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Analysis of rice microbial communities under different storage conditions using culture-dependent and -independent techniques

Xiaoying He[1,2], Hairong Liu[3], Chong Lv[3], Feng Wang[1,2], Chaoqun Zhao[1,2], Rui Tao[1,2], Jianpeng Li[4] , Zhu Liu[1,2]*, Lihui Du[5]*

[1]Zhejiang Institute for Food and Drug Control, Hangzhou, China PR; [2]Key Laboratory of Quality and Safety of Functional Food for State Market Regulation, Hangzhou, China PR; [3]Zhejiang Branch of China Grain Reserves Group Ltd. Company, Hangzhou, China PR; [4]Shandong Sinograin Quality Control Center of Grain and Oil Co., Ltd, Jinan, China PR; [5]College of Food Science and Engineering, Nanjing University of Finance and Economics, Nanjing, China PR

***Corresponding Authors**: Lihui Du, College of Food Science and Engineering, Nanjing University of Finance and Economics, Nanjing 210023, Jiangsu, China. Email: ddabc_2000@163.com; Zhu Liu, Zhejiang Institute for Food and Drug Control, Hangzhou 310052, Zhejiang, China. Email: zliu82@126.com

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RESEARCH ARTICLE

Abstract

Rice is a staple food for over half of the world's population, and fungal spoilage in stored rice may occur when the moisture content and temperature are conducive. *Aspergillus* sp. and *Penicillium* sp. are the most harmful toxigenic species that produce harmful mycotoxins. Molds pose a potential threat to public health and cause a huge economic loss. Therefore, it is of great importance to find out how molds multiply in rice. This study focused on the isolation and identification of fungi presented in rice and their evolution in rice with different moisture contents stored for varying periods of time and under different temperatures. Mold community was detected every month using the culture-dependent and -independent method of polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Significant differences were detected by the traditional culture method under different storage conditions. For potato dextrose agar (PDA) media, high temperature and moisture were suitable for the dominant strains including *Penicillium aurantiogriseum* and *Penicillium oxalicum*. In particular, *P. oxalicum* competitively inhibited the other fungi. For Rose Bengal medium, no difference was observed under different storage conditions, and only typical strains such as *Aspergillus candidus* and *Alternaria* were detected. PCR-DGGE identified some uncultured strains such as *Trichoderma* sp. and *Cladosporium* sp., the dominant strains and the flora diversity such as *Aspergillus restrictus* and *Eurotium athecium*. These results indicated that storage conditions greatly shape fungal growth. This study provides a foundation for the evolution of fungal flora in rice during storage in China and may help in developing biological control methods to prevent mold contamination in rice.

Keywords: mold community; PCR-DGGE; rice; traditional culture

Introduction

Rice is one of the most important food in the world, and due to its high nutrition attributes such as protein, carbohydrates, minerals, and vitamins, it has become the main staple food in most places of the world (Cortés-Rojas et al., 2021; Naseer *et al.*, 2014). To supply rice throughout the year, harvested rice need to be safely stored for a long time, even for years. Different from other cereals, most type of rice before harvest is cultivated in flooded conditions, which helps breeding of molds. Microbial species invade rice and its accessories (Park *et al.*, 2005;

Sempere and Santamarina, 2010). As the most populous country in the world, China needs to increase rice yield by approximately 20% by 2030 (Dong *et al.*, 2017; Paterson and Lima, 2010).

Fungi play an important role in rice spoilage, and molds produce mycotoxins that may contaminate cereal grains at any stage during harvest, storage, or processing (Ali, 2016; Park *et al.*, 2005). Various edible commodities from diverse meteorological regions are contaminated by different types of molds that produce distinct mycotoxins (Bennett and Klich, 2003; Sultana *et al.*, 2015), such as aflatoxin B1 (AFB1), fumonisin B1 (FB1), ochratoxin A (OTA), trichothecenes, and zearalenone (ZEN), which can cause important social and economic concerns. Mold growth is influenced by multiple variables, such as water activity, temperature, and time. In general, moisture and temperature are the most critical factors during storage (Mohapatra *et al.*, 2017; Gock MA *et al.*, 2003). There are few studies that have focused on understanding and minimizing rice contamination. Lahouar *et al.* found that water activity and temperature can seriously affect *Aspergillus flavus* growth, and this result was confirmed by Somjaipeng and Ta-uea (Lahouar *et al*, 2016). Therefore, investigating the mechanism of mold evolution in rice might be beneficial in increasing rice production.

Culture-dependent analyses are efficient in determining fungi diversity in ecological studies during harvest and storage. *Alternaria* sp*., Cladopoium* sp*., Fusarium* sp., and *Pullularia* sp. are common field fungi species that contaminate grains, the genus of *Aspergillus* sp., *Penicillium* sp., and *Fusarium* sp. species was the most frequent species that is called storage fungi (Louis, 1969). *Penicillium citrinum* and *Aspergillus candidus* were the most prevalent species infecting the polished rice, while *Fusarium proliferatum* was found as the dominant (Park *et al.*, 2005). All the above results are using cultural methods that are laborious and time-consuming. These include isolation, cultivation, and characterization of each species. Microorganisms are difficult to cultivate in the laboratory. In addition, the minor populations present are difficult to detect using cultural methods because they may be masked on the plates (Cocolin *et al.*, 2013).

