Molecular and morphological traits of *Pyricularia oryzae* Cav. in the south of Russia

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Abstract. Based on molecular genetic approaches, a study of the biodiversity of the phytopathogenic fungus *Pyricularia oryzae* Cav. was carried out in the south of Russia, the classification of morphological and cultural traits of the pathogen was carried out. In 2020, monitoring was carried out and 9 strains of the pathogen were isolated from the affected herbarium material collected from the fields of five ecological rice-growing zones of Krasnodar region (Krasnoarmeisky, Kalininsky, Abinsky, Slavyansky districts, Krasnodar). Using a multiplex PCR system based on fragment analysis, among the studied strains of *Pyricularia oryzae* Cav. 5 genotypes were identified in 2020, each of which is characterized by a unique genetic profile. Their «DNA-passports» have been compiled. The studied isolates of the blast pathogen were characterized by morphological and cultural traits. 6 morphotypes of the pathogen were identified. Classification of the pathogen by morphological, cultural and genetic traits will allow obtaining scientifically grounded and comparable data that can be used in the analysis of the interaction of blast strains with rice plants on different varieties and in different agro-technological conditions in order to improve and rationalize agricultural activities. In addition, the studied approaches open up the possibility of using data in breeding, allowing the identification of pathogen forms that infect certain varieties.

1 Introduction

Study of genetic structure and biological diversity of highly variable fungi phytopathogens as well as identification of effective resistance genes of cultivated plants to diseases remain an urgent problem. [1, 2, 3].

According to the degree of harmfulness for rice, blast disease (the causative agent - the imperfect fungus *Pyricularia oryzae* Cav. (*Magnaporthe grisea* (Herbert) Barr)) takes the first place. The World Institute of Mycology has registered this disease in more than 80 countries, and in recent years, a high severity of this disease has been noted in all rice-growing regions of the world [2, 4, 5].

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P. oryzae Cav. pathogen affects all aboveground plant organs and is dangerous during the entire rice growing season. Small caryopses develop on the affected plants, the setting of seeds in the panicle decreases, the grain yield lowers to 40%, and the quality of products deteriorates sharply. In the presence of favorable conditions, the blast pathogen may reduce the rice yield by up to 45%, and in the years of epiphytotic yield damage can reach 100% [6-8].

Breeding for resistance to phytopathogenic fungi is more difficult than breeding for any other economically valuable trait. This difficulty is determined by the race-forming processes continuously going on in the pathogen. With a change in the cultivated assortment of rice, a strict selection takes place for the survival of the *P. oryzae* pathogen itself, as a result of which new races, pathotypes of the blast pathogen appear [6, 8]. To conduct successful breeding for resistance to this disease, it is necessary to have a clear understanding of the evolution of the pathogen; knowing its intrapopulation structure, virulence gene pool and interaction features in the host-parasite system; constantly conducting a wide search for new sources of resistance and creating a bank of resistance genes and pathogen strains. These studies are essential for understanding what the *P. oryzae* pathogen is at the present stage. Only on the basis of all this information the correct approach to the selection of resistance types and the most effective method for developing resistant genetic resources to the disease can be developed.

Purpose of the work: to study the genetic diversity of *P. oryzae* pathogen strains by DNA polymorphism and morphological and cultural characteristics of the south of Russia.

Research tasks:

1. To characterize the biodiversity of *P. oryzae* by morphological and cultural traits.
2. To conduct genetic certification of pathogen field strains using PCR (microsatellite) markers based on fragment analysis.
3. To draw up their "DNA-passports".
4. To organize the results obtained in the database [19].
5. To determine effective genes for resistance to blast disease, as well as genes for virulence of *Pyricularia oryzae* Cav. pathogen.

In the future it will allow to carry out a comparative characterization of the microsatellite profile of each newly isolated field strain of the pathogen with those from the database [19], thus assessing the degree of their genetic similarity or difference, as well as to control the variability of the pathogen by determining the so-called "lines" or "pathotypes" of a pathogen.

The implementation of the obtained results will lay the foundations for solving the problem of zonal distribution of rice varieties in the south of Russia, that will reduce crop yield losses and increase the socio-economic development of the rice growing industry.

