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The Assessment of Genetic Diversity of *Venturia inaequalis* Isolates Obtained from Turkey and Europe by Molecular Markers

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ABSTRACT

Apple scab (*Venturia inaequalis* (Cke) Wint) is the main disease of apple trees not only in the world but also in Turkey. The disease can cause severe yield losses in all Turkish apple orchards. Genetic diversity and relatedness of apple scab (*Venturia inaequalis* (Cke) Wint) isolates collected from Turkey and Europe were investigated in this study through molecular markers. RAPD, ISSR, SSR and SRAP markers were used in the molecular studies. Results obtained by marker system revealed that isolates were quite separated from each other and it is appeared to exist a variation between them. Genetic relatedness between the isolates are very close to each other, and difference among the groups is not significant due to host specificity and geographical location. The groups are consisted of more number of isolates when SSR and SRAP markers are used compared with ISSR and RAPD markers. Using SSR and SRAP markers are preferable to provide more informative outcomes because of ease of use, repeatability and specificity.

Keywords: Apple; Genetic difference; ISSR; RAPD; SRAP; SSR; *Venturia inaequalis*

Avrupa ve Türkiye’den Toplanan *Venturia inaequalis* İzolatlarının Genetik Farklılığının Moleküler Markırlar ile Değerlendirilmesi

ESER BİLGİSİ

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ÖZET

Elma kara lekesi hastalığı (*Venturia inaequalis* (Cke) Wint) dünyada olduğu gibi ülkemizde de elmanın ana hastalığıdır. Hastalık, yurdumuzda elma yetiştirilen tüm bahçelerde önemli ürün kaybına neden olabilmektedir. Bu çalışmada moleküler markırlar ile Türkiye’den ve Avrupa’dan toplanan Elma kara lekesi izolatlarının genetik farklılıkları ve akrabalık ilişkileri araştırılmıştır. Moleküler çalışmalarda RAPD, ISSR, SSR ve SRAP markırları kullanılmıştır. Markır

sistemlerinden elde edilen sonuçlara göre, izolatların tamamı birbirinden ayrılmış ve bunlar arasında bir varyasyon olduğu görülmüştür. Genetik benzerlikler birbirine yakın bulunmuş, gruplar arasında konukçu seçimi ve coğrafik konum yönünden farklılıklar oluşmamıştır. SSR ve SRAP markıklarında gruplar daha toplu oluşmuş ve uygulamanın kolaylığı, tekrarlanabilirliği ve spesifikliğı bakımından daha bilgi verici bulunmuştur.

Anahtar Kelimeler: Elma; Genetik farklılık; ISSR; RAPD; SRAP; SSR; *Venturia inaequalis*

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1. Introduction

Apple scab caused by *Venturia inaequalis* is a fungal disease and mostly resulted from filamentous fungus. It is among the most significant threats to commercial orchards located in cool and precipitated regions of the world (MacHardy 1996). Lesions caused by pathogenic infections are commonly superficial and developed between the cuticle and the cell wall of epidermal layers (MacHardy et al 2001). Therefore they are not life threatening. However, such lesions significantly reduce the market values of apples, thus considered as serious concerns for commercial growers (MacHardy et al 2001). Generally fungicides are applied several times throughout the growing season to prevent apples from *V. inaequalis* (Soriano et al 2009).

Virulence and evolutionary structures of the pathogen should be well-comprehended to develop new cultivars resistant to *V. inaequalis*. Gladioux et al (2008) and Tenzer & Gessler (1997) analyzed microsatellite profiles of *V. inaequalis* samples collected from five different continents and indicated the origin of the fungus as Central Asia and reported the widespread of the fungus worldwide. Barbara et al (2008) carried out AFLP analysis on isolates taken from different cultivars and reported significant differences in virulence of the fungus even in the same orchard established with different apple cultivars in UK, but Xu et al (2008) was not able to observe significant differences in isolates taken from different cultivars or regions of China. Xu et al (2009) indicated the need for more knowledge about inner-population variability effecting host pathogen co-evolution.