Culture-independent techniques have been widely applied for the efficient analysis of total mold diversity. Denaturing gradient gel electrophoresis (DGGE) is a culture-independent microbial technique that has been used to study microbial diversity, such as in kefir grains, animals, cheese and milk (Delgado *et al.*, 2013; Demirci *et al.*, 2021; El Sheikha, 2019; Jiang *et al.*, 2010; Leite *et al.*, 2012; Maturano *et al.*, 2016). For example, the 28S rDNA fingerprinting of fungal communities determined by PCR-DGGE has been applied to detect the geographical origin of fruits (Sheikha *et al.*, 2011). However, no attempt has been made to survey mycoflora i n r ice i n C hina. Therefore, the use of a combined approach may be advantageous to obtain additional detailed information.

This study explores fungal community and dominant mold strains under different conditions. To overcome the limitation of the culture-dependent method and get the rice microbial community from a culture-independent method, the microbial diversity of rice was investigated using polymerase chain reaction (PCR)-DGGE. The use of culture-independent method to investigate rice microbial community has theoretical and practical relevance in understanding community structure and rice microbe mechanism.

Materials and Methods

Rice sample preparation

Rice was harvested in Huaian Jiangsu province, China on October 10, 2020. Then the tests of the Huaidao No. 5 rice samples were sealed humidifier quenched at 14.5%, 18.5% moisture content and were stored for 1 year at 25°C, 30°C, and 35°C, respectively. Mold variation was detected every month using culture-dependent techniques and PCR-DGGE.

Isolation of fungal species

Culturable fungal strains were isolated from freshly harvested rice sample according to China National Standard (GB 4789.15-2010) and placed in Rose Bengal and potato dextrose agar (PDA) media. For the preparation of a single colony, the isolated strains were further purified under the same conditions. For inoculum preparation, sterilized water was used to wash spores from plates after good sporulation (10 days). The spore suspension was stored at 4 °C for further studies.

Identification of fungal species

The fungal species were identified into species level on the basis of the morphological observation of colonies, hyphae, and spores in reference to <Flora fungorum sinicorum>. All isolates of *Aspergillus* were assessed at 3, 5, 7, and 10 days of age to observe their morphological characteristics under different growth periods.

Fungal DNA extraction

The isolates of pure fungal strains for DNA extraction were subcultured on PDA and Rose Bengal media and incubated for 7 days at 37 °C. Small hyphae with micro-loop were selected and placed in 50 μL of lysis buffer at 80 °C for 15 min. The purified DNA was stored at 4 °C until further analysis (Iheanacho *et al.*, 2014).

PCR reaction to amplify the ITS and 28S gene of molds

Taq DNA Polymerase was used for PCR following manufacturer's instructions (Dongsheng Biotech, Guangzhou, China). Amplification was carried out as follows: initial denaturation at 94°C for 10 min, 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 10 min (Dongsheng Biotech, Guangzhou, China). All PCR primers are listed in Table 1. Primers EF4 and Fungi5 were used to amplify the ITS regions of the molds. Primers NL1 and NL4 were used to amplify the 28S regions of the molds. PCR was performed with the Taq DNA polymerase kit from Dongsheng Biotech (Guangzhou, China). The samples were amplified in a T1 Whatman Biometra (Applied Biosystems, Shanghai, USA) using the following program: 95°C for 5 min, and 30 cycles at 95 °C for 1 min, 50 °C for 1 min, 72 °C for 30 s, and 72 °C for a final 10 min extension. A horizontal electrophoresis apparatus (Bio-Rad, Shanghai, USA) on 2.0% (w/v) agarose gels in 0.5 TAE buffer was used to verify the PCR products. The gels were stained with ethidium bromide and visualized under UV light. PCR products were purified and sequenced by pEASY-T3 Cloning Vector (TransGen Biotech, Beijing, China) using an ABI3730 XL automatic DNA sequencer (Table 1).

The Basic Alignment Search Tool (BLAST) search was conducted to compare the obtained sequences with those published in National Center for Biotechnology Information (NCBI) database. The identification was verified as correct when the gene sequences showed ≥99% identity (Maturano *et al.*, 2016).

