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Reproductive Biology Factors Hampering Lemon [*Citrus limon* (L.) Burm. f.] Genetic Improvement

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Abstract: Background: Floral sterility and nucellar embryony are peculiar traits of several *Citrus* species and represent an obstacle to traditional breeding. Morphological sterility mainly results in pistil abortion and anther atrophy, while polyembryony is due to a mechanism known as sporophytic apomixis, which consists of the presence of embryos in the seed generated from the nucellar (maternal) tissue alongside the zygotic embryo (sexual origin). Considering the growing interest in lemon [*Citrus limon* (L.) Burm. f.] breeding, and the lack of information on floral sterility and the polyembryony trait among different lemon cultivars, a morphological and molecular characterization of these traits of interest was performed on forty Sicilian and international lemon cultivars available in the citrus germplasm collection of Catania University (Italy). Methods: Eight traits related to the reproductive biology were assessed on the selected lemon cultivars, namely: pistil abortion and anther atrophy, number of seeds per fruit, number of embryos per seed, percentage of seeds showing polyembryony, germination, percentage of seeds resulting in more than one plantlet, and average seed weight. Moreover, seedlings recovered after the germination assay were genotyped with SNP and SSR markers for ascertaining their nucellar or zygotic origin. In addition, PCR analysis were performed to assess the allele combination of the miniature inverted-repeat transposable element (MITE) insertion in CitRKD1, a gene associated with the occurrence of apomixis in citrus. Results: All traits showed high variability among the accessions analyzed. As for polyembryony, lemon 'Adamopoulos' scored the highest percentage of polyembryonic seeds (67.6%), whilst lemon 'Lunario' showed the lowest value (8.7%). Conclusions: Insights on the level of polyembryony within lemon varieties will represent a valuable tool for breeders for the set-up of novel mating schemes. In fact, when a polyembryonic female parent is used in cross breeding, the selection of the zygotic individual is hampered by the presence of a nucellar one.

Keywords: citrus; floral sterility; polyembryony; molecular markers; MITE

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1. Introduction

The genus *Citrus* encompass several species of great economic interest such as mandarins (*C. reticulata* Blanco), sweet oranges (*C. sinensis* L. Osbeck), grapefruits (*C. paradisi* Macf), lemons (*C. limon*), and limes (*C. aurantifolia* Swingle, *C. latifolia*), that are widely appreciated worldwide for fresh consumption thanks to the high content of nutraceutical compounds (e.g., flavonoids) and their organoleptic features. The main challenge for citrus breeding is the obtainment of new genotypes coupling high yields, fruit quality and tolerance (or resistance) to biotic and abiotic stresses. Nevertheless, the generation of improved cultivars is hampered by several biological and technical constraints: (1) the long juvenile phase of citrus seedlings makes the selection and evaluation phases a process that can last more than 15 years; (2) the occurrence, in some genotypes, of morphological (or cytological)

sterility and self-incompatibility; (3) the high heterozygosity resulting in a strong variability in the phenotypes of seedlings arising from a controlled cross, especially when the trait of interest is under polygenic control; (4) the nucellar polyembryony hindering the use of superior cultivars as a potential seed parent [1].

Morphological female sterility in citrus is mainly reported in lemon, citron (*C. medica* L.) and lime, whose flowers can be sterile due to either abnormal development or the abortion of the pistil [2,3]. This phenomenon is poorly studied and can vary greatly from bloom to bloom. Physiological conditions affecting pistil development (ringing, defruiting and defoliation) were investigated by [4], who demonstrated the correlation between starch content in the shoots and the development of perfect flowers. Instead, structural aspects of female sterility in lemon, in terms of the morphological development of the pistil, were investigated by light and scanning electron microscopy by [5], comparing normal and female sterile flowers.

Male sterility is widely spread within citrus varieties and species, and it is one of the main causes of seedlessness in citrus [6,7]. In fact, while this feature limits the application of traditional breeding approaches (e.g., hybridization), it is also a trait of major interest, when coupled with parthenocarpy, for the obtainment of high-quality seedless fruits for fresh consumption. In *Citrus*, environmental stresses can severely affect reproductive organ development. It was demonstrated that male gametophyte differentiation is highly affected by temperature during flower bud development and anthesis, causing a drastic reduction in pollen performances [8].

Regarding self-incompatibility, this mechanism prevents self-fertilization and was demonstrated to be determined by multi-allele S-RNase in the pistil which arrests incompatible pollen tube growth during pollen–pistil interaction [9].

The high level of heterozygosity in citrus genotypes was confirmed by Whole Genome Sequence (WGS) approaches [10], and more recently, also in the haploid genome of lemon that is characterized by a heterozygosity level of 3.56% [11].

Nucellar embryony is a peculiar trait of citrus, which consists of the presence of embryos generated from the nucellar (maternal) tissue alongside the zygotic embryo, due to a mechanism known as sporophytic apomixis. Consequently, the offspring generated from nucellar embryos are genetically identical to the female parent. Although polyembryony is a favorable feature for the obtainment of genetically uniform rootstocks, to propagated sowing seeds only, it hinders the selection of zygotic embryos from sexual crosses when cultivar, or rootstock, breeding is pursued through traditional approaches (e.g., hybridization, [1]).

Since zygotic embryos must compete for space and nutrients with several nucellar embryos, the latter grow preferentially [12]; thus, limiting the genetic variability that can be selected on a progeny obtained via sexual cross [13]. Consequently, seed parents in cross breeding are chosen among monoembryonic varieties and the available mating combinations are reduced [14].

