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A First Epidemiological Survey of Skin Fungus in Free Range *Pelophylax Nigromaculatus* in Hunan Province, China

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Abstract

Epidemiological investigation of fungus in amphibian was conducted from Hunan province of China to isolate fungus from skin of amphibians. Specimens from the skin of local *Pelophylax Nigromaculatus* were collected and analyzed using molecular biology techniques. The sequences were analyzed using phylogenetic tree. From the *Pelophylax Nigromaculatus* specimen collected a total of 58 fungal strains representing 10 fungi species from 8 genera were identified. The detection rate of *Penicillium* spp. was highest (11%), followed by *Yarrowia* spp. (1.5%) and detection rate of other genera varied from 0.25% ～ 0.75%.Therefore, long-termmonitoring should be implemented to realize ecological risk for Hunan province and vital to establish a necessary management measures for monitoring and prevention of pathogenic fungus which have great significance in protecting ecological risk in China as well as eastern Asian regions.

Keywords: **E**cological risk; Amphibian; Epidemiology; Fungus; PCR; Hunan

1. INTRODUCTION

From the historical view of evolution, amphibians may become an important part of the 6th great extinction [1]. Amphibians are experiencing threatened and population decline more than birds and mammals and many of them are on the brink of extinction [2]. Their extinction rate is 211 times the background of extinction[3]. As the indicators of environmental degradation [4,5], more and more attention have been attracting to the declines of Amphibians populations [6]. Many evidences showed that diseases were one of the most important factors that causes decline in populations of amphibian in addition to habitat destruction, introductions of predators and competitors, stronger ultraviolet radiation, climate change and other factors [7].

In recent decades, disease, especially fungus threatened animal healthy, but usually underestimated. These had been confirmed by the events that wildlife populations declines caused by pathogenic fungus [8]. For example, in 2009, a psychrophilic fungus caused White-nose syndrome in bat and resulted in mass mortality of the hibernating bats [9]. Moreover Epizootic ulcerative syndrome caused by A. *invadans* also resulted in mass mortality of fish in Zambezi river system in southern Africa[10]. Furthermore *Fusariumsolani*is responsible for mass mortality in nests of loggerhead sea turtle *Carettacaretta*in Boavista, Cape Verde[11]. In particular, in 1998, a fatal fungus *Batrachochytriumdendrobatidis* caused mass mortality of anura amphibians in Queensland,

Australia and Panama attracted the global attention [12].

Among these species of fungus of amphibians, *Batrachochytriumdendrobatidis* are one of the well documented species, which raised great attention around the world bysignificant population decline[13,14], and altered our scope on the aspects of hosts species (amphibians, reptiles, aquatic animals)[15, 16, 17] and distributed range[18, 19, 20, 21, 22, 23, 24, 25]. While its protection level raised to biological protection class II by Office International des Epizootic (OIE). Therefore its ecological significance and importance in public security of fungal disease is more important than before.

Many Chinese endemic species of amphibians with large population size are widely distributed in Hunan province[26]. This province also an important prevention and control area of notifiable wildlife diseases such as avian influenza in which there is no surveillance on fungal disease in amphibians up to now. It is confirmed that Changsha in Hunan province, the provincial capital is the main distribution center for the amphibians' trade with an annual sales amount of six million tones[31]. Most of them were finally sold to Guangdong province which may finally involved in international entrepot trade of amphibians. The activities of artificial amphibians trafficking increased and complicated the regional uncertainty of amphibians' fungal diseases distribution to some extent and the fungal diseases risk was globalized passively and increased the ecological risk. Hunan province is a bridge that connects the eastern coastal provinces to the western inland area, showing a very important ecological status. The Yangtze River with one of its seven major tributaries Xiangjiang River flows through the north of Hunan province. This create suitable environment for the spread of fungus by water body[27]. Hence the local inherent amphibians' fungal infection or imported pathogens along the water body after engraftment will further threaten the ecological safety of the whole Yangtze River basin and provinces in the east and south parts of China such as Jiangxi, Anhui, Jiangsu and Shanghai City. Therefore it is necessary to carry out epidemiological investigation of fungus in amphibian to distinguish whether the fungus in amphibian's skin is environmental or pathogenic.