Electrophoretic conditions and identification of bands

After the a culture-dependent experiment, a suspension was centrifuged at 15,000×g at 4°C for 30 min. In brief, 50 μL of supernatant was mixed with 50 μL of lysis buffer at 80°C for 15 min in accordance with supplier's instructions. The 18Sr DNA region of molds was amplified using the universal primer pair NS1/GCFung, Taq DNA Polymerase (Dongsheng Biotech, Guangzhou, China) in the PCR reaction. Amplification was carried out with a PCR T1 Whatman Biometra (Applied Biosystems, Shanghai, USA) using a final volume of 25 μL containing 12.5 μL of Taq DNA polymerase, 1 μL of each primer, 1 μL of DNA template, and 9.5 μL of dd H_2O under the same conditions as above. DGGE electrophoresis was carried out by using a DCode apparatus (Bio-Rad, Shanghai, USA) at 60°C and employing 8% polyacrylamide gels with a denaturing range of 25%–35% for fungi. Electrophoresis was performed at 75 V for 7 h for fungal amplification.

Bands were stained with ethidium bromide (0.5 mg/mL), visualized under UV light, then photographed. Sequences were used to identify the bands; bands were cut from the gels and bands with DNA eluted overnight in 50 μL of sterile water at 4°C. Same primer pairs without the GC-clamp were used to re-amplify and the DNA by the above condition. The identity of the sequences was determined by the BLAST algorithm in the GenBank database (Leite *et al.*, 2012).

Results

In terms of fungal diversity in initial Huaidao No. 5 rice, 33 strains of fungi were isolated from the 2013 Huaidao No. 5 rice. These species were mainly *Penicillium* sp., *Aspergillus* sp., and *Fusarium* sp., including 16 strains of *Penicillium* sp. and 15 strains of *Aspergillus* sp. accounting for 48.5% and 45.5%, respectively. Therefore, these two fungus genera are dominant in 2013 Huaidao No. 5 rice. Table 2 shows the detailed results. As shown in Figures 1 and 2, the different fungal genera were chosen as examples. Their morphological characteristics, hyphae, and spores were the preliminary determined in reference to <Flora fungorum sinicorum>. On the basis of ITS and 28S rDNA results, the precise conclusion was obtained as shown in Table 2.

Table 1. Primers used in this study.

Primer name	Sequence	Sequence length (bp)
NS1	5′- GTAGTCATATGCTTGTCTC -3′	340
GCFung	5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCATTCCCCGTTACCCGTTG -3′	
EF4	5′-GGAAGGGRTGTATTTATTAG-3′	
Fungi5	5′-GTAAAAGTCCTGGTTCCCC-3′	580
NL1	5′- GCATATCAATAAGCGGAGGAAAAG -3′	
NL4	5′- GGTCCGTGTTTCAAGACGG-3′	560

Table 2. BLAST results of initial rice sample based on the strains ITS and 28S rDNA sequences.

Strain	Comparison result with ITS	Similarity (%)	Comparison result with 28S	Similarity (%)
m23	*Fusarium* sp.	99	*Fusarium fujikuroi*	99
Pr	*Fusarium oxysporum*	99	*Fusarium* sp.	99
pe	*Penicillium* sp.	99	*Penicillium* sp.	99
Pj	*Penicillium* sp.	99	*Penicillium aurantiogriseum*	100
Pd	*Penicillium* sp	100	*Penicillium aurantiogriseum*	99
Pp	*Penicillium* sp.	100	*Penicillium aurantiogriseum*	99
m31	*Penicillium chrysogenum*	100	*Penicillium chrysogenum*	100
m32	*Penicillium chrysogenum*	100	*Penicillium chrysogenum*	99
pD	*Penicillium decumbens*	99	*Penicillium oxalicum*	100
Pb	*Penicillium* sp.	99	*Penicillium oxalicum*	100
Pv	*Penicillium* sp.	99	*Penicillium oxalicum*	99
Mf	*Penicillium* sp.	99	*Penicillium oxalicum*	99
Mn	*Penicillium* sp.	99	*Penicillium* sp.	99
p11	*Penicillium* sp.	99	*Penicillium* sp.	99
p1	*Penicillium* sp.	100	*Penicillium aurantiogriseum*	99
p36	*Penicillium* sp.	100	*Penicillium* sp.	99
p12	*Penicillium* sp.	99	*Penicillium* sp.	99
pg	*Penicillium* sp.	99	*Penicillium solitum*	99
m27	*Aspergillus* sp.	99	*Aspergillus protuberus*	100
mk	*Aspergillus fumigates*	100	*Aspergillus fumigatus*	99
m5	*Aspergillus niger*	99	*Aspergillus protuberus*	99
ma	*Aspergillus niger*	100	*Aspergillus niger*	99
p6	*Aspergillus sydowii*	100	*Aspergillus sydowii*	99
mh	*Aspergillus niger*	99	*Aspergillus protuberus*	99
p2	*Aspergillus* sp.	99	*Aspergillus protuberus*	100
m003	*Aspergillus sydowii*	99	*Aspergillus sydowii*	100
p5	*Aspergillus niger*	99	*Aspergillus protuberus*	100
ps	*Aspergillus niger*	99	*Aspergillus sydowii*	100
p10	*Aspergillus niger*	99	*Aspergillus versicolor*	99
m30	*Aspergillus niger*	99	*Aspergillus niger*	100
m22	*Aspergillus niger*	99	*Aspergillus niger*	100
m15	*Aspergillus niger*	99	*Aspergillus protuberus*	100
m16	*Aspergillus* sp.	99	*Aspergillus versicolor*	100

