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Morphological and Molecular Characterization of a New Self-Compatible Almond Variety

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Abstract: Almonds are one of the most popular nuts, cultivated in countries with Mediterranean climates. In an almond orchard of the self-incompatible cultivar 'Ferragnes' in Greece, a tree with different morphological characteristics and signs of self-compatibility was observed. The aim of this study was to study the phenotype, investigate the self-compatibility trait, and elucidate the phylogenetic background of this tree, named 'Mars'. Morphological traits and kernel and nut characteristics were measured in 'Mars', 'Ferragnes', 'Tuono', and 'Lauranne' cultivars. The self-compatibility trait of almonds is attributed to the S_f allele; thus, its existence was investigated in 'Mars' by PCR amplification. Moreover, the *S*-RNase genes of all the cultivars were sequenced. The genetic profile of 'Mars' was identified using eight SSR molecular markers and compared with the 'Ferragnes', 'Ferraduel', 'Texas', 'Tuono', and 'Lauranne' cultivars. The morphological traits suggest that 'Mars' is more similar to the 'Ferragnes' cultivar, while it bears the S_f allele. *S*-RNases sequencing revealed that 'Mars' has the genotype S_1S_f, and the SSR markers showed that it is differentiated genetic material, suggesting it is a cross between 'Ferragnes' and 'Tuono'. Therefore, 'Mars' is evaluated as a self-compatible variety with interesting agronomic traits for use in new mono-cultivar almond plantations.

Keywords: *Prunus dulcis*; SSR markers; *S*-RNase genes; agronomical traits

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1. Introduction

Almonds, *Prunus dulcis* Miller (D.A. Webb) synonym *Prunus amygdalus* Batsch, are one of the most popular nuts, second in global consumption after peanuts [1]. They are referred to for their sweet taste and high nutritional value. *Prunus dulcis* is a diploid species belonging to the Rosaceae family, widely distributed in the world, and cultivated in hot, arid Mediterranean climate regions. It is closely related to peach (*Prunus persica*), both belonging to the Prunoideae subfamily of the Rosaceae family, and has evolved from a wild species (*P. webbii*) that originated in Asia [2]. Although almonds are not native to the U.S., nowadays the U.S., especially California due to its climate, is the top almond producer, covering about 80% of global consumption, followed by Spain and Australia [3]. More than 30 cultivars are used in California, but 13 of them cover 98% of production. The dominant cultivar is 'Nonpareil', followed by 'Carmel' and 'Texas' [4]. Greece produced 22,750 tons of shelled almonds in 2021 [5], with the two most popular cultivars being 'Ferragnes' from France and 'Texas' from the U.S., both self-incompatible [6].

Almonds have been consumed as a snack since ancient times, as they are a nutrient-dense food rich in protein and fatty acids, containing mostly unsaturated fat, low in saturated fat, without cholesterol, but high in dietary fiber and essential minerals [3]. Various studies have demonstrated the significant health effects that regular almond consumption offers humans. Specifically, almond consumption has a beneficial impact on health problems such as obesity [7], hypertension [8], diabetes [9], and cardiovascular diseases [10]. The nutritional properties of the almonds depend on the cultivar. For instance, cultivar 'Ferragnes' has a protein content of 11 g/100 g fresh weight, while 'Lauranne' has 17 g/100 g fresh weight, and 'Tuono' has 21 g/100 g fresh weight [11].

Morphological assessment of almond traits is necessary for cultivar selection, while in the past it was the only tool for cultivar discrimination [12]. This assessment is based on phenotypic observations and includes nut and kernel characteristics like weight, length/width ratio, and thickness, as well as characteristics of the fruits, leaves, flowering, and harvest time [13]. The establishment of molecular techniques and especially of DNA-based markers, like AFLPs, RAPD, SSR, ISSR, and microarrays, led to a shift from morphological to molecular characterization, as it is more reliable and not influenced by environmental factors [14]. SSR (Simple Sequence Repeats) molecular markers have become the markers of choice in *Prunus* species genotyping, as they are highly polymorphic, co-dominant, multi-allelic, and relatively simple to interpret [15–17].

Most flowering plants have bisexual flowers and are capable of self-pollination. However, inbred populations show reduced fitness and several other deficiencies; thus, during evolution, plants have adopted various mechanisms to avoid self-fertilization and promote outcrossing [18]. The almond flower is predominantly self-incompatible, a trait controlled by a series of *S*-alleles designated by a series of numbers [19]. The *S*-alleles code for haplotype-specific *S*-RNases (ribonucleases) expressed in the style [20] and for SFBs (F-box proteins) expressed in the pollen [21]. If pollen's *S*-allele matches one of the two alleles of the pistil, then it is perceived as pollen from the plant itself, and fertilization does not proceed. If the pollen carries a different allele, pollen tube growth is allowed, and pollination occurs [22]. Most almond cultivars are self-incompatible, but a mutation found in the *S*-allele caused a loss of ribonuclease activity, resulting in self-compatible individuals [23]. This *S*-allele, designated S_f, was found in wild-grown almond *P. webbii* in the Puglia region of Italy and in some sweet almond cultivars in that area like 'Tuono', 'Genco', 'Falsa Barese', and 'Filippo Ceo', possibly from a cross between the two species [24]. These cultivars, especially 'Tuono', have been extensively used in breeding schemes in order to produce new self-compatible cultivars with desirable traits [25,26]. One of the most successful self-compatible cultivars, coming from a cross between 'Ferragnes' (S_1S_3) and 'Tuono' (S_1S_f), is 'Lauranne' (S_3S_f), produced at INRA, France, in 1978 [20,27].

Self-compatibility and late flowering time are important traits for almond breeding [28]. Self-compatible cultivars are preferred by farmers since they allow for monovarietal orchards, which have several advantages. Some of these advantages are lower orchard management costs, a lower effect of unfavorable climatic conditions at bloom, which usually decrease cross-pollination, resulting in low fruit set and yield losses, and no need for flowering synchronization of the main and pollinating cultivars to achieve fertilization [29]. Moreover, the market prefers nuts with the same morphological characteristics, so after-harvest mixing of different cultivars should be avoided to ensure uniformity and quality of the final product and to facilitate the processing operation [30].

