

Yeasts in amphibians are common: isolation and the first molecular characterization from Thailand

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Abstract. A survey of the presence of yeasts in frogs and toads in Thailand was conducted using standard mycological examination techniques. The results, which were confirmed with molecular techniques, revealed the presence of five yeast species – *Cryptococcus liquefaciens*, *C. heveanensis*, *Pseudozyma hubeiensis*, *Rhodotorula mucilaginosa* and *R. minuta* – in the bile of these amphibians. Although previous works have isolated yeasts from amphibian gastrointestinal tracts and skin, it is questionable whether these yeasts were acquired by ingestion or were commensals on adult individuals. Frog farms, an urban area and protected natural areas were surveyed and all tested positive for yeasts, which shows their ubiquity in both wild and farm-reared frogs. Additionally, the finding of yeasts in five different species of frogs and toads shows that there is a wide spectrum of hosts in this vertebrate group. Our results thus suggest that yeasts are likely to be widespread among amphibians in different habitat types and in a wide range of host species.

Keywords. Amphibian, yeast, *Rhodotorula*, *Pseudozyma*, *Cryptococcus*

Frogs are parasitized by several taxa, the most-studied of which include helminths, bacteria, viruses and the chytrid fungus *Batrachochytrium dendrobatidis*. Parasites cause high mortality rates in wild amphibian populations (Densmore and Green, 2007), while infectious diseases are a concern in amphibians kept in captivity as pets or reared on farms as a food source. Although not strictly pathogenic, symbiont organisms have been found on frog skins, where they are reported to act as a barrier against chytridiomycosis, and have also been isolated in the guts of these vertebrates (Walke et al., 2014). Anecdotal reports of yeasts – opportunistic pathogens that are ubiquitous in nature – exist for tadpoles and adult amphibians. Steinwascher (1979) found that *Candida humicola* was abundant in the faeces of some large tadpoles of *Rana clamitans* in Australia, where it acts as a mutualistic symbiont. *Rhodotorula glutinis* was isolated from the tadpoles of marsh frogs (*Limnodynastes peronii*) in Australia, where it plays a role in suppressing the growth of

the mosquito larvae that inhabit the same pond (Mokany and Shine, 2003). In the study by Sammon et al. (2010), yeast were isolated from the faeces and the skin of adult Australian green tree frogs (*Litoria caerulea*), although no subsequent molecular confirmation was undertaken. As all studies of yeast from the gastrointestinal tracts of amphibians (tadpoles and adults) to date have only focused on faeces, it is still unclear whether or not yeasts form part of the natural microflora of amphibians or whether they are acquired from the environment through the ingestion of water and thus appear in faeces. The yeast *Candida humicola* can modify the behavior of tadpoles (Kiesecker et al., 1999) and so should be considered pathogenic rather than symbiotic, as has been previously reported (Steinwascher, 1979). Consequently, the distribution of yeasts in both wild and farm-reared populations of amphibians is of interest. In the course of a parasitological survey of adult frogs and toads in Thailand, the observation under the microscope of yeast-like cells

in bile led us to suspect that yeasts were present in these amphibians. We thus conducted culture and molecular characterization to confirm the validity of these microscopic observations.

Fieldwork was carried out in March–June 2014. Frogs were collected by hand in three types of habitats: frog farms, urban areas and protected natural areas. On Farm 1, where chicken and fish are also bred, frogs are raised in walled concrete enclosures at high densities. On Farm 2, frogs are raised in shallow ponds which were covered by plastic on the floor and net as a wall, it also has chickens in the very close area. On Farm 3, frogs are raised in ponds and coexist with fish and ducks, although they are physically separated by netting. All farms are located in Udon Thani Province.

The second habitat surveyed was an urban area on the Udon Thani Rajabhat University campus, where there are many buildings and green spaces. The third environment surveyed were two protected forest areas, Na Yung-Nam Som Natural Park (344 km², Udon Thani Province) and Phu Wua Wildlife Sanctuary (186.5 km², Bueng Kan Province), that both harbour great floral and faunal biodiversity. Once collected, all samples were immediately transported alive to a laboratory at Udon Thani Rajabhat University and identified following Taylor (1962) and the nomenclature used in an online database of the world's amphibians (Frost, 2015).

