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Exploring the Genetic and Morphological Variation and Disease Resistance in Local and Foreign *Prunus persica* (L.) Batsch Cultivars

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Abstract: Local germplasm may be the basis for genetic improvement for sustainability and resilience, yet little is known about Greek peach [*Prunus persica* (L.) Batsch] local genetic resources. The aims of the present study were to entail a prospection in the mainland and islands of Greece for local traditional and underutilized germplasm and study their genetic and morphological variation and susceptibility to shoot blight from *Cylindrocarpon destructans* and *Monilinia laxa*. A total of 32 peach cultivars/accessions were prospected from the mainland (Imathia and Magnesia) and islands (Andros, Ikaria, Kythira, Lesvos and Samos) of Greece. Leaf and fruit morphological characterization was made in situ and ex situ using 42 qualitative and quantitative morphological descriptors. Nine clones of 'Lemonato', isolated in Magnesia, with differing ripening times, were found to vary in leaf and fruit phenotypic traits. The local peach genotypes were separated from 12 old foreign cultivars, in 8 out of the 42 leaf and fruit phenotypic traits studied. We observed greater lesion damages from *C. destructans* than *M. laxa*. Local cultivars had greater resistance to *Cylindrocarpon destructans* than foreign cultivars, with 'Lemonato Andrea', 'Daggalakou' and 'Papagianni' exhibiting the greatest resistance. Genetic characterization was performed in the studied local and foreign peach cultivars/accessions, using eight Inter Simple Sequence Repeats (ISSRs), resulting in a total of 404 bands. Analysis of molecular variance and principal coordinates analysis revealed moderate to low genetic diversity among the peach cultivars, and three distinct clusters were formed. Furthermore, multiple regression analysis was implemented for the association study between morphological traits and the ISSR markers, revealing several markers that are statistically and significantly correlated with fruits' traits. The obtained results could be valuable for breeding programs and future research on peach.

Keywords: *Cylindrocarpon destructans*; genetic resources; inter simple sequence repeat; leaf and fruit characters; 'Lemonato' peach; *Monilinia laxa*

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1. Introduction

The peach [*Prunus persica* (L.) Batsch] tree is believed to originate in East and South-East Asia and spread through Europe along trade routes through Persia in the Ancient and Middle Ages [1]. In the early peach cultivation, the farmers isolated and propagated the chance seedlings, resulting in a large number of different ecotypes and forms adapted to diverse edaphic and climatic conditions in each country. A dramatic varietal improvement was achieved by the modern breeding programs with controlled crosses initiated in early US breeding programs and later in the second half of the 20th century in European breeding programs [2]. This resulted in a rapid replacement of landraces and local accessions and their consequent loss. The genetic narrowing in peach further occurred since the modern cultivars shared a few common ancestors [3,4]. Awareness of genetic erosion in modern plant breeding is rising, thus it is important to protect and exploit the local genetic diversity.

Little is known about Greek peach local genetic resources [5,6]. A traditional peach cultivar is 'Lemonato', being a series of clones separated by the growers in Magnesia, central Greece. It has been cultivated since the beginning of the 19th century mainly in mountainous areas of Western Pelion and it was after 1957 it was spread to more low-elevation areas [5]. In different islands of Greece, the white flesh peach 'Breasts of Venus' has been documented with reference in the end of 19th century [7,8].

Moreover, in the main peach producing areas Imathia and Pella (with >90% of total peach growing), northern Greece, in an area that commercial orchards were initially established after the liberation from the Ottoman occupation in 1912, and mainly after the Second World War [9], many white and yellow flesh local cultivars are known. Local peach cultivars commercially grown nowadays is 'Opsimo Naoussas', a yellow peach cultivated in 6.8 hectares [10] separated for having the slow-ripening trait, similar to 'Big Top' and 'Royal Gem' [6]. Moreover, the 'Papagianni' (white-fleshed peach) and 'Princess Anne' (yellow-fleshed peach) are commercially grown in Imathia (6.2 and 3.7 hectares, respectively). Little is known on the origin of local cultivars. The peach 'Papagianni' is known to have been a chance seedling of an old cultivar named 'Doukissa' and spread after 1980. In situ phenotypic observations on morphological traits are useful for preliminary evaluation of germplasm and can be used as a first approach for assessing genetic diversity and cultivar improvement. As a second step, the selection and establishment of an ex situ collection could serve effectively to characterize germplasm under the same environmental conditions and serve as a comparison for local and introduced genetic material.

The use of resistant cultivars is the most important mean to control damages from biotic stresses, taking into account that fungicide resistant fungus isolates appear, the mish of available fungicides and the economic and environmental need to reduce sprays. An important environmentally friendly method for plant disease management is host specificity. The fungi of genus *Monilinia* is a common pathogen in stone fruit species, causing shoot blights and fruit rots [11–14]. Information for variable phenotyping susceptibility in different cultivars or segregation progenies to *Monilinia* in peach or other stone fruit is previously reported [15–17]. Little is known on the importance of *Cylindrocarpon destructans* as a fungus causing shoot blight on peach trees [18]. This fungus is reported to cause damages in fruit trees of the Malus genus [19,20]. Numerous methods have been developed to evaluate fruit trees for resistance to wound pathogens. In the field, cultivars are frequently tested for resistance to wood pathogens using the stem inoculation method.

Peach is one of the most described species within the Rosaceae family in terms of genetics. The emergence of the genomic era has brought about new possibilities in the improvement of cultivars through molecular breeding and genetic engineering. Molecular markers could facilitate the identification of genomic regions or even genes responsible for important agronomic traits, whether caused by a single gene or multiple genes. These markers are beneficial for marker-assisted selection (MAS) as they help to reduce the time and effort required to produce exceptional genotypes by allowing the analysis of many offspring at an earlier stage. Peach linkage maps have identified numerous traits, making MAS possible for this particular species as well [21]. So far, at least 28 morphological genes of various Prunus species have been identified on a single map [22]. These genes regulate important fruit traits influencing the peach industry (peach vs. nectarine, flat vs. round fruit, clingstone vs. freestone pit, melting vs. non-melting flesh, subacid vs. acid flavor and yellow vs. white flesh), tree architecture (columnar vs. normal tree), disease resistance (root-knot nematode, powdery mildew and brown rot), flower morphology (non-showy/showy) and fruit development causing early abortion [23–28].

Segregation of the local cultivars for their phenotypic traits would provide an insight into the local variability [29]. The combination of phenotypic and molecular characterization is extremely important for peach cultivar identification and relatedness in order to ensure better knowledge of Greek cultivars, since these have been inadequately described until now. Similar studies have been performed on foreign cultivars using several types of markers: Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) [30], Restriction Fragment Length Polymorphism (RFLP), isozymes [31], Simple Sequence Repeats (SSR) [32,33], Expressed Sequence Tag-derived Simple Sequence Repeat (EST-SSR) [34], and Single Nucleotide Polymorphisms (SNP) markers [35].

ISSR markers, compared to other categories of molecular markers, such as SSR or RAPD, are simple to use (do not require prior sequence information) and capable of producing a high number of polymorphisms, especially when studying closely related individuals, which exhibit low levels of polymorphism [36].

Today, the peach market has very demanding objectives in terms of fruit quality characteristics, while the increase in production costs and climate change are challenges to deal with. Exploring the local biodiversity facilitates the creation of advanced and resilient cultivars, and the incorporation of molecular, morphological and biochemical data offer an integrated approach to determine the genetic diversity [37–41]. Furthermore, evaluating the resistance to stress conditions could contribute to the creation of resilient varieties. The purpose of this work was to entail a prospection for local peach germplasm in the mainland and islands of Greece and study their variability in genetic, leaf and fruit phenotypic traits and resistance to shoot blight diseases. ISSR markers were implemented to determine the genetic diversity and relatedness among Greek genotypes and some important old foreign cultivars.

