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Molecular markers to evaluate genetic diversity among *Venturia inaequalis* isolates obtained from apple plantations in Isparta Province

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Abstract: Turkey, the third leading apple producer in world, produces approximately 2.6 million tons of apples per year. *Venturia inaequalis*, the causal agent of apple scab, is a major fungal disease among apples varieties. This study was conducted in accordance with the plant protection method for isolates collected from the apple production areas in Isparta Province. The purpose of this study was to determine genetic differences among *V. inequalis* isolates causing apple scab, and to discriminate the effects of varieties and geographical origins on genetic diversity. For this purpose, a total of 83 samples from the leaves and fruits of infected plants were collected to obtain isolates from apple scab. Of these, 67 isolates were obtained by isolating single conidia. Genetic differences and the relationship of the isolates were evaluated using random amplified polymorphic DNA (RAPD), intersimple sequence repeat (ISSR), simple sequence repeat (SSR), and sequence-related amplified polymorphism (SRAP) markers. The marker techniques SRAP and ISSR were used for the first time in this study to determine the genotype of the isolates of *V. inaequalis*. There was no relationship between apple varieties and geographic isolation or source of cultivar patterns. Variance analysis and molecular data of clustering from the isolates at district level revealed highly genetic similarity among populations. Additionally, SSR and SRAP markers were found to be more informative and consistent than other marker techniques. This study was the first report on population genetics of *V. inaequalis* identified with molecular markers in Turkey.

Key words: Apple, genetic diversity, ISSR, RAPD, SRAP, SSR, *Venturia inaequalis*

1. Introduction

Apple scab is a major problem in both Turkish and global apple markets. This disease is responsible for a decrease in the total market value of the fruit by 70%, resulting in production losses ranging from 30% to 60% (Türkoğlu, 1978; Agrios, 1997). On average, apple producers in Turkey apply fungicides 20–30 times per season in order to control the incidence of the causal pathogen, *Venturia inaequalis*, which is attributed to excess environmental pollutants (Boyraz et al., 2005; Soriano et al., 2009).

V. inaequalis has a high degree of genetic variability due to recombination, thus leading to selective pressures on the fungal pathogen to overcome host resistance. An example of this selective pressure leading to host plant susceptibility was observed in Golden Delicious apple varieties. During the 1900s, Golden Delicious apples were highly resistant to apple scab, whereas today this variety is considered one of the most susceptible apple varieties (Gessler et al., 2006).

There are 11 host genes (*Va*, *Vb*, *Vbj*, *Vd*, *Vf*, *Vg*, *Vh2*, *Vh4*, *Vh8*, *Vm*, and *Vr2*) responsible for the resistance observed among apple varieties. Eight of the 11 genes were identified as being primarily responsible for host resistance against apple scab (Roberts and Crute, 1994; Janick and Moore, 1996).

Renaming of *V. inaequalis* races was previously based on a numerical system in which the pathogen was able to overcome resistance; however, later nomenclature sought to improve this system and base it on their source of resistance. Regardless, these systems caused confusion and did not account for relationships between host resistance genes and pathogen avirulence genes (Bus et al., 2011). Currently, *V. inaequalis* is defined according to the avirulence genes it is lacking. Here class 0 is defined as a host that does not carry any resistance genes or universal susceptibility, meaning it is susceptible to all *V. inaequalis* isolates (Bus et al., 2011).

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In apple varieties, genes such as the Gala avirulence (Avr) gene are considered nonsensitive types. The presence of an Avr gene in a variety identifies it as resistant. Apple varieties were observed as lacking symptoms when apples containing the Avr gene were inoculated with the pathogen *V. inaequalis*. Therefore, several apple scab resistance genes isolated from wild apples were identified, and these genes were inoculated into cultivated apples by classical growing methods. In other studies, 17 resistance genes were determined on apple scab (Jha and Thakur, 2009; Bus et al., 2011).

Sierotzki et al. (1994), Sierotzki and Gessler (1998), Tenzer and Gessler (1999), Melounova et al. (2004), and Padder et al. (2011) used RAPD markers for identification of global isolates of *V. inaequalis*, while Tenzer et al. (1999), Boehm et al. (2003), Guérin et al. (2004), and Xu et al. (2008) used simple sequence repeats (SSRs) markers for similar isolates. Unlike previous research, in the present study, intersimple sequence repeats (ISSRs) and sequence-related amplified polymorphisms (SRAPs) were used for the first time for molecular characterization of apple scab.

Although disease-resistant varieties were grown by apple producers in Turkey, no study has characterized the molecular genes of *V. inaequalis* isolates from the apple growing regions in Turkey (Kaymak et al., 2012). Therefore, this research was conducted to investigate genetic differences between *V. inaequalis* isolates based on geographical and cultural growing practices in Turkey.

