Original Research Paper

APPLICATION OF SIMPLIFIED-AFLP TO ASSESS GENETIC DIVERSITY OF *Pseudomonas syringae* PV. *syringae* STRAINS ISOLATED FROM RICE AND WHEAT HOSTS

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Abstract

Pseudomonas syringae pv. *syringae* is a plant pathogen bacterium causing bacterial leaf blight in wheat and sheath rot in Rice. In this study, simplified AFLP (Amplified Fragment Length Polymorphism) was tested in order to evaluate genetic diversity of 60 *P.s.* pv. *syringae* strains isolated from wheat and rice fields of Guilan province-Iran by 12 AFLP primers. The products resulting from AFLP were separated using agarose gel electrophoresis. The maximum number of PIC among all tested primers belonged to primer 36. Based on UPGMA method, using NTSISpc. Software, all *P.s.* pv. *syringae* strains were divided to four distinct clusters at 70% similarity level. Genetic analysis of studied populations (pop1: isolates of rice field, pop2: isolates of wheat, pop3: isolates of rice panicle, pop4: isolates of rice nursery) determined that the high genetic similarity was seen among isolates obtained from wheat and rice panicle, while strains isolated from field of rice had the most genetic distance with the other populations. Results of this study showed that AFLP is an effective marker in evaluating genetic diversity within and among studied populations while all of them had the same pathogenesis characteristics.

Keywords: *Pseudomonas syringae* pv. *syringae;* genetic diversity; AFLP; rice; wheat; genetic similarity; genetic distance.

INTRODUCTION

Pseudomonas syringae van Hall (Palleroni 1984) is a plant-associated bacterium species which has been divided into more than 50 pathovars (Young et al., 1992). One of the important pathovar of this bacterium is *Pseudomonas syringae* pv. *syringae* with a very wide host range, which includes economically important crops such as wheat, rice, beans, clover, stone fruits and citrus (Bradbury, 1986). This bacterium causes bacterial leaf blight in wheat and sheath rot in rice (Niknejad Kazempour et al., 2010; Zeigler and Alvarez, 1990). All of the *P.s.* pv. *syringae* strains show a high level of similarity in biochemical and serological tests (Cirvilleri et al., 2005). Nonetheless, there is a kind of host-specialty and genetic diversity among strains, which those general biochemical tests cannot recognize. As regards improvement of using molecular techniques, it seems using molecular techniques rely on PCR can highly assess genetic diversity and host range of different strains of *P.s.* pv. *syringae.* Various molecular techniques have been used to characterize *P.s.* pv. *syringae* strains. AFLP is a genomic fingerprinting method with an effective taxonomic resolution from species to strain level (VanDamme et al., 1996) and has been used to study the taxonomy and genetic diversity of a number of organisms including bacteria (Coenye et al., 1999; Jassen et al., 1996; Janssen et al., 1997; Keim et al., 1997; Rademaker et al., 2000; Sławiak et al., 2005). Clerc et al. (1998) used both simplified-AFLP and RAPD techniques to discriminate *Pseudomonas syringae* pathovars (Genospecies ш). They reported that AFLP method was more efficient for assessing intrapathovar diversity than RAPD analysis and allowed clear delineation between intraspecific and interspecific genetic distances. Sławiak et al. (2005) found that Simplified-AFLP analysis seems to be the method, which is more effective and easier to perform than PCR-RFLP for the genetic differentiation of *Pseudomonas* species and pathovars. Cirvilleri et al. (2006) showed that the fAFLP analysis revealed a high genetic heterogencity in the *P.s.* pv. *syringae* strains and variability was observed among isolates from the same host plants as well as among isolates within the same antagonistic group or with similar pathogenic activity. In this study, genetic diversity of 60 *P.s.* pv. *syringae* strains isolated from wheat and rice field of Guilan province-Iran was evaluated by Simplified AFLP to determine if there were differences that may be related to the host species or site of sampling on plant tissues and weather condition where the strains were isolated.