2 Methods

To study the genetic diversity of the blast pathogen by molecular genetic methods, the fungus DNA was isolated from a mixture of mycelium and conidia of the monoconidial culture *Pyricularia oryzae* Cav. by the CTAB method [9]. The method consists in using hexadecyltrimethylammonium bromide as a detergent in lysis buffer.

The composition of the lysis CTAB buffer: 0.2 M Tris pH 8; 2 M NaCl; 0.05 M EDTA; 2% CTAB.

The growth of the mycelium for DNA isolation was carried out in Petri dishes on liquid carrot agar medium. When the required biomass was reached, the mycelium was removed from the dishes and grounded in 700 µL of lysis CTAB buffer in a sterile ceramic mortar. Then the resulting mixture was transferred to an Eppendorf tube and incubated at 65 °C for an hour. To the resulting mixture, 500 µL of chloroform were added and centrifuged for 10

min. at 13,000 rpm Then, using a pipetman, 500 µL of the supernatant liquid were taken with a 1000 µL pipetman, another 500 µL of chloroform were added to it, and centrifugation was repeated. To the obtained supernatant, 400 µl of isopropanol were added and the solution was placed in a freezer for 2 hours. After it was centrifuged for 10 min at 13,000 rpm. The formed DNA precipitate was washed with cooled 70% ethanol and resuspended in 50 µl of buffer TE for storage at -20 ° C.

DNA amplification of the phytopathogenic fungus *Pyricularia oryzae* Cav. was carried out in a volume of 25 µl containing 1 PCR-buffer, 1.25 units of Taq DNA-polymerase, 100 µmol of dNTP (DNA-Technology llc, Russia), 10 pmol of each primer, and 5 µl of DNA solution in a detecting thermal cycler (CFX-BioRad, USA) according to the program: 95oC - 10 min, (94 ° C - 30 s, 55 ° C - 30 s, 72 ° C - 30 s) 30 cycles and final elongation - 72 ° C 5 min.

Sequence of SSR-markers used in the study is presented in Table 1 [10, 11].

Table 1. Microsatellite markers used for studying biodiversity of blast agent.

Mar ker nam e	Origin	Chro mo some (geno me conti g)	Refere nce for genomi c sequen ce	Repe at motif	Primers	Ta (°C)	Multi plex pool	Allele size range, bp
pyrms 07-08	Sequenc ed BAC	7 (557)	Kaye et al. 2003	CT/ GA29	F: (NED)- GCAAATA ACAGGAA AACG R: AGAAAGA GAC AAAACAC TGG	55	1	116/162
pyrms 43-44	BAC end	4 (864)	Kaye et al. 2003	TA12	F:(FAM)- CAGTAGG CTTGGAA TTGA AAAA R: CTTGATT GGTG GTGGTGT TG	55	2	205/233
pyrms 47-48	BAC end	4 (781)	Kaye et al. 2003	TA15	F: (HEX)- TCACATT TGCTTGC TGGAGT R:AGACA GGGTTGA CGGCTAA A	55	2	164/196
pyrms 83-84	BAC end	2 (1895)	Kaye et al. 2003	TCA/ AGT1 3	F:(FAM)- GTCTGCC TCGACTC CTTCAC R:	55	2	168/189

					AGCCCAA AAACA GAAAGCA A			
pyrms 99- 100	BAC end	5 (718)	Kaye et al. 2003	ACC/ TGG2 0	F: (NED)- CACCACT TTATGGC GCAGT R: ACCTAGG TAGG TATACAT GTTGTT	55	2	178/222
pyrms 427- 428	Genome sequenc e	5 (718)	Dean et al. 2005	AT/ TA16	F:(HEX)- CTGTCAC CACAACC AAGACG R: TTGCCCT GATTT GTCAGTC A	55	2	212/220

Fragment analysis was carried out for visualization of PCR products on an automatic genetic analyzer "NANOPHOR-05" (Institute of Analytical Instrumentation, Russia) with the support of Research Equipment Sharing Center "Biotechnology" of All-Russian Research Institute of Agricultural Biotechnology.

Data processing is performed using GeneMapper software.