For an effective selection of zygotic embryos, morphological, biochemical, and molecular markers have been investigated. Unfortunately, morphological seed traits, such as weight, size, and color, are not fully effective for the selection of monoembryonic seeds [15,16], while isozyme analysis had a very limited application [17,18]. On the other hand, several molecular markers were set up to determine the zygotic or nucellar origin of seedlings from sexual cross. Firstly, six Quantitative Trait Loci (QTLs) were reported to control apomixis in citrus [19]. Then, AFLP (Amplified fragment length polymorphism; [20,21]), RAPD (Random Amplification of Polymorphic DNA; [22–24], CAPS (Cleaved Amplified Polymorphic Sequence; [25]), SSR (Single Sequence Repeats; [26–29]), and SNP (Single Nucleotide Polymorphism; [30,31]) markers were applied for determining the zygotic or nucellar origin of seedlings in many breeding programs.

As for the genetic determinism of polyembryony in citrus, it was firstly investigated by [32], who identified 70 candidate genes in a locus associated with the trait of interest and isolated it through BAC clones. More recently, CitRKD1, encoding an RWP-RK domain-containing protein, was identified as a candidate gene responsible for citrus so-

matic embryogenesis in satsuma mandarin (*C. unshiu* Marc.; [33]). CitRKD1 comprises two alleles (mg1 and mg2) at the locus controlling embryonic type and they differ for the insertion in the upstream region of a miniature inverted-repeat transposable element (MITE). In particular, the allele CitRKD-mg2 with the MITE insertion was demonstrated to be predominant when somatic embryogenesis occurs [14,33]. Therefore, it was possible to verify the CitRKD1 allele arrangement through genotyping the PCR in a huge selection of citrus genotypes [14].

With a special focus on lemon, polyembryony makes for particularly challenging breeding. Among the most important goals for lemon breeding, particular efforts have been made for the obtainment of new varieties coupling excellent fruit quality (high juice content, seedless) and tolerance or resistance to Mal Secco disease, a tracheomycosis caused by *Plenodomus tracheiphilus* [34]. The most recent advance in lemon genetic improvement regards the study of a segregating population (the mildly resistant lemon 'Interdonato' x the susceptible lemon 'Femminello Siracusano 2Kr') through a genomic breeding approach and a genotype-phenotype association study [35]. When parents for the segregating population were chosen, it became clear how little was known about the distribution of the polyembryony trait among lemon cultivars, since it appeared that this trait has a quantitative feature comparing several accessions [12,36].

In this work, eight traits related to reproductive biology were evaluated on a selection of 40 lemon accessions (listed in Table 1) over three years. In addition, polyembryony was assessed using molecular markers and verifying the MITE insertion in a subset of samples.

Results revealed that nucellar embryony trait differs among lemon varieties despite the uniformity of the allelic constitution of the CitRKD1 gene, thus underlining the importance of knowing the phenotypic variability of this character for time- and cost-effective breeding programs.

Table 1. Results of the three-year evaluation of eight traits related to reproductive biology in lemon accessions considered in the present work.