Culture-dependent analysis of fungal communities

As shown in Figure 1, the community composition in Rose Bengal medium varied at different storage stages. *A. candidus* (band 7) and yeast (band 6) were the dominant species throughout the entire storage time. All strains were replaced by *A. candidus* at the last 3 months of storage. *Alternaria* sp. (band 3), *Penicillium chrysogenum* (band 2), *Aspergillus niger* (band 5), *Aspergillus protuberus* (band 4), and *Aspergillus fumigatus* (band 1) were also detected under different storage conditions. *Alternaria* sp. was one of the field fungi and disappeared after 2–4 months of storage. However, none of these mold strains were the dominant strain.

Figure 2 shows that in the PDA media, no dominant strains appeared in the initial 4 months of storage under all conditions, except at 18.5%/30°C and 18.5%/35°C. At the 5th month, *P. aurantiogriseum* (band 9) was detected in samples under 14.5%/25°C and 14.5%/30°C and persisted for 4 months. When the sample was cultured for 8 months, yeast (band 12) species were detected under 14.5%/35°C and persisted for 1 month. Yeast was detected in PDA and Rose Bengal media and persisted from 3 months to 6 or 9 months under 30°C. The emergence of yeast was earlier at 18.5% than at 14.5%. *P. oxalicum* (band 10) was detected in the initial sample. After storing for 10 months, the dominant mold was replaced by *P. oxalicum*. Under 14.5%/35°C, *Aspergillus glaucus*

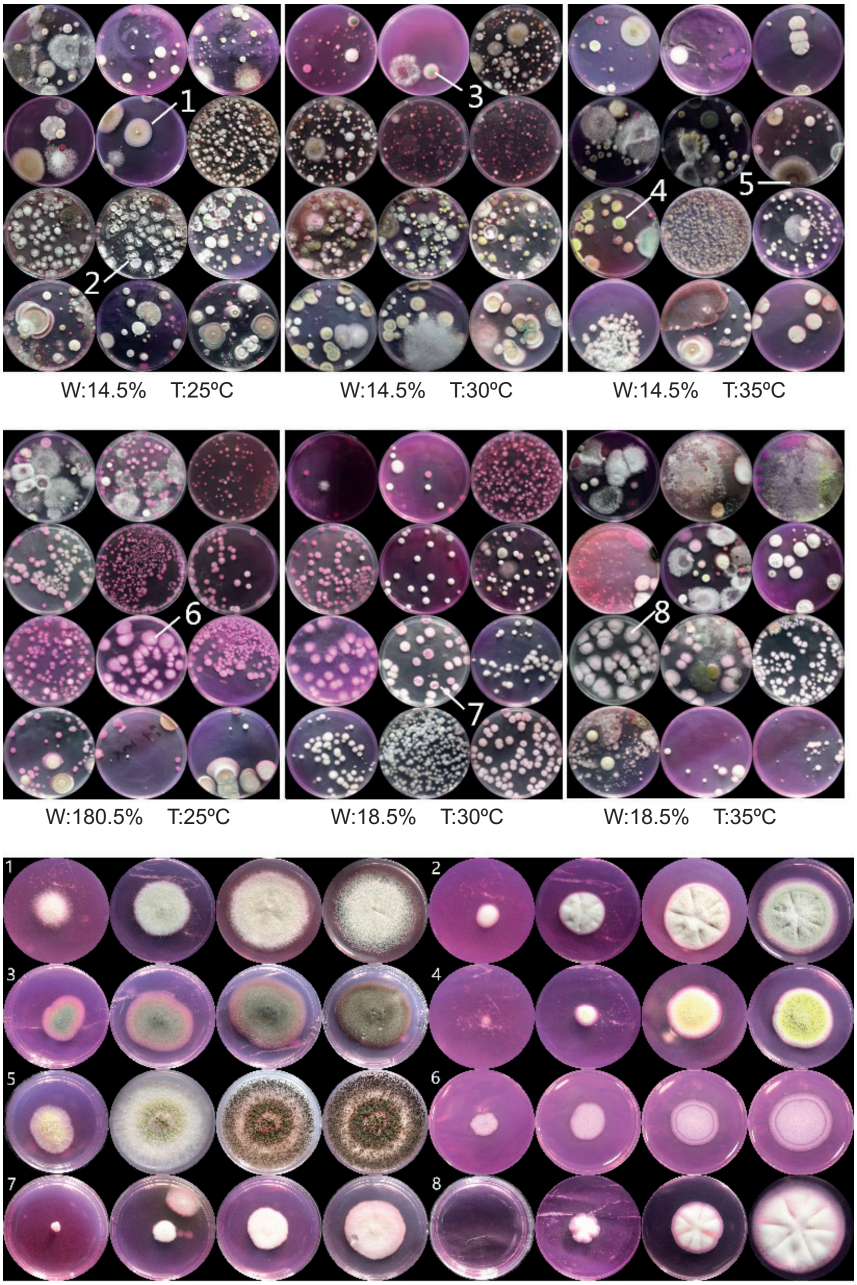


Figure 1. Pictures of the mold community on Rose Bengal medium from rice samples at storage time 1 to 12 months. Single colonies indicated by numbers 1 to 8 were collected and identified. Morphology of a single colony at 3, 5, 7, and 10 days is shown in the last part of the picture.