In Greece, as mentioned before, the most popular almond cultivars are 'Ferragnes' and 'Texas' (S_1S_5) [6]. 'Ferragnes' is preferred for fresh consumption due to its exceptional kernel characteristics, while 'Texas' is preferred for processing [6]. The 'Ferraduel' (S_1S_4) cultivar is used as the main pollinator of 'Ferragnes', but its kernel has different commercial characteristics. Both cultivars come from a cross between 'Cristomorto' and 'Ai' [31,32]. The 'Tuono' cultivar was introduced in Greece by several farmers in the 1970s as a promising self-compatible cultivar, but it was not until 2010 that monovarietal 'Tuono' orchards were

cultivated. However, 'Tuono's nut quality was inferior to that of 'Ferragnes' or 'Texas', so its cultivation is now limited.

Currently, climate change is leading to more extreme weather conditions, making more important the cultivation of at least two inter-compatible and simultaneous blooming cultivars, pollinating insects for pollen transfer, and proper weather conditions for bee activity [33]. Another problem observed in recent years in the Greek main areas of almond cultivation is the infestation of 'Ferragnes' trees by serious fungal diseases that cause extensive yield losses. All these reasons contribute to the shrinkage of 'Ferragnes' cultivation, while new cultivars tend to replace it after limited evaluation of their agronomic traits under Greek conditions and with uncertain productivity and kernel quality.

The aim of this study was to characterize a new almond selection, thereafter called 'Mars'. The 'Mars' tree was first observed in 2012 by the Greek nursery VITRO HELLAS S.A. in a field of 'Ferragnes' trees due to its unique characteristics, such as self-compatibility, nut production in one-year-old branches, regular yields even under adverse weather conditions for cross-pollination, and early maturation of the nuts (5–7 days earlier than 'Ferragnes'). The cultivar typically used for 'Ferragnes' pollination was 'Ferraduel', but the empirically observed self-compatibility trait led to the hypothesis that 'Mars' came from a spontaneous cross between 'Ferragnes' and a self-compatible cultivar, potentially 'Lauranne' or 'Tuono', that were available in the area. The 'Mars' tree was propagated through budding, and its morphological and nut characteristics were assessed and compared with those of other cultivars. Then, the presence of the S_f allele was investigated to verify the empirically observed self-compatibility, and the *S*-RNase genes of all cultivars were sequenced. Moreover, 'Mars' genetic profile was studied using SSR markers and compared with the cultivars 'Ferragnes', 'Lauranne', 'Tuono', 'Ferraduel', and 'Texas', in order to elucidate its genetic background. The results of this study suggest that 'Mars' is a new genetic material, coming from a cross between 'Ferragnes' and 'Tuono', and will assist in classifying this genotype as a new cultivar.

2. Materials and Methods

2.1. Plant Material

Kernels from a 'Ferragnes' tree were planted directly into soil in a field with 'Ferragnes' trees in the area of Kavala, Greece, in the 1980s. Thirty years later, one tree that had germinated from the nuts mentioned above displayed distinguished characteristics. Vegetative buds from this tree, named 'Mars', were grafted on GF677 rootstocks, trained in vase form, and plants were grown in a nursery before being transferred to soil.

Trees of 'Lauranne', 'Tuono', 'Ferraduel', and 'Texas' were also grafted onto GF677 rootstock and trained in vase form in a nursery before being transferred to soil in the area of Kavala, Greece.

2.2. Morphological Characterization

Morphological characterization between 'Mars' and 'Ferragnes' was conducted during the cultivation period of 2022 on shoots, leaves, flowers, fruit, nuts, and kernels. Flowers were collected in full bloom (20 March 2022). Ten flowers were taken from each of the three trees studied (each tree is one replication), and in closed flowers, bud shape, bud color of tip of petals, bud color of sepals, bud pubescence of sepals, and in flowers, petal shape, petal color of inner side, petal undulation of margin, number of stamens, stamen anthocyanin coloration of filament, stigma position in relation to anthers, and stigma size were assessed. Leaves were collected at the adult stage in early August. Six leaves were sampled per tree replication, and petiole length (mm), leaf blade length (mm), width (mm) and their ratio, leaf blade intensity of green color, and blade margin incisions were measured or evaluated. Shoot coloration was observed on the sunny side of the one-year-old shoot. Fruits were harvested at maturity, when the fruit pericarp was fully dried and split along the fruit suture. Fruit morphological characteristics were measured in five replications of 10 fruits for 'Ferragnes' and 'Mars' cultivars and included qualitative measurements.

Fruit size, fruit shape (in lateral view), fruit shape of apex, and fruit pubescence were assessed. Fruit pericarp was removed, and nuts were left to dry naturally. Nut (endocarp with kernel) shape (in lateral view), nut shape of apex, nut thickness (mm), nut resistance to cracking, nut keel development, kernel (seed) size, kernel intensity of brown color, and kernel rugosity of surface were measured or evaluated. The time of harvest was also logged.

Nut and kernel quantitative characteristics were assessed at maturity stage in five replications of 10 left to dry naturally fruits of 'Ferragnes', 'Mars', 'Tuono', and 'Lauranne' cultivars. The measurements were performed in 2022 at the same trees, as mentioned above, for 'Mars' and 'Ferragnes', and at three trees of 'Lauranne' and 'Tuono' cultivars from local commercial orchards. All sampled trees were mature (above 7 years old) and regularly produced the typical form of nuts for each cultivar. The three nut farms used for nut collection of the four cultivars in Kavala, Greece, were intensively planted and cultivated similarly with locally applied irrigation, fertilization, and pruning practices. The productivity of each cultivar for the study year was normal, and nut size was representative of the cultivar for the specific area and depended on the yield of each cultivar. Nut and kernel weight (g) were measured using a scale (OHAUS® GA200D, Parsippany, NJ, USA), kernel dimensions [length (mm), width (mm), thickness (mm)] using a digital caliper (Powerfix Profi Electronic Digital Caliper, Owim GmbH &Co. KG, Neckarsulm, Germany), and the ratio length/width for nut and kernel, kernel percentage (kernel weight/nut weight), and the percentage of double kernels were logged or calculated. The dendrogram for the morphological data was plotted using https://www.bioinformatics.com.cn/en (accessed on 30 June 2023), a free online platform for data analysis and visualization.

