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Occurrence and quantification of fungi and detection of mycotoxigenic fungi in drinking water in Xiamen City, China

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HIGHLIGHTS

- Different species fungi were detected in water samples with high frequency.
- The ITS region was the best for fungal diversity study than 18S region.
- We detected strains of fungi that are well-known for the production of mycotoxins.
- Some detected strains may directly cause human health problems.

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ABSTRACT

Fungi are known to play an important role in nutrient and carbon cycling, and the occurrence of fungi in the water supply may result in a variety of human health problems. This study aimed to investigate the occurrence and frequency of various fungi in drinking water over a one-year period. The study also aimed to quantify the fungal presence using real-time PCR, and to effectively detect mycotoxigenic fungi in a variety of water sources. Water samples were collected from different water systems (surface water, public system water, house water, and tank water), from different sites (n = 15) in Xiamen, China. Each month from February 2011 to January 2012, 22 water samples were collected and analyzed. The results showed that surface water samples possessed a higher frequency of fungi than did the other water samples. Identification of fungal species was conducted using morphological and molecular methods. The most dominant fungi found were *Aspergillus* spp., *Fusarium* spp., *Pencillium* spp., *Trichoderma* spp., *Mucor* sp., and *Rhizopus* sp. Notably, the more-frequency observed fungi in the tap water of houses and public systems were *Fusarium* sp., *Exophiala* sp., and *Phialophora* sp. Meanwhile, mycotoxigenic fungi were detected in some water samples at different times. The strains isolated from samples collected in September to November had the aflatoxigenic fungi, and for fumonisin and trichothecenes, the fungi strains were re-isolated from water in November and December.

1. Introduction

Fungi can be found almost everywhere in the environment, and are able to grow in large quantities in water (Kanzler et al., 2007). Recently, more attention has been given to the study of fungi in drinking water (Arvanitidou et al., 1999; Hageskal et al., 2006; Pereira et al., 2010), as fungi and their metabolites are generally accepted to be some of the most hazardous drinking-water contaminants (Hageskal et al., 2009). With the aim of investigating the health problems associated with the fungi present in water, many studies have been conducted in different countries, ultimately focusing on the occurrence of fungi in different water sources, including surface water, groundwater, waterworks, and tank water (Gottlich et al., 2002; Kanzler et al., 2007; Pereira et al., 2010). Pereira et al. (2010) investigated fungal presence in spring water, and the most frequently-isolated fungi included *Phialophora* spp., *Exophiala* spp., *Verticillium* spp., *Fusarium* spp., *Phoma* sp., *Aspergillus* spp., *Chalara* sp., *Acremonium* spp., *Penicillium* sp., and *Cladosporium* spp. Other studies have used raw water, treated water, and water from private homes and hospital installations to determine the occurrence of fungi (Hageskal et al., 2006; Grabinska-Loniewska et al., 2007). Sammon et al. (2010) collected samples from inlets, different parts of treatment plants, as well as outlets to investigate the presence of fungi, and found *Candida*, *Aspergillus*, *Cladosporium*, *Fusarium* spp., *Penicillium* sp., and *Trichoderma* sp. Fungi like *Penicillium* spp., *Cladosporium cladosporioides*, and *Alternaria alternata* were also investigated in bottled mineral water by Cabral and Pinto (2002). Tap water and hospital water, too, have been analyzed for the presence and

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frequency of fungi (Arvanitidou et al., 1999; Panagopoulou et al., 2002; Hageskal et al., 2006; Goncalves et al., 2006a; Hayette et al., 2010), and reports have shown that the most dominant fungi in those sources included *Aspergillus* spp., *Trichoderma*, and *pencillium* spp. Even school tap water has been found to be contaminated with some of the most prevalent isolated species of terrestrial fungi, such as *Trichoderma*, *Aspergillus* sp., and *C. cladosporioides* (Nasser, 2004). Some previous studies have also investigated the formation of biofilms in municipal water distribution systems (Doggett, 2000) where the presence of *Aspergillus* spp., *Penicillium* spp., *Cladosporium* sp., *Phoma* sp., and some other species was reported.

The study of fungi in water is very important, because many health problems are caused by fungal occurrences in water and because fungi are also involved in the production of water tastes and odors (Hageskal et al., 2006; Goncalves et al., 2006a). The health problems that result from the presence of fungi possibly originate from mycotoxins and allergies (Marr et al., 2002; Kanzler et al., 2007; Basilico et al., 2007). Exposure to filamentous fungi may result in a wide variety of health problem in humans (Fanga et al., 2005; Hageskal et al., 2006), and may also contribute to the contamination of food and beverages, skin irritations and allergic reactions, as well as an increased occurrence of opportunistic systematic mycosis in immune-compromised patients (Spreadbury et al., 1993). Furthermore, aflatoxins (G2 and B2) produced by *Aspergillus flavus* have been detected in stored water (Paterson et al., 1997).

In the past, some research groups have only worked on frequency and identification of fungi, but no one studied if the strains found could produced toxins or not, such as the strains in genera *Aspergillus* spp., and *Fusarium* spp. Because some of fungal strains, occurred in water but do not have the cluster gene in toxin biosynthesis, so can't produce the mycotoxins. This study investigated both the occurrences of fungal strains and gene responsible for toxin production using advanced molecular technique.

According to the authors' knowledge, seldom studies have so far been done on the comprehensive survey of the occurrence and quantification of fungi in the water in a high population density area such as Xiamen. The advanced molecular technique, such as real-time PCR, is seldom used, too. Therefore, this study aimed to investigate the occurrence and frequency of fungi in different sources of water collected from Xiamen, China on monthly basis throughout the year, using the isolation technique of media culture (starting from February, 2010 to January, 2011). Further, this study focused on the quantification of fungal diversity using Real Time-PCR, as well as the investigation of mycotoxigenic fungi using the common PCR method.

2. Materials and methods

2.1. Study area description and water sampling

Xiamen City is located at 24° 28′ 47.41″ N and 118° 05′ 21.91″ E in Fujian Province, China. Its district covers an area of about 1573 km^2 and has a total population of 3.5 million. For the study, a total of 15 different sites were selected in such a way to adequately represent the area's surface water (number of samples (n) = 6), bus stop water (n = 3), house water (n = 7), hospital water (n = 2), university water (n = 2), and tank water (n = 2). From the study area, 424 water samples were collected during the one-year study period. Water samples were collected in one-liter clean polyethylene bottles. From surface water bodies, water samples were collected from the edge of rivers and lakes, while water samples were taken directly from the tanks. Samples collected from the public network (hospitals, bus stations, and universities) were collected from bathrooms, and in the case of bus stations were also taken from tap water that was used for drinking. Before samples were taken from the tap, water was discarded continuously for 5 min. Following collection, samples were directly transferred to the lab at the Institute of Urban Environment, CAS, for analysis.

2.2. Water quality

Water samples were analyzed for physical characteristics, such as pH and dissolved oxygen (DO), by using dissolved oxygen meter HQd field case (HACH, Colorado, USA). Turbidity was measured using a turbidity meter (Orion AQUAfast Field Kit, Thermo Electron Corporation, Beverly, USA). The total organic carbon (TOC) and total nitrogen (TN) were investigated using a TOC analyzer (TOC-V CPH, Shimadzu, Japan), while free Cl_2 and total Cl_2 were measured using a pocket colorimeter, (HACH, Colorado, USA).