(band 11) was also detected after 6 months of storage. All above fungi are shown in Figures 1 and 2 and Table 3.

PCR-DGGE analysis of fungal communities

A PCR-DGGE analysis of the fungi 18S rRNA gene was conducted on 12 RM and the derived rice samples, with universal primers, were used to analyze the community structure of the dominant fungi using a culture-independent technique.

Figure 3 and Table 4 show that band patterns differed for the rice samples under varying conditions and were almost identical for those under the same conditions. Twenty-eight high-intensity bands were cut and identified by re-amplification, sequencing, and sequence comparison (bands with a number on top in Figure 3). Table 4 summarizes the band identification results. Most bands were identified at the species level, and only seven bands were identified at the genus level. Some bands were labeled as uncultured fungi. Five bands belonged to *Aspergillus* sp., three to *Penicillium* sp., and two to

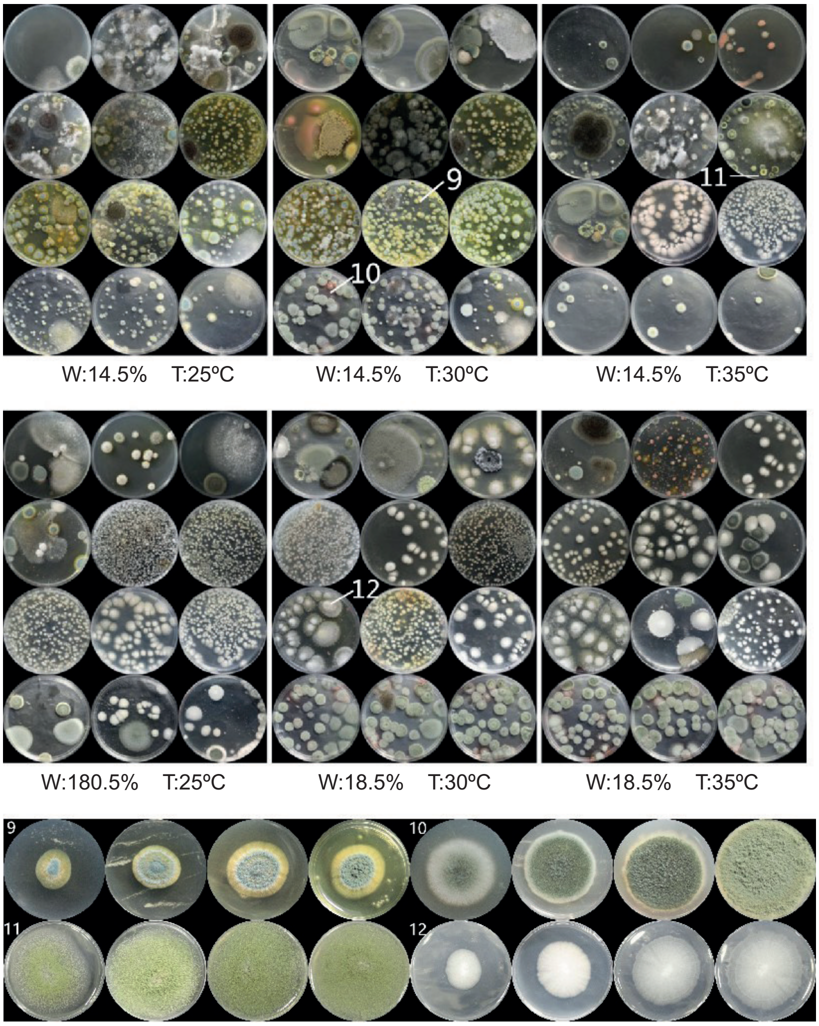
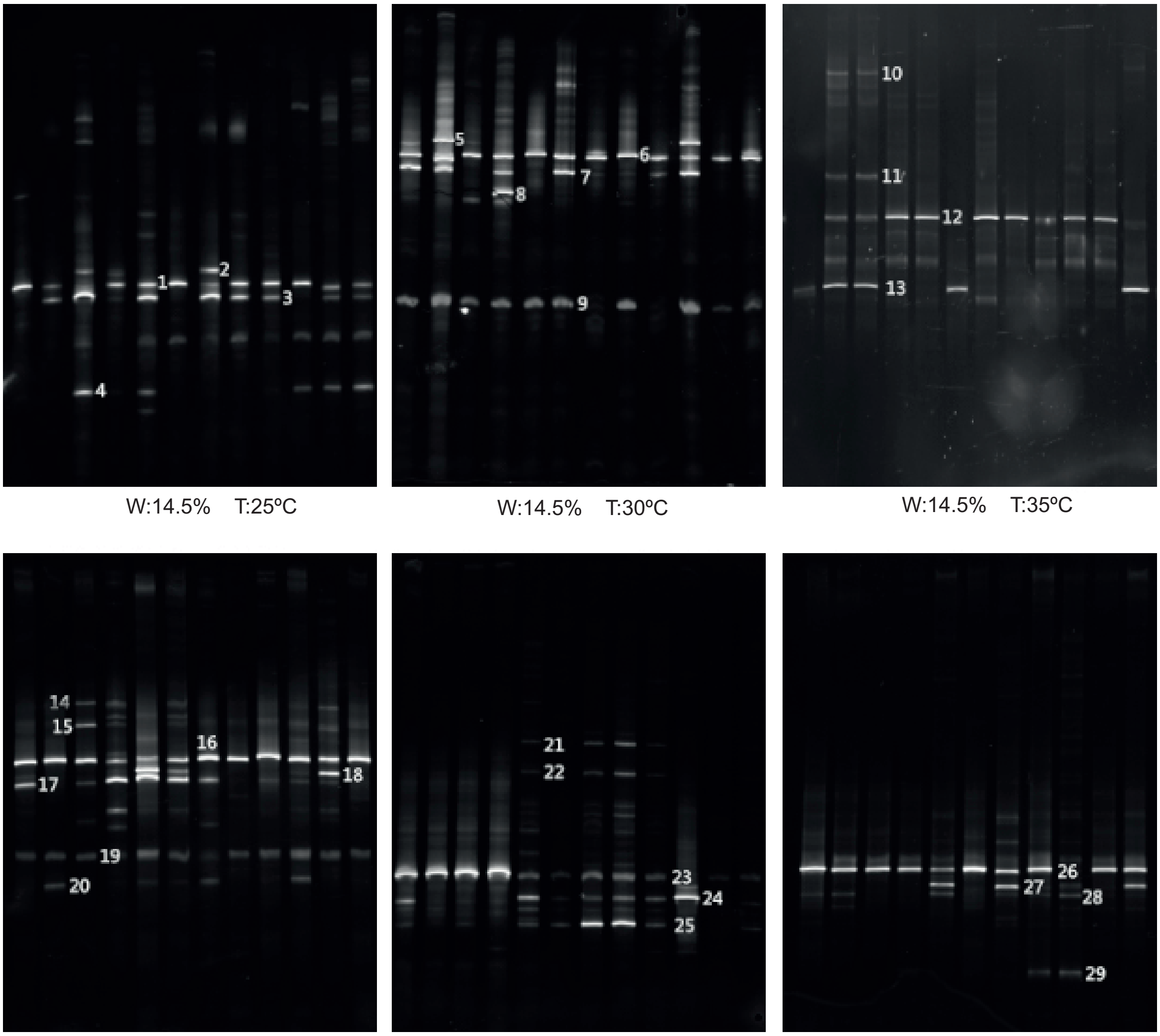