Table 1: strains of *P. syringae* pv. *syringae* used in this study

strain	Host of origin	Sampling site	Area of origin	Year of sampling	Population num.	cultivar	Strain	Host of origin	Area of origin	Year of isolation	Population num.
PRF1	Rice	Field	Rasht	2005	1	Alikazemi	PW1	Wheat	Roudbar	2005	2
PRF2	Rice	Field	Rasht	2005	1	Alikazemi	PW2	Wheat	Roudbar	2005	2
PRF3	Rice	Field	Lahijan	2005	1	Alikazemi	PW3	Wheat	Roudbar	2005	2
PRF4	Rice	Field	Roodsar	2005	1	Alikazemi	PW4	Wheat	Siahkal	2005	2
PRF5	Rice	Field	Astane	2005	1	Alikazemi	PW5	Wheat	Siahkal	2005	2
PRF6	Rice	Field	Astane	2005	1	Hashemi	PW6	Wheat	Siahkal	2005	2
PRF7	Rice	Field	Kalachay	2005	1	Hashemi	PW7	Wheat	Roudbar	2005	2
PRF8	Rice	Field	Kalachay	2005	1	Khazar	PW8	Wheat	Amalsh	2006	2
PRF9	Rice	Field	Kouchesfahan	2005	1	Khazar	PW9	Wheat	Amalsh	2006	2
PRF10	Rice	Field	Kouchesfahan	2005	1	Khazar	PW10	Wheat	Talesh	2006	2
PRF11	Rice	Field	Kalachay	2005	1	Khazar	PW11	Wheat	Talesh	2006	2
PRP1	Rice	Panicle	Astane Astane	2006	3	Hashemi	PW12	Wheat	Astara	2005	2
PRP2	Rice	Panicle	Astane	2006	3	Khazar	PW13	Wheat	Astara	2005	2
PRP3	Rice	Panicle	Astane	2006	3	Khazar	PW14	Wheat	Astara	2005	2
PRP4	Rice	Panicle	Bandarkiashahr	2006	3	Khazar	PW15	Wheat	Roudbar	2005	2
PRP5	Rice	Panicle	Bandarkiashahr	2006	3	Khazar	PW16	Wheat	Roudbar	2005	2
PRP6	Rice	Panicle	Bandarkiashahr	2006	3	Alikazemi	PW17	Wheat	Siahkal	2005	2
PRP7	Rice	Panicle	Bandarkiashahr	2006	3	Alikazemi	PW18	Wheat	Siahkal	2005	2
PRP8	Rice	Panicle	Lahijan	2006	3	Alikazemi	PW19	Wheat	Amalsh	2006	2
PRP9	Rice	Panicle	Lahijan	2006	3	Alikazemi	PW20	Wheat	Roudbar	2006	2
PRP10	Rice	Panicle	Rasht	2006	3	Alikazemi	PW21	Wheat	Roudbar	2006	2
PRP11	Rice	Panicle	Rasht	2006	3	Alikazemi	PW22	Wheat	Roudbar	2006	2
PRP13	Rice	Panicle	Hasanrood	2006	3	Khazar					
PRP14	Rice	Panicle	Anzali	2006	3	Hashemi					
PRP15	Rice	Panicle	Anzali	2006	3	Hashemi					
PRP16	Rice	Panicle	Foman	2006	3	Hashemi					
PRN1	Rice	Nursery	Rasht	2005	4	Hashemi					
PRN2	Rice	Nursery	Rasht	2005	4	Hashemi					
PRN3	Rice	Nursery	Lahijan	2005	4	Hashemi					
PRN4	Rice	Nursery	Roodsar	2005	4	Hashemi					
PRN5	Rice	Nursery	Astane	2005	4	Hashemi					
PRN6	Rice	Nursery	Astane	2005	4	Alikazemi					
PRN7	Rice	Nursery	Kalachay	2005	4	Alikazemi					
PRN8	Rice	Nursery	Kalachay	2005	4	Alikazemi					
PRN9	Rice	Nursery	Kouchesfahan	2005	4	Alikazemi					
PRN10	Rice	Nursery	Kouchesfahan	2005	4	Alikazemi					
PRN11	Rice	Nursery	Kalachay	2005	4	Khazar					
PRN12	Rice	Nursery	Kalachay	2005	4	Khazar					

