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Molecular screening of domestic apple cultivars for scab resistance genes in Greece

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Abstract: Apple scab caused by *Venturia inaequalis* has the most destructive effects among other phytopathogens in apple crops all over the world. The integration of resistance genes from local and domestic cultivars is a prerequisite for the efficient control of this disease and is a main target in efficient breeding approaches. Across Greece, many domestic apple cultivars are reported without deep knowledge about the presence and diversity of scab resistance genes. In this study, the presence of five resistance genes (*Rvi2*, *Rvi4*, *Rvi6*, *Rvi8* and *Rvi11*) was evaluated across twenty local and domestic apple genotypes, employing twelve molecular markers closely linked to known apple scab resistance loci. Significant differences and polymorphisms among the tested cultivars were detected suggesting that some of them carry a sufficient number of resistance genes. This observed genetic diversity could be exploited in ongoing breeding approaches as a natural source of polygenic resistance against apple scab.

Keywords: apple scab; disease resistance; marker-assisted selection; *Venturia inaequalis*

Apple scab caused by *Venturia inaequalis* (Cooke) G. Winter is characterised as the most devastating disease of apple crops (Bowen et al. 2011). Most of the commercial apple cultivars are susceptible and the pathogen has already developed resistance to many classes of fungicides (Cova et al. 2015). Thus, the development of resistant cultivars is the most efficient and environmentally friendly approach to cope with apple scab reducing the side effects of the fungicides (Kumar et al. 2014). Several resistance genes were identified from wild apple cultivars and small-fruited Asiatic progenies, while twenty relevant loci were identified across the apple genome (Khajuria et al. 2018). In general, apple scab resistance can be monogenic or polygenic (Bodea et al. 2008). However, it is important to pyramid several

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resistance genes in apple cultivars through breeding programmes towards piled up durable resistance against *V. inaequalis*. Besides, resistance alleles against apple scab must also be related to adequate phenotypic resistance evidence. Molecular markers linked with resistance genes are important tools for the selection of germplasms with high reproducibility and accuracy (Parita et al. 2018). Thus, the aim of this study was to screen and genotype twenty Greek domestic apple cultivars for the presence of five apple scab resistance genes employing a polymorphic sequence characterised amplified region (SCAR) and simple sequence repeat (SSR) markers.

Cultivars from domestic apple genotypes were collected from various cultivation locations across three geographical regions of Greece (Table 1). Genomic DNA was extracted from each cultivar using the cetyl trimethylammonium bromide (CTAB) method (Doyle & Doyle 1987). Multi-locus genotyping of five apple scab resistance genes (*Rvi2*, *Rvi4*, *Rvi6*, *Rvi8*, *Rvi11*) was employed in all the cultivars using seven SCAR markers, four SSR markers and a specific marker for the *Rvi6* gene (Table 2). PCR amplifications were performed using an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 40 s at 94 °C, 1 min at annealing temperatures based on the primers used for each marker (Table 2), 1.3 min at 72 °C, and a final extension at 72 °C for 10 min. The amplification PCR products were visualised in electrophoresis. The bands on the gels were transformed in binary data by being scored as 1 and 0 for the presence and absence, respectively, of alleles for each cultivar per marker. The sizes of the fragments corresponding to the allelic sizes were accurately scored based on a molecular weight DNA ladder of 100 bp (NEB). A Jaccard similarity co-efficient test was employed in the data matrix and a cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). A principal component analysis (PCA) was performed with a bootstrap value of 1000 replicates using the PAST software (Ver. 3.26, Hammer et al. 2001).

For all the markers that were employed, samples M6 and M15 produced no fragment, while some markers produced genotype specific and unique allelic fragments (Table 3). Based on these molecular data, the apples genotypes grouped into five major clusters (Figure 1). The clustering results indicated

Table 1. Domestic apple cultivars collected from various locations across three geographical regions of Greece along with phenotypic data regarding the apple scab resistance

