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DIFFERENTIATION BY SIMPLIFIED AFLP OF *PSEUDOMONAS SYRINGE* PV. *SYRINGAE* ISOLATES FROM FIELDS, PANICLES AND NURSERIES OF THE GUILAN PROVINCE – IRAN

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Abstract: *Pseudomonas syringae* pv. *syringae* is a plant pathogen bacterium in rice which causes bacterial sheath rot. In this study, simplified AFLP (Amplified fragment length polymorphism) was tested in order to evaluate genetic diversity of 38 *Pseudomonas syringae* pv. *syringae* strains isolated from rice fields of the Guilan province, by 12 AFLP primers. The products resulted from AFLP were separated using agarose gel electrophoresis. The maximum number of PIC among all tested primers belonged to primer 36. Based on the Unweighted Pair Group Method with Arithmetic (UPGMA) method, using NTSISpc Software, all *Pseudomonas syringae* pv. *syringae* strains were divided into three distinct clusters which had a 70% similarity level. Cluster analysis of studied populations (isolates of fields, panicles, and nurseries) determined that a high genetic identity could be seen among isolates obtained from the panicles and nurseries, while strains isolated from the fields and nurseries had the most genetic distance with each other. The result of this study showed that sampling site and weather conditions play an important role in genetic evolution of strains. It was also found that AFLP is an effective marker in evaluating genetic diversity within and among isolates being studied, while all of them had the same host and pathogenesis characteristics.

Key words: genetic diversity, simplified AFLP, site of sampling, weather conditions, genetic distance

INTRODUCTION

Rice is an economically important cereal crop providing food for more than half of the world's population (Tae soon *et al.* 2008). Bacterial sheath rot caused by *Pseudomonas syringae* pv. *syringae* Van Hall (*Pss*) was first reported by Klement (1955) in Hungary, as a new rice bacterial disease affecting the panicle leaf sheath. Khoshkdaman *et al.* (2008) identified causal agent of sheath rot of rice based on morphological, phenotypical, nutritional characteristics, pathogenicity tests and polymerase chain reaction (PCR) using such specific primers as *P. syringae* pv. *syringae,* in the Guilan province of Iran. As reported by Cirvilleri *et al*. (2005), all of the *Pss* strains showed a high level of similarity in biochemical and serological tests. Nonetheless, there was a kind of host-specificity and genetic diversity among strains which those general biochemical tests could not recognize. Hildebrand *et al*. (1982) reported that the general biochemical tests could not separate isolates at the pathovar or intrapathovar level. Kałużna *et al*. (2009) based on repetitive-polymerase chain reaction (REP-PCR) analysis, reported that *Pss* is a heterogenic pathovar. Civilleri *et al*. (2006) showed that the fAFLP analysis revealed a high genetic heterogeneity in the *Pss* 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. Various molecular techniques have been used to characterize *Pss* strains. Recently, AFLP has been applied to study epidemiological and taxonomical perspectives of microorganisms and differentiate genera, species, and even strains at the pathovar level (Jaimes *et al*. 2006). Clerc *et al*. (1998) used both simplified-AFLP and Random Amplified Polymorphism DNA (RAPD) techniques to discriminate *Pseudomonas syringae* pathovars (Genospecies III). They reported that simplified AFLP method was more efficient to assess intrapathovar diversity than RAPD analysis and allowed clear delineation between intraspecific and interspecific genetic distances. Slawiak *et al*. (2005) found that simplified AFLP analysis seems to be a better, more effective, and easier method to perform than PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) for the genetic differentiation of *Pseudomonas* species and pathovars. Studies on genetic variability of phytopathogenic bacteria are important to elucidate

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possible relationships between certain populations of pathogen and the area from where they were originally isolated (Scortichini 2005). In this study, the genetic diversity of 38 Pss strains isolated from rice fields of the Guilan province, Iran, was evaluated by simplified AFLP. The evaluation was done to determine if there were differences that may be related to the site of sampling or growth stage of rice during sampling and weather conditions where the strains were isolated.

MATERIALS AND METHODS

Bacterial strains

The 38 *Pss* strains used in this study were isolated and identified from rice fields of the Guilan province, Iran, during the 2005 and 2006 time period (Khoshkdaman *et al.* 2008). The bacterial strains used in this study are listed in table 1. Bacterial strains were grown in NA (nutrient agar) media, at 28°C, for long-term storage. Bacteria were kept at –80°C in LP medium mixed with 30% glycerol.