2. Materials and Methods

2.1. Plant Material

A series of expeditions were made in the summer periods of 2018–2021 in the islands Andros (Cyclades), Kythira (Attika), Ikaria, Samos and Lesvos (Northern Aegean) and the mainland of Imathia (Central Macedonia) and Magnesia (Thessaly) in Greece (Figure 1; Table 1). A total of 32 peach cultivars/accessions were selected from farms, home yards or already maintained at the Department of Deciduous Fruit Trees in Naoussa. A selection was considered when there was information that it was grown for at least two generations, while those selected in Imathia may have been spots from old foreign cultivars. Thirteen old foreign cultivars were also studied for comparison. Where there was no local denomination, the accessions were named after the area, village or the owner where they had been collected from.

Figure 1. Geographic locations of the prospected plant material.

Table 1. Name, location, flesh type and flesh color in 32 local peach cultivars/accessions prospected from various areas in Greece, and 13 old foreign peach cultivars grown in an ex situ collection. MPE, melting peach; NMPE, non−melting peach; NE, nectarine; YE, yellow; W, white.

Name	Location	Cultivar/Accession	Flesh Type	Flesh Color
Psychakis	Andros	Accession	MPE	YE
Mikros giarmas (July peach)	Ikaria	Accession	MPE	YE
Petrorodakino Kataphygi	Ikaria	Accession	MPE	YE
Andross late	Imathia	Selection	NMPE	YE
Catherina late	Imathia	Selection	NMPE	YE
Evert selection	Imathia	Selection	NMPE	YE
PI-A37	Imathia	Selection	NMPE	YE
PI-A39	Imathia	Selection	NMPE	YE
PI-IB42	Imathia	Selection	NMPE	YE
Daggalakou	Imathia	Cultivar	MPE	YE
Opsimo Naoussas	Imathia	Cultivar	MPE	YE
Princess Anne	Imathia	Cultivar	MPE	YE
Prophet Ilias	Imathia	Cultivar	MPE	YE
Kaliga	Imathia	Cultivar	MPE	W
Papagianni	Imathia	Cultivar	MPE	W
Queen of October	Imathia	Cultivar	MPE	W
Strantza	Imathia	Accession	MPE	YE
Afrodite's breast	Kythira	Cultivar	MPE	W
Kythiron yellow	Kythira	Accession	MPE	YE
Charazani	Lesvos	Accession	MPE	YE
Lemonato Afrodite	Magnesia	Accession	MPE	W
Lemonato Andrea	Magnisia	Accession	MPE	W
Lemonato August-1	Magnisia	Accession	MPE	W
Lemonato August-2	Magnisia	Accession	MPE	W
Lemonato Kountoupi	Magnisia	Accession	MPE	W
Lemonato late	Magnisia	Accession	MPE	W
Lemonato lemon	Magnisia	Accession	MPE	W
Lemonato medium	Magnisia	Accession	MPE	W
Lemonato very early	Magnisia	Accession	MPE	W
Magiatiko	Samos	Accession	MPE	YE
Pandrosou	Samos	Accession	MPE	YE
Sourliotiki	Samos	Accession	MPE	YE
Fantasia	Foreign	Cultivar	NE	YE
Andross	Foreign	Cultivar	NMPE	YE
Catherina	Foreign	Cultivar	NMPE	YE
Evert	Foreign	Cultivar	NMPE	YE
Romea	Foreign	Cultivar	NMPE	YE
Early May Crest	Foreign	Cultivar	MPE	YE
Fayette	Foreign	Cultivar	MPE	YE
Golden Jubilee	Foreign	Cultivar	MPE	YE
H.D. Hale	Foreign	Cultivar	MPE	W
June Gold	Foreign	Cultivar	MPE	YE
Maria Bianca	Foreign	Cultivar	MPE	W
Red Haven	Foreign	Cultivar	MPE	YE
Spring crest	Foreign	Cultivar	MPE	YE

A total of 19 local genotypes together with 12 foreign peach and nectarine cultivars were propagated vegetatively using GF677 rootstock and established in an ex situ collection at the Department of Deciduous Fruit Trees in Naoussa, Greece (40°37′13.40″ N; 22°06′59.80″ E, at 119 masl) in 2019 (Table 2). Cultivars/genotypes were planted with a spacing of 5 × 3 m in soil with a medium–heavy mechanical composition and a neutral pH (pH 7.4). Trees were managed according to an integrated management system.

Table 2. Cultivar names, ripening day (Julian day), fruit fresh weight (g), soluble solid content (°Brix), titratable acidity (g malic acid 100^{-1} mL juice) and ripening index (soluble solid content/titratable acidity) in fruit from 19 local and 12 foreign peach and nectarine cultivars harvested from an ex situ collection at the Department of Deciduous Fruit Trees in Naoussa. Mean values for the local and foreign cultivars are presented. CV%, coefficient variation; LSD, least significant difference.

Cultivar	Ripening Day	Fruit Fresh Weight	Soluble Solid Content	Titratable Acidity	Ripening Index
Local					
Lemonato very early	184	178.7	12.3	0.7	18.3
Lemonato Andrea	202	208.7	11.6	1.2	9.8
Kalliga	205	224.7	12.3	1.5	8.0
PI-A37	206	173.1	10.7	1.1	9.5
Papagianni	209	359.2	10.8	1.1	9.5
Lemonato medium	213	303.6	11.6	0.8	14.2
PI-A39	218	174.6	12.0	0.7	16.6
PI-IB42	223	203.2	10.5	0.5	19.2
Daggalakou	225	337.1	14.0	0.9	14.9
Lemonato August-2	229	177.0	15.2	0.7	22.6
Princess Anne	231	238.6	14.9	0.7	20.6
Andross late	234	192.3	10.8	0.6	17.6
Lemonato Kountoupi	238	203.7	15.6	0.7	22.6
Opsimo Naoussas	238	274.5	12.5	0.6	20.6
Lemonato late	241	235.3	13.4	0.6	23.5
Evert selection	242	196.5	12.5	0.6	19.6
Prophet Ilias	245	268.1	16.5	0.9	17.7
Lemonato Afrodite	247	171.7	15.8	0.6	25.1
Queen of October	265	222.9	11.9	0.6	21.0
Foreign					
Spring crest	160	137.5	9.1	1.2	7.9
June Gold	176	255.0	12.9	1.2	10.8
Red Haven	182	252.0	10.3	1.2	8.9
Maria Bianca	189	275.0	11.3	1.0	10.8
Romea	189	158.6	11.5	0.5	21.3
Golden Jubilee	194	253.3	11.6	1.2	9.6
Catherina	195	179.1	13.1	0.4	30.3
Andross	218	292.8	11.8	0.7	16.1
Fantasia	221	184.3	16.6	0.6	27.1
H.D. Hale	225	198.9	14.3	0.7	19.1
Fayette	233	147.6	11.5	0.8	15.0
Evert	243	171.7	10.0	0.7	14.6
Mean	217	220.9	12.5	0.8	16.9
CV%	11	25	16	32	35
LSD		48.8	1.9	0.3	6.1

2.2. Leaf and Fruit Morphological Characterization

The trees were evaluated for 35 morphological traits, which included 10 leaf descriptors and 25 fruit descriptors, recommended by CPVO TP14/1 (Table 3). In the 12 foreign cultivars, leaf morphometric characteristics were evaluated ex situ for 2–3 years, and for the 32 local cultivars/accessions, leaf characterization was made in situ and repeated for the 19 cultivars/accessions grown in the ex situ collection.

The leaf blade length (LBL), leaf blade width (LBW) and stalk length (SL) were measured using a caliper. The ratios LBL/LBW and LBL/SL were calculated. The above leaf dimensions, despite their quantitative nature, were transformed into a qualitative ordinal variable with three classes owing to the lack of accuracy in its measurement: LBL (small < 14.5 cm, medium = 14.6–17.2 cm and long > 17.3 cm), LBW (narrow < 3.4 cm, medium = 3.5–4.4 cm and large > 4.5 cm) and SL (small < 0.9 cm, medium = 1.0–1.2 cm add long > 1.3 cm). The leaf morphological measurements were made in 5 fully developed leaves collected from the middle part of the previous year's grown shoots.

Table 3. List of leaf and fruit phenotypic traits measured in local and foreign peach and nectarine cultivars. Mean values, percentage coefficient variation (CV%) and *p* values are presented.