Molecular techniques and new analytical methods have been developed for better understanding of population biology of plant pathogens (Taylor et al 1999a; 1999b; 2000). Multi-locus DNA datasets are

now used in phylogenetic and population genetic methods to find out the species and populations and to identify their mating system and population structure (Taylor et al 1999a).

Currently, molecular markers are also employed in researches on population structure of *V. inaequalis*. In the studies carried out on population genetics of *V. inaequalis*, neutral markers of four *V. inaequalis* populations from Switzerland (Tenzer & Gessler 1997) and 11 populations from the other European countries (Tenzer & Gessler 1999) were evaluated to investigate relationship between the geographic population structure and host origin. Those findings revealed a high level gene flow in both short and long-distances in Switzerland (Tenzer & Gessler 1997) and throughout Europe (Tenzer & Gessler 1999).

Gladioux et al (2008) carried out a broad study including 1,273 isolates from 28 orchards of 5 continents with microsatellite markers to identify the origin of *V. inaequalis*. Researchers gathered isolates under 17 clusters representing distinct groups of geographic locations. Analysis of Molecular Variance (AMOVA) revealed about 88% variation within populations. Depending on such a high genetic variation and random association of alleles from different microsatellite loci, researchers concluded that regular sexual recombination occurred in all sampled sites.

While Sierotzki et al (1994), Sierotzki & Gessler (1998), Tenzer & Gessler (1999), Melounova et al (2004) and Padder et al (2011) used RAPD markers in molecular studies related to *Venturia inaequalis*, Tenzer et al (1999), Boehm et al (2003), Guérin et al (2004) and Xu et al (2008) used SSR markers in similar studies. However, ISSR and SRAP markers haven't been used in molecular characterization of *V. inaequalis*.

The present study was conducted to investigate the genetic diversity and relatedness of 54 *Venturia inaequalis* genotypes through RAPD, ISSR, SSR and SRAP molecular markers.

2. Material and Methods

2.1. Isolation

Venturia inaequalis isolates were isolated as single spore from apple orchard at different ages and types (Sierotzki et al 1994). Isolates of *V. inaequalis* were obtained from infected apple tree leaves were washed with sterile distilled water. Spore suspensions were adjusted in sterile distilled water to 15×10^3 conidia mL^{-1} and maintained on agar surface to obtain pure culture in petri dishes. Petri dishes were kept at 20 °C for 24 h in the dark, and the conidia were transplanted to Potato Dextrose Agar (PDA) medium. Conidia on the PDA were taken from petri dishes and ground in liquid nitrogen using a mortar and pestle. About 100 mg of fungal powder was added to centrifuge cups and used in DNA isolation. DNA isolation was performed with

a genomic QIAGEN® DNA extraction kit (OIGEN, Roche) along with the recommendations of producer firm. Single spore isolates of 43 *Venturia inaequalis* supplied from 22 provinces of Turkey and 11 from 5 different European countries in April and June when the disease is active were used in this study (Table 1).

2.2. PCR analysis

For PCR analyses, a total of 24 μL PCR mixture was prepared with 1xPCR buffer solution (50 mM KCl, 10 mM Tris HCl, 25 °C, pH 9.0, 1% Triton X-100), 2.5 mM MgCl_2 , 0.2 mM dNTP, 0.8 μM primer/primers and 30-50 ng (1 μL) DNA with 1.25 U Taq DNA polymerase (Takara Ex Taq) enzyme. For DNA amplification, the prepared PCR mixture was placed into PCR device (BioRad, C1000 Thermal Cycler USA) and gene amplification was performed. All isolates were screened through by selecting 11 RAPD primers (Tenzer & Gessler 1997; Meleunova et al 2004), 6 ISSR primers (Baysal et al 2009), 7 SSR primers (Tenzer et al 1999) and 14 SRAP primer combinations (Li & Quiros 2001).

