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Molecular epidemiology of canine histoplasmosis in Japan

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A recent case of canine histoplasmosis, the first confirmed case of disseminated infection accompanied by carcinoma in Japan, was diagnosed by clinical characteristics, histopathological examination, chest radiographs, ocular fundoscopy and molecular biological data. The clinical manifestations were not limited to cutaneous symptoms but were referable to disseminated infection, similar to human autochthonous cases. The partial sequences of the internal transcribed spacer (ITS1/2) regions of the ribosomal DNA genes of this and other Japanese canine histoplasmosis strains were 99–100% identical to the sequence AB211551 derived from a human isolate in Thailand, and showed a close relationship to the sequences derived from Japanese autochthonous systemic and cutaneous human cases. The phylogenetic analysis of 97 sequences of the ITS1/2 region disclosed six genotypes. The genotypes derived from Japanese autochthonous human and dog cases belonged to the cluster consisting of *Histoplasma capsulatum* var. *capsulatum* and *H. capsulatum* var. *farciminosum* sequences, indicating that these varieties might cause not only cutaneous but also systemic histoplasmosis, regardless of their host species. The current status of the 3 varieties of *Histoplasma capsulatum* according to the host species remains a subject of further investigation.

Keywords dog, *Histoplasma capsulatum*, histoplasmosis, Japan

Introduction

Histoplasmosis is a highly pathogenic fungal infection, which is endemic to the basins of certain large rivers in tropical, subtropical and temperate zones all over the world [1–4]. The disease in Japan is generally considered to be an imported mycosis; however, human cases in our country are both imported and autochthonous infections [5]. On the other hand, all cases in dogs [6–9], horses [10,11] and sea otters [12] were autochthonous infections.

The disease is caused by *Histoplasma capsulatum*, a thermally dependent dimorphic fungus. The fungus takes a mycelial form at room temperature and transforms to a yeast form in host tissue or at 35–37°C in certain culture media [1–4].

Histoplasmosis has been categorized into three types: histoplasmosis capsulati caused by *H. capsulatum* var. *capsulatum*, histoplasmosis duboisii caused by *H. capsulatum* var. *duboisii*, and histoplasmosis farciminosi caused by *H. capsulatum* var. *farciminosum* (formerly *H. farciminosum*) and is known as "epizootic lymphangitis or pseudofarcy" in horses. The difference between *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *duboisii* is based on endemic areas and parasitic forms. The former is endemic all over the world, causing pulmonary and systemic infections with small-sized yeast-form cells, while the latter is endemic in continental Africa and Japan [1–4,13] and causes large sized yeast-like cells accompanied by lymphadenopathy, and dissemination to the skin and bones. *H. capsulatum* var. *farciminosum* affects the skin and

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subcutaneous lymph nodes and is found in horses, but has been recovered in cases involving humans and dogs [1–4].

Canine histoplasmosis has been reported in many countries, such as the USA [14–23], Canada [24], Italy [25], Brazil [26,27], Australia [28] and Japan [6–9]. The disease in Japanese dogs typically involves skin ulcers with purulent exudates but no pulmonary involvement, while cases from other countries have been systemic or superficial infections invariably involving pulmonary lesions [29] except for one case reported from Australia [28]. Canine histoplasmosis in Japan has been thought to be an infection caused by *H. capsulatum* var. *farciminosum* as a heteroecism, on the basis of its clinical manifestations, the historical background of equine epizootic lymphangitis, and epidemiological studies [7–9]. The fact of a 100% sequence identity between a sequence from a human autochthonous case limited to cutaneous lesions in Japan [30] (submitted to the GenBank as AB071841) and a sequence derived from *H. capsulatum* var. *farciminosum* in the USA (AB071838) indicated that autochthonous human cases have been caused by the same genotype of *H. capsulatum* var. *farciminosum* as that found in dogs [8].

However, we recently encountered a case of systemic canine histoplasmosis, which raised the question of whether canine histoplasmosis in Japan can be caused by *H. capsulatum* var. *farciminosum* as a heteroecism or not. The present study describes two new cases of canine histoplasmosis, including one disseminated case, and mentions the varieties of *H. capsulatum* based on phylogenetic relationships of the internal transcribed spacer (ITS1/2) region.

Materials and methods

Cases

The cases of canine histoplasmosis reported in Japan are shown in Table 1 [6–9].