The fungus culture was isolated from diseased rice tissues (stem nodes, leaves, panicle parts) collected from rice fields, in accordance with the guidelines developed at the All-Russian Research Institute of Phytopathology [12]. Samples of the affected tissue were washed with sterile water and placed on sterile carrot-sugar agar medium in Petri dishes, which were then placed in a humid (100%) chamber at a temperature of $27 \pm 1^{\circ}\text{C}$, which was optimal for the formation of conidia of the fungus *Pyricularia oryzae* Cav. The resulting pure culture was cultivated in a thermostat until the fungal colony was distributed over the entire surface of the agar medium, then the selected isolates were described by morphological and cultural traits [13-16].

3 Results

3.1 Studing biodiversity of *Pyricularia oryzae* Cav. on the basis of microbiology methods in the South of Russia

Recently, the growing distribution and harmfulness of the phytopathogenic fungus *Pyricularia oryzae* Cav have been observed in the rice-growing regions of Russia. Therefore, the question of studying the genetic structure, determining the pathotype of the local population of *P. oryzae* is very relevant, especially for the main rice-producing region of Russia - Krasnodar region [17, 18].

Such knowledge is of particular interest, since it makes it possible to predict the emergence of new *P. oryzae* races and pathotypes, and is also a necessary theoretical basis for development of genetic sources with long-term blast resistance. [19, 20].

For this purpose, we annually conduct monitoring (survey) of rice-growing farms in the south of Russia for the damage of rice crops by blast. In 2020, 10 rice growing farms were

surveyed in five ecological zones of Krasnodar region (Krasnoarmeisky, Abinsky, Slavyansky, Kalininsky districts and the city of Krasnodar). Herbarium material with traits of disease was collected. Nine strains of *Pyricularia oryzae* Cav with different sporulating ability were isolated from the affected leaves, stem nodes, and panicles of rice plants. All obtained *P. oryzae* strains have a unique morphotype.

Tables 2, 3 show their origin and description by morphological and cultural traits (the nature of the colony growth, the color of the upper mycelium and spores, the color of the lower mycelium) [22].

Table 2. Origin of *Pyricularia oryzae* Cav. strains, 2020.

№	Strain number	District	Selection date	Variety	Part of plant
1	1.20	Krasnoarmeisky	06.07	Cahika	leaf
2	5.20	Krasnoarmeisky	15.07.	Favorit	leaf
3	7.20	Krasnoarmeisky	15.07.	Polevik	leaf
4	10.20	Krasnoarmeisky	20.07	Rapan	leaf
5	11.20	Kalininsky	21.07	Istok	leaf
6	12.20	Krasnoarmeisky	22.07	Istok	leaf
7	13.20	Krasnoarmeisky	22.07	Istok	leaf
8	16.20	Slavyansky	12.08	Rapan	panicle stalk
9	20.20	Krasnodar, FSBSI "FSRC"	21.08	KP-20	node

Table 3. Morphological and cultural traits of *P. oryzae* Cav. strains, 2020.

№	Growth pattern	Structure	Relief	Profile	Mycelium color	Substrate color
1.20	Multidirectional	Flocky	Conical	Low	Typical	Brown
5.20	Zoned	Flocky	Nodular	Low	Light	Black
7.20	Unidirectional	Felted	Conical	Medium	Olive	Black
10.20	Unidirectional	Felted	Conical	Medium	Olive	Black
11.20	Zoned	Flocky	Nodular	Low	Light	Black
12.20	Unidirectional	Felted	Uniform	Low	Typical	Gray
13.20	Zoned	Flocky	Nodular	Low	Light	Black
16.20	Concentric	Felted	Conical	Medium	Olive	Black
20.20	Zoned	Flocky	Nodular	Low	Light	Black

The strains of *Pyricularia oryzae* Cav. pathogen can be divided into the following morphotypes (groups):

1. Strains with a unidirectional or concentric growth pattern, a felt structure of a conical relief and a low growth profile of olive-gray mycelium and a black substrate;

2. Strains with a zoned growth pattern, a flocky structure of nodular relief and a low growth profile of light gray mycelium and a black substrate;

- 3. Strains with a unidirectional growth pattern, a felt structure of a uniform relief and a low growth profile of the mycelium of a typical gray color and a gray substrate;
- 4. Strains with multidirectional growth pattern, a flocky structure of conical relief and a low growth profile of the mycelium of a typical gray color and a brown substrate.