This study also assess ecological risk scientifically to suggest necessary control and prevention measures for the fungus identified from amphibians skin to secure the health of amphibians. For this study the local amphibian species *Pelophylax Nigromaculatus*, which is a broad distributed species, was selected with an object to screen and analyze the genetic information of the skin fungus strains preliminary by molecular biology techniques.

2. MATERIALS AND METHODS

2.1 Study area

Hunan province is located in the southeastern China in the middle reaches area of the Yangtze River (E 108° 47′ -114° 15′ , N 24° 39′ -30° 08′), having an area of 2.118×105 square kilometers with continental monsoon humid subtropical climate. The mean precipitation is approximately 1300 – 1700mm per year [28]. The average temperature in May and June is about 21 – 26 ℃ [29]. Hunan province was covered by rivers and lakes with a continental subtropical humid monsoon climate which is suitable for survival of fungus. Among the lakes, Dongting Lake is the second largest fresh water lake in China is located in the northeastern Hunan province. This lake occupies an area of 1310 km2 on the average and can expand up to 2691 km2 during the rainy season and shrink to 709.9 km2 in dry season [30].

2.2 Field Sampling

In this survey a total of 12 sampling regions in Hunan province were set up. The five regions around the Dongting Lake include Ningxiang County (A), Wangcheng County (C), Taoyuan County (H), Hanshou County (J) and Yueyang City (G). The other seven regions with Changsha City as the center are Liling City (B), Changsha County (D), Xiangtan City (E), Liuyang City (F), Loudi City (I), Hengyang City (K), and Shaoyang City (L) (Figure 1). In each sampling region, 20 to 50 samples were randomly collected near the streams or the ponds. Accordingly a total of 400 adult frogs, *Pelophylax Nigromaculatus* were sampled (Supplementary Table 1).

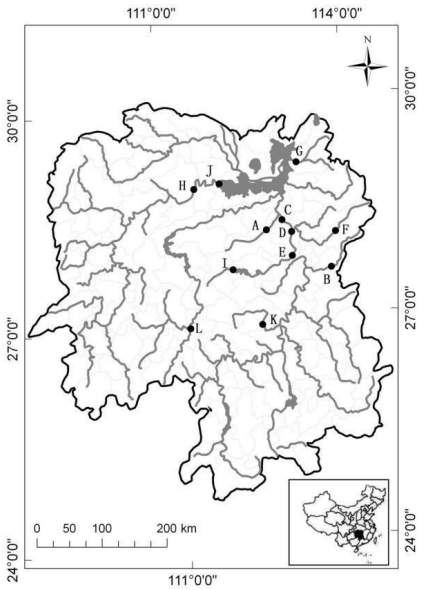

Figure 1： Map and location of sampling regions in and around Dongting Lake and some Important Trade Centers in Hunan Province, China

Frogs were captured using a hand-net placed in disposable plastic bags transported to the laboratory and kept individually under humid conditions. A sterile pair of gloves was used during handling of each frog to avoid cross-contamination. The swab method was used to collect samples by scripting repeat on the surface of the whole body of frogs and kept for laboratory analysis.

2.3 Fungal culture

The swab was cut into pieces using sterile scissors and then rubbed on Potato Dextrose Agar (Medium) and incubated routinely.

