Iran J Biotech. 2014 November;12(3): e1007. DOI:10.15171/ijb.1007
Published online 2014 November 25. **Research Article**

Characterization and Phylogenetic Analysis of *Magnaporthe* spp. strains on Various Hosts in Iran

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Received: March 17, 2013; **Revised:** August 03, 2014

Background: Populations of *Magnaporthe*, the causal agent of rice blast disease, are pathotypically and genetically diverse and therefore their interaction with different rice cultivars and also antagonistic microorganisms are very complicated.
Objectives: The objectives of the present study were to characterize phylogenetic relationships of 114 native *Magnaporthe* strains, isolated from rice and different weeds in the North region of Iran and to study their interaction with the fungal and bacterial antagonists.
Materials and Methods: Phylogenetic studies (lineage structure, cluster analysis and gene flow) were performed using AFLP DNA fingerprinting. Antagonistic effects of the native fungal (*Trichoderma harzianum*) and bacterial (*Bacillus subtilis* and *Pseudomonas fluorescens*) against *Magnaporthe* strains were assayed at *In vitro* levels using factorial experiments based on completely randomized designs (CRD) and mean comparison tests.
Results: In total, 39 clonal lineages including 48 haplotypes were identified among the strains of *M. grisea* and designated here as A-Z. AFLP marker could finely differentiate the strains isolated from various hosts. The strains isolated from *Setaria* sp. were much close to those from rice (*Oryza sativa* L.). *Magnaporthe* strains isolated from *Digitaria* sp. showed higher genetic variation than other strains. Genetic distances revealed by the AFLP markers could be finely differentiated *M. grisea* and *M. salvinii*. The rate of gene flow was an evidence of low gene transferring among *Magnaporthe* populations and the existence of a complex species for *Magnaporthe* strains. The fungal and bacterial antagonists showed different reactions against different *Magnaporthe* strains. These results confirmed high genetic diversity between the *Magnaporthe* strains which was also previously determined by the AFLP experiments.
Conclusions: It was concluded that the Magnaporthe populations in Iran have a complex genetic diversity, and therefore, to achieve an efficient control of the different strains and pathotypes of *Magnaporthe* sp, it is necessary to use different bacterial and fungal biocontrol agents as a dynamic and integrated control system.

Keywords: AFLP; *Bacillus subtilis*; DNA fingerprinting; *Magnaporthe*; *Pseudomonas fluorescens;* Rice blast; *Trichoderma harzianum*

1. Background

Pyricularia grisea Sacc. (*Magnaporthe grisea* (T. T. Hebert) M. E. Barr), the causal agent of rice blast disease, is a widespread fungus which causes considerable loss in most rice-growing areas of the world (1-3). Populations of this pathogen are pathotypically diverse, which must be related to the continuous generation of novel pathogenic variation as demonstrated by several researches. Special attention was paid to pathotype analysis of *Magnaporthe* when the primarily inbred resistant cultivar of rice against *M. grisea* became susceptible in the field. Numerous pathotypes of this pathogen have been mainly characterized based on their capability to cause disease on a set of differential cultivars (2, 4).

DNA fingerprinting has often been considered as an effective tool to analyze the population structure of *Magnaporthe*. AFLP and SSR markers are the most appropriate DNA fingerprinting methods for providing enough information required for the rational deployment of resistance genes due to its high sensitivity and precise results provided, for this reason they has been widely

used for the analysis of the genetic diversity of *Magnaporthe* populations, and also for mapping plant resistance genes to this pathogen (5-8). In addition to markers, the phylogenetic species concept (PSC) based on the concordance of multilocus DNA sequence data has recently become popular among filamentous fungi. For example, phylogenetic analysis using actin, beta-tubulin, and calmodulin gene sequences were used as useful tools for phylogenetic studies in the *Magnaporthe* complex (1, 9).

In Iran, the rice blast was reported for the first time in 1966 in Guilan province (10). At present, this disease is the most important disease of rice in two major rice-growing areas of Iran, namely Guilan and Mazandaran provinces (north of Iran). The loss caused by this fungus on rice in Guilan has been estimated around 15.45% (11). Several investigations in Iran have been conducted on pathotypes variation of *Magnaporthe*, which have mainly showed that the population structure of this fungus is highly variable in different geographical areas (6, 11-14), but these studies were not designed to determine the genetic structure of Iranian *P. grisea* populations. Analyses of the population structure of *M. grisea* isolated from rice in Iran was primarily performed by Javan-Nikkhah *et al*. (15) using rep-PCR marker, which subsequently followed by Hemati (16) and Bargnil (17) on the strains isolated from rice and weeds.

1. Objectives

The objectives of the present study were: (i) determining the genetic relationship among *Magnaporthe* spp. strains isolated from rice and other hosts such as weeds, mostly belonging to *Poaceaea* in Iran, using an AFLP marker, (ii) determining the phylogenetic relationship among the identified genotypes of *M. grisea* and *M. salvinii* (Catt.) R.A. Krause and R.K. Webster using an AFLP marker, (iii) *in vitro* evaluation of *M. grisea* genotypes reactions against the fungal and bacterial antagonists.