2. Materials and methods

2.1. Field studies

A total of 67 *V. inaequalis* isolates obtained from more than 1000 leaves and fruits were collected from apple orchards in Isparta Province, Turkey (Figure 1), where active pest management strategies were applied. Surveys were conducted in April and June, which are known to be the most favorable periods for the pathogen. Infected leaves showing symptoms were placed in zip-lock plastic bags and carried in cold storage to the laboratory.

Isolates were collected based on several considerations including varieties, management methods (organic or conventional control methods), and geographical distribution. Various agricultural practices such as organic farming, commercial growing, and monoculture and polyculture applications have been considered to evaluate the formation of genetic adaptations. When considering geographical distribution, apple samples were grouped based on their respective varieties. Host pathogen relationships were identified with genetically similar analysis, according to Dice (1945). For each collected specimen, a code number, which included the plane number of city/town/village-neighborhood/year/host variety, was given. For example, the isolate coded/named "32EgG09st" means Isparta/Eğirdir/Gökdere/2009/Starking Delicious. The collected samples are shown in Table 1.

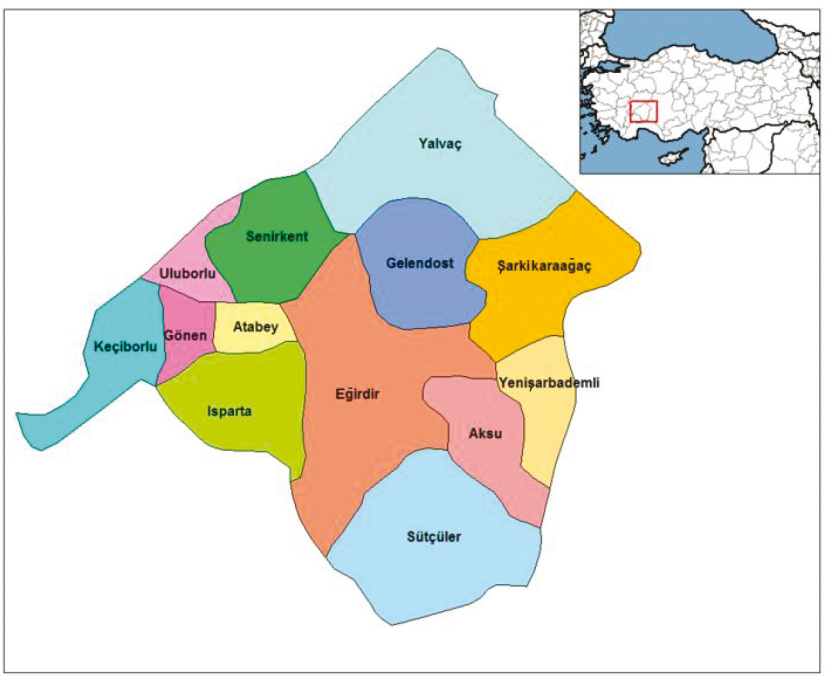

Figure 1. Map of Isparta Province.

Table 1. The number of samples by host.

N.	Samples	N.	Samples	N.	Samples	N.	Samples
1.	32A10go	18.	32EgP09st	35.	32EnTst1209st	52.	32GeM09st
2.	32A10st	19.	32EgT09go	36.	32EnTsT1309st	53.	32GeYK09st
3.	32ABB10st	20.	32EgT09st	37.	32EnTsT1409st	54.	32HO09st
4.	32ABS10st	21.	32EgT09st1	38.	32EnTsT209st	55.	32KBK10st
5.	32EgA09go	22.	32EgY09st	39.	32EnTsT309st	56.	32KO09st
6.	32EgA09st	23.	32EK09st	40.	32EnTsT409st	57.	32KV09st
7.	32EgAKK09st	24.	32EKs09st	41.	32EnTsT509st	58.	32Pe09st
8.	32EgB09st	25.	32En10st	42.	32EnTsT609st	59.	32SB09st
9.	32EgB09st2	26.	32EnGK09a	43.	32EnTsT709st	60.	32SDU09st
10.	32EgC09go	27.	32EnGK09gr	44.	32EnTsT809st	61.	32SeG09st
11.	32EgC09gr	28.	32EnKA09st	45.	32EnTsT909st	62.	32SK10go
12.	32EgG09go	29.	32EnOr09go	46.	32EnTsTKo09st	63.	32SK10gr
13.	32EgG09st	30.	32EnOr09gr	47.	32ESp09st	64.	32SK10st
14.	32EgHR09st	31.	32EnOr09st	48.	32GD09st	65.	32UB10st
15.	32EgK09go	32.	32EnTsT1009st	49.	32GeBa09go	66.	32YaÇ09st
16.	32EgK09st2	33.	32EnTsT109st	50.	32GeBag09st	67.	32YB09st
17.	32EgKB09st	34.	32EnTsT1109st	51.	32GeK10st		

2.2. Isolation

Single spore isolates were obtained from apple orchards that have several ages and variations. The conidiospores located around lesions on the primary leaves were washed with sterile distilled water and the concentration of conidial suspension was adjusted to 15×10^3 conidia/mL in sterile H_2O. A 5-µL suspension was pipetted on the culture medium comprising 1.2% agar, 1.5% malt extract, and Terramycin 25 µg/mL. After incubation at 20 °C for 24 h, the morphology of the germinated conidia was observed under a stereo microscope and some germinated conidia were selected. A single conidium was transferred onto potato dextrose agar (PDA) (Sierotzki et al., 1994). A genomic DNA extraction kit from QIAGEN (Oige, Roche) was used for DNA isolation according to the manufacturer's recommendations.

