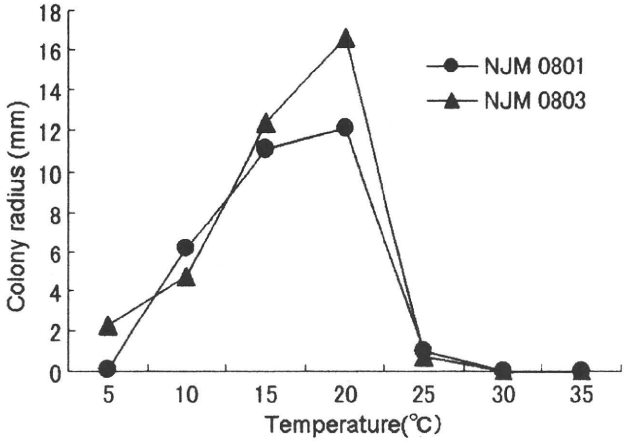

Fig. 6 Effects of temperature on the hyphal growth of isolates NJM 0801 and NJM 0803 cultured on glucose–yeast (GY) agar with 0.5% sodium chloride (NaCl)

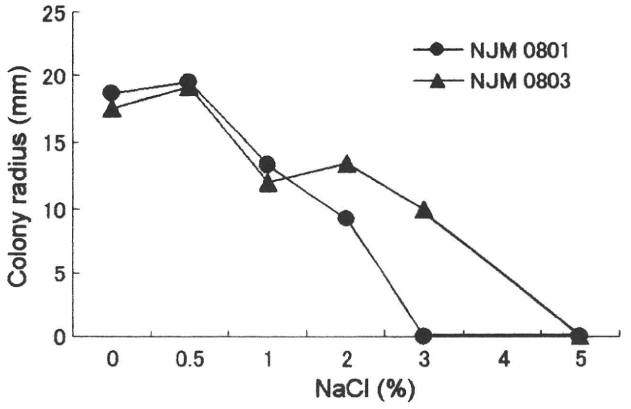

Fig. 7 Effects of sodium chloride (NaCl) concentrations on the hyphal growth of isolates NJM 0801 and NJM 0803 cultured at 20°C for 7 days

Physiology

Vegetative growth of isolates NJM 0801 and NJM 0803 was robust at temperatures ranging from 10 to 25°C, with maximal growth at 20°C. At temperatures >25°C, mycelial growth immediately declined, and there was no growth at 30°C (Fig. 6).

Isolates NJM 0801 and NJM 0803 grew well on GY agar supplemented with 0.5% NaCl, and they grew slowly on the media containing 1.0% or 2.0% NaCl (Fig. 7). Isolates NJM 0801 and NJM 0803 did not grow on GY agar of 3% and 5%, respectively.

Phylogenetic analysis

The sequences were submitted to the DNA database through DDBJ (DNA Data Bank of Japan, Mishima, Shizuoka, Japan). The accession numbers of all five strains, NJM 0801, 0802, 0803, 0804, and 0805, were AB510348, AB510349, AB510350, AB510351, and AB510352, respectively. All five isolates, NJM 0801–0805, formed an independent cluster in the phylogenetic analysis. This cluster had a significant boot strap value at 100% from the related species *A. astaci* (Fig. 8).

Discussion

We confirmed that a February 2008 outbreak of cotton-like lesions in the ice fish was caused by *Aphanomyces* sp. based on clinical, histopathological, morphological, physiological, and molecular biological data. As numerous Gram-negative short rods were observed in these lesions, it was suggested that the outbreak was caused by simultaneous infections of bacteria and *Aphanomyces* sp. Nevertheless, the fish tissues were severely damaged by the mycelial penetrations, indicating that the primary causal agent for the disease was *Aphanomyces* but not the bacteria. Interestingly the clinical symptoms associated with this outbreak were different from those resulting from *A. invadans*, the causal agent of EGA in menhaden (Dykstra et al. 1986; Noga and Dykstra 1986; Johnson et al. 2004). The outbreak we studied was a water-mold disease, whereas the disease caused by *A. invadans* results in skin ulcers known as eEUS and affects a wide range of freshwater and brackish-water animals (Baldoc et al. 2005). The lesions observed in the ice fish were associated with a minimal granulomatous reaction when inspected using microscopy. Nevertheless, hyphae did penetrate the skin, muscles, and bone, indicating significant damage to the hosts. The most striking characteristic of this disease is mycelial growth into the host tissue. In contrast, *A. invadans* infections formed mycotic granuloma in menhaden (Noga and Dykstra 1986) and ayu (Miyazaki and Egusa 1973; Hatai 1980).

In March, the ice fish were in the terminal phase of their life because of their 1-year life span, and might have been fatigued, had weakened host defense mechanism, were aged, and susceptible to various opportunistic pathogens. Therefore, the ice fish have been infected with new *Aphanomyces* sp. pathogen. Two primary factors, host—parasite specificity and compromised host condition—might be correlated to the simultaneous infection of the *Aphanomyces* sp. as the outbreak.

As *A. stellatus* and *A. laevis* are reported as fish pathogens and produce similar sexual reproductive organs (Johnson et al. 2002), we morphologically compared our isolates with the two species (Table 4). Superficial structures of the spherical oogonia, with irregular short papillate projections of the NJM isolates, resembled those of *A. stellatus*, and this papillate surface of oogonia was different from the smooth surface of *A. laevis*. In contrast, the straight oogonial stalks

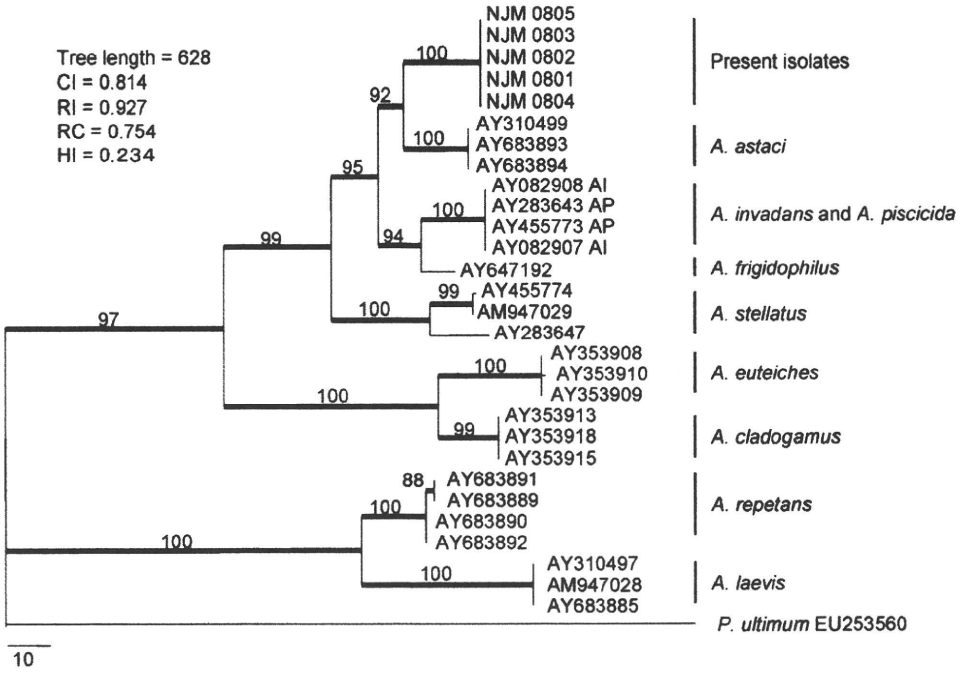

Fig. 8 Phylogenetic tree of *Aphanomyces* including isolates NJM 0801–0805. One of the 52 most parsimonious trees obtained from heuristic searches based on 659 base pair of internal transcribed spacer (ITS) 1-5.8S-ITS 2 rDNA sequence. Clusters were supported by bootstrap values >88%. The *bar* indicates ten base differences. Data are shown with accession number and fungal species. EU253560 derived from *Pythium ultimum* was used as an outgroup sequence. *CI* consistency index, *RI* retention index, *RC* rescaled consistency index, *HI* homoplasy index

Table 4 Comparison of morphological feature between isolate *Aphanomyces* sp. NJM 0801 and *Aphanomyces laevis* and *A. stellatus* showing closer morphological similarities

	Aphanomyces sp. NJM 0801	*A. laevis*[a]	*A. stellatus*[a]
Oogonia wall ornamentation	Irregular short papillate	Smooth	Irregular short papillate
Basal portion of the oogonium	Broad	Broad	Tapered
Many antheridial branch formations	Androgynous	Monoclinous and diclinous	Monoclinous and diclinous

[a] Johnson et al. (2002)

of the isolates were more similar to the straight stalks of *A. laevis* than to the tapered oogonial stalks of *A. stellatus*. Finally, the antheridial branches of *A. laevis* and *A. stellatus* were more similar to one another than to those of NJM isolates 0801–0805. Specifically, the origin of the antheridial branch of the isolates was different from those of *A. laevis* and *A. stellatus* (Table 4). As *A. astaci* is a pathogen of crayfish, not fish, and does not produce sexual organs, we did not compared it with our isolates.

The optimal growth temperature for NJM isolates 0801 and 0803 was 20°C, and growth rate decreased when the temperature was raised to 25°C. Isolate NJM 0803 was able to grow at 5°C, indicating that the effects of low temperature on growth were mild when compared with the effects of higher temperatures. Furthermore, the growth rates of isolates 0801 and 0803 increased progressively with the incremental increases in temperature from 10°C to 20°C (Fig. 5). Optimal growth temperatures of NJM isolates may have been significant in the onset of the outbreak. The water temperature in the aquarium during the outbreak was 13.4°C, whereas the water temperature of Lake Shinji, when measure on 6 February 2009, was 8.2°C. These observations suggest that the nursing environments of the ice fish in the aquarium were suitable for aggressive growth of the isolated *Aphanomyces* sp., whereas the temperature of Lake Shinji was too low for aggressive growth. Therefore, no outbreak of this fungal species has ever been recorded in Lake Shinji. Water temperature control may be an effective strategy to prevent fungal outbreaks in ice fish housed in tanks.

Future studies are necessary to determine the natural habitat of the *Aphanomyces* sp. responsible for the disease outbreak in these ice fish. We propose several possibilities: (1) Ice fish were infected with the fungi while in the lake before capture, and the disease state emerged because of some stress. (2) The species were introduced from the sand-filtered lake water used during the exhibition. (3) Fish were infected after feeding on brine shrimp harboring the species. (4) Fungi were introduced from some other unidentified source.

The optimal growth temperatures for other *Aphanomyces* spp. have been established. *A. astaci* grows best at temperatures ranging from 20 to 25°C and halt growth at temperatures of 30°C and above (Unestam 1965). In

Table 5 Comparison of identities between the present isolate *Aphanomyces* sp. NJM 0801 and the other *Aphanomyces* spp.