Table 1- List of samples collected from European countries and different provinces of Turkey

Çizelge 1- Türkiye'nin farklı şehirlerinden ve Avrupa ülkelerinden toplanan örneklerin listesi

No	Sample no	No	Sample no	No	Sample no
1	03D09st	19	32EgHR09st	37	53ID10kr
2	05M09a1	20	32GeBag09st	38	55M09a
3	05M09a2	21	32EnTst1209st	39	59M09go
4	07E109st	22	32EnTst709s	40	59M09st
5	07E110st	23	33M09st	41	60M09st
6	17Br09st	24	33M10an	42	70M09st
7	17BI09st	25	33Mut09st	43	77AE10st
8	17L09ga	26	38Y10st	44	AlKln10kr(Germany/Cologne)
9	17Lap09st	27	38YS09st	45	BelAE10kr(Belgium/Resc. Inst.)
10	17MS09st	28	42BS09st	46	BelBrM10kr(Belgium, Brussel)
11	17Un09st	29	42BS10st	47	BelBrO10kr(Belgium/Oaslt)
12	20COm09go	30	42E10st	48	BelGe10kr(Belgium/Gent)
13	20Om09st	31	43To09br	49	BelLux10se(Belgium-Luxemburg)
14	20Om09st2	32	43To09go	50	Bos210kr(Bosnia/University)
15	22Lb10pl	33	43To09gr	51	Bos310kr(Bosnia/University)
16	24CK10st	34	43To09st	52	Hol10kr(The Netherland)
17	24M09a	35	48U109go	53	HolRot10se(The Netherland/Rotterdam)
18	26MhIS10st	36	50M09st	54	LuxM10se(Luxemburg/Center)

PCR products of RAPD, ISSR and SRAP markers were separated by electrophoresis method in Tris boric acid EDTA (TBE) buffer solution by using 2% Agar (Sigma, A5093 Agarose) and PCR products of SSR markers by using 2% High Resolution Agar (LONZA Metaphor Agarose). Then dyed with ethidium bromide (0.5 mg mL⁻¹) and imaged in a gel imaging system (Gel Logic 200 Imaging System) under ultraviolet light and scored as 0 indicating the absence of a band and 1 indicating existence of a band.

Resultant data were analyzed through NTSYS (Numerical Taxonomy Multivariate Analysis System, NTSYS-pc version 2.1, Exeter Software, Setauket, N.Y. USA) software. Similarity indexes were calculated in accordance with Dice (1945) method and dendrograms was created in accordance with UPGMA (Unweighted Pair-Group Method with Arithmetic Average) method (Rohlf 1993). With all these analyses, variation and similarity levels among investigated scab isolates were identified and characteristics of genetic structure were put forth.

3. Results and Discussion

Phylogenetic analyses of 54 isolates were performed by using RAPD, ISSR, SSR and SRAP markers to identify genetic relatedness of apple scab single

spore isolates supplied from different production sites.

With RAPD markers, number of bands per primer was found to be 5.9 and number of polymorphic band was found to be 3.9. The highest number of band (8 bands) was observed in OPG05 and M2 RAPD primers. The greatest number of polymorphic band (7 bands) was observed in M2 primer and polymorphism ratio was identified as 88%. The highest Polymorphism Information Content (PIC) value (0.51) was observed again in OPG05 and M2 RAPD primers and general average was observed as 0.42. RAPD marker-based dendrograms revealed that scab isolates separated into an initial group with a ratio of 0.83 and it was followed by several sub-groups. A distinct difference was not observed in host-specificity and geographic origin (Figure 1). With regard to relativeness, the samples 50M09st and 42BS09st were the closest ones (0.97) to each other. In general, European isolates formed collectively and genotypic similarity was around 0.84.