Table 1. Strains of *P. syringae* pv. *syringae* isolated from rice fields, panicles and nurseries of the Guilan province in Iran

Number of strain	Strain	Host of origin	Sampling site	Area of origin	Year of sampling	Cultivar
1	PRF1	rice	field	Rasht	2005	Alikazemi
2	PRF2	rice	field	Rasht	2005	Alikazemi
3	PRF3	rice	field	Lahijan	2005	Alikazemi
4	PRF4	rice	field	Roodsar	2005	Alikazemi
5	PRF5	rice	field	Astane	2005	Alikazemi
6	PRF6	rice	field	Astane	2005	Hashemi
7	PRF7	rice	field	Kalachay	2005	Hashemi
8	PRF8	rice	field	Kalachay	2005	Khazar
9	PRF9	rice	field	Kouchesfahan	2005	Khazar
10	PRF10	rice	field	Kouchesfahan	2005	Khazar
11	PRF11	rice	field	Kalachay	2005	Khazar
12	PRP1	rice	panicle	Astane Astane	2006	Hashemi
13	PRP2	rice	panicle	Astane	2006	Khazar
14	PRP3	rice	panicle	Astane	2006	Khazar
15	PRP4	rice	panicle	Bandarkiashahr	2006	Khazar
16	PRP5	rice	panicle	Bandarkiashahr	2006	Khazar
17	PRP6	rice	panicle	Bandarkiashahr	2006	Alikazemi
18	PRP7	rice	panicle	Bandarkiashahr	2006	Alikazemi
19	PRP8	rice	panicle	Lahijan	2006	Alikazemi
20	PRP9	rice	panicle	Lahijan	2006	Alikazemi
21	PRP10	rice	panicle	Rasht	2006	Alikazemi
22	PRP11	rice	panicle	Rasht	2006	Alikazemi
23	PRP13	rice	panicle	Hasanrood	2006	Khazar
24	PRP14	rice	panicle	Anzali	2006	Hashemi
25	PRP15	rice	panicle	Anzali	2006	Hashemi
26	PRP16	rice	panicle	Foman	2006	Hashemi
27	PRN1	rice	nursery	Rasht	2005	Hashemi
28	PRN2	rice	nursery	Rasht	2005	Hashemi
29	PRN3	rice	nursery	Lahijan	2005	Hashemi
30	PRN4	rice	nursery	Roodsar	2005	Hashemi
31	PRN5	rice	nursery	Astane	2005	Hashemi
32	PRN6	rice	nursery	Astane	2005	Alikazemi
33	PRN7	rice	nursery	Kalachay	2005	Alikazemi
34	PRN8	rice	nursery	Kalachay	2005	Alikazemi
35	PRN9	rice	nursery	Kouchesfahan	2005	Alikazemi
36	PRN10	rice	nursery	Kouchesfahan	2005	Alikazemi
37	PRN11	rice	nursery	Kalachay	2005	Khazar
38	PRN12	rice	nursery	Kalachay	2005	Khazar

Deoxyribonucleic acid (DNA) extraction

Initially, suspension of each strain was streaked on nutrient agar (NA) medium and kept at 28°C. After 24 hours, the grown colonies of bacterium were used for DNA extraction. Bacterial DNA was extracted according to the CTAB (Cetyl Trimethyl Ammonium Bromide) method described by Ausubel *et al*. (1987).

Simplified AFLP

The simplified AFLP method was based on the AFLP method described by Clerc *et al*. (1998). All template DNAs were amplified by single 16-bp primers
(primer 3: 5′CCAGGATCCTCGGCCA3′
primer 4: 5′CCAGGATCCTCGGCCT3′
primer 7: 5′CCAGGATCCTCGGCAG3′
primer 8: 5′CCAGGATCCTCGGCAA3′
primer 10: 5′CCAGGATCCTCGGCTC3′
primer 12: 5′CCAGGATCCTCGGCTA3′
primer 15: 5′CCAGGATCCTCGGCGG3′
primer 18: 5′CCAGGATCCTCGGAGC3′
primer 35: 5′CCAGGATCCTCGGGAG3′
primer 36: 5′CCAGGATCCTCGGGAA3′
primer 38: 5′CCAGGATCCTCGGGTC3′ and
primer 42: 5′CCAGGATCCTCGGGGC3′)
with constant peak complementary to the adaptor sequence and the *MspI* site and a 3-nucleotide variable portion at the 3′-OH end.

Electrophoresis

Amplified DNA fragments were examined by horizontal electrophoresis in 1.5% agarose gel in TBE buffer with PCR products. Gels were stained with ethidium bromide and were photographed under UV light (312 nm).