2.3. Isolation of fungi

For fungal isolation, 100 ml water was filtered through a membrane filter with a pore diameter of 0.45 μm (Millipore, MA, USA). Filters were then placed on melt extract agar (MEA) (Cabral and Pinto, 2002; Kanzler et al., 2007; Sammon et al., 2010) and sabroud dextrose agar (SDA) (Arvanitidou et al., 1999; Panagopoulou et al., 2002; Kanzler et al., 2007), to which antibiotics were added (chloramphenicol and streptomycin sulfate, 100 g/1L and 50 g/1L, respectively) to prevent bacterial growth. Subsequently, the plates were incubated in darkness at 28 °C ± 1 °C for four weeks. The number of colonies was then determined and expressed as the number of CFU/100 ml in the water sample.

For isolation of pure single colonies, positive cultures were further sub-cultured on potato dextrose agar and incubated at 28 °C ± 1 °C for seven days. The representative isolates were stored on potato dextrose agar slants at 4 °C.

2.4. Identification of fungi

For identification, the fungi were sub-cultured on potato dextrose agar (PDA) and Czapek media. The fungi were phenotypically identified to the species level based on macroscopic and microscopic characteristics identified under light microscopy (Lesli and Summerell, 2006; Pitt and Hocking, 2009; Johnson et al., 2010). Slides were stained with lacto-fuchsine in distilled water to ensure adequate viewing of all parts of the fungi on the prepared slide culture according to the method of Johnson et al. (2010). Briefly, a block of PDA sized at approximately 1 cm^2 was inoculated with the fungus of interest around the edges of the disk. It was then incubated in a moist chamber for a week or more until fungi were visible. The cover-slip was harvested by placing it on a drop of mounting fluid on a glass slide. The block of agar was discarded, and a drop of mounting fluid was placed on the original slide and covered with a cover-slip.

A few isolates that could not be identified morphologically were identified by sequencing of the rRNA gene internal transcribed spacer region (ITS), comprising the ITS1, 5.8S, ITS4, and 18S nu-ssu regions. After seven days, DNA was extracted from fresh mycelium and spores from PDA media using commercial kits following manufacturer instructions, with some modification (Fungi DNA kits, Omega Company, USA). DNA was extracted using glass beads and a pestle plate to grind the fungi, and the DNA was purified. In brief, phenol, chloroform, and isoamyl alcohol (25:24:1 v/v), with isoamyl alcohol (24:1) were used to re-extract DNA. DNA was precipitated with 0.7 ml volume of 2-propanol, washed with 70% ethanol, dried, and re-suspended in 25–50 μL of double deionized water (Płaza et al., 2004; Kuo et al., 2005; Manonmani et al., 2005).

The PCR protocol consisted of an initial denaturation at 95 °C for 10 min; 35 cycles of 94 °C for 1 min, and 55.5 °C for 1 min (annealing), and 72 °C for 1 min (extension); and a final elongation of 72 °C for 10 min. After that, gel purification was performed using a commercial kit to purify the PCR products, sequenced in both directions with the primers, as previously mentioned (Poly Technology Company, Xiamen, China).

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2.5. Phylogenetic analysis

The ITS and 18S rRNA sequences of fungal isolates were used for BLAST searches within the EMBL/GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). The ITS and 18S rRNA isolate sequences were further aligned and compared with published ITS and 18S rRNA sequences, using the taxonomy browser of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) and GenBank. A phylogenetic tree was constructed using a neighbor-joining algorithm in Geneious basic software 3.6.1, New Zealand.

2.6. Diversity of fungi

2.6.1. Extraction of DNA

For DNA extraction, water samples were individually collected for eight months at two-month intervals (four times total) to effectively sample during all prevailing seasons (summer, autumn, and winter) in Xiamen, China. Following DNA extraction with a one-liter water sample, a preliminary study to determine the most suitable volume for DNA extraction from water was conducted. To analyzed the effectiveness of 100 ml, 500 ml, and 1000 ml water samples for extraction. For the 100 ml and 500 ml samples which used for extracting DNA, the observed DNA concentration was very low, and the results of PCR experiment were negative (undefined).

One liter of water was filtered through a filter membrane (0.45 μm) (Millipore, MA, USA). DNA was then extracted from the membrane using water DNA kits, following kit protocol with some modification (Omega Company, USA). Briefly, the filter membrane was taken from the filter adapter and cut into small pieces. To this, 100 mg of glass beads was added. The test tube was then placed into liquid nitrogen and vortexed. This process was repeated three times to ensure that the spores or any mycelium fraction in the filter membrane was properly ground. Subsequently, DNA was extracted and the concentration was measured using a Qubit Flouorometer, according to manufacturer instruction (Qubit Fluorometer, USA). The extracted DNA was stored at −20 °C for further analyses, including quantification through real-time-PCR.

2.6.2. Quantification using Real Time-PCR

The extracted DNA was used for quantification of fungi. Specifically, gene expression of the ITS and 18S regions was used for the purposes of comparing and selecting the best primer for studying the diversity of fungi in water using real-time PCR (ABI PRISM 7500 Fast, Applied Biosystems, USA). Sequence Detection System software v.1.4 was used to analyze the data Ct value. Microsoft Excel software 2007, USA was used to calculate the samples and make a standard curve. The Ct value was calculated to DNA concentration using two primers from two regions. SYBR Green dye was used as the amplification reporter, and the total reaction volume was 20 μl, containing the following: 0.4 μl SYBR Green II Sigma; 0.2 μl each of primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCCGCTTATTGATATGC) (White et al., 1990), and nu-ssu-0817-5 (TTAGCATGGAATAATRRAATAGGA) and nu-ssu-1196-3 (TCTGGACCTGGTGAGTTTCC) (Borneman and Hartin, 2000); 10 μl DNA polymerase; and a variable quantity of DNA (1 μl for environmental samples was used as template). The cycling conditions consisted of the following: 95 °C for 30 s, followed by 40 cycles with denaturation at 95 °C for 5 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 32 s. An additional elongation step of: 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s was finally performed. Fluorescence data were collected at each PCR cycle during the elongation step.

2.7. Detection of mycotoxigenic fungi

Re-isolation of many strains of fungi, such as *A. flavus and Fusarium* spp., was done using three media cultures: PDA, Czapek, and Yeast extract sucrose agar. The strains were cultured on PDA and incubated at 28 °C for 24 h. After the appearance of colonies, a single spore was transferred to the Czapek media for all isolations. The following was used for isolation of *A. flavus* and *A. parasiticus*: YEA consisting of sucrose (20 g), yeast extract (4 g), KH_2PO_4 (1 g), $MgSO_4.7H_2O$ (0.5 g), and agar (15 g) per liter. DNA was extracted from the culture plate after one week as mentioned earlier and stored in freezer (−20 °C) for further testing with some specific primers in cluster genes: aflatoxin, fumonisin, and trichothecene biosyntheses (Table 1). Two primers, *AFLR*, and *OMT-A*, were used for aflatoxins (Shapira et al., 1996); gene *Fum1* (primer *PQF1*) and β-tubulin gene TUB2 (primer *PQTUB*) were used for Fumonisin biosynthesis (Errasquín et al., 2007); and primer *Tri5* was used for trichothecenes (Doohan et al., 1999). PCR was conducted to amplify DNA using different conditions for different primers. The conditions for *aflr* were as follows: 95 °C for 5 min, and 35 cycles at 94 °C for 30 s, 63 °C for 1 min, and 72 °C for 40 s. Conditions for *OMT-A* were: 95 °C for 5 min, 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min. Conditions for Tri5 were: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Conditions for *PQF1* were: 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 40 s; and for *PQTUB*, only the annealing temperature was changed to 57 °C for 1 min. All were finally elongated at 72 °C for 10 min.