2. Materials and Methods

2.1. Fungal and Bacterial strains

One hundred and eight *M. grisea* isolates, previously isolated from rice (28 strains) and weeds (including *Setaria* sp. (17 strains), *Digitaria* sp. (40 strains), and *Echinochloa* sp. (23)) in different regions of Guilan and Mazandaran provinces, were used (13-16). Six *M. grisea* strains isolated from other hosts, including *Eleusine coracana* and *Hordeum vulgare* in Tailand, India and Uganda, were used as standards. Four strains of *M. salvinii* isolated from rice were also used to investigate the phylogenetic relationships between these two species. Four *Bacillus subtilis* and nine *Trichoderma harzianum* strains (provided by Microbial Gene Bank of Agricultural Biotechnology Research Institute of Iran) and eight *Pseudomonas fluorescens* strains (provided by Bacterial Culture Collection of Tehran University), were used for antagonistic tests (Table 1).

2.2. AFLP fingerprinting

Genomic DNA of *Magnaporthe* strains was extracted according to the method of Dellaportha *et al*. (18). AFLP technique was performed according to Tredway *et al*. (8) with minor modifications. Template DNA(125 ng) was digested with 1.25 units of *Eco* RI, 1.25 units of *Mse*I, and 2.5 µL of 5× reaction buffer (50 mM Tris-HCl [pH 7.5], 50 mM Mg-acetate, 250 mM K-acetate) at 37°C for 2 h, followed by heat deactivation of the enzymes at 70°C for 10 min. Double-stranded *Eco*RI and *Mse*I adapters were ligated with 0.5 units of T4 DNA ligase and 12 µL of adapter/ligation solution (*Eco*RI/*Mse*I adapters, 0.4 mM ATP, 10 mM Tris-HCl [pH 7.5], 10 mM Mg-acetate, 50 mM K-acetate) at 20ºC for 2 h and then diluted 10×. Preamplification was performed using primers complimentary to the adapter sequences with no selective nucleotides. Diluted ligation reaction (2 µL) was added to 2 µL of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl),1.5 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase, 50 ng of *Eco*RI primer, 50 ng of *Mse*I primer, 0.2 mM of each dNTPs, and water to a final volume of 20 µL. Thermal cycling conditions included an initial denaturation step at 94ºC for 1 min followed by 30 cycles at 94ºC for 30 s, 56ºC for 1 min, 72ºC for 1 min, and a final extension step at 72ºC for 1 min. The preamplification products were diluted 20× for use in selective amplification reactions. Selective amplification reactions were performed with *Eco*RI and *Mse*I primers that included 3 selective nucleotides. A single *Eco* RI primer (E-ATA) was used in combi-

Table 1. The Bacterial and fungal strains used in this study

Isolates	Brief name	Host	Place
B. subtilis	DSMZ	Rice	Germany
B. subtilis	Kbzl	Soil	Mazandaran
B. subtilis	9	Soil	Fuman
B. subtilis	6	Soil	Guilan
P. flourescencs	Chao	Soil	Fuman
P. flourescencs	P61	Soil	Karaj
P. flourescencs	Prah4	Soil	Karaj
P. flourescencs	Prh2	Soil	Karaj
P. flourescencs	Prh4	Soil	Karaj
P. flourescencs	Prh7	Soil	Semnan
P. flourescencs	Prs2	Soil	Semnan
P. flourescencs	Prs3	Soil	Semnan
P. flourescencs	Prs7	Soil	Guilan
T. harzianum	Gl-69-3	Soil	Guilan
T. harzianum	T3-3	Soil	Mazandaran
T. harzianum	M14	Soil	Mazandaran
T. harzianum	Khb3	Soil	Esfahan
T. harzianum	G21	Soil	Nour
T. harzianum	Mtrec1	Soil	Polsefid
T. harzianum	G124-1	Soil	Pareseh
T. harzianum	37r4s	Soil	Guilan
T. harzianum	G50-8	Soil	Guilan

nation with three *Mse*I primers (E-AAT, E-AAA, and E-TTT) to yield three primer pair combinations. Selective PCRs (4-µL volume) contained 1 µL of diluted preamplification product, 1.5 ng of *Eco* RI primer, 9 ng of *Mse*I primer, 0.15 unit of *Taq* DNA polymerase, 0.4 µL of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl), 1.5 Mm $MgCl_2$, and 0.2 mM of each dNTPs. Thermal cycling conditions included an initial cycle at 94ºC for 30 s, 65ºC for 30 s, 72ºC for 1 min, followed by a touchdown phase where the annealing temperature was lowered to 0.7ºC, each cycle for 12 cycles, and finally 23 cycles at 94ºC for 30 s, 56ºC for 30 s, and 72ºC for 1 min. For each *Magnaporthe* strain, 1.5 µL of selective amplification product from each of the three primer combinations was combined with 0.85 µL of formamide, 0.30 µL of GS-500 ROX internal size standard (Applied Biosystems), and 0.35 µL of loading dye (Applied Biosystems, Foster City, USA). The mixture was denatured at 95ºC for 5 min, and then separated on a 5% polyacrylamide gel on a Perkin-Elmer ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, USA).

