V.4 PATHOGENICITY AND MOLECULAR DIVERSITY OF NEW ISOLATES OF RICE BLAST FUNGUS COLLECTED FROM DIFFERENT HOSTS

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Abstract

Rice blast disease, caused by the fungal pathogen *Magnapor the grisea*, is one of the most destructive diseases that severely affects rice crop production in Egypt, where japonica rice is mainly grown. In the following study, 34 blast fungal isolates were characterized and investigated. These isolates were collected from different rice areas and comprise three groups, first group included 22 isolates that collected individually from susceptible international monogenic lines and the highly susceptible recurrent parent LTH. The second group has 9 isolates collected from Egyptian old cultivar (Giza 171, Sahka 101, Sakha 104), and the third group has two isolates collected from weeds. All isolates were artificially used to inoculate local cultivars and some GZ lines under greenhouse conditions. The results showed highly significant differences in resistance reactions among the tested cultivars. The resistant reaction percentage of tested cultivars to all isolates (34) varied from 59.1% to 100%. The promising lines GZ 6296 and GZ 7955 (Sakha 106) showed high resistance percentage to all tested isolates (34 isolates), which suggested that these two lines may have new resistance genes. This data recommended that these two promising lines can be utilized as good donors for blast resistant genes in breeding programs. Genetic diversity of blast isolates was investigated using molecular markers. Five ISJ markers were used to analyze the genetic diversity among those isolates. The isolates were classified based on the molecular similarity of the ISJ markers profiles to avirulent, mild virulent and highly virulent. The ISJ5 and ISJ9 markers generated the highest polymorphisms, which can be utilized as specific markers to identify the genetic diversity of *M. grisea* pathogen. This study investigated the molecular diversity among the new isolates of *M. grisea*. Hence, these results will be an important consideration in breeding program to develop durable resistance for blast disease in rice.*

Keywords: *Oryza sativa* L., *Magnaporthe grisea*, races, molecular diversity, monogenic lines.

* Results of ISJ markers revealed close relation between isolate of Echinoclowa colonum and rice isolates and its role as alternative host and source of primary inoculum for blast fungus.

INTRODUCTION

Rice blast disease caused by *Magnaporthe grisea* (Hebert) Barr. (anamorph, *Pyricularia grisea* Sacc.), is one of the most damaging plant diseases and the main limiting factor for rice production worldwide (Couch *et. al.,* 2005, Ou, 1985, Skamnioti and Gurr, 2009). Extensive and uncontrolled use of fungicides to prevent blast disease possess a significant concern for human health and environmental safety. Therefore, utilization of multiple R genes with overlapped resistance spectra is one of the most powerful strategies for managing blast disease (Wang *et. al.,* 2010, Roy Chowdhury *et. al.,* 2012a). Management of rice blast through the breeding of blast resistant cultivars has only limited success due to the frequent breakdown of resistance under field conditions due to the frequent variation of race in pathogen populations (Ou, 1980, Kiyosawa, 1981, and Correa-victoria and Zeigler, 1993). Various potential mechanisms, including heterokaryosis, parasexual recombination, and aneuploidy (Suzuki, 1965, Ou, 1980), have been proposed to explain frequent race changes. The application of molecular genetic tools to study the fungus, ranging from its genes controlling host specificity to its population structures and dynamics, have begun to provide new insights into the potential mechanisms underlying race variation. Also, *M. grisea* is pathogenic on a wide range of cultivated and wild gramineous hosts, but the species is considered to consist of host-limited forms (Borromeo *et. al.,* 1993, Dobinson *et. al.,* 1993). In Egypt, the blast disease is primarily controlled using resistant cultivars. The extensive use of blast resistant cultivars has led to release new pathogen races. As a result of massive and wide range of variability of blast fungus, race shifting and appearance of new specific virulent races, the fungus caused several breakdown of Egyptian resistant cultivars. So, it must make huge changes in genetic background of promising lines to induce cultivar diversity. To create a wide range of diversity in resistance to blast disease, it must use in crosses new resources of resistance cultivars, which have new resistance genes. To understand the mechanisms of frequent breakdown of resistance in blast-resistant cultivars, studies on the extent of genetic diversity present in the population of *M. grisea* in a specific geographical region is important (Levy *et. al.,* 1993). Consequently, detailed genetic information on population structure is essential for understanding the DNA fingerprint haplotypes and pathotypes of the pathogen and devising more effective strategies to reduce the impact of rice-blast disease. Monogenic resistant lines can be utilized as donor for different blast resistant genes (Fukuta *et. al.,* 2004, Kobayashi, 2007, Wang *et. al.,* 2010).

The objectives of the current study were to analyze *M. grisea* populations from the susceptible rice monogenic lines, old commercial cultivars and non-rice hosts through ISJ markers to address the following issues: (i) assessing population structure and genetic variation among different *M. grisea* isolates and (ii) to clarify the role of weeds isolates in the initiation of inoculum, (iii) Investigate the response of resistance genes in some promising lines. Such this investigation will have implications for sustainable disease management.

MATERIALS AND METHODS

Fungal isolates and race identification: 34 *M. grisea* isolates were selected according to their pathogenicity from avirulent to highly aggressive from different hosts (rice and weeds) during 2013 season. *M. grisea* Isolates were collected from 23 susceptible monogenic lines differentials carrying 24 major blast resistance genes Pia, Pib, Pii, Pik, Pik-h, Pik-m, Pik-p, Pik-s, Pish, Pit, Pita, Pita-2, Piz, Piz-t, Pi1, Piz-5, Pi3, Pi5(t), Pi7(t), Pi9, Pil2(t), Pi11(t), Pi19, Pi20 and the highly susceptible recurrent parent, Lijiangxintuanheigu (LTH), from Gemmiza province and 9 isolates from culture collections of different old Egyptian rice cultivars and two isolates from weed hosts. Isolates were purified using single spore and hyphal tip techniques. Leaves containing sporulating lesions were incubated overnight in a moist chamber at room temperature to induce sporulation. Spores from a single lesion were streaked on water agar and incubated overnight to allow spores to germinate. Germinating spore was sub-cultured on banana dextrose agar. Isolates were maintained on BDA. Stored isolates were inoculated on filter paper and colonized by the fungus, then dried and stored in a desiccator at -20^0C. During 2014 season, international differential set of eight cultivars was used to characterize 34 isolates for race identification under greenhouse conditions at Rice Research and Training Center (RRTC) (Atkins *et. al.,* 1967).