2.3. Statistical Analysis

For the flower, leaf, and fruit qualitative characteristics data, Student's *t*-test at 5% level of significance was performed, while for nut and kernel quantitative characteristics, kernel percentage, and percentage of double kernels, Tukey test at 5% level of significance was prepared using the statistical package SPSS (SPSS Statistics for Windows, Version 26.0, IBM Corporation, Armonk, NY, USA).

2.4. Preparation of Genomic DNA and Self-Compatibility Assay

Leaves from 'Mars', 'Ferragnes', 'Tuono', 'Lauranne', 'Ferraduel', and 'Texas' trees were collected from the area of Kavala, Greece, and stored immediately at −20 °C until further processing. The leaves were ground to fine powder using liquid nitrogen and mortar, and then the DNA was extracted using the GRS Genomic DNA Kit-Plant (Grisp, Porto, Portugal), following the kit protocol. DNA quality and quantity were assessed by gel electrophoresis and spectrophotometry, respectively.

For the investigation of the self-compatibility of each cultivar, the S_f gene was PCR-amplified using the primers P8 and P2 [34], which specifically amplify from C1 to C5 regions of the S_f gene, as described in Marchese et al., 2008 [18]. For each PCR reaction of total volume of 20 µL, 100 ng of genomic DNA were amplified using 1 unit of Xpert Fast DNA polymerase (Grisp, Porto, Portugal), 4 µL of 5×Xpert Fast Reaction Buffer (that includes 5 mM dNTPs), 0.4 µL of each 10 µM primer, and the following cycling conditions: 95 °C for 1 min, 40 cycles of 95 °C for 15 s, 15 s at 58 °C, 72 °C for 30 s, and one final extension step at 72 °C for 3 min on a Thermocycler (Thermo Fisher Scientific, Waltham, MA, USA). Amplification products were separated on agarose gel.

For the determination of the *S*-RNase allele of each cultivar, the relative locus was PCR-amplified using the primers Pru_C2 and Pru_C5 [35], which specifically amplify from C2 to C5 regions of S_1, S_7, S_8, and S_f alleles [36,37]. PCR reactions were performed as described before at Ta 51 °C. Amplification products were separated on agarose gel. The PCR products were then purified using the PureLink™ PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). For the samples 'Mars' and 'Tuono', where two PCR products were present in the gel, each band was excised from the agarose gel using a scalpel, and the DNA

was extracted using the Monarch® DNA Gel Extraction Kit (New England Biolabs, Ipswich, MA, USA). All the cleaned products were then subjected to Sanger sequencing using Pru_C2 primer on the ABI3730xl platform (Cemia, SA, Larissa, Greece). The sequences were aligned by Muscle with MEGA, version X software (https://www.megasoftware.net/, accessed on 30 June 2023) [38] and identified using the Nucleotide Blast feature of NCBI. A Neighbor-Joining phylogenetic tree [39] was created using Molecular Evolutionary Genetics Analysis (MEGA, version X) software with bootstrap test (1000 replicates).

2.5. SSR Analysis

For the SSR analysis, PCRs were performed in a volume of 20 µL including 30 ng genomic DNA extracted as described in Section 2.3, 0.4 µL of each 10 µM primer, 1 unit of Xpert Fast DNA polymerase (Grisp, Porto, Portugal), and 4 µL of 5× Xpert Fast Reaction Buffer (that includes 5 mM dNTPs), following the cycling program: 95 °C for 1 min, 40 cycles of 95 °C for 15 s, 15 s at annealing temperature (Ta) (Table 1), 72 °C for 3 s, and one final extension step at 72 °C for 20 min, on a Thermocycler (Thermo Fisher Scientific, Waltham, MA, USA). Eight pairs of primers were used: UDP98410, Pchgms1, Pchgms3, Ps8e8, Ps9f8 [15], UDP96008, UDP96018 [40], and BPPCT010 [16] (Table 1). Forward primers were 5′-end fluorescently labeled with either FAM or HEX, according to each dye's absorption and emission wavelength and the size of the amplified product, in order to avoid overlapping during fragment analysis.

PCR fragments were separated using capillary electrophoresis in an ABI 3500 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). Data analysis, sizing, and genotyping were performed using the Peak Scanner (version 4.0) software (Thermo Fisher Scientific, Waltham, MA, USA). The SSR alleles were converted to a 1/0 matrix, with the presence of an allele being scored as 1 and the absence as 0. Allele scoring generated a table for GenAlex (version 6.5) [41] that was used for the calculation of the total number of alleles, number of polymorphic alleles, effective number of alleles (Ne), Shannon information index (I), observed heterozygosity (Ho), and expected heterozygosity (He). A Neighbor-Joining phylogenetic tree [39] was created using Molecular Evolutionary Genetics Analysis (MEGA-X) software [38]. The information content of each primer (PIC, Primers Information Content) was calculated based on the formula PICi = 2fi (1 − fi) [42], where PICi is the polymorphic information content of marker 'i', fi is the frequency of the amplified allele, and 1 − fi is the frequency of the null allele (band absent).

Table 1. List of SSR primers used. F: forward. R: reverse. Ta: annealing temperature. Reference: the study that designed the primers.