2.3. Analysis of lineage structure

DNA fingerprints were primarily divided into groups based on visual similarities among amplified DNA profiles. Subsequently, the identified groups were analyzed together on the same gel to confirm an overall fingerprint identity and to quantify the number of shared amplicons among strains. DNAs from a couple of strains representing a particular lineage were separated on electrophoresis gels, together with representatives of other putative lineages to allow scoring of individual amplicon positions. Binary codes were used to score the bands for presence (1) and absence (0). The binary data was used to calculate genetic distances between all pairs of strains based on the mean character difference using the distance option in NTSYSpc version 2.10 (NTSYSpc, Numerical Taxonomy System, Exeter Software).

2.4. Cluster Analysis

Cluster analysis was conducted based on the genetic distances, and a dendrogram was constructed using UPGMA (unweighted pair group method with arithmetic average) method. A cophenetic value was calculated using NTSYS. Based on visual assessments, similarity coefficients, and the stability of each group in the dendrogram, a similarity level of ≥70% was used to define lineage groups.

Some indices such as the number of effective alleles (A_e) were measured using software POPGen 32. F statistics for measurement of differentiation levels between or within the populations were performed by POPGen32; molecular variance analyses (19) by GenALEX version 6.1; standard genetic distance by POPGen32 and GenALEX version 6.1 and analyses of Principal Coordinates by GenALEX version 6.1. Phylogenetic analysis of *Magnaporthe* strains based on AFLP data was carried out using DARwin 5 version: 5. 0. 146 with 1000 bootstrap replicates, followed by phenograms drawing.

2.5. Assessment of reactions of Magnaporthe strains against antagonists

To investigate reaction of *Magnaporthe* strains against antagonistic organisms and to determine its association with AFLP groups, one strain from each clonal lineage was selected based on occurrence on each host, including *Echinochloa* sp., *Digitaria* sp., *Setaria* sp., *E. coracana* and *H. vulgare*. Four native *Bacillus subtilis* strains, nine *Pseudomonas fluorescens* native strains and nine native fungal strains of *Trichoderma harzianum* were used in the experiments. The experiments with bacterial strains were performed according to the method of Expert and Digat (20). Strains of bacterial antagonists were co-cultured with *Magnaporthe* strains in Petri plates in triplicates. The experiments with *Trichoderma* strains was performed according to Bell *et al*., (21) method.

The antagonistic effect of fungal and bacterial strains against *Magnaporthe* strains was evaluated using factorial experiments (bifactorial) based on completely randomized designs (CRD) with mean comparison tests (Duncan's test) performed. Statistical analysis was performed using the software SAS version 9. 1.

3. Results

3.1. DNA fingerprinting

The *Magnaporthe* isolates were divided into thirty nine lineages designated as A-Z. These lineages were obtained by scoring 41 resolvable DNA amplicons, ranging from 220 to 2500 bp (Figure 1). DNA banding similarity among strains within a putative lineage ranged from 70 to 100%. The rate of r-coph was consistently variable between 89 and 90%. Due to high similarity among haplotypes (more than 95%), only 48 unique haplotypes were used in AFLP statistical test and subsequent phenogram drawing (Table 2). The resultant phenogram showed that *Magnaporthe* strains were mainly differentiated according to their hosts (Figure 2).

The *M. salvinii* strains (33s and 6s) were separated from strain d90 on *Digitaria* sp. and *M. grisea* with 77% and 50% similarity coefficient, respectively. Furthermore, *M. salvinii* strains were differentiated from each other with a percentage higher than 95% similarity coefficient. In a phenogram drawn on *Magnaporthe* strains from

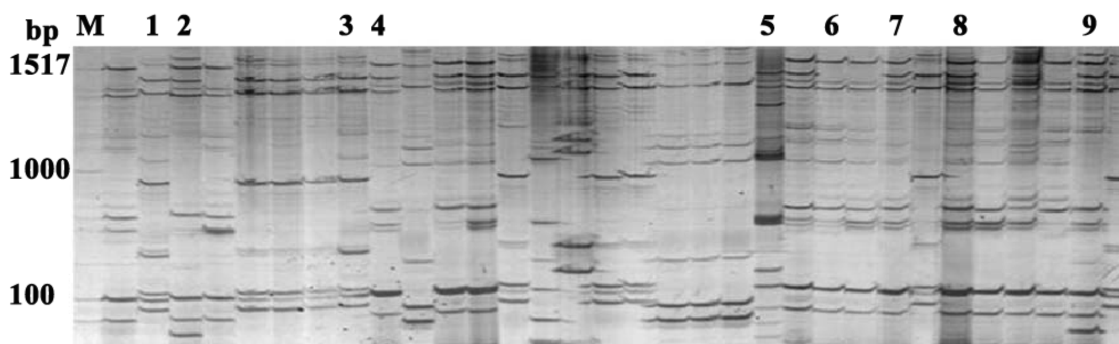

Figure 1. DNA banding pattern of *Magnaporthe* strains amplified by AFLP method on PAGE. 1. Strain 13d on *Digitaria* sp., 2. Strain S3 on *Setaria* sp., 3. Strain 5r on *O. sativa*, 4. Strain KA9 on *E. coracana*, 5-9. Strains of *M. salvinii* on *O. sativa*. DNA ladder 100 bp

Table 2. Some characteristics of 48 selected haplotypes after removing the similar haplotypes