2.3. PCR analysis

RAPD marker analysis was carried out according to the PCR conditions described by Tenzer and Gessler (1997) and Meleunova et al. (2004). ISSR marker analysis was carried out according to the PCR conditions described by Baysal et al. (2009). SRAP marker analysis was conducted according to the PCR conditions described by Li and Quiros (2001). A total of 14 primer combinations were used in this analysis. Additionally, 7 SSR primers were used according to Tenzer et al. (1999). Amplified DNA fragments were visualized in 2.5% agarose gel with ethidium bromide.

2.4. SSR analysis

PCR amplified DNA fragments were separated on a 2% High Resolution Agarose gel containing 1X TBE (45 mM Tris–borate, 1 mM EDTA) and a 0.5 mg/mL aqueous solution of ethidium bromide. SSR primer pairs used in this study were available only for scoring RAPD marker.

2.5. Data analysis

Amplified bands from each primer were scored as present (1) or absent (0). Only those bands that were consistently amplified were considered. Smeared or weak bands were excluded from the analysis. Similarity coefficient (Dij) was determined between each pair of strains (Dice, 1945). The estimates of similarity between strains were used for cluster analysis by UPGMA (Unweighted Pair-Group Method With Arithmetic Average) using the NTSYS (Numerical Taxonomy Multivariate Analysis System, NTSYS-pc version 2.11, Exeter Software, Setauket, NY, USA, Rohlf, 2000). The studies were carried out to assess the possible genetic differences among the isolates of *V. inaequalis*.

3. Results

3.1. Field studies

The samples were collected based on their geographic features and their cultural growing practices, and were recorded as Starking Delicious (53), Golden Delicious (9), Granny Smith (4), and Anna (1). It was observed during surveys that commercial apple orchards were established with susceptible varieties.

3.2. Isolation

Surveys were conducted in April and June when the incidence of disease was high. A total of 67 isolates were collected from the 83 samples. Isolating the pathogen spore at the early stage was more efficient because contamination of the leaves and fruits with other saprophytes was very high at later stages.

3.3. PCR analysis

The primers of RAPD, ISSR, SRAP, and SSR markers were used with end point PCR. High resolution agarose for SSR markers could not be used with capillary electrophoresis (CE); therefore, SSR primer pairs were used only in scoring, similar to the use of RAPD. Both ISSR and RAPD markers were able to discriminate *V. inaequalis*; however, SRAP and SSR primers provided better specificity. The method using SRAP and SSR primers was more informative, simple, and reproducible with a higher degree of specificity than the others. Additionally, this was the first study in which ISSR and SRAP markers were used for molecular characterization of *V. inaequalis* isolates.

3.3.1. Assessment of genetic differences between pathogen isolates by RAPD markers

The average number of bands for each primer was 5.9, while that of polymorphic bands was 3.9. The maximum number of eight bands was determined with OPG05 and M2 RAPD primers. The maximum number of seven polymorphic bands was also determined with M2 primer, the polymorphism rate of which was calculated to be 88%. Polymorphism information content (PIC) values of 0.51 was determined with the OPG05 and M2 RAPD primers, while the overall average number was determined to be 0.4 (Table 2).

Cluster analysis was carried out using Dice's similarity coefficient, which was also used to generate a dendrogram in order to show the relationship among isolates (Figure 2). This dendrogram illustrates that all isolates were separated with two main branching nodes. The genetic similarity coefficient, 0.81, was used to separate each isolate.

Group 1 isolates contained clustering of 32EgBo09st, 32EgKB09st, 32EnOr09go, and 32GeYK09st. The other isolates were separated into group 2, which showed low genetic similarity. Host specificity was not the case and there was no difference between geographical aspects given that the distance between samples was only 30 km from the farthest points. This cluster analysis revealed that the observed genetic differences could be linked to the race or to the species, but not the geographical distances among isolates. In terms of genetic similarity, the identified samples that were closest to each other were 32EnTsT709st

Table 2. RAPD primers used to analyze the genetic diversity between *Venturia inaequalis* isolates. Maximum and minimum band size (bp), number of amplified loci (n), number of polymorphic loci (NPL), percentage of polymorphic loci (PPL), polymorphism information content (PIC).