Accession	Pistil Abortion (%)		Atrophic Anthers (%)		Number of Seeds per Fruit		Average Seed Weight (g)		Number of Embryos per Seed		Polyembryonic Seeds (%)		Germination (%)		Seeds Giving More than One Plantlet (%)	
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
Adamopoulos	72.3	0.22	5.3	0.05	7.47	2.02	0.20	0.002	2.98	0.18	67.6	0.22	85.0	0.13	46.1	0.30
Akragas	16.0	0.07	5.5	0.06	2.50	2.12	0.15	-	2.43	-	22.0	-	70.0	-	14.3	-
Cerza	68.3	0.19	10.0	0.09	-	-	NA	-	NA	-	NA	-	60.0	-	0.0	-
Chaparro	53.7	0.19	10.3	0.17	14.23	2.25	0.15	0.0007	2.65	0.16	49.5	0.14	80.0	0.13	24.9	0.19
Cirò	80.0	-	20.0	-	1.05	0.07	0.18	-	2.00	-	9.0	-	25.0	-	0.0	-
Erice	17.7	0.02	7.1	0.03	3.45	1.48	0.15	0.03	2.62	0.13	57.9	-	67.5	0.11	20.8	0.18
Femminello Adamo (seedless)	22.4	0.26	76.0	0.40	1.00	0.00	NA	-	NA	-	NA	-	NA	-	NA	-
Femminello apireno Continella m84	NA	-	NA	-	6.80	-	0.11	-	2.50	-	32.3	-	80.0	-	18.8	-
Femminello Campisi (seedless)	92.2	0.09	71.7	0.38	NA	-	NA	-	NA	-	NA	-	47.5	0.11	26.1	0.02
Femminello Cucuzzaro	18.5	0.08	2.5	0.02	17.54	4.37	0.15	0.03	2.82	0.34	37.6	0.04	66.7	0.08	29.9	0.06
Femminello Dosaco m503	53.4	0.01	20.0	0.11	5.00	2.26	0.14	0.01	2.68	0.01	44.9	0.10	92.5	0.11	16.3	0.02
Femminello Fior d'Arancio	72.9	0.23	4.3	0.06	11.70	2.96	0.16	0.04	2.55	0.47	39.4	0.13	66.7	0.10	42.0	0.19
Femminello Germanà (seedless)	34.3	0.18	96.1	0.01	1.80	1.13	0.14	-	2.40	-	19.2	-	NA	-	NA	-
Femminello Greco apireno	72.1	0.13	6.3	0.05	5.77	4.99	0.15	0.00	3.03	0.04	44.8	-	70.0	-	85.7	-
Femminello Pennisi Carrubbaro	42.3	0.23	6.7	0.08	10.65	0.35	0.11	0.02	2.57	0.61	37.2	0.20	75.0	0.07	16.5	0.03
Femminello S01	NA	-	NA	-	7.30	-	NA	-	NA	-	19.4	-	NA	-	NA	-
Femminello S02	NA	-	NA	-	5.70	-	NA	-	NA	-	30.3	-	NA	-	NA	-
Femminello Santa Teresa	7.7	0.03	4.0	0.04	15.13	3.80	0.14	0.02	2.76	0.08	38.3	0.02	73.3	0.13	52.1	0.20
Femminello Scandurra (seedless)	89.8	0.09	48.3	0.43	1.00	0.00	NA	-	NA	-	NA	-	NA	-	NA	-
Femminello Siracusano 2Kr	78.0	-	12.0	-	5.97	1.46	0.13	-	NA	-	53.0	0.14	77.5	0.18	25.4	0.03
Femminello Siracusano m296	48.9	0.25	5.6	0.08	20.33	3.04	0.13	0.03	3.42	0.68	51.5	0.06	80.0	0.13	14.0	0.05
Femminello Zagara Bianca Fragalà	62.9	0.22	10.1	0.07	7.53	1.18	0.17	0.02	2.73	0.32	39.3	0.12	78.3	0.20	20.2	0.06
Fino Iniasel 49	66.0	0.17	40.0	0.24	13.00	0.71	0.13	0.05	2.64	0.52	45.8	0.13	77.5	0.11	22.5	0.01
Fino Iniasel 95	58.3	0.15	35.7	0.31	11.03	4.66	0.14	0.004	2.50	0.06	34.8	0.02	91.7	0.06	12.5	0.06
Incappucciato m504	17.0	-	0.0	-	10.30	-	0.10	-	2.33	-	26.0	-	NA	-	NA	-
Interdonato	46.0	0.17	11.5	0.06	7.95	1.91	0.16	-	2.33	-	13.5	0.09	65.0	-	23.1	-
Kamarina	26.3	0.14	96.1	0.05	2.62	2.38	0.11	0.01	2.33	0.24	16.3	0.06	100.0	-	15.0	-
Lemox (seedless)	63.0	-	12.0	-	1.00	0.00	NA	-	NA	-	NA	-	NA	-	NA	-
Lisbon	62.7	0.18	5.7	0.07	12.27	0.78	0.14	0.03	3.03	0.31	49.7	0.11	75.0	0.13	21.6	0.07
Lunario	75.0	0.17	5.7	0.06	2.78	0.74	0.11	0.02	2.17	0.23	8.7	0.02	50.0	0.22	12.0	0.07
Messina old line	70.5	0.37	52.5	0.60	NA	-	0.14	-	NA	-	NA	-	70.0	-	35.7	-
Meyer ((*C. maxima* × *C. reticulata*) × *C. medica* hybrid)	99.0	-	12.0	-	11.83	1.65	0.14	0.02	1.10	1.56	9.7	0.09	76.7	0.03	10.7	0.10
Monachello Continella old line	31.0	-	3.0	-	2.30	-	0.17	-	2.60	-	42.0	-	NA	-	NA	-
Monachello nucellar line	89.0	-	18.0	-	3.05	0.49	NA	-	2.27	-	25.0	0.11	67.5	0.04	3.6	0.05
Ovale di Sorrento	70.0	0.19	6.7	0.04	13.60	5.40	0.15	0.05	2.75	0.49	45.1	0.10	88.3	0.03	26.5	0.04
Quattrocchi	50.5	0.26	51.0	0.52	5.74	0.80	0.15	-	2.43	-	23.0	-	NA	-	NA	-
Segesta (seedless)	42.0	-	0.0	-	1.00	0.00	NA	-	NA	-	NA	-	NA	-	NA	-
Selinunte	52.9	0.15	7.7	0.07	2.80	1.13	0.13	0.03	2.88	-	38.0	0.11	72.5	0.04	34.5	0.02
Sfusato Amalfitano	47.3	0.23	4.7	0.07	12.17	3.68	0.21	0.03	3.15	0.21	43.1	0.09	88.3	0.03	11.5	0.06
Verna	70.3	0.14	6.0	0.08	6.70	3.90	0.15	0.03	2.85	0.05	32.2	0.05	78.3	0.20	17.2	0.01

2. Materials and Methods

2.1. Plant Material

In the present work, 39 lemon varieties and one hybrid were used (Table 1). Fruits and flowers were harvested from the citrus germplasm collection of Catania University maintained at its experimental farm (Contrada Primosole, Catania—Sicily, N 37°41′02.0091, E 15°05′95.3542). The adult trees of the collection were managed according to the usual cultural practice for lemon in this area. For each variety, a total of 40 fruits were collected in winter (main production) at the ripening stage and 100 flowers in spring (main flowering flux), just before flowering. All the analyses were repeated for three years (2020–2022). For DNA extraction, young leaves were collected in spring.

2.2. Flower and Seed Analysis

On one hundred flowers per accession, the percentage of those showing the aborted pistil and/or with atrophic anthers (all dry, whitish anthers without traces of pollen) was assessed (Figure 1a–c). Flowers were collected one day before anthesis: the petals were removed and the flowers were left for 24 h at room temperature for the dehiscence of the anthers. On the seeds, six traits were assessed: the number of seeds per fruit, the average seed weight, the number of embryos per seed (Figure 1d,e), the percentage of seeds showing polyembryony, in vitro germination, the percentage of seeds giving more than one plantlet. The number of seeds per fruit was measured by counting the number of seeds in 20 ripened fruits; then 30 seeds for each variety were weighed, the tegument was removed and the number of embryos in each seed was considered to determine the seed-type: monoembryonic or polyembryonic. For measuring the germination rate in vitro, 20 seeds were set in MS medium with added McCown salt (2.5 g/L), sucrose (30 g/L) and Gel Rite (2.2 g/L), after 3–4 weeks, the germinated seeds and number of seedlings produced by every seed were counted.

