Mycopathologia (2010) 170:21–30
DOI 10.1007/s11046-010-9292-7

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

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Received: 6 June 2009 / Accepted: 11 February 2010 / Published online: 7 March 2010

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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Keywords Hot spring · New species · *Ochroconis calidifluminalis* · Virulence

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

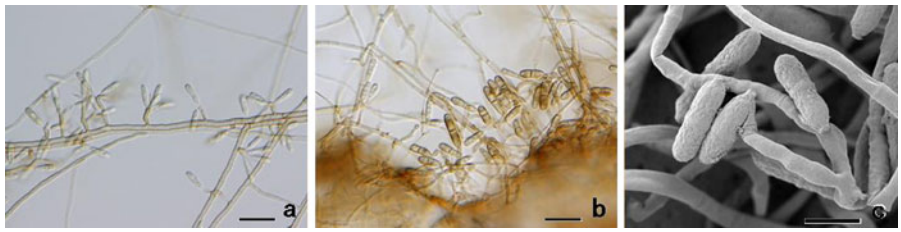

Fig. 2 Conidia of *Ochroconis calidifluminalis* IFM 54738. **a**, **b** Conidia formation on cornmeal agar at 25°C for 4 weeks, lactophenol fixation, ×600; the *bars* indicate 10 μm. **c** SEM picture image of conidia on OA at 35°C for 5 weeks, ×5000; the *bar* indicates 5 μm. SEM image was taken by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

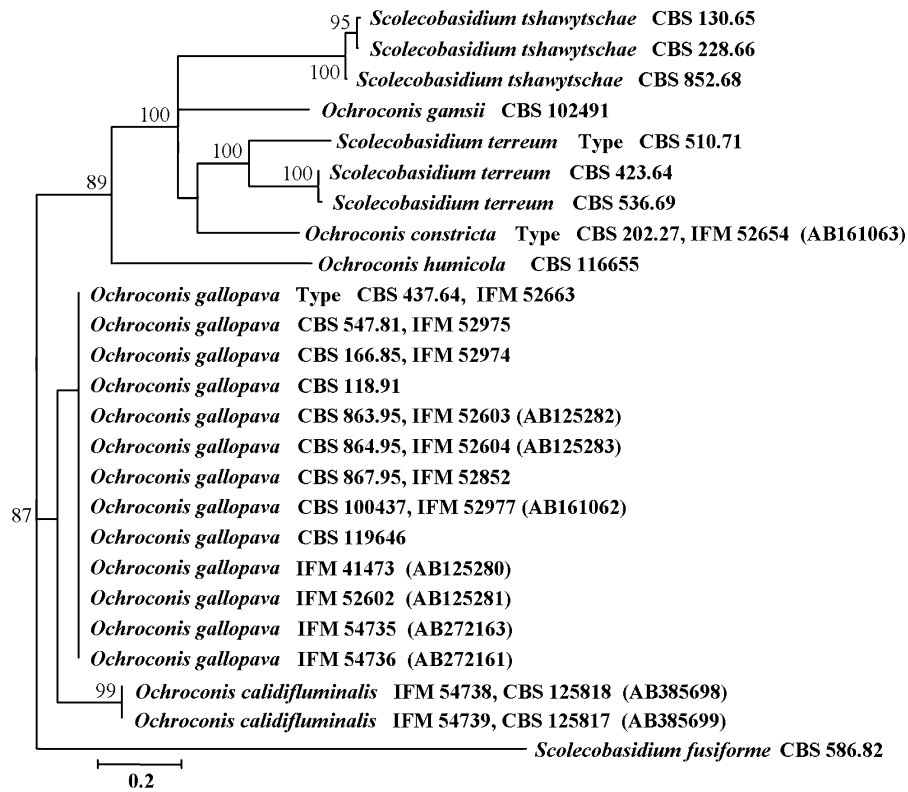

Fig. 3 Phylogenetic analysis of *Ochroconis calidifluminalis* based on ITS 1 - 5.8S - ITS 2 regions of rRNA gene sequences. (Accession numbers of GenBank)