RAPD primers	bp	n	NPL	PPL	PIC
OPK14	550–1250	5	2	40	0.39
OPBO8	300–800	5	4	80	0.38
OPG03	200–1250	7	4	57	0.41
OPG05	330–1250	8	6	75	0.51
OPG17	400–1100	7	5	71	0.41
OPH04	500–1050	4	2	50	0.21
OPN11	600–1200	6	5	83	0.45
M2	300–1100	8	7	88	0.51
RAPD1	400–1100	5	2	40	0.33
P49	250–800	6	4	67	0.45
F04	500–1100	4	2	50	0.45
Total		65	43	66	27.48
Mean		5.9	3.9	66	0.42

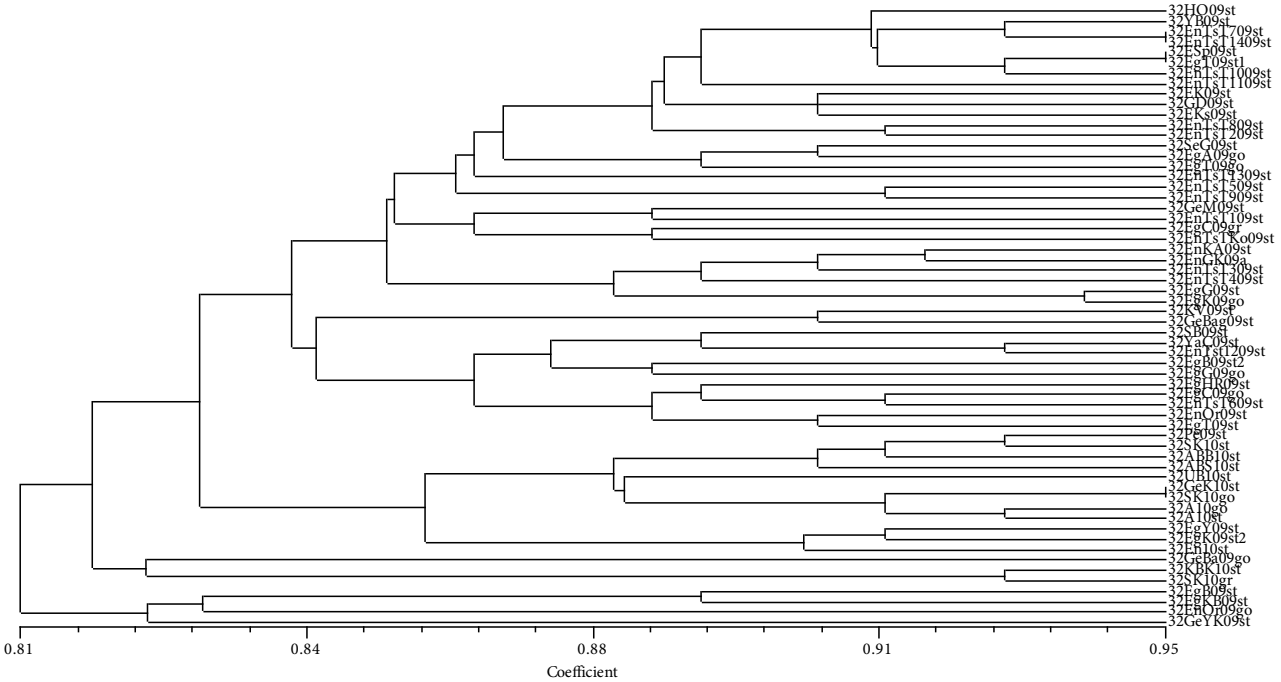

Figure 2. The UPGMA dendrogram of analyzed isolates obtained from the Dice's coefficient similarity matrix using RAPD markers data.

with 32EnTsT1409st, 32Esn09st with 32EgT09st1, and 32SK10go with 32GeK10st at 0.95 each.

3.3.2. Assessment of genetic differences in pathogen isolates by ISSR markers

The average number of bands for each ISSR primer was six and two, with the average number of polymorphic bands being four and two. The maximum number of 8 bands was determined by using the UBC 885 ISSR primer. The maximum number of 7 polymorphic bands was also found using the same primer UBC 885 ISSR. The polymorphism rate was calculated at 88%. PIC values were found using the maximum UBC 887 ISSR primer at 0.47, while the overall average was determined to be 0.36 (Table 3). The overall average of the lowest PIC analysis was obtained with ISSR marker.

Dice's similarity coefficient, as observed in Figure 3, was used to carry out cluster analysis in order to generate a dendrogram illustrating the relationship among isolates. This dendrogram demonstrates that all isolates could be distinctly separated and contained by two main branching nodes. A genetic similarity coefficient of 0.82 was obtained with the isolates. While group 1 had the 32EgK09st gene, the remaining isolates were separated into group 2. Group 2's contained a genetic similarity of 0.84 on two subbranching nodes. 32EgA09st, 32EnTsT1109st, 32EnTsT1209st, 32EgG09go, 32EgA09go, and 32EgB09st2

Table 3. ISSR primers used to analyze the genetic diversity between *Venturia inaequalis* isolates. Maximum and minimum band size (bp), number of amplified loci (n), number of polymorphic loci (NPL), percentage of polymorphic loci (PPL), polymorphism information content (PIC).