ID	Cultivar	Location	Region	Disease phenotype to apple scab
M1	Wines	Imathia	North Greece	–
M2	Reneta	Magnesia	Central Greece	–
M3	Rodochori	Imathia	North Greece	–
M4	Firiki Vermiou	Imathia	North Greece	moderately resistant
M5	Belfort	Lesvos	Greek Island	moderately resistant
M6	Evropis-A	Lesvos	Greek Island	–
M7	Evropis-B	Lesvos	Greek Island	–
M8	Skioupia	Magnesia	Central Greece	–
M9	Italika	Lesvos	Greek Island	moderately susceptible
M10	Oal	Magnesia	Central Greece	moderately resistant
M11	Gkekika	Magnesia	Central Greece	–
M12	Kaliga Aiginiou	Pieria	North Greece	–
M13	Megas Alexandros	Imathia	North Greece	moderately resistant
M14	Karlat	Pieria	North Greece	–
M15	Chamomilia-A	Samos	Greek Island	moderately susceptible
M16	Chamomilia-B	Samos	Greek Island	–
M17	Xinomilia	Samos	Greek Island	–
M18	Firiki Volou	Magnesia	Central Greece	moderately resistant
M19	Firiki Faslatiria	Lesvos	Greek Island	moderately resistant
M20	Firiki Asomatos/Megali Vrisi	Lesvos	Greek Island	moderately resistant

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Table 2. List of the molecular markers (7 SCARs, 4 SSRs and a specific PCR marker) for the five apple scab resistance genes (*Rvi2*, *Rvi4*, *Rvi6*, *Rvi8*, *Rvi11*) along with their primer sequences, amplified fragment sizes and annealing temperatures (Ta) as employed for the screening of the Greek domestic apple cultivars

R gene	Marker name	Forward sequence	Reverse sequence	Marker type	Fragment size (bp)	Ta (°C)
Rvi2	OPB18	CCACAGCAGTCATTGGGA	CCACAGCAGTGCATAAAC	SCAR	620	58
Rvi2	CH02b10	AAGGAAATCATCAAAGATTCAAG	CAAGTGGCTTCGGATAGTTG	SSR	122	55
Rvi4	AD13	GGTTCCTCTGTAAAGCTAG	GGTTCCTCTGCCCAACAA	SCAR	950	58
Rvi4	S22	GTCGTGGAAGAGGACCGA	GTCGTGGAAATCCTCGTGAG	SCAR	1300	65
Rvi4	CH02c02a	CTTCAAGTTCAGCATCAAGACAA	TAGGGCACACTTGCTGGTC	SSR	183	55
Rvi6	AL07	TGGAAGAGATCCAGAAAGTG	CATCCCTCCACACAAATGCC	SCAR	466	60
Rvi6	AM19	CGTAGAACGGAATTTGACAGTG	GACAAAGGGCTTAAGTGCTCC	SCAR	526	60
Rvi6	HcrVf2	TCAATCTCAGTAGTTTCTATGGA	CCCCCGAGATTAAGAGTTG	PCR	505	57
Rvi6	CH-Vf1	ATCACCACCAGCAGCAAAG	CATACAAATCAAAGCACACACCC	SSR	166	60
Rvi8	OPL19	ACCTGCACTACAATCTTCACTAATC	GACTCGTTTCCACTGAGGATATTTG	SCAR	433	55
Rvi11	K08	GAACACTGGGCAAAGGAAAC	TAAAAGCCACGTTCTCTCGC	SCAR	743	64
Rvi11	CH05e03	CGAATATTTTCACTCTGACTGGG	CAAGTTGTTGTACTGCTCCGAC	SSR	160	60

that the genotypes showed reasonable variability and high genetic diversity, which may be exploited for selecting parents for breeding purposes. The results of the PCA analysis (Figure 2) resembled the results of the clustering dendrogram, although some genotypes have been diverted on the PCA plot. Thus, five distinct groups formed on the PCA plot again, while some genotypes were scattered from the clusters observed in the dendrogram. Overall, these results indicate that the informative markers being employed in the present study were polymorphic and capable of assessing the genetic diversity in terms of the apple scab resistance genes.