2.8. Statistical analysis

Analysis of variance was conducted for all parameters using the SPSS software program, version 11.5, Chicago, USA. Means of the media culture MEA and SDA at CFU/100 ml were compared using one-way ANOVA analysis and T-tests ($p \leq 0.05$). The correlation between the media and the different water parameters was also determined. Similarly, the ITS and 18S regions were compared to ensure good results to quantify fungi in water. The correlation between concentrations of DNA and the different water parameters was also determined.

3. Results and discussion

3.1. Basic characteristics of water

Table 2 summarizes the characteristics of the water collected from the study area. At each collection time, water samples were analyzed for pH, Cl_2, TCl_2 DO, TOC, TN, temperature, and turbidity. The pH variation was not too great (6.59–7.15), and therefore may not affect the occurrence of fungi in water. In comparison to other sources, the concentration of Cl_2 was higher in water samples collected from the public system and houses (0.43 mg/l and 0.47 mg/l, respectively). However, some public system and house samples showed lower free Cl_2 concentrations, and the occurrence of fungi was higher than

Table 1
The primers used for detection of mycotoxigenic fungi which isolated from drinking water.

Gene	Primer (5′–3′)	Target	Reference
Aflr	APA-450-TATCTCCCCCCGGGCATCTCCCGG APA-1482 CCGTCAGACAGCCACTGGACACGG	Aflatoxin biosynthesis	Shapira et al. (1996)
Omt-A	OMT-208 -GGCCCGGTTCCTTGGCTCCTAAGC OMT-1232 -CGCCCCAGTGAGACCCTTCCTCG	Aflatoxin biosynthesis	Shapira et al. (1996)
Fum1	PQF1-F GAGCCGAGTCAGCAAGGATT PQF1-R AGGGTTCGTGAGCCAAGGA	Fumonisin biosynthesis	Errasquín et al. (2007)
β-tubulin	PQTUB-F CCCCGAGGACTTACGATGTC PQTUB-R CGCTTGAAGAGCTCCTGGAT	Fumonisin biosynthesis	Errasquín et al. (2007)
Tri5	Tr5F- AGCGACTACAGGCTTCCCTC Tr5R- AAACCATCCAGTTCTCCATCTG	Trichothecenes	Doohan et al. (1999)

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in other samples. Samples collected from bus station showed low Cl_2 concentrations. For all samples, the DO concentrations ranged from 6.12 to 8.02 mg/l, the lowest DO concentration was observed in surface water, and the highest was observed in the samples collected from houses. TOC concentrations ranged from 1.71 to 4.12 mg/l, with the highest concentration (4.12 mg/l) observed in surface water and the lowest (1.71 mg/l) in the tank water sample. TN contents ranged from 2.86 to 4.92 mg/l, with the highest value (4.92 mg/l) observed in surface water and the lowest value (2.86 mg/l) in tank water. Significant turbidity (24.25 NTU) was observed only in surface water, and other sources showed very low turbidity. The correlation results showed that there were significant relationships between all parameters, with the exception of pH with Cl_2, and pH with TOC, both of which were not significant.

3.2. Isolation and identification of fungi

Fungi occurrence was noted monthly in the different water sources, using SDA and MEA culture media (Fig. 1). Both SDA and MEA were employed with the aim of selecting the most suitable culture for the proper isolation of all species of fungi. Results observed that there was no significant difference between the results of the MEA and SDA media cultures. However, the colonies on SDA were more clear and the growth was a little slower in comparison to the MEA results. All fungal growth on both media cultures was similar, and no difference was found between the genera of fungi grown on these media.

As the results have indicated, a total of 18 different genera of fungi were identified from the collected water samples (Table 3). The most dominant fungi observed were *Aspergillus* spp., *Fusarium* spp., *Pencillium* spp., *Phialophora* spp., and *Trichoderma* spp., and these findings are similar to those reported previously (Arvanitidou et al., 2000; Cabral and Pinto, 2002; Hageskal et al., 2006; Kanzler et al., 2007; Abdel-hameed et al., 2008; Sammon et al., 2010). In the present study, the most frequent species were *Asergillus flavus* and *Aspergillus terrus*, and these findings are consistent with those reported by Nasser (2004); Hageskal et al. (2006); Goncalves et al. (2006a); and Sammon et al. (2010). The species *Fusarium oxysporum*, *Fusarium proliferatum*, *Trichoderma* spp., *Phialophora* spp., and *Exophiala* spp. were observed in the samples collected from houses and public systems, while *Phialophora* spp. and *Exophiala* spp. were noted at a higher frequency in tap water than in other water sources. These results agreed with the results of a previous study (Gottlich et al., 2002). However, in the surface water, besides those fungi, *Mucar* spp., *Aspergillus* spp., and *Rhizopus* sp. were also observed each month in both media cultures. The highest frequency for *Trichoderma* spp. was observed in surface water. These results are similar to those found in a study conducted by Hageskal et al. (2006). The frequency of *Fusarium* spp. was higher than that of *Aspergillus* spp. and *Penicillium* spp. in surface water, which supported the results of the study conducted by Cabral and Pinto (2002). Meanwhile, the occurrence and frequency of *Acremonium* sp. were similar to previously reported results (Vesper et al., 2008; Pereira et al., 2010).

Tap water samples collected from houses showed low/no frequency of fungi, with the exception of two samples, in which the frequency of fungi was high. The most common fungi appearing in house water included *Fusarium*, *Exophiala* spp., and *Trichoderma* spp., fungi which were not reported by Goncalves et al. (2006a). *A. flavus* and *A. niger* were found only in two house samples, both of which had low concentrations of Cl_2 and TCl_2 in comparison to those of other house

Table 2
The basic properties of different drinking water collected from the study area (February 2011–January 2012).