MATERIALS AND METHODS

Bacterial strains

The 60 *P.s.* pv. *syringae* strains used in this study were isolated and identified from wheat and rice fields of Guilan province-Iran during 2005 and 2006 (Assemaninejad et al., 2008; Kheyrgo et al., 2008; Khoshkdaman et al., 2008) (Table 1). Bacterial strains were grown in nutrient agar (NA) media, at 28 °C. For long-term storage, bacteria were kept at -80 °C in Lauria Pepton (LP) medium mixed with 30% glycerol.

DNA extraction and Simplified- AFLP

For bacterial DNA extraction, the isolates were grown overnight, in nutrient broth (Merck, Darmstadt, Germany), at 26 °C and the DNA was extracted as described by Ausubel et al. (2005). The simplified-AFLP method was based on AFLP method described by Clerc et al. (1998). In this study the *MspI* endonuclease and discriminating primers with three additional nucleotides were used, which allowed selective amplification of an average of four to seven products. To prepare temple DNA, bacterial DNAs were digested with *MspI* and were ligated to adaptors in a single step. Adaptors were constructed with a 22-bp oligonucleotide. In order to obtain double-stranded 11-bp oligonucleotides complementary to the *MspI* sites, the adaptors were digested with *TaqI* endonuclease. Original *MspI* sites were not restored after the adaptors were ligated which prevented digestion of ligation products. *MspI* restriction fragments tagged with the specific adaptors were used as template DNAs for selective PCR amplification directed by single 16-bp primers (Table 2) with constant peak complementary to the adaptor sequence and the *MspI* site and a 3-nucleotide variable portion at the 3´-OH end.

Table 2. selected primers used for the AFLP analysis

Primer	Sequence
3	5´CCAGGATCCTCGGCCA3´
4	5´CCAGGATCCTCGGCCT3´
7	5´CCAGGATCCTCGGCAG3´
8	5´CCAGGATCCTCGGCAA3´
10	5´CCAGGATCCTCGGCTC3´
12	5´CCAGGATCCTCGGCTA3´
15	5´CCAGGATCCTCGGCGG3´
18	5´CCAGGATCCTCGGAGC3´
35	5´CCAGGATCCTCGGGAG3´
36	5´CCAGGATCCTCGGGAA3´
38	5´CCAGGATCCTCGGGTC3´
42	5´CCAGGATCCTCGGGGC3´

PCR and electrophoresis

Amplification was carried out in a 25 µl volume in 0.5 ml micro tube using a Hybaid programmable thermal controller. Each 25 µl PCR reaction mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl2, 0.2 mM of each nucleotide (dATP,dCTP, dGTP, and dTTP), 0.25 µM of each primer, 100 ng DNA and 2 U of *Taq* DNA polymerase (Promega Corp., Madison, WI). DNA amplification was carried out in a PTC-100 programmable DNA thermal cycler (MJ Research, Watertown MA). The amplification was performed for *P. s*. pv. *syringae* as follows: initial denaturation at 93 °C for 3 min; 37 cycles of 93 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min; and 3 min extension at 72 °C. For *P. s*. pv. *syringae*: initial denaturation at 93 °C for 1 min; 37 cycles of 93 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min; and 5 min extension at 72 °C. Amplified DNA fragments were examined by horizontal electrophoresis in 2% agarose gel in TBE buffer with 10 µl aliquots of PCR products (Clerc et al., 1998). Gels were stained with ethidium bromide and were photographed under UV light (312 nm), see Fig. 1.