Melounova et al (2004) identified the genetic similarity with RAPD markers as 79%. The value was quite close to the similarity (88%) observed in this study with RAPD markers. Padder et al (2011) also carried out phylogenetic analyses with RAPD molecular markers and were not able to identify

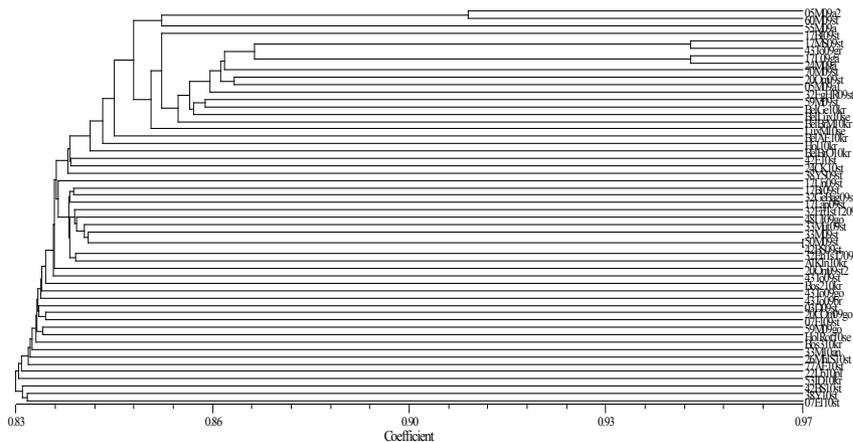


Figure 1- Phylogenetic dendrograms for *Venturia inaequalis* isolated based on RAPD markers

Şekil 1- RAPD markerler kullanılarak Venturia inaequalis için elde edilen filogenetik dendogramlar

distinctive relationships among the groups with regard to geographical and host specificity. Current findings were parallel to those earlier ones.

With ISSR makers, average number of band per primer was found to be 6.2 and average number of polymorphic band was identified as 4.2. The highest number of band was observed in UBC 885 ISSR primer (8 bands) and the highest number of polymorphic band was observed in the same primer (7 bands) and polymorphism was identified as 88%. The greatest Polymorphism Information Content was observed in UBC 887 ISSR primer (0.47) and general average was identified as 0.36. ISSR marker-based dendrograms (Figure 2) revealed that samples were separated into two groups with a ratio of 0.80. The first group was formed only by the isolate taken from Konya-Beyşehir (42BS10st) and the rest formed the second group. The second group was divided into sub-groups with 0.83 similarity ratio and the isolates 50M0st and 42Bs09st, the isolate 24CK10st and German isolate AIKln10kr were identified as close relative with each other with a genetic similarity ratio of 0.98. The use of ISSR markers in identification of genetic diversity in *V. inaequalis* isolates haven't been reported, yet. However, Baysal et al (2009) performed genetic race

separation in *Fusarium oxysporum* f. sp. *lycopersici* by using 6 ISSR primer markers. Researchers obtained 23 bands (average 3.83 bands per primer) with a band widths between 190 bp and 900 bp and identified the separation capacity as $\sum \delta: 1.08 \pm 0.59$. Of the ISSR primers, UBC 880 (1.56), UBC 886 (1.54) and UBC 887 (1.52) were identified as the most effective primers in separation.

Yalim (2005) in a study selected 10 ISSR primers based on polymorphism ratio and produced 75 bands of which 51 were polymorphic. As an indicator of polymorphism level, the researcher identified the highest PIC value as 0.844 and the lowest as 0.354 with an average value of 0.611. In this study, the least PIC value (0.36) was observed in analyses carried out with ISSR markers. Such findings were found to be parallel to those earlier ones. In this marker system, it was observed that variation among genotypes came from a single source and variations were not observed based on geographic or host origin.

With SSR markers, average number of band per SSR primer pair was observed as 1.9 and average number of polymorphic band was observed as 1.7. The highest PIC value (0.71) was observed in 1tc1a and 1tc1b SSR primer pair and general average