Figure 2. Pictures of the mold community on PDA medium from rice samples at storage time 1 to 12 months. Single colonies indicated by numbers 9 to 12 were collected and identified. Morphology of a single colony at 3, 5, 7, and 10 days is shown in the last part of the picture.

yeast. In addition to *Aspergillus* sp. and *Penicillium* sp., a culture-independent PCR-DGGE analysis detected other species such as *Phoma, Cladosporium, Aureobasidium, Soliformovum, Hyphopichia burtonii*, and *Eurotium athecium.* Most of these fungi cannot be detected by culture-dependent methods. *Cladosporium* is an intermediate mycoflora in most cereal grains and was detected in the rice sample stored for 3 months (Fleurat-Lessard, 2017). The sample under 14.5%/25°C produced 1–4 bands, where band 1 was *A. versicolor*, one of the initial fungi, and appears only under 14.5%/25°C. Therefore, this condition is suitable for the growth of *A. versicolor*. Different dominant molds were found under various temperatures. Under 18.5% moisture, the microbial communities were simple, especially when the temperature was high. Therefore, dominant strains can easily grow under high temperature and moisture. Under 18.5%/30°C and 18.5%/35°C, the same dominant strains were identified. Bands 23 and 26 were attributed to *Aspergillus flavus*, which produces toxic metabolites called aflatoxins that are potential threat human. Hence, inhibiting *A. flavus* growth or aflatoxin synthesis is crucial (Iqbal *et al.*, 2006). Bands 24 and 27 correspond to *Myrothecium roridum*, a fungal plant pathogen. Myrotoxin B has been isolated from this organism. The sample under 14.5%/30°C produced 5–9 high-intensity bands. Band 6 was *A. candidus*, which was also detected by the culture-dependent method, and band 9 was *Penicillium commune*. The sample under 14.5%/35°C produced 10–13 high-intensity bands when stored for 1 year. Band 10 was *Penicillium purpurogenum*, band 12 was *Aspergillus ustus,* and band 18 was *Penicillium islandicum*. Ji Yeon Oh reported that when the relative humidity increases, the abundance of *P. islandicum* also increases up to 40%–70% of total fungi in the stored rice sample (Oh *et al.*, 2008). The sample under 18.5%/25°C produced 14–20 high-intensity bands. Band

Table 3. BLAST results based on the strains ITS and 28S rDNA sequences of typical strains.