	Species	Accession no.	bps	1	2	3	4	5	6	7	8	9	10	11	12
1	This isolate	AB510348	659												
2	*A. astaci*	AY683894	663	93.5											
3	*A. cladogamus*	AY353918	655	79.2	80.4										
4	*A. euteiches*	AY353908	651	79.5	80.8	92.8									
5	*A. frigidophilus*	AY647192	650	92.9	93.4	82.5	82.4								
6	*A. invadans*	AY082907	634	91.3	92.4	81.9	81.9	96.5							
7	*A. laevis*	AY683885	657	78.3	78.0	76.5	75.9	79.5	79.4						
8	*A. piscicida*	AY283643	648	90.9	91.4	81.8	81.8	96.2	99.7	79.6					
9	*A. repetans*	AY683889	655	77.0	76.9	75.8	75.6	79.1	77.8	90.6	78.0				
10	*A. repetans*	AY683890	654	76.2	75.7	75.3	75.0	78.1	77.5	90.2	77.6	98.6			
11	*A. stellatus*	AY283647	661	89.8	89.3	80.4	80.3	90.2	89.8	77.6	89.7	77.6	76.5		
12	*A. stellatus*	AY455774	662	89.6	90.1	79.9	79.0	91.1	90.1	77.8	90.1	77.2	76.2	95.1	
13	*P. ultimum*	EU253560	684	62.2	62.4	60.8	60.8	62.1	61.9	61.1	60.7	61.1	59.8	60.7	63.3

contrast, *A. frigidophilus* NJM 9500 has a narrow optimal growth temperature of 25°C, and this fungal species stops growing at temperature immediately above 25°C (Kitancharoen and Hatai 1997). Sinmuk et al. (1996) reported that optimal growth temperature of *A. invadans* NJM 8997 isolated from ayu was also 30°C, whereas *Aphanomyces* sp. NJM 9525 isolated from juvenile soft-shelled turtles, *Pelodiscus senensis*, was the same as that of *A. frigidophilus*. Interestingly, isolates NJM 0801 and 0803 showed maximum growth rates on GY medium supplemented with 0.5% NaCl, suggesting they might be accustomed to brackish water. *A. piscicida* isolated from ayu and *Aphanomyces* sp. isolated from dwarf gourami cannot grow on GY agar with 1.5% NaCl (Hatai et al. 1994). In contrast, the *Aphamnomyces* sp. isolated in this study were able to grow, albeit slowly, on media with 1% and 2% NaCl, and they grew well on 0.5% NaCl, suggesting that the isolates were adapted to blackish conditions. *A. invadans* also grow in brackish water but not at high NaCl concentration. The morphological differences between isolates NJM 0801–0805 and *A. invadans*, including the absence of sexual structure in *A. invadans*, suggest that these are separate species.

According to Diéguez-Uribeondo et al. (2009), Scott (1961), and Johnson et al. (2002), *Aphanomyces* taxa correspond to the host taxa. For example, all *Aphanomyces* spp. that parasitize animals are more closely related to one another than they are to any species that does not parasitize an animal host. The isolates NJM 0801–0805 are limited to fish hosts. According to Scott (1961), Dick (2001), and Johnson et al. (2002), *Aphanomyces* spp. occur in very different ecological niches and include host-specific plant or animal parasites and saprotrophic species growing on decaying animal and plant debris. The host specificities of *Aphanomyces* spp. most closely related to isolates NJM 0801–0805 were as follows: *A. astaci* parasitize crayfish, *A. invadance* and *A. piscida* parasitize fish, *A. frigidophilus* parasitize crayfish and/or fish egg, and *A. stellatus* live on fish and in soil. The sequence identities based on 659 base pairs of the ITS rDNA for isolates NJM 0801–0805 were 93.5% (*A. astaci*), 92.9% (*A. frigidophilus*), 91.3% (*A. invadans*), 89.6% or 89.8% (*A. stellatus*), 79.5% (*A. euteiches*), 79.2% (*A. cladogamus*), 78.3% (*A. laevis*), 77.0% (*A. repetans*), and 62.2% (*Pithium ultimum*) (Table 5). The species was consequently regarded to represent a hitherto undescribed taxon based on morphology, physiology, and molecular biology.

Isolates NJM 0801–0805 formed an independent phylogenetic cluster separated from closely related *Aphanomyces* spp., all of which are isolated from the other sources. The bootstrap value of the cluster consisted of NJM 0801–0805 in this analysis also supported the observation that the isolates might belong to a separate, as-yet unknown, species of *Aphanomyces*. As mentioned above, this isolate was identified as a new species and new name is proposed in the genus *Aphanomyces*—*A. salsuginosus*—which was also confirmed as the pathogen causing the disease outbreak in the ice fish. The new name of *A. salsuginosus* has been registered with the Mycobank under the accession number MB 516781.

Aphanomyces salsuginosus sp. nov. Takuma, Hatai & A. Sano Figs. 4, 5

Mycelium aseptatum, subtile, 5–10 μm diametro, laeve, leviter undulatum, modice ramosum; zoosporangia isodiametra diam hyphae aequantia; zoosporae prope orificio emergentes et incystatae, conglobatae in globum; oogonia sphaero vel subsphaerica raro pyriformia, 21–33 μm diametro, oospore singulars, 19–27 μm diametro.

A congeneribus sequentiis nucleotidi distinctus, a specie proxima *A. astraci* 93.5 per centum identitati regionis ITS.

Holotypus: NJM 0801, colonia exsiccata ex cultura ex rostro *Salangichitys microdon* aqualio, Izumo, Shimane Pref., Japoniaia, 18-II-2008 a D. Takuma isolata, in collectione culturae Universitatis Scientiae Veterinariae et Animalis Nipponensis (NJM) conservata.

Type specimen: On rostrum of ice fish *Salangichitys microdon* on 18 Feb. 2008, the aquarium, Izumo-shi, Shimane prefecture, Japan, collected by D. Takuma (ex-type culture NJM 0801 = NBRC 106578). Figures 4 and 5 showing that the strain NJM 0801 is designated as the holptype because there are technical difficulties in preserving the type specimen: i.e., during slide preparation of hyphae with zoospore and sexual reproductive organs, their characteristic structures of this species are easily destroyed.

The vegetative mycelium was delicate, about 5–10 μm in diameter, aseptate, smooth, slightly wavy, moderately branched. Zoosporangia were slender and the same diameter as hyphae, namely, isodiametric; primary encysted zoospores were produced in a single row within zoosporangium and were encysted in a cluster at the top of the zoosporangium. Primary zoospores were about 8–11 μm in diameter. Oogonia were abundant, usually spherical or subspherical, rarely pyriform, from 21 to 33 μm in diameter with irregular short papillate, even in young oogonia. Pits were not found on the oogonial wall. Singly sprouted oospores were dominantly spherical, from 19 to 27 μm in diameter. Oogonial stalks were unbranched. Antheridial cells were simple, vermiform, often irregular. Antheridial branches, when present, were dominantly androgynous, infrequently monoclinous, and diclinous. Single antheridium was dominant; however, there were a few double or triple ones. The sequence identity based on 659 base pairs between the present isolates and *A. astaci*, closest species, was 93.5%.

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References

Abliz P, Fukushima K, Takizawa K, Nishimura K (2004) Identification of pathogenic dematiaceous fungi and related taxa based on large subunit ribosomal DNA D1/D2 domain sequence analysis. FEMS Immunol Med Microbiol 40:41–49

Andrew TG, Huchzermeyer KD, Mbeha BC, Nengu SM (2008) Epizootic ulcerative syndrome affecting fish in the Zambezi river system in southern Africa. Vet Rec 163:629–631

Baldoc FC, Blazer V, Callinan R, Hatai K, Karunasagar I, Mohan CV, Bondad-Reantaso MG (2005) Outcomes of a short expert consultation on epizootic ulcerative syndrome (EUS): reexamination of causal factors, case definition and nomenclature. In: Walker P, Bondad-Reantaso MG (eds) Diseases in Asian aquaculture V. Fish Health Section, Asian Fisheries Society, Manila, pp 555–585

Ballesteros I, Martín MP, Diéguez-Uribeondo J (2006) First isolation of *Aphanomyces frigidophilus* (Saprolegniales) in Europe. Mycotaxon 95:335–340

Dick MW (2001) The saprolegniales. In: Straminipilous fungi: systematics of the Peronosporomycetes including accounts of the marine Straminipilous Protists, the Plasmodiophorids and similar organisms. Kluwer Academic Publisher. Dordrecht, pp 160–165

Diéguez-Uribeondo J, García MA, Cerenius L, Kozubíková E, Ballesteros I, Windels C, Weiland J, Kator H, Söderhäll K, Martín MP (2009) Phylogenetic relationships among plant and animal parasites, and saprotrophs in *Aphanomyces* (Oomycetes). Fungal Genet Biol 46:365–376

Dykstra MJ, Noga EJ, Levine JF, Moye DW (1986) Characterization of the *Aphanomyces* species involved with ulcerative mycosis (UM) in menhaden. Mycologia 78:664–672

Fraser GC, Millar SD, Calder LM (1992) *Aphanomyces* species associated with red spot disease: an ulcerative disease of estuarine fish from eastern Australia. J Fish Dis 15:173–181

Harada Y, Kuwamura K, Kinoshita I, Tanaka M, Tagawa M (2005) Histological observation of the pituitary-thyroid axis of a neotenic fish (the ice fish, *Salangichtys microdon*). Fish Sci 71:115–121

Hatai K (1980) Studies on pathogenic agents of saprolegniasis in fresh water fishes. Spec Rep Nagasaki Pref Inst Fish 8:1–95

Hatai K (1989) Fungal pathogens/parasites of aquatic animals. In: Austin B, Austin DA (eds) Methods for the microbiological examination of fish and shellfish. Ellis Horwood Ltd., West Sussex, pp 240–272

Hatai K, Nakamura K, Rha SA, Yuasa K, Wada S (1994) *Aphanomyces* infection in Dwarf Gourami (*Colisa lalia*). Fish Pathol 29:95–99

Hendrickson DA (1985) Reagents and stains. In: Lennette EH (ed) Manual of clinical microbiology, 4th edn. American Society for Microbiology, Washington, pp 1093–1107

Hoshina T, Sano T, Sunayama M (1960) Studies on the saprolegniasis of eel. J Tokyo Univ Fish 47:59–79

Jeanmougin F, Thomson JD, Gouy M, Higgins DG, Gibson TJ (1998) Multiple sequence alignment with Clustal X. Trend Biochem Sci 23:403–405

Johnson TW Jr, Seymour RL, Padgett DE (2002) Biology and the systematics of the Saprolegniaceae. http://dl.uncw.edu/digilib/biology/fungi/taxonomy%20and%20systematics/padgett%20book/

Johnson RA, Zabrecky J, Kiryu Y, Shields JD (2004) Infection experiments with *Aphanomyces invadans* in four species of estuarine fish. J Fish Dis 27:287–295

Kitancharoen N, Hatai K (1997) *Aphanomyces frigidophilus* sp nov. from eggs of Japanese char, Salvelinus leucomaens. Mycoscience. Mycoscience 38:135–140

Kurtzman CP, Robnett CJ (1997) Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 50 end of the large-subunit (26S) ribosomal DNA gene. J Clin Microbiol 35:1216–1223

Levenfors JP, Fatehi J (2004) Molecular characterization of *Aphanomyces* species associated with legumes. Mycol Res 108:682–689

Lilley JH, Callinan RB, Chinabut S, Kanchanakhan S, MacRae IH, Phillips MJ (1998) Epizootic ulcerative syndrome (EUS) technical handbook. Aquatic Animal Health Research Institute, Department of fisheries, Bangkok

Miyazaki T, Egusa S (1973) Studies on mycotic granulomatosis in fresh water fishes-II Mycotic granulomatosis in ayu, Plecoglossus. Fish Pathol 7:25–133

Noga EJ, Dykstra MJ (1986) Oomycete fungi associated with ulcerative mycosis in menhaden, *Brevoortia tyrannus* (Latrobe). J Fish Dis 9:47–53

Oidtmann B, Schaefers N, Cerenius L, Soderhall K, Hoffmann RW (2004) Detection of genomic DNA of the crayfish plague fungus *Aphanomyces astaci* (Oomycete) in clinical samples by PCR. Vet Microbiol 100:269–282