Data analysis

Each amplification band was treated as a unit character and was scored as 1 (present) or 0 (absent) for all strains. The NTSYSpc ver**.** 2**.**02e software was used to calculate a similarity matrix, using Simple match's coefficient of similarity. Cluster analysis was done with the unweighted pair group method with averages (UPGMA). In order to assess genetic identity and distance between major groups (Pop 1: strains isolated from Rice fields, Pop 2: strains isolated from wheat, Pop3: strains isolated from Rice panicle, Pop 4: strains isolated from Rice nursery), the PopGene ver. 1.31 software was used. Genetic diversity within and among pathovars with analysis of molecular variance was determined by GeneAlex ver. 6.2.

RESULTS

Diversity indexes

In this study, we assessed the genetic diversity of 60 *P.s.* pv. *syringae* strains by AFLP. The number of scorable DNA bands observed after electrophoresis of PCR products obtained from 12 primers was 288 which all of them were polymorphic. Absence of monomorphic bands and the large number of polymorphic amplified fragments indicates considerable differences among studied strains (Maurilio et al., 2006). The maximum (30) and minimum

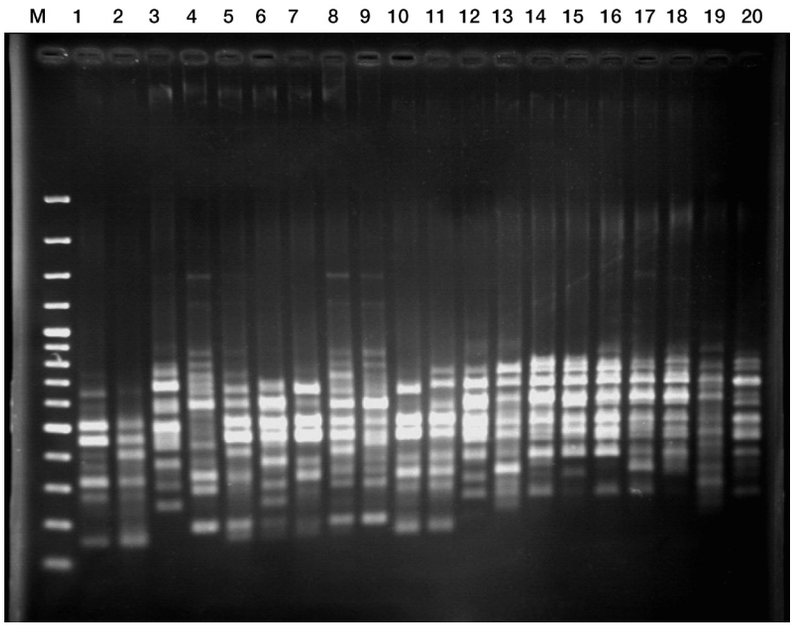

Figure 1:. Agarose gel electrophoresis of the AFLP products obtained for *P. syringae* pv. *syringae* strains by primer 12. (M (Ladder100bp.)، 1 (PW13)، 2 (PW14)، 3 (PW15)، 4 (PW16)، 5 (PW17)، 6 (PPW18)، 7 (PW19)، 8(PW20)، 9 (PW21)، 10 (PW22)، 11 (PRP1)، 12 (PRP2)، 13 (PRP3)، 14 (PRP4)، 15 (PRP5)، 16 (PRP6)، 17 (PRP7)، 18 (PRP8)، 19 (PRP9 and) 20(PRP10.