Data analysis

Each amplification band was treated as a unit character, and 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 using the unweighted pair group method with averages (UPGMA). 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 calculated. In order to assess genetic identity and distance between major groups, 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

In this study, we assessed the genetic diversity of 38 *Pss* strains by AFLP. The number of scorable DNA bands observed after electrophoresis of PCR products obtained with 12 primers was 233, of which 222 were polymorphic. The maximum (25) and minimum (16) number of polymorphic bands was seen in primer 35 and 10, 38, respectively (Table 2).

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′

Diversity indexes

Polymorphic information content (PIC), one of the most important indexes to determine the effectiveness of primes, was calculated for all of them. The minimum number of PIC (0.2162) was observed in primer 15, while the maximum number (0.3505) belonged to primer 36. Moreover, Heterozygosity (H), another index to determine the genetic variation among strains, was calculated for each primer. The minimum amount of H (0.2162) which belonged to primer 15, was the same as the PIC value. But the maximum number of H (0.34) was observed in primer 42 (Table 2).

The results from genetic variation indexes showed 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 PssF (isolates from the fields) and population PssN (isolates from the nurseries), respectively (Table 3).

Table 3. Value of Polymorphic Information Content (PIC) and Heterozigosity (H)

Primer	Number of polymorphic bands	PIC	H
3	19	0.2537	0.2537
4	22	0.2711	0.2685
7	19	0.2878	0.2877
8	17	0.2224	0.2224
10	16	0.3229	0.2905
12	21	0.2966	0.2966
15	20	0.2162	0.2162
18	17	0.3038	0.2615
35	25	0.2868	0.2868
36	20	0.3505	0.33
38	16	0.3260	0.32
42	20	0.3486	0.34

Table 4. Mean of observed number of alleles (Na), effective number of alleles (Ne) and Shannon's Information index (I) calculated for populations

Population	Number of members	Na	Ne	I
PssF	11	1.664	1.428	0.359
PssP	15	1.422	1.379	0.34
PssN	12	0.996	1.211	0.19

Table 5. Analysis of molecular variance of simplified-AFLP data related to 4 populations of 60 P. syringae pv. syringae strains

Source	df	SS	MS	Obs. Variance [%]
Among Pops	2	326.862	163.431	86
Within Pops	35	919.743	26.278	14
Total	37	1246.605		100

df: degrees of freedom; SS: sum of square; MS: mean of square; Obs. Variance%: percentage of observed variance

Cluster analysis

Cluster analysis of the three studied populations based on genetic identity (Nei 1973) determined that the most genetic distance or the least genetic identity were seen between panicle and nursery populations. In contrast, the least genetic distance or the most genetic identity was observed between field and nursery populations (Fig. 1).

In addition to cluster analysis of populations based on the isolation site (fields, panicles and nurseries), another analysis was performed for all the studied strains. A dendrogram obtained from cluster analysis by using the Simple Match similarity coefficient and UPGMA method at a 70% similarity level, was drawn. All strains were divided into 3 groups. Cluster I included only strains isolated

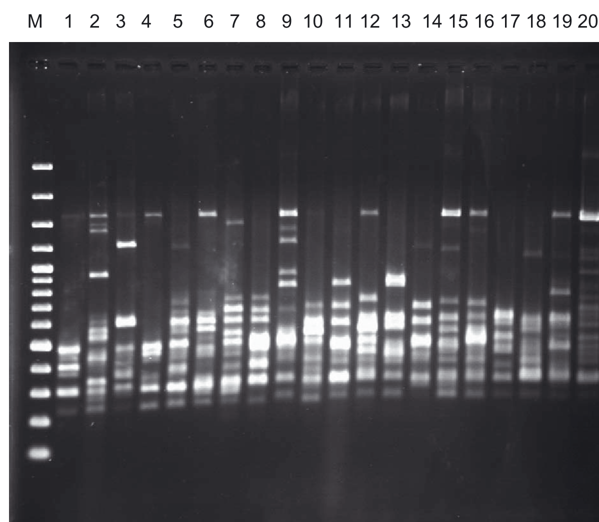
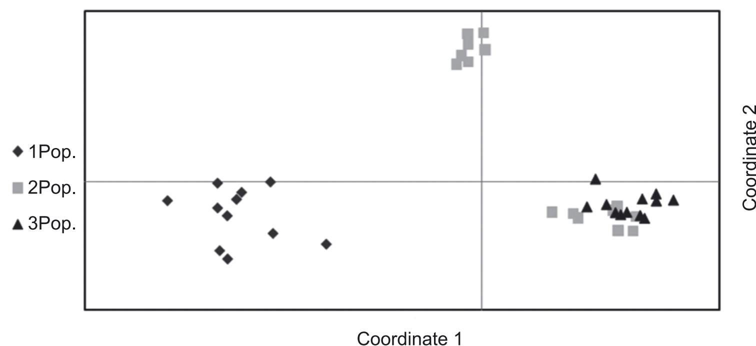


