultivar within the three orchards sampled in this study.

	'Celina'	'Fritjof'	'Anna'	'Clara Frijs'	'Herzogin Elsa'
Orchard 1	17%	50%	33%	-	-
Orchard 2	92%	*	4%	2%	2%
Orchard 3	87%	*	3%	5%	5%

* Branches in bloom collected from pear cultivar 'Fritjof' and placed in buckets of water were distributed in every tree row of the Orchard 2 and 3 during the flowering of 'Celina' pear.; - not present in the orchard.

The commercial pear orchards were traditionally designed with single rows of 'Celina' trees spaced 1 × 4 m and planted in 2014. The cultivars were grafted on Quince 'Adams'. Orchard floor management consisted of frequent mowing of the inter-rows, and a 1 m wide vegetation free strip was maintained in the intra-row. Trees were irrigated by drip irrigation when water deficits occurred. All trees received the same amount of fertilizers based on soil and leaf analysis.

2.2. Genetic Analyses

S-genotyping was conducted on the cultivar 'Celina', as well as on its five potential pollinizers, using consensus and allele specific primers for *S* alleles ranging from S1 to S24 [17]. The applied PCR protocols are detailed in the study by Sanzol [17]. In order to confirm the accuracy of *S*-allele genotyping conducted in 2017, all obtained *S*-alleles (except for *S101* and *S102*, which due to their length are unambiguous) were sequenced in both directions, with an ABI PRISM 3500 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Raw sequencing data was collected by 3500 series data collection software v.3.1 (Microsoft, Redmond, WA, USA). Automated base calling was done by KB Basecaller v.1.4.1. (Applied Biosystems, Foster City, CA, USA) and secondary analyses was done with Seqscape v.3.0 (Microsoft, Redmond, WA, USA). In case of 'Celina', only partial sequence of one of its *S*-alleles was successfully sequenced in spite of repeated attempts. Obtained sequences were identified by identity and similarity scores in local databases using the FASTA program [23] and in GenBank at NCBI (Bethesda, MD, USA) using the BLAST network service [24].

In order to quantify pollen donor contributions from individual pollinizers, 30 mature fruits were collected each season from 'Celina' trees within the individual orchards. The sampling was conducted entirely randomly, throughout the examined orchards. All sampled fruits were cut open and all pear seeds were extracted. The seeds collected within individual orchards were pooled together in order to obtain a homogenous sample for each location, during each experimental year. Additionally, tissue samples (fully developed extension shoot leaves) for SSR analyses were collected during the summer of 2017, from a single tree of 'Celina', as well as the other pear cultivars present in the examined orchards ('Clara Frijs', 'Herzogin Elsa', 'Anna' and 'Fritjof') and bordering sites ('Kristina').

Genomic DNA was isolated from 70–80 mg of leaf powder using CTAB (hexadecyltrimethy lammonium bromide) method [25,26]. Extraction and isolation of genomic DNA from pear seeds was conducted according to Padmalatha et al. [27].

Eleven SSR primer pairs, previously published by Gianfranceschi et al. [28], Liebhard et al. [29], and Fernández-Fernández et al. [30], were used according to the protocol described by Gasi et al. [31]. After the genetic characterization, based on the mentioned 11 microsatellite markers, a database was created with the SSR profiles of 'Celina', for all potential pollinizers and seeds samples collected in 2017 and 2018. The obtained database was used for the assignment or exclusion of individuals [32] within GeneClass 2 software [33] in order to identify the pollen contribution of each potential pollinizer. The purpose of the genetic assignment is to assign or exclude reference group as the origin individuals on the basis of multilocus genotype data [33]. The Bayesian approach was implemented as it has been described by Rannala and Mountain [32]. It included calculation of relative scores with –log values of the likelihoods without Monte Carlo resampling algorithms.

2.3. Weather Data

Meteorological data, before, during and after the pear flowering in 2017 and 2018 was collected from meteorological stations located in Ullensvang (available at: https://lmt.nibio.no/agrometbase/getweatherdata_new.php).

The temperature registered during the month of May, when all the investigated pear cultivars flowered, was on average 4 °C higher in 2018 (15 °C) compared to that of 2017 (11 °C). Warm and dry weather during flowering period is completely unusual for this location in Norway. However, higher post bloom temperatures, which can significantly influence the decrease of fruit set in pear [34], were recorded in 2018. The average temperature during the 7-day post bloom period in 2017 was 12.5 °C (with a max daily temperature of 21.7 °C), whereas, in 2018, it was 16.3 °C (with a max daily temperature of 25.6 °C). The precipitation was much higher in May of 2017 (52.6 mm) compared to 2018 (15.0 mm), but most of it was concentrated in 3 to 4 days, thus not disrupting the pollination of pear flowers, which on average lasted more than ten days in 2017.

3. Results and Discussion

3.1. Phenology

The results of the following flowering observations: dates of first bloom (BBCH 60), full bloom (80% of blossoms open, BBCH 65), and petal fall (80%, BBCH 67) [35] for 'Celina' in the three commercial orchards, as well as for five pollinizer cultivars, during 2017 and 2018 are presented in Table 2. It confirms that there was sufficient overlap between all pollinizers and 'Celina' in both years.