Case 1. A 12-year-old male Shih Tzu from Tokyo, Japan showed several interdigital purulent skin ulcers (0.5–0.8 cm in diameter) on his front and hind paws. The dog had no history of importation or travel to an endemic area of histoplasmosis. The cutaneous lesions were characterized by repeated exascerbations and remissions for 3 years. There was no response to antibiotic, corticosteroid or antiseptic treatment. In August 2003, impression smears of the pus were collected from the skin ulcers on the front and hind paws. After being fixed with methanol, the samples were stained with Giemsa solution (Merck & Co., Inc., NJ, USA) and observed under light microscopy, which showed macrophages and a few yeast-like cells, 1–4 μm in diameter. An immunodiffusion test for *Histoplasma capsulatum* antibody performed with the Histoplasma Immunodiffusion Reagents kit (Immuno-Mycologics, Inc., Norman, OK, USA) was negative. The pus was cultured on potato dextrose agar plates (Becton Dickinson and Company, Sparks, MD, USA) supplemented with 100 mg/liter of chrolamphenicol (PDAC) at 25°C for 8 weeks. No fungal growth from the pus was detected. Topical treatments with antibiotics, antifungal agents, antiseptics and corticosteroids for the wounds were tried again; however, they had no effect. Five mg/kg/day of itraconazole given orally for 2 weeks induced a remission for one month. A topical treatment with cream including ketoconazole (20 mg/g) and amphotericin B (1 mg/g) mixed in the clinic was applied on the skin lesions for the remainder of the dog's lifetime. In November 2003, the ulcers appeared again. Impression smears stained with Giemsa solution showed macrophages containing 5–10 small yeast cells of 1–4 μm in diameter (Fig. 1). Culture on PDAC at 25°C for 8 weeks was again negative. Chest radiographs and haematological examinations were normal. This case was diagnosed as histoplasmosis based on clinical manifestations, histopathology and PCR sequence data. The dog suffered several aggravations and remissions until he died of old age in February 2005. Postmortem examination was refused.

Case 2. An 8-year-old spayed female Boston terrier from Mobara, Chiba prefecture Japan showed multiple skin ulcers of various sizes appearing on the left front paw with purulent exudate followed by dissemination of breast cancer (Fig. 2). The dog had no history of importation from, or travel to, an endemic area of histoplasmosis. Fungal culture of the biopsy tissues on PDAC at 25°C for 8 weeks was negative. Histopathological observation of the biopsied skin samples showed macrophages in the granulomatous tissue containing periodic acid Schiff (PAS) positive yeast-like cells, 1–4 μm in diameter (Fig. 3). Chest X-ray and physiological examinations of blood were normal at the onset of the multiple skin ulcers. An immunodiffusion test for *Histoplasma capsulatum* antibody performed with the Histoplasma Immunodiffusion Reagents kit was negative. The ulcers became worse and were treated by surgical excision from the humerus one month after the onset of multiple skin ulcers. The dog recovered and remained in good condition for one month after the operation. However, multiple ulcers appeared again on the right front paw, the back, the abdomen and the legs. After the reappearance of the ulcers amphotericin B

Table 1 Canine histoplasmosis in Japan.

Case No.	Breed	Age	Sex	Symptom	Diagnosis	Outcome	Sequence length	Reference
1	Mongrel	8	F	Gingiva, skin	H, IA	Cured	ND	[6] J Vet Med Sci 60:863–865, 1998
2	Min Dac	3	M	Skin	H, MB	Cured	516	[7] Jpn J Med Mycol 42:229–235, 2001
3	SZ	3	M	Skin	H, MB	Remission	518	[7] Jpn J Med Mycol 42:229–235, 2001
4	Shiba	5	F	Skin	H, MB	Euthanized	518	[8] Vet Microbiol 94:219–224, 2003
5	SH	4	M	Skin	C, H, MB	Remission*	518	[9] J Vet Med A Physiol Pathol Clin Med 52: 478–480, 2005
6	SZ	12	M	Skin	C, MB	aggravations and remissions *†	518	Case 1 (present study)
7	BT	8	F	Skin, systemic	H, MB	Died	518	Case 2 (present study)

Isolation of the causative agent failed in all of the above cases. Except for the [first] case, dogs were diagnosed by clinical manifestation, cytology or histopathology and molecular biological techniques. Min Dac, Miniature Dachshund; SZ, Shih Tzu; SH, Siberian Husky; BT, Boston Terrier; H, histopathological observation; MB, molecular biological technique; C, cytological observation; *, fungal DNA detected from pus; †, died in February 2005 of old age.

was administrated intravenously (0.5 mg/kg/day for 2 weeks), and itraconazole (5 mg/kg) was given orally twice daily for 11 weeks. These antifungal drugs produced no effect, and the dog developed cachexia. In addition the dog developed diarrhea 2 weeks after the start of antifungal treatments. The rectal smear did not contain small yeast cells. The ocular fundus observed by a indirect opthalmoscopy indicated retinochoroiditis with granules in the right eye (Fig. 4), and a chest radiograph taken 3 days before the death showed multiple focal shadows (Fig. 5a). The dog died 4 months after the onset of the multiple skin ulcers. A soybean-sized hard nodule in the third left mammary gland, abundant rice-sized nodules in the lungs (Fig. 5b), and a broad bean-sized nodule in the liver were observed in postmortem examination. The nodule in the third left mammary gland was diagnosed as the primary lesion of mammalian adenocarcinoma, and those in the visceral organs were diagnosed as its metastases. Cancerous tissue also contained granulomatous cells with or without PAS-positive yeast-like cells in the lungs, spleen and liver (Fig. 6). This case was diagnosed as a disseminated histoplasmosis following blast carcinoma leading to immunosuppression on the basis of clinical manifestations, histopathology and molecular biology.