		Local		**Foreign**		
		Mean	**CV%**	**Mean**	**CV%**	***p***
Leaf blade: Length	3—short; 5—medium; 7—long	5.8	24	4.2	25	0.001
Leaf blade: Width	3—narrow; 5—medium; 7—large	5.6	27	5.5	23	0.802
Leaf blade: Ratio length/width	3—small; 5—medium; 7—large	5.1	9	3.8	27	0.000
Leaf blade: Shape in cross section	1—concave; 2—flat; 3—convex	1.5	52	2.1	43	0.054
Leaf blade: Angle at base	1—acute; 2—approximately right angle 3—obtuse	1.4	36	1.4	36	0.982
Leaf blade: Angle at apex	3—small; 5—medium; 7—large	4.3	28	3.7	27	0.159
Leaf: Red mid-vein on the lower side	1—absent; 9—present	1.8	137	3.0	121	0.302
Petiole: Length	3—short; 5—medium; 7—long	5.5	27	5.5	23	0.959
Petiole: Shape of nectaries	1—round; 2—reniform	1.6	32	1.6	42	0.984
Petiole: Predominant number of nectaries	1—two; 2—more than two	1.7	26	1.2	49	0.005
Fruit: Size	1—very small; 3—small; 5—medium; 7—large; 9—very large	5.9	23	6.5	19	0.272
Fruit: Shape (in ventral view)	1—broad; oblate; 2—oblate; 3—round; 4—ovate; 5—elliptic	3.2	30	3.2	30	0.902
Fruit: Shape of pistil end	1—prominently; pointed; 2—weakly pointed; 3—flat; 4—weakly depressed; 5—strongly depressed	2.1	57	2.7	49	0.185
Fruit: Symmetry (viewed from pistil end)	1—asymmetric; 2—symmetric	1.4	36	1.4	36	0.982
Fruit: Prominence of suture	3—weak; 5—medium; 7—strong	4.9	32	4.8	28	0.911
Fruit: Depth of stalk cavity	3—shallow; 5—medium; 7—deep	6.6	13	5.7	17	0.010
Fruit: Width of stalk cavity	3—narrow; 5—medium; 7—broad	5.0	27	5.3	27	0.515
Fruit: Ground color	1—green; 2—cream; green; 3—greenish white; 4—cream white; 5—cream; 6—pink white; 7—greenish yellow; 8—cream yellow; 9—yellow; 10—orange yellow	6.3	54	7.9	33	0.159
Fruit: Overcolor	1—absent; 9—present	6.9	52	7.7	41	0.547
Fruit: Hue of overcolor	1—orange red; 2—pink; 3—pink red; 4—light red; 5—medium red; 6—dark red; 7—blackish red	3.5	73	4.8	48	0.190
Fruit: Pattern of overcolor	1—solid flush; 2—striped; 3—mottled; 4—marbled	1.9	58	3.2	32	0.011
Fruit: Extent of overcolor	1—very small; 3—small; 5—medium; 7—large; 9—very large	2.8	93	4.7	54	0.056
Fruit: Density of pubescence	1—very sparse; 3—sparse; 5—medium; 7—dense; 9—very dense	6.9	15	6.6	40	0.644
Fruit: Adherence of skin to flesh	1—absent; or; very; weak; 3—weak; 5—medium; 7—strong; 9—very; strong	5.3	26	5.3	22	0.971

Table 3. *Cont.*

		Local		Foreign		
		Mean	**CV%**	**Mean**	**CV%**	***p***
Fruit: Firmness of flesh	1—very soft; 3—soft; 5—medium; 7—firm; 9—very firm	6.3	22	6.8	26	0.326
Fruit: Ground color of flesh	1—greenish white; 2—white; 3—cream white; 4—light yellow; 5—yellow; 6—orange yellow; 7—orange; 8—red	3.7	58	5.1	34	0.081
Fruit: Anthocyanin directly under skin	1—absent or very weakly expressed; 2—weakly expressed; 3—strongly expressed	1.1	29	1.5	60	0.090
Fruit: Anthocyanin in flesh	1—absent or very weakly expressed; 2—weakly expressed; 3—strongly expressed	1.3	44	1.4	56	0.686
Fruit: Anthocyanin around stone	1—absent or very weakly expressed; 2—weakly expressed; 3—strongly expressed	1.7	50	1.5	53	0.453
Fruit: Texture of the flesh	1—not fibrous; 2—fibrous	1.8	20	2.0	0	0.158
Stone: Size compared to fruit	3—small; 5—medium; 7—large	4.9	25	4.8	28	0.897
Stone: Shape (in lateral view)	1—oblate; 2—round; 3—elliptic; 4—obovate	3.3	20	2.8	31	0.050
Stone: Intensity of brown color	3—light; 5—medium; 7—dark	6.3	16	5.7	27	0.201
Stone: Relief of surface	1—small pits; 2—large pits; 3—grooves; 4—pits and grooves	3.9	10	3.8	10	0.444
Stone: Degree of adherence to flesh	3—weak; 5—medium; 7—strong	5.7	29	5.3	35	0.536
Ripening day	Julian day	226	9	202	13	0.006
Fruit fresh weight	g	228.6	25	208.8	26	0.339
Soluble solid content	°Brix	12.9	15	12.0	17	0.260
Titratable acidity	g 100 mL^{-1}	0.8	33	0.9	33	0.590
Ripening index	Soluble solid content/Titratable acidity	17.4	30	16.0	45	0.540
Cylindrocarpon destructans	Shoot lesion in mm	32.5	16	37.9	20	0.005
Monilinia laxa	Shoot lesion in mm	11.6	29	11.9	22	0.687

The fruit fresh weight (FFW) was measured. The soluble solid content (SSC) and total acidity (TA) were determined from juice extracted using a food processor in three replicates for four peaches. The SSC was determined using a digital refractometer (model PR-1, Atago, Japan) and expressed as °Brix. TA was measured using an automatic titrator (Titrometic 25; Crison Instruments SA, Barcelona, Spain) and determined by titrating 5 mL of juice with 0.1 N NaOH to a pH end point of 8.2. Results were expressed as g malic acid 100 mL^{-1}. Ripening index was calculated as the SSC/TA ratio. The fruit morphological traits were measured on a 15-fruit sample per cultivar harvested at the commercial stage based on size, color and firmness.

2.3. Susceptibility to Cylindrocarpon destructans and Monilinia laxa

An isolate of *C. destructans* from the shoot of cherry trees with canker symptoms, and of *M. laxa* from the shoot of peach trees with blossom blight symptoms were used. The fungi were identified by using several taxonomic keys [42,43] and the internal transcribed spacer (ITS) region of ribosomal DNA with the universal primers ITS1 and ITS2 (QIAGEN DNA Mini Kit, HB-1166, Hilden, Germany).

Experiments were conducted in 3- and 4-year-old trees from the ex situ collection of peach and nectarine cultivars described in Section 2.1. Annual shoots from peach and nectarine cultivars were inoculated by removing a 6 mm strip of bark, exposing the cambium, and placing a mycelium disk of *M. laxa* or *C. destructans* directly on each wound. Following that, petroleum jelly was applied to the wound and adhesive tape was used to close it. There were 20 annual shoots for each peach cultivar, 10 for each fungus tested.

The results were collected by scraping the bark and measuring the resulting canker 40 days after inoculation. The fungi were recovered from the margin of the canker by isolating on potato dextrose agar.

Inoculations were made in September 2021 and 2022 so that meteorological parameters were favorable for the mycelia growth; the mean air temperature was 21.4 °C and 20.7 °C, mean relative humidity was 63.6% and 59.3%, and total rainfall was 41 and 69 mm, respectively.