Virulence Test and disease assessment: The 34 isolates were characterized for virulence diversity on additional 23 rice cultivars. Greenhouse inoculation tests were conducted by growing plants to the 3–4 leaf-stage (approximately 2–3 weeks) prior to inoculation. Seeds of each cultivar were directly sown in plastic trays (30 x 20 x15 cm.) which comprised 20 rows. Conidia were collected from 7-day-old cultures grown on banana dextrose agar medium by washing the agar surface with sterile water. The concentration of conidia was adjusted to 4×10^{-5} conidia/ml. Gelatin was added to the spore suspension at a concentration of 2.5 g L^{-1} (Bastiaans, 1993) to enhance the adhesion of spores on leaf surfaces. Each tray was sprayed with 100 ml of inoculum for each isolate with a compressed-air sprayer. Artificially inoculated

plants were incubated in a dew chamber at 100% relative humidity (RH) at approximately 22–25°C for 24 h. Plants were then placed back into the greenhouse at approximately 28–30◦ C and scored for disease symptoms. Inoculated rice seedlings were evaluated for disease reaction 7 days after inoculation using a 0–9 scale (IRRI Standard evaluation system 1996).

Virulence percentage was assessed as a number of infected cultivars compared with total number of tested materials. Resistance % was calculated as a response of a cultivar to inoculated isolates which represents the number of avirulent isolates to the total.

Sample Collection for DNA extraction: The isolates of *M. grisea* were inoculated into 50 ml liquid medium of potato dextrose at 25°C for 7 days in an orbital shaker (120 rpm). The fresh mycelia mass were harvested two weeks after incubation using Whatman filter paper No. 3 and immediately frozen in the liquid nitrogen. The frozen mycelium was pulverized, freeze-dried and ground to a fine powder using a sterile pestle and mortar. The mycelia powder was stored at -20°C until needed.

DNA extraction: To assess the genetic variability among *M. grisea* isolates an initial screening with ISJ primers (Table 1) was carried out. DNA isolation and purification was carried out using CTAB method (Murray and Thompson, 1980). The DNA was quantified using gel assay method and then PCR was performed. A total of five ISJ primers were used for the screening purpose.

PCR Reaction: The PCR was performed in 10µl PCR volume containing 50 ng of template DNA, 5 pmole of each of forward and reverse primers, 0.1mM dNTP`s, 1x PCR buffer (10mM Tris,pH 8.0, 50mM KCl and 50mM ammonium sulphate), 1.8 mM MgCl2 , and 0.2 units of Taq DNA polymerase. Initial denaturation at 94 oC for 5 minutes was followed by 35 cycles of amplification with template denaturation at 94 oC for 1 minute, primer annealing at 55.7oC for 1 min and primer extension at 72 oC for 2 min. After the end of the 35th cycle, a final extension at 72 oC for 7 min was given followed by storage at 4.0 oC. The PCR products were separated using 1.5% agarose gel stained with Et Br solution (1 mg L^{-1}). The banding pattern was then scored and used to prepare the matrix.

Molecular analysis: All the numerical analyses were performed using the computer program NTSYS-pc, version 2.1 (Exeter Software, New York). (Ralf, 1998), Jaccord`s similarity coefficients were calculated and used to establish genetic relationship among the genotypes based on unweighted pair group method of arithmetic averages (UPGMA) and sequential agglomerative hierarchical nested (SAHN) clustering.

Table 1. list of ISJ primers used in the current study.

No.	Name	Sequence	base pair (bp)
5	ISJ 7	5'-TGCAGGTCAGGACCCT-3'	16
6	ISJ 8	5'-GACCGCTTGCAGGTAAGT -3'	18
7	ISJ 9	5'-AGGTGACCGACCTGCA-3'	16
8	ISJ-10	5'-ACTTACCTGCATCCCCCT-3'	18
9	ISJ-11	5'-TGCAGGTCAAACGTCG-3'	16

RESULTS AND DISCUSSION

Race identification of rice blast populations

Twenty-three isolates of *M. grisea* were collected from the susceptible monogenic lines at Gemmiza, El-Gharbia governorate and 11 isolates were collected from old commercial cultivars and weeds. The isolates were identified to race level using the eight international differential cultivars. The 23 collected-isolates from susceptible monogenic lines were categorized in five race-groups, six races belonging to group IB, representing 26.1% from the total tested isolates, one race follow group IC (4.3%), five races follow IF group race (21.7%), eight races IH (34.9%) as most common race group and three races representing avirulent group II with 13.0% (Table 2). The eleven isolates of *M. grisea* were collected from old commercial cultivars and weeds were classified into six race groups, two isolates belong to group IB representing 18.1%, three isolates fellow group IC races with 27.3%, one isolates fellow group ID races with 9.1%, three isolates from group IG races with 27.3%, one isolates belong to group IH races 9.1% and one isolate representing avirulent group II with 9.1% (Table 2). The most common races in monogenic lines group were IH and IB, while the group IC and IG were the majority with old rice cultivars.

Table 2. Race identification of rice blast populations from monogenic lines, Egyptian cultivars and different hosts

No. isolate	Cultivar/line	Pi-Genes	Race
1	IRBLa-A	Pia	IB-63
2	IRBLa-C	Pia	IH-1
3	IRBLi-F5	Pii	IF-1
4	IRBLks-F5	Pik-^{s}	IH-1
5	IRBLks-S	Pik-^{s}	IH-1
6	IRBLk-ka	Pik	IC-28
7	IRBLkp-K60	Pik-p	IH-1
8	IRBLkh-K3	Pik-h	IB-63
9	IRBLzt-T	Piz-t	IB-39
10	IRBLb-B	Pib	IH-1
11	IRBLt-K59	Pit	IH-1
12	IRBLsh-S	Pish	IF-1
13	IRBL1-CL	Pi1	IB-57
14	IRBL3-CP4	Pi3	IF-1
15	IRBL7-M	Pi7 (t)	IB-59
16	IRBL12-M	Pi12 (t)	IH-1
17	IRBL19-A	Pi19	IF-1
18	IRBLkm-Ts	*Pik-^{m}*	IF-3
19	IRBL20-IR24	Pi20	IH-1
20	IRBLta2-Pi	Pita2	IB13
21	IRBLta-CP1	Pita	II
22	IRBL11-Zh	Pi11 (t)	II
23	LIJIANG XINTUAN HEIGU(LTH)	-	II
24	Giza171- Kafr sakr	*Pi-a, Pi-k^s	II
25	Giza171- Sherbien	*Pi-a, Pi-k^s	IH-1
26	Giza171-Sakha	*Pi-a, Pi-k^s	IC-1
27	Sakha101- Beheira	*Pi-ta2	IG-1
28	Sakha104 –Sakha	Unknown	IB-45
29	Giza171 Abu Kabeer	*Pi-a, Pi-k^s	ID-15
30	*Echinocloa Colona-Gemmiza*		IG-1
31	Giza171-Basion	*Pi-a, Pi-k^s	IC-13
32	*Cyperus rotundus-Gemmiza*		IG-1
33	Giza 159-sakha	Unknown	IC-5
34	Giza181-Sakha	*Pi-20, Pi-b, Pi-k^s	IB-63