Isolates studied

Fifteen clinical isolates of *H. capsulatum* including one isolate derived from autochthonous disseminated histoplasmosis in Japan [31], five isolates from Thailand, five from Nigeria and four from unknown sources stored in the USA and brought to our center in Japan via Shiga University Medical School, Shiga, Japan were added to the present study, to search for sequences related to the ones obtained from Japanese canine cases (Table 2).

Sequencing of the ITS 1-5.8S-ITS 2 region of rDNA gene cluster

The sequences of the ITS 1-5.8S-ITS 2 region of rDNA gene cluster were obtained by routine methods [30]. Briefly, DNA was extracted with a DEXPAT® kit (TaKaRa, Ohtsu, Japan) using a modified procedure. Approximately 100 μl of fungal cells cultured at 25°C for 2 months on potato dextrose agar (Difco, MO, USA) slants were placed in a sterilized microtube (1.5 ml); then 0.5 ml of DEXPAT® solution was added, and the mixture was homogenized with a plastic pestle. It was then incubated at 100°C for 10 min and centrifuged at 12,000 rpm (13,201 *g*) for 10 min. The supernatants were used as DNA samples. Although this

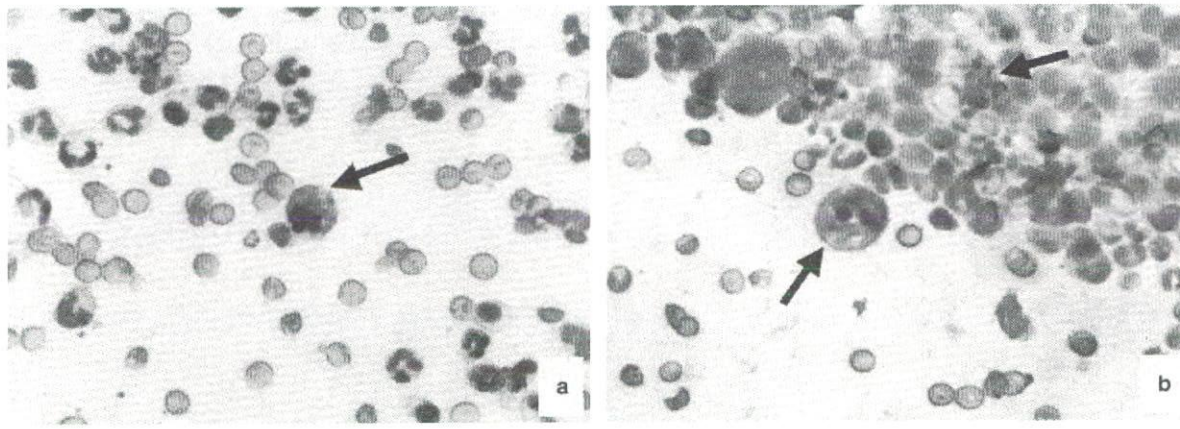


Fig. 1 Pus stamps of Case 1 stained with Giemsa solution showing macrophages containing small yeast cells, 1–4 μm in diameter (arrows); ×400.

type of kit is designed for extracting DNA from paraffin-embedded tissue samples, we routinely use it for the isolation of genes from fungal cultures including *H. capsulatum* isolates because of its convenience [33–35].

We mixed 2.5 μl of the DNA extract with a Ready-to-Go bead (Amersham Pharmacia Tokyo, Japan), 2.5 μl of 10 pM of the primers ITS-5 (5′- GGA AGT AAA AGT CGT AAC AAG G-3′) and ITS-4 (5′- TCC TCC GCT TAT TGA TAT GC-3′) [36], and 17.5 μl of distilled water. The reaction mixture was subjected to 1 cycle of denaturation at 95°C for 4 min, 30 cycles of amplification at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, and a final extension cycle at 72°C for 10 min with a PCR Thermal Cycler MP (TaKaRa, Ohtsu, Japan).

The PCR products were confirmed by electrophoresis in 1.0% agarose in 1 × TBE buffer (0.04 M Tris-boric