ISSR primers	Bp	N	NPL	PPL	PIC
UBC 880	200–950	7	3	43	0.26
UBC 885	350–1000	8	7	88	0.44
UBC 886	300–1250	5	4	80	0.45
UBC 887	250–1100	6	5	83	0.47
UBC 888	300–1250	7	4	57	0.18
UBC 890	500–1000	4	2	50	0.41
Total		37	25	68	12.91
Mean		6.2	4.2	68	0.36

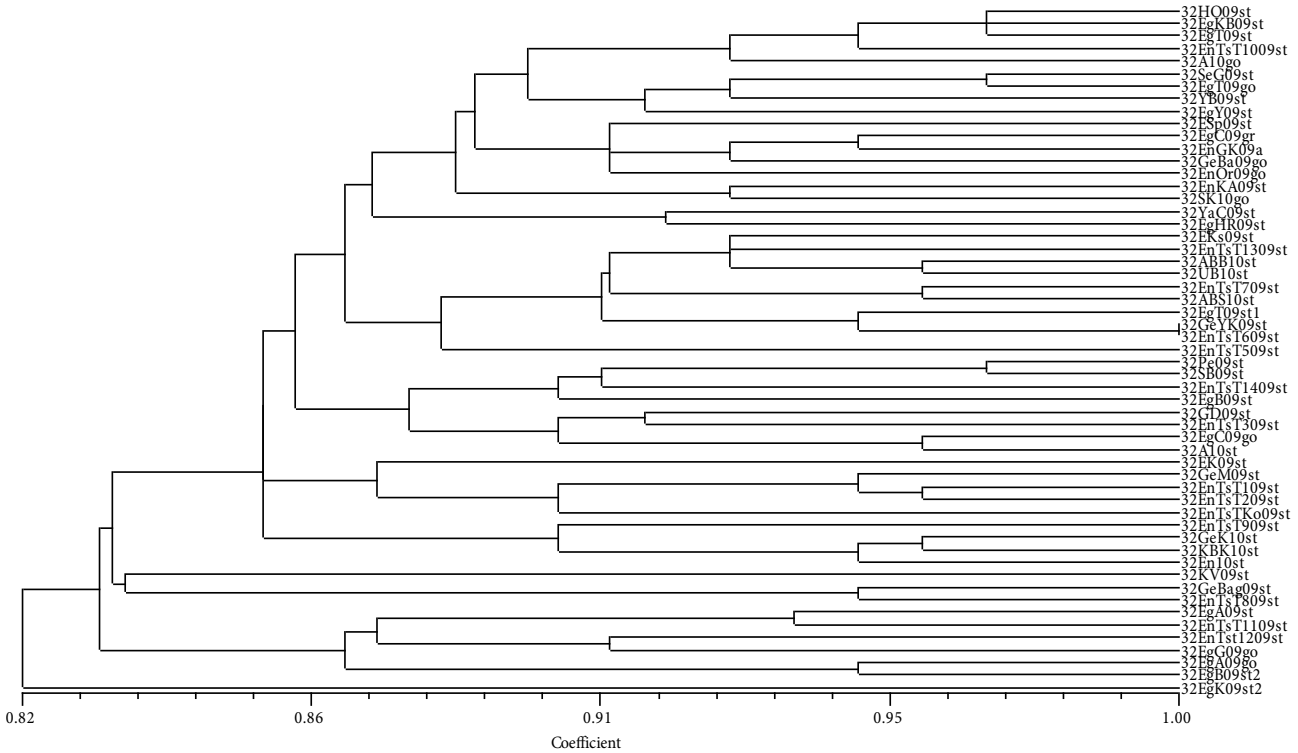

Figure 3. The UPGMA dendrogram of analyzed isolates obtained from the Dice's coefficient similarity matrix using ISSR markers data.

isolates were clustered into small groups. Isolates were separated based on a genetic similarity coefficient of 0.74. Genetic similarities were based on levels of 0.67 to 1.00. Genetic analysis results revealed isolates 32GeYK09st and 32EnTsT609st were identical.

3.3.3. Assessment of genetic differences in pathogen isolates by SSR markers

The average number of bands for each primer was 1.9, while that of polymorphic bands was 1.7. Only one band was obtained using the 1aac4h primer pair with a polymorphic rate equal to 0% being observed. PIC values were found at a maximum of 0.71 while using 1tc1a and 1tc1b primers. The overall average was determined to be 0.56 (Table 4). Using the SSR markers, the highest overall average was obtained with the PIC.