No	Strain	Host	Isolation place	Mating Type
1	33s	Rice	Rasht	Undetermined
2	6s	Rice	Rasht	Undetermined
3	D12	Digitaria	Nowshahr	Mat1-2
4	D7	Digitaria	Rasht	Mat1-1
5	73D	Digitaria	Rasht	Mat1-1
6	U3	Echinochloa	Sowme'eh Sara	Undetermined
7	D2	Digitaria	Rasht	Mat1-1
8	D2a	Digitaria	Rasht	Mat1-2
9	D81	Digitaria	Rasht	Mat1-2
10	S7	Setaria	Sowme'eh Sara	Mat1-1
11	11-3D	Digitaria	Rasht	Undetermined
12	13D	Digitaria	Rasht	Undetermined
13	D12-5	Digitaria	Rasht	Undetermined
14	D22	Digitaria	Rasht	Mat1-1
15	D3	Digitaria	Rasht	Mat1-1
16	D90	Digitaria	Rasht	Mat1-1
17	D13	Digitaria	Rasht	Mat1-1
18	3D	Digitaria	Rasht	Mat1-1
19	D9a	Digitaria	Rasht	Mat1-2
20	19D	Digitaria	Rasht	Mat1-2
21	71D	Digitaria	Rasht	Mat1-2
22	14u	Echinochloa	Sowme'eh Sara	Undetermined
23	D5	Digitaria	Rasht	Undetermined
24	12-5D	Digitaria	Rasht	Undetermined
25	D23	Digitaria	Rasht	Mat1-2
26	D9-9	Digitaria	Rasht	Undetermined
27	D29	Digitaria	Kordi Kala	Mat1-1
28	U33	Echinochloa	Sowme'eh Sara	Undetermined
29	D8	Digitaria	Noor	Mat1-2
30	D21	Digitaria	Rasht	Mat1-2
31	D70	Digitaria	Rasht	Undetermined
32	5r	Rice	Sowme'eh Sara	Undetermined
33	90r	Rice	Noor	Mat1-1
34	67r	Rice	Chaboksar	Mat1-2
35	4r	Rice	Amol	Mat1-1
36	121r	Rice	Mahmoud abad	Mat1-2
37	M2r	Rice	Salman Shahr	Mat1-1
38	20r	Rice	Rasht	Undetermined
39	145r	Rice	Amol	Mat1-1
40	S50-1	Setaria	Rasht	Mat1-2
41	S3	Setaria	Sowme'eh Sara	Undetermined
42	11u	Echinochloa	Sowme'eh Sara	Mat1-2
43	121u	Echinochloa	Sowme'eh Sara	Mat1-2
44	S71	Setaria	Sowme'eh Sara	Mat1-1
45	S18	Setaria	Sowme'eh Sara	Mat1-1
46	S50a	Setaria	Sowme'eh Sara	Mat1-1
47	S5	Setaria	Ghadikala	Mat1-2
48	S41	Setaria	Sowme'eh Sara	Mat1-2

four hosts, namely *Digitaria* sp., *Setaria* sp., *O. sativa* and *E. coracana*, 39 clonal lineages and 48 haplotypes were recognized in the similarity coefficient of 80% (Table 2). The strains S7 and S3 of *M. grisea* on *Setaria* sp. were separated from those on rice with 63% and 46 % similarity coefficient, respectively. Three clonal lineages *A*, *B* and *C* were differentiated from strains on rice with 34% similarity coefficient. Clonal lineages *A*, *B*, *C*, *D*, *G*, *J*, *N* and *O*, each with one haplotype, include *M. grisea* strains on *Setaria* sp. Clonal lineages *E*, *F*, *K*, *L*, *M*, are also with one haplotype and accommodate *M. grisea* strains on rice. Clonal lineages *H* and *I*, including those strains on *Echinochloa sp*., were finely separated from those on rice with 48% similarity.

It is noteworthy to mention that the standard strain br-114 isolated from *E. coracana* was separated from strains on *Digitaria* sp. with 43% similarity. The strains isolated from *Digitaria* sp. were differentiated from each other with 45% similarity. However, the strains isolated from rice and *Digitaria* sp. were placed in two separate groups, which imply on the far genetic distance between these strains. Those strains obtained from *Digitaria* sp. were differentiated from others on *Setaria* sp. with 47% similarity coefficient and were placed in two separate groups. Those strains obtained from *Setaria* sp. were differentiated from each other with 27% similarity coefficient. Furthermore, the strains isolated from *Digitaria* sp. were differentiated from each other with more than 50% similarity coefficient.

These results indicate that genetic variation within the populations of *Magnaporthe* is more significant (73%) than between populations (27%). AFLP banding patterns analysis of the populations revealed that there is a specific band in the populations on *Setaria* sp., which is absent in other populations. This analysis indicated that this specific band belongs to strain S7.