Strain	Comparison result with ITS	Similarity (%)	Comparison result with 28S	Similarity (%)
1	*Aspergillus fumigatus*	99	*Aspergillus fumigatus*	99
2	*Penicillium chrysogenum*	99	*Penicillium chrysogenum*	99
3	*Alternaria*	99	*Alternaria*	99
4	*Aspergillus protuberus*	99	*Aspergillus protuberus*	100
5	*Aspergillus niger*	100	*Aspergillus niger*	99
6	Yeast	100	Yeast	99
7	*Aspergillus candidus*	100	*Aspergillus candidus*	100
8	*Penicillium chrysogenum*	100	*Penicillium chrysogenum*	99
9	*Penicillium aurantiogriseum*	99	*Penicillium aurantiogriseum*	100
10	*Penicillium oxalicum*	99	*Penicillium oxalicum*	100
11	*Aspergullus glaucus*	99	*Aspergullus glaucus*	99
12	Yeast	99	Yeast	99



W:14.5% T:25ºC

W:14.5% T:30ºC

W:14.5% T:35ºC

W:180.5% T:25ºC

W:18.5% T:30ºC

W:18.5% T:35ºC

Figure 3. DGGE profiles of the mold community from DNA directly extracted from rice samples at storage time 1 to 12 months. Bands indicated by numbers 1 to 29 were excised and, after re-amplification, subjected to sequencing. The bands are discussed in the text, and the positions of the bands are indicated on the right.

Table 4. Strains and DGGE bands identified in this study using partial 18S rRNA gene sequencing.

Bands	Closest relative	Identity	Accession No.
1	*Aspergillus versicolor*	100%	AB002064.1
2	*Cladosporium* sp.	100%	JQ824844.1
3	*Talaromyces purpurogenus*	100%	KC009578.1
4	*Hyphopichia burtonii*	99%	AB018177.1
5	*Eurotium athecium*	100%	KJ126879.1
6	*Aspergillus candidus*	99%	KJ126884.1
7	*Apergillus restrictus*	99%	KR123678.1
8	*Aureobasidium*	100%	KJ561257.1
9	*Talaromyces purpurogenus*	100%	KC009578.1
10	Uncultured Archaeospora	100%	KT923272.1
11	*Soliformovum*	90%	FJ766479.1
12	*Aspergillus ustus*	99%	KR063174.1
13	*Aspergillus penicillioides*	99%	DQ985959.1
14	Uncultured Ascomycota	99%	KF650047.1
15	Uncultured eukaryote	100%	JX099065.1
16	*Aspergillus ustus*	99%	KR063174.1
17	*Penicillium commune*	99%	KF018446.1
18	*Penicillium islandicum*	100%	AY373919.1
19	*Penicillium purpurogenum*	100%	JX022616.1
20	Fungal sp.	100%	HQ132705.1
21	*Rhizopus oryzae*	99%	AY213624.1
22	Uncultured plosporales	98%	KF650048.1
23	*Penicillium oxalicum*	99%	KR063176.1
24	*Myrothecium roridum*	98%	KR063175.1
25	Uncultured fungus	100%	KR906021.1
26	*Penicillium oxalicum*	99%	KR063176.1
27	*Myrothecium roridum*	98%	KR063175.1
28	*Phoma*	99%	KM387394.1
29	*Hyphopichia burtonii*	99%	AB018177.1

28 was *Phoma*, one of the field strains. The sample under 18.5%/35°C produced four bands, but only the bright ones were evident. Bands 23 and 26 were attributed to *P. oxalicum*.

Discussion

The microbial diversity and community structure of rice were analyzed using both culture-dependent and -independent techniques under different storage time and conditions. The results provide a complete view of the overall composition of rice communities.