2.4 PCR Assay

Fungal DNA was extracted from the cultured strains with Lysis buffer for microorganism using direct PCR (TaKaRa Co. Ltd., Dalin, China). PCR Amplification using direct PCR (TaKaRa). PCR Amplification was conducted by fungal universal primers (InvitrogenCo. Ltd., Shanghai, China). ITS1:5'-TCCGTAGGTGAACCTGCGG-3'; ITS4:5'-TCCTCCGCTTATTGATATGC-3'. Reaction file: template 2 μl, ddH2O 6 μl, 2× Taq PCR StarMix (Genestar Co. Ltd., Shanghai, China) 10 μl, primer ITS1 1 μl and primer ITS4 1 μl to make a 20 μl reaction system. The strain *Yarrowialipolytica*stored in laboratory was used as positive control.

2.5 Sequencing and Phylogenic analysis

Positive amplified products of PCR were supplied for sub-clone and the clones were sent to Invitrogen Co. Ltd., Shanghai, China, BGI Tech, Shenzhen, China and TaKaRa Co. Ltd., Dalian, China respectively for thrice sequencing. Then the sequences were compared with sequences available in GenBank. Subsequently, MegAlign component of the MEGA5.5 for sequence alignment and phylogenic analysis, and neighbor-joining method for phylogenetic analysis were used. Phylogenetic tree was constructed based on the sequences of amplified products. The length of each branch pair represents the distance between sequence pairs and the units on the horizontal axis indicate the number of

substitution events in the phylogenetic tree.

3. RESULTS

A total of 58 fungal strains representing 10 fungi species from 8 genera were identified from 400 specimens collected from 12 sampling regions. These include *Penicillium, Cladosporium, Chaetomium, Yarrowia, Debaryoyces, Candida* and *Trichosporon, Aspergillus* genera (Supplementary Table 1). The detection rate of *Penicillium spp.* was highest (11%), followed by *Yarrowia spp.* (1.5%) and detection rate of other genera varied from 0.25%-0.75% (Figure 2). In terms of geographic distribution, *Penicillium spp.* were detected in nine sampling regions (Supplementary Table 1), and not detected in Liuyang (F), Hengyang (K) and Shaoyang (L) cities.

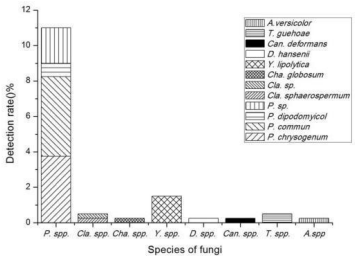

Figure 2：Fungi and its detection rate in amphibians in Hunan

Phylogenetic tree were constructed by sequences of 58 PCR positive samples (Figure 3). This includes *Penicillium spp.*: 15 *Penicilliumchrysogenum*(D36-1, E13, E15, E17, E18, H25, H2, I8, J1, J3, J7, J8, J10, J14 and J15)； 18*Penicilliumcommun* (A3, A41, A45, A47, B3, B6, B8, B9, B26, D30, D36-2, D37, D38-1, D38-2, E16, G12, H1 and I22)； 3*Penicilliumdipodomyicol*(D34, D38 and E14), however 8 strains were not identified to species level (A1, A8, A42, B20, C19, G18, G20 and J5) ; 2 *Cladosporium spp.*: *Cladosporiumsphaerospermum*(B4) and the strain were not identified to species level (C17); 1 *Chaetomium spp.*: *Chaetomiumglobosum* (A48); 6 *Yarrowia spp.*: *Yarrowialipolytica* (G13, G14, G19, J27, K21 and L26); 1 *Debaryomyces spp.*: *Debaryomyceshansenii*(F20); 1 *Candida spp.*: *Candida deformans*(G16); 2 *Trichosporon spp.*: *Trichosporonguehoae* (H8 and H10); 1 *Aspergillusversicolor*(E50).