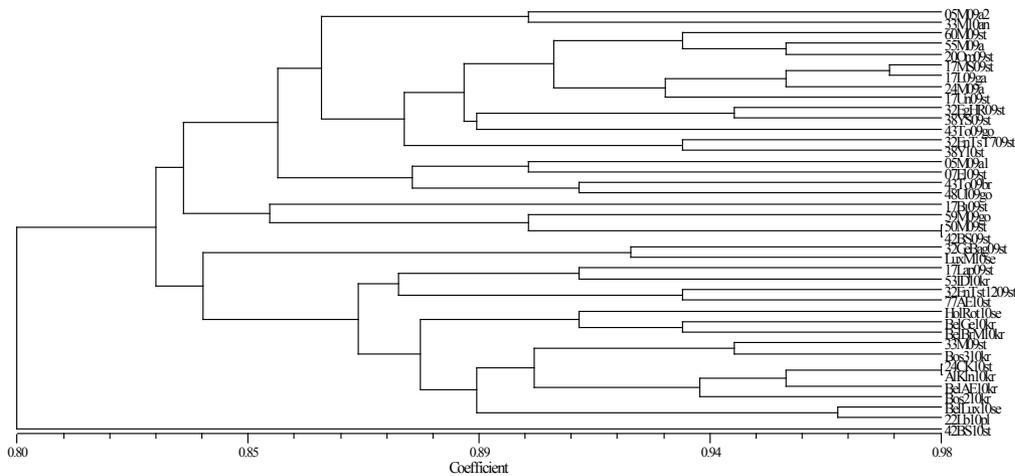


Figure 2- Phylogenetic dendrograms for *Venturia inaequalis* isolated based on ISSR markers

Şekil 2- ISSR markerler kullanılarak *Venturia inaequalis* için elde edilen filogenetik dendogramlar

was observed as 0.56. In SSR-based dendrograms, genetic similarity of entire genotypes varied between 0.57-1.00. The dendrograms (Figure 3) revealed 2 groups with a similarity ratio of 0.74. These two main groups were then separated into 7 sub-groups with a relatedness ratio of 0.81 within themselves. Considering the genetic similarities between the sub-groups, it was observed that even European isolates were placed separately in sub-groups and distinctive differences were not able to be observed between the isolates. Populations were separated better and groups were clearer with SSR markers.

Similarly, Tenzer et al (1999) carried out a study screened 350 *V. inaequalis* isolates collected from 11 European countries with 7 SSR markers. Researchers identified genetic diversity as between 0.52-0.96 and population diversity as between 0.28-0.49. On the other hand, Guérin et al (2004) obtained 21 microsatellite (SSR) markers from *Venturia inaequalis* isolate and analyzed 44 isolates to identify host-pathogen and geographical differences. Each primer pair was found to be polymorphic and average 9.1 alleles (2-24) were obtained from the loci.

Current findings did not reveal distinctive genetic differences among the isolates. The differences

among the isolates were probably because of inner-race variations.

As it was in ISSR primer system, the use of SRAP markers in identification of genetic diversity in *V. inaequalis* isolate haven't been also reported before. However, Baysal et al (2009) carried out genetic race separation of *Fusarium oxysporum* f. sp. *lycopersici* by using 4 SRAP primer markers and reported higher separation capacity ($\Sigma\hat{c}$: 1.28±0.97) for ISSR markers. Phylogenetic dendrograms created based on SRAP markers is presented in Figure 4. Similar to analyses with SSR markers, more clear outcomes were obtained with SRAP markers. The dendrograms revealed 2 main groups with a relatedness ratio of 0.66. Of these groups, the first one included only 1 isolate (17B109st) and the rest were placed in the second group. The second group was then separated into 2 sub-groups with a relatedness ratio of 0.77. The first sub-group was composed of 33M09st and 38Y10st and the samples supplied from Bosnia were placed in the second sub-group with a relatedness ratio of 0.94. The isolates 17MS09st and 24M09a and the isolates from The Netherlands (Hol10kr) and Belgium (BelAE10kr) were found to be the closest isolates with a relatedness ratio of 0.96. A low level variation was observed among the samples and