Figure 1 shows photographs of these *Pyricularia oryzae* Cav. morphotypes isolated in 2020.

Fig. 1. Strains of blast agent *Pyricularia oryzae* Cav.

Note to Figure 1: the figure shows the cultures of the most characteristic representatives of the morphotype group (their characteristics are presented in Table 2).

This information was entered into the previously developed database “Strains of the *Pyricularia oryzae* Cav. pathogen in the south of Russia” [21], which contained factual material on the strains of the *P. oryzae* pathogen. These data can be used to analyze and monitor populations and pathotypes of the blast pathogen in the rice-growing regions of southern Russia. They presents the characteristics of the *P. oryzae* population by morphological and cultural traits, "morphological" and "DNA-passports" of the isolated strains of the blast pathogen from different ecological zones of rice cultivation in the south of Russia were compiled.

3.2. Genetic certification of pathogen field strains using PCR (microsatellite) markers based on fragment analysis

For the genetic classification of the *Pyricularia oryzae* Cav. strains found in Krasnodar region, we used the previously developed multi-primer PCR system [3, 17], which allowed simultaneous analysis of 6 microsatellite loci. This approach allows each DNA sample to be analyzed simultaneously for all six microsatellite loci. Amplification of each microsatellite locus is carried out with specific primers, one of which is labeled with a certain fluorescent dye (FAM, R6G, or ROX), which makes it possible to analyze PCR fragments separately using the corresponding detection channel.

The results of the analysis for microsatellite loci in duplicate are presented in Table 4.

Table 4. Classification of strains of *Pyricularia oryzae* Cav. causative agent of blast based on the obtained genetic profiles and their "DNA-passport".

Genotyp e	A	B	C	D	E	F
	07-08	427-428	83-84	43-44	99-100	47-48
1.1	137	172	177	209	186	220
1.2	137	172	177	209	186	220
5.1	137	172	180	205	211	220
5.2	137	172	180	205	211	220
7.1	139	172	180	203	211	220
7.2	139	172	180	203	211	220

10.1	139	172	180	203	211	220
10.2	139	172	180	203	211	220
11.1	137	172	180	203	211	220
11.2	137	172	180	203	211	220
12.1	137	172	180	203	211	218
12.2	137	172	180	203	211	218
13.1	139	172	180	203	211	220
13.2	139	172	180	203	211	220
16.1	139	172	180	203	211	220
16.2	139	172	180	203	211	220
20.1	137	172	180	203	211	220
20.2	137	172	180	203	211	220
K1-50	129	174	177	219	217	222
K2-31	137	172	180	203	211	220
K3-V	131	172, 176	177	221	219	222

Note to Table 4: blue - shows a fluorescent dye FAM; green - fluorescent dye R6G; red - fluorescent dye ROX; A, B, C, E, D, F – the designation of loci; numbers – the fragment length detected in each locus.

As a result of molecular genetic studies, 5 genotypes of the pathogen were identified. Their “DNA-passports” were compiled (Fig. 2, Table 5).

Fig. 2. Genetic profiles of the studied isolates of *Pyricularia oryzae* Cav., identified as a result of DNA analysis; 1 - genotype 1 (sample 1); 2- genotype 2 (sample 5); 3 - genotype 3 (samples 7, 10, 13, 16); 4 - genotype 4 (samples 11, 20, K2).

On the digitized genetic profiles of *Pyricularia oryzae* Cav. strains, each peak is a PCR fragment of a certain length. The color of the peak corresponds to the detection channel and determines the belonging of the fragment to one or another locus (Table 5).

Table 5. Classification of strains of *Pyricularia oryzae* Cav. causative agent of blast based on the obtained genetic profiles.