2.3. DNA Extraction

Genomic DNA was extracted from young leaves using a modified protocol from Doyle and Doyle (1987) as described in [30]. Briefly, approximately 30 mg leaf samples were homogenized in a 300 µL CTAB extraction buffer (2% CTAB, 100 mM Tris–HCl, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl, and 0.1% 2-mercapthoethanol) using a TissueLyser (Qiagen, Valencia, CA, USA) and incubated at 65 °C for 1 h. To each sample, 100 µL of chloroform was added, and the tube was vortexed and centrifuged for 10 min at 10,000 rpm. DNA was precipitated by mixing 200 µL of supernatant with 500 µL 95% ethanol and incubated for 20 min on ice. The pellet was washed with 70% ethanol, dried and dissolved in water. Quantities and qualities of the extracted DNA samples were determined using a Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA) spectrophotometer and agarose gel electrophoresis. DNA samples were stored at −20°C. DNA for Illumina sequencing was extracted following the protocol described in [11].

2.4. Identification of Nucellar and Zygotic Seedlings

SSR and SNPs analyses were performed on in vitro germinated seedlings; plantlets from 5 seeds for 9 varieties, including all groups of multiple plantlets produced by a single seed, were analyzed. Ten SSR primer pairs [37] and ten SNPs primer pairs [38], all showing heterozygosity in lemon, were screened on five samples and eleven primer pairs (5 SSR and 6 SNPs, Tables 2 and 3, respectively) were selected on the basis of amplification efficiency and for the clearness and reproducibility of results.

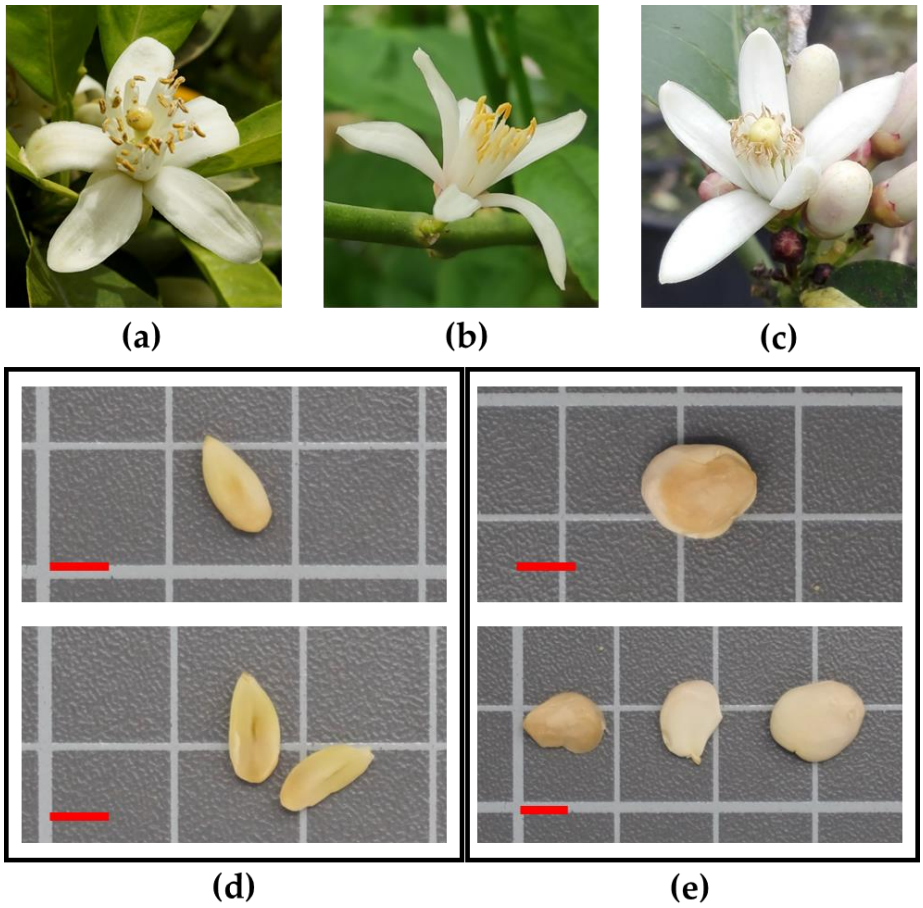

Figure 1. (**a**) Normal lemon flower; (**b**) lemon flower showing pistil abortion; (**c**) lemon flower showing atrophic (dry and whitish) anthers; (**d**) monoembryonic lemon seed; (**e**) polyembryonic lemon seed (bars are 0.5 cm).

Table 2. SSR markers used in the present work for assessing seedlings of nucellar origin [37].

SSRs Marker	Primer Sequences	Size
INRA 1388	F: AAAACAAAGCACCC AGATCG R: ACGGCAGCAACGAG ATAAGT	139
INRA 1210	F: GCCAAAATGCATGT TCAAGA R: GTGCCAATGATGAT CACGTC	175
INRA 818	F: GTAGATTCGTTCAA GGCCCA R: GTGAAGCTGGAAGA GATGGC	134
INRA 116	F: GAATTGGGAGGACG AACTGA R: CGAGCCCTAGACAG AGATGG	252
INRA 338	F: TTTCTAAAATTTCCT TCATGGC R: CAGGTGAAATCTCA TCGCCT	204

PCR reactions were performed in a 15 µL volume containing approximately 60 ng of genomic DNA, 1× PCR buffer (Bioline–Meridian Bioscience, Memphis, TN, USA), 2.5 mM $MgCl_2$, 0.2 mM dNTP, 300 nM forward and reverse primers, 1.5 mM Syto® 9 (Life Technologies, Gaithersburg, MD, USA) and 0.5 U Biotaq DNA polymerase (Bioline). HRM genotyping was performed on a Rotor-Gene Q real-time PCR (Qiagen) and the data were analyzed by Rotor Gene Q Series 2.0.3 software. PCR conditions were as follows: denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 5 s and 55–57 °C for 20 s; a final extension at 72 °C for 2 min; 95 °C for 1 min; and 40 °C for 1 min. HRM analysis was performed at a ramp of 16 °C (from 72 °C to 88 °C) with 0.1 °C increments every 2 s. The fluorescent data were acquired at each of the HRM steps and subjected to automatic gain optimization.