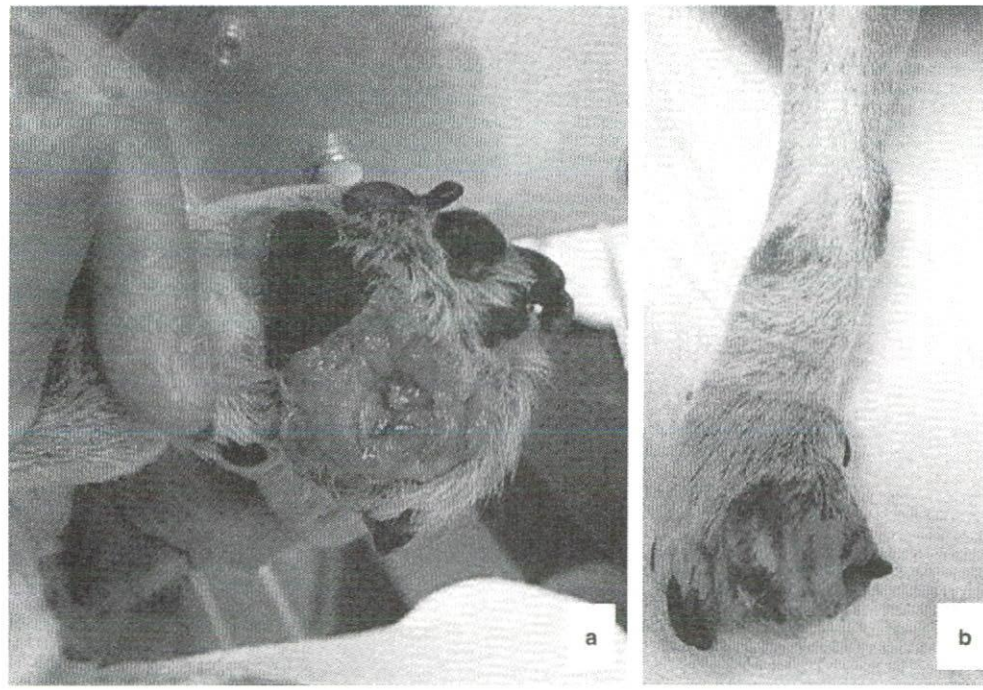


Fig. 2 Multiple skin ulcers of various sizes and with purulent exudates appeared on the left front paw of Case 2 (a and b).

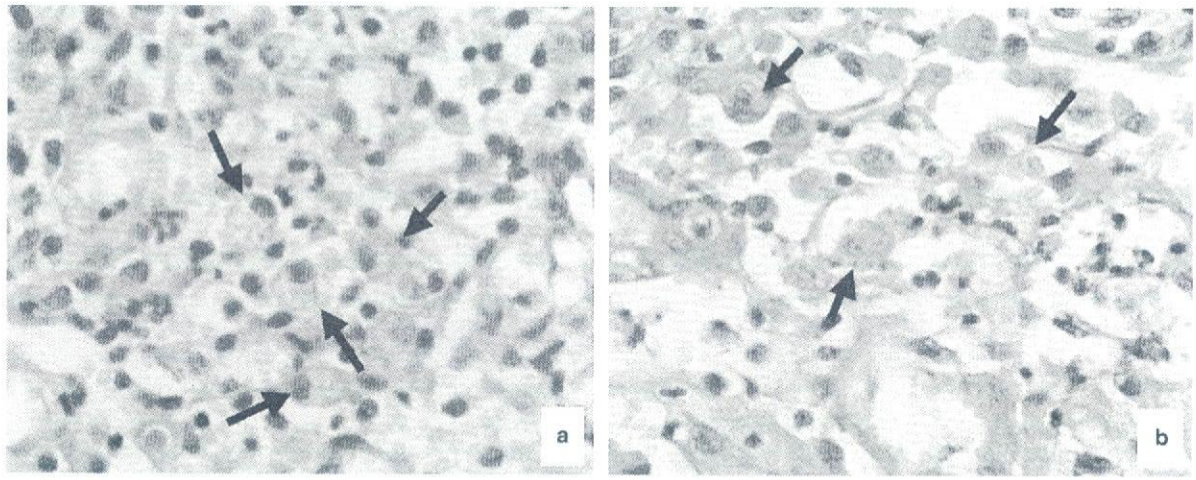


Fig. 3 Histopathological observation of the skin biopsy sample of Case 2 showed macrophages in the granulomatous tissue containing yeast-like cells, 1–4 μm in diameter (arrows): (a) hematoxylin-eosin (HE); (b) periodic acid Schiff (PAS); ×400.

acid, 0.001 M EDTA [pH 8.0]) followed by ethidium bromide staining. The PCR samples were purified using a PCR purification kit (QIAquick®, Qiagen Co. Ltd., Tokyo, Japan), and labeled with BigDye® Terminator Ver. 1.1 (Applied Biosystems, Foster City, CA., USA) following the manufacturer's protocol. The labeled samples were directly sequenced on an ABI PRISM® 3100 sequencer (Applied Biosystems, Foster City, CA., USA) using the primers ITS-5, ITS-4, ITS-2 (5′- GCT GCG TTC TTC ATC GAT GC-3′) and ITS3 (5′- GCA TCG ATG AAG AAC GCA GC-3′) [36]. DNA sequences were aligned using GENETEX-MAC genetic information processing software (Software Development Co., Ltd., Tokyo, Japan). The origins of the isolates and their GenBank accession numbers are shown in Table 2.

Detection of the ITS 1-5.8S-ITS 2 region of the rDNA gene cluster of H. capsulatum by nested PCR

Partial ITS 1/2 region sequences from pus and paraffin-embedded tissue samples of canine histoplasmosis were evaluated.