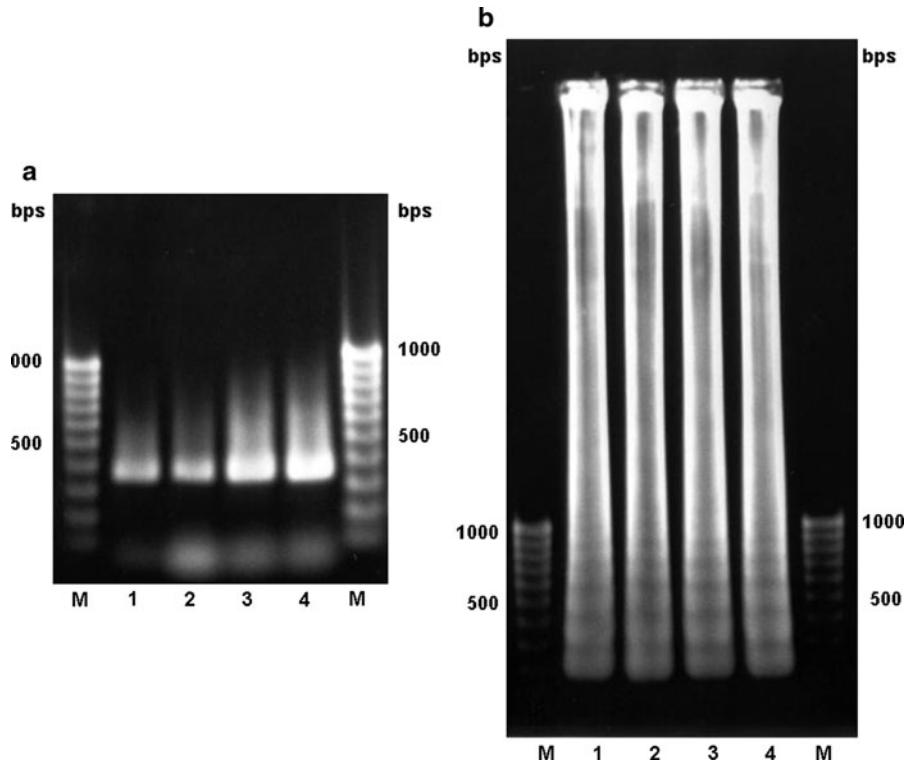

Fig. 4 PCR banding patterns amplified by two identification methods designed for *O. gallopava*, **a** the species-specific PCR primer set, **b** the species-specific LAMP method. 1: *Ochroconis calidifluminalis* IFM 54738, 2: *O. calidifluminalis* IFM 54739, 3: *O. gallopava* IFM 41473, 4: *O. gallopava*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

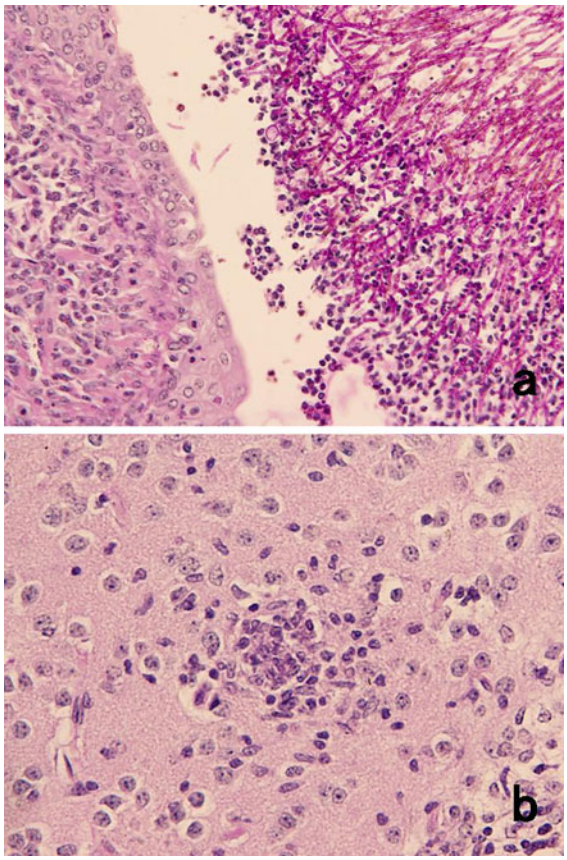

Fig. 5 Representative histopathology at day 28 in a mouse infected with *Ochroconis calidifluminalis* IFM 54738. **a** A hyphal mass and polymorphonuclear leukocytes in the renal calyx, and granulomatous reaction in the renal parenchyma, PAS, ×200. **b** A small lesion in the brain, PAS, ×400

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.

Acknowledgments This study was supported in part by the Special Research Fund for Emerging and Re-emerging Infections from the Ministry of Health, Labour, and Welfare, Japan, to Drs. Katsuhiko Kamei and Ayako Sano. We are grateful to Dr. Hitoshi Okada and Dr. Masako Osumi, Integrated Imaging Research Support, Tokyo, Japan, for providing SEM pictures.

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