The melting data were normalized by adjusting the start and end fluorescence signals, respectively, of all samples to the same levels. The data were recorded and analyzed using the Rotor-Gene 6500 series software (Corbett Research, Sydney, Australia). The HRM curve for each individual was visually scored, identifying hetero (nucellar genotype) and homozygous (zygotic genotype obtained by segregation) samples through both the normalized and derivative melting plots. The zygotic origin of the sample was demonstrated if, in at least one marker, the HRM results showed a heterozygous profile different from the maternal one, or, more frequently, a homozygous profile.

Table 3. SNPs markers used in the present work for assessing seedlings of nucellar origin [38].

SNPs Marker	Base Variation	SNP Position	Primer Sequences	Size
U455	C/T-C/T	907–933	F: ACTTCCGTGAGC CAGTGAAC R: GATAGGTAGCTT CTTGTCCTCAAA	98
U513	A/T-C/G	340–345	F: AATAACGAATACGC ACACGGA R: CAGTGTCAGAAG CGAAAGATTG	124
U555	C/T-A/G-A/G-C/T	802–806–864–879	F: GTCCCAATCCAA GTGGCTTA R: GGAGTCTGAGGT ATCCTTCATTAG	124
U10304	G/T-C/G-G/TG/C-A/G	368–390–400–411–426	F: AGAAGAAGCATA CGGGCTCA R: GCTCAGTCCCT TTGAACCAA	146
U7190	A/T-A/G-C/G-C/T-C/GC/T	636–655–665–667–678–687	F: GCTTTCATTTGG TTTGCTGC R: GGTGCCTATTTT GTCCCTGAT	132
U56	A/G-AG	514–531	F: GCCACATCCC AGTTTAGCC R: ATATTCAGCG GAAAGCAAGG	104

2.5. Mono/Poly Embryonic Allelic Genotypes Discrimination by PCR Genotyping

CitRKD1 allelic constitution and MITE insertions responsible for polyembryonic phenotype were evaluated by PCR genotyping on a subset of lemon cultivars, as indicated in Table 1. In addition, the Elongation Factor (EF) gene was amplified for verifying DNA quality using a primer from [39]. Moreover, pummelo (*C. maxima*) and bitter orange (*C. aurantium* L.) were included as reference monoembryonic and polyembryonic genotypes [33]. Genomic DNA isolated from young leaves was amplified using MITE primers with the following sequences: sense primer 5′-TCTCTGGTTCATTGAGAATCC-3′ in the upstream region, antisense primer 5′-CTGAGCACCAGGCAACAACTAC-3′ in the second exon [14]. Allelic genotyping PCR was carried out using Biotaq DNA polymerase (Bioline) with a program of 30 cycles of 45 s at 94 °C, 45 s at 60 °C, and 60 s at 72 °C in a 20 µL reaction solution. The PCR product for each reaction was analyzed using electrophoresis in a 1.5% (*v*/*v*) agarose gel.

2.6. Data Analysis

Statistical analysis on the collected data considered the calculation of the standard deviation, correlation coefficient and ANOVA test by using the software Excel (Microsoft) and R [40]. The ANOVA test was performed considering a p critical value of 0.05.

2.7. Sequencing

Illumina reads of Interdonato were aligned against the lemon reference genome [35] using the Burrows-Wheeler Aligner (BWA; [41]) and variant calling was performed with BCFtools software [42].

3. Results

Overall, eight phenotypic traits were assessed on the 40 lemon accessions in analysis (Tables 4 and S1): the occurrence of pistil abortion or atrophic anthers, the number of seeds per fruit, the average seed weight, the number of embryos per seed, the percentage of seeds showing polyembryony (on 30 seeds), germination (sowing 20 seeds) and percentage

of seeds giving more than one plantlet. Then, the mono- or polyembryonic origin of the plantlets was tested using SNP molecular markers. Then, the MITE insertion was verified on a subset of samples through genotyping the PCR. Since MITE amplification failed for lemon 'Interdonato', we further investigated the occurrence of single-nucleotide or structural variations at the primer annealing sites hampering the PCR assay and the whole-genome of this accession was sequenced employing Illumina pair-ends technology. Reads were aligned against the lemon genome [11], but the primer annealing sites did not show any SNPs or INDELS (insertions-deletions) compared to the reference.

Table 4. Results of the considered 11 molecular markers analyzed on 69 seedlings from 9 genotypes in order to identify zygotic and nucellar plantlets.