Table 3: Frequency of amplified loci in primers

Primer	Number of polymorphic bands	Minimum Frequency	Maximum Frequency	Mean Frequency
3	21	0.5	0.967	0.788
4	28	0.5	0.966	0.788
7	23	0.5	0.966	0.789
8	27	0.5	0.967	0.792
10	23	0.5	0.963	0.784
12	22	0.5	0.967	0.788
15	23	0.521	0.968	0.786
18	22	0.5	0.964	0.784
35	26	0.5	0.967	0.788
36	24	0.5	0.962	0.781
38	19	0.5	0.964	0.787
42	30	0.516	0.967	0.787

Table 4: Value of PIC and H

Primer	Number of polymorphic bands	PIC	H
3	21	0.241892	0.240714
4	28	0.295833	0.27175
7	23	0.302729	0.302726
8	27	0.196008	0.195996
10	23	0.45599	0.332509
12	22	0.363737	0.363727
15	23	0.31378	0.290313
18	22	0.431843	0.323836
35	26	0.36735	0.367338
36	24	0.489792	0.320729
38	19	0.440117	0.357289
42	30	0.380944	0.337517

Table 5: Mean of na, ne, I and Nei calculated for populations

Population	Observed number of alleles (na)	Effective number of alleles (ne)	Shannon's Information index (I)	Nei gene diversity (Nei)
Pop. 1	1.232	1.351	0.302	0.203
Pop. 2	1.86	1.489	0.44	0.29
Pop. 3	1.61	1.408	0.373	0.245
Pop. 4	1.035	1.213	0.205	0.133

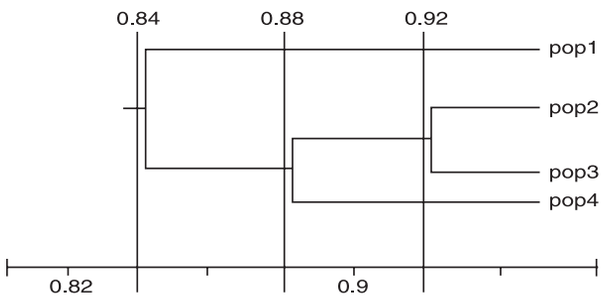

Figure 2: Dendrogram obtained from cluster analysis of populations according to Nei similarity coefficient by PopGene software (pop1: isolates obtained from rice field, pop2: isolates from wheat, pop3: isolates from rice panicle and pop4: isolates from rice nursery)

(19) number of polymorphic bands were seen in primer 42 and 38, respectively (Table 3). PIC (polymorphic information content), one of the important index for determining effectiveness of primes, was calculated in all primers (Table 4). The minimum number of PIC (0.196) was observed in primer 8, while the maximum number of it (0.49) belonged to primer 36. Moreover, Heterozygosity (H), the other index to determine genetic variation among strains, was calculated for each primer. Like PIC value, the minimum amount of H (0.196) belonged to primer 8. Nonetheless, the maximum number of H was observed in primer 35.

Furthermore, Effective number of alleles, Observed number of alleles (Kimura and Crow 1964), Nei gene diversity (Nei 1973) and Shannon's Information index (Lewontin 1974), considered as genetic variation indexes in asexual propagation, were shown (Table 5). The results showed that the maximum and minimum number of the effective number of alleles (ne), Observed number of alleles (Na), Shannon's Information index (I) and Nei gene diversity (Nei) were seen in population 2 (isolates from wheat) and population 4 (isolates from nursery of rice), respectively.

Cluster analysis

Cluster analysis of the four studied populations based on genetic identity (Nei 1973) determined that strains within population 1 (isolated from rice field) showed most genetic distance or least genetic identity with the other 3 populations. In addition, between all strains isolated from rice and wheat, the most genetic identity was seen between strains isolated from wheat and rice panicle (pop2 and pop3), whereas the least genetic identity was observed between isolates from field and nursery of rice (Fig. 2). Moreover cluster analysis of populations according to site of isolation, the other one was performed for all studied strains. Dendrogram was constructed from cluster analysis by using the simple match similarity coefficient and UPGMA (unweighted pair group method with average) method at 70% similarity level which clustered all strains into 4 groups (Fig. 3). Cluster I included strains isolated from rice field, cluster II isolates from wheat, cluster III isolates from rice panicle and nursery of rice and cluster IV included only stain PW12 which was isolated from wheat. Transferring of bacteria from nursery to panicle by seed and seedling probably was the cause of observing nursery strains among panicle strains, but these two groups were separated at 81% similarity level. Also, some strains isolated from wheat were clustered with isolates from rice field and separated into 2 subclusters at 75% similarity level. The value of coffinetic coefficient was 0.9 indicating a very good fit of the clustering with the original similarity matrix. Moreover genetic variation among and within populations were calculated. The value of diversity within and among populations was 17% and 83%, respectively (Table 6).