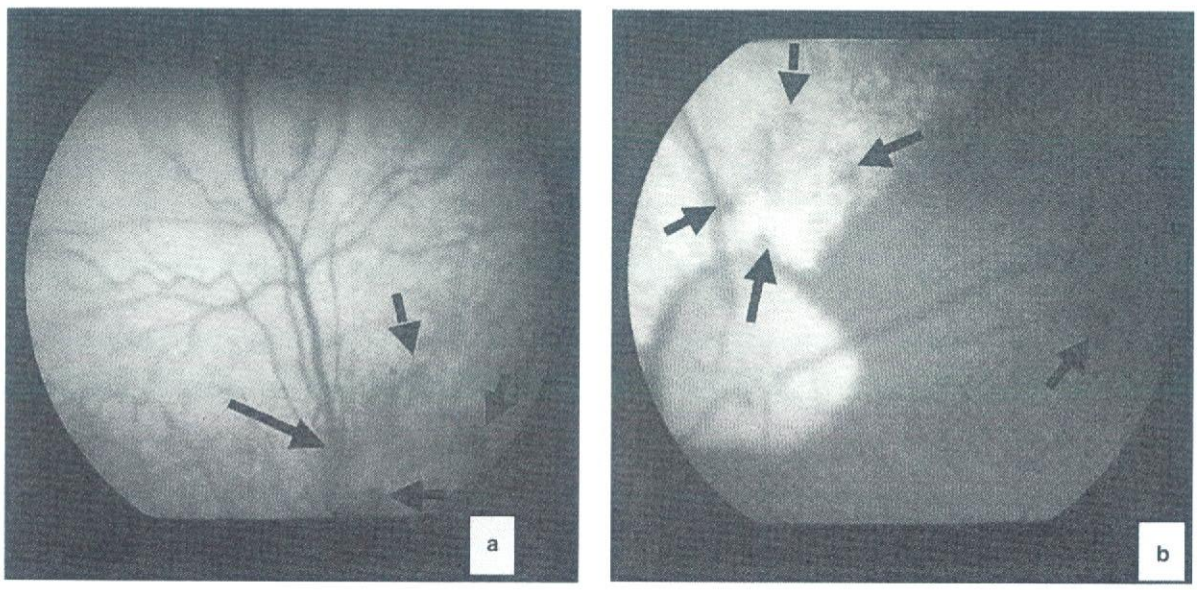


Fig. 4 The right ocular fundus (a and b) observed by indirect othalmoscopy indicated retinochoroiditis with granules in Case 2 (arrows).

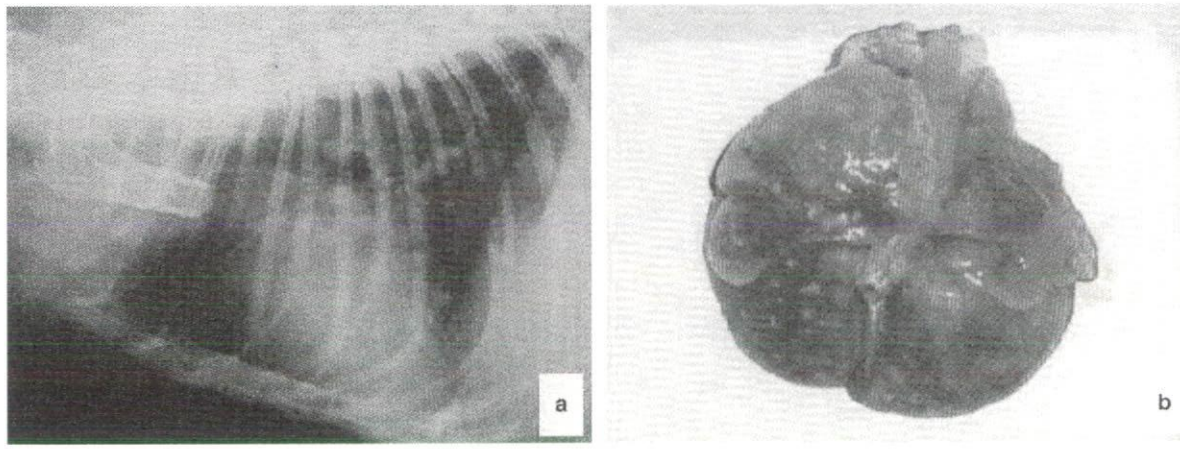


Fig. 5 A chest x-ray showed multiple foci 3 days before death (a), and abundant rice-sized nodules in the lungs found upon postmortem examination (b) in Case 2.