Fig 1. Agarose gel electrophoresis of the AFLP products obtained for *P. syringae* pv. *syringae* strains by primer 12. M(100bp DNA ladder), 1(PW10), 2 (PW11), 3 (PW12), 4 (PW13), 5 (PW14), 6 (PW15), 7 (PW16), 8 (PW17), 9 (PW18), 10 (PW19), 11 (PW20), 12 (PW21), 13 (PW22), 14 (PRP1), 15 (PRP2), 16 (PRP3), 17 (PRP4), 18 (PRP5) , 19 (PRP6), 20 (PRP7)



The plot of the first two principle coordinates

Fig. 2. Principal component analysis (PCA) using GeneAlex (ver. 6.2) based on three populations; fields (Pop.1), panicles (Pop. 2) and nurseries (Pop. 3)

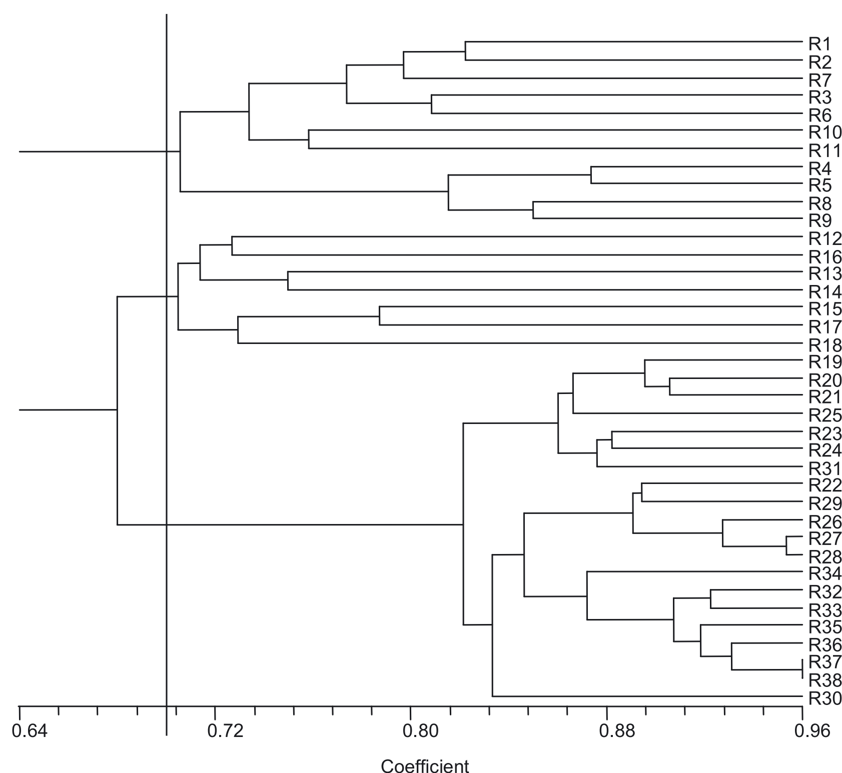


Fig. 3. Dendrogram based on simplified-AFLP data of 60 *P. syringae* pv. *syringae*. Dendrogram was constructed by using UPGMA method and simple match similarity coefficient

from rice fields, cluster II isolates only from panicles, and cluster III isolates from panicles and nurseries (Fig. 2).

Molecular variance

Genetic variation among and within populations was also calculated. The value of diversity within and among populations was 14% and 86%, respectively.

DISCUSSION

In this study, the ability of the AFLP technique for molecular separation of *P. syringae* pv. *syringae* isolates of rice was assessed based on the sampling site. The large number of polymorphic amplified fragments indicates considerable differences among studied strains (Maurilio *et al*. 2006)

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. In a study by Slawiak *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. This difference in suitable primers means 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 an inconsistency in the maximum number of H and PIC. The maximum number of PIC relates to primer 36, while the maximum number of H is observed in primer 42. This finding can be related to the method of calculating these indexes. Due to the fact that, in most loci related to primer 36, the number of strains with bands compared to those without bands, which was almost equal. Therefore, according to the 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 42 among the studied primers, were the most effective primers according to PIC and H, respectively.