Parameters	Water types	Minimum	Maximum	Mean	Std. deviation
pH	Surface water	6.66	9.76	7.15	0.46
	Public system	6.14	9.40	6.89	0.43
	House water	6.41	7.90	7.07	0.38
	Tank water	3.99	7.62	6.59	1.19
DO (mg/l)	Surface water	3.33	8.66	6.12	1.40
	Public system	4.08	9.24	7.41	1.23
	House water	6.65	9.61	8.02	0.79
	Tank water	6.49	8.78	7.60	0.68
Cl_2 (mg/l)	Surface water	0.00	0.60	0.10	0.13
	Public system	0.00	1.90	0.43	0.53
	House water	0.00	2.20	0.47	0.43
	Tank water	0.00	0.30	0.12	0.08
TCl (mg/l)	Surface water	0.00	0.70	0.18	0.21
	Public system	0.00	2.20	0.57	0.62
	House water	0.05	1.50	0.61	0.42
	Tank water	0.00	0.60	0.30	0.17
TOC (mg/l)	Surface water	2.00	19.60	4.12	2.76
	Public system	0.05	14.12	2.02	1.46
	House water	0.11	8.36	2.36	1.75
	Tank water	1.12	3.80	1.71	0.45
Temp (°C)	Surface water	15.22	30.50	23.03	4.86
	Public system	15.10	30.90	23.13	4.62
	House water	14.90	31.10	22.90	4.62
	Tank water	17.60	30.20	23.80	3.99
TN (mg/l)	Surface water	0.20	9.25	4.92	2.50
	Public system	0.00	10.20	3.65	2.22
	House water	0.00	8.40	3.68	2.36
	Tank water	0.00	4.29	2.86	1.15
TURBI (NTU)	Surface water	6.82	65.55	24.45	17.56
	Public system	0.00	5.00	0.36	0.89
	House water	0.00	1.17	0.25	0.25
	Tank water	0.00	0.86	0.36	0.24

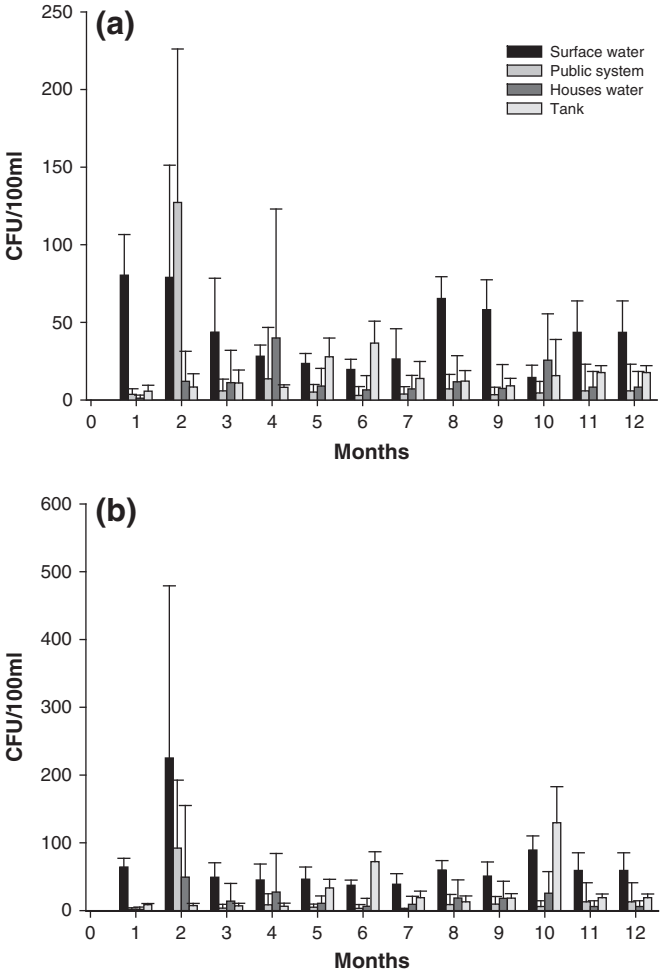

Fig. 1. Frequency of various fungi in drinking water through the year (from February 2011 to January 2012). a) Using SDA media culture, and b) using MEA media culture.

Table 3
Identification of fungi species, which were isolated from the water.

Fungi	Sample sources	Fungi	Sample sources
Didymella sp.	s. water	*Pseudallescheria boydii*	s. water & tap water
Cladosporium cladosporioides	s. water & tap water	*Mucor* sp.	s. water
Phialophora europaea	s. water & tap water	*Rhizopus* spp.	s. water
Phoma sp.	s. water	*Aspergillus terreus*	s. water & tap water
Microsphaeropsis arundinis	s. water	*Aspergillus fumigatus*	s. water
Penicillium spp.	s. water & tap water	*A. penicillioides*	s. water
Penicillium glabrum	s. water & tap water	*A. clavatus*	s. water
Eupenicillium sp.	s. water & tap water	*A. ustus*	s. water
Penicillium janthinellum	s. water & tap water	*Alternaria* sp.	s. water
Trichoderma longibrachiatum	s. water	*A. niger*	s. water & tap water
Hypocrea lixii	s. water	*A. flavus*	s. water & tap water
Trichoderma sp.	s. water & tap water	*Eurotium amstelodami*	s. water & tap water
Trichoderma viride	s. water & tap water	*Fusarium verticillioides*	s. water
Scolecobasidium sp.	s. water & tap water	*Fusarium chlamydosporum*	s. water
Cladosporium sp.	s. water & tap water	*Fusarium oxysporum*	s. water & tap water
Fusarium sp.	s. water & tap water	*Exophiala pisciphila*	s. water & tap water
Acremonium sp.	s. water	*Exophiala spinifera*	s. water and tap water
Fusarium proliferatum	s. water & tap water	*Plectosphaerella* sp.	s. water