- LIJIANG XINTUAN HEIGU (LTH) free from R genes

* Pi-gene in Egyptian cultivars were identified according to Imbe 1998

In Egypt, the *M. grisea* fungus recorded a frequent breakdown for 8 cultivars during 1964-2010. As a result of high race shifting and big change in prevalence of specific races, cultivar Sakha 101 was broken down in some locations and this cultivar was completely susceptible in 2005 till present season due to appearance of specific virulent races IG-1. The race shifting was coupled with the extension of the cultivated areas of cultivars Sakha101 and Sakha104, whereas, these races appeared when the cultivated area by both cultivars covered 75% of the total (EL-Shafey, 2002 and Sehly *et. al.,* 2008). So, the monitoring of race groups and race shifting is very vital in management of rice blast. Sehly *et. al.* (2000) inoculated forty-five isolates of P. grisea on eight international differential cultivars. The most common races were IH-1 (36. 6%), I D-race group (17.8%), I A (13.3%), I G-1 (13.3%) and a virulent race group II (9.0%). El-wahsh *et. al.,* 2007 categorized 24 blast isolates into six race groups and fingerprint patterns for the 24 tested isolates revealed the presence of 20 haplotypes. Many pathogenic races of *M. grisea* may occur in a blast nursery at any time of the year. The kind and frequency of these races vary greatly. This may explain why cultivars showing a resistant reaction in one test may become susceptible in another (Quamaruzzaman and Ou, 1970).

Table 3. Blast reaction of 23 rice cultivars with blast isolates derived from monogenic lines under greenhouse conditions.

No.	Isolate no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	Resistance %
	Monogenic line	IRBLa-A	IRBLa-C	IRBLi-F5	IRBLks-F5	IRBLks-S	IRBLk-ka	IRBLkp-K60	IRBLkh-K3	IRBLzt-T	IRBLb-B	IRBLt-K59	IRBLsh-S	IRBL1-CL	IRBL3-CP4	IRBL7-M	IRBL12-M	IRBL19-A	IRBLkm-Ts	IRBL20-IR24	IRBLta2-Pi	IRBLta-CP1	IRBL11-Zh	LIJIANG XINTUAN HEIGU	
	Pi-gene	Pia	Pia	Pii	Pik^s	Pik^s	Pik	Pik-p	Pik-h	Piz-t	Pib	Pit	Pish	Pi1	Pi3	Pi7 (t)	Pi12 (t)	Pi19	Pik-m	Pi20	Pita2	Pita	Pi11 (t)		
	Race	IB-63	IH-1	IF-1	IH-1	IH-1	IC-28	IH-1	IB-63	IB-39	IH-1	IH-1	IF-1	IB-57	IF-1	IB-59	IH-1	IF-1	IF-3	IH-1	IB-13	II	II	II	
1	cultivar: Giza 159	4	3	4	5	3	2	4	1	1	7	4	4	5	4	2	4	6	1	3	7	1	1	2	45.5
2	Giza 171	6	4	4	7	5	5	4	1	6	7	7	4	5	4	6	5	5	1	7	8	1	1	1	22.7
3	Reiho	2	4	2	1	1	4	1	1	3	2	2	1	5	4	3-4	2	4	2	3	8	4	1	1	68.2
4	Giza 176	5	2	4	7	4	2	7	4	4	7	5	5	7	4	2	4	7	1	2	8	1	4	4	27.3
5	Giza 177	2	2	2	2	2	2	3	5	1	2	2	1	3	2	2	2	2	1	2	3	1	1	1	95.5
6	Giza 178	1	2	2	1	2	1	1	1	1	1	2	3	2	2	2	1	2	1	1	1	1	1	1	100
7	Giza 181	4	2	2	2	2	1	4	4	1	3-4	2	1	2	2	2	3-4	1	1	1	5	1	1	1	77.3
8	Sakha 101	5	2	2	3	4	2	7	4	1	3-4	5	1	2	2	2	6	1	1	1	7	4	4	1	59.1
9	Sakha 102	1	2	3	2	1	2	2	4	3-4	6	2	1	2	1	2	1	2	2	1	2	1	1	1	91.0
10	Sakha 103	1	1	2	2	1	2	3	3	1	2	2	1	2	1	2	1	1	1	1	2	1	2	1	100
11	Sakha 104	2	3	4	2	1	5	1	1	1	2	2	6	7	5	7	1	6	2	7	1	1	4	1	63.6
12	Sakha 105	2	2	2	2	1	1	2	4	1	2	2	3	2	2	1	1	2	1	2	3	1	2	1	95.5
13	GZ 7764-38-1-3-3	2	1	2	2	1	1	1	1	1	2	2	2	2	2	2	1	2	1	2	2	1	1	1	100
14	GZ 7769-2-1-1-2	2	1	4	3	1	1	1	1	1	1	2	3	2	1	3	1	2	1	2	2	1	1	1	95.5
15	GZ 7955-13-2-1-1	2	1	3	1	1	3	1	1	1	1	2	3	3-4	2	2	1	3	1	2	2	1	2	1	100
16	GZ 8126-1-3-1-2	2	1	2	2	1	1	2	1	1	1	2	4	3	2	2	2	3	1	2	2	1	1	1	95.5
17	GZ 8479-6-2-3-1	2	1	2	2	1	1	2	1	1	3-4	2	1	2	1	2	2	1	1	2	7	1	1	1	90.9
18	GZ 7576-10-3-2-1	2	1	2	3	1	2	2	1	1	2	2	1	2	1	2	2	1	1	2	4	1	1	1	95.5
19	GZ 8455-9-1-1-2	3	4	2	5	1	1	4	3-4	1	7	7	3	2	1	2	2	1	1	4	4	3	1	1	63.6
20	GZ 8450-4-2-3-3	2	4	2	5	1	1	4	2	1	6	6	1	2	1	2	3	1	1	1	6	1	1	1	77.3
21	GZ 6903	4	2	5	5	3	1	4	1	1	4	3	6	4	4	2	2	5	1	4	4	1	1	1	54.5
22	Egyptian hybrid 1	2	2	1	1	1	2	2	1	2	1	2	4	2	2	1	2	2	1	1	2	1	1	1	95.5
23	GZ 6296-12-1-2-1	1	2	2	1	1	1	2	1	1	1	1	1	1	2	2	2	1	1	1	1	2	2	2	100
	No. of infected cultivars	6	4	6	6	3	3	8	6	2	7	6	8	6	6	2	4	6	0	4	11	2	3	1	
	Virulence %	26.1	17.4	26.1	26.1	13.0	13.0	34.8	26.1	8.7	30.4	26.1	34.8	26.1	26.1	8.7	17.4	26.1	0	17.4	47.8	8.7	13.0	4.3	