The overlap in full bloom dates, between 'Celina' and its potential pollinizers, ranged from complete overlap ('Fritjof') to five days difference ('Anna') in 2017 and from one day ('Fritjof') to three days difference ('Anna') in 2018. Aside from a notable difference in full bloom dates between 'Celina' and 'Anna' in 2017, the overall flowering period overlap among all analyzed pear cultivars was very high.

Table 2. Dates of first bloom, full bloom (80% of flowers open), and end of flowering (80% of petals fallen) for 'Celina' and five pollinizer pear cultivars in Ullensvang, Norway, in 2017 and 2018.

Season		'Celina'	'Fritjof'	'Anna'	'Clara Frijs'	'Herzogin Else'	'Kristina'
2017	First flower open	15 May	15 May	11 May	12 May	13 May	13 May
	Full bloom	19 May	19 May	14 May	15 May	16 May	18 May
	Petal fall	28 May	27 May	21 May	21 May	22 May	23 May
2018	First flower open	13 May	12 May	11 May	12 May	12 May	11 May
	Full bloom	15 May	14 May	12 May	13 May	13 May	13 May
	Petal fall	26 May	23 May	20 May	21 May	21 May	20 May

3.2. S-Genotyping

S-genotyping of 'Celina', which to our knowledge has not been conducted earlier, revealed that this cultivar possesses the following *S*- allele composition: S101 and S115, labelled in accordance with the numeration proposed by Goldway et al. [36] (Table 3). Identical analyses on the five other pear cultivars included in this study revealed that all of them, with the exception of 'Anna', are in fact only semi-compatible with 'Celina', while 'Anna' is the only fully cross-compatible cultivar used as a pollinizer in the examined orchards.

Table 3. The results of S genotyping of the six pear cultivars included in this study.

Genotype	*S* Allele Profile	
'Celina'	S101	S115
'Fritjof'	S101	S119
'Anna'	S105	S108
'Clara Frijs'	S101	S105
'Herzogin Elsa'	S101	S111
'Kristina'	S101	S121

Due to the current climate change, which can cause more frequent, severe seasonal variations in weather conditions, it is recommended to seek fully compatible pear pollinizers, in order to guarantee satisfactory yields [14]. Meteorological data obtained within this study indicate weather patterns uncharacteristic for this region, such as warm and dry conditions during bloom and high post bloom temperatures. In the light of this finding, it is relevant to note that one of the features of global climatic warming is the increase in the frequency of extreme temperatures, which have an impact on the reduced success rate of fertilization, thereby causing a low fruit set [37].

3.3. Allele Polymorphism

Eleven primer pairs amplified 45 distinct alleles among 'Celina', the five analyzed pollinizer cultivars, as well as the 'Celina' seeds collected in 2017 and 2018 (Table 4).

The average number of alleles per locus (4.1) was slightly higher in comparison to the values reported in the only similar study on pear, conducted on the cultivar 'Ingeborg', its pollinizers and seeds (3.75) [2].

Table 4. Allele size range (bp), number of alleles per locus (Na), and gene diversity (He) [38] for pollinizer cultivars ('Fritjof', 'Anna', 'Clara Frijs', 'Herzogin Elsa', and 'Kristina') and the main commercial cultivar ('Celina'), as well as for 'Celina' seeds, collected in 2017 and 2018, based on 11 SSR loci.

Locus Code	Pollinizers and 'Celina' Seeds			'Celina' Seeds 2017		'Celina' Seeds 2018	
	Size Range (bp)	Na	He	Na	He	Na	He
CH02b10	120/155	5	0.60	2	0.50	2	0.50
EMPc117	97/129	6	0.76	4	0.67	2	0.50
CH05e03	164/184	2	0.33	2	0.28	2	0.44
CH02c02	130/154	4	0.65	3	0.50	3	0.61
EMPc11	138/149	3	0.23	2	0.28	2	0.28
CH03d12	107/111	3	0.57	3	0.61	2	0.50
CH02c11	213/239	4	0.72	2	0.50	3	0.61
CH01d03	133/155	4	0.71	4	0.67	2	0.50
CH01f07a	175/190	4	0.73	4	0.67	2	0.50
CH04e03	178/202	2	0.44	2	0.44	2	0.50
CH01d09	135/160	8	0.77	3	0.61	3	0.61
Mean		4.1	0.59	2.8	0.52	2.3	0.51

There was a substantially difference in the average number of alleles per locus, detected among genotyped 'Celina' seeds, between the 2017 (2.8) and 2018 (2.3) season, although the overall gene diversity varied minimally. This can be explained with the fact that the cultivar 'Kristina' served as a pollen donor, albeit a small one, exclusively in 2017, when its full bloom almost completely overlapped with 'Celina' (Table 2). In addition, one of the possibilities which can explain lower number of alleles per locus in 2018 is a self-pollination of 'Celina'. A breakdown of the self-incompatibility mechanism has been reported for 'Celina' under extremely high temperatures [39], which might influence its receptiveness to pollen from a less then fully compatible source due to RNase deactivation. However, the same authors have indicated that in normal seasonal temperatures the same cultivar shows close to non-existent fruit set after self-pollination. It is therefore unwise to rely on this phenomenon in commercial cultivation of 'Celina'.