Amphibians were anesthetized, killed using MS222 (ethyl-4-aminobenzoate) and then dissected. Their gall bladders were removed and placed on sterile Petri dishes. Syringes were used to suck bile from the gall bladders, which was then transferred onto Yeast Malt agar (HiMedia Laboratories) and cultured using a spread-plate technique. The YM culture plates were incubated at 25°C for 5–7 days. Single colonies were selected, streaked onto YM agar plates and then incubated at 25°C for 5–7 days. The purified colonies were examined microscopically and those with yeast-like shapes were subjected to further molecular studies.

Isolation of DNA was carried out by boiling of cells with lysis buffer according to the methods of Maniatis et al., (1982) with slight modification. A loopful of yeast cells was transferred to 1.5 ml Eppendorf tube. The 100 µl of lysis buffer was added. Cell suspensions were boiled in water bath or metal block bath for 15 min. After boiling, 100 µl of 2.5 M potassium acetate (pH 7.5) was added and placed on ice for 1 hour, and centrifugated at 14,000 rpm for 5 min. Supernatant was extracted twice with 100 µl of chloroform: isoamyl alcohol (24:1 v/v). DNA was precipitated with isopropanol, placed at 20°C for 10 min and centrifugated at 15,000 rpm for 15 min. DNA pellet was rinsed with 70% and 90% ethanol and then dried up

(15–30 min at room temperature). The dried DNA was dissolved in 30 µl milli Q water.

The amplification and sequencing of 13 isolates were performed at the National Center for Genetic Engineering and Biotechnology (BIOTEC), Bangkok (Thailand). The divergent D1/D2 domain of 26S rDNA was amplified with primers NL-1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') (Kurtzman and Robnett, 1998). Amplification was carried out in a 100 µl reaction mixture containing 100 ng of genomic DNA, 2.5 U of *Taq* polymerase (Thermo Scientific), 20 mM of each dNTP, 40 mM of each primer, 10 mM Tris-HCl and 1.5 mM MgCl₂. The reaction was pre-denatured at 94°C for 5 min, then repeated for 30 PCR cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2.5 min and then final extension at 72°C for 10 min. The amplified DNA was purified using a QIAquick PCR Purification Kit according to the manufacturer's instructions. The nucleotide sequences of D1/D2 domain of 26S rDNA were directly determined using PCR products following Kurtzman and Robnett (1998) with slight modifications. Cyclic sequencing of the D1/D2 domain was conducted with an NL1 forward primer (59-GCA TAT CAA TAA GCG GAG GAA AAG-39) and an NL4 reverse primer (59-GGT CCG TGT TTC AAG ACG G-39) using an ABI Prism™ BigDye™ Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems, Stafford, USA) following the manufacturer's instructions.

The thirteen obtained sequences (576 to 612 base pairs) of D1/D2 domain of 26S rDNA were compared using a BLAST Homology Search (<http://www.ncbi.nlm.nih.gov/blast>): AF070432, AF181515, AF189948, DQ008953 and FJ534905. Our obtained sequences were deposited in Genbank, accession numbers KU893128 to KU893140.

Growth of yeast colonies on YM agar (pink and white) was observed in samples from 12 amphibians from 3 of the 90 farms (3.33%), 2 of the 7 amphibians from the urban area (28.57%) and 7 of the 31 amphibians (21.87%) from the wild (total=129 specimens). One specimen of *Hoplobatrachus rugulosus* from a farm harboured two gross colony morphologies (Table 1). Molecular characterization confirmed the presence of *Cryptococcus heveanensis*, *C. liquefaciens*, *Pseudozyma hubeiensis*, *Rhodotorula minuta* and *R. mucilaginosa* (see Table 1).

The yeasts isolated from the amphibians are common in the environment and have been reported as opportunistic pathogens in vertebrates. *Cryptococcus liquefaciens* is a common yeast that is able to survive in extreme habitats such as the deep sea (Buzzini and Margesin, 2013) and can be found in the microbiota of human

Table 1. Distribution of the yeast species confirmed by molecular analysis according to habitat and host.