P. oxalicum and *P. aurantiogriseum* were detected from the initial and stored samples and on PDA media regardless of the condition. The dominant strain was replaced by *P. oxalicum* in the last few storage months. PCR-DGGE results show that *P. oxalicum* was the dominant strain under the condition of 18.5%/30°C and 18.5%/35°C, indicating that this species can grow at higher temperatures and humidity than other fungi. *P. oxalicum* is an effective biological control agent against other fungal species, such as *Fusarium oxysporum* (Cal *et al.*, 2001, 2010) and *Verticillium dahliae* (Larena *et al.*, 2010; Sabuquillo *et al.*, 2005, 2006). An analysis on the antagonistic effect of *P. oxalicum* on *A. alternata* in rice under different temperature and water activity found that *P. oxalicum* is a good biological control agent of rice fungi pathogens (Sempere and Santamarina, 2010). This antagonistic agent was also tested for strawberry, pea, and tomato fungal strains, and other crop pathogens with good results (Cal *et al.*, 2001, 2008; Windels and Kommedahl, 1982). In the present work, *P. oxalicum* produced red liquid; further work must explore the specific mechanisms of this red liquid. This finding may provide a new research direction for the control and prevention of rice mold contamination (Li *et al.*, 2015). *P. aurantiogriseum* is commonly found in stored cereals and is a potential producer of mycotoxins and a broad spectrum of volatile metabolites (Brjesson T *et al.*, 1990). This fungus produces a type of liquid that can inhibit other strains. Additional experiments are needed to study the specific mechanism. *Aspergillus* spp. are the most typical fungal species responsible for cereal spoilage. These species include *A. fumigatus*, *A. protuberus*, *A. candidus*, *A. glaucus*, *A. versicolor*, *A. restrictus*, *A. ustus*, and *A. penicillioides*. *Aspergillus* species are most dominant among food deteriorating molds. These fungi widely occur in food, particularly in starchy cereal grains such as wheat, maize, rice, and barley and may produce toxic secondary metabolites called aflatoxins. The current findings are in agreement with Mannaa and Kim (2017). *A. protuberus* SP1 was isolated from Marine Sediments of the South Indian Coast and showed activity against human pathogens (Mathan *et al.*, 2011). Oh identified three predominant *Aspergillus* species from five regions in Korean stored rice, namely, *A. candidus, A. flavus*, and *A. fumigatus* (Oh *et al.*, 2010). In this work, *A. candidus* was detected under 18.5%/30°C. This mold is common in stored food grains (Chattopadhyay *et al.*, 1987). Humidity is one of the crucial factors affecting the biodiversity and evolution of mold species during storage. The present results reveal that *P. aurantiogriseum* prefers a lower humidity than yeast species and is significantly positively correlated at 14.5%/25°C and 14.5%/30°C. By contrast, yeast species require a high humidity (18.5%). *P. aurantiogriseum* cannot survive when the moisture content is above 18.5% (Fleurat-Lessard, 2017). The optimal conditions for molds are extremely important: *P. oxalicum* performed and acclimatized best at 14.5%/30°C, 18.5% (30°C–35°C), and *A. candidus* grew optimally at high temperature and humidity of 18.5% (30°C–35°C). During storage, the dominant strains benefited from the high storage temperature and humidity. The present results reveal that *P. oxalicum* is strongly dominant in the PDA media. Water activity is the main environmental factor governing the predominance of fungal groups on grains, and this finding is in harmony with the current study.

Differences in results may be attributed to several reasons. Some uncultured species are unable to grow on artificial media. The number of fungi identified by DGGE is smaller than that by traditional culture (Duong *et al.*, 2006). In this work, the dominant strains *P. oxalicum*, *P. aurantiogriseum*, and *A. candidus* were detected through cultivation but not by PCR-DGGE. This bias may be caused by differences in the efficiency of DNA extraction or the preferential amplification of the primers for some sequences (Wang *et al.*, 2015). Meanwhile, not all species are efficiently amplified with PCR-DGGE primers. *P. oxalicum* exhibited poor PCR efficiency with the 18S primer set. Therefore, *P. oxalicum* was not detected in DGGE gels but was observed in the culturable community. A similar situation was observed for two other species, *P. aurantiogriseum* and *A. candidus*. The dominant strain was detected by PCR-DGGE probably because it was a part of the major population in the grains. Rapidly growing fungi can absorb more nutrition than others and thus inhibit their growth, leading to species diversity loss. In general, PCR-DGGE using a part of the 18S rRNA gene is often seen as an alternative or complement of culture-independent approach in investigating the biodiversity of microbial ecosystems. Owing to the technical deficiencies of PCR-DGGE, however, some elements of the microbial community might not be detected. Other new technologies, such as T-RLFP, MLST, and high-throughput sequencing, must be adopted for further studies (Paola *et al.*, 2016).

In conclusion, the combination of these two methods provide a complete overview of the rice community. *P. oxalicum* was identified as the dominant strain and detected even in the initial sample. During storage, *P. oxalicum* competitively inhibits the other fungi. Field fungi (especially *Fusarium*) and stored fungi (especially *Penicillium* and *Aspergillus*) have different suitable storage conditions. Culture-independent and -dependent techniques must be combined to obtain total counts and provide a complete overview of the ecosystem biodiversity. This study contributes to the understanding of rice evolution at different storage conditions and provides scientific data and theoretical support for further research on stored grain fungi.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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