DISCUSSION

Regarding the importance of the PIC index in polymorphic information content value of primers, the maximum number of the PIC is shown by primer 36, thus this primer has more ability to distinguish genetic diversity between strains than the other primers, while in a study by Sławiak et al. (2005), which used the same primes as in our study, primer 8 was selected as the most suitable primer for *Pseudomonas* sp. analysis. Thus AFLP marker has various applications in bacterial studies.

Generally, the result of H and PIC must be similar which is approximately seen in this study. But there was

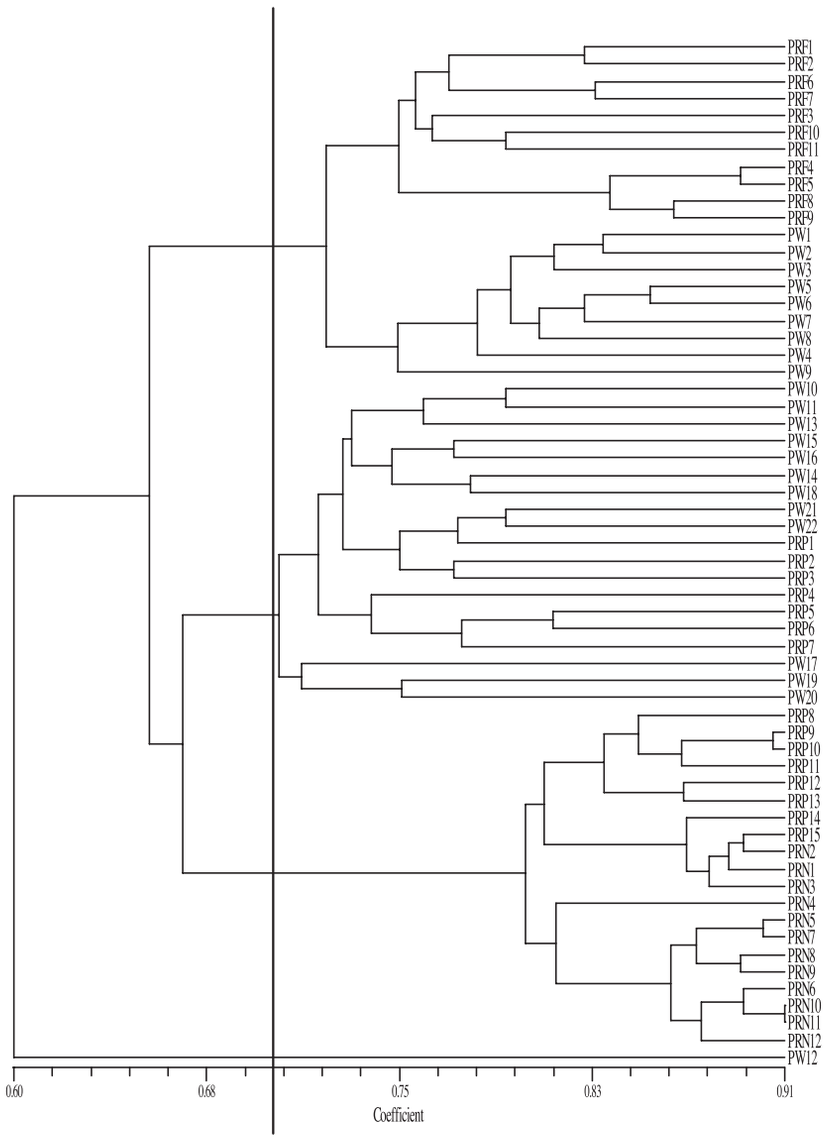

Figure 3: Dendrogram based on simplified-AFLP data of 60 *P. syringae* pv. *syringae*. Dendrogram was constructed by using UPGMA method and simple match similarity coefficient