Royo F, Andersson G, Bangyeekhun E, Muzquiz JL, Soderhall K, Cerenius L (2004) Physiological and genetic characterisation of some new *Aphanomyces* strains isolated from freshwater crayfish. Vet Microbiol 104:103–112

Scott WW (1961) A monograph of the genus *Aphanomyces*. Virginia Agri Exp Stat Tech Bull 151

Shah KL, Jha BC, Jhingran AG (1977) Observations on some aquatic phycomycetes pathogenic to eggs and fry of freshwater fish and prawn. Aquaculture 12:141–147

Shanor L, Saslow HB (1944) *Aphanomyces* as a fish parasite. Mycologia 36:413–415

Sinmuk S, Suda H, Hatai K (1996) *Aphanomyces* infection in juvenile soft-shelled turtle, *Pelodiscus sinensis*, imported from Singapore. Mycoscience 37:249–254

Sparrow FK (1960) Aquatic Phycomycetes, 2nd edn. Univ. Michigan Press, Ann Arbor

Srivastava GC, Srivastava RC (1976) A note on the destruction of the eggs of *Cyprinus carpio* var. Communis by the members of Saprolegniaceae. Sci Cult 42:612–614

Swofford DL (2001) PAUP phylogenetic analysis using parsimony (and other methods) (version 4.0) Sinauer Associates, Sunderland

Unestam T (1965) Studies on the crayfish plague fungus *Aphanomyces astaci* Part I. Some factors affecting growth in vitro. Physiol Plant 18:483–505

Unestem T (1972) On the host range and origin of the crayfish plague fungus. Rep Inst Freshw Res Drottningholm 52:192–198

Vralstad T, Knutsen AK, Tengs T, Holst-Jensen A (2009) A quantitative TaqMan^R MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. Vet Microbiol 137:145–155

Willoughby LG, Robert RJ (1994) Improved methodology for isolation of the *Aphanomyces* fungal pathogen of epizootic ulcerative syndrome (EUS) in Asian fish. J Fish Dis 17:237–248

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Ochroconis calidifluminalis, a Sibling of the Neurotropic Pathogen *O. gallopava*, Isolated from Hot Spring

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Abstract Two strains resembling the neurotropic fungus *Ochroconis gallopava* were isolated from hot spring river water (IFM 54738 and IFM 54739). The isolates showed optimal growth at 42°C, while the maximum growth temperature was 49°C, thus having temperature relationships similar to those of *O. gallopava*. Colonies were light olive green, with a color change to dark reddish brown after several passages, which was also observed in *O. gallopava*. Conidia were indistinguishable from those of *O. gallopava*. The antifungal susceptibility profile of the isolates was also similar to that of *O. gallopava*, except for a lower susceptibility to micafungin. The two isolates had 100% homologous rRNA genes including the internal transcribed spacer (ITS) region and the D1/D2 region of the large subunit. The gene fragments, as *O. gallopava*, could be amplified with species-specific rDNA primers, and loop-mediated isothermal amplification designed for *O. gallopava* yielded positive results in the two isolates. However, homologies with *O. gallopava* in ITS and D1/D2 regions were 79.2 and 95.9%, respectively, widely exceeding generally accepted species boundaries. These differences were corroborated in virulence tested in experimental infection. The two isolates did not kill a mouse even until 28 days. However, mortalities of four *O. gallopava* strains ranged from 40 to 100%. The new isolates mainly affected the kidneys; whereas *O. gallopava* had a strong preference for the brain. We therefore propose a new species, *Ochroconis calidifluminalis*, for the two isolates.

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Abbreviations

5-FC	Flucytosine
AMB	Amphotericin B
CBS	CBS-KNAW Fungal Biodiversity Centre (Centraalbureau voor Schimmelcultures)
CMA	Cornmeal agar
DDBJ	Center for Information Biology and DNA Data Bank of Japan
FCZ	Fluconazole
ICZ	Itraconazole
ITS	Internal transcribed spacer
LAMP	Loop-mediated isothermal amplification
LSU	Large subunit
MCF	Micafungin
MCZ	Miconazole
MIC	Minimal inhibitory concentration
MOPS	3-(N-Morpholino) propanesulfonic acid
OA	Oatmeal agar
PDA	Potato dextrose agar
SEM	Scanning electron microscope
SSU	Small subunit
VCZ	Voriconazole

Introduction

Ochroconis gallopava is a melanized fungus potentially causing cerebral infections in warm-blooded animals, including birds and humans, occasionally regardless of the host's immune status [1]. Between 1986 and April 2009, 44 human cases were reported, with a high proportion being detected in organ transplant recipients [2–5]. The environmental niche of *O. gallopava* comprises low pH and high temperature habitats worldwide, particularly hot springs [2]. Hot spring bathing is popular in Japan, not only for recreational purposes but also for the treatment of chronic diseases. We recently isolated four strains of the species from hot spring water in Japan [2]. They were found to be mortally virulent to experimentally infected immunocompetent mice, with death rates of 40–100%. This finding suggests that caution is needed while using hot spring facilities.

Together with the *O. gallopava* isolates, we simultaneously obtained two morphologically similar, equally thermotolerant, melanized fungi from the same water sample, a hot spring river water. In this study, we reported on the morphology, physiology, and molecular biology of these two isolates. Furthermore, we tested their susceptibility to antifungal agents and established the virulence of these isolates in intravenously infected normal and corticosteroid-treated mice.

Materials and Methods

Isolation

Hot spring water samples from one river and 14 bathtubs were collected at various spa towns in Japan, from 2004 to 2006 [2]. The samples were stored at 4°C for 2 days, then 500 ml each of them was filtered with 0.22-μm-pore-sized filter. The filters were placed on potato dextrose agar (PDA) plates and cultured at 42°C for 2 weeks. Olive-green to brownish-green and/or black-brown colonies were transferred to PDA slants and maintained at room temperature during the course of the experiments.

Mycology

The second transfer isolates were used for the following studies. Macrocultures were observed on PDA plates at 25, 37, and 42°C, and microcultures on cornmeal agar (CMA) plates at 25°C for 4 weeks. Maximum growth temperatures were determined with PDA slants up to 51°C. Gelatin liquefaction was determined at 35°C on gelatin slants [6] containing 0.1% yeast extract. Sensitivity to cycloheximide was tested on PDA plates containing 0.05% cycloheximide. In addition, the size, structure, and surface structure of conidia were observed on PDA, CMA, and oatmeal agar (OA) plates with light and scanning electron microscopy (SEM). Mean lengths and maximum widths of apical cells from 30 conidia were calculated for each culture.

Molecular Biology

A routine method was used to generate the partial sequence of the small subunit (SSU), the complete sequence of the internal transcribed spacer (ITS)

region, and the partial sequence of the large subunit (LSU) including the D1/D2 region of the rRNA gene [2, 7]. The sequences were compared with the GenBank database and with a dedicated database maintained at the CBS that included all available strains of *Ochroconis* and *Scolecobasidium* [8, 9]. In addition, PCR banding patterns of the isolates were compared with those of *O. gallopava* reference strains using a species-specific PCR primer set and a species-specific loop-mediated isothermal amplification (LAMP) method designed for *O. gallopava* [10].

Antifungal Susceptibility Testing

Testing was performed according to the broth microdilution modified method of the Clinical and Laboratory Standards Institute (CLSI) M38-A2 standard [11]. Two commercial kits: Dryplate (Eiken, Tokyo, Japan) and ASTY (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan), with RPMI 1640 medium (GIBCO, Invitrogen Corporation, Carlsbad, USA) buffered to pH 7.0 with MOPS (Dojindo Laboratories, Kumamoto, Japan), were used. The two kits included the following antifungal agents: amphotericin B (AMB), flucytosine (5-FC), fluconazole (FCZ), miconazole (MCZ), itraconazole (ICZ), voriconazole (VCZ), and micafungin (MCF). As a control, six *O. gallopava* strains chosen among hot spring isolates from Japan and clinical isolates from Japan and the U.S.A. were tested simultaneously.

Conidial suspensions were obtained from 4-week-old cultures on OA which were inoculated with 10^4 conidia/ml in RPMI 1640 medium on microdilution plates. Readings was done after 48 h of incubation at 37°C. The minimal inhibitory concentrations (MICs) for AMB, ICZ, and VCZ were read visually as the lowest drug concentration that prevented any discernible growth (IC_{100}). For MCF, this was determined as the concentration required for 80% growth inhibition compared with the drug-free control (IC_{80}). For the remaining antifungal agents, the MICs were determined as the concentration required for 50% growth inhibition (IC_{50}) and IC_{80}.

Experimental Infection

Twenty genetically identical 5-week-old male ddY mice (Nihon SLC, Shizuoka, Japan) were housed at 25 ± 1°C with 55 ± 5% humidity. They were provided with clean drinking water ad libitum and fed a commercial chow (Nihon CLEA, Tokyo). The mice were divided into four groups of five mice each: 8−; 8+; 9−; and 9+. Mice from groups 8+ and 9+ were subcutaneously injected with 150 mg/kg of body weight of hydrocortisone (Hydrocortone; Banyu Pharmaceutical, Merck, Tokyo, Japan) at 1, 3, 5, and 7 days before and 1, 3, 5, and 7 days after inoculation with conidia. Mice from groups 8− and 9− were not treated with hydrocortisone. The conidial suspensions were prepared with sterilized physiological saline under sterile conditions in the same manner as that used for the antifungal susceptibility tests. Mice from groups 8− and 8+ were inoculated with 5×10^5 IFM 54738 conidia/10 g of body weight intravenously at 6 weeks of age; mice from groups 9− and 9+ were inoculated with the same amount of IFM 54739 conidia. A control group of four mice received hydrocortisone without fungal inoculation. Body weights, behavioral changes, and survival rates of all mice were recorded up to 28 days after the fungal inoculation. On day 28, survived mice were killed by ether anesthesia.

The livers, kidneys, spleens, hearts, lungs, and brains of all mice were macroscopically examined. The organs were cut into pieces approximately $5 \times 5 \times 5$ mm^3, placed the pieces on PDA plates, and cultured the plates at 37°C for 2 weeks. Fungal sprouts from each organ were noted. Virulence scores were recorded as recovery ratio of each fungal strain from cultivated organs in percentage, being calculated as the number of mice with fungal-positive organs per total mice in each group [12].

The remaining organs were fixed in buffered 10% formalin, processed by routine histopathological methods (including H&E and PAS staining) and observed under a light microscope. The animal experiments complied with all relevant guidelines and policies of the Animal Welfare Committee of the Faculty of Medicine of Chiba University, Japan.

Results

The two *Ochroconis gallopava*-like isolates were obtained from one of the water samples, a hot spring river water. The sampling point was located at latitude 35°20′ north and longitude 139°06′ in Kanagawa Prefecture, Japan, where is the closest spa resort town to the Tokyo metropolitan area. The

water temperature was 41–42°C, and the pH values were in the range of 5.6–5.8. The two isolates were deposited in the culture collections of the Medical Mycology Research Center, Chiba University, Japan, and the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, as IFM 54738 = CBS 125818 and IFM 54739 = CBS 125817, respectively. From the same water sample, an *O. gallopava* isolate, IFM 54736, was also obtained. From the bathtub samples, no *O. gallopava*-like isolate but three *O. gallopava* isolates were obtained [2].