Genotype	A	B	C	E	D	F	Sample
	07-08	427-428	83-84	99-100	43-44	47-48	
1	137	172	177	186	209	220	1.1
2	137	172	180	211	205	220	5.20
3	139	172	180	211	203	220	7.20, 10.20, 15.20, 16.20
4	137	172	180	211	203	220	K2, 11.20, 20.20
5	137	172	180	211	203	218	12.20
K1	129	174	177	217	219	222	50-2016
K2	137	172	180	211	203	220	31-2016
K3	131	172, 176	177	219	221	222	V-2016

Among the studied strains, 5 variants of genotypes were identified. The genotype of samples 11 and 20 corresponded to the genotype of the previously studied strain 31 [17], which was used as a positive control.

Strain No. 1.20 was assigned to the first genotype; 5.20 - to the second, which had a unique genetic profile and morphotype. Strains No. 7.20, 10.20, 15.20, 16.20 from Krasnoarmeysky, Kalininsky and Slavyansky districts entered the third group and had the same DNA- profile. In strains No. 11.20, and 20.20, the genotype of the positive control K2 was revealed. They were assigned to the 4th genetic group. Strain sample No. 12.20 had a fifth genotype. This strain had a unique genetic profile and morphotype.

Thus, all obtained and studied strains of *Pyricularia oryzae* Cav. when describing by morphological and cultural characteristics, were divided into 6 morphotypes and, according to the results of DNA analysis, 5 genotypes of the pathogen were identified.

The obtained results were clustered using the Ward method [23] according to 12 characters (6 genetic characters and 6 morphological and cultural ones, Fig. 6).

Fig.3. Clustering of *P. oryzae* Cav. strains by the Ward method.

It can be seen from the figure that when a hierarchical dendrite is cut at a distance of 3 units (along the x axis), all studied strains are separated into 4 clusters with a unique genetic DNA- profile and morphotype. The first cluster included strain 12.20; the second - 1.20; the third: strains No. 20.20, 11.20, 5.20 and 13.20; the fourth cluster included pathogen strains No. 10.20, 7.20, 16.20. These results are entered into the Database "Strains of *Pyricularia oryzae* Cav. pathogen, in south of Russia" [19] and can be used in phytopathological studies to control the variability of the pathogen.

3.3. Determination of effective blast resistance genes in the south of Russia

One of the goals of our work was to determine effective blast resistance genes [24], as well as genes for the virulence of *Pyricularia oryzae* Cav. pathogen. To achieve it, we have identified resistance genes of the fungus *Pyricularia oryzae* Cav. that are effective for southern Russia using the International Set of Differentiator Varieties of *Pyricularia* races obtained from the International Rice Research Institute (IRRI, Philippines) and the Institute of Agricultural Genetics (Hanoi, Vietnam). (Table 6): Pi-sh, Pi-b, Pi-z-5, Pi-z-t, Pi-5, Pi-1, Pi-7, Pi-kh, Pi-km, Pi-kp, Pi-ks, Pi-ta, Pi-ta2.

Table 6 shows the results of phytopathological testing for resistance to the Krasnodar population of the pathogen during artificial infection of rice differentiator varieties (Fig. 7).

Fig. 4. Artificial infection of differentiating varieties with the phytopathogenic fungus *P.oryzae* Cav.

Table 6. Blast resistance of rice differentiator varieties with resistance genes (IDB laboratory, 2020).

№	Registration number	Variety name	Resistance gene	Variety reaction	Virulence gene
1	11366	Sha-tiao-tsao	<i>Pi-ks</i>	R	
2	11367	Ishikari-shiroke	<i>Pi-i</i>	S	Av-i+
3	11370	Usen	<i>Pi-a</i>	R	
4	11373	Yaschiro-mochi	<i>Pi-ta</i>	S	Av-ta+
5	11375	Toride-1	<i>Pi-zt</i>	S	Av-zt+
6	11377	Minehikari	<i>Pi-m</i>	R	