1-2 Resistant , 3 Moderathy res Blant, 4-6 Suscepfible, 7-9 highly susceptible.

Virulence variation among rice blast populations:

The virulence of the obtained isolates was determined by the inoculation of each individual isolate on twenty-three of rice cultivars. The percentage of virulent reactions of tested isolates was ranging from 0 as avirulent to 47.8%. Results in Tables (3 & 4) show that the two rice blast isolates no. 20 and 26 were highly virulent to almost fifty percent of tested cultivars (47.8%) and have the similar bands. Also, isolate 33 is more closely related to the previous isolates in virulence (39.1%) and similar bands (Fig. 2). So, the genetic diversity can reflect the virulence type. Evaluation of virulence indicates the occurrence at some virulence diversity within each of the race group and also among different groups. Hamer *et. al.* (1989) identified a family of dispersed repetitive DNA sequences within the genome of *P. oryzae*, and these repetitive elements have become useful tools for examining genetic diversity within and between populations of the rice blast pathogen. Levy *et. al.* (1991) initially examined the relationship between virulence and distinct genetic groups or lineages.

The data indicate that the population virulence is strongly influenced by host genotype. The isolates of Sakha 101 (26) and Sakha 104 (27) were disabling to infect Reiho cultivar and other resistant cultivars. These results clarified the high levels of specificity of blast population. ISJ5 primer revealed that both highly virulent isolates no. 26 and no. 27, which induced the breakdown of both high yielding cultivars Sakha 101 and Sakha 104 were located in the same cluster and have some similar bands. On the other hand, ISJ9 indicated that both isolates are located in various clusters. The weakest isolates 23 from cultivar Giza171 and 24 from LTH were located in the same cluster with both ISJ 5 and ISJ9, Fig.s (2 and 3).

Effect of location on variation of virulence, concerning isolates of rice cultivar Giza 171 from different locations, it were significantly varied in their virulence. Isolate no. 24 derived from Kafr Saker -Sharkia governorate was avirulent (II) and exhibited low level of virulence (4.3%) compared with isolate no. 26 (IC-1) from Sakha Kafr Elsheikh, which recorded high level of virulence (47.8%) and low level (8.7%) of isolate no. 31 (IG-1) from Basion district. The isolates 24 and 26 have the similar genetic structure and bands, also, isolate 31 have some similar bands with these isolates. This result exhibited the ability of molecular diversity to reflect the geographical and virulence variation of the blast fungus isolates. Depending on virulence and no. of alleles in each isolate, all isolates were distributed in principal component analysis into three major genetic groups and one minor was observed (Fig. 1). One group corresponds to 12 isolates of monogenic lines from no. 4 to no. 15. This group exhibited significant virulence variation and wider spectrum of

virulence ranging from 8.7-34.8% with the majority of high virulence rate 26.1% and same location. The second group gathers 6 isolates from monogenic lines (16-22) except no.18 and no. 4 from rice cultivars and 2 weeds (26-34) except 29, 30 and 31. This group comprises the highly virulent isolates, 20 and 26 with (47.8%) and 33 (39.1%). The third group contains 6 isolates, 18, 23, 24,25,29,30 and 31.

All isolates in this group recorded the low level of virulence (0-13%). The minor group have 3 isolates 1, 2 and 3, which have almost the same virulence level 26.1%. Ngueko *et. al.* (2004) obtained M. grisea isolates from different blast nurseries with different pathotypes that were clustered together into the same genetic lineages, in comparing genetic diversity on large scale 101 isolates collected from the Guangdong province generating 14 genetic lineages while 45 blast isolates from Fujian generated 17 lineages among which 13 lineages comprised only one isolate each and one main lineage comprising 26 isolates. Some races from different geographic origin clustered in the same lineage and these races might be genetically closed.

On the other hand, races from the same blast nursery are clustered in different groups. Therefore, the geographic origin does not seem to be the only factor on blast differentiation. The genetic diversity in blast nursery could be attributed to diversified rice cultivars grown there. Genetic lineage is not corresponding to their pathotypes. The variation in the pathotypes of blast could be due to the cultivar diversity. The virulence variation was clearly depending on various resistance genes from the same location.

These results indicated that the virulence significantly differed among monogenic lines isolates depend on their infected individual genes. Isolates 18 (IF-3), 20 (IB-13) and 21(II) derived from monogenic lines, which carried genes Pikm, Pita2 and Pita exhibited different level of virulence 0, 48.7 and 8.7% from blast nursery of Gemmiza Gharbia, respectively. While, some isolated 8 and 12 from different genes exhibited the same virulence level 34.8%. Isolates 18 and 20 almost have the same similar bands while 21 have some bands. This variation maybe contributed to that those isolates carrying different Avr-genes, these results in agreement with Jia *et. al.* (2009). From the virulence results, data indicated that resistance genes Pi-ta2, Pik-p, Pi-sh, Pi-b, Pi-ks, Pi-a and Pi-i the most common genes in Egyptian cultivars, whereas the races which infected and isolated from monogenic lines carrying these genes able to infect 47.8, 34.8, 34.8, 26.1, 26.1, 26.1 and 26.1 respectively of all tested cultivars.