Phylogenetic groups were generated using SSR marker analysis of groups more distinct from other markers. This analysis suggests that the second main group formed a relative rate of 0.74, while each major group was approximately 0.80 in genetic closeness to each other from a branching. This gave rise to 7 subgroups (Figure 4). Host specificity of genetic similarities among subgroups was

Table 4. SSR primers used to analyze the genetic diversity between *Venturia inaequalis* isolates. Maximum and minimum band size (bp), number of amplified loci (n), number of polymorphic loci (NPL), percentage of polymorphic loci (PPL), polymorphism information content (PIC).

SSR primers	Bp	n	NPL	PPL	PIC
1tc1a	109–187	2	2	100	0.71
1tc1b	149–210	2	2	100	0.71
1tc1g	111–185	2	2	100	0.69
1aac3b	118–174	2	2	100	0.50
1aac4b	166–177	2	2	100	0.50
1aac4f	96–116	2	2	100	0.50
1aac4h	198–201	1	-	0	0
Total		13	12	600	7.24
Mean		1.9	1.7	86	0.56

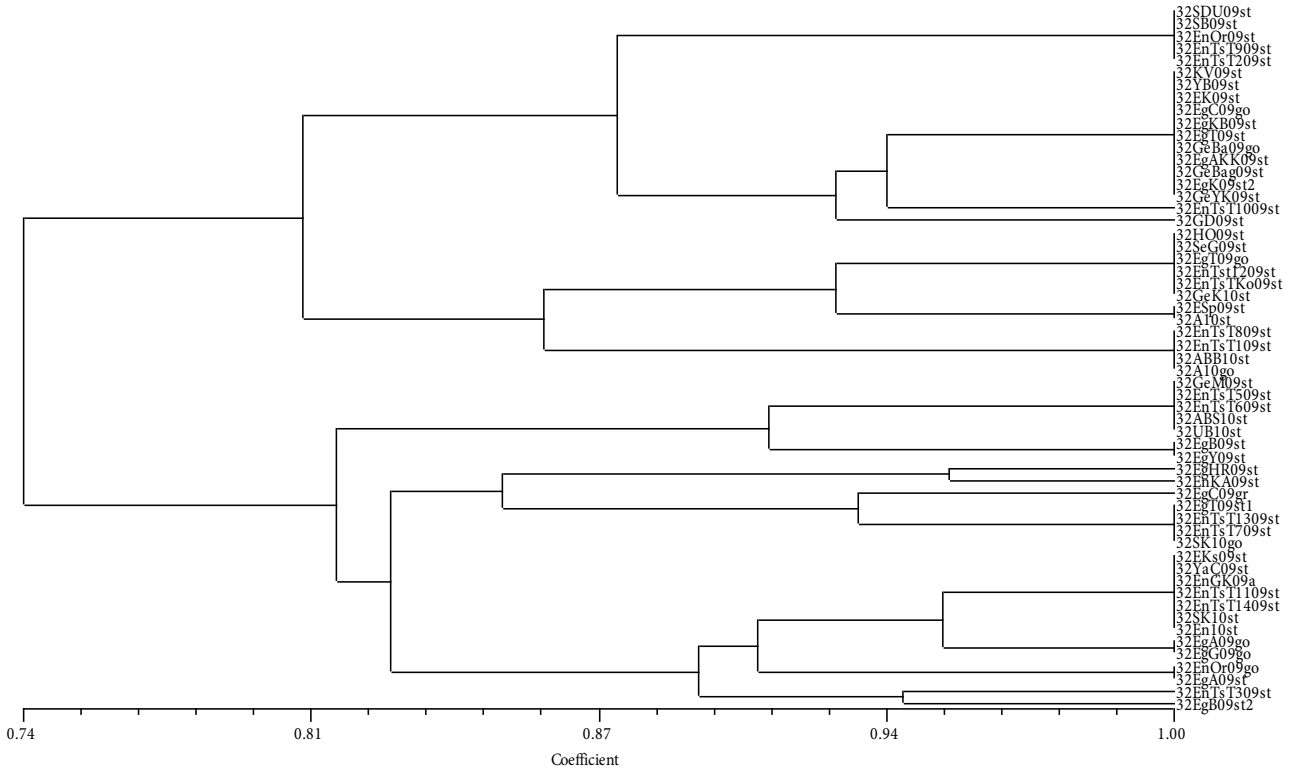

Figure 4. The UPGMA dendrogram of analyzed isolates obtained from the Dice's coefficient similarity matrix using SSR markers data.

analyzed and no differences in the relationship between the geographical area and the application were established.

3.3.4. Assessment of genetic differences in pathogen isolates by SRAP markers

The average number of bands for each primer was 6.5, with an average of polymorphic bands at 4.8. The maximum number of bands was eight, determined by using the Em1Me4, Em2Me5, Em9Me12, and Em10Me12 SRAP primers. The maximum number of polymorphic bands was seven, found by using the Em10Me12 primer, with a polymorphic rate calculated at 100%. A PIC value was determined at a maximum value of 0.76 for the Em5Me10 primer, whereas the overall average was determined to be 0.54 (Table 5).