7	11382	CO39	<i>No R gene</i>	S	
8	11385	IRBIK-KU [CO]	Pi-k	R	
9	11388	IRBIKm-TS [CO]	<i>Pi-k-m</i>	S	Av-k-m+
10	11389	IRBIKp-K60 [CO]	<i>Pi-k-p</i>	MS	
11	11390	IRBI1 -La [CO]	Pi-1	R	
12	11391	IRBI7-M [CO]	Pi-7(t)	R	
13	11392	IRBI sh-Ku [CO]	<i>Pi-sh</i>	S	Av-sh+
14	11395	IRBI ta - Ja [CO]	Pi-ta	R	
15	11396	IRBI ta - ME [CO]	<i>Pi-ta</i>	MS	
16	11399	IRBI ta2 - IR64 [CO]	<i>Pi-ta2</i>	S	Av-ta2+
17	11400	Pi-z5	<i>S</i>	Av-z5+	
18	11405	IRBI9 - W [LT]	<i>Pi-9</i>	S	Av-9+
19	11406	IRBI3 - CP 4 [LT]	<i>Pi-3</i>	S	Av-3+
20	11409	IRBI K3 [LT]	<i>Pi-kh</i>	S	Av-kh+
21	11431	IRBI -Fu	Pi-z	R	
22	11433	IRBI zt-T	<i>Pi-zt</i>	S	Av-zt+
23	11436	IRBL b-B	Pi-b	R	
24	11437	IRBI t- K59	<i>Pi-t</i>	S	Av-t+
25	11445	IRBI12 - M	Pi-12(t)	R	
26	11446	IRBI19 - A	<i>Pi-19</i>	S	Av-19+
27	11451	IRBI11-Zh	Pi-11 (t)	R	
28	St	Novator	<i>S</i>		
29	–	Sonet	<i>R</i>		

The effective blast resistance genes identified in this work are recommended for use in breeding programs for the development of disease-resistant rice varieties in rice-growing zones of southern Russia [24].

4 Discussion

The use of molecular markers is a new, developing science. At the present time, it will spread to all types of agricultural animals, plants and microorganisms.

In this work, we have demonstrated the possibilities of using molecular marking technologies in the study of biodiversity of the most dangerous pathogen in all areas of the world rice cultivation, *Pyricularia oryzae* Cav.

Introduced genotyping of strains of the pathogen *P.oryzae* Cav. allowed to create their genetic morphological and "ДНК-passport", which were entered into the database "Strains of the patogen *Pyricularia oryzae* Cav. south of Russia [21]. It represents a collection of data reflecting information on the biodiversity of phytopathogenic fungi and specimens of *Pyricularia oryzae* Cav strains isolated from herbarium material with signs of harmlessness to the crop.

This will allow in the future to carry out a comparative characteristic of the microsatellite profile outside the selected field strain of the pathogen *P.* monitor the variability of the fungus and predict the spread of the disease.

Identified genes for resistance to blast infection to the Krasnodar population of the pathogen *P. oryzae* Cav. recommended for use in breeding programs for the creation of disease-resistant rice varieties in the rice-growing areas of the south of Russia. Thus, the strategy of applying new methods of DNA marking is based on the reliability, efficiency, information and interoperability of the above approaches. Knowledge of the interaction

between the pathogen and the plant-host will allow, over time, to switch to intensive farming, to produce plants and animals with greater productivity and more resistant to various environmental factors.

5 Conclusions

Based on molecular genetic approaches, a study of the biodiversity of the phytopathogenic fungus *Pyricularia oryzae* Cav. was carried out in the south of Russia, the classification of morphological and cultural traits of the pathogen was carried out.

In 2020, monitoring was carried out and 9 strains of the pathogen were isolated from the affected herbarium material collected from the fields of five ecological rice-growing zones of Krasnodar region (Krasnoarmeisky, Kalininsky, Abinsky, Slavyansky districts, Krasnodar).

Using a multiplex PCR system based on fragment analysis, among the studied strains of *Pyricularia oryzae* Cav. 5 genotypes were identified in 2020, each of which is was characterized by a unique genetic profile. Their "DNA- passports" have been compiled.

The studied isolates of the blast pathogen were characterized by morphological and cultural traits. 6 morphotypes of the pathogen were identified.

Classification of the pathogen by morphological, cultural and genetic traits will allow obtaining scientifically grounded and comparable data that can be used in the analysis of the interaction of blast strains with rice plants on different varieties and in different agro-technological conditions in order to improve and rationalize agricultural activities. In addition, the studied approaches open up the possibility of using data in breeding, allowing the identification of pathogen forms that infect certain varieties.

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