These results in agreement with results of Imbe (1998) as shown in Table 2, who identified the resistance genes in some Egyptian cultivars. These results explained the reason of breakdown of Sakha 101, which is carrying and depending on Pita2 resistance gene.

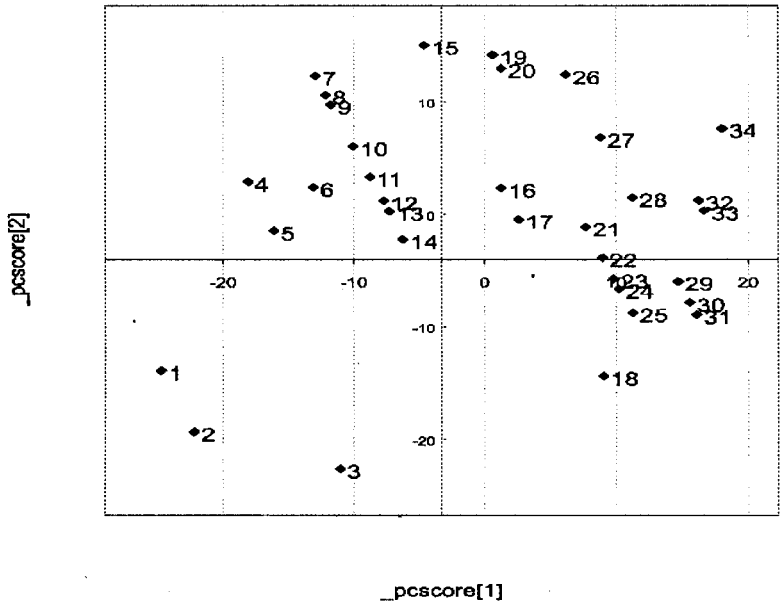

Fig. 1 principal component analysis *of Magnaporthe gresia* populations' structure according their virulence and no. of alleles to each isolate with marker ISJ5 and ISJ9, each point represents an isolate.

Genetic diversity among rice blast *"Magnaporthe"* populations:

Five primers of ISJ (Table 1) were screened to analyze the population structure of the causal organism of rice blast. The genetic variation in this investigation, which existing among the isolates of blast fungus from the susceptible monogenic lines and old commercial cultivars was studied by analyzing the DNA polymorphism in isolates that recovered from different rice cultivars. The five ISJ primers were amplified and gave reproducible results. These 5 primers reproduced monomorphic as well as polymorphic bands for 34 representative isolates of *Magnaporthe*. The maximum number of polymorphisms was shown with primer ISJ 9 (Fig. 4). Out of Five primers, four primers ISJ5, ISJ6, ISJ8 and ISJ9, revealed the best profiled of isolates. The ISJ primers profiled showed a high level of genetic variability among the monogenic lines isolates. Higher genetic variability was found among the tested isolates of monogenic lines and from old commercial cultivars. Higher genetic variability and polymorphism were found among populations of rice blast and isolates of weeds from *Cyprus rotunds* and *Echinochloa colonum*.

All tested markers revealed a high level of genotypic diversity in different populations of the pathogen. ISJ 5 and ISJ 9 detected a higher diversity in isolates of monogenic lines, old commercial cultivars and weed isolates.

Table 4. Blast reaction of 23 rice cultivars with blast isolates derived from rice cultivars and weed hosts under greenhouse conditions.

No.	Isolate no.	24	25	26	27	28	29	30	31	32	33	34	Resistance
	Race	II	IH-1	IC-1	IG-1	IB-45	ID-15	IG-1	IC-13	IG-1	IC-5	IB-63	%
1	**Cultivar:** Giza 159	4	4	6	4	4	7	4	3	4	4	4	9.1
2	Giza 171	2	4	6	4	4	2	4	4	4	4	4	18.2
3	Reiho	2	3	5	2	2	2	2	2	2	2	2	90.9
4	Giza 176	2	4	6	4	4	5	4	3	1	4	4	27.3
5	Giza 177	1	1	4	2	2	2	2	2	2	5	2	81.8
6	Giza 178	2	1	4	2	2	2	2	4	2	6	2	72.7
7	Giza 181	2	1	1	2	2	2	2	2	2	2	4	90.9
8	Sakha 101	2	1	2	7	2	2	2	2	2	4	2	81.8
9	Sakha 102	2	1	4	2	2	2	2	2	2	2	2	90.9
10	Sakha 103	2	2	2	2	2	2	2	2	2	7	2	90.9
11	Sakha 104	1	2	5	2	7	2	2	2	2	4	2	81.8
12	Sakha 105	1	2	1	2	2	1	1	1	1	2	2	100
13	GZ 7764-38-1-3-3	1	2	1	1	1	2	1	2	1	2	1	100
14	GZ 7769-2-1-1-2	1	2	1	1	2	1	1	2	1	1	1	100
15	GZ 7955-13-2-1-1	1	1	2	1	2	2	1	1	1	1	2	100
16	GZ 8126-1-3-1-2	1	1	2	1	1	2	1	2	1	2	1	100
17	GZ 8479-6-2-3-1	1	2	4	2	2	1	1	2	1	1	1	90.9
18	GZ 7576-10-3-2-1	1	1	1	1	1	2	1	2	1	1	2	100
19	GZ 8455-9-1-1-2	1	3	4	3	3	2	1	1	1	4	1	81.8
20	GZ 8450-4-2-3-3	1	3	4	3	2	1	1	1	1	3	1	90.9
21	GZ 6903	1	1	2	1	1	2	1	1	1	1	1	100
22	Egyptian hybrid 1	1	2	1	1	1	2	1	1	1	1	2	100
23	GZ 6296-12-1-2-1	1	1	1	1	1	2	1	1	1	2	2	100
	No. of infected cultivars	1	3	11	3	3	2	3	2	2	9	4	
	Virulence %	**4.3**	**13.0**	**47.8**	**13.0**	**13.0**	**8.7**	**13.0**	**8.7**	**8.7**	**39.1**	**17.4**	

1 -2 Resistant, 3 Moderatly resistant, 4-6 susceptible and 7-9 Highly susaphible.