Mycology

Colonies of the two *Ochroconis gallopava*-like isolates on PDA were floccose, light olive green, corrugate (IFM 54738) or crateriform (IFM54739) at the surface, and dark brown on the reverse side of the plates at 37 and 42°C for 7 days. Both isolates were olive green and produced a reddish pigment into the medium at 25°C (Fig. 1). In addition, the colonies became felty, dry, and brownish-black and produced reddish-brown pigment into PDA slants after four passages, at intervals of 6 months.

The two *O. gallopava*-like isolates grew better at 42°C than at 37 and 25°C (Fig. 1). Their maximum growth temperature was 49°C. Both isolates did not liquefy gelatin and showed no growth on a medium supplemented with cycloheximide.

Hyphae of the two isolates were brown and with somewhat thickened walls. Both isolates produced two-celled, light or dark brown colored conidia with detectable hilums. The shape of the conidia was cylindrical to clavate, with or without constrictions at the septa. In isolate IFM 54738, the conidiophores were short, dark-brown, straight or flexuous with pronounced denticles, producing 1–5 conidia. The conidia were 13.2 (10.3–18.0) × 3.6 (2.2–4.5) μm in size on PDA or CMA plates, and 12.5 (9.5–20.5) × 3.5 (2.5–5.0) μm on OA plates; they showed a smooth surface structure under SEM (Fig. 2). The conidiophores were lost after 3 years of preservation at room temperature (Fig. 2c). In isolate IFM 54739, the conidiophores were hypha-like, not well differentiated, and produced a few conidia only on PDA. Conidiogenesis was lost after 3 years. The conidia were 11.4 (8.8–20.0) × 2.5 μm on OA.

Molecular Biology

The concatenated sequences of the two *O. gallopava*-like isolates from the partial SSU to the D1/D2 region of the LSU consisting of 1696 bps were 100% identical. Their homologies with *O. gallopava* at the

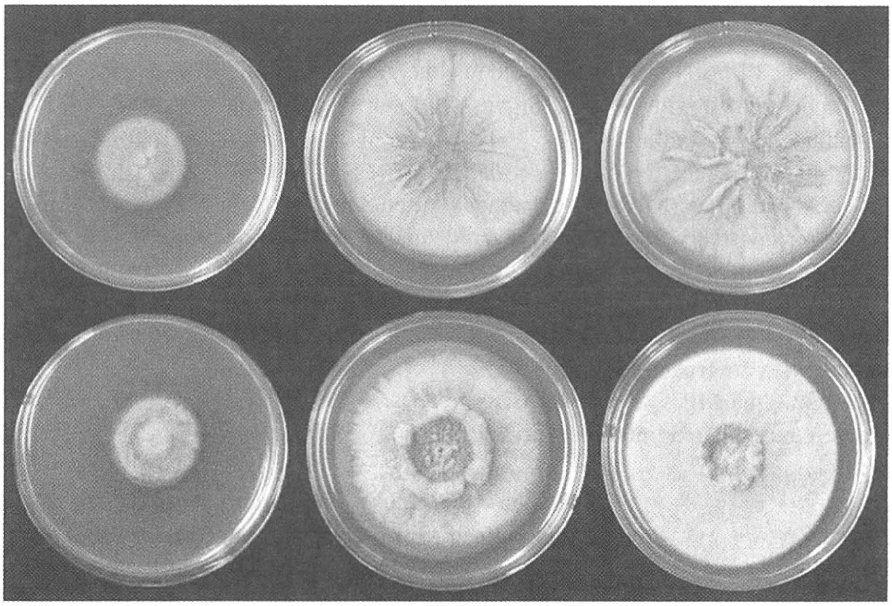

Fig. 1 *Ochroconis calidifluminalis* IFM 54738 (*upper*) and IFM 54739 (*lower*) cultured at 25°C (*left*), 37°C (*center*), and 42°C (*right*) for 7 days on PDA

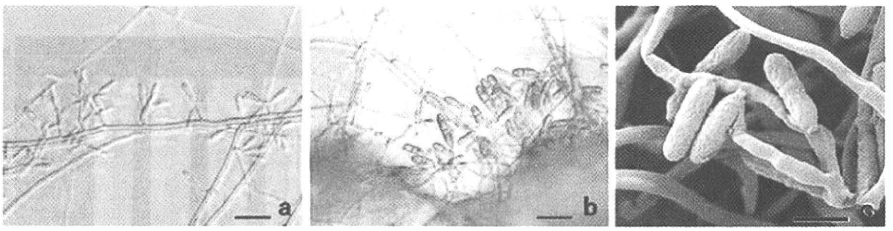

Fig. 2 Conidia of *Ochroconis calidifluminalis* IFM 54738. **a**, **b** Conidia formation on cornmeal agar at 25°C for 4 weeks, lactophenol fixation, ×600; the *bars* indicate 10 μm. **c** SEM picture image of conidia on OA at 35°C for 5 weeks, ×5000; the *bar* indicates 5 μm. SEM image was taken by Integrated Imaging Research Support, Tokyo, Japan

ITS and D1/D2 regions was 79.2 and 95.9%, respectively. A phylogenetic analysis demonstrated that the two *O. gallopava*-like isolates constituted a sister species of *O. gallopava* (Fig. 3). The sequences were registered in the Center for Information Biology and DNA Data Bank of Japan (DDBJ, Mishima, Shizuoka Japan) as *Ochroconis* sp. with accession numbers AB385698 for IFM 54738 and AB385699 for IFM 54739.

PCR banding patterns of the *Ochroconis* sp. isolates and *O. gallopava* strains were very similar to each other and were indistinguishable by either the species-specific PCR primer set (Fig. 4a) or the species-specific LAMP method (Fig. 4b).

Antifungal Susceptibility Testing

Antifungal susceptibility profiles of the two *Ochroconis* sp. isolates and five *O. gallopava* strains are shown in Table 1. Range of MICs for the *Ochroconis* sp. isolates with AMB, 5-FC, FCZ, MCZ, ICZ, VCZ, and MCF was 0.25 μg/ml, 0.25–0.5 μg/ml, 2–16 μg/ml, 0.5–1 μg/ml, 0.03–0.25 μg/ml, 0.25 μg/ml, and 4 μg/ml, respectively. Those against *O. gallopava*

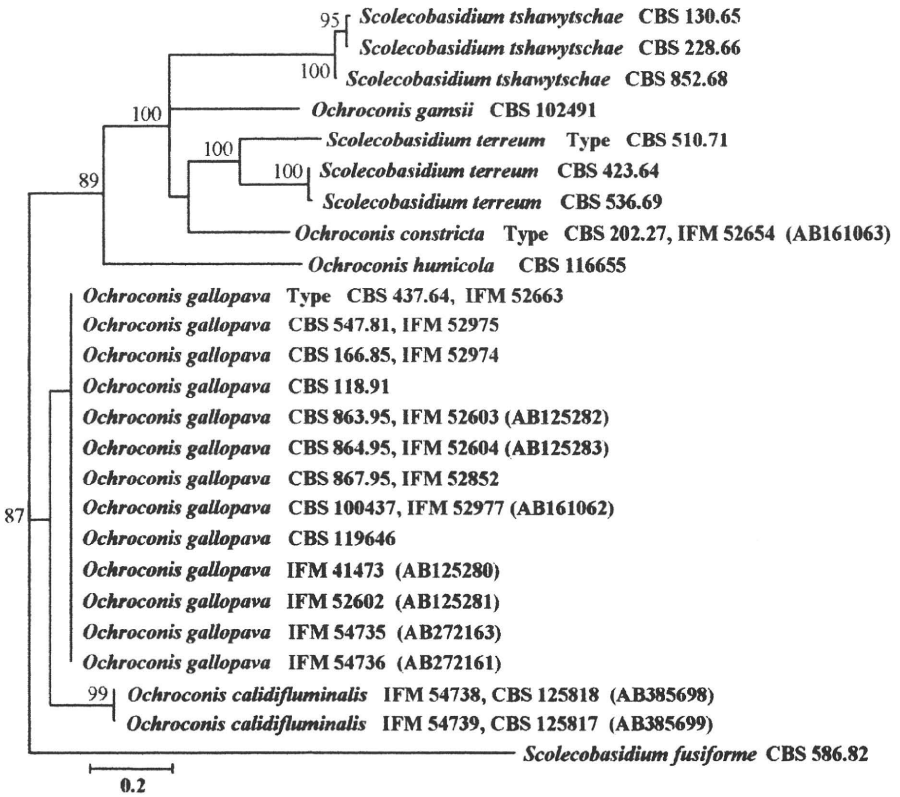

Fig. 3 Phylogenetic analysis of *Ochroconis calidifluminalis* based on ITS 1 - 5.8S - ITS 2 regions of rRNA gene sequences. (Accession numbers of GenBank)

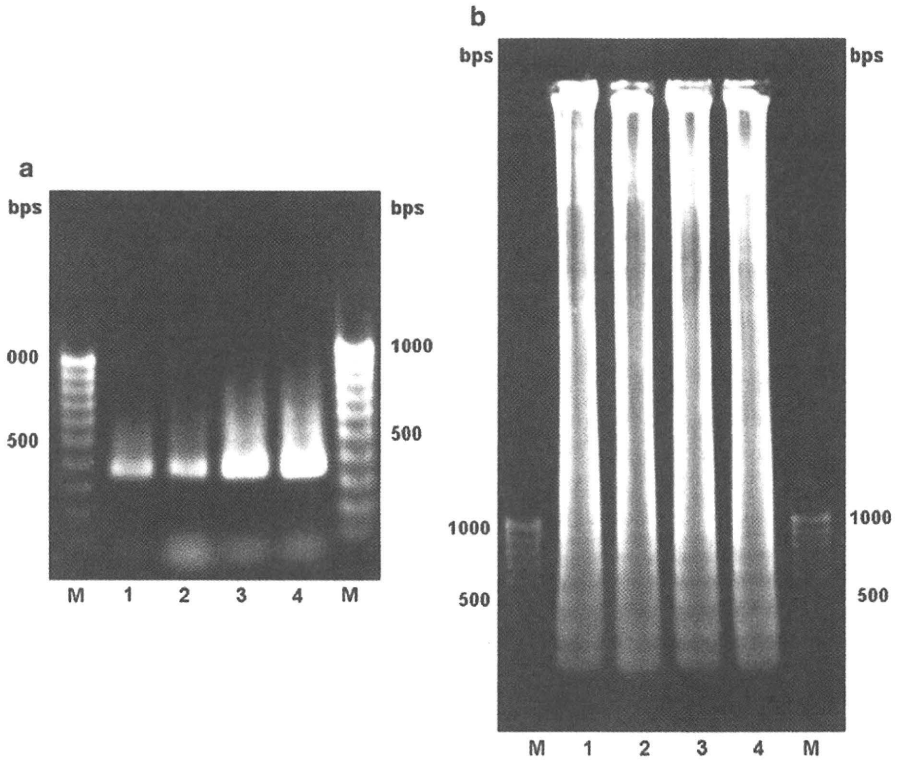

Fig. 4 PCR banding patterns amplified by two identification methods designed for *O. gallopava*, **a** the species-specific PCR primer set, **b** the species-specific LAMP method. 1: *Ochroconis calidifluminalis* IFM 54738, 2: *O. calidifluminalis* IFM 54739, 3: *O. gallopava* IFM 41473, 4: *O. gallopava* IFM 54736, M: Marker

strains were 0.25–1 μg/ml, 0.25–4 μg/ml, 8–64 μg/ml, 0.5–2 μg/ml, 0.25–1 μg/ml, 1–2 μg/ml, and ≤0.03 –0.125 μg/ml, respectively.