The dendrogram in Figure 5 shows that all isolates could be distinctly separated with the presence of two main branching nodes. Isolates could be separated at a genetic similarity coefficient of 0.74. Levels of genetic similarity were determined to vary between 0.67 and 0.94. Only the 32EnTsT1309st isolates were clustered together into group 1, while the other isolates were separated into group 2 with a lower genetic similarity of 0.82. Differences in terms of geographical and chemical application were not found among isolates. The closest related genetic isolates were 32GeBa09go with 32A10st and 32EgK09go with 32EnTsT1009st.

4. Discussion

Apples are cultivated in nearly all provinces in Turkey; however, Isparta has the largest apple growing area with 549,371 tons of apple production, corresponding to 20% of the country's apple supply. The risk and damage caused by apple scab are increased by the Mediterranean climate of this region due to favorable growing conditions for the causal fungal pathogen *V. inaequalis.* Therefore, in an effort to decrease economic damage and assist in controlling *V. inaequalis*, extremely large amounts of fungicide are applied. Typically, the use of large volumes of fungicide has prevented the disease from exceeding the damage threshold where growers cannot produce enough yields to maintain profitability. However, this long-term use of fungicides poses a risk to the environment due to increased pollutants from run-off (Boyraz et al., 2005).

Melounova et al. (2004), in the Czech Republic, performed the first genetic characterization of isolates of *V. inaequalis* on one spore. Their reported isolates were identified with genetic similarities between 0.16 and 0.79. In our study a similar analysis was performed by using the same RAPD primers; however, an elevated range of 0.83–0.97 was found. When the isolates were genetically characterized, it was observed that geographic differences were the case in terms of features and host specificity.

Tenzer and Gessler (1997), in Switzerland, identified

Table 5. SRAP primers used to analyze the genetic diversity between *Venturia inaequalis* isolates. Maximum and minimum band size (bp), number of amplified loci (n), number of polymorphic loci (NPL), percentage of polymorphic loci (PPL), polymorphism information content (PIC).

SRAP primer combinations	bp	n	NPL	PPL	PIC
Em1Me4	400–1250	8	6	75	0.65
Em2Me5	250–1100	8	5	63	0.62
Em2Me9	230–1150	7	5	71	0.69
Em3Me13	200–1000	6	4	67	0.58
Em4Me9	400–1200	5	3	60	0.38
Em4Me11	300–1000	6	5	83	0.71
Em5Me10	300–1150	5	4	80	0.76
Em6Me2	300–900	5	5	100	0.59
Em6Me3	200–700	6	4	67	0.48
Em7Me3	250–1000	6	5	83	0.56
Em7Me8	200–1200	7	6	86	0.53
Em8Me11	200–700	6	3	50	0.18
Em9Me12	150–1100	8	5	63	0.30
Em10Me12	200–900	8	7	88	0.49
Total		91	67	74	49.4
Mean		6.5	4.8	74	0.54