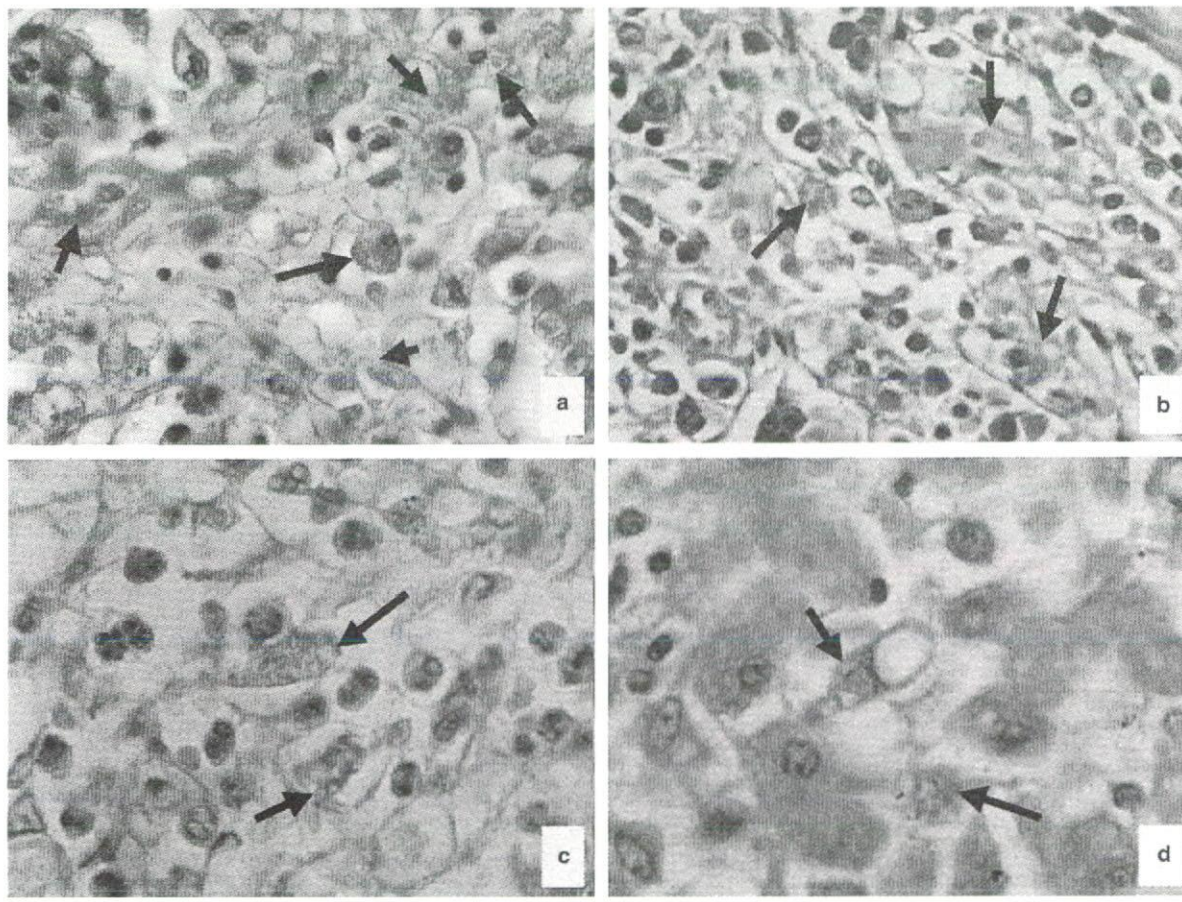


Fig. 6 PAS-positive yeast-like cells (arrows) in the neoplastic tissue in the lung (a), spleen (b), liver (c and d) in Case 2, ×400.

Table 2 Accession numbers and backgrounds of the sequences.