In Greece, there is a large variety of apple genetic resources encompassing traditional cultivars whose

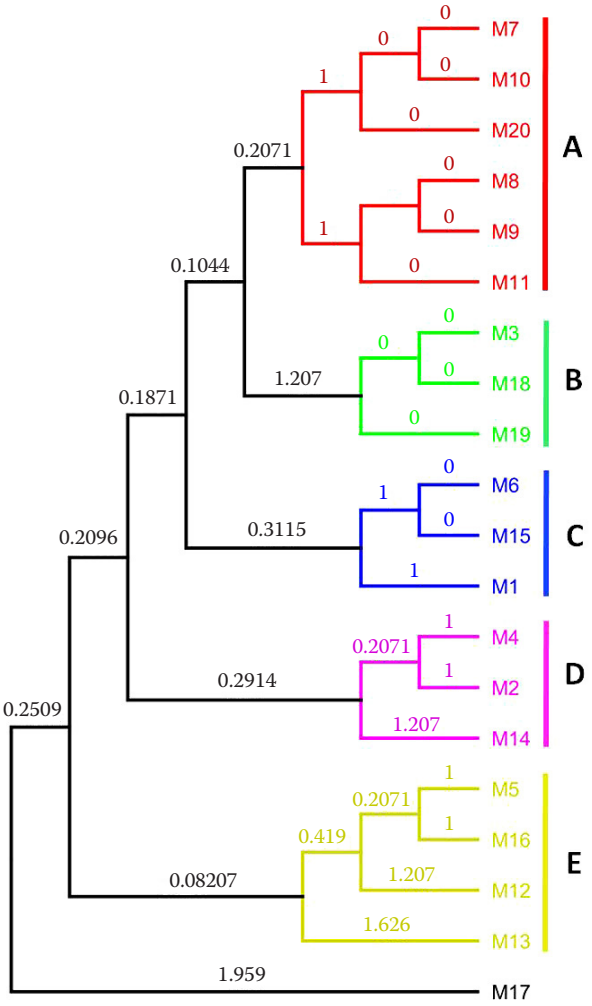

Figure 1. Unrooted UPGMA-based dendrogram showing the main clusters of the cultivars using Jaccard's similarity coefficients after the employment of molecular screening for scab resistance in the Greek domestic apple cultivars

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Table 3. Results of the molecular screening of the Greek domestic apple cultivars for scab resistance using molecular markers; in the binary data matrix, it is indicated with +/– the presence/absence of the respective amplified allelic fragment linked to each resistance gene (*Rvi2*, *Rvi4*, *Rvi6*, *Rvi8*, *Rvi11*) in each cultivar

Cultivar	*Rvi2*		*Rvi4*			*Rvi6*				*Rvi8*	*Rvi11*	
	OPB18	CH02b10	S22	AD13	CH02c02a	AL07	AM19	CH-Vf1	HcrVf2	OPL19	K08	CH05e03
M1	+	–	–	–	+	–	–	–	–	–	–	–
M2	+	–	–	–	+	–	–	–	–	+	–	–
M3	+	–	–	–	+	–	–	–	–	–	–	–
M4	+	–	–	–	+	–	–	–	–	+	+	–
M5	+	+	–	–	–	–	–	–	–	–	+	–
M6	–	–	–	–	–	–	–	–	–	–	–	–
M7	+	–	–	–	–	–	–	–	–	+	–	–
M8	+	–	–	–	–	–	–	–	–	–	–	–
M9	+	–	–	–	–	–	–	–	–	–	–	–
M10	+	–	–	–	–	–	–	–	–	+	–	–
M11	+	–	–	–	–	–	–	–	–	–	–	–
M12	+	+	–	–	–	–	–	–	–	+	–	–
M13	+	–	–	–	–	–	–	–	–	+	+	+
M14	+	–	+	–	+	–	–	–	–	+	–	–
M15	–	–	–	–	–	–	–	–	–	–	–	–
M16	+	+	–	–	–	–	–	–	–	+	+	–
M17	+	+	+	–	+	–	–	–	–	–	+	–
M18	+	–	–	–	+	–	–	–	–	–	–	–
M19	+	–	–	–	+	–	–	–	–	–	–	–
M20	+	–	–	–	–	–	–	–	–	+	–	–