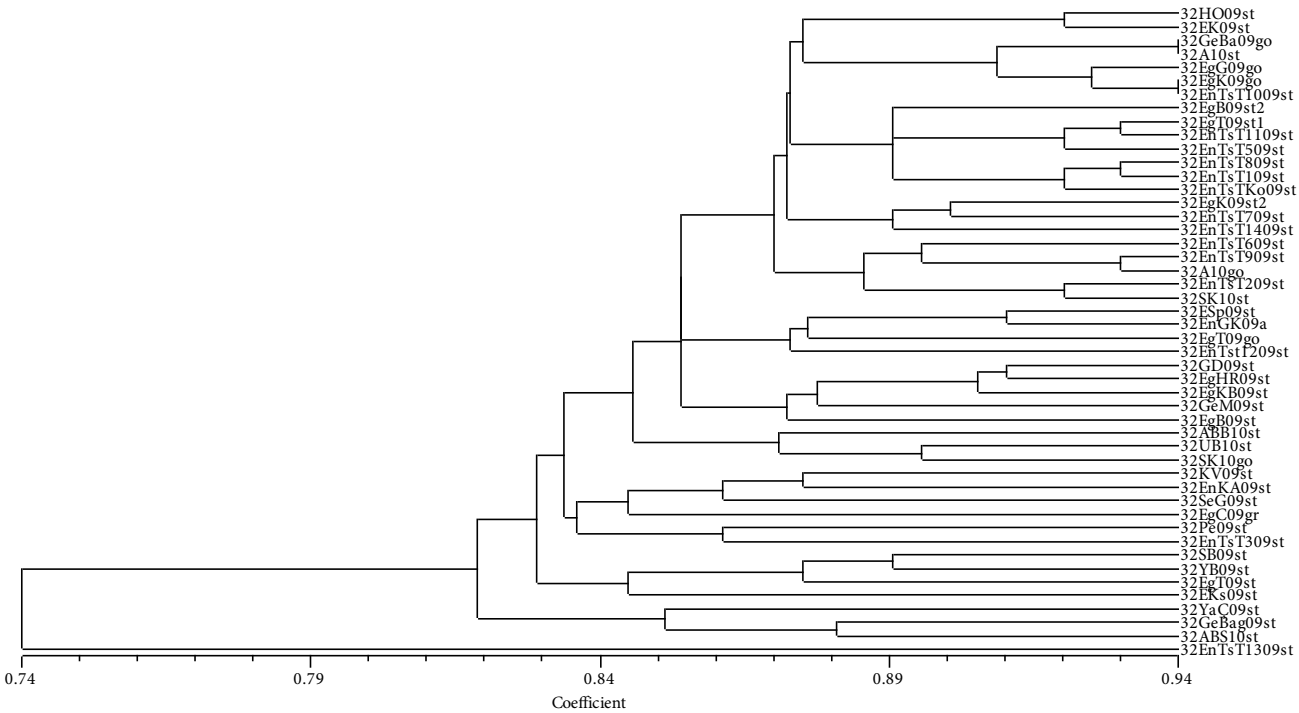

Figure 5. The UPGMA dendrogram of analyzed isolates obtained from the Dice's coefficient similarity matrix using SRAP markers data.

four new resistant varieties (*Vf*) and the propagation velocity of the moving type, which could overcome the pathogenicity of *V. inaequalis*. All four populations were found to be similar at 0.95. These results suggested that the natural gene flow would be enhanced by human activities. Therefore, it is very difficult to predict the continuity of *Vf*. Additionally, similar results were determined in this study.

Tenzer and Gessler (1999) reported that five European countries were investigated for the genetic diversity of populations of *V. inaequalis* by using 18 ITS-rDNA regions compared using RAPD markers. Their results indicated that gene flow occurs among the European regions being investigated. Additionally, these researchers explored the possibility of spread of the pathogen by falling leaves, rain, and wind between the Northern and Southern Alps. It was determined that the genetic diversity of *V. inaequalis* isolates in the Alps was attributed to greater gene flow from individuals carrying spores from one locale to another. Accordingly, our findings confirmed those of Tenzer and Gessler (1999) and Melounova et al. (2004). Interestingly, our variation was found to be at the lowest levels with all genotype dendrograms being similar at levels of 0.73 to 0.95. This analysis, using RAPD markers, indicated that the lowest rate of polymorphism was 66%, suggesting the original genetic mutation was from samples closely related, therefore giving similar banding. Additionally, polymorphism was thought to contribute to the cause of the low rate of emergence.

Boehm et al. (2003) obtained 38 isolates of *V. inaequalis* from two varieties of apples (Top Red and Starking Delicious) in the Golan Heights and Hula Valley, located along the coastal regions. SSR marker analysis was used to profile these populations. It was found that all conidia spores collected from the seaside were 100% genetically uniform, while Top Red and Starking Delicious varieties of samples collected from the Golan Heights were less genetically similar at 74%. Similar results were obtained in our study.

Levels of genetic similarity of *V. inaequalis* were found to vary between 0.53 and 1.00 according to SSR markers. The highest rate of SSR polymorphic markers was 86%. In this respect, the studies were quite promising in determining genetic similarity.

Apple producers apply chemicals 20–25 times for prevention and control of apple scab per season. This is an extremely large volume when compared to the amount applied in apple orchards in other countries, which are reported at a rate of 10–12 applications per season (Boyraz et al., 2005). Despite the higher number of fungicide applications, the disease still occurs and continues to increase, and the pathogen continues to propagate. This increase in apple scab along with numerous fungicide applications suggests a more virulent strain of fungus has evolved.

As a result of selective pressure on the pathogen, from increased pesticide use, new apple cultivars could potentially lack adequate resistance to the pathogen. However, considerable efforts are being made to develop novel genes that would provide resistance against the pathogen.

The SRAP analysis resulted in low levels of variation due to the high level of genetic similarity among isolates. The differences in pathogenicity between the isolates might have been caused by single nucleotide mutations, thus leading to a summarization that the genetic structure would be the same.

This study was the first report on molecular characterization of *V. inaequalis* isolates by using ISSR and SRAP markers. As a result, it was found that isolates were genetically similar to each other. There were no differences between groups on account of host selection or in geographical location. SSR and SRAP markers formed collective groups. These markers seemed to be more informative, easily applicable, reproducible, and specific.

Like RAPD, SSR primer pairs could only be used in scoring. The results obtained were accurate and reproducible due to a co-dominant marker. In this study, *V. inaequalis* isolates collected from different regions of Turkey were determined to be genetically diverse and to differ among isolates by using molecular markers. Given the results, it can be said that all of the isolates could be separated from one another genetically. However, further work is needed to explore and detail the diversity and distribution of the *V. inaequalis* race. The findings of this study were in accord with those reported in the literature.

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