Sampling Point	Habitat	Anuran Species	Species of Yeasts	% Similarity
Farm 2	Farm	<i>Hoplobatrachus rugulosus</i>	<i>Rhodotorula mucilaginosa</i>	100.00%
Farm 2	Farm	<i>Hoplobatrachus rugulosus</i>	<i>Rhodotorula mucilaginosa</i>	100.00%
UDRU	Urban Area	<i>Kaloula pulchra</i>	<i>Rhodotorula mucilaginosa</i>	100.00%
Phu Wua Wildlife Sanctuary	Wild	<i>Sylvirana faber</i>	<i>Rhodotorula mucilaginosa</i>	99.80%
UDRU	Urban Area	<i>Duttaphrynus melanostictus</i>	<i>Rhodotorula mucilaginosa</i>	99.50%
Phu Wua Wildlife Sanctuary	Wild	<i>Kaloula pulchra</i>	<i>Rhodotorula minuta</i>	99.70%
Farm 2	Farm	<i>Hoplobatrachus rugulosus</i>	<i>Cryptococcus liquefaciens</i>	100.00%
Phu Wua Wildlife Sanctuary	Wild	<i>Sylvirana faber</i>	<i>Cryptococcus liquefaciens</i>	100.00%
Na Yung-Nam Som protected area	Wild	<i>Duttaphrynus melanostictus</i>	<i>Cryptococcus liquefaciens</i>	100.00%
Na Yung-Nam Som protected area	Wild	<i>Kaloula pulchra</i>	<i>Cryptococcus liquefaciens</i>	100.00%
UDRU	Urban Area	<i>Duttaphrynus melanostictus</i>	<i>Cryptococcus heveanensis</i>	99.80%
Na Yung-Nam Som protected area	Wild	<i>Fejervarya limnocharis</i>	<i>Pseudozyma hubeiensis</i>	99.80%
Na Yung-Nam Som protected area	Wild	<i>Duttaphrynus melanostictus</i>	<i>Pseudozyma hubeiensis</i>	99.80%

skin (Zhang et al., 2011). It has also been reported as an opportunistic pathogen (Takemura et al., 2015) and is able to cause fatalities in humans (Conde-Pereira et al., 2015), but has never before been reported in amphibians. *Cryptococcusuniculus* has previously been reported from rabbits (Shin et al., 2006).

Pseudozyma hubeiensis, a basidiomycetous yeast, was recently isolated in China from the leaves of a number of plant species (Wang et al., 2006), while Liou et al. (2009) have described a new species of this genus in Thailand from the region in which the current study was carried out. To our knowledge, there are no previous reports of this genus of yeast in vertebrates.

The genus *Rhodotorula* is a common yeast found in air, soil, lakes, ocean water, milk and fruit juice (Wirth and Goldani, 2012), and is able to survive in adverse conditions. *Rhodotorula* spp. including the two species found in amphibians in the present study have been observed as opportunistic pathogens in humans (Wirth and Goldani, 2012). Recent studies have reported the presence of *Rhodotorula* yeasts in vertebrates (Chitko-Mckown et al., 2013) including a female lamb (but with no associated pathology), in skin lesions in sea lions in an aquarium (Alvarez-Perez et al., 2010) and as a normal component of the microbiota of salmonids (Raggi et al., 2014). Our results suggest that the role of yeasts in wild and farmed amphibians as pathogens has to date been underestimated and it is likely that extrinsic and intrinsic factors induce their pathogenicity. Our results show a high diversity of yeast species (5) in amphibians compared to the number of species found in vertebrates. However, only a few studies of yeasts in vertebrates have ever been conducted. It is possible that the aquatic habitat of adult amphibians (partially dependent on water and

varying according to the species) may contribute to the life cycle of these yeasts.

Our examination of bile samples revealed that yeasts colonize the internal organs of frogs. Although the presence of *Candida* spp. in human gall bladders has previously been reported (Diebel et al., 1996), we believe that this is the first report of yeast from the gall bladders of non-human vertebrates. The study by Sammon et al. (2010) found a minimum of six species of yeasts (*Rhodotorula* sp., *Trichosporon* spp., *Candida* sp., *C. famata* and *C. glabrata*) in faeces from five frogs and our findings confirm this high diversity. We isolated five species of yeasts, which suggests that the biodiversity of yeasts in frogs has been insufficiently studied. Additionally, our study confirms the ubiquity of yeasts in these amphibians since they were found in two wild protected areas, in an urban area and on frog farms. Our study also shows that yeasts are widespread in a number of species of amphibians, including both those associated with aquatic habitats and those with more terrestrial habitats (toads).

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