3.4. Identifying Most Successful Pollinizers

Overall, the most successful pollinizer cultivars across all orchards and in both investigated seasons were 'Fritjof' and 'Anna' (Table 5). Concurrent study on the success rate of individual 'Celina' pollinizers, conducted through controlled crossings and the monitoring of pollen tube growth using fluorescence microscopy, also identified 'Fritjof' and 'Anna' as the recommended pollinizer for 'Celina' [38]. Both cultivars were ubiquitous in the examined orchards, either as planted trees or as branches introduced during the flowering period. Although, the abundance of a pollinizer cultivar within an orchard has previously been reported as a major factor in pollinizer success among pear orchards in Norway [2], it is important to note that both 'Anna' and 'Fritjof' were the most abundant pollinizers in just one orchard each (Orchard 2 and 1, respectively) (Table 1). However, 'Fritjof' was introduced to Orchard 2 and 3 with branches in bloom, while 'Anna' was present in Orchard 1 and 3, but in a very small percentage.

Both 'Anna' and 'Fritjof' had two major advantages and two disadvantages as 'Celina' pollinizers in comparison to all other examined genotypes. Namely, 'Anna' is the only genotype fully cross-compatible with 'Celina'. Considering that semi-compatibility can cause significant fruit yield reductions in adverse environmental conditions for pollination [12], use of a fully compatible 'Anna' as a pollinizer is obviously highly prudent in orchards which have the disadvantage of a Nordic spring with low temperatures and high precipitation. However, among all the examined pollinizer genotypes 'Anna' also has the smallest flowering overlap with 'Celina'. This drawback has nevertheless done little to hinder the success of 'Anna' as a pollinizer in most of the examined orchards and seasons. Further examination of the pollen contribution results obtained through genotyping of 'Celina' seeds also

revealed that the pollinizer efficacy of 'Anna' is not fully consistent. Namely, during the 2017 growing season, pollen contribution from this cultivar was nonexistent in orchard 2 and varied greatly between the seasons in the two other orchards.

The main advantage that 'Fritjof' held as a pollinizer, was its very high bloom overlap with 'Celina'. However, 'Fritjof' is only semi-compatible and not fully compatible in terms of *S*-alleles. 'Fritjof', like 'Anna', also displayed inconsistences in pollinizer efficacies across the seasons.

Table 5. Pollen contribution of individual pollinizer cultivars in three orchards and two seasons, identified through gene assignment within GeneClass 2 software [32]. X% of progeny was derived from Y pollinizer cultivar.

		ASSIGNMENT–Score (%)				
	Season	**'Fritjof'**	**'Anna'**	**'Clara Frijs'**	**'Herzogin Elsa'**	**'Kristina'**
Orchard 1	2017	47.10	42.73	-	-	10.17
	2018	15.28	84.71	-	-	-
Orchard 2	2017	56.06	-	-	43.93	-
	2018	11.03	72.29	12.36	4.32	-
Orchard 3	2017	6.10	72.03	21.55	-	-
	2018	79.66	10.65	9.10	-	-

- no contribution detected.

Before ascribing the success in fertilizing 'Celina' flowers, exclusively to gametophytic compatibility or flower overlap, additional factors, such as the attraction of pollinators to floral resources of individual cultivars, needs to be mentioned. The quantity and quality of the floral resources can differ among pear cultivars [4], which can in turn affect the flower visitation rates by pollinators. Namely, the secretion of nectar, which together with pollen serves as source of nourishment for pollinator insects, has been shown to vary among different cultivars, clones, across seasons [3], and under different stresses, and not just in pear but in other fruit species [3,40,41]. Additionally, pollen which is rich in carbohydrates, polypeptides, amino acids, phytosterols, and minerals varies in quality and quantity among pear cultivars and affects pollinators foraging behavior [4].

Determining the gene flow within the examined orchards has been simplified by the absence of nearby seed propagated pear trees that could serve as a very diverse set of pollinizers and confound the gene assignment approach. Recent study, which relied on SSR markers to, among other things estimate gene-flow, detected a significant presence of alleles from such feral populations [42]. This is something that needs to be considered when setting up a similar trial.

4. Conclusions

The results of *S*-genotyping of 'Celina' revealed that this cultivar is having S101, S115 *S*-allele composition, which to our knowledge have not been published earlier. This will serve as an important tool for future orchard design involving this cultivar worldwide.

Overall, it can be concluded that pollinizer that had a very high flowering period overlap with 'Celina' pear cultivar, in this case 'Fritjof' and a pollinizer that showed fully cross-compatibly ('Anna'), are the most effective in fertilizing 'Celina' ovules. Therefore, it is possible to summarize that, in this case, the level of cross-compatibility and flower overlaps are more important for pollinizer success than the abundance of a pollinizer within an orchard.

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