Experimental Infection

All mice infected with the *Ochroconis* sp. isolates survived during the observation period of 28 days. All mice from groups 8− and 8+ showed transient decreases in their body weight 1 week after infection, regardless of hydrocortisone treatment. In group 8+, one mouse showed a rotating movement. On the 16th day after inoculation, its head and body leaned to the right. At day 19, the occasional rotating movement started and continued until the end of the experiment at day 28. When we picked up the animal from the cage, it also showed a tremor. At day 22, its head became swollen and the right eye remained closed. Around this time, the mouse would lie still for several seconds after handling. Although its body weight was the lowest in group 8+, it was not significantly different (*t*-test). The body weights and behaviors of groups 9− and 9+ were not statistically different from those of the control mice, regardless of hydrocortisone treatment.

Range of the recovery ratios from six kinds of organs for all four groups of the *Ochroconis* sp. isolates were as follows: 0–60% from the liver or kidney; 20–80% from the lung; 20–100% from the spleen, heart, or brain (Table 2).

Marked macroscopic alterations were observed on the kidneys from groups 8− and 8+, regardless of hydrocortisone treatment. Barely visible sized dents or white spots on the surface of the kidneys were observed in 80 and 100% of kidneys from groups 8− and 8+, respectively. There was no macroscopically marked change in mice from groups 9− and 9+.

Remarkable histopathological changes included fungal colonization surrounded by polymorphonuclear leucocytes in the renal calyces and pelvises of mice from groups 8− and 8+, shown in 60% and 100% of mice, respectively. In addition, granulomatous lesions and cicatricial lesions without fungal elements were observed in the renal parenchyma of all mice, regardless of hydrocortisone treatment (Fig. 5). As for the kidneys of mice from groups 9− and 9+, small granulomatous lesions and cicatricial lesions without fungal elements appeared in 40% and 20% of mice, respectively. In the brains of infected mice, small granulomatous lesions were thinly scattered in

Table 1 Susceptibilities of *Ochroconis calidifluminalis* and *O. gallopava* strains to antifungal agents with the micro dilution method

Organism	IFM No.	Origin	MIC (μg/ml)						
			AMB IC_{100}	5-FC IC_{50} (IC_{80})	FCZ IC_{50} (IC_{80})	MCZ IC_{50} (IC_{80})	ICZ IC_{100}	VCZ IC_{100}	MCF IC_{80}
Ochroconis calidifluminalis	54738	Hot spring river	0.25	0.5 (1)	2 (4)	0.5 (1)	0.03	0.25[a]	4
O. calidifluminalis	54739	Hot spring river	0.25	0.25 (0.5)	16 (32)	1 (2)	0.25	ND	4
O. gallopava	54736	Hot spring river	1	1 (2)	8 (32)	0.5 (1)	0.25	ND	≤0.03
O. gallopava	54735	Hot spring bath	0.5	0.25 (0.5)	8 (16)	0.5 (1)	0.25	1[a]	≤0.03
O. gallopava	54737	Hot spring bath	1	2 (4)	32 (64)	2 (4)	0.5	2[a]	0.06
O. gallopava (Type)	52663	Turkey	0.25[a]	4 (16)[a]	64 (64)[a]	1 (4)[a]	1[a]	2[a]	0.125[a]
O. gallopava	41473	Human	0.25[a]	2 (16)[a]	64 (>64)[a]	1 (4)[a]	1[a]	2[a]	0.125[a]
O. gallopava	52602	Human	0.25[a]	2 (32)[a]	16 (64)[a]	0.5 (1)[a]	0.5[a]	2[a]	0.125[a]

Ochroconis calidifluminalis and *O. gallopava* were inoculated with 10^4 conidia/ml in RPMI 1640 medium and incubated for 48 h at 37°C using 2 commercial kits; the Dryplate and ASTY kits

The MICs were determined according to CLSI M38-A2

AMB: amphotericin B, 5-FC: flucytosine, FCZ: fluconazole, MCZ: miconazole, ITZ: itraconazole, VCZ: voriconazole, MCF: micafungin

IC_{100}: 100% inhibitory concentration, IC_{50}: 50% inhibitory concentration, IC_{80}: 80% inhibitory concentration

[a] The data were obtained with the ASTY kit

ND Not done

All isolates, except the type strain, were obtained in Japan

Table 2 Virulence of 2 *Ochroconis calidifluminalis* isolates from hot spring river water

Group name	Isolate	Hydro-cortisone	Virulence scores (%)[a]					
			Liver	Kidney	Spleen	Heart	Lung	Brain
8−	IFM 54738	−	60	60	100	80	80	100
8+	IFM 54738	+	20	40	60	100	40	40
9−	IFM 54739	−	0	0	40	20	20	20
9+	IFM 54739	+	20	0	20	40	20	40

The mice received 5×10^5 conidia/10 g of body weight suspended in sterilized normal saline intravenously at 6 weeks of age and were observed up to 28 days after the inoculation

[a] Virulence scores were calculated as the number of mice with fungal-positive organs per total mice in each group and are shown in percentage

20–40% of all four groups. Two mice, one each from groups 8+ and 9−, included a few filamentous cells within their brain lesions. There were no histopathological changes in the livers, spleens, hearts, or lungs of mice in any of the four groups.

Discussion

We isolated two thermotolerant isolates of an *Ochroconis* sp., IFM 54738 and IFM 54739, from hot spring river water flowing in a spa town in Japan; one strain of *O. gallopava*, IFM 54736, was isolated from the same sample [2]. The reason is not clear why this new entity was not isolated from any bathtub samples unlike *O. gallopava*. Morphological phenotypes of the two isolates were obviously different from each other but within the variation of those of *O. gallopava*. Their physiological characteristics were very close to those of *O. gallopava*. However, genetic analysis of the partial rRNA gene with *O. gallopava* suggested that the two isolates could not be identical to *O. gallopava* [9],

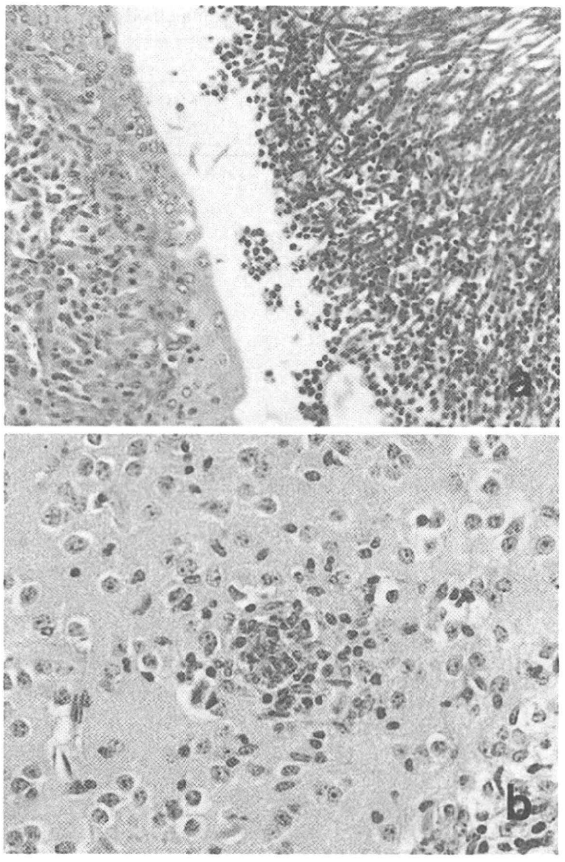

Fig. 5 Representative histopathology at day 28 in a mouse infected with *Ochroconis calidifluminalis* IFM 54738. **a** A hyphal mass and polymorphonuclear leukocytes in the renal calyx, and granulomatous reaction in the renal parenchyma, PAS, ×200. **b** A small lesion in the brain, PAS, ×400

despite indistinguishableness by PCR banding patterns using species-specific primers designed for *O. gallopava*.

The two *Ochroconis* sp. isolates from a hot spring river water resembled *O. gallopava* in terms of their morphological characteristics such as the olive green color of fresh isolates on PDA and the formation of 2-celled clavate conidia within the size range of *O. gallopava* [1, 2, 4, 13]. Colonies of fresh isolates of the *Ochroconis* sp. were lighter than those of fresh isolates of *O. gallopava*; however, differences disappeared after repeated transfers on artificial media. Their colony surfaces and thermotolerance characteristics were comparable with those of *O. gallopava* [1], showing excellent growth at 42°C and no growth at 50°C. Their gelatin liquefaction and tolerance to cycloheximide were also the same as those of *O. gallopava*. Therefore, it was impossible to distinguish the *Ochroconis* sp. isolates from *O. gallopava* on the basis of conventional morphology and physiology. We supposed that this indistinguishableness might be one of the reasons why this new entity had not been recognized up to now.

The two *Ochroconis* sp. isolates were 100% homologous in partial rRNA gene sequences including the ITS and D1/D2 regions. On the other hand, their homologies with *O. gallopava* at the both regions were 79.2 and 95.9%, respectively. We recently devised and published rapid identification methods of *O. gallopava* using two primer sets designed from sequence in the D1/D2 region [10]. However, the *Ochroconis* sp. and *O. gallopava* had similar primer-binding sites and equal lengths of amplified rRNA gene fragments. That is, there was only one bp difference each at the forward and reverse primer binding sites for a species-specific PCR primer set, OgF3 and OgB3, between the two species. As for LAMP primers, sequences of the two species at the forward and reverse primers were completely identical. Therefore, the two *Ochroconis* sp. isolates and *O. gallopava* were indistinguishable by that diagnostics.

The results of various tests for identification suggested that the two *Ochroconis* sp. isolates and *O. gallopava* are closely related, when compared to other species of the genera *Ochroconis* and *Scolecobasidium*. DNA homologies of the two species at the ITS regions was 79.2%. Separation of the two species using an ITS-based phylogenetic tree was robust with 96% bootstrap support (Fig. 3). The *Ochroconis* sp. and *O. gallopava* formed a monophyletic group. On the other hand, strains of the genera *Ochroconis* and its relative *Scolecobasidium* were located at different branches with significant bootstrap values. On the basis of these data, we propose the present *Ochroconis* sp. as a new species according to the concept of species outlined by Balajee et al. [9].

Susceptibilities to antifungal agents of the two *Ochroconis* sp. isolates were approximately equivalent to those previously reported for *O. gallopava* strains [1, 2, 4, 5, 14–22], except for MCF. MCF was not as effective against the *Ochroconis* sp. isolates, showing 4 μg/ml MIC, as against *O. gallopava* strains showing ≤0.03–0.125 μg/ml.

No mice died during observation period of experiment infection with either of the *Ochroconis* sp.

isolates regardless of hydrocortisone treatment, although one mouse inoculated with IFM 54738 and treated with hydrocortisone showed rotating movements that might suggest neurological lesions. In contrast, after inoculation with four *O. gallopava* strains isolated from hot springs, this behavioral change occurred in all mice, and the death rate ranged from 40% to 100% in intact mice [2]. The main lesions observed in mice infected with the *Ochroconis* sp. isolates were in the kidneys, but not as severe as those caused by *O. gallopava* infection. Moreover, the lesions by *O. gallopava* were primarily located in the brain. It is suggested that the strong neurotropism of *O. gallopava* strains caused high mortalities in contrast to the *Ochroconis* sp. isolates. There were slight differences in virulence between the two *Ochroconis* sp. isolates. Concerning the kidneys, mice inoculated with isolate IFM 54738 had severe lesions with massive hyphal growth surrounded by polymorphonuclear leucocytes in the renal pelvises. On the other hand, some of mice inoculated with isolate IFM 54739 had small granulomatous or cicatricial lesions without hyphae in the parenchyma; while the others had no lesions. As for the brain, there was no obvious difference between the two isolates.