2.4. Molecular Analysis

Molecular analysis was made in 30 local and 13 foreign peach and nectarine cultivars. DNA extraction from fresh leaves was performed according to the CTAB (cetyl trimethylammonium bromide) protocol as described by Doyle and Doyle [44]. DNA quality evaluation and quantification was performed by using a Quawell UV-Vis Spectrophotometer (Q5000). Eight ISSR molecular markers (UBC811-UBC823-UBC826-UBC827-UBC834-UBC841-UBC873-UBC880) were selected after a systematic review of the available scientific literature for the genotypic characterization of the peach cultivars. PCR amplifications for the ISSR markers were performed in 20 µL reaction volumes containing 20 ng of genomic DNA, 1× PCR buffer, 0.4 µM of primer, 0.2 mM of DNTPs and 1 U of Kapa Taq polymerase. PCR reactions for each ISSR marker were performed using a SureCycler 8800 thermocycler (Agilent Technologies, Santa Clara, CA, USA) under the cycling profile: first step at 94 °C for 4 min, followed by 35 cycles segmented in 30 s at 94 °C, 30 s at varied temperature based on primers (Table 4) and 40 s at 72 °C and a final extension at 72 °C for 7 min. PCR products were visualized by ethidium bromide stained 1.5% agarose gels in 1× TAE buffer. A 1000 bp DNA ladder was used as a molecular weight size marker in each gel.

Table 4. Details of ISSR used, number of different alleles (Na), number of effective alleles (Ne), Shannon's Information Index (I), gene diversity (GD), polymorphism information content (PIC) and resolving power (Rp) from 43 peach cultivars/accessions.

Primer	Annealing Temperature (°C)	Fragment Size Range (bp)	Na	Ne	Shannon Index (I)	Gene Diversity (GD)	PIC	Rp
ISSR (UBC)								
811	58	350–1700	1.860	1.726	0.574	0.399	0.360	4.065
823	49	500–2500	0.930	1.233	0.238	0.154	0.356	5.721
826	54	500–1800	0.953	1.363	0.272	0.191	0.346	6.465
827	47.5	600–2000	0.977	1.426	0.318	0.225	0.350	7.116
834	47.5	380–1800	2.000	1.894	0.661	0.469	0.349	4.140
841	51.5	230–2050	2.000	1.865	0.643	0.454	0.363	6.605
873	51.5	650–1700	0.953	1.240	0.241	0.156	0.380	5.442
880	54.5	280–1700	2.000	1.905	0.663	0.471	0.404	3.860
Mean			1.459	1.582	0.451	0.315	0.364	5.427

2.5. Statistical Analysis

2.5.1. Phenotypic Data

Means were used for the statistical analysis, including the descriptive statistic and multivariate analysis. For descriptive traits, variation was assessed by frequency distribution and expressed as a percentage. For metric traits, the following parameters were calculated: mean, minimum and maximum value, maximum/minimum ratio, standard deviation and the coefficient of variation (CV%). The data were subject to one-way analysis of variance (ANOVA) with local and foreign cultivars as the treatment. Linear correlation analysis was performed.

The data were also subjected to multivariate analysis using principal component analysis (PCA) and cluster analysis. PCA was used to study the patterns of variation in a set of interrelated traits through the identification of sub-sets of these traits, called factors. As a criterion to extract the main principal components, an eigenvalue greater than 1 was taken, and to determine which PC scores had the greatest contribution to variation, the eigenvalues of these components were compared for each trait. Cluster analysis used to evaluate the relationships among the accessions was conducted using Ward's method using the Euclidean distances. Statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

2.5.2. Molecular Data

The bands of DNA fragments generated by ISSR were scored and coded as 1 for the presence of a band in specific loci or 0 for the absence of a band. All data were further analyzed with an applicable Excel add-in that is specialized in the genetic analysis, GenAlEx 6.5 (Australian National University, Cambera, AU, Australia) [45]. For each group of peach cultivars, band frequencies and genetic distance matrices were calculated along with further genetic analyses that included: Analysis of Molecular Variance (AMOVA) and Principal Coordinates Analysis (PCoA). The genetic distance matrix was used for the construction of the Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree with MEGA X (Pennsylvania State University, State College, PA, USA) [46].

ISSR markers' information on the number of different alleles (Na), number of effective alleles (Ne), Shannon's Information Index (I) and gene diversity (GD) were obtained by GenAlEx 6.5 [45], while polymorphism information content (PIC) and resolving power (Rp) were obtained by the online program Online Marker Efficiency Calculator (iMEC) [47] (Table 4).

To estimate the association between ISSR markers and the morphological traits, stepwise Multiple Regression Analysis (MRA) was performed [41], where every morphological trait was treated as a dependent variable and DNA fragments produced by ISSRs were treated as independent variables. The MRA analysis was performed using SPSS version 28.0.0 (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Prospections

In Imathia and Pella, seven local peach cultivars and one accession, with yellow ('Daggalakou', 'Opsimo Naoussas', 'Princess Anne', 'Prophet Ilias' and 'Strantsa') or white ('Kalliga', 'Papagianni' and 'Queen of October') flesh were isolated (Table 1). Three of the local cultivars were named after the grower who separated and propagated them ('Daggalakou', 'Kalliga' and 'Papagianni'). Considering that in the regions of Imathia and Pella (Central Macedonia, northern Greece) are traditionally the peach growing regions (with more than 90% of the country's production), starting from the beginning of the 20th century, the greater number of local cultivars and spots remaining until nowadays is probably related to the grower's interest for peach propagation material. In previous studies, the white-flesh peaches 'Agrio Giannakochoriou' and 'Giatsou' and the yellow-flesh peach 'Kontoni', all ripening in July, were described [48] (Tsipouridis, unpublished data), yet it was not possible to find them during the present prospections, indicating a loss of local genetic resources.

In Greece, a traditional peach cultivar is 'Lemonato' being a series of clones with different ripening times, separated by the growers in Magnesia, central Greece. 'Lemonato' is easily discriminated from other cultivars having green skin that turns into yellow when ripening with no or very little overcolor and a white and aromatic flesh. Nine selections of 'Lemonato' were separated; seven clones were prospected in Agios Vlasios, Magnesia, after being already separated by growers; and two clones were prospected from nurseries ('Lemonato medium' and 'Lemonato late'). The names of the selections were indicated either from the shape of fruit (e.g., lemon), time of ripening (early, medium or late) or the grower who isolated it (e.g., Andrea, Kountoupi). In Greece, the 'Lemonato' peach was been cultivated during the 19th century [5] when only white-fleshed peach was available.

Nine cultivar/accessions were isolated from the Greek islands: one from Andros (Cyclades), two from Ikaria (northern Aegean), two from Kythira (Attika prefecture), one from Lesvos and three from Samos (northern Aegean) (Figure 1; Table 1). From the above prospected plant material, previous information was available only for the white peach cultivars 'Breasts of Venus' grown in Kythira with reference to the end of 19th century [7,8]. During the expeditions, local germplasm of other deciduous fruit species was also collected, but the number of prospected peach local accessions was relatively small compared with that found for pear and plum (data not presented). The fact that peach is more prone to disease and pests than other fruit crops and that tourism has changed the resident priorities [49], may be related to the low number of prospected peach accessions from the islands studied.

3.2. Leaf Morphological Characterization

The local cultivars/accessions were separated from the foreign cultivars in 3 out of the 10 leaf phenotypic traits evaluated, having a more elongated leaf blade (32.3% vs. 0% cultivars with long leaf blade) and a higher number of nectaries (64.3% vs. 27.3%, respectively) (Table 3; Figure 2).