Accession No.	Variety	Clade (major variety)	Host	Area or country	Remarks
AB055228	capsulatum	I (C)	Human	USA	IFM 41329
AB055229	capsulatum	IV (C)	Human	USA	IFM 40752
AB055230	capsulatum	IV (C)	Human	USA	IFM 41659 = ATCC 26032
AB055231	capsulatum	V (C)	Human	Argentina	IFM 50248
AB055232	capsulatum	VI (C)	Human	Colombia	H59
AB055233	capsulatum	V (C)	Human	Brazil	H150
AB055234	capsulatum	V (C)	Human	Brazil	H196
AB055235	capsulatum	IV (C)	Human	Indonesia	H141
AB055238	capsulatum	IV (C)	Human	Thailand	HP3
AB055241	capsulatum	IV (C)	Human	Thailand	HP13
AB055244	capsulatum	I (C)	Human	Japan or Thailand	IFM 49109
AB055245	capsulatum	I (C)	Human	Japan or China	IFM 47750
AB071770	capsulatum	I (C)	Human	USA	H79 = ATCC11408
AB071821	capsulatum	IV (C)	Human	USA	H5
AB071822	capsulatum	IV (C)	Human	USA	H84
AB071823	capsulatum	IV (C)	Human	Colombia	H60
AB071824	capsulatum	IV (C)	Human	Colombia	H74
AB071825	capsulatum	IV (C)	Human	USA	H152
AB071826	capsulatum	V (C)	Human	Brazil	H200
AB071827	capsulatum	IV (C)	Human	Ecuador	E1
AB071830	capsulatum	I (C)	Human	Guatemala	EH332
AB071831	capsulatum	I (C)	Human	Mexico	EH372
AB071832	capsulatum	V (C)	Human	Argentina	IFM 50249
AB071842	capsulatum	V (C)	Human	USA	IFM 41330
AB071844	capsulatum	I (C)	Human	Australia	H157
AB211550	capsulatum	I (C)	Human	Thailand	IFM 54225 = DMST 19742
ACU18363	capsulatum	I (C)	Human	North America	No remark
AF038353	capsulatum	I (C)	Human	Canada	UAMH 7141
AF038354	capsulatum	IV (C)	Human	North America	UAMH 3536 = CDC B-1392
AF129538	capsulatum	I (C)	Human	USA	type A-1
AF129539	capsulatum	I (C)	Human	USA	type A-2
AF129540	capsulatum	I (C)	Human	USA	type A-3
AF129541	capsulatum	I (C)	Human	USA	type B
AF129542	capsulatum	I (C)	Human	USA	type C
AF129543	capsulatum	I (C)	Human	USA	type D
AF129544	capsulatum	I (C)	Human	USA	type E
AF129545	capsulatum	I (C)	Human	USA	type F
AF129546	capsulatum	I (C)	Human	USA	type G
AF129547	capsulatum	I (C)	Human	USA	type H
AF156892	capsulatum	IV (C)	Human	USA	ATCC 12700
AF322377	capsulatum	IV (C)	Human	USA	H2
AF322378	capsulatum	I (C)	Human	USA	H9
AF322379	capsulatum	IV (C)	Human	Colombia	H62
AF322380	capsulatum	I (C)	Human	Colombia	H64
AF322381	capsulatum	V (C)	Human	Colombia	H67
AF322382	capsulatum	VI (C)	Human	Colombia	H68
AF322383	capsulatum	VI (C)	Human	Colombia	H70
AF322384	capsulatum	IV (C)	Human	Colombia	H71
AF322385	capsulatum	IV (C)	Human	Panama	H81
AF458086	capsulatum	IV (C)	Human	Mexico	CDC-B6206
AB055246	duboisii	II (D)	Human	South Africa	H143
AB055247	duboisii	II (D)	Human	Senegal	H147
AB055248	duboisii	II (D)	Human	USA	IFM 5415
AB061781	duboisii	II (D)	Human	Belgium	H88 = ATCC 32281
AB071833	duboisii	II (D)	Human	Africa	H87 = ATCC 24294
AB071834	duboisii	II (D)	Human	USA	IFM 41332
AB071835	duboisii	II (D)	Human	Uganda	IFM 50954
AB214321	duboisii	II (D)	Human	Nigeria	IFM 52673
AB214322	duboisii	II (D)	Human	Nigeria	IFM 52674

Table 2 (*Continued*)

Accession No.	Variety	Clade (major variety)	Host	Area or country	Remarks
AB214323	duboisii	II (D)	Human	Nigeria	IFM 52675
AB214324	duboisii	II (D)	Human	Nigeria	IFM 52676
AF162917	duboisii	II (D)	Human	Guinea-Liberian border	ATCC 24294
AF322386	duboisii	II (D)	Human	Belgium	ATCC 32281
AF458085	capsulatum	II (D)	Human	Mexico	CDC-B6160
AB055236	capsulatum	III (C or F)	Human	United Kingdom	H142 = CBS 214.53
AB055237	capsulatum	III (C or F)	Human	China	H177 = D16a
AB055239	capsulatum	III (C or F)	Human	Thailand	HP4
AB055240	capsulatum	III (C or F)	Human	Thailand	HP12 = NIH 36-395-15
AB055242	capsulatum	III (C or F)	Human	Thailand	HP18 = NIH 37-466-131
AB055243	capsulatum	III (C or F)	Human	Thailand	HP23
AB055249	farciminosum	III (C or F)	Horse	Egypt	H95 = ATCC 58333
AB071828	capsulatum	III (C or F)	Human	Mexico	EH315
AB071836	farciminosum	III (C or F)	Horse	Poland	H174 = ATCC 32138
AB071837	farciminosum	III (C or F)	Horse	Egypt	H194 = ATCC 58335
AB071838	farciminosum	III (C or F)	Horse	Algeria	IFM 5418
AB071839	farciminosum	III (C or F)	Horse	USA	IFM 41334
AB071840	capsulatum	III (C or F)	Human	China	H178
AB071841	farciminosum*	III (C or F)	Human	Japan	IFM 41612
AB071843	capsulatum	III (C or F)	Human	Australia	A4a
AB211529	capsulatum	III (C or F)	Human	Japan	IFM 53166
AB211548	capsulatum	III (C or F)	Human	Thailand	IFM 54223 = DMST 19740
AB211549	capsulatum	III (C or F)	Human	Thailand	IFM 54224 = DMST 19741
AB211551	capsulatum	III (C or F)	Human	Thailand	IFM 54226 = DMST 19743
AB211552	capsulatum	III (C or F)	Human	Thailand	IFM 54227 = DMST 19744
AB214319	farciminosum	III (C or F)	Horse	origin unknown, stored in USA	IFM 41333
AB214320	farciminosum	III (C or F)	Horse	origin unknown, stored in USA	IFM 41335
AB214325	duboisii	III (C or F)	Human	Nigeria	IFM 52677
AB214326	farciminosum	III (C or F)	Horse	origin unknown, stored in USA	IFM 52876
AB214327	farciminosum	III (C or F)	Horse	origin unknown, stored in USA	IFM 52877
AB220055	ND	III (C or F)***	Dog	Japan	[7]
AB220056	ND	III (C or F)***	Dog	Japan	[7]
AB220057	ND	III (C or F)***	Dog	Japan	[8]
AB220058	ND	III (C or F)***	Dog	Japan	[9]
AB220059	ND	III (C or F)**	Dog	Japan	Added at the present study
AB220060	ND	III (C or F)**	Dog	Japan	Case 1
AB220061	ND	III (C or F)***	Dog	Japan	Case 2
AF322387	farciminosum	III (C or F)	Horse	Egypt	H90 = ATCC 58332