SSR marker	Ta	Sequence (5′-3′)	Reference
Pchgms1-F Pchgms1-R	59 °C	GGGTAAATATGCCCATTGTGCAATC GGATCATTGAACTACGTCAATCCTC	Sosinski et al., 2000 [43]
Pchgms3-F Pchgms3-R	59 °C	ACGGTATGTCCGTACACTCTCCATG CAACCTGTGATTGCTCCTATTAAAC	
UDP98410-F UDP98410-R	50 °C	AATTTACCTATCAGCCTCAAA TTTATGGCAGTTTACAGACCG	Testolin et al., 2000 [44]
Ps8e8-F Ps8e8-R	50 °C	CCCAATGAACAACTGCAT CATATCAATCACTGGGATG	Joobeur et al., 2000 [45]
Ps9f8-F Ps9f8-R	45 °C	GGTTCTTGGTTATTATGA ACATTTCTATGCAGAGTA	
UDP96008-F UDP96008-R	58 °C	TTGTACACACCCTCAGCCTG TGCTGAGGTTCAGGTGAGTG	Cipriani et al., 1999 [40]
UDP96018-F UDP96018-R	58 °C	TTCTAATCTGGGCTATGGCG GAAGTTCACATTTACGACAGGG	
BPPCT010-F BPPCT010-R	55 °C	AAAGCACAGCCCATAATGC GTACTGTTACTGCTGGGAATGC	Dirlewanger et al., 2002 [46]

3. Results

3.1. Morphological Characterization

Shoot, leaf, flower, fruit, nut, and kernel morphological characteristics were assessed for 'Mars' and compared with those of the 'Ferragnes' cultivar, both grown in the same field (Table 2). In 'Mars', one-year-old shoot anthocyanin coloration on the sunny side was weak, while in 'Ferragnes', it was strong. Leaf dimensions, shape, color, blade incisions at the margin, and petiole length were similar to those of 'Mars' and 'Ferragnes'. Flower morphological characteristics were similar between 'Mars' and 'Ferragnes', except for the petal shape, which was medium elliptic for 'Mars', while for 'Ferragnes', it was circular. In 'Mars', the color at the base of the filament of the stamen was characteristically reddish, while in 'Ferragnes', the color was green, and the flowers of 'Mars' had a significantly larger number of stamens compared with 'Ferragnes'. Macroscopically, the pistil of the flowers of 'Mars' was smaller compared with 'Ferragnes'. Regarding the qualitative traits of fruit, the fruit of 'Mars' was more elliptic and the nut keel development was more intense compared with 'Ferragnes', while the rest of the fruit's qualitative characteristics were similar between 'Mars' and 'Ferragnes'. The time of harvest was earlier for 'Mars' (late August) compared with 'Ferragnes' (early September) (Table 2).

Table 2. Tree, shoot, leaf, flower, and fruit morphological traits of 'Mars' and 'Ferragnes'.

Trait	'Mars'	'Ferragnes'
One-year-old shoot anthocyanin coloration	Weak	Strong
Leaf blade width (mm)	26.2 a	26.6 a
Leaf ratio length/width	3.18 a	3.20 a
Leaf blade intensity of green color	Medium	Medium
Leaf blade incisions of margin	Crenate	Crenate
Petiole length (mm)	28.0 a	28.3 a
Flower bud shape	Triangular	Triangular
Flower bud color of tip of petals	Pink	Pink
Flower bud color of sepals	Red	Red
Flower bud pubescence of sepals	Medium	Medium
Petal shape	Medium elliptic	Circular
Petal color of inner side	Light pink	Light pink
Petal undulation of margin	Weak	Weak
Flower number of stamens	42 a	32 b
Stamen anthocyanin coloration of filament	Moderate	Absent
Stigma position in relation to anthers	Above	Above
Stigma size	Small	Small
Fruit size	Large	Large
Fruit shape (in lateral view)	Elliptic	Ovate
Fruit shape of apex	Obtuse	Obtuse
Fruit pubescence	Dense	Dense
Nut shape (in lateral view)	Ovate	Ovate
Nut shape of apex	Acute	Acute
Nut thickness of endocarp	Medium	Medium
Nut resistance to cracking	Strong	Strong
Nut keel development	Medium	Weak
Kernel size	Large	Large
Kernel intensity of brown color	Light	Light
Kernel rugosity of surface	Weak	Weak
Time of harvest	Early	Medium

Different letters on values of the same trait indicate significantly different changes, according to Student's *t*-test at $p \leq 0.05$.

Nut and kernel quantitative characteristics of the 'Mars' selection were compared with those of 'Ferragnes', 'Lauranne', and 'Tuono' in 2022 (Table 3). The highest nut weight was found for 'Ferragnes' and 'Mars', followed by 'Lauranne', while 'Tuono' had the lowest one. Concerning the nut dimensions, nut length was similar among the genotypes,

while 'Mars' and 'Ferragnes' had slightly bigger nut widths than 'Lauranne' and 'Tuono', with 'Lauranne' having the smallest. Nut thickness was significantly higher in 'Mars' and 'Ferragnes' compared with 'Tuono' and 'Lauranne'. Nut length/width ratio was highest for 'Lauranne', while 'Mars' had a slightly lower than 'Lauranne', but similar nut length/width ratio with 'Ferragnes' and 'Tuono'. Kernel weight was highest for 'Ferragnes', 'Mars' had slightly lower, followed by 'Lauranne' and 'Tuono'. Kernel length was significantly higher in 'Ferragnes', followed by 'Mars' and 'Lauranne', while 'Tuono' had the smallest kernel length. Kernel width was similar among all the genotypes. Kernel thickness was similar for 'Ferragnes' and 'Mars' and significantly higher compared with 'Lauranne' and 'Tuono'. Kernel ratio length/width was similar among 'Mars', 'Ferragnes', and 'Lauranne', while 'Tuono' had the lowest value. Shelling percentage (% kernel) had the highest value for 'Tuono', while 'Mars', 'Ferragnes', and 'Lauranne' had similar lower values. Double kernels were found only in 'Mars' and 'Tuono', and 'Mars' had a lower percentage than Tuono (Table 3). Moreover, it was observed that the 'Mars' shell presented two concentric layers, with the outer layer easily removed from the nut, while this trait was not observed in any other cultivar. In addition, in the 'Mars' shell, the suture line was not always well sealed. The dendrogram of the morphological characteristics of the four cultivars has two branches (Figure 1). In one branch, the cultivars 'Tuono' and 'Lauranne' are observed, while in the other, 'Ferragnes' and 'Mars', are. This result suggests that 'Mars' is phenotypically closer to the 'Ferragnes' cultivar.