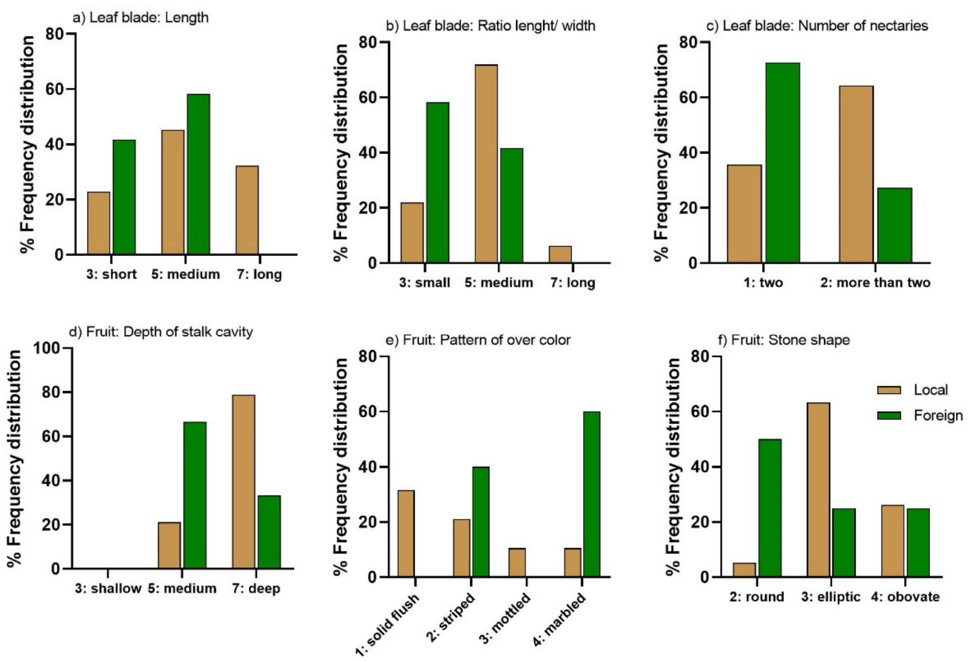

Figure 2. Frequency distribution of leaf: (**a**) length, (**b**) ratio length/width, and (**c**) number of nectaries, and fruit: (**d**) depth of stalk cavity, (**e**) pattern of over color, and (**f**) stone shape, traits in the local and foreign peach cultivars studied.

Results from a frequency distribution study made on leaf morphological traits, showed that the majority of the local peach had medium LBL (43.8%), LBW (50.0%) and ratio LBL/LFW (71.9%) (Supplementary Table S1). The leaf blade shape in cross section was usually concave (65.8%) and recurvature of the apex was present (90.6%). The angle of the leaf blade at the base was acute (59.4%) and the angle at the apex was medium (59.4%). There was no red mid-vein on the lower side in any genotype. The petiole length was long

(43.8%). Nectaries were present in 84.4% with a reniform shape in 56.3% and there were more than two in 56.3% of the local peach genotypes studied.

3.3. Ripening Time and Fruit Morphological Characterization

The local cultivars/accessions were separated from the foreign cultivars in 4 out of the 30 fruit-related traits evaluated in the present study (Table 3; Figure 2). Fruit from the local cultivars had later ripening (mean 226 vs. 202 Julian day) and a deeper stalk cavity (78.9% vs. 33.3% with deep stalk cavity), which may suggest it being more prone to fungal development as it may hold water after a rainfall. Moreover, in local cultivars, the skin overcolor pattern was more often characterized as solid flush and mottled and less as striped and marbled.

Regarding the fruit phenotypic traits studied, most local cultivars/accessions had medium-sized fruit (47.4%) with a round shape (52.6%) (Table S1). The shape of the pistil end was weekly pointed (42.1%) and the fruit was asymmetric (57.9%) with medium prominence of suture (42.1%) and deep stalk of cavity (78.9%). Fruit round shape without protruding tips and/or sutures are required by the markets and, therefore, fruit growers [50,51]. The fruit from peach 'Kalliga', 'Princess Anne' and 'Lemonato Late' was ovate or elliptic, which is a less favorable trait. Prominent pistil end was in 'Daggalakou', 'Kalliga', 'Princess Anne' and four 'Lemonato' clones, while prominence of suture was strong in 'Daggalakou' and four 'Lemonato' clones.

The fruit in most local cultivars had overcolor (73.7%) with a blackish-red hue (31.6%), solid flush (31.6%), small extent of overcolor (26.3%), dense pubescence (73.7%), medium adherence to stone (52.6%) and firm flesh (57.9%) (Table S1). Anthocyanin was usually absent or very weakly expressed directly under the skin (89.5%), flesh (73.7%) or around the stone (52.6%). The coefficient of variation in the studied traits was usually higher for the skin color parameters (hue, pattern and extent of overcolor) (Table 3) evaluated in the local compared with the variation observed for the foreign cultivars, probably driven by having cultivars without overcolor, such as 'Lemonato'.

The stone size compared to fruit was medium (57.9%), the shape was elliptic (63.2%), intensity of the blown color was dark (66.7%) and the relief of surface was with pits and grooves (84.2%) (Table S1). In most cultivars the stone was strongly adhered to flesh (57.9%).

In local compared with foreign cultivars the stone shape was more often elliptic and obovate, whereas in foreign cultivars it was round (Table 3; Figure 2). The lack of any difference in the fruit shape, but differences in the stone shape, between local and foreign peach cultivars suggests an unequal mesocarp growth. Similarly, in the study by Quilot et al. [52], peach cultivars with different domestication (*Prunus davidiana* compared with modern cultivars and hybrids) were studied and there was no difference in the fruit shape, but there were variations in the stone shape.

In the cultivars isolated in Imathia, ripening varied from late July (205 Julian Day (JD) in 'Kalliga') to late September (265 JD in 'Queen of October') (Table 2). Ripening in the 'Lemonato' clones spanned from early July (184 JD in 'Lemonato very early') to early September (247 JD in 'Lemonato Afrodite').

Soluble solid content was 10.8 °Brix in the table peach 'Papagianni' being close to 11 °Brix, which is considered as the minimum to be acceptable to consumers (<11 °Brix) [53]. In the non-melting peach cultivars 'PI-A37', 'PI-IB42' and 'Andross late', SSC was in the lower range (10.5–10.8 °Brix), yet it is a character with less importance for the canning industry where sugar is added to the final product.

In the 'Lemonato' clones, TA usually ranged from 0.6 to 0.8 g malic acid 100^{-1} mL, yet it was 1.2 g malic acid 100^{-1} mL only in the early ripening of 'Lemonato Andrea'. The ripening index (SSC/TA ratio) ranged from 8.0 ('Kalliga') to 25.1 ('Lemonato Afrodite'), being a key factor influencing the taste perception and consumer acceptance [53].

3.4. Susceptibility to Cylindrocarpon destructans and Monilinia laxa

In the present study, both *C. destructans* and *M. laxa* were pathogenic to peach trees, with *C. destructans* being more aggressive than *M. laxa* as suggested by greater lesion damages in the shoots (mean 35.2 vs. 11.8 mm, respectively; Table 3 and Figure 3). This is the first report on *C. destructans* causing pathogenic damages in the wood of peach trees, while only the species *C. obtusisporum* was previously identified as a pathogen causing stem canker in peach trees [18]. *C. destructans* is reported to cause damages in fruit trees of the Malus genus [19,20] and was also identified as the causal agent of fruit mold in apricot (*Prunus armeniaca*), peach (*P. persica*) and pear (*Pyrus communis*) during cold storage [54].