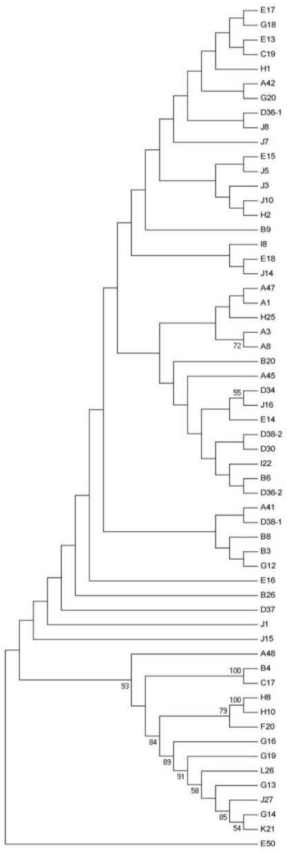

Figure 3：Phylogenetic tree constructed by 58 tested fungi sequences

4. DISCUSSION

There is no research conducted yet in Hunan province to isolate fungus from the amphibian’s skin. This study is the first to conduct a research on a large scale in amphibian’s skin fungus in Hunan province, China. The result of this research indicates that *Penicillium spp.* and *Yarrowia spp.* were the

dominant genera on the amphibian skin while fungal distribution in the amphibian's skin was affected by many internal and external factors.

According to Figure 4, although the overall trends of detection rate for fungi in the amphibians skin samples from west to east was not obvious (figure 4:4-1), the trends from north to south gradually reduced (figure 4:4-2).

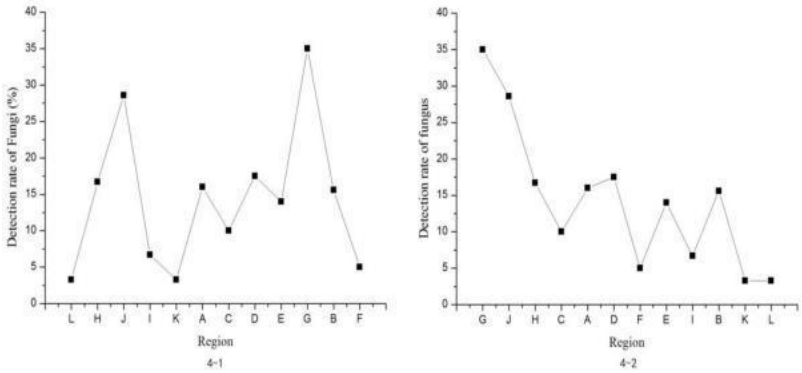

Figure 4：4-1 and 4-2 are tendency chart of detected rate for fungi in different regions

This is probably due to climate related fungal distribution. As indicated on figure 3, *Yarrowia spp.*（G13, G14, G19, J27, K21 and L26）and Candida spp.（G16）are in one Clade. However *Trichosporonguehoae* (H8 and H10) and*Debaryomyces spp.*（F20）have relatively high similarities where as the 44 fungi from *Penicillium spp.* are the monophyletic clade. This study found that fungus in the same genera from the same sampling site has high levels of genetic diversity in that J3, J10 and J8 are on the different clades although they belongs to *Penicilliumchrysogenum*. So it is with E18 and E17 (figure 1). The genetic diversity detected in this study may be due to strong environmental influence. With the environmental pollution and ecological deterioration, it is worthwhile to pay attention for the ecological factors as a potential risk in this study area.

5. Conclusions

The current study focuses on isolation of the pathogenic skin fungus, and several species of skin fungus had been isolated in which *Penicillium spp.* were the dominant. Phylogenetic tree sequences of PCR positive samples were also constructed. Whether the fungi isolated in this study have the symbiotic relationship with amphibians or play an important role in amphibiansimmuno-prophylaxis were unclear. This finding suggested that extensive surveillance and monitoring are needed to prevent the introduction of this lethal amphibians disease to this province. It is also important to formulate a necessary surveillance and monitoring measures for pathogenic fungus, which have great significance in protecting ecological risk in China as well as east Asia regions. Furthermore the pathogenicity of those isolated skin fungus should be studied to understand their effect on amphibians population.

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