Based on the obtained results from diversity indexes, the reason for the highest value of Nei, Ne, Na and I indexes in population PssF may be exposure to different weather conditions than the nursery and panicle isolates. Ochiai *et al*. (2000) noted that clustering of *Xoo* strains is partially associated with climatic conditions. Strains of fields were isolated, as well, from different rice tissues during the long growth term of the plant. Modern rice varieties and intensive growing of modern rice cultivars in fields may also play an important role in pathogen variation as reported by Kosawang *et al*. (2006). However, in rice nurseries, there is a microclimate with stable condition (high humidity and medium temperature). These factors provide a good term for bacterial growth. In such condition, genetic variation may occur in low levels. Further, despite the population field, isolation in the nurseries took place during the short growth term, and only from leaf tissue. The above information proves that changing of genotype may occur due to various weather conditions at collection sites (Kolliker *et al*. 2006).

Genetic distance between isolates from the fields compared to the rice nurseries may be due to the sampling of these strains from the transplant stage to the anthesis stage, and different tissues. Whereas, strains obtained

from the nurseries were isolated from leaves during the short phase of the growing stage. Also, growing conditions in a nursery are different than in the rice field.

Bacteria which has been transferred from nurseries to panicles by seeds and seedlings is probably the cause of the observed panicle strains among nursery strains, but these two groups were separated at a 83% similarity level. In conclusion, a dendrogram constructed from AFLP data could separate strains according to their site of sampling. The effect of weather conditions during different rice growth stages on strain genotype can be taken into account. Our results were consistent with Scortichini (2005), who noticed that environmental factors have a major influence on the bacterial population structure fluctuation. The value of the coefficient was 0.93 indicating a very good fit of the clustering with the original similarity matrix.

The isolation of strains from a limited area, same host (rice), special growth stages, and a small number of strains within a population are presumably the reasons for low levels of variation within populations. In addition, the homogeneity within the host population is reflected in the pathogen population (Restrepo *et al.* 1999). In contrast, two reasons may explain the high level of diversity among populations. First, the isolation sites (fields, panicles and nurseries) were varied among populations. Second, this apparent high diversity might be expected in an area with a long history of cultivating rice. Presumably, the associated micro flora would have evolved with and adapted to the various rice hosts over time (Little *et al*. 1998). This study confirms that considerable genetic variability exists among the *P. syringae* pv. *syringae* complex, while no significant differences were observed in biochemical, physiological and pathogenecity tests between all strains (Khoshkdaman *et al.* 2008). Genetic variability is also present when the pathogen(s) solely attacks one host (rice) plant (Natalini *et al*. 2006). As described by Scortichini (2005), host selection combined with different rice cultivation practices and different weather conditions play an important role in a population structure of a pathogen.

Overall, there was a high genetic similarity as most strain pairs showed genetic identity between 0.6–0.7. There are a few reasons for this similarity among strains. First of all, the studied strains were isolated from a restricted geographical region (the Guilan Province). 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. Such similarity may be the result of a recent adaptation to a specialized niche (the Guilan province) (Little *et al*. 1998). Sisto *et al*. (2007) reported that strains from the same geographical regions, in the majority of cases are genotypically more closely related to one another than those strains isolated from different geographical regions. The other factor is host speciality in *Pss* strains. Accordingly, high genetic similarity between isolates of rice, which were grown in almost the same weather conditions, is not unexpected.

Our study has confirmed the results of Cirvilleri *et al*. (2005). They found that genetic variability exists within *Pss* strains which are isolated from the same host plants or with the same antagonistic characters that revealed AFLP marker ability in distinguishing genetic variation and its effectiveness.

Our results proved that AFLP analysis is the best technique to complement other molecular fingerprinting techniques for analysis of closely related populations (Yu *et al*. 2003). Because our sample size was limited, additional samples from a broad geographical region are needed to fully understand the population structure of this pathogen.

Our study revealed that genetic variation exists within *P. syringae* pv. *syringae* strains isolated from rice in the Guilan province of Iran. There were also differences between studied populations (isolates of fields, panicles and nurseries) based on site of sampling and weather conditions where the strains were isolated. Interestingly, apparent genetic distance was seen between isolates of the fields and nurseries while all of them belonged to the same pathovar (pv. *syringae*) and were isolated from the same host (rice).

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