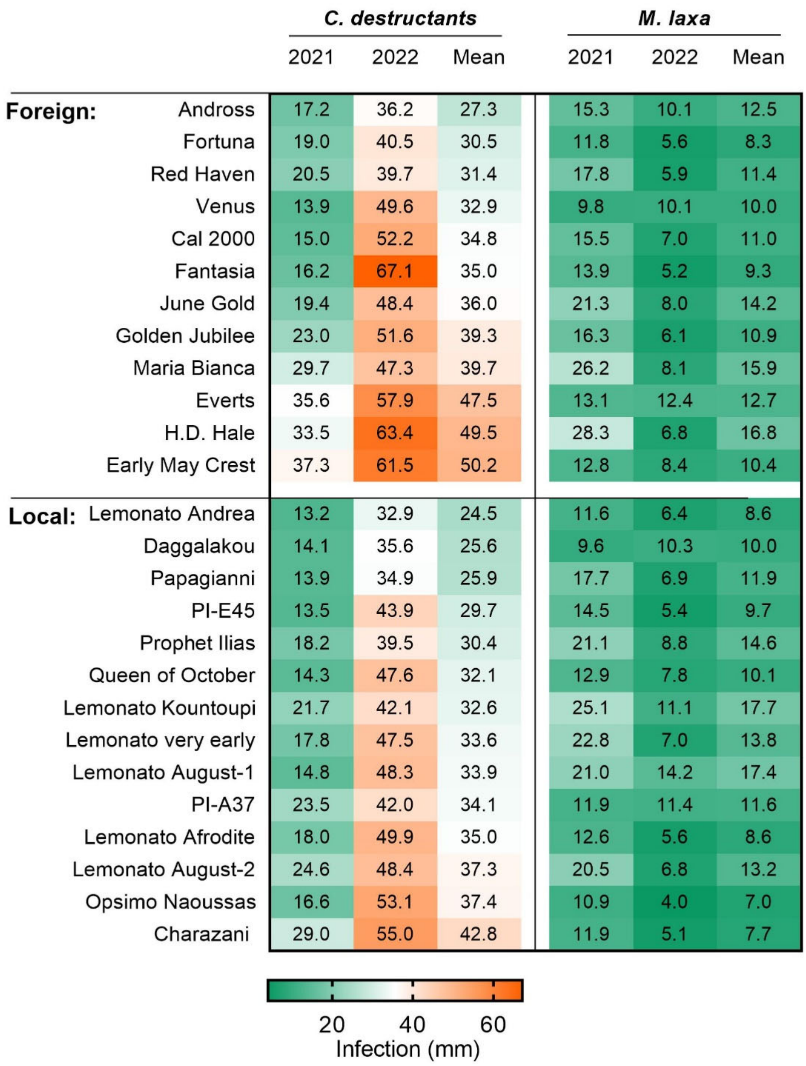

Figure 3. Length of lesion (mm) in one-year-old shoots after inoculation with *C. destructants* and *M. laxa* in different peach local and foreign cultivars evaluated in 2021 and 2022. $LSD_{C.\ destructants} = 8$; $LSD_{M.\ laxa} = 5.3$.

Damages from *C. destructans* in shoots were less pronounced in local cultivars (32.5 mm) than in foreign cultivars (37.9 mm) ($p = 0.005$) (Table 3). The local peach cultivars 'Lemonato Andrea', 'Daggalakou' and 'Papagianni' showed a tendency to limit the extension of the lesion in inoculated shoots, whereas higher damages were found in 'Early May Crest', 'H.D. Hale', 'Everts' and 'Charazani' (Figure 3). Damages from *C. destructans* were higher in 2021 compared with 2022 (mean 47.5 mm vs 20.5 mm, respectively) ($p < 0.001$). There was no significant interaction between cultivar and year ($p = 0.245$) suggesting that the response of cultivars was similar in the two studied years.

The studied peach/nectarine genotypes also differed in the susceptibility to shoot blight from *Monilinia laxa*; lower susceptibility was recorded in 'Opsimo Naoussas' and higher in 'Lemonato August-1', 'Lemonato August-2' and 'H.D. Hale' (Table 3, Figure 3). There was no significant difference in the susceptibility between local and foreign cultivars ($p > 0.050$). Damages were higher in 2021 compared with 2022 (mean 7.9 mm vs. 16.4 mm, respectively), yet the response of cultivars was not similar across the two studied years ($p < 0.001$). Thomidis and Michaildies [17] also found different levels of susceptibility to shoot blight caused by *Monilinia* spp. among 24 peach and nectarine cultivars when evaluated in field trials. From the above studied plant material, 11 cultivars were also included in the present study, yet they exhibited different levels of susceptibility. It is possible that susceptibility is affected by the species and the isolates of *Monilinia*; the most extended species with the greatest degree of damage is *M. laxa* (which was used in the present study), and its occurrence is currently at the same frequency as it is for *M. fructicola* [55–57]. Moreover, it cannot be excluded that there was an inversion in the behavior of peach genotypes to brown rot among years. This was reported in the study by Pacheco et al. [24] and Pascal et al. [58] suggesting that phenotypic instability can also result in a response to environmental factors, such as nutrients, water availability and temperature [16,59].

3.5. Correlation Analysis

Correlation analysis of all measured parameters was made for 19 peach and nectarine genotypes. There was a positive correlation between the ratio of LBL/LBW and LBL ($r = 0.726$) suggesting that leaf shape was mainly influenced from leaf length and, to a lesser extent, from the leaf width.

More dark-red shades of the hue of overcolor was positively correlated with greater extent of overcolor ($r = 0.832$), marbled pattern of overcolor ($r = 0.669$), yellow shades of ground color ($r = 0.692$) and more firm flesh ($r = 0.692$).

Fruit size was positively correlated with fruit shape ($r = 0.686$) suggesting that larger fruit had a more elongated shape. Fruit size and shape are important agronomical and pomological attributes, and recent cultivars more often have a nearly round form or are slightly oblate as a consequence of the strong selection activities towards the consumers' preferred attributes [50,51,60].

Ripening day was positively correlated with ripening index ($r = 0.665$), and negatively correlated with titratable acidity ($r = -0.719$) suggesting that the latter ripening cultivars were sweeter. Previous studies also reported that SSC is associated with harvest day of peach [61,62], apricot [62–64] and plum cultivars [65]. There was no significant interaction ($p > 0.05$) between FFW and other fruit quality parameters.

3.6. Principal Component Analyses (PCA)

PCA was applied to the leaf and fruit phenotypic traits and resistance to diseases to determine the most important variables that explain the correlations between cultivars and to identify group patterns. The first six principal components explained a sum of 67.9% of the variability with variations of 18.7, 14.5, 10.7, 9.8, 7.7 and 6.5%, respectively (Figure 4). The parameters with significant positive correlation to PC1 (values > 0.60) were the presence of mid-vein on the lower side, fruit ground color, hue of overcolor, pattern of overcolor and extent of overcolor, while there was a negative correlation with the LBL/LBW ratio, leaf angle at apex and ripening index; the foreign cultivars 'Red Haven', 'Maria Bianca' and 'Golden Jubilee' were separated from the rest of the studied cultivars (Figure 5). The second component (PC2) had a positive correlation with the leaf angle at the base, shape of nectaries, fruit size compared to stone and ripening day. There was a negative correlation with the stone shape. The cultivar 'Fantasia' was separated. Most local cultivars had low values for PC1.