Table 3. Nut and kernel weight, length, width, thickness, length/width ratio, kernel percentage, and percentage of double kernels of 'Mars', 'Ferragnes', 'Lauranne' and 'Tuono'. Means with different letters within the row are significantly different, according to Tukey test at $p \leq 0.05$.

Trait	'Mars'	'Ferragnes'	'Lauranne'	'Tuono'
Nut weight (g)	5.15 a	5.58 a	3.86 b	2.97 c
Nut length (mm)	37.9 a	37.6 a	36.6 a	34.3 a
Nut width (mm)	24.1 ab	25.0 a	22.3 b	23.3 ab
Nut thickness (mm)	17.6 a	17.1 a	14.4 b	15.5 b
Nut ratio length/width	1.57 ab	1.51 b	1.64 a	1.47 b
Kernel weight (g)	1.57 ab	1.83 a	1.3 bc	1.25 c
Kernel length (mm)	27.2 b	29.4 a	26.3 b	23.9 c
Kernel width (mm)	15.2 a	15.3 a	13.7 a	14.0 a
Kernel thickness (mm)	8.33 a	8.85 a	7.20 b	7.21 b
Kernel ratio length/width	1.80 ab	1.93 a	1.92 a	1.71 b
Kernel (%)	30.6 b	32.9 b	33.5 b	42.0 a
Double kernels (%)	9.2 b	0 c	0 c	20 a

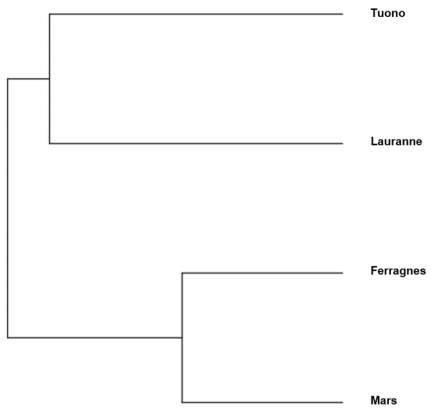

Figure 1. Dendrogram of the phylogenetic relationships of 'Mars', 'Ferragnes', 'Lauranne', and 'Tuono' trees based on their morphological characteristics.

3.2. Self-Compatibility Analysis

The S_f gene was PCR amplified using the primers P8-P2, revealing a product in 'Tuono', 'Lauranne', which are self-compatible cultivars, and 'Mars' (1.2 kb), while it was absent from 'Ferragnes', 'Ferraduel', and 'Texas' samples, as these are self-incompatible cultivars (Figure 2). These results suggest that 'Mars' is self-compatible.

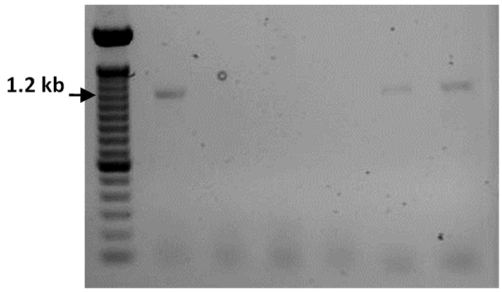

Figure 2. Gel electrophoresis of the amplification products of the samples 'Mars' (1), 'Ferragnes' (2), 'Ferraduel' (3), 'Texas' (4), 'Tuono' (5), and 'Lauranne' (6) using the primers P8–P2. The DNA ladder used was 100 bp (Invitrogen, Thermo Scientific, Waltham, MA, USA). Arrow points at the 1.2 kb band.

S-genotyping by PCR, from the C2 to C5 regions of S_1, S_7, S_8, and S_f alleles, using the primers Pru_–Pru_C5, revealed two bands for the samples 'Mars' and 'Tuono' at about 1 kb and 1.1 kb, while for cultivars 'Ferragnes', 'Ferraduel', and 'Texas', one product was observed at about 1 kb, and for 'Lauranne' one product at 1.1 kb (Figure 3).

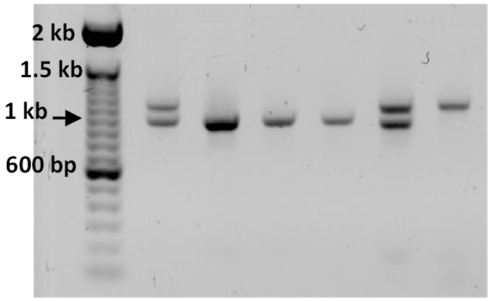

Figure 3. Gel electrophoresis of the PCR amplification products of the samples 'Mars' (1), 'Ferragnes' (2), 'Ferraduel' (3), 'Texas' (4), 'Tuono' (5), and 'Lauranne' (6), using the primers Pru_C2–Pru_C5. The DNA ladder used was 100 bp (Invitrogen, Thermo Scientific, Waltham, MA, USA). Arrow points at the 1 kb band.

The sequencing of all the PCR products of Pru_C2–Pru_C5 primer amplification revealed that the lower bands of the 'Mars' and 'Tuono' samples (at 1 kb) and the products of 'Ferragnes', Ferraduel', and 'Texas' shared the same sequence, with some discrepancies attributed to the sequencing quality. This sequence was identified as the S_1 RNase allele (accession number AF149039.1) in NCBI (Figure 4). The rest of the PCR products, the higher bands of the 'Mars' and 'Tuono' samples (at 1.1 kb), and the 'Lauranne' sample shared the same sequence, identified as the Tuono S_f allele (accession number AF157009.1) in NCBI (Figure 5). The phylogenetic relationships between the amplified fragments are depicted in a phylogenetic tree (Figure 6), where all the S_1 alleles form one cluster while all the S_f are located on a separate branch.