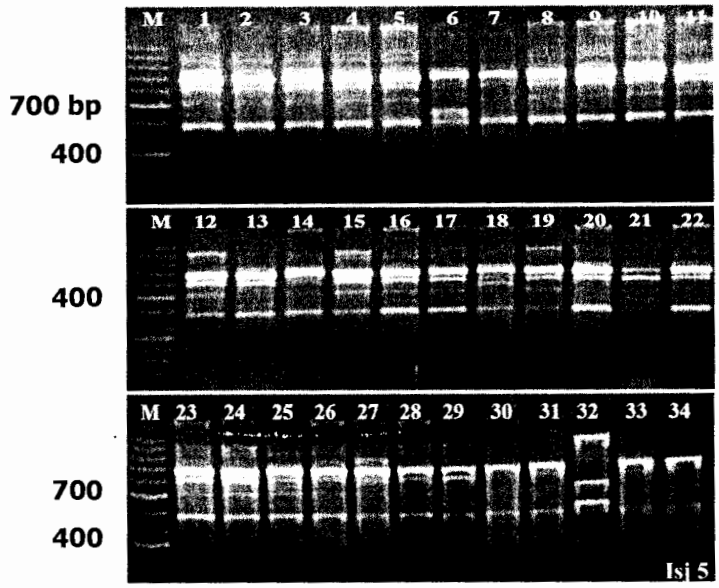

Fig. 2. Amplification results of ISJ5 primer with tested isolates using PCR, M marker 50-1000

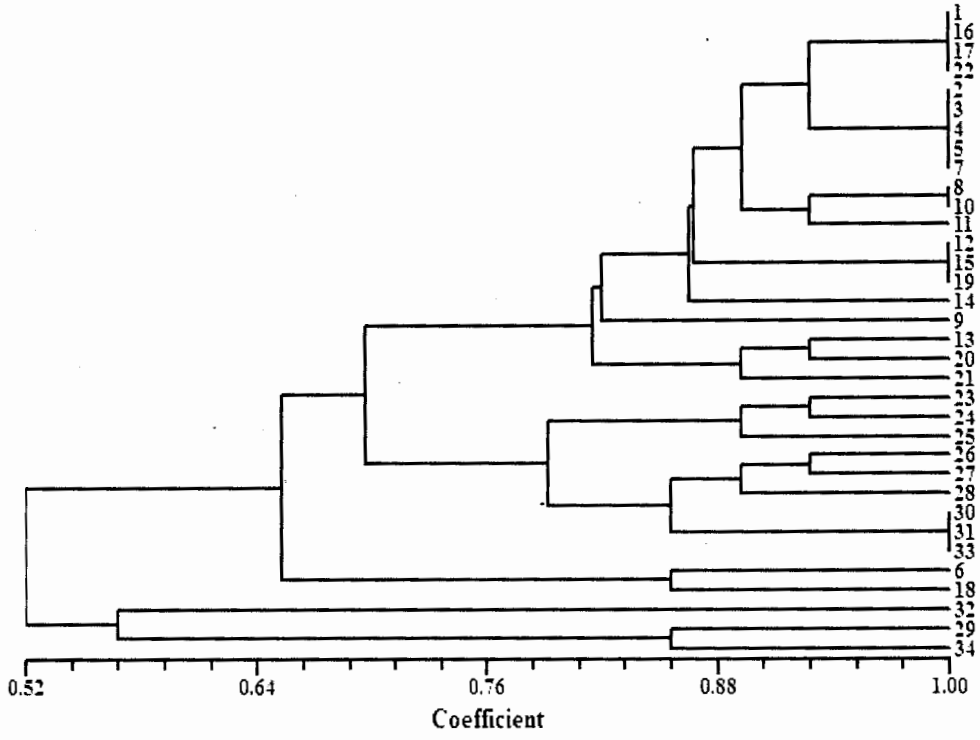

Fig. 3. Dendrogram of genetic relationship among the tested isolates using Jaccord`s coefficient. (ISJ5).

Concerning the genetic variability among isolates of monogenic lines, there are a significant variation among isolates 6,18 and 1, 16, 17, 22. Isolates no. 2, 3, 4, 5

are more similar to each other and have the same genetic pattern. Unique bands of 400 bp and 700 bp in size were presented in the isolates of monogenic lines except isolates no. 21 (Fig. 2). Also, bands of 400 bp and 700 bp were presented in all the isolates of old commercial cultivars except isolate no. 32 from Cyprus rotunds (Fig.'s 2 & 4). This indicated that ISJ5 and ISJ9 can be considered as specific primers for differentiation among blast fungus populations.

In UPGMA cluster analysis (Fig. 3) based on molecular scoring of ISJ primers, the fingerprint patterns for the 34 tested isolates thus revealed the presence of 22 haplotypes. At 52% similarity, all the 34 isolates formed two clusters or two main lineages were observed, the first lineage contains three isolates, 29, 34, in the same cluster and no. 32 in another one. The isolate no. 34 was specific for indica cultivar Giza 181 which is rare compared with the most common of japonica cultivars isolates under Egyptian condition. So, this isolate is more diverse than japonica isolates from both monogenic and old commercial cultivars. Also, isolate no. 32 derived from Cyprus rotundus as a weed host for blast fungus and more diversed than all isolates of rice host. The value of genetic similarity varied from 0.52 to 1.0 (Fig's 3 & 5).

M. grisea has a broad host range and besides rice, it is known to infect 50 graminaceous species including economical important crops, such as wheat (*Triticum aestivum*), maize (*Zea mays*), finger millet (*Eleusine coracana*), common millet (*Panicum repens*), foxtail millet (*Setaria italica*) and various feral grasses. Of the grass species that are known to be the host for *M. grisea*, crabgrass (*Digitaria sanguinalis*), jungle rice (*Echinochloa colonum*), goose grass (*Eleusine indica*) and *Cyperus rotundus* are the major rice associated weed flora of Himachal Pradesh. Most of these grasses are often found infected with blast even before the onset of blast symptoms in the contiguous rice fields (Ou, 1985, Rathour *et. al.,* 2006). El-Shafey (2002) in Egypt, isolated for first time fifteen Pyricularia grisea isolates from different weeds and identified four races as IG-1 (10 isolates), two isolates for each of IB-57 and IB- 61 and one was identified as ID-13. Also, he detected significant differences in morphological and virulence traits among weed and rice blast isolates.

The second lineage or main cluster comprised the rest of 31 isolates representing 19 haplotypes and were further subdivided into two sub-clusters. The first sub-cluster has isolates 6 and 18 from monogenic lines. While, the second sub-cluster contains two sub-sub clusters, the first one involved the isolates from old commercial cultivars Giza 171, Sakha 101 and Sakha 104 from isolate no. 23-28 and 30, 31 and 33. The second sub-sub cluster comprised the most isolates of monogenic lines isolated from one location (Gemmiza, Gharbia governorate). The ISJ5 marker exhibited high level of genetic diversity among all population of rice blast fungus.