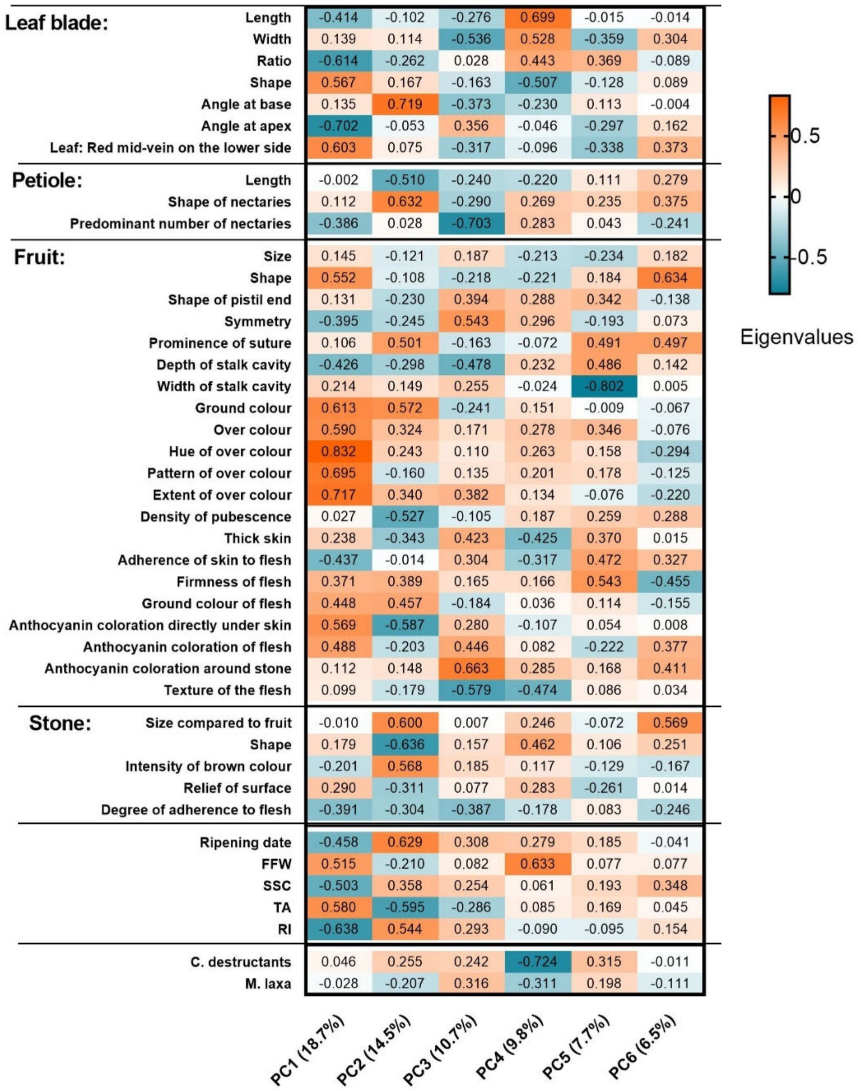

Figure 4. Eigenvalues of the first six principal component axes from the PCA on leaf, fruit and resistance to diseases traits for 18 local and foreign peach cultivars.

In the third factor (PC3), traits with higher scores were the anthocyanin correlation around the stone and traits with lower scores were the predominant number of nectaries. The fourth factor (PC4) was positively correlated with the LBL and FFW and negatively correlated with *C. destructants* infection. The fifth factor (PC5) had a negative correlation with the width of stalk cavity. The sixth factor (PC6) was positively correlated with fruit shape.

FFW was not related in any of the six PCs suggesting that this trait was genetically independently regulated, which was also shown in local vineyard peach cultivars [63]. Nevertheless, fruit ground and flesh color traits were related in the present study but not in the study by Bakić et al. [66].

3.7. Genetic Characterization

All eight ISSR markers used in this study produced clear and reproducible bands that ranged from 230 bp to 2500 bp. A total of 404 bands were generated for all markers that were applied across the 43 peach cultivars/accessions with an average of 50 bands per ISSR marker. A total of 32.98% of the ISSR fragments were polymorphic, and five private bands were detected across peach cultivars/accessions.

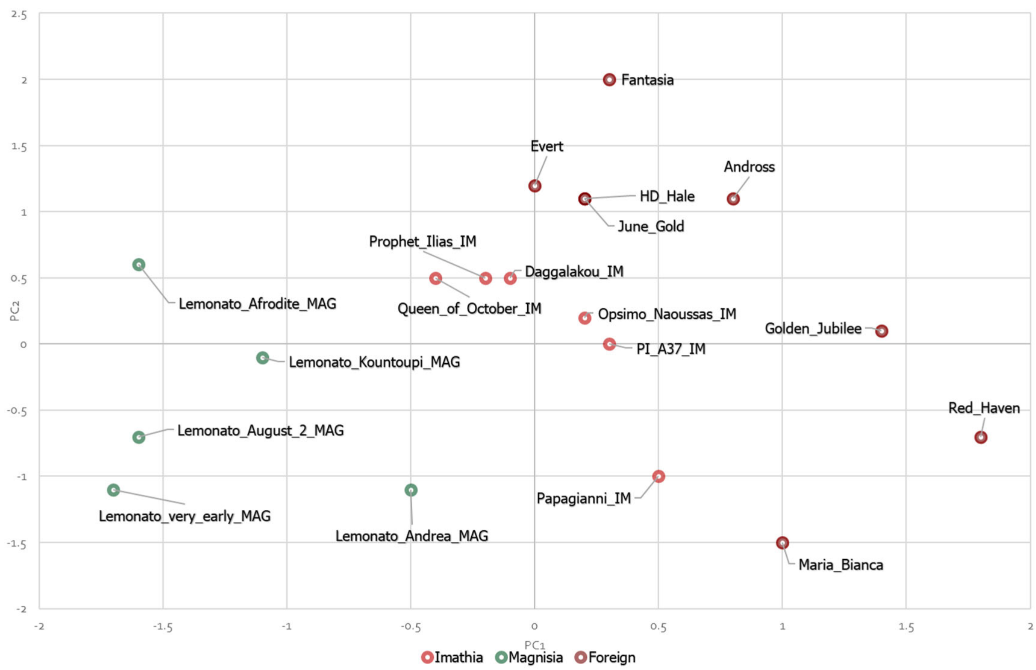

Figure 5. Segregation of 19 local and foreign peach cultivars on the basis of leaf and fruit physical and chemical characteristics determined by principal component analysis.

All selected ISSR markers were characterized, in general, by high values of PIC, with the highest PIC value of 0.404 for primer UBC880 and the lowest PIC value of 0.349 for primer UBC834, with an average PIC value of 0.364 per primer. An average Rp of 5.427 per primer was obtained with the highest Rp value of 7.116 being that of primer UBC827 and the lowest value of 3.860 for primer UBC880 (Table 4).

Results of AMOVA for the ISSR markers revealed a percentage of 39% among populations and 61% within populations. PCoA resulted in clustering the peach cultivars as shown in Figure 6, which agree with the obtained clusters from the UPGMA dendrogram in Figure 7.

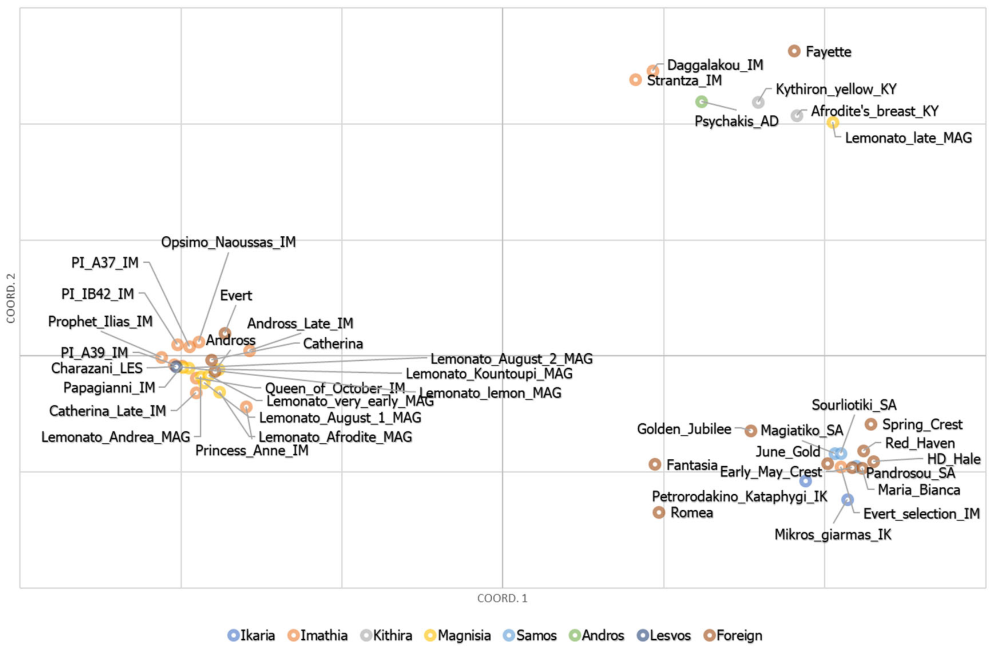

Figure 6. Principal Coordinates Analysis (PCoA) for ISSR molecular markers for 43 local and old foreign peach and nectarine cultivars/accessions.