susceptibility or partial resistance against apple scab has not yet been determined. In our study, the frequencies of the detected resistance alleles were 90%, 40%, 45% and 20% for *Rvi2*, *Rvi4*, *Rvi8* and *Rvi11*, respectively, whereas no alleles were observed to correspond to the *Rvi6* gene in any of the tested cultivars even using the specific HcrVf2 marker that is tightly linked to this gene. This particular gene originates from the wild crab apple species *M. floribunda 821* and has been extensively targeted in breeding programmes towards apple scab resistance (Bus et al. 2009). The cultivars tested in this study are not evolved and linked with this wild species. In our study, all the cultivars, except M6 and M15, carried at least one resistance gene from the *Rvi2, Rvi4, Rvi8* and *Rvi11* pool. In five cultivars, at least a combination of three resistance genes were detected, while in nine cultivars, two resistance genes were identified. The M4 cultivar Firiki Vermiou, which is a small-fruited apple tree, was the only cultivar where four resistance genes were identified (*Rvi2*, *Rvi4*, *Rvi8* and *Rvi11*) and could be considered as a relative resistant genotype. This cultivar seems to be a natural source of polygenic resistance and can possibly contribute in future breeding programmes. In Greece, many small-fruited apple cultivars with the name Firiki mostly exist in mountainous areas and they are generally considered as moderately resistant to apple scab (Table 1), serving as crucial resistance resources against apple scab. Apart from these cultivars, the disease phenotypes of other cultivars based on previous data and various observations are shown in Table 1. The usage of co-dominant SCAR and polymorphic SSR markers enabled the sensitive and reliable assessment of the encrypted genetic diversity of resistance genes against *V. inaequalis* in the tested germplasm. Precisely, our results indicate that distinct groups of cultivars were evident, which might imply that a significant and divergent genetic pool for apple scab resistance genes is evident across the cultivars. Besides, there is no regional correlation in the clustering of these cultivars, implying that perhaps they evolved independently across previous introgression events during their evolutionary

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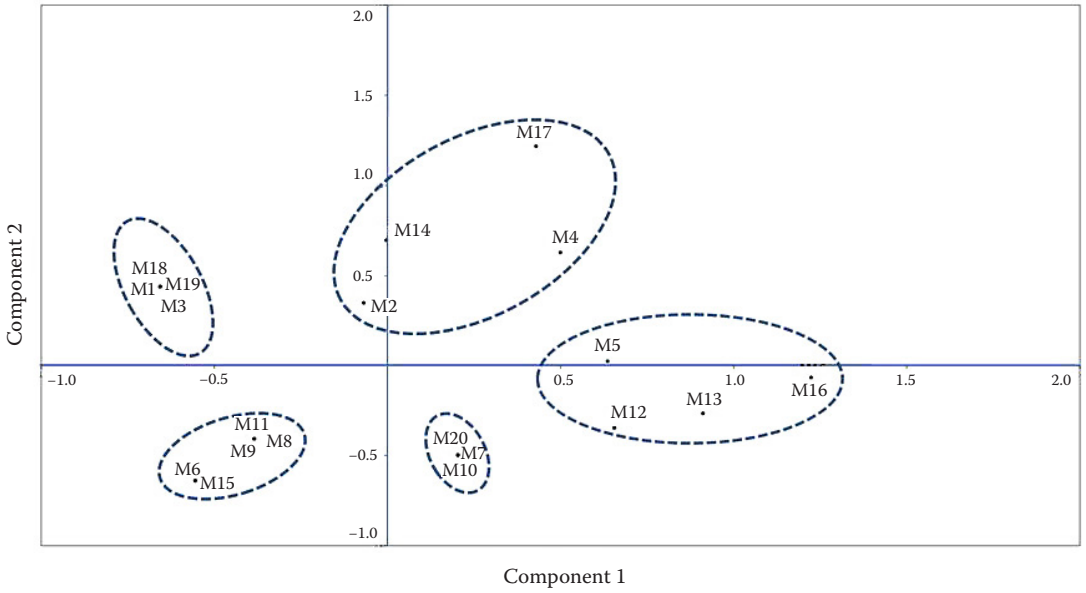

Figure 2. Principle component analysis (PCA) performed with a bootstrap value of 1 000 replicates based on the molecular screening for scab resistance in the Greek domestic apple cultivars

history. However, in order to employ any marker-assisted selection on apple scab resistant cultivars, it is necessary to broaden the screening of wild and domestic apple germplasms.

These SCAR and SSR markers could be undoubtedly valuable in enhancing breeding programmes to detect pyramidisation linked to apple scab resistance across the cultivars. Our results suggest that domestic Greek apple cultivars can be a useful polymorphic germplasm that could contribute, in some extent, to effective breeding programmes for resistance to apple scab. However, in order to validate our results, more phenotypic data and further studies upon the evaluation of these cultivars should be performed towards their putative exploitation.

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