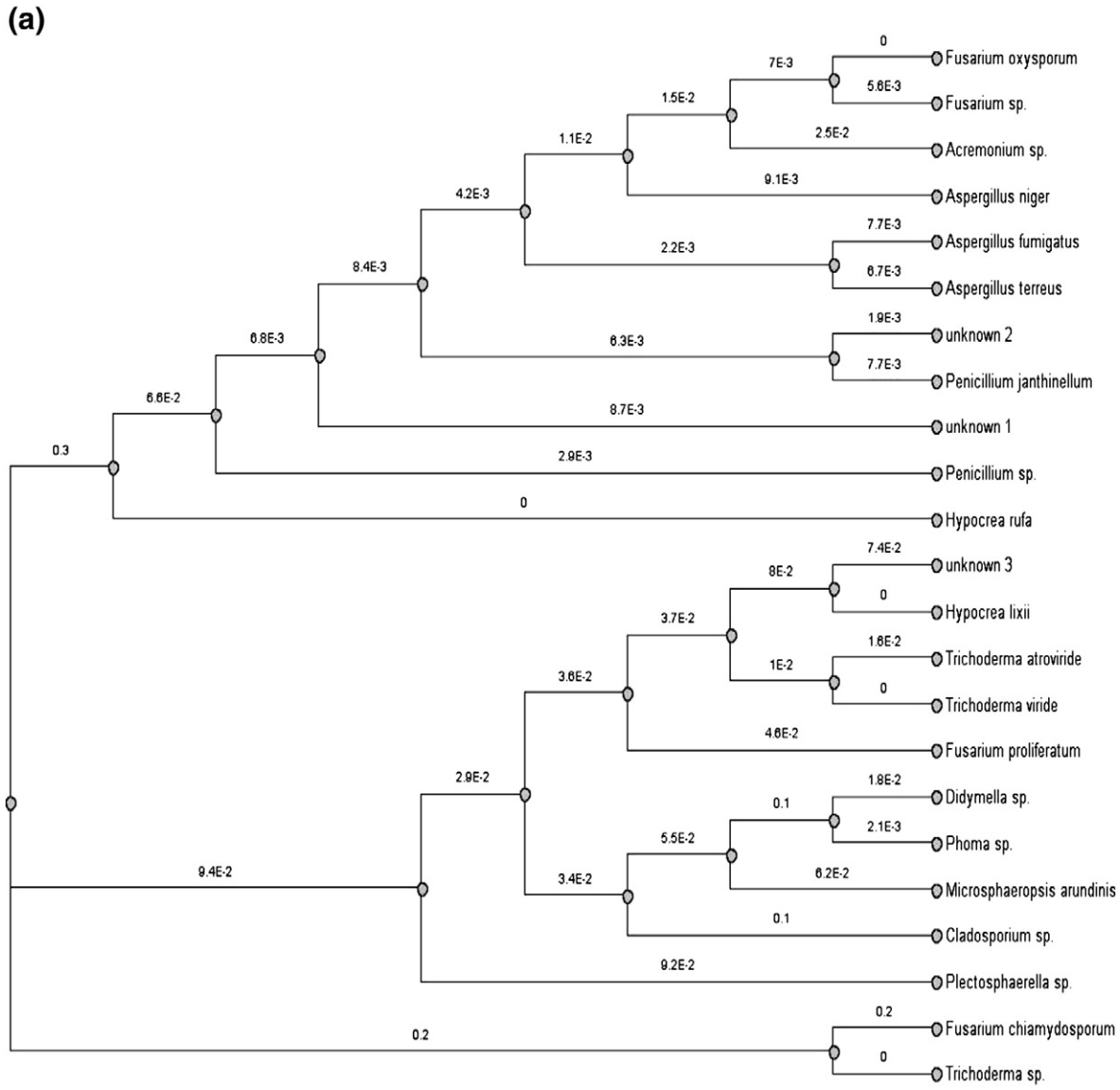

Fig. 2. Neighbor joining of phylogenetic tree for the fungi species isolated from, a): surface water, b): Tap water by using ITS and 18S sequence.

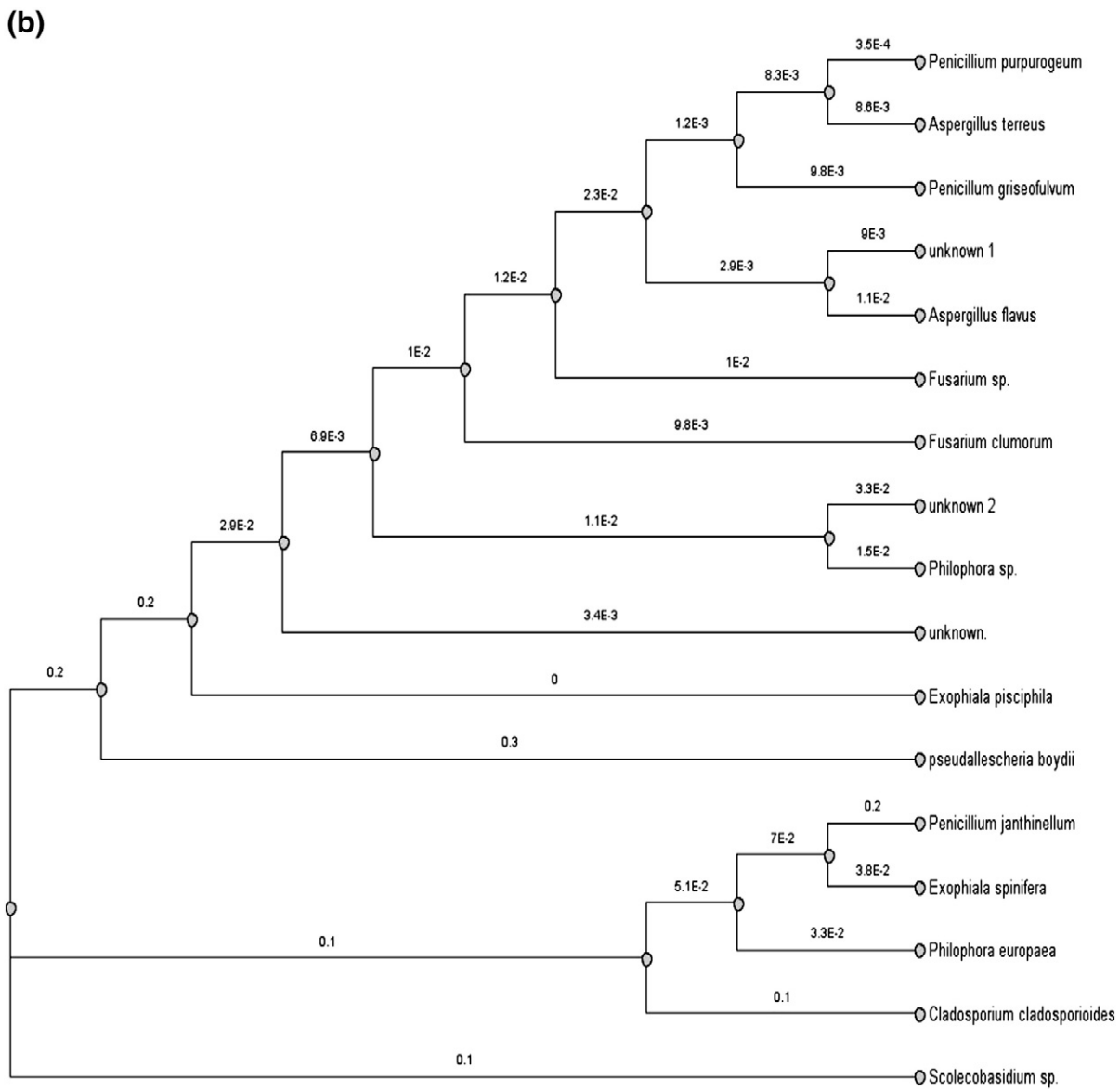

Fig. 2. (*continued*).

water samples. The frequency of these two strains was 0.3% in tap water samples as reported in the past study (Goncalves et al., 2006a). In those two samples, the frequency of fungi was significantly higher ($P = 0.05$) in comparison to that of other water samples collected from houses. This may be a result of several factors, such as the presence of bio-films in pipes, which may be a source of water contamination (Doggett, 2000; Saad et al., 2004; Goncalves et al., 2006b; Grabinska-Loniewska et al., 2007). Furthermore, the high-frequency occurring fungi in tap water were *Exophiala* spp. and *Phialophora* spp., both of which may be a cause of certain forms of skin hypersensitivity, as reported in previous studies (Sughayer et al., 1991; Rossmann et al., 1996; Gottlich et al., 2002).

Like other water sources, the hospital water samples were also investigated for the frequency of fungi, but only *Exophiala* spp. was observed in all samples collected throughout the study duration.

The water samples collected from universities showed significantly ($P = 0.05$) higher frequency of fungi in comparison to other sources, like tap water, public system, bus station, and hospital samples. The fungi in this source mostly belonged to the *Exophiala* spp., *Aspergillus* spp., and *Penicillium* spp. genera, results which are similar to those reported by Arvanitidou et al. (1999). Sometimes, *Fusarium* spp. was observed and with a highly significant difference from the other water samples.

Fig. 1 shows the effect of time on the frequency of fungi present in surface water in the months of February, April, May, June, July, August, and September in both media cultures. Results of the SDA culture showed high frequency in the months of January, February, August, September, November, and December, while results of the MEA culture showed high frequency in January, February, October, November, and December. Furthermore, a significant difference was observed in the occurrence of fungi between months throughout the year. These findings are in agreement with those mentioned in a past study (Pereira et al., 2010), which also noted a high fungi frequency in November and December in surface water.