Gene flow rate was low and estimated around 0.9%. PcoA analysis additionally confirmed that the strains were mainly separated from each other according the hosts they have occupied. In another analysis based on Nei coefficient in NTSYS pc version 2.10, used to show the genetic differences between *M. salvinii* and *M. grisea*, it was demonstrated that these two species are differentiated from each other with 35.7% similarity, and standard strains which are placed in a separate group, are differentiated from these two species with 45 % similarity. In addition, the strains isolated from *Digitaria* sp. were separated from those on rice, *Setaria* sp. and *Echinochloa sp.* with 23% similarity. The strains isolated from *Setaria* sp. and *Echinochloa sp*. were much closer to each other. The lowest genetic distance was recorded for strains isolated from *Setaria sp*. and *Echinochloa sp*. These strains were differentiated from those on

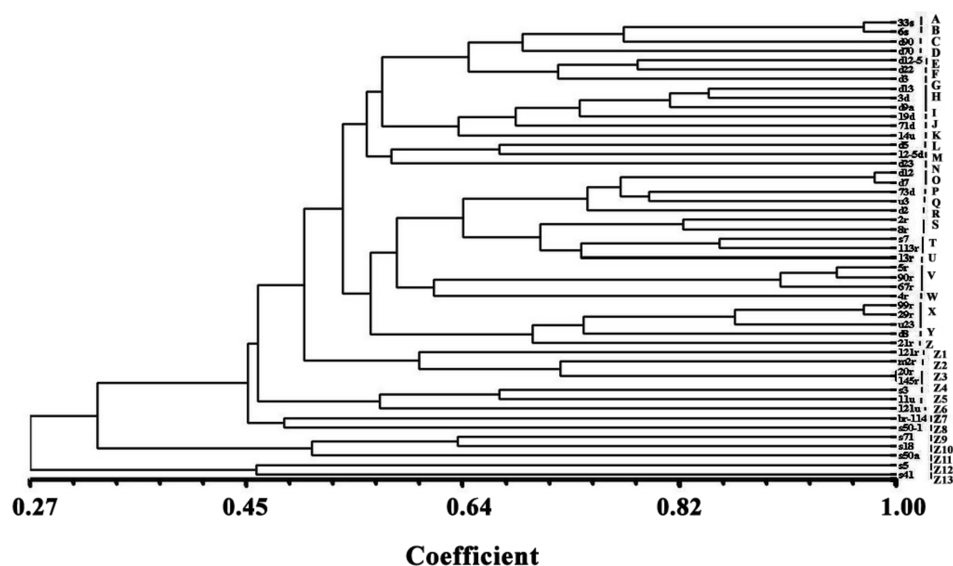

Figure 2. Phenogram of *Magnaporthe* strains on different hosts constructed with UPGMA method, with primers MAT, M22, MTC. Similar haplotypes determined by AFLP marker are excluded (33s and 6s:*M. salvinii*; d: *Digitaria* sp.; s: *Setaria* sp. ; U: *Echinochloa sp.* ; br-114: *E. coracana* ; r: Rice)

rice with 18 % similarity (Figure 3). The Shanon and Vier index and gene flow rate was estimated around 0.6 and 0.59, respectively.

Phylogenetic analysis of *Magnaporthe* strains based on AFLP marker, placed strains of *M. salvinii* as an out group for the clade including the strains of *M. grisea*. The robustness of phylogenetic dendrogram was evaluated with 1000 replicates of bootstrap (Figure 4). The resultant phylogenetic phenograms also confirmed the differentiation of *Magnaporthe* strains mainly according to their hosts. These phenograms revealed that the strains of *M. salvinii* are much closer to those strains isolated from *Digitaria* sp., although they are less closer to strains isolated from *Setaria* sp. Strain S7 showed high similarity with strains on rice as was also shown by AFLP. Consequently, the strains isolated from *Setaria* and *Echinochloa sp*. were much closer to each other.

3.2. Antagonistic effect of bacteria against Magnaporthe strains

In vitro antagonistic essays showed that *P. fluorescens* strains had more inhibitory effects on *Magnaporthe* strains than *B. subtilis* strains. The strains Prs3 and Prah4 (*P. fluorescens*) represented the highest antagonistic effects, 7.16 and 5.33 mm, respectively. Among the strains of *B. subtilis*, strain 6 had higher inhibitory effect, while the strains Kbzl and 9 had less significant inhibitory effects (Figure 5A). It was also showed that *M. salvinii* and *M. grisea* strains isolated from *Echinochloa* sp. leaves were more resistant than other strains against bacterial antagonists. In contrast, strains isolated from *Setaria* sp. were the most sensitive strains against bacterial antagonist; with strains on rice and *Digitaria* sp. in the second and third position (data was not shown). These results showed that different fungus strains from different hosts had different responses against the antagonistic bacteria.

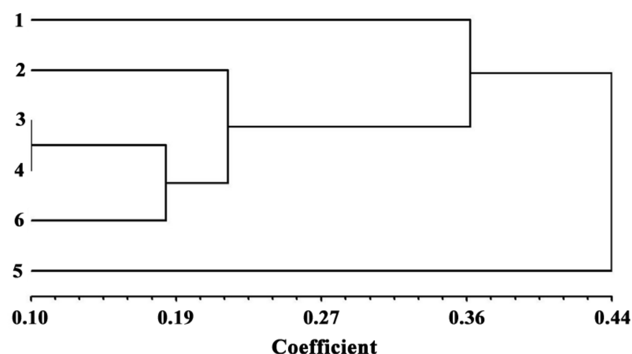

Figure 3. Phenogram of genetic distance between populations of *M. grisea* and *M. salvinii* based on Nei similarity coefficient. (1: *M. salvinii*; 2: *Digitaria*; 3: *Setaria*;4: *Echinochloa sp.*; 5: *E. coracana* and *H. vulgare*; 6: *O. sativa*)

3.3. Antagonistic effect of T. harzianum against Magnaporthe strains

The colonies of *Trichoderma* were divided into five groups according to the indices presented by Bell *et al*., (1982) (21). Score 1 was given to the most effective strains and the scores 4 and 5 to weakest strains of *Trichoderma*. Finally, the strains scored ≤ 1 were selected. The strains mtrec 1, m14 and G21 were the most effective antagonists against *Magnaporthe* strains, and showed 98, 97 and 97% colonization and sporulation inhibition, respectively (Figure 5B). However, the strain G1-69-3 was classified among the weak strains with 72% colonization rate and without sporulation. The fungal strains of *M. salvinii* and those strains of *M. grisea* isolated from *Digitaria* sp. were more resistant than other strains against *Trichoderma* in culture media. However, the strains of *M. grisea* on rice and *Setaria* sp. were classified as the most sensitive strains (Figure 5B)
.

4. Discussion

Over the last three decades, rice blast has become the most important pathogen in rice-growing areas of Iran. Therefore, several investigations have been conducted to evaluate the pathotypes diversity of *M. grisea* in various rice-growing regions of Iran (12-13, 15-17). It seems that genotype flow of *M. grisea* from outside north region of Iran has not been enough to introduce some new genotypes. Short growing season and good environmental conditions are favor for sporadic blast epidemics and genetic drift more likely. This phenomenon could maintain diversity in the fungal population at low level. The widespread distribution of common haplotypes is consistent with regionally significant gene flow. Evolution in these populations is likely characterized by selection among different mutants that occur within the existing clonal lineages followed by movement of regionally selected genotypes.

The low level of pathotype diversity (22) and lack of correlation between DNA fingerprint and

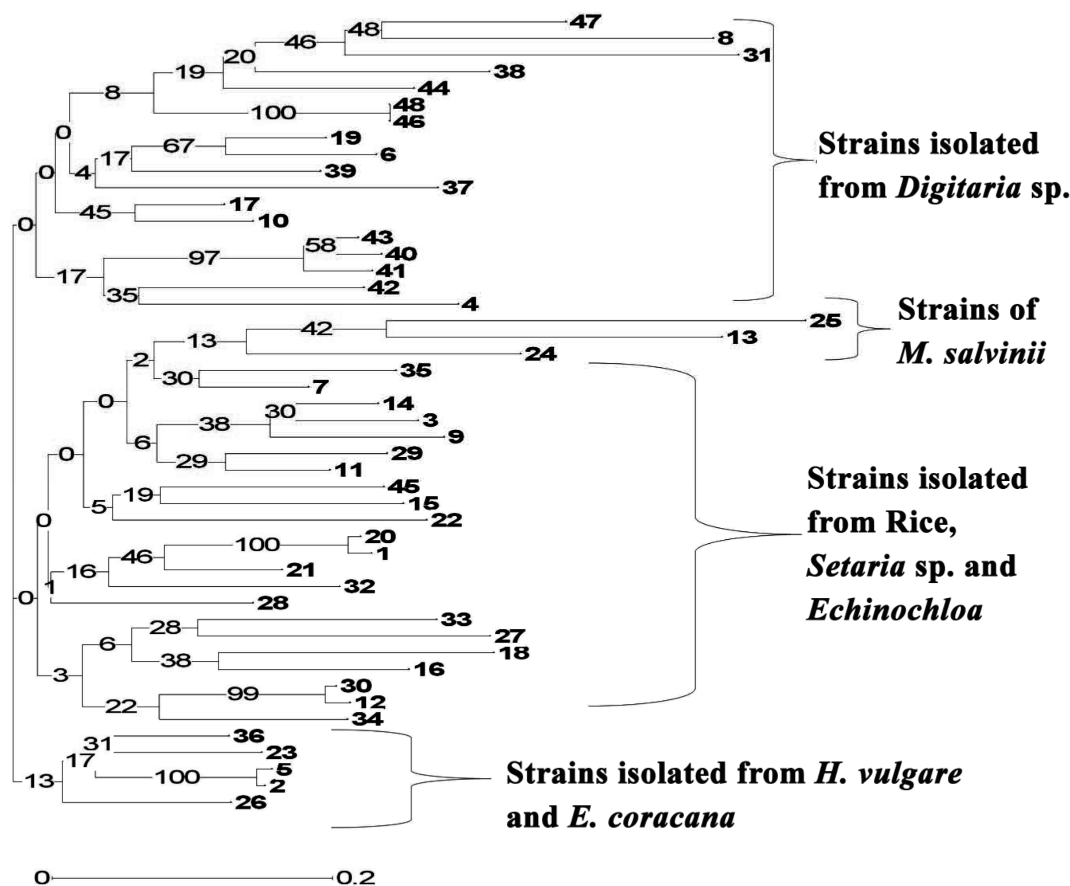

Figure 4. Phenogram of phylogenetic relationships among 48 strains of *Magnaporthe* isolated from different hosts based on AFLP marker. *M. salvinii* is outgrouped

pathotypes suggest that selection has favored the same spectrum of virulence in each clonal lineage. There was no evidence for sexual recombination in the studied population which is consistent with the dominant asexual reproduction observed in most rice-growing areas of the world. However, further characterization of *M. grisea* strains in Iran with mating-type or multi-allelic, co-dominant, neutral genetic markers could probably provide evidence for the role of sexual or parasexual recombination in maintaining haplotype diversity of this fungus in Iran (15).

Evaluation of genetic variation of *M. grisea*, based on DNA fingerprinting using AFLP markers determined the genetic variation among the *M. grisea* strains on *Digitaria* sp., rice and *Setaria* sp. The *Magnaporthe* strains were mainly differentiated according to their hosts which confirm that the cross infectivity has not occurred or is at a low level. Gene flow rate was estimated around 0.9%, which implies a low rate of gene transfer or genotypes throughout populations of *M. grisea* on various hosts, as it was demonstrated by low genetic distance between some populations and high genetic distance between the others.

These results were consistent with those results obtained by Couch and Kohn (1) using PCR-RFLP technique. Therefore, it seems that the clonal lineages are mainly separated according to the host they have been isolated from. Higher genetic similarity among *M. grisea* strains on *Digitaria* sp. was commonly observed which was also shown by Kato *et al*. (23). The far genetic distance between strains of *M. grisea* on *Digitaria* sp., rice and *Setaria* sp. confirmed the distant phylogenetic relationship between these two groups. However, the strains isolated from *Setaria* sp. and a strain from *Echinocloa* sp. were much closer to those strains on rice (more than 70%). It is evidence that there is a close genetic relationship between strains isolated from *Setaria* sp. and rice, as grouped in the same species.

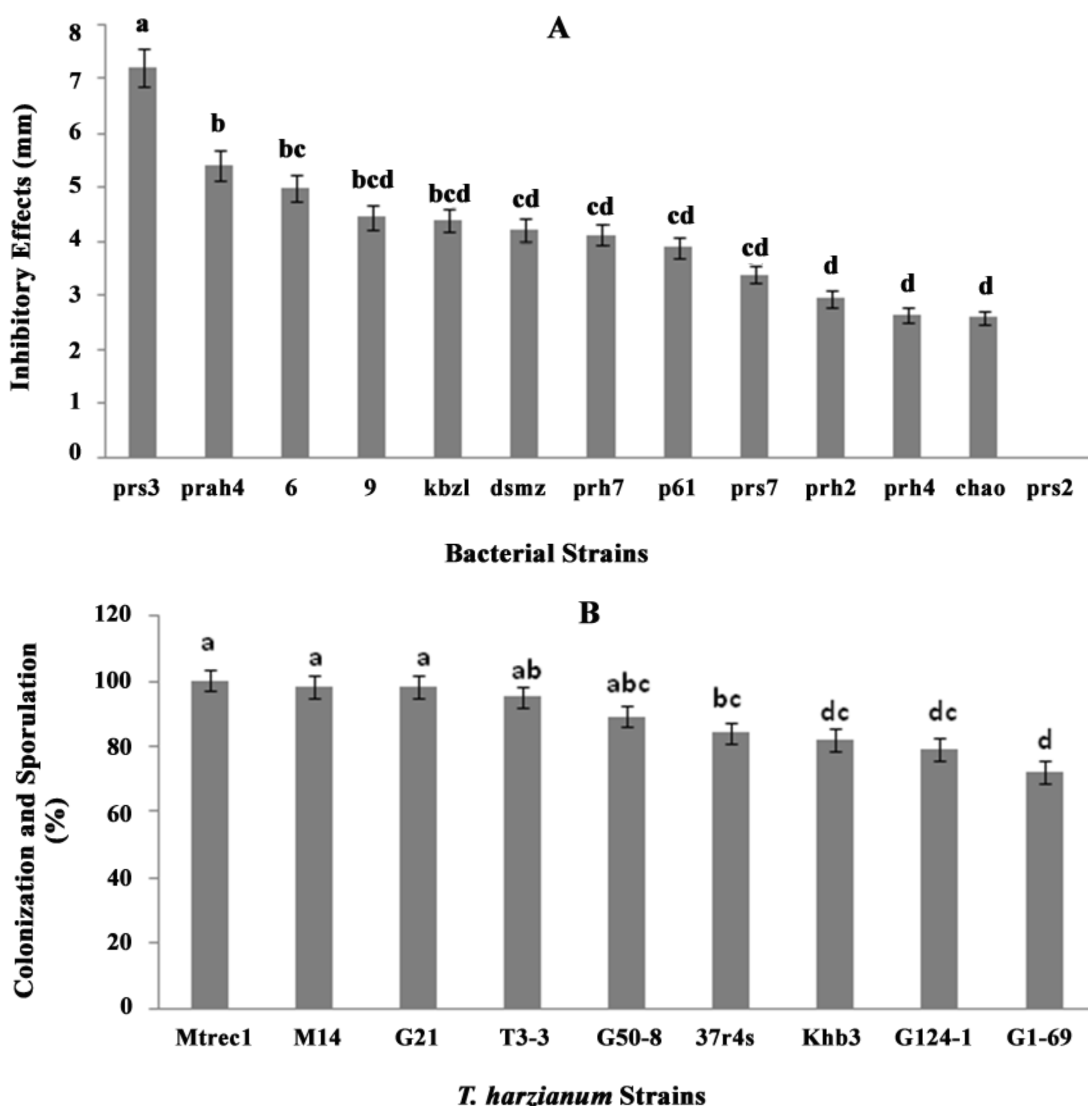

Figure 5. The results of antagonistic effects of the bacteria (*P. fluorescens* and *B. subtilis)* (A) and fungus (*T. harzianum*) (B) against *Magnaporthe* strains

The far distance among DNA fingerprinting groups of *M. grisea* strains on *Digitaria* sp., rice and *Setaria* sp. could be evidence of far relationship between these two groups of strains which supports the inclusion of these strains in two separate species groups, *M. grisea* and *M. oryzae*. In the case of accepting *M. grisea* and *M. oryzae* as separate species, based on the AFLP data (far genetic distance) it can be concluded that the possibility of cross reactions between these two species would be rare or even impossible. Furthermore, the lack of genetic variation within each clonal lineage can be an evidence of *in situ* asexual (clonal) reproduction of strains. Therefore, it seems that the role of sexual reproduction in genetic variation within the populations of *Magnaporthe* to be less significant. Choe *et al*. (2013) studied 103 isolates from different grass species and analyzed their phylogenetic relationships and pathogenicity. Phylogenetic analyses of multilocus sequences and DNA fingerprinting revealed that the haplotypes of most isolates were associated with their hosts, whereas only six isolates showed potential host shift in nature.

The current research confirmed results of Taheri and Irannejad (6) and Hemati (16) which implied on high genetic variation among strains on rice and weeds, respectively. It was also concordant with Bargnil (17) which showed high genetic similarity between strains isolated from

Setaria sp. and rice and genetic differences between strains on *Digitaria* sp. and those on rice and *Setaria* sp. However, this study questioned arguments of Bargnil (17) on the existence of low genetic variation among the strains on *Digitaria* sp., as these strains showed higher genetic variation than other strains here.

AFLP marker was also recognized here to be the most useful DNA fingerprinting tool for resolving the high genetic variation of this fungus compared with other fingerprinting techniques applied by several previous researchers such as RAPD and rep-PCR (6, 15, 22-24). However, this research confirmed results obtained by Javan-Nikkhah (15) showing the low genetic variation among strains isolated from rice in Iran. All *M. grisea* strains showed high genetic variation and distant genetic relationship, which was also shown by Kato *et al.* (23) implying that there is less possibility of cross reactions between these two groups of fungi. The strains isolated from *Setaria* sp. revealed higher genetic variation than those on rice, which is concordant with Javan-Nikkhah (15).

The rate of gene flow was estimated here around 0.9%, which imply on low gene transfer among strains of *Magnaporthe*, as was shown by Couch and Kohn (1). Therefore, we also suggest the existence of complex species for strains of *Magnaporthe*, as Takan et al. (24) and Couch and Kohn (1) did. It is noteworthy to mention that the high amount of recombination (nearly 41%) was observed among strains of *Magnaporthe*. Shanon and Vier index was estimated around 0.6 which shows that AFLP marker could successfully differentiate this fungus strains up to 60%. The rate of Gst, 0.35, shows the high genetic variation among populations of *Magnaporthe*. The results of phylogenetic analysis were also highly concordant with those of AFLP marker.

Previously the possibility of biocontrol of *Magnaporthe* using antagonists was confirmed (3, 25-26). The fungal and bacterial antagonists showed different reactions against different Magnaporthe strains. These results confirmed high genetic diversity between the Magnaporthe strains which was also previously determined by AFLP experiments. In the bioassays, it was shown that native *P. fluorescens* strains had more significant inhibitory effect on *Magnaporthe* strains than *B. subtilis*. The strains of *M. salvinii* represented the different reactions than those of *M. grisea* and proved to be more resistant against both fungal and bacterial antagonists. The strains isolated from *Digitaria* sp., *E. coracana* and *H. vulgare*, had similar antagonistic reactions against fungal and bacterial antagonists. In addition, the strains on *Setaria* sp., *Echinochloa* sp., and rice represented similar reactions against antagonistic agents.

5. Conclusions

The results of this study can form the basis for a breeding program to improve new resistant cultivars to all clonal lineages of *Magnaporthe*. Based on the obtained results, it was shown that *M. grisea* populations in Iran have high genetic diversity, and the representative of each group was selected which can be used during the plant breeding and microbial biological control programs. Also, it is possible to conclude that because of the complex genetic diversity of the studied fungus strains in Iran, we need to use different bacterial and fungal biocontrol agents to have an efficient control of different strains and pathotypes of *Magnaporthe* sp.

Acknowledgments

This study was partly supported by Agriculture and Biotechnology Research Institute of Iran, Karaj (ABRII). We are grateful to Dr. Kowsari, Mrs Mousivand, Mrs Kazemi and Mrs. Vasfi for kindly assistance and advice.

Authors’ Contribution

Masoud Niknam-Galejugi: AFLP analysis and antagonistic effects, Gholamreza Salehi Jouzani: elaboration of AFLP and bioassay results, Mohammad Javan-Nikkhah: designing of the experiments.

Financial Disclosure

There is no conflict of interest.

Funding/Support

This work was partially supported by a grant from Agricultural Biotechnology Research Institute of Iran (ABRII).

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