Ochroconis calidifluminalis Yarita, Sano, de Hoog *et* Nishimura sp. nov.

Haec species nova *Ochroconidi gallopavae* proxima, difficiliter diversa secundum morphologicam vel physiologicam, sed distincta characteribus sequentibus nucleotiditis; ITS regione 79 per centum et D1/D2 regione 96 per centum homologa.

Status teleomorphosus ignotus.

Holotypus: IFM 54738; isolatus a K. Nishimura ex flumine de fonte calido, Hakone, Kanagawa Prefecture, Japan, Martius 2004, depositus in collectione Chiba University Medical Mycology Research Center, Japan.

Ochroconis calidifluminalis Yarita, Sano, de Hoog *et* Nishimura sp. nov.

The new species is closely related to *Ochroconis gallopava*, with identical morphological and physiological characteristics. This species differs from *O. gallopava* in its DNA sequence: 79% homologous for the ITS region and 96% homologous for the D1/D2 region of the ribosomal RNA gene.

Teleomorph unknown.

Holotype: IFM 54738; isolated by K. Nishimura from hot spring river water, Hakone, Kanagawa Prefecture, Japan, March 2004, and deposited in the collection of Medical Mycology Research Center, Chiba University, Japan.

Etymology: The name of this fungus is derived from the Latin words *calidus*, which means "warm" or "hot" and *fluminalis*, which means "of river" or "of stream."

We isolated a new species, *Ochroconis calidifluminalis*, together with an *O. gallopava* strain from a single water sample of a hot spring river in Hakone, Kanagawa Prefecture, Japan. The fact that these two species have drifted so widely apart in their ribosomal gene although occupied an identical slot in a highly specific ecosystem is remarkable from an evolutionary viewpoint. The new species showed lower virulence and neurotropism than *O. gallopava* even though the two species have identical temperature relations. Despite indefiniteness of its virulence owing to few strains existing, there is a possibility that the fungus may cause an opportunistic infection.

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References

1. de Hoog GS, Guarro J, Gené J, Figueras MJ. Atlas of clinical fungi. Utrecht, The Netherlands: Centraalbureau voor Schimmelcultures; 2000. p. 82–3.
2. Yarita K, Sano A, Murata Y, Takayama A, Takahashi Y, Takahashi H, et al. Pathogenicity of *Ochroconis gallopava* isolated from hot springs in Japan and a review of published reports. Mycopathologia. 2007;64:135–47.
3. Hollingsworth JW, Shofer S, Zaas A. Successful treatment of *Ochroconis gallopavum* infection in an immunocompetent host. Infection. 2007;35:367–9.
4. Shoham S, Pic-Aluas L, Taylor J, Cortez K, Rinaldi MG, Shea Y, et al. Walsh. Transplant-associated *Ochroconis gallopava* infections. Transpl Infect Dis. 2008;10:442–8.
5. Mayer N, Bastani B. A case of pulmonary cavitary lesion due to *Dactylaria constricta* var. *gallopava* in a renal transplant patient. Nephrol. 2009;14:262.
6. Al-Doory Y. Laboratory medical mycology. Philadelphia, USA: Lea & Febiger; 1980. p. 117.
7. Takahashi H, Takahashi-Kyuhachi H, Takahashi Y, Yarita K, Takayama A, Inomata T, et al. An intrafamilial transmission of *Arthroderma benhamiae* in Canadian porcupines (*Erethizon dorsatum*) in a Japanese zoo. Med Mycol. 2008;46:465–73.

8. Stamatakis A, Hoover P, Rougemont J. A rapid bootstrap algorithm for the RAxML web servers. Syst Biol. 2008;57:758–71.
9. Balajee SA, Borman AM, Brandt ME, Cano J, Cuenca-Estrella M, Dannaoui E, et al. Sequence-based identification of *Aspergillus*, *Fusarium*, and *Mucorales* species in the clinical mycology laboratory: where are we and where should we go from here? J Clin Microbiol. 2009;47: 877–84.
10. Ohori A, Endo S, Sano A, Yokoyama K, Yarita K, Yamaguchi M, et al. Rapid identification of *Ochroconis gallopava* by a loop-mediated isothermal amplification (LAMP) method. Vet Microbiol. 2006;14:359–65.
11. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi—second edition: Approved standard M38-A2. Wayne, Pennsylvania, USA: Clinical and Laboratory Standards Institute (CLSI); 2008.
12. Sano A, Miyaji M, Nishimura K, de Franco MF. Studies on the relationship between the pathogenicity of *Paracoccidioides brasiliensis* in mice and its growth rate under different oxygen atmospheres. Mycopathologia. 1991;114: 93–101.
13. Dixon DM, Salkin I. Morphologic and physiologic studies of three dematiaceous pathogens. J Clin Microbiol. 1986;24:12–5.
14. Vukmir RB, Kusne S, Linden P, Pasculle W, Fothergill AW, Sheaffer J, et al. Successful therapy for cerebral phaeohyphomycosis due to *Dactylaria gallopava* in a liver transplant recipient. Clin Infect Dis. 1994;19:714–9.
15. Kralovic SM, Rhodes JC. Phaeohyphomycosis caused by *Dactylaria* (human dactylariosis): report of a case with review of the literature. J Infect. 1995;31:107–13.
16. Bowyer JD, Johnson EM, Horn EH, Gregson RM. *Ochroconis gallopava* endophthalmitis in fludarabine treated chronic lymphocytic leukemia. Br J Ophthalmol. 2000;84:117.
17. Mazur JE, Judson MA. A case report of a dactylaria fungal infection in a lung transplant patient. Chest. 2001;119: 651–3.
18. Malani PN, Bleicher JJ, Kauffman CA, Davenport DS. Disseminated *Dactylaria constricta* infection in a renal transplant recipient. Transpl Infect Dis. 2001;3:40–3.
19. Wang TK, Chiu W, Chim S, Chan TM, Wong SS, Ho PL. Disseminated *Ochroconis gallopavum* infection in a renal transplant recipient: the first reported case and a review of the literature. Clin Nephrol. 2003;60:415–23.
20. McGinnis MR, Pasarell L. In vitro testing of susceptibilities of filamentous ascomycetes to voriconazole, itraconazole, and amphotericin B, with consideration of phylogenetic implications. J Clin Microbiol. 1998;36: 2353–5.
21. Meletiadis J, Meis JFG, Horré R, Verweij PE. Short communication: in vitro antifungal activity of six drugs against 13 clinical isolates of *Ochroconis gallopava*. Stud Mycol. 1999;43:206–8.
22. Espinel-Ingroff A. In vitro fungicidal activities of voriconazole, itraconazole, and amphotericin B against opportunistic moniliaceous and dematiaceous fungi. J Clin Microbiol. 2001;39:954–8.

Article/Artigo

High molecular mass fraction in clinical isolates of *Paracoccidioides brasiliensis*

Fração de alta massa molecular em isolados clínicos de *Paracoccidioides brasiliensis*

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ABSTRACT

Introduction: Different serum levels of the IgG/IgE for *Paracoccidioides brasiliensis* high mass molecular (hMM) fraction (~366kDa) in the acute and chronic forms of the disease have been reported. Considering the nonexistence of hMM fraction investigation involving clinical isolates of *P. brasiliensis*, the present study aimed to investigate the presence of the hMM fraction (~366kDa) in cell free antigens (CFA) from *P. brasiliensis* clinical isolates. **Methods:** CFA from 10 clinical isolates and a reference strain (Pb18) were submitted to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by gel image capturing and densitometer analysis. Additionally, CFA from 20 isolates and Pb18 were analyzed by capture ELISA (cELISA) using polyclonal (polAb) or monoclonal (mAb) antibodies to the hMM fraction. **Results:** The presence of the hMM component was observed in CFA of all samples analyzed by SDS-PAGE/densitometry and by cELISA. In addition, Pearson's correlation test demonstrated stronger coefficients between hMM fraction levels using pAb and mAb (R = 0.853) in cELISA. **Conclusions:** The soluble hMM fraction was present in all the *P. brasiliensis* clinical isolates analyzed and the reference strain Pb18, which could be used as a source of this antigen. The work also introduces for first time, the cELISA method for *P. brasiliensis* hMM fraction detection. Analysis also suggests that detection is viable using polAb or mAb and this methodology may be useful for future investigation of the soluble hMM fraction (~366kDa) in sera from PCM patients.

Key-words: Paracoccidioidomycosis. Soluble antigen. Capture ELISA. Monoclonal antibodies. IgG.

RESUMO

Introdução: Diferentes níveis sorológicos de IgG/IgE contra a fração de alta massa molecular (hMM) (~366kDa) de *Paracoccidioides brasiliensis* têm sido encontrados na PCM aguda e crônica. Considerando a inexistência de investigação sobre esta fração em isolados clínicos de *P. brasiliensis*, o objetivo deste estudo foi investigar a presença da fração hMM (~366kDa) no preparado livre de células (CFA) de *P. brasiliensis* obtidos de isolados clínicos. **Métodos:** CFA de 10 isolados e de cepa de referência (Pb18) foram submetidas à eletroforese em gel de SDS-poliacrilamida (SDS-PAGE) seguida de captura de imagem e análise por densitometria. Adicionalmente, CFA de 20 isolados e de Pb18 foram analisados por ELISA captura (cELISA) utilizando anticorpos policlonal (polAb) ou monoclonal (mAb) para fração hMM. **Resultados:** A presença do componente de hMM foi observada em todas as amostras analisadas por SDS-PAGE/densitometria e por cELISA. Adicionalmente, o teste de correlação de *Pearson* demonstrou forte relação entre os níveis de fração hMM usando pAb e mAb (R = 0.853) no cELISA. **Conclusões:** Conclui-se que a fração hMM está presente em todos os isolados clínicos de *P. brasiliensis* analisados e no isolado referencial, sugerindo a possibilidade dos mesmos serem utilizados como fonte desta fração antigênica. Este trabalho também introduz pela primeira vez o método de cELISA para detecção da fração hMM de *P. brasiliensis*, sugerindo que detecção utilizando anticorpos polAb ou mAb é viável e essa metodologia poderá ser útil para investigação futura desta fração solúvel (~366kDa) em soros de pacientes com PCM.

Palavras-chaves: Paracoccidioidomicose. Antígeno solúvel. ELISA de captura. Anticorpos monoclonais. IgG.

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INTRODUCTION

Paracoccidioidomycosis (PCM), a deep mycosis endemic in Latin America, is caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*, which develops as yeast at body temperature and as mycelium at room temperature. *P. brasiliensis* causes natural infections by inhalation of conidia or mycelial elements[1]. Most exposed subjects develop an asymptomatic infection, although some individuals present clinical manifestations that can vary from benign and localized to severe and disseminated forms[2]. Two forms of the disease are distinguished: the acute or subacute and chronic form. The acute form is more severe and rare, while the chronic form occurs more frequently and mostly affects adult males[3,4].

Usually, PCM diagnosis is inferred from indirect evidence obtained via serological tests and clinically relevant antigens have been identified and adapted for use in immunoassays for the detection of specific antibodies[5]. For this purpose, several fungal components have already been identified. The antigens most frequently identified in PCM patient sera are the glycoproteins of 43kDa (97-100%)[6-8], the main PCM diagnostic antigen[6,7,9,10], 160kDa (78%) and 70kDa (60%)[8].