The results indicated that surface water normally showed a higher frequency than did other water sources, while public system samples showed a higher frequency in February on both media cultures (SDA and MEA). In tank water, a higher frequency of fungi was observed in the months of May and June for the SDA media culture, and in June and October for the MEA culture. Likewise, the results showed a significant correlation ($p < 0.01$) between the frequency of fungi and the temperature. These results agreed with the findings of Sammon et al. (2010).

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Fig. 2 shows the phylogenetic tree for all fungi isolated from surface water and tap water, as well as those sequenced from the ITS1 and ITS4, and 18S (nu-SSU-0817 and nu-SSU-1196) regions. The clone libraries were most closely related to fungal 18S rRNA, ITS1, 5.8S, ITS4, and 28S sequences within the GenBank and EMBL + DDBJ + PDB databases. The results showed that these sequences clustered further into three smaller clades for all water sources.

Statistical analyses for the occurrence of fungi and certain water parameters showed that the Cl_2 concentration may affect the occurrence of fungi. Results showed that in some public system and house samples, the concentration of free Cl_2 was slightly lower, but the occurrence of fungi was higher than other samples in which the level of free chlorine was high. However, for the samples collected from bus station, the free Cl_2 concentration was low, but the frequency of fungi was also low. Meanwhile, in the water samples collected from hospitals, the free Cl_2 concentration was always the highest and the occurrence of fungi was low. These results disagreed with those of a previous study, in which no difference in the frequency of fungi was found between chlorinated and untreated water samples (Gottlich et al., 2002). Results agreed with those of another study, which noted a negative correlation between chlorine concentration and the diversity of fungi in water samples (Sammon et al., 2010).

3.3. Quantification of fungi

3.3.1. Standard curve

For standard curve, genomic DNA were obtained from *A. terrus* pure culture. Ten fold diluted was made, and run on real-time PCR system (Applied Biosystems, USA) using different primers such as ITS1, ITS4, and nu-ssu1196, nu-ssu-0817. The result showed a linear relationship between $\log_{10}$ input DNA and Ct value, and their R^2 values were 0.9995 and 0.9946 for ITS and nu-ssu primers, respectively. The value of y was also calculated using the following equation: $y = (n \times 6.02 \times 10^{20}/G\ da)$, where n represents the concentration of DNA, and G is the size of the whole genome in the gene bank.

3.3.2. Diversity of fungi

3.3.2.1. Comparison of the primers. Results from the comparison of the two primers showed that ITS was more sensitive than nu-ssu. Further, the 7500 System SDS software Ct value analysis showed better results using the ITS primer than it did for the nu-ssu primer for all water samples. Likewise, for many of samples, the Ct value remained undetermined when the nu-ssu primer was used, whereas the ct value was determined for all samples when the ITS primer was used. The nu-ssu primer had a higher concentration in September in all samples, and also showed higher concentrations in surface water samples collected in September and November. Meanwhile, the nu-ssu primer showed a low concentration in January in all water samples, with the exception of tank water, which showed a higher concentration in January than did the samples collected from houses and public systems. Furthermore, for many tap water samples, the Ct value was undetermined in Real Time-PCR, but the level of DNA for the samples was lower when the nu-ssu primer was used than it was when the ITS primer was used, indicating that the ITS was higher than the 18S. Results of the statistical analysis also showed that there was a significant difference between the Real Time-PCR using ITS and the Real Time-PCR using the nu-ssu at $p \leq 0.05$. This study showed that the primers (ITS1, ITS4) were more suitable for studying diversity of fungi in water as compared to nu-ssu-1196 and nu-ssu-0817 primers. These results matched those of the study by Lord et al. (2002), which also showed that the ITS region was better predicted and observed to generate a greater richness than was the 18S region.

3.3.2.2. Quantification of fungi. Fig. 3 shows that the diversity of fungi was higher in surface water than in other sources. Similarly, a higher concentration was observed in November and January in comparison to other times of the year, using the ITS primer. A higher concentration was found in January for tank water, while public system water samples showed a higher concentration in July than at other sampling times, but the concentration was still lower than that in surface water. Meanwhile, tap water collected from houses showed a higher concentration in September than in other months. A significant difference was observed between all samples, and also between the collected sample times, as well as the concentration of DNA in surface water and tap water. However, in house water samples, no significant difference between different sampling times was observed, with the exception of the month of September. In tank water samples, a greater significant difference was observed in January, but no significant difference was observed at other sampling times. This quantification of fungi by Real Time-PCR confirmed the frequency of fungi by time, as observed in January and November in surface water samples.

3.4. Detection of mycotoxigenic fungi

Many of the strains in this study were re-isolated each month using YES and Czapek culture media. The re-isolated genera included *A. flavus*, *Aspergillus parasiticus*, and *Fusarium* spp., and the results illustrated that many strains possess cluster genes that are involved in mycotoxin biosynthesis. Some strains, such as *A. flavus* and *A. parasiticus*, were re-isolated each month and kept in PDA. They were also re-isolated using YES for DNA extraction. The results showed that the strains isolated from samples collected in September to November had the genes, and thus may produce aflatoxins in water (Fig. 4). These results are supported by a previous study,

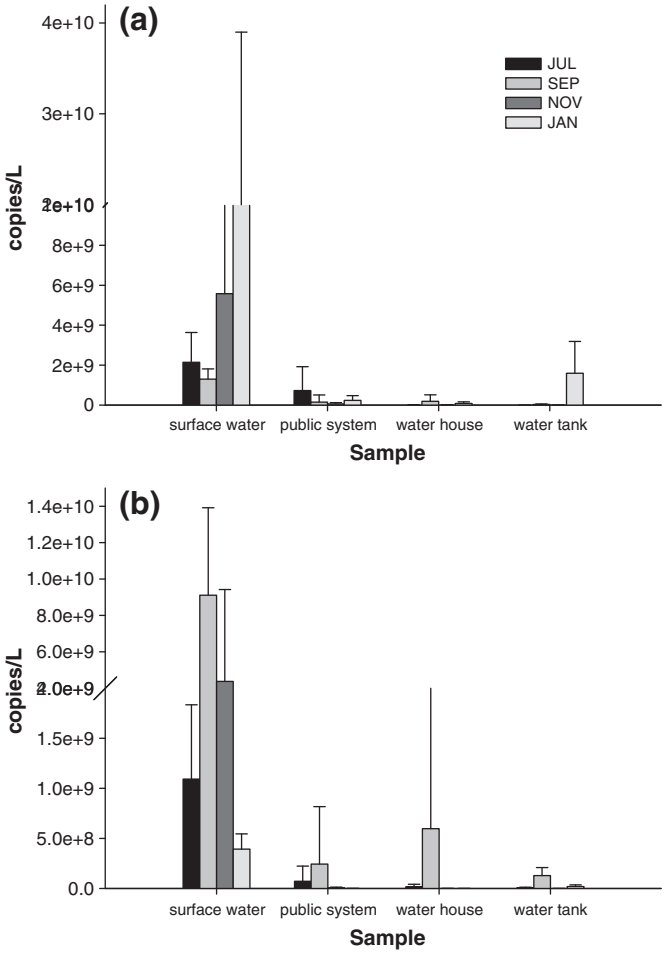

Fig. 3. RT-PCR during eight months (July2011–January2012), a): using (ITS1, ITS4) primers, b): using (nu-ssu-1196, nu-ssu-817) primers.

which detected *Apsergillus flavus* in cold tank water, and for which Aflatoxins B2 and G2 were also detected (Paterson et al., 1997). In this study, *Fusarium* spp. was also investigated for gene clusters involved in regulating fumonisin and trichothecenes. The isolates, which were re-isolated from water in November and December were identified, did have the relevant gene clusters. Another previous study also investigated the production of zearalenone in drinking water by *Fusarium graminearuum*|, and found that this fungus could, in fact, produce this toxin (Paterson, 2007). Furthermore, Gromadzka et al. (2009) also investigated the production of zearalenone in water. These metabolites are known for their high carcinogenicity in humans and animals, and are also the cause of many health problems (Hageskal et al., 2006).