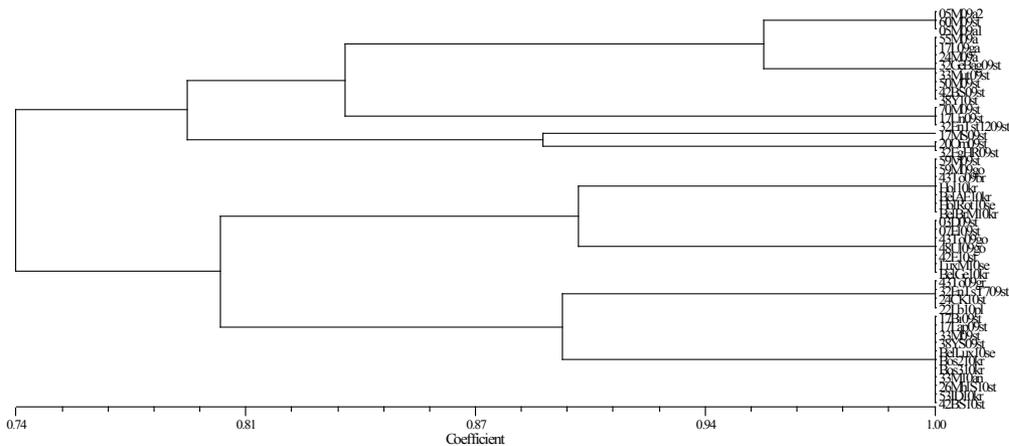


Figure 3- Phylogenetic dendrograms for *Venturia inaequalis* isolated based on SSR markers

Şekil 3- SSR markerler kullanılarak Venturia inaequalis için elde edilen filogenetik dendogramlar

the dendrograms revealed the similarity levels of the genotypes as between 0.54-0.96. The average number of band per primer was observed as 6.5 and number of polymorphic band was observed as 4.8. The highest PIC value was observed in Em5Me10 primer combination (0.76) and general average was observed as 0.54. Current findings were similar to earlier reports of the researchers and SRAP markers were found to be more effective in separation than RAPD and ISSR markers.

Current findings pointed out that the analyses carried out with SRAP markers may reveal a regional relationship. Also, SRAP markers were used for the first time worldwide in molecular analyses of *V. inaequalis* isolates and SRAP markers were found to be quite promising in such analyses.

Boehm et al (2003) investigated the genetic diversity in *V. inaequalis* (Cke.) Wint. populations with neutral markers and indicated that genetic diversity was mostly related to ecological conditions of the places from where isolates supplied in Israel. The researchers identified that the populations supplied from coastal sections of the country were quite uniform and the populations supplied from Golan Heights were quite diverse. The researchers also indicated that this pathogen did not reproduce

well in regions with mild winters and existed in such regions as clonal lineages.

Tenzer & Gessler (1999) investigated the genetic diversity among 11 *V. inaequalis* populations supplied from five European countries through allele frequencies of 18 random amplified polymorphic DNA markers and the internal transcribed spacer region of the ribosomal DNA. The diversity within each population was observed as between 0.26-0.33. The average differentiation among populations was identified as 0.11 and populations were isolated by distance ($r^2=0.50$, $P<0.01$). Such results indicated an extensive short-distance gene flow in Europe and dispersal over longer distances enough to prevent differentiation due to genetic drift (Tenzer & Gessler 1999).

Xu et al (2008) pointed out the significance of identification of pathogen population structure for breeding resistant cultivars. The researchers supplied *V. inaequalis* isolates from China, India and the United Kingdom to investigate the differences in pathogen populations through AFLP markers. The researchers reported significant differences in *V. inaequalis* populations supplied from China and the United Kingdom and insignificant differences in populations supplied from China.