The other alternative laboratory approach for diagnosis of PCM is the detection of circulating *P. brasiliensis* antigens. The inhibition ELISA methodology (inh-ELISA) is able to detect gp43 in 96.3% of PCM patients, mainly in those with the acute form of the disease (100%)[11]. Gp70 has also been detected in the urine or in cerebrospinal fluid (CSF) of PCM patients[12,13]. Gómez et al[14,15] reported the use of inh-ELISA and detected gp87 circulating antigen in sera from patients with active disease.

Puccia et al[9] demonstrated polydispersed high-molecular mass glycoprotein, with heterogeneous electrophoresis migration. From this heterogeneous electrophoresis migration Marquez et al[16] isolated fractions ranging from 278kDa to 466kDa (mean approximately 366kDa) obtained from sonicated

or cell free antigens (CFA) preparations. Moreover, different serum levels of the IgG/IgE to the hMM fraction was verified in sera of acute and chronic PCM patients and the authors suggested the analysis as a new characteristic to differentiate between these two clinical forms of the disease[16].

Taking into account the lack of data regarding the soluble hMM fraction (~366kDa) in *P. brasiliensis* clinical isolates and the immunological methodology for identifying it, in the present study, the hMMAg antigen was investigated in different clinical isolates and capture ELISA (cELISA) was introduced. In principle, observation verified that all the *P. brasiliensis* samples analyzed produced the hMM fraction (~366kDa). In addition, this hMM fraction was detected by cELISA using monoclonal or polyclonal antibodies to the hMM fraction.

METHODS

Fungal isolates

Clinical isolates of *P. brasiliensis* of the chronic form of PCM disease were obtained: 17 isolates (LDR1 to LDR17) from Londrina State University (MOOI/CCS, HC, HC, Londrina State University, Londrina, Paraná) patients (2000 to 2006); two isolates (RC-Wang and RC-Hori) from Chiba University, Chiba, Japan; one isolate (EPM-01) and reference strain (Pb18) from UNIFESP, São Paulo, Brazil. *P. brasiliensis* strains were maintained on potato dextrose agar (Difco Laboratories, MI, USA) slants at room temperature. Prior to experiments, samples of the isolates were inoculated onto a slant of brain heart infusion agar (BHI, Difco Laboratories) supplemented with 1% dextrose and cultured at 35°C to produce the yeast form and maintained by subculturing at 35°C at 5-day intervals on Sabouraud agar (Micromed, Rio de Janeiro, RJ, Brazil).

Cell free antigens preparation

Yeast cells were collected and the CFA samples were obtained according to Camargo et al[17], modified by the addition of PMSF protease inhibitor at 2.5mM to the supernatant. The protein concentration was determined by the Lowry method[18], adjusted to 3mg/mL and stored in -80°C freezer until ready to use.

Cell free antigens analysis by SDS-PAGE

Cell free antigens (3mg/mL) samples, obtained as described above, were mixed with the reducing sample buffer (62.5mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 10% β-mercaptoethanol and 0.05% bromophenol blue) and boiled for 3 min. The antigens were separated by SDS-polyacrylamide gel electrophoresis (7.5%), in tris-glycine buffer, pH 8.2, at 125v. Protein standards with the following molecular masses were used: myosin (201.1kDa); β-galactosidase (115.7kDa); bovine serum albumin (93.6kDa); ovalbumin (50.3kDa); carbonic anhydrase (37.3kDa). Using specific densitometer software, Glob-Al Scan (Cellogel Electrophoresis Co, Milan, Italy), silver stained dehydrated SDS-electrophoresis gel image was captured, the specific cutoff point of that defines the area of the hMM fraction band (~366kDa) was indicated and the result automatically calculated and expressed in percentages.

Capture ELISA for IgG-hMM fraction

ELISA immunoplates sensitized with rabbit IgG anti-hMM fraction (25μg/ml) were incubated with CFA samples at 30μg/ml at 37°C for 1h and then with polyclonal mouse IgG anti-hMM (IgG pAb) (30μg/ml), anti-mouse IgG peroxidase conjugate and OPD (100μL well). The absorbance was read at 492nm. Additionally, cELISA for hMM fraction level determination was performed as described, substituting polyclonal mouse IgG anti-hMM fraction for monoclonal IgG anti-hMM fraction (IgG mAb) as secondary antibodies. Polyclonal rabbit and mouse antibodies were produced by using the hMM fractions obtained, according to Pavanelli et al[19] for immunizations. A monoclonal antibody was obtained by spleen cells (from immunized BALB/c mice with the hMM fraction) fused with P3U1 cell line by using PEG. Hybridomas were screened by ELISA with hMM fraction (~366kDa) and cloned by limiting dilutions. Pristane-primed BALB/c mice were injected i.p. with hybridoma and IgG purified (Sepharose-G protein column) from ascite fluid. The cutoff was determined as the mean plus 2 standard deviations of the absorbance obtained with control: primary rabbit IgG anti-hMM, secondary mouse pAb or mAb IgG and peroxidase conjugate without the CFA sample.

Statistical analysis

Statistical comparisons were performed by analysis of variance (ANOVA) and by the Tukey test. All values are reported as the mean ± SD of the mean, with significance assumed in the range of $p<0.05$. Pearson's correlation was applied between hMM fraction levels using IgG pAb and mAb anti-hMM fraction and a significant correlation was considered when $r \geq 0.50$.

Ethical

This study was approved by the Internal Scientific Commission and the Bioethics in Research Committee of the State University of Londrina (Londrina, PR, Brazil).

RESULTS

CFA analysis by SDS-PAGE

The presence of the hMM fraction was observed in CFA from all the clinical isolates and reference Pb18 by SDS-PAGE. The SDS-PAGE was submitted to densitometry in an attempt to quantify the hMM fraction. The results show that 20.5 ± 5.7% (clinical isolates) and 16.7% (Pb18) from CFA correspond to the hMM fraction (**Figure 1**). This study indicates that this antigen is present in different clinical isolates from chronic patients and could be used as source of the hMM fraction.

Capture ELISA for hMM fraction levels in CFA samples

Various researchers have attempted to detect circulating antigens in PCM patients using polyvalent antigens or antibodies in different assays. For this purpose, the presence of the hMM fraction in CFA samples was determined using cELISA expressed as optical density units (OD). Analysis of the result verified the presence of the hMM fraction in Pb18, a strain characterised as highly virulent, and in all the clinical isolates of *P. brasiliensis*, with the use of polAb or mAb to the hMM fraction (**Table 1**). All values greater than the values obtained in the control systems of polyclonal/polyclonal or monoclonal/monoclonal with free samples of CFA plus 2 x standard (0.118 ± 0.008 and 0.086 ± 0.005, respectively) were considered as positive.

Correlation analysis

Analysis of the results confirmed a strong correlation between hMM fraction levels with IgG pAb or mAb anti-hMM as the secondary antibody ($r = 0.853$) (**Figure 2**). This result demonstrates that the hMM fraction can be detected by cELISA using monoclonal or polyclonal antibodies to hMM, as a secondary antibody.

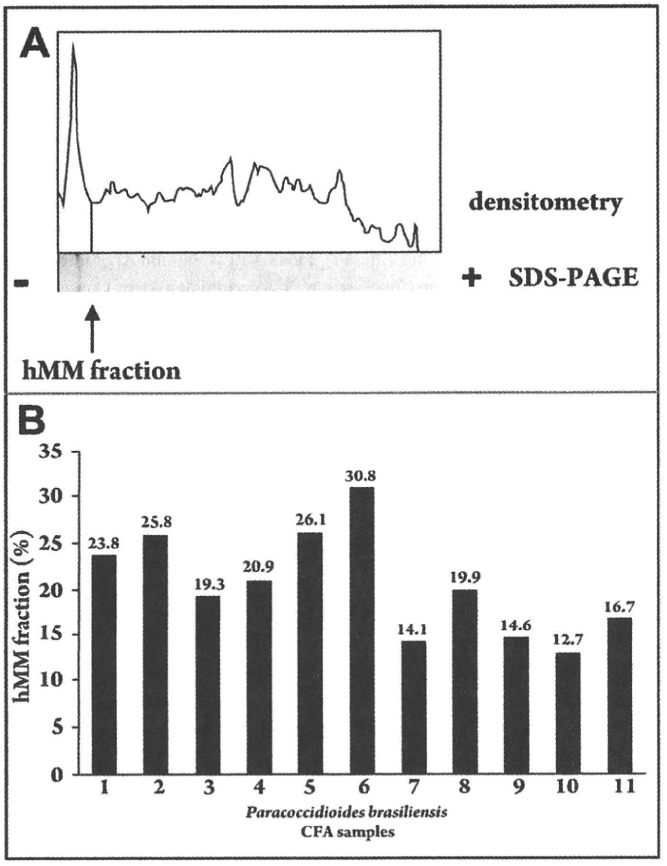

FIGURE 1 - The hMM fraction in CFA from *P. brasiliensis* by SDS-PAGE and densitometry. A) *P. brasiliensis* CFA sample was separated using 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), silver stained and dehydrated. The gel image was captured and the specific cutoff point of hMM fraction (~366kDa) was marked and equivalent area was automatically calculated and expressed in percentages of hMM fraction. B) Percentage of hMM fraction in relation to all other fractions in CFA from clinical isolates of *P. brasiliensis*: (1) LDR13, (2) LDR11, (3) EPM-01, (4) LDR16, (5) LDR17, (6) RC-Hori, (7) RC-Wang, (8) LDR12, (9) LDR14, (10) LDR10 and reference strain (11) Pb18.

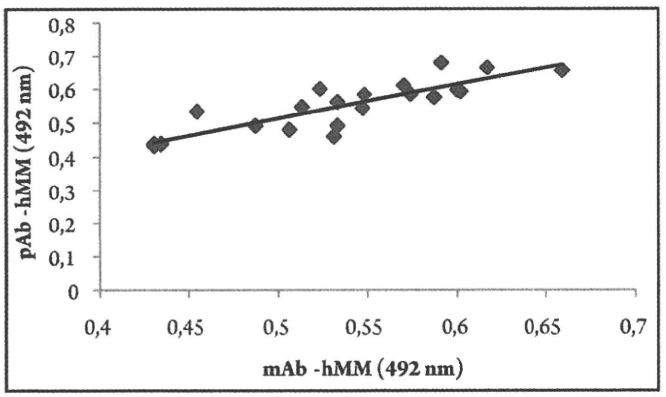

FIGURE 2 - Pearson's correlation tests between hMM fraction levels by capture ELISA (cELISA) using monoclonal (mAb) or polyclonal (polAb). The cELISA results showed a stronger correlation between hMM fraction levels with IgG pAb or mAb anti-hMM as a secondary antibody (r= 0.85).

DISCUSSION

In this work, the hMM fraction was detected in all the *P. brasiliensis* clinical isolates and the reference strain Pb18 by SDS-PAGE, suggesting that all these *P. brasiliensis* could be used as a source of this fraction.

TABLE 1 - High molecular mass fraction (~ 366 KDa) levels in CFA from clinical isolates *Paracoccidioides brasiliensis* (Pb) by capture ELISA, expressed in optical density units.