Accessions	Seedlings	Origin	Number of Recombinant Markers	Accessions	Seedlings	Origin	Number of Recombinant Markers
'Femminello Siracusano m296'	1	zygotic	5	**'Chaparro'**	1A	nucellar	0
	2	zygotic	6		1B	zygotic	3
	3	zygotic	2		2	zygotic	1
	4	zygotic	7		3	zygotic	2
	5A	zygotic	2		4	nucellar	0
	5B	nucellar	0		5	nucellar	0
	6A	nucellar	0		6A	nucellar	0
	6B	nucellar	0		6B	zygotic	1
	6C	zygotic	6	**'Femminello Siracusano 2Kr'**	1	nucellar	0
'Femminello Zagara Bianca'	1	zygotic	7		2A	zygotic	1
	2	zygotic	4		2B	nucellar	0
	3	zygotic	3		3	zygotic	2
	4	zygotic	3		4	zygotic	1
	5	zygotic	8		5	zygotic	2
'Lisbon'	1A	nucellar	0	**'Fino Iniasel 95'**	1	zygotic	3
	1B	zygotic	6		2	zygotic	3
	2	zygotic	5		3	nucellar	0
	3	nucellar	0		4	zygotic	2
	4	zygotic	5		5A	zygotic	1
	5	nucellar	0		5B	nucellar	0
'Verna'	1	zygotic	3	**'Adamopoulos'**	1	nucellar	0
	2	zygotic	3		2A	nucellar	0
	4	zygotic	9		2B	nucellar	0
	5	zygotic	8		3	zygotic	2
	6A	zygotic	9		4A	nucellar	0
	6B	nucellar	0		4B	nucellar	0
'Femminello Santa Teresa'	1	nucellar	0		5	nucellar	0
	2A	nucellar	0		6	zygotic	5
	2B	zygotic	4				
	3	zygotic	5				
	4	zygotic	6				
	5	nucellar	0				

In general, the average of pistil abortion incidence was lower in 2021 (40%) than in 2019 (74%) and 2020 (53%); while atrophic anther incidence was lower in 2019 (15%) than in 2020 and 2021 (both 25%) (Table S1). In particular, pistil abortion was more frequent

in 'Meyer' (99.0%), 'Femminello Campisi' (92.2%) and 'Femminello Scandurra' (89.8%), while it was rarer in 'Femminello Santa Teresa' (7.7%), 'Akragas' (16%) and in 'Incappucciato m504' (17.0%). On the other hand, the presence of atrophic anthers was observed most frequently in 'Kamarina' (96.1%) and in the seedless varieties 'Femminello Germanà' (96.1%), 'Femminello Adamo' (76.0%) and 'Femminello Campisi' (71.7%). According to the ANOVA test ($p < 0.05$), a quite good variability was observed between the years 2019–2020 and 2020–2021.

The average number of seeds per fruit ranged from 20.3 ('Femminello Siracusano m296' lemon) to 1.0 ('Femminello Adamo', 'Femminello Scandurra', 'Segesta', all considered seedless). Globally, those cultivars highly appreciated on the market showed the highest presence of seeds in the fruit: 15.1 in 'Femminello Santa Teresa', 13.6 in 'Ovale di Sorrento', 13.00 in 'Fino Inasel 49', 12.27 in 'Lisbon', while those with a lower fruit quality also contained a lower number of seeds: 7.95 in 'Interdonato', 3.05 in 'Monachello nucellar line', 2.5 in 'Akragas'. As an exception to this trend, the lemon 'Femminello Siracusano 2Kr' showed an average value of 5.97.

Moreover, a moderate correlation (38.6%, Figure S1) was found between the number of seeds per fruit and the percentage of seeds showing polyembryony, a trait that was higher in 'Adamopoulos' (67.6%), 'Erice' (57.9%), 'Femminello Siracusano 2Kr' (53%), 'Femminello Siracusano m296' (53%) and 'Lisbon' (49.7%). On the other hand, 'Meyer', 'Cirò' and 'Lunario' showed a ratio of polyembryonic seeds under 10%.

Instead, a high correlation (75%, Figure S1) was calculated by comparing the percentage of seeds showing polyembryony and the effective number of embryos counted on a subset of 30 seeds per genotype analyzed. The latter ranged from 3.42 in 'Femminello Siracusano 2kr' to 1.1 in 'Meyer'.

With regard to the number of seeds per fruit, in lemons 'Verna', 'Femminello Fior d'Arancio', 'Meyer', 'Ovale di Sorrento', 'Sfusato Amalfitano' and 'Kamarina', this trait varied between the years 2020 and 2021 (ANOVA test with p value < 0.05). Similarly, in lemons 'Femminello Fior d'Arancio', 'Limone Pennisi Carrubbaro', 'Ovale di Sorrento', 'Fino Iniasel 49' and 'Lisbon', the number of embryos per seed was highly variable when comparing the years 2020 and 2021 (ANOVA test with p value < 0.05, Table S1).

Seed germination, assessed on 20 seeds per variety per year, ranged from 100% in 'Kamarina' to 25.0% in 'Femminello Campisi', but most of the genotypes showed values between 80% and 70%. Instead, the percentage of seeds giving more than one plantlet was globally lower and the values ranged between 85.7% for the 'Femminello Greco Apireno' and 3.6% in the 'Monachello nucellar line'. This trait had a moderate correlation with the average seed weight (37%, Figure S1), which values ranged from 210 mg in 'Sfusato Amalfitano' to 100 mg in 'Incappucciato m504'. At the same time, seed weight was not strongly correlated with the number of embryos per seed (19%).

Molecular markers were applied on 60 plantlets from the germination test and on a subset of nine genotypes: 'Femminello Siracusano m296', 'Femminello Zagara Bianca', 'Lisbon', 'Verna', 'Femminello Santa Teresa', 'Adamopoulos', 'Chaparro', 'Femminello Siracusano 2Kr' and 'Fino Iniasel 95' (Figure 2; Table 4). When only a seedling occurred from a seed, it was of zygotic or nucellar origin, and this trait was variable according to the genotype. Instead, if two or three seedlings per seed were observed, in particular in 'Femminello Santa Teresa', 'Chaparro' and 'Femminello Siracusano m296', one of them had a zygotic origin and the others were nucellar clones.