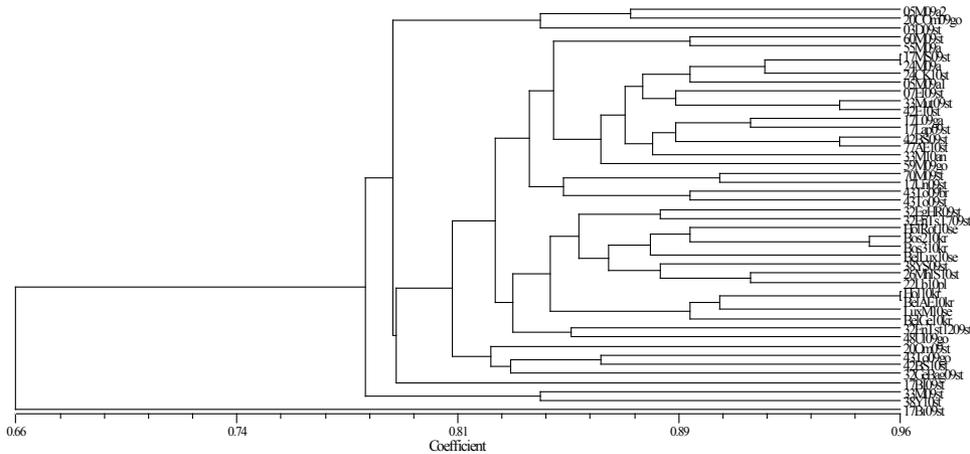


Figure 4- Phylogenetic dendrograms for *Venturia inaequalis* isolated based on SRAP markers

Şekil 4- SRAP markerler kullanılarak *Venturia inaequalis* için elde edilen filogenetik dendogramlar

Guérin & Le Cam (2004) indicated that *V. inaequalis* population structure might be greatly influenced by genetic background of host cultivars. The recent breakdown of the Vf resistance gene by *V. inaequalis* in France allowed the comparison of *V. inaequalis* populations and consequently identification of resistant and susceptible cultivars. The researchers genotyped 133 isolates of *V. inaequalis* from Vf cultivar Judeline and non-Vf cultivars Petit Jaune, Juliana, and Judor from a commercial orchard through AFLPs and four microsatellite loci and indicated significantly lower genetic diversity for *V. inaequalis* populations than for non-Vf cultivars. The researchers also reported that isolates from Vf cultivars were belong to the same clonal lineage.

Guérin & Le Cam (2004) investigated three susceptible apple cultivars and one cultivar carrying the Vf gene by using AFLPs and four microsatellite loci. Xu et al (2008) analyzed *V. inaequalis* populations supplied from different apple cultivars from the UK, China and India and reported significant differences between populations of Asia and Europe based on cluster analysis of more than 100 isolates. The researchers also indicated significant differences among the isolates supplied from three apple cultivars (cvs. Cox, Bramley, Worcester) of a single orchard in the UK. Xu et al (2008) in the same study reported that *V. inaequalis* populations within China (Shaanxi Province) were much more homogenous and no population differentiation was detected on isolates obtained from different cultivars (cvs. Gala, Fuji and Qingquan).

Tenzer et al (1999) used 7 microsatellite markers with (TC)_n and (AAC)_n repeats and tested them on 350 *V. inaequalis* isolates. The researchers reported that three markers with the (TC)_n motif out of seven were highly diverse with Nei's expected genetic diversity value of between 0.52-0.96. Guérin et al (2004) developed 21 new polymorphic microsatellite markers to be used genetic analyses of *V. inaequalis* isolates. All above mention studies were capable of detecting high level of genetic diversity for *V. inaequalis*.

4. Conclusions

ISSR and SRAP markers were used for the first time in this study for molecular characterization of *V. inaequalis* isolates. The groups were better clustered with SSR and SRAP markers and were more informative considering the easy use, repeatability and specificity of the markers.

Chemical control practices may sometimes create new races resistant to fungicides. Commercial cultivars are sensitive to pathogen most of the time since an obstruction is not experienced in spread of disease based on sensitivity of the cultivar, a specificity is not also observed based on the host pathogen (Kaymak et al 2008). Whenever the orchards are established with resistant cultivars, the fungus will experience a powerful obstruction and will try to specialize to adapt itself to changing conditions. Then in time, resistant cultivars will turn into sensitive ones. An effective use of gene sources will allow the identification of new resistance genes and provide significant contributions in control of the fungus.

Considering the entire results obtained from marker system together, it was observed that all of the isolates were separated from each other and a variation was observed among them. The present study is the first study in this subject matter and further more comprehensive and detailed studies will reveal significant information about diversity and distribution of scab races.

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