*Identified as *H. capsulatum* var. *farciminosum* based on the internal transcribed spacer (ITS1/2) region, **; derived from pus.***; derived from paraffin-embedded tissue sample.

Major varieties are indicated as C; clade mainly consisted of *H. capsulatum* var. *capsulatum*, as D; *H. capsulatum* var. *duboisii*, and as C or F; *H. capsulatum* var. *capsulatum* or *H. capsulatum* var. *farciminosum*, respectively.

The abbreviations for culture collections: ATCC; American Type Culture Collection, USA, CBS; Centraalbeaurau voor Schimmelcultures, The Netherlands, CDC; Center for Disease Controls and Prevention, USA, DMST; National Institute of Health Thai, Thailand, IFM is indicating the abbreviation of "Institute of Food Microbiology, Chiba University", which is the former name of Research Center for Pathogenic Fungi and Microbial Toxicoses.

The pus sample from Case 1 was collected with sterile cotton buds. The cotton tip was cut off and placed in a sterilized microtube of 1.5 ml in volume to which 0.5 ml of 70% ethanol was added before vortexing. The sample was washed 3 times with sterile water by centrifuging at 12,000 rpm (13,201 *g*) for 5 min, and 0.5 ml of DEXPAT™ solution was added. The cotton tip and the solution were incubated together at 100°C for 10 min and centrifuged at 12,000 rpm (13,201 *g*) for 10 min. The supernatant was processed for PCR.

Three sections of 10 micrometer-thick paraffin tissue were placed into a microtube (1.5 ml), and 0.5 ml of DEXPAT™ solution was added. After being boiled for 10 min, the tube was centrifuged at 12,000 rpm (13,201 g) for 10 min. The supernatant was processed for PCR.

We placed 2.5 μl of the sample, 2.5 μl of 20 pM of the primers ITS 4 and ITS 5 and 17.5 μl of distilled water in

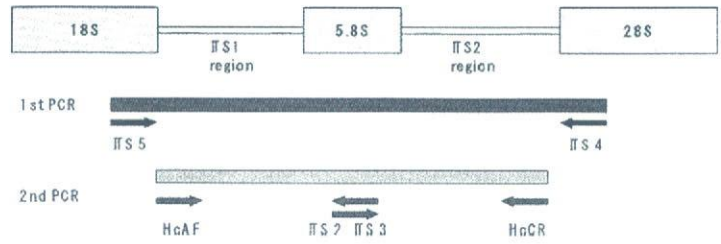


Fig. 7 A primer map for detecting partial sequences of the ITS1/2 region from paraffin-embedded tissue samples or pus of canine histoplasmosis. Primers ITS-5 and ITS-4 were used for the first PCR, and HcAF and HcCR were used for the second one. In addition, primers ITS-2 and ITS-3 were used for labeling at the sequencing.

a 0.2-ml PCR tube with one Ready-to-Go bead (Amersham Pharmacia, Tokyo, Japan). The reaction mixture was subjected to 1 cycle of denaturation at 95°C for 4 min, 40 cycles of amplification at 94°C for 1 min, 50°C for 1.5 min, and 72°C for 2 min, and a final extension cycle at 72°C for 10 min with PCR Thermal Cycler MP (TaKaRa, Ohtsu, Japan). For amplification in the second-round PCR, we used 2.5 μl of the first PCR products diluted 10 or 100 times with 2.5 μl of 20 pM of the following internal primers, designed on the basis of the ITS1/2 region of the rDNA gene cluster of *Ajellomyces capsulatus*, the teleomorph of *H. capsulatum*, published as GenBank accession numbers AB055230 and AF322381: HcAF (5′-CAC GCC GTG GGG GGC TGG GAG CCT-3′, corresponding to AB055230 at bp 1–24 and AF322381 at bp 33–56) and HcCR (5′-ATG GTG GGC RGG AGC CGG CC-3′, with a complementary sequence of 5′-GGC CGG CTC CYG CCC ACC AT-3′, corresponding to AB055230 at bp 492–511 and AF322381 at bp 531–550.). The primer map is shown in Fig. 7.

The PCR