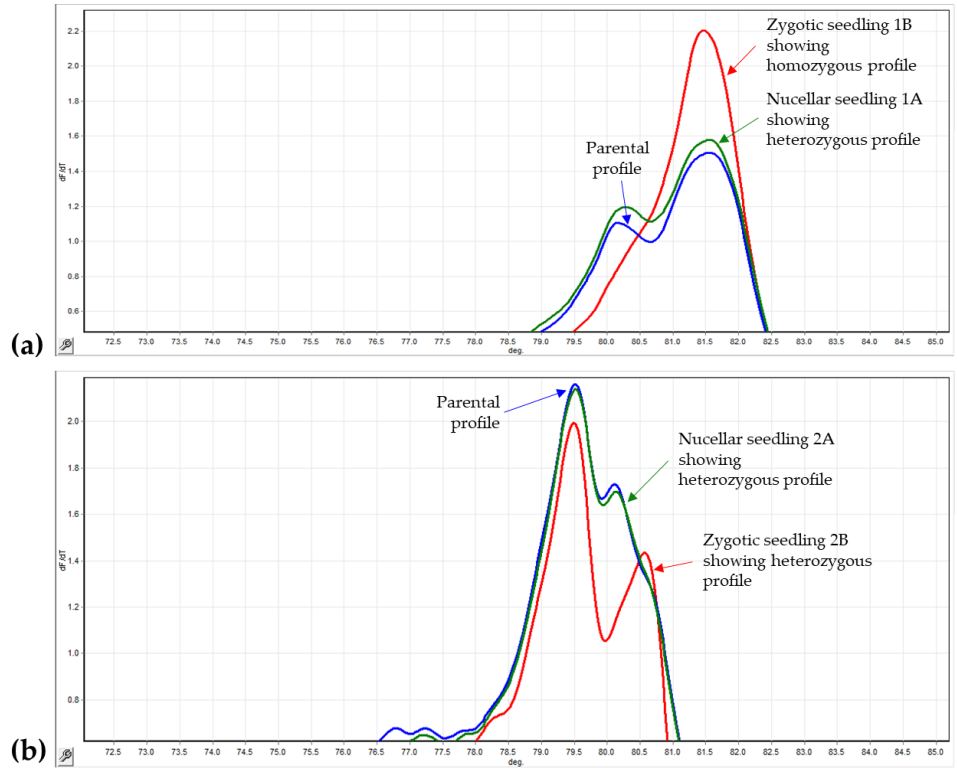

Figure 2. (**a**) Marker SNP U455 on parental DNA of lemon 'Lisbon' and two seedlings grown from the same seed (1A and 1B); (**b**) marker SSR INRA 338 on parental DNA of 'Femminello Siracusano m296' and two seedlings grown from the same seed (2A and 2B).

PCR genotyping for assessing the allelic configuration of the gene responsible for the polyembryony trait revealed in all lemon accessions the presence of one fragment at approximately 1.3 kbp including the MITE insertion site in the CitRKD1 gene, and one fragment at 0.7 kbp not including the transposable element. The lemon 'Meyer' was the only one revealing the presence of one single band at approximately 0.7 kbp, thus meaning the absence of the CitRKD1 allele including the MITE insertion and the monoembryonic phenotype. In Table 5, the PCR genotyping results are shown for a representative subset of genotypes, while in Figure S2, gel agarose is shown.

Table 5. Results of the allelic genotyping PCR for the MITE insertion in the CitRKD1 gene on a subset of citrus accessions included in the present work.

Accession	Polyembryony	Allelic Constitution of MITE Gene	
		1.3 kbp Band	0.7 kbp Band
Pummelo (*C. maxima*)	monoembryonic	-	+
Sour orange (*C. aurantium)*	polyembryonic	+	+
Adamopoulos	polyembryonic	+	+
Akragas	polyembryonic	+	+
Chaparro	polyembryonic	+	+
Femminello Cucuzzaro	polyembryonic	+	+
Femminello Dosaco m503	polyembryonic	+	+
Femminello Fior d'Arancio	polyembryonic	+	+
Femminello Greco apireno	polyembryonic	+	+
Femminello Pennisi Carrubbaro	polyembryonic	+	+
Femminello Santa Teresa	polyembryonic	+	+
Femminello Siracusano 2Kr	polyembryonic	+	+
Femminello Siracusano m296	polyembryonic	+	+
Femminello Zagara Bianca Fragalà	polyembryonic	+	+
Fino Iniasel 49	polyembryonic	+	+
Fino Iniasel 95	polyembryonic	+	+
Kamarina	polyembryonic	+	+

Table 5. *Cont.*

Accession	Polyembryony	Allelic Constitution of MITE Gene	
		1.3 kbp Band	0.7 kbp Band
Lisbon	polyembryonic	+	+
Lunario	polyembryonic	+	+
Meyer	monoembryonic	-	+
Monachello Continella old line	polyembryonic	+	+
Monachello nucellar line	polyembryonic	+	+
Ovale di Sorrento	polyembryonic	+	+
Quattrocchi	polyembryonic	+	+
Segesta (seedless)	polyembryonic	+	+
Selinunte	polyembryonic	+	+
Sfusato Amalfitano	polyembryonic	+	+
Verna	polyembryonic	+	+

4. Discussion

Sterility and polyembryony are peculiar traits of many citrus accessions (sweet orange, lemon, mandarin, grapefruit) and represent significant limiting factors for citrus breeding through traditional approaches [1]. Although morphological female sterility is poorly studied in citrus, the mechanism causing male sterility has been widely investigated in natural seedless mutants of several citrus accessions through cytologic, transcriptomic, and proteomic approaches [43–48], but only recently, a small RNA sequence (the miR399-CsUBC24 module) was demonstrated to negatively affect floral development, stamen morphology, anther dehiscence and pollen fertility [49].