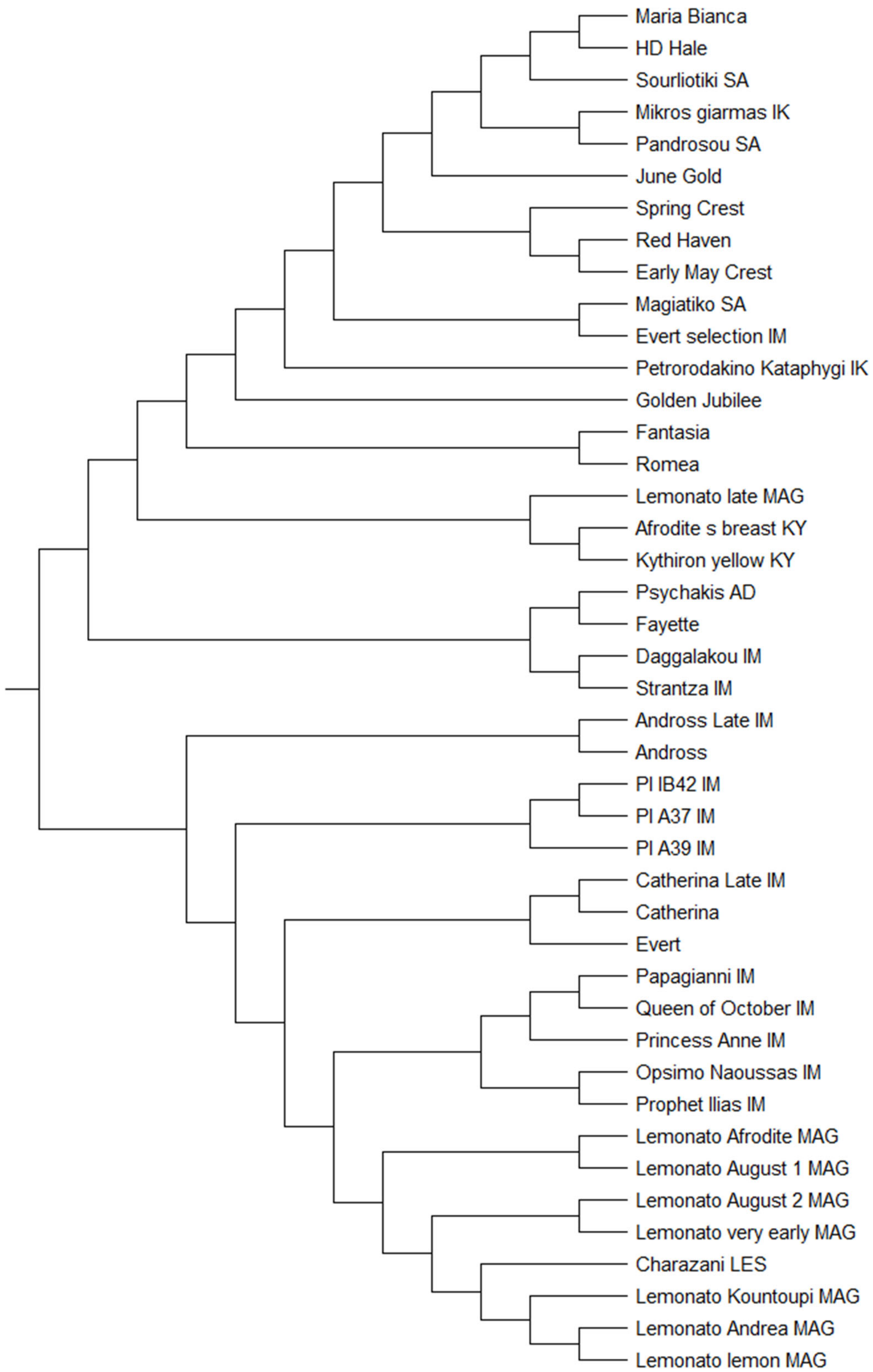

Figure 7. UPGMA dendrogram of 43 local and old foreign peach and nectarine cultivars/accessions based on eight ISSR markers. AD = Andros; MAG = Magnesia; LES = Lesvos; SA = Samos; IK = Ikaria; IM = Imathia; KY = Kythira.

The distribution of the genetic variation in the peach cultivars/accessions was performed by AMOVA analysis. Observed differences within populations resulted in a moderate to low variability found in the studied peach cultivars/accessions. Low genetic variation has also been reported in other studies [4]. The dendrogram based on population pairwise genetic distances (FST) between regions showed the distribution of genetic variability for the peach cultivars and differentiates three main clusters. The first cluster contains foreign cultivars/accessions along with those from Ikaria and Samos. The second cluster contains cultivars/accessions from Magnisia, Imathia and Lesvos. Finally, the third cluster contains a mix of foreign cultivars along with cultivars/accessions from Imathia, Kythira, Andros and one cultivar from Magnisia.

The MRA analysis with ISSR markers revealed many markers associated with phenotypic traits that are statistically significant ($R^2 > 0.5$). Two markers (UBC841_750 and UBC841_1620) were associated with RD; UBC841_750 showed a strongly negative and statistically significant correlation (beta coefficient = −0.535, $p = 0.002$), while UBC841_1620 showed an average correlation. One marker was associated with FFW (UBC834_1030) and showed an average correlation. A total of three markers were identified for SSC (UBC841_390, UBC827_1190 and UBC834_550) and showed an average correlation. For TA, a total of four markers were identified (UBC823_650, UBC841_1050, UBC841_450 and UBC827_600); UBC823_650 showed a strongly positive and statistically significant correlation (beta coefficient = 0.738, $p = 0.002$), while the other markers showed an average correlation. Finally, three markers (UBC823_650, UBC841_350 and UBC841_1050) were identified for the ripening index; UBC823_650 showed a strongly negative and statistically significant correlation (beta coefficient = −0.637, $p < 0.001$), while the others showed an average correlation. All MRA results are shown in Table S3 followed by a list of all alleles. The MRA method is an easy and quick approach for associating traits with markers and is suitable for tree crops and MAS breeding programs [41]. Thus, the ISSR markers used here presented high PIC numbers suggesting that they could be candidates for genetic relationship analysis and parent selection in the breeding programs. Furthermore, the markers used here also present correlation, either positive or negative, with important traits, such as ripening day, FFW, SSC, TA and ripening index, rendering them suitable for the selection of promising progeny in a breeding program.

4. Conclusions

The results from the present study give insight into the genetic and phenotypic variability in leaf and fruit traits and disease resistance in local peach cultivars/accessions prospected from the mainland (Imathia and Magnesia) and islands (Andros, Ikaria, Kythira, Lesvos and Samos) of Greece. A total of 19 local peach cultivars/accessions were found. A higher number of local cultivars/accessions were prospected in Imathia, likely related to the more extensive peach growing occurring in this area. Nine clones of 'Lemonato', isolated in Magnesia, central Greece, with differing ripening times were found to vary in leaf and fruit phenotypic traits. The local cultivars/accessions were separated from the old foreign cultivars studied in in 8 out of the 42 leaf and fruit phenotypic traits studied, suggesting low variability among the local and foreign cultivars.

There was no peach cultivar immune against *M. laxa* or *C. destructans;* higher resistance was found in the cultivars 'Lemonato Andrea', 'Daggalakou' and 'Papagianni' for *C. destructans* and in 'Opsimo Naoussas', 'Charazani' and 'Fortuna' for *M. laxa*. The local cultivars/accessions studied were less susceptible to *C. destructans* compared with foreign studied cultivars. The lack of host specificity for the above pathogens shows the risk of spores produced in neighboring cherry fruit orchards could be inocula for peach orchards.

Results in our study from molecular analysis with ISSR markers indicated a moderate to low percentage of polymorphism, which could be a result of the low diversity among the material used. The low genetic diversity among the plant material that originated from different areas of Greece suggests that these plants either have common ancestors and/or are propagated by cuttings and not seeds. The clusters that were formed according to the PcoA analysis are identical to the clusters of the generated UPGMA dendrogram. MRA analysis with ISSR revealed many markers associated with phenotypic traits, which are strongly positively and negatively statistically significant ($R^2 > 0.5$).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13040800/s1, Table S1: Frequency distribution of leaf morphological characters in 32 local peach cultivars and fruit morphological traits in 19 local peach cultivars. Table S2. Pearson correlation analyses between leaf and fruit morphological quantitative and qualitative parameters, and susceptibility to shoot blight diseases. Absolute linear correlations $\geq |0.60|$ are marked in bold. Table S3. ISSR markers associated with phenotypic traits in peach cultivars as revealed by MRA and the coefficients.

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