3.5. Statistical analysis

One-way ANOVA statistical analysis showed that there was no significant difference between the SDA and MEA media cultures. The correlation analysis between the water parameters and media culture (using SPSS) showed that SDA did have a correlation with pH at the level of $p \leq 0.05$, and at a level of $p \leq 0.01$ with all other parameters, with the exception of DO. The MEA had a correlation with all parameters at a level of $p \leq 0.01$, with the exception of DO. The statistical analysis showed that there was, in fact, a relationship between the occurrence of fungi and the quality of water. A negative correlation was observed between the occurrence of fungi and the concentration of free Cl_2. Furthermore, a negative correlation ($p \leq 0.01$) was observed between media culture and temperature. However, a significant positive correlation ($p \leq 0.01$) was found between media culture and other water quality parameters, such as TOC, turbidity, and TN, while a positive correlation ($p \leq 0.05$) was shown between media culture and water sample pH.

4. Conclusions

This study, which is the first report from the Xiamen region in China, concluded that drinking water samples possessed a high frequency of fungi, and contained many strains of fungi that are well-known for the production of metabolites like mycotoxins, as

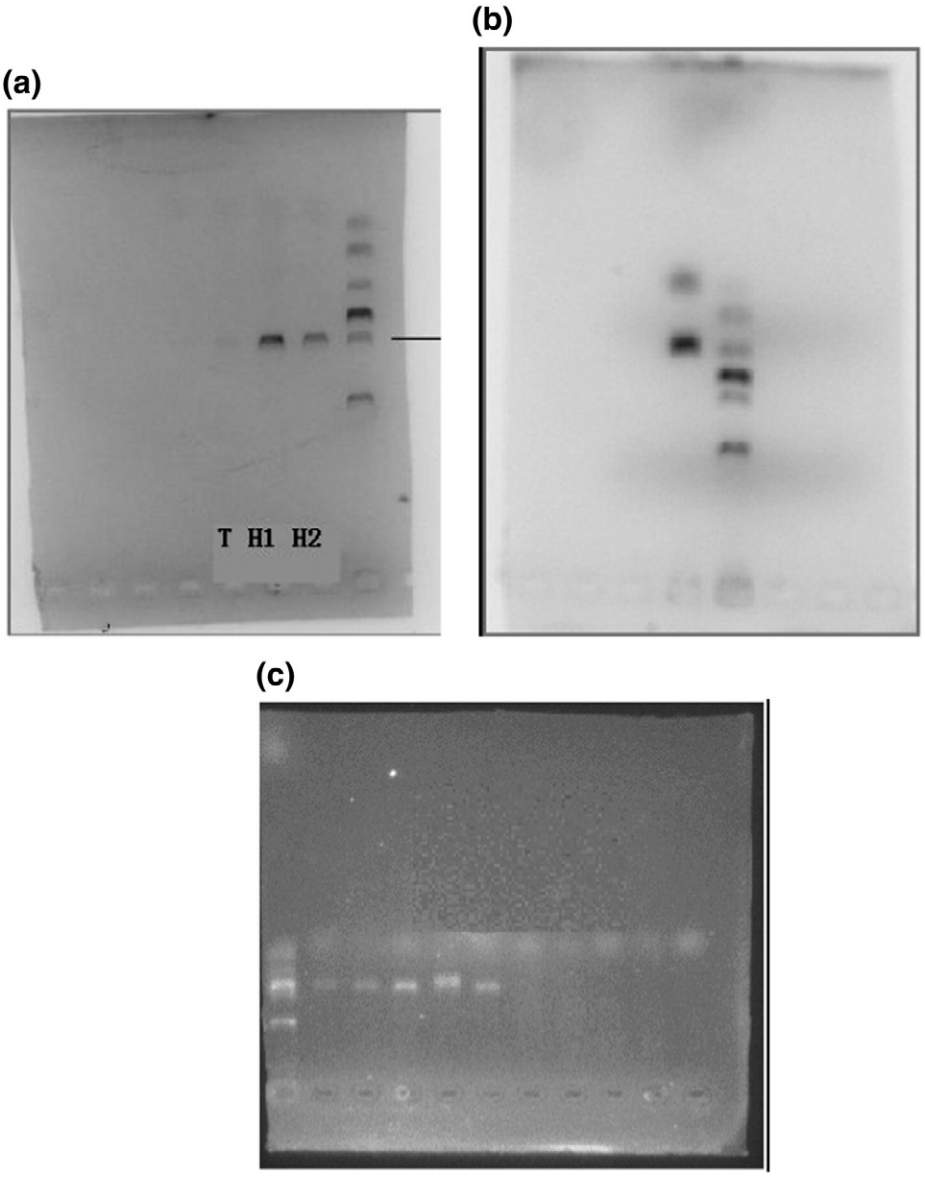

Fig. 4. a. *Aflr* gene found in *Aspergillus flavus* isolated from the house water (H1) and tank water (T), the DNA about 1000 pb in agarose gel 1.5% samples. Isolated from YES for 7 days and extracted DNA. b. PCR product for *PQTUB* gene for *Fusarium verticillium* isolated from surface water samples, the size of the fragment is about400–500 pb. The Czapek media was used to re-isolated fungi species for 7 days for extracting DNA. c. PCR product for *OMT-A* gene in *Aspergillus flavus*, *Aspergillus parasiticus* isolated from surface water, house water, and tank water, the fragment size was between 800 and 900 pb.

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well as some strains that may directly cause health problems. The high frequency of fungi that was found in samples collected from houses requires further study to investigate the occurrence of bio-film in the water supply system. The study concluded that SDA and MEA media cultures were more suitable for the study of fungi frequency in water, but that the growth on SDA was slower than that on MEA culture media. The comparison between the ITS and 18S regions using Real Time-PCR showed that ITS was the best region for the study of the diversity of fungi.