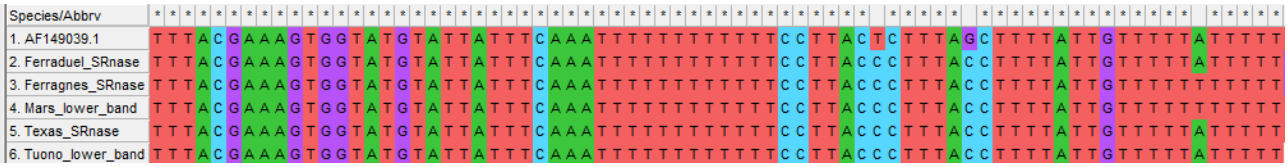

Figure 4. Alignment of the first 74 nucleotides of the sequences of 'Mars' and 'Tuono' (1 kb band), 'Ferragnes', Ferraduel', 'Texas', and *Pr. dulcis* S_1 RNase allele (accession number AF149039.1) as reference. Asterisks indicate conserved nucleotides. Different nucleotides are marked with different colors.

Figure 5. Alignment of the first 66 nucleotides of the sequences of 'Mars' and 'Tuono' (1.1 kb band), 'Lauranne' PCR products, and *Pr. dulcis* S_f allele (accession number AF157009.1) as reference. Asterisks indicate conserved nucleotides. Different nucleotides are marked with different colors.

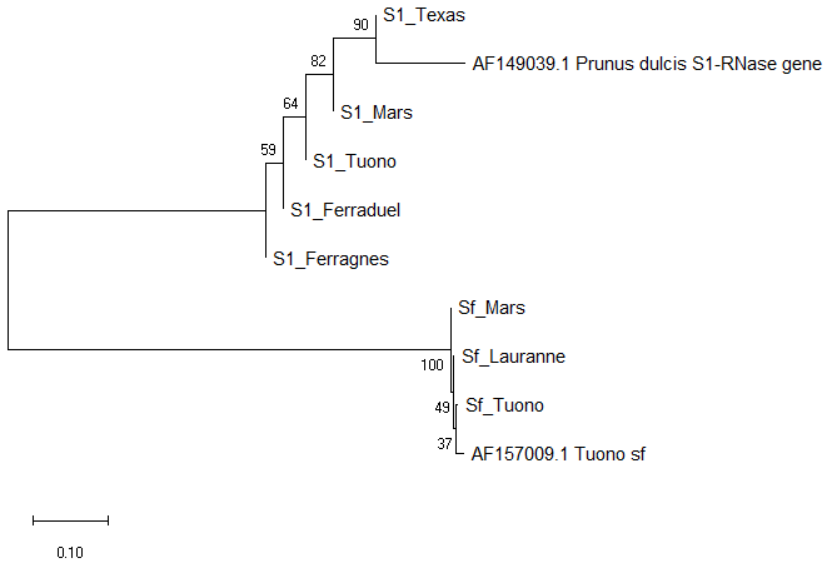

Figure 6. Neighbor-Joining phylogenetic tree representing the evolutionary relationships between the amplified sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

3.3. SSR Analysis

For the evaluation of the genetic profile of six almond trees of different cultivars/genotypes, eight sets of SSR markers were used, generating a total of 25 alleles. The analysis was informative since all analyzed loci were polymorphic among almond accessions, resulting in an average of three alleles per locus. The genetic variation of the eight SSR loci was calculated based on the number of alleles, the number of effective alleles, Shannon's information index, observed heterozygosity (Ho), expected heterozygosity (He), and polymorphic information content (PIC) (Table 4).

Table 4. Indices of genetic diversity calculated for each primer used in the analysis. Ne: number of effective alleles; I: Shannon's Information Index; Ho: observed heterozygosity; He: expected heterozygosity; PIC: Polymorphic information content. Reference: the study that designed the primers.

SSR Marker	Allele Size Range	No of Bands		Ne	I	Ho	He	PIC
		Total	Polymorphic					
Pchgms1	179–212 bp	6	6	4.000	1.583	1.000	0.750	0.365
Pchgms3	166–186 bp	5	5	3.789	1.445	1.000	0.736	0.320
UDP98410	131–143 bp	3	3	1.946	0.824	0.333	0.486	0.211
Ps8e8	170–174 bp	2	1	1.600	0.562	0.500	0.375	0.147
Ps9f8	133–165 bp	4	4	3.429	1.309	0.667	0.708	0.269
UDP96008	130–134 bp	2	1	1.385	0.451	0.333	0.278	0.147
UDP96018	226–232 bp	4	4	3.789	1.358	0.667	0.736	0.269
BPPCT010	131–141 bp	4	4	3.789	1.358	0.500	0.736	0.269
Mean		3.75	3.5	2.966	1.111	0.625	0.601	0.250

The values of Ho ranged from 33.3% to 100%, with a mean of 62.5%. Ho is defined as the number of individual heterozygotes per locus [47]; the higher the Ho values, the higher the genetic variability. The He, or gene diversity, ranged between 27.8% and 75%, with a mean value of 60.1%. The maximum number of fragments was amplified by the primer Pchgms1 (N = 6), while the minimum was generated by the primers Ps8e8 and UDP96008 (N = 2). PIC reflects the discriminating ability of the marker and depends on the number of known alleles and their frequency distribution, thus representing genetic diversity. The highest PIC values were observed for the markers Pchgms1 and Pchgms3, as well as the highest Shannon's Information Index, in accordance with the highest number of alleles detected by these markers.

It is worth mentioning that of the 16 loci amplified using eight SSR markers, 'Mars' exhibited two unique alleles, one amplified by Pchgms1 and one by the Ps9f8 marker, that were not identified in any of the other cultivars. The phylogenetic relationship of 'Mars' with the other five cultivars was depicted in a phylogenetic tree constructed using the Neighbor-Joining method (Figure 7). The 'Mars' sample had a distinct genetic profile from 'Tuono', showing differences in five out of the eight markers, confirming that it is a different self-compatible cultivar in genetic proximity with 'Tuono'. 'Ferragnes' was depicted in the same branch as 'Ferraduel', while 'Lauranne', was shown in phylogenetic proximity to them. 'Texas' had the greatest distance from all the other cultivars, forming a separate branch.