Moreover, male gametophyte development, in citrus as well as in other superior plants, represents a phase of the plant reproduction cycle particularly sensitive to environmental stresses [8]. Temperature stresses compromise anthers and pollen grain functionality. In our assays, the variable results among the considered accessions and between the years confirmed how sterility is a genetically and environmentally dependent trait (Tables 1 and S1). Interestingly, and as a general trend, pistil abortion and the presence of atrophic anthers were more predominant in those varieties with a lower level of polyembryony. In fact, underdeveloped anthers containing a few dysfunctional or no pollen grains are due to microsporogenesis breakdown and, combined with parthenocarpy, led to seedless fruit in many citrus genotypes [43].

Regarding polyembryony, much research has been performed to set up molecular markers useful for the selection of citrus zygotic individuals [19–31] and the genetic mechanism responsible for this trait has been unlocked [14,33].

Nevertheless, there is a lack of information about the variability of these traits of interest in the main citrus species. Seedlings from germination tests were tested with SSR and SNP markers, and it was verified that when more than one seedling occurred there was both a zygotic individual and a nucellar one. However, it is worth mentioning that exceptions can occur. For example, in citrus, there have been reports of polyembryonic seeds in non-apomictic genotypes as a consequence of 2x × 4x hybridizations and in vitro cultures of isolated nucelli [27]. In addition, despite what is reported in literature [12], according to our molecular analyses results, nucellar seedlings do not always prevail on the zygotic one, as shown for 'Femminello Zagara Bianca' and 'Femminello Siracusano m296' compared with 'Adamopoulos', where most of the plantlets were derived from somatic embryogenesis.

Also, genotyping PCR was performed on a subset of genotypes to verify the presence of the MITE insertion in at least one of the two CitRKD1 alleles, which was demonstrated to be responsible for the polyembryony trait [14,33]. In [33], a PCR-based analysis of the MITE insertion was performed in 786 citrus accessions. Among these, 12 lemon accessions were analyzed, demonstrating that their allelic configuration is consistent with the presence of both the CitRKD1 alleles mg1, containing the MITE insertion (band at approximately 1.3 kbp), and mg2, which lacks the MITE insertion (0.7 kbp). This data was confirmed in the lemon cultivars considered in the present work by PCR genotyping performed

according to Shimada et al. (2018) (Table 5). Only lemon 'Meyer', which is not a true lemon but a (*C. maxima* × *C. reticulata*) × *C. medica* hybrid [44], both alleles were lacking the MITE insertion and only allele mg2 occurred, confirming that it is a monoembryonic genotype. Only in lemon 'Interdonato' did the MITE amplification fail, even though the occurrence of structural variations was verified; thus, ongoing studies aimed at the de novo sequencing of this lemon accession will help to clarify the genetic mechanism behind the lack of amplification.

Interestingly, all lemon cultivars are polyembryonic with the same CitRKD1 allelic constitution (only one of the two alleles contain the MITE insertion), but by phenotyping the trait of interest, we highlighted that it is variable among the genotypes and blooms.

In the present work, the polyembryonic attitude was investigated by determining the number of seeds showing more than one embryo and the number of embryos per seed; a high correlation between these two parameters was found. For both traits, lemon 'Adamopoulos', 'Erice', 'Femminello Siracusano 2Kr' and 'Femminello Siracusano m296' showed the highest values, while lemon 'Lunario', 'Monachello nucellar line' and 'Interdonato' showed the lowest scores. Being monoembryonic, lemon 'Meyer' showed one of the lowest polyembryony rates (9.7%) even if in one year (2020) it revealed a number of embryos per seed higher than one on average (Table 1), suggesting a possible variability of the polyembryony trait according to the season (Table S1). In fact, most of the studied accessions revealed variability in both traits (number of seeds showing polyembryony and number of embryos per seed) between the different years. In contrast with previous reports, there was a low correlation (19%, Figure S1) between the seed weight and the number of embryos per seed [15]; thus confirming that morphological features are not fully effective for the selection of zygotic seeds compared with the use of molecular markers on the seedling.

Since most of lemon accessions under study were selected through clonal and nucellar selection by both citrus growers and breeders during genetic improvement programs [45], we speculate that, despite the same allelic constitution at the genetic locus determining polyembryony, other genetic or environmental factors could affect the presence and amount of nucellar embryos in the seed. For example, we do not exclude that other genetic variations occurring in the CitRKD1 locus could affect the polyembryony phenotype in lemon and should be investigated in more depth in the future, taking advantage of the recent release of the lemon genome [11]. Overall, polyembryony is considered a quantitative trait [13,15] and the characterization of this trait exists also among cultivars of the same species, representing a keystone for increasing the time and cost-effectiveness of breeding programs.

5. Conclusions

In the present work, eight traits related to reproductive biology were quantified on thirty-nine lemon accessions and one lemon hybrid, in order to investigate sterility and polyembryony features among cultivars of the same species. Moreover, molecular markers were implemented for assessing the nucellar or zygotic origin of seedlings from the in vitro germination test, while PCR genotyping at the CitRKD1 locus, which is responsible for polyembryony in citrus, confirmed the same allelic configuration for all true lemon genotypes under study. This is in contrast with the high variability results for polyembryony traits, as well as for sterility features; thus, suggesting the influence of the environment or for other genetic factors in determining these reproductive traits, which are so important for breeding programs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12122020/s1, Table S1: Data for the three years of evaluation of the eight traits related to reproductive biology in lemon accessions considered in the present work; Figure S1: Correlation plot of the eight traits related to reproductive biology in lemon accessions considered in the present work; Figure S2: Results of the MITE-CitRKD1 allele constitution analysis on a subset on the analyzed genotypes.

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