The role of weed isolates in initiation of blast primary inoculum, isolate 30 derived from jungle rice *Echinocloa colonum* is more similar to aggressive isolates 33 and 31. In Egypt, El-Shafey, 2002 and Sehly, 2009 revealed a significant cross infection between isolates of both jungle rice and old rice commercial cultivars Giza 159, Giza 171 and Giza 176 under artificial inoculation. The isolate of jungle rice has the same morphological traits of rice blast isolates. Also, jungle rice *Echinochloa* colonum was more severely infected with blast as a common weed in rice fields. Therefore, an important concern is to know whether the *M. grisea* isolates from jungle rice and susceptible rice contribute inoculum for the initiation of rice blast infection. (Mackill and Bonman, 1986, Kumar and Singh, 1995) demonstrated the capacity of non-rice isolates of *M. grisea* to infect rice and vice versa. (Rathour *et. al.,* 2004) have indicated a strong possibility of gene flow between the different host-limited forms of the pathogen.

The same results were revealed with ISJ 9 marker except the fingerprint patterns for the 34 tested isolates thus revealed the presence of 8 haplotypes. Also this marker revealed low level of genetic diversity among monogenic lines isolates. The most of monogenic lines isolates have the same bands and almost grouped in one cluster and contributed to the same geographical location Gemmiza, Gharbia. This result exhibited the ability of molecular diversity assessment to reflect the geographical variation of the blast fungus isolates. Correll *et. al.* (2009) Samples of the 3000 rice blast pathogen populations over the past 17 years in Arkansas which indicated a relatively consistent persistence of the predominant MGR586 fingerprint groups and/or VCGs. Although eight groups have been identified in the U.S. (Correll *et. al.,* 2000c, Levy *et. al.,* 1991, Xia *et. al.,* 1993), only four groups (Groups A, B, C, and D) appear to persist in the more current population.

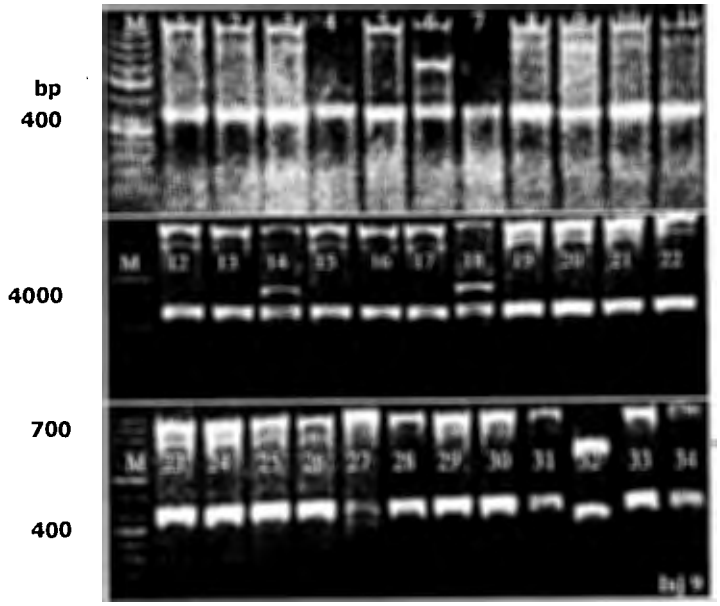

Fig. 4. Amplification results of ISJ9 primer with tested isolates using PCR, M marker 50-1000bp.

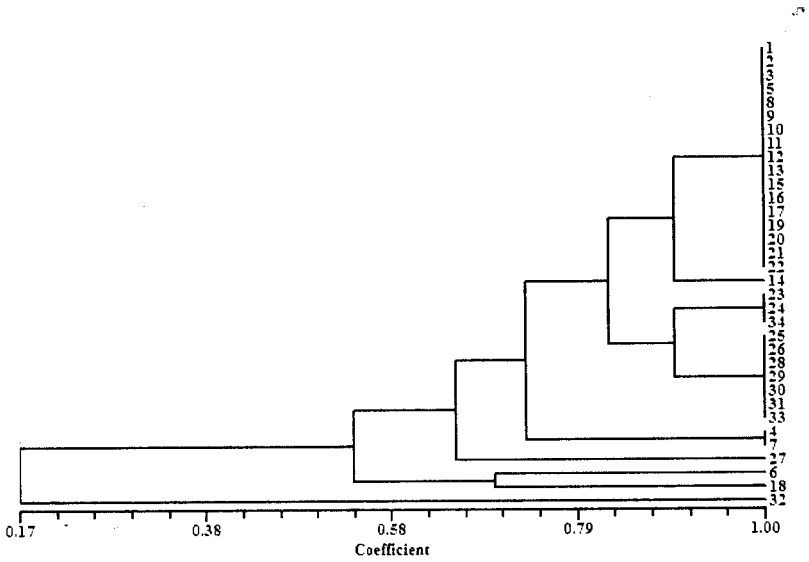

Fig. 5. Dendrogram of genetic relationship among the tested isolates using Jaccord`s coefficient (ISJ9).

All rice specific markers ISJ used were successfully amplified with the blast fungus isolates, indicating a sufficient level of similarity between the rice plant and the blast fungus in these genetic loci. These results demonstrate the usefulness of molecular diversity in assessment and differentiation of virulence among blast fungus populations.