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Reference

Abdel-Hameed A, El Hawarry S, Kamel MM. Prevalence and distribution of airborne and waterborne fungi and actinomycetes in the Nile River. Aerobiologia 2008;24:231–40.
Arvanitidou M, Kanellou K, Constantinides TC, Katsouyannopoulos V. The occurrence of fungi in hospital and community potable waters. Lett Appl Microbiol 1999;29:81–4.
Arvanitidou M, Spaia S, Velegraki A, Pazarloglou M, Kanetidis D, Pangidis P, et al. High level of recovery of fungi from water and dialysate in haemodialysis units. J Hosp Infect 2000;45:225–30.
Basilico MD, Chiericatti C, Aringoli EE, Althaus RL, Basilico JC. Influence of environmental factors on airborne fungi in houses of Santa Fe City Argentina. Sci Total Environ 2007;376:143–50.
Borneman J, hartin RJ. PCR primers that amplify fungal rRNA genes from environmental samples. Appl Environ Microbiol 2000;66:4356–60.
Cabral D, Pinto VEF. Fungal spoilage of bottled mineral water. Int J Food Microbiol 2002;72:73–6.
Doggett MS. Characterization of fungal biofilms within a municipal water distribution system. Appl Environ Microbiol 2000;66:1249–51.
Doohan FM, Weston G, Rezanoor HN, Parry DW, Nicholson P. Development and use of a reverse transcription-PCR assay to study expression of *Tri5* by *Fusarium* species in vitro and in planta. Appl Environ Microbiol 1999;65:3850–4.
Errasquín EL, Vazquez C, Jimenez M, Gonzalez-jaen MT. Real-Time PCR assay to quantify the expression of fum1 and fum19 genes from the fumonisin-producing *Fusarium verticillioides*. J Microbiol Methods 2007;68:312–7.
Fanga Z, Ouyang Z, Hu L, Wang X, Zheng H, Lin X. Culturable airborne fungi in outdoor environments in Beijing, China. Sci Total Environ 2005;350:47–58.
Goncalves AB, Paterson RRM, Lima N. Survey and significance of filamentous fungi from tap water. Int J Hyg Environ Health 2006a;209:257–64.
Goncalves AB, Santos IM, Paterson RM, Lima N. Fish and calcofluor staining techniques to detect in situ filamentous fungal biofilms in water. Rev Iberoam Micol 2006 bb;23:194–8.
Gottlich E, Lubbe WVD, Lange B, Fiedler S, Melchert I, Reifenrath M, et al. Fungal flora in groundwater derived public drinking water. Int J Hyg Environ Health 2002;205:269–79.
Grabinska-Loniewska A, Konillowicz-Kowalska T, Wardzynska G, Boryn K. Occurrence of fungi in water distribution system. Polish J Environ Stud 2007;4:539–47.
Gromadzka K, Waskiewicz A, Golinski P, Swietlik J. Occurrence of estrogenic mycotoxin — zearalenone in aqueous environmental samples with various NOM content. Water Res 2009;43:1051–9.
Hageskal G, Knutsen AK, Gaustad P, Hoog GSD, Skaar I. Diversity and significance of mold species in Norwegian drinking water. Appl Environ Microbiol 2006;72:7586–93.
Hageskal G, lima N, Skaar I. The study of fungi in drinking water. Mycol Res 2009;113:165–72.
Hayette MP, Christiaens G, Mutsers J, Barbier C, Huynen P, Melin P, et al. Filamentous fungi recovered from the water distribution system of a Belgian university hospital. Med Mycol 2010;48:969–74.
Johnson EM, Borman AM. The importance of conventional methods microscopy and culture. In: Alessandro CP. Aspergillosis: from diagnosis to prevention, New York: E-ISBN 978-90-481-2407-7; 2010. P 56–72.
Kanzler D, Buzina W, Paulitsch A, Haas D, Platzer S, Marth E, et al. Occurrence and hygienic relevance of fungi in drinking water. Mycoses 2007;51:165–9.
Kuo HC, Su YL, Yang HL, Chen TY. Identification of Chinese medicinal fungus *Cordyceps sinensis* by PCR-single-stranded conformation polymorphism and phylogenetic relationship. J Agric Food Chem 2005;53:3963–8.
Lesli JF, Summerell BA. The *Fusarium* laboratory manual. First ed. Iowa: Blackwell Publishing; 2006 [ISBN-13: 978-0-8138-1919-8].
Lord NS, Kaplan CW, Shank P, Kitts CL, Elrod SL. Assessment of fungal diversity using terminal restriction fragment (TRF) pattern analysis: comparison of 18S and ITS ribosomal regions. FEMS Microbiol Ecol 2002;42:327–37.
Manonmani HK, Anand S, Chandrashekar A, Rati ER. Detection of aflatoxigenic fungi in selected food commodities by PCR process. Biochemistry 2005;40:2859–64.
Marr KA, Carter RA, Crippa F, Wald A, Corey L. Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients. Clin Infect Dis 2002;34:909–17.
Nasser LA. Incidence of terrestrial fungi in drinking water collected from different schools in Riyadh region, Saudi Arabia. Pak J Biol Sci 2004;11:1927–32.
Panagopoulou P, Filioti J, Petrikkosy G, Giakouppiy P, Anatoliotakiz M, Farmaki E, et al. Environmental surveillance of filamentous fungi in three tertiary care hospitals in Greece. J Hosp Infect 2002;52:185–91.
Paterson RRM. Zearalenone production and growth in drinking water inoculated with *Fusarium graminearum*. Mycol Prog 2007;6:109–13.
Paterson RRM, Kelleyl J, Gallagher M. Natural occurrence of aflatoxins and *Aspergillus flavus* (Link) in water. Lett Appl Microbiol 1997;25:435–6.
Pereira VJ, Fernandes D, Carvalho G, Benoliel MJ, Romao MV, Crespo MT. Assessment of the presence and dynamics of fungi in drinking water sources using cultural and molecular methods. Water Res 2010;44:4850–9.
Pitt JI, Hocking AD. Fungi and Food Spoilage. 4th ed. New York: Springer Science and Business Media, ISBN: 978-0-387-92206-5; 2009.
Płaza GA, Upchurch R, Brigmon RL, Whitman WB, Ulfig K. Rapid DNA extraction for screening soil filamentous fungi using PCR amplification. Polish J Environ Studies 2004;13:315–8.
Rossmann SN, Cernoch PL, Davis JR. Dematiaceous fungi are an increasing cause of human disease. Clin Infect Dis 1996;22:73–80.
Saad DS, Kinseyb GC, Kimb S, Gaylarde CC. Extraction of genomic DNA from filamentous fungi in biofilms on water-based paint coatings. Int Biodeter Biodegr 2004;54:99-103.
Sammon NB, Harrower KM, Fabbro LD, Reed RH. Incidence and distribution of microfungi in a treated municipal water supply system in subtropical Australia. Int J Environ Res Public Health 2010;7:1597–611.
Shapira R, Paster N, Eyal O, Menasherov M, Mett A, Salomon R. Detection of aflatoxigenic molds in grains by PCR. Appl Environ Microbiol 1996;62:3270–3.
Spreadbury C, David H, Agnes AB, Bainbridge B, cohen J. Detection of *Aspergillus fumigatus* by polymerase chain reaction. J Clin Microbiol 1993;31:615–21.
Sughayer M, DeGirolami PC, Khettry U, Korzeniowski D, Grumney A, Pasarell L, et al. Human infection caused by *Exophiala pisciphila*: case report and review. Clin Infect Dis 1991;13:379–82.
Vesper S, McKinstry C, Hartmann C, Neace M, Yoder S, Vesper A. Quantifying fungal viability in air and water samples using quantitative PCR after treatment with propidium monoazide (PMA). J Microbiol Methods 2008;72:180–4.
White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and application. San Diego: CA: Academic Press Inc.; 1990. p. 315–22. [Academic Press].

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