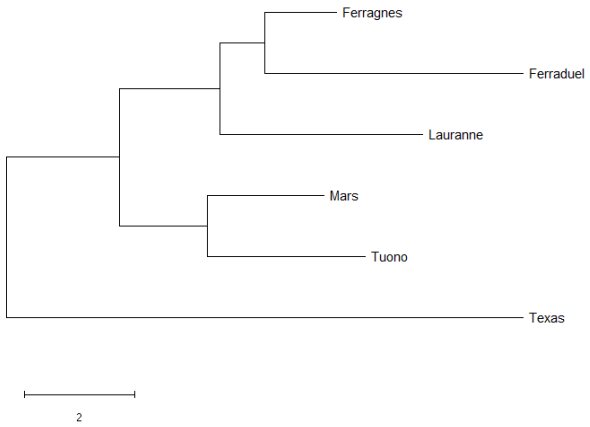

Figure 7. Neighbor-Joining phylogenetic tree representing the evolutionary relationships between the 6 almond samples analyzed.

4. Discussion

This work aimed to elucidate the genetic profile and morphological characteristics of a new almond genotype, named 'Mars', coming from a 'Ferragnes' seedling that grew in a field of 'Ferragnes' almond trees, macroscopically exhibiting unique properties. Our first goal, after the phenotypical assessment of various characteristics, was to genetically verify the self-compatibility trait that was observed in the field. Then, the phylogenetic relationship of 'Mars' with the other cultivars in the area was evaluated, in order to verify that it is a new genotype, excluding the possibility of being a 'Tuono' or a 'Lauranne' tree since 'Mars' proved to be self-compatible.

The assessment of shoot, leaf, flower, and fruit morphological characteristics of 'Mars' in comparison with 'Ferragnes', 'Lauranne', and 'Tuono' traits revealed that 'Mars' flowers had a characteristically higher number of stamens (42 stamens) compared with 'Ferragnes' (32 stamens). In several almond cultivars, the number of stamens may range between 20 and 30, but may reach 40 [48], with the usual number being 30–33 [49]. Even though the stigma of the flower of both 'Mars' and 'Ferragnes' was characterized as small macroscopically, the pistil of the flowers of 'Mars' was smaller compared with 'Ferragnes'. The stigma position in relation to anthers was above for both 'Mars' and 'Ferragnes'. According to Bernad et al., 1995 [50], the stigma relative to anthers position is critical for self-pollination in the absence of pollinating insects, as self-compatibility alone is not enough for ensuring a crop in commercial farms, and, thus, 'Mars' should be evaluated to ensure a good nut set in the absence of pollinating insects.

In general, the size and unique nut shape of certain almond cultivars must be assessed, as they tend to establish specific marketing categories and uses [49]. Whole fruit, nut, and kernel qualitative traits showed many similarities between 'Mars' and 'Ferragnes', such as the hard shell, an important trait as it helps maintain kernel quality during nut storage [51], weight, and dimensions. The size and shape of almond kernels, as well as their specific nutritional qualities, are influenced by specific cultivars and the growing environment [52]. In another study, 'Ferragnes' nut and kernel weight were found to be 3.67 g and 1.19 g, respectively [53], while other studies showed that 'Ferragnes' nut and kernel weight were 4.3 g and 1.6 g, in 'Lauranne' 3.6 g and 1.2 g, and in 'Tuono' 4.3 g and 1.5 g, respectively [54]. Average kernel weight is an important parameter affected by tree yield [49]. Socias i Company et al., 2017 [55] indicated that the range of kernel weight varies between 0.5 and 1.5 g, with those that exceed 1.2 g being preferred for most uses. They also commented that the general trend in the industry is the preference for large kernels in order to facilitate cracking and blanching [55]. In our study, all the cultivars, including 'Mars', showed relatively high kernel weights, while 'Mars' nut and kernel weights exceeded those of the other self-compatible cultivars, 'Lauranne' and 'Tuono'. Kernel shape, determined by the ratio of length/width, is characteristic of the cultivar and, together with linear dimensions, is a major commercial trait. Industry needs different sizes of kernel for manufacturing, processing, and marketing operations [56]. Maldera et al., 2021 [57] found that the kernel percentage for 'Tuono' and 'Lauranne' was 36% and 38%, respectively, while in our study they were measured at 42% and 33.5%, respectively.

Shelling is used to obtain a quantitative measure of shell density and is utilized commercially to calculate the kernel yield of different cultivars [49]. Socias i Company et al., 2008 [56] classified shelling proportions, according to shell hardness, from 10 to 30% for very hard shells, 30 to 50% for hard shells, and 50 to 70% for soft shells. All the cultivars examined in the current study were characterized as having a hard shell, and therefore kernel percentages belong to the above-proposed range of 30–50%. Double kernels were not observed in 'Ferragnes' and 'Lauranne', while 'Mars' and 'Tuono' presented 9.2 and 20% double kernels, respectively. Maldera et al., 2021 [57] found that 'Tuono' had a significantly higher double kernel percentage than 'Lauranne' (14.7% and 2.33%, respectively). Lovicu et al., 2002 [54] found that the double kernel percentage for 'Ferragnes' was 0.3%, for 'Lauranne' 0%, and for 'Tuono' 26.3%. Double kernel occurrence is considered a negative trait that may be characteristic of the variety, may be affected by conditions that favor

pollination and fruit set, or could be affected by lower than normal temperatures before or during blooming [58]. The concentric layers observed in 'Mars' shell are also considered a negative trait from an industrial point of view, as they may cause repetition of cracking, leading to kernel breakage and decreased nut quality.