Reference strain	Pb	pAb-hMM	mAb-hMM
	Pb18	0.600	0.596
	LDR1	0.659	0.655
	LDR2	0.617	0.664
	LDR3	0.587	0.574
	LDR4	0.531	0.458
	LDR5	0.434	0.437
	LDR6	0.574	0.583
	LDR7	0.548	0.582
	LDR8	0.591	0.679
Clinical isolates	LDR9	0.570	0.610
	RC-WANG	0.547	0.542
	LDR10	0.513	0.545
	EPM-01	0.533	0.490
	LDR11	0.454	0.532
	RC-HORI	0.430	0.431
	LDR12	0.506	0.478
	LDR13	0.533	0.560
	LDR14	0.487	0.490
	LDR15	0.430	0.437
	LDR16	0.523	0.599
	LDR17	0.602	0.591
Mean±SEM		**0.537±0.064**	**0.549±0.076**

ELISA immunoplates sensitized with rabbit IgG anti-hMM were incubated with CFA samples and then with polyclonal mouse IgG anti-hMM (pAb-hMM) or with monoclonal mouse IgG anti-hMM (mAb-hMM). After incubation with anti-mouse IgG peroxidase conjugate, absorbance was read at 492nm. All values higher than the controls: polyclonal/polyclonal (0.118 ± 0.008) or polyclonal/monoclonal (0.086 ± 0.005), with free CFA sample, plus 2 x standard were considered positive.

For diagnostic purposes, the presence of the target antigen in all isolates of the *P. brasiliensis* is important. The present study indicates that this antigen is present in different clinical isolates from chronic patients as the common *P. brasiliensis* antigen. However, further studies involving larger sample of isolates are required to confirm these findings.

Panunto-Castelo et al[8] identified hMM antigens with 172 or 160kDa in exoAg from three *P. brasiliensis* isolates (DGO, C-9 and BAT). In this study, the considered hMM band identified in CFA from the *P. brasiliensis* isolates presented ~366kDa and is, therefore, a different hMM antigen.

The detection of circulating antigens is a useful approach for serodiagnosis for monitoring PCM treatment. The gp43 glycoprotein, one of the most important immunodominant antigens of *P. brasiliensis*, has been extensively investigated, particularly in relation to diagnosis[20-22]. Gp43 has been detected at higher levels in PCM patient sera as circulating antigens[22] and in CSF and bronchoalveolar lavage (BAL) fluid samples from PCM patients[11]. However, recently the existence of a *P. brasiliensis* isolate presenting differences in this major antigen coding gene gp43[23,24] has been demonstrated and speculation of the possibility of a new species in the genus Paracoccidioides has been noted. Considering genetic differences in gp43 according to *P. brasiliensis* isolate, it has become important to investigate other *P. brasiliensis* antigens for diagnosis.

Besides gp43[11], the 87-kDa molecule[15], gp70[13] and the high molecular mass antigen with 160kDa[25] were also introduced as potential candidates for diagnosis and/or for follow-up of patients with PCM.

Panuto-Castelo et al[8] demonstrated that the hMM antigens (172 or 160kDa) are highly reactive with serum IgG of patients with acute or chronic PCM, indicating their potential application in the diagnosis and follow-up of the disease. In addition, Coltri et al[25] characterized this antigen as a protein of 160kDa, designated paracoccin, with selective binding to immobilized GlcNAc and able to interact with laminin.

In this study, the association between SDS-PAGE and densitometry analysis shows that approximately 16-20% of *P. brasiliensis* CFA antigens correspond to the hMM fraction. Considering the proportion and as a soluble antigen present in CFA, we speculate that its presence may also be observed as a soluble antigen during infection, similar to other antigens, such as gp43 or gp70, present in CFA and in the serum[11,13,22,26], with the potential for diagnosis and follow-up of the PCM patients. Thus, the hMM fraction is also important when considering the distinct isotypic humoral immune response to hMM antigens with ~336kDa observed in the acute and chronic forms of the PCM disease, which suggest that it has potential as a new biomarker for differentiating these two clinical forms[16].

In this study cELISA was introduced to detect the hMM fraction in clinical isolates and the reference Pb18 strain was introduced and the presence of the hMM fraction was detected in all the samples analyzed, in agreement with SDS-PAGE. This study is qualitative and the results are expressed as optical density. cELISA has the advantage of being able to process large numbers of samples at the same time and presents high sensitivity and a high specificity. The analysis was performed using polAb or mAb to the hMM fraction as a secondary antibody and the results showed a stronger correlation between hMM fraction levels obtained using pAb or mAb anti-hMM. cELISA with specific polAb and mAb was used in previous studies by our group to determine circulating soluble gp43 levels in PCM[22] and determining plasmatic hMM fraction levels in PCM patients by cELISA will be the object of future investigations.

Unexpectedly, the results of ELISA showed homogeneity. We believe that regardless of differences in isolates or strains, these components are better preserved and produced more homogeneously, as observed with other antigens reported in the literature[8]. The same isolates showed less homogeneity in relation to gp43 determined by capture ELISA (data not shown) and the heterogeneity observed in the percentage of the hMM fraction in the electrophoresis was due to variations in the other components present in CFA (data not shown).

In conclusion, the soluble hMM fraction was present in all the *P. brasiliensis* clinical isolates analyzed and the reference strain Pb18, which could be used as source of this fraction. The work also introduced for first time the capture ELISA method for *P. brasiliensis* hMM fraction (~366kDa) detection.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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REFERENCES

1. Restrepo A, Tobón AM. *Paracoccidioides brasiliensis. In:* Mandell GL, Bennett JE, Dollin R, editors. Principles and Practice of Infectious Diseases. 6th ed. Philadelphia: Elsevier; 2005. p. 3062-3068.
2. Borges-Walmsley MI, Chen D, Shu X, Walmsley AR. The pathobiology of *Paracoccidioides brasiliensis*. Trends Microbiol 2002;10:80-87.
3. Shikanai-Yasuda MA, Telles Filho FQ, Mendes RP, Colombo AL, Moretti ML, Grupo de Consultores do Consenso em Paracoccidioidomicose. Consenso em Paracoccidioidomicose. Rev Bras Med Tropical 2006; 39:297-310.
4. Franco M, Montenegro MR, Mendes RP, Marques SA, Dillon NL, Mota NGS. Paracoccidioiodomycosis: a recently proposed classification of its clinical forms. Rev Soc Brasil Med Trop 1987; 20:129-132.
5. Ferreira-da-Cruz MF, Galvão-Castro B, Daniel-Ribeiro CT. Isolation and antigenicity of a 45-kilodalton *Paracoccidioides brasiliensis* immunodominant antigen. Infect Immun 1992; 60:2667-2671.
6. De-Camargo Z, Unterkircher C, Campoy SP, Travassos LR. Production of *Paracoccidioides brasiliensis* exoantigens for immunodiffusion tests. J Clin Microbiol 1988; 26:2147-2151.
7. Taborda CP, Camargo ZP. Diagnosis of paracoccidioidomycosis by passive haemagglutination assay of antibody using a purified and specific antigen-gp43. J Med Vet Mycol 1993; 31:155-160.
8. Panunto-Castelo A, Freitas-da-Silva G, Bragheto IC, Martinez R, Roque-Barreira MC. *Paracoccidioides brasiliensis* exoantigens: recognition by IgG from patients with different clinical forms of paracoccidioidomycosis. Microbes Infect 2003; 5:1205-1211.
9. Puccia R, Schenkman S, Gorin PA, Travassos LR. Exocellular components of *Paracoccidioides brasiliensis*: identification of a specific antigen. Infect Immun 1986; 53:199-206.
10. Puccia R, Travassos LR. 43-kilodalton glycoprotein from *Paracoccidioides brasiliensis:* immunochemical reactions with sera from patients with paracoccidioidomycosis, histoplasmosis, or Jorge Lobo's disease. J Clin Microbiol 1991; 29:1610-1615.
11. Marques-da-Silva SH, Colombo AL, Blotta MHSL, Lopes LD, Queiroz-Telles F, Camargo ZP. Detection of circulating gp43 antigen in serum, cerebrospinal fluid and bronchoalveolar lavage of patients with paracoccidioidomycosis. J Clin Microbiol 2003; 41:3675-3680.
12. Salina MA, Shikanai-Yasuda MA, Mendes RP, Barravieira B, Mendes-Giannini MJS. Detection of circulating *Paracoccidioides brasiliensis* antigen in urine of paracoccidioidomycosis patients before and during treatment. J Clin Microbiol 1998; 36:1723-1728.
13. Marques-da-Silva SH, Mattos Grosso D, Lopes JD, Colombo AL, Blotta MHSL, Queiroz-Telles F, et al. Detection of *Paracoccidioides brasiliensis* gp70 circulating antigen and follow-up of patients undergoing antimycotic therapy. J Clin Microbiol 2004; 42:4480-4486.
14. Gómez BL, Figueroa JL, Hamilton AJ, Ortiz B, Robledo MA, Hay RJ, et al. Use of monoclonal antibodies in diagnosis of paracoccidioidomycosis: new strategies for detection of circulating antigens. J Clin Microbiol 1997; 35:3278-3283.
15. Gómez BL, Figueroa JI, Hamilton AJ, Diez S, Rojas M, Tobón AM, et al. Restrepo A. Antigenemia in patients with paracoccidioidomycosis: detection of the 87-kilodalton determinant during and after antifungal therapy. J Clin Microbiol 1998; 36:3309-3316.
16. Márquez AS, Vicentini P, Ono MA, Watanabe MAE, Camargo ZP, Itano EN. Reactivity of antibodies from patients with acute and chronic paracoccidioidomycosis to a high-mass molecular antigen from *Paracoccidioides brasiliensis*. J Clin Labor Anal 2005; 19:199-204.
17. Camargo ZP, Taborda CP, Rodrigues EG, Travassos LR. The use of cell-free antigens of *Paracoccidioides brasiliensis* in serological tests. J Med Vet Mycol 1991; 29:31-38.
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Colorimetric assays: Lowry's method for protein determination. J Biol Chem 1951; 193:265-275.
19. Pavanelli WR, Kaminami MS, Gerez JR, Sano A, Ono MA, Camargo ICC, et al. Protection induced in BALB/c mice by the high-molecular-mass (hMM) fraction of *Paracoccidioides brasiliensis*. Mycopathologia 2007; 163:117-128.

20. Camargo ZP, Unterkircher C, Travassos L. Identification of antigenic polypeptides of *Paracoccidioides brasiliensis* by immunoblotting. J Med Vet Mycol 1989; 27:407-412.

21. Brummer E, Castañeda E, Restrepo A. Paracoccidioidomycosis: an update. Clin Microbiol Rev 1993; 6:89-117.

22. Miura CSN, Estevão D, Lopes JD, Itano EN. Levels of specific antigen (gp43), specific antibodies, and antigen-antibody complexes in saliva and serum of paracoccidioidomycosis patients. Med Mycol 2001; 37:423-428.

23. Carrerro L, Nino-Vega G, Teixeira MM, Carvalho MJA, Soares CM, Pereira M, et al. New *Paracoccidioides brasiliensis* isolate reveals unexpected genomic variability in this human pathogen. Fungal Genet Biol 2008; 45:605-612.

24. Takayama A, Itano EN, Sano A, Ono MA, Kamei K. An atypical *Paracoccidioides brasiliensis* clinical isolate. Med Mycol 2009; 47:01-09.

25. Coltri KC, Casabona-Fortunato AS, Gennari-Cardoso ML, Pinzan CF, Ruas LP, Mariano VS, et al. Paracoccin, a GlcNAc-binding lectin from *Paracoccidioides brasiliensis*, binds to laminin and induces TNF-alpha production by macrophages. Microbes Infect 2006; 8:704-713.

26. Stambuk BU, Puccia R, De-Almeida ML, Travassos LR, Schenkman S. Secretion of the 43kDa glycoprotein antigen by *Paracoccidioides brasiliensis*. J Med Vet Mycol 1988; 26:367-373.