an inconsistency in maximum number of H and PIC. The maximum number of PIC relates to primer 36 while the maximum number of the H is observed in primer 35. This can be related to the method of calculating these indexes. Due to the fact that in the most loci related to primer 36, the number of strains with bands compared to those without bands was almost equal. Therefore according to PIC formula, the amount of PIC value is high in this primer. In contrast to PIC, the number of strains with and without bands has no significant influence on heterozygosity value. Our results therefore support the idea that, primer 36 and primer 35 among the studied primers were the most effective primers according to PIC and H, respectively.

Table 6: Analysis of molecular variance of simplified-AFLP data related to 4 populations of 60 *P. syringae* pv. *syringae* strains

Variance %	Est. Var.	MS	SS	df	Source
83%	8.446**	155.578	466.735	3	Among Pops
17%		32.363	1812.332	56	Within Pops
100%			2279.067	59	Total

**: Significant difference at 1% level.

Probably different weather conditions at the area under wheat culturing compared to rice culturing is the main reason for the highest value of Nei, ne, na and I indexes in population 2. In fact, strains obtained from wheat were isolated from both humid and dry areas with different growing conditions, as regards better bacterial growth in humid conditions; thus variation between strains was due to genetic changing because of consistency to dry weather. Sisto et al. (2007) reported that strains from the same geographical regions are, in the majority of cases, genotypically more closely related to one another than those isolated from different geographical regions.

The high level of genetic similarity between isolates from wheat and panicle of rice may be explained by two hypotheses: (i) growing conditions of these strains were similar. Generally, rice crop in anthesis stage is in dry conditions; thus strains isolated from mature panicle had growing conditions the same as strains isolated from wheat grown in upland conditions; (ii) number of strains in both populations is higher than in the other populations. Overall, high genetic similarity (most strain pairs showed genetic identity between 0.6-0.7) among strains could be due to some reasons. First of all, the studied strains were isolated from a restricted geographical region, as concerns one of the important factors in population variation especially in microorganisms including fungi and bacteria is geographical distance and separation, therefore high genetic similarity among strains in this study is not unlikely and it may be the result of a recent adaptation to a specialized niche (Guilan province) (Little et al., 1998). Despite our result, the other factor is host specifity in *P.s.* pv. *syringae* strains.

Cirvilleri et al. (2006) reported that there was no strict correlation between the strain and the host of the origin. Accordingly, high genetic similarity between isolates from wheat and rice which were grown in closely same weather condition is not unexpected. In fact, this apparent high diversity might be expected in an area with a long history of cultivating rice and wheat species, presumably the associated microflora would have evolved with and adapted to the various rice and wheat hosts over time (Little et al., 1998).

In conclusion, dendrogram constructed from AFLP data could not separate strains according to their host origin which it may be due to effect of weather condition on strain genotype.

The isolation of strains from limited area, same host (wheat or rice) in special growth stage and the few in number of strains within populations are presumably the reasons of low level of variation within populations. In addition, the homogeneity within the host population is reflected in the pathogen population (Restrepo et al., 1999). In contrast, high level of diversity among populations may be explained by 2 reasons. First, the isolation sites (wheat and rice) were varied in both hosts. Second, changing of genotype may be occurred due to distinct weather conditions at collection sites (Kolliker et al., 2006).

This study has confirmed that genetic variability exists within *P.s.* pv. *syringae* strains which is isolated from same host plants or with same antagonistic characters that revealed effectiveness of this marker.

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