The chosen approach for the verification of the observed self-compatibility trait of 'Mars', was the PCR amplification of the S_f allele. This technique is the only molecular method described so far in literature and has been implemented in the self-compatible cultivars 'Supernova', 'Tuono' [18], 'Soleta' [23], and the self-incompatible 'Ferragnes' [34], 'Cristomorto' [59], 'Nonpareil', and 'Texas' [60]. The application of the Marchese et al., 2008 [18] protocol in the samples examined in this research verified the absence of the S_f allele from 'Ferragnes', 'Ferraduel', and 'Texas' cultivars, as they are known self-incompatible cultivars, and its presence in the self-compatible cultivars 'Tuono' and 'Lauranne'. The detection of the S_f allele in the 'Mars' genotype verifies its self-compatibility trait and suggests that the 'Mars' paternal cultivar was self-compatible, possibly 'Tuono' or 'Lauranne'.

The PCR products obtained by the primers Pru_C2–Pru_C5 were in accordance with the *S*-genotype of each cultivar. As these primers amplify only the alleles S_1, S_7, S_8, and S_f, it was expected to observe one PCR product in the cultivars that have the S_1 allele, meaning 'Ferragnes' (S_1S_3), 'Ferraduel' (S_1S_4), 'Texas' (S_1S_5), or the S_f allele, meaning 'Lauranne' (S_3S_f), and two products in 'Tuono' (S_1S_f). The fact that two products were observed in 'Mars', in the same size as 'Tuono' is a suggestion that 'Mars' genotype is also S_1S_f. This was verified by sequencing of the PCR products, which confirmed that all products at 1 kb were the S_1 allele, while the PCR products at 1.1 kb were the S_f allele.

The analysis of all the samples using SSR markers revealed a unique profile for each genotype, resulting in their clear discrimination. The 'Mars' genetic profile, as generated by the eight SSR markers of this study, showed differences in eight out of the sixteen loci amplified compared with 'Tuono' and 'Lauranne' cultivars, verifying that it is a different self-compatible genotype. 'Ferragnes' (S_1S_3) and 'Ferraduel' (S_1S_4) were in close genetic proximity in the phylogenetic tree (Figure 7) as anticipated, since they come from the same cross, 'Cristomorto' × 'Ai', as mentioned before. 'Lauranne' (S_3S_f) comes from a cross between 'Ferragnes' and 'Tuono' (S_1S_f) and was placed closer to 'Ferragnes', while 'Texas' (S_1S_5) formed a completely distinct branch since it is a cultivar developed in the U.S. as a selection of an early American cultivar known as Languedoc, originally coming from France [31]. 'Tuono' has been used in breeding schemes for the production of self-compatible cultivars [25], so its genetic proximity with the 'Mars' genotype implies that it was the pollinator of 'Ferragnes' for the production of 'Mars'. The cross 'Ferragnes' × 'Tuono' has given rise to numerous self-compatible almond cultivars, like 'Steliette', 'Cambra', 'Antoneta', 'Marta' in Spain, 'Lauranne', and 'Mandaline' in France, which show differences in shell hardness, ripening time, and other morphological characteristics [26,28]. Interestingly, a recent study suggested that 'Tuono' has been the founding genotype for self-compatible cultivars, with 24.7% of the total genetic contribution to modern cultivars [26].

The occurrence of two unique alleles in 'Mars' may be attributed to differences in 'Tuono' genotype between the tree that actually pollinated 'Ferragnes', leading to 'Mars' production, and the tree that was sampled for this analysis. As mentioned before, 'Tuono' was first introduced in Greece in the 1970s, and the crossing event with 'Ferragnes' dates back to the 1980s. The trees that could have been 'Ferragnes' pollinators are no longer available; thus, the 'Tuono' sample for this analysis was taken from a tree grown by a nursery. It is possible that these two trees exhibit discrepancies in some loci, causing the differences we observed. This phenomenon has been reported in a study where an SSR analysis of two different 'Tuono' accessions, one from Puglia and one from Sicily, indicated a difference of two base pairs in one of the nine markers used between the two accessions [18]. This was attributed to intra-cultivar variation as a result of somatic mutation, suggesting that this type of difference may be noted between different 'Tuono' trees.

The use of SSR markers for the genotyping of almond varieties and their relatives is well established in the literature [15–18,61–68]. The great sample variability in these

reports does not allow a comparison of diversity indices with the values of the current study. Regarding the six samples analyzed here, it could be mentioned that the fact that the observed heterozygosity was higher than the expected heterozygosity might indicate an isolate-breaking effect where two previously isolated populations were mixed [47].

Six of the eight markers used in this study were also used in Xu et al., 2004 [15], in combination with one more. The size range of the alleles generally agrees between the studies, with the exception of the UDP96008 marker. In Xu et al., 2004, the markers employed failed to distinguish 'Ferragnes' from 'Ferraduel', which was accomplished in our study. Xie et al., 2006 [16] also failed to distinguish these two cultivars using 16 SSR markers, three of them in common with our study. In a study of Turkish almond cultivars, the phylogenetic relationship of 'Ferragnes' and 'Ferraduel' was in accordance with our findings, being depicted in the same branch [62]. To capture all information derived from SSR fragments, the PCR products were analyzed in an automated capillary electrophoresis system. Studies have shown that the amount of data obtained using automated detection systems exceeds that obtained using the conventional method of agarose gel electrophoresis, eliminating factors affecting the results originating from gel preparation, imaging, and analysis as well as the subjectivity of each user at scoring [69].

5. Conclusions

This research provides a useful protocol, implementing well-established and highly accurate molecular techniques that could assist researchers and producers in identifying the phylogenetic background and self-compatibility trait of any almond tree without the need for field observations by an expert. The new 'Mars' selection was identified as a cross between 'Ferragnes' and 'Tuono' and as a self-compatible genotype. Although phylogenetically it is closer to 'Tuono', its interesting agronomic characteristics were found closer to 'Ferragnes'. The quality of the producing nuts, compared with their self-compatibility, could lead to the replacement of other almond cultivars with lower marketability. Further evaluation of the stability of the agronomic traits and productivity in subsequent years and under different environmental conditions, as well as quality/nutritional aspects, disease sensitivity, and post-harvest handling, needs to be assessed before the commercial cultivation of the new 'Mars' selection and its acceptance by the almond market.

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