Varietal resistance variation under artificial inoculation

The old commercial cultivars Giza 159, Giza 171 and Giza 176 were highly susceptible to all tested rice blast races, this result indicated that their resistance

genes are compatible with prevalent races and must replace with more new incompatible R-genes. The percentage of resistant reactions of local cultivars to the 22 isolates of monogenic lines was found ranging from 59.1% to 100%. The cultivars Giza 177, Giza 178, Sakha 102, Sakha 103, Sakha 105 still recorded high level of resistance ranging from 81.8 to 100 % and promising lines from 54.5 to 100 %. These results on the line of the Rice Research & Training Center (RRTC) strategy to start produce resistant cultivars after the epidemic and breakdown of Reiho in 1984 (RRTC, 2006) and According to Imbe, 1998 and Sehly, 2008 the Egyptian cultivars carry different blast resistance genes. The genes Pi-a, Pi-ks, Pi-z and even unknown genes in Giza 171, Giza 172 and Giza 176 were ineffective under Egyptian condition because these cultivars were highly susceptible to blast disease. Pi-a gene showed resistant level ranging from 14.3 to 20% and Pi-Ks showed 30.7 to 85% during 1994 up to 2003 seasons. While, the Egyptian resistant cultivars Giza177, Giza178, Sakha102, Giza182 and Sakha103 were resistant and carry different resistance genes. Promising lines GZ 6296, GZ7576, GZ 7764, GZ 7769, and GZ 7955 and GZ 8126 were resistant to all tested isolates, Tables (3 & 4). These lines may have new resistance and highly effective genes. These lines were resistant to all races isolated from monogenic lines which carry individual resistance genes. Therefore, it can be utilized as a good source as new resistance genes donors to blast in breeding program to extend the narrow genetic background of Egyptian rice cultivars. The obtained results are in agreement with results of El-Refaee *et. al.* (2011) who evaluated the genetic diversity and relatedness among some Egyptian rice genotypes based on important agronomic traits and some biotic stress using SSR markers and indicated that Still Giza 177, Sakha 102 and Sakha 103 shown resistant and became good sources for blast resistance. Giza 178 and other indica rice appeared to be resistant. Also, they reported that all new promising lines were resistant (GZ 6522, GZ6903 and GZ7955) and all tested cultivars showed a wide variation in blast reaction. High race shifting and big change in prevalence of specific races for highly susceptible old rice cultivars is very clear from the reaction of Reiho, however, this cultivar recorded the first serious epidemic and breakdown during season 1984 and now exhibited high level of resistance ranging from 68.8 to 95.9 % compared with the two major virulent genotypes Giza 159 and Giza 171 (9.1 and 18.2 %), respectively (Tables 3 and 4). This result is in compatible with Sehly, 2008.

Data in Tables (2 & 3) show that the isolate no. 20 (race IB-13), which isolated from line IRBL-Pi ta2 and carrying gene Pi-ta2 was able to infect 47.8 % from tested cultivars. These results proved that 50% of the tested cultivars maybe have this gene.

On the other hand, the race IF-3 from line IRBL km-Ts (Pi-Km gene) was avirulent and disable to infect any tested cultivar, which indicates that these cultivars may not have Pi-Km gene and carrying another. In addition, race II (20- IRBLta-CP1) derived from this line, which have gene Pita, was only able to infect Reiho and Sakha 101, Reiho have already had this identified gene (Fukuta *et. al.,* 2004). Therefore, these results and same pathological behavior indicated that Sakha 101 might have the same gene. Also, according to Imbe, 1998 and Sehly, (2008) concerning Sakha 101, it has Pi-ta2 with low level of resistance ranging from 21.1 up to 70% from 1997 up to 2006 seasons, these were the same period of its release and breakdown (1999 to 2003 seasons).This reaction of different cultivars and the ability of infection to some new commercial cultivars revealed high level of variability among rice isolates. These results are in agreement with the findings of Ou (1975), Sehly *et. al.* (1993 and 2000). They reported a wide variation among isolates of P. grisea in Pathogenicity to rice cultivars and identified many pathogenic races belonging to different race groups.

In a conclusion, rice blast fungus has a wide range of variability in virulence and response to different cultivars. Therefore, it must extend the genetic background and induces the varietal resistance diversity through incorporation of new resistance genes. GZ 6296 and GZ 7955 were predicted to be a highly effective, as none of the isolates infected these lines. These blast- resistant rice lines can be used in resistance breeding for the effective management of rice blast in Egypt. Therefore, utilization of multiple R genes with overlapped resistance spectra is one of the most powerful strategies for managing blast disease

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٥-٤ التنوع المرضي والجزيئي لعزلات جديدة من فطر اللفحة في الأرز مجمعة من عوائل مختلفة

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يعتبر مرض اللفحة في الأرز المتسبب عن الفطر *Magnaporthe grisea* من أكثر الأمراض المدمرة للأرز في مصر حيث يؤثر على محصول الأرز بشدة خاصة في الأصناف اليابانية الأكثر انتشارا. وفي هذه الدراسة تم استخدام ٣٤ عزلة تم تصنيفها إلى ثلاث مجموعات: المجموعة الأولى شملت ٢٢ عزلة من الأصناف المفرقة فردية الجينات، والمجموعة الثانية شملت ١٠ عزلات من الأصناف المصرية القديمة، جيزة ١٧١ وسخا ١٠١ وسخا ١٠٤، أما المجموعة الثالثة فقد احتوت على حشائش أبو ركبة والسعد. وقد تمت العدوى بهذه السلالات في الصوبة على الأصناف المحلية والسلالات المبشرة. وأظهرت النتائج اختلافا شديد المعنوية في المقاومة بين هذه الأصناف المختبرة. وتراوحت نسب المقاومة للعزلات المختبرة من ٥٩ إلى ١٠٠%. وكانت السلالات المبشرة GZ6296 والسلالة GZ7955 (سخا ١٠٦) مقاومة لجميع السلالات المختبرة مما يدل على أنه قد تحتوي على جينات مقاومة جديدة. وتوصي هذه النتائج بإمكانية استخدام هذه السلالات كمصادر لجينات المقاومة في برنامج التربية. كما تم دراسة التنوع الوراثي لعزلات اللفحة باستخدام المعلمات الجزيئية حيث تم استخدام خمس معلمات جزيئية من ISJ. وتم تصنيف عزلات اللفحة بهذه المعلمات على أساس درجة القرابة الوراثية إلى عزلات عديمة القدرة المرضية وعزلات ضعيفة وعزلات قوية. أظهرت المعلمات ISJ5 و ISJ9 درجة كبيرة من الاختلاف بين العزلات وبذلك يمكن استخدامها كمعلم جزيئي متخصص لتحديد مدى التنوع بين عزلات اللفحة. وتناولت هذه الدراسة أيضا مدى التنوع الوراثي بين عزلات جديدة لفطر اللفحة وسوف تكون هذه النتائج هامة وتؤخذ في الاعتبار في برنامج التربية لاستنباط أصناف مستديمة مقاومة لهذا المرض المدمر.

أظهرت نتائج المعلمات الجزئيئية وجود قرابة بين عزلة حشيشة أبو ركبة وعزلات الأرز ودور هذه الحشيشة كعائل بديل ومصدر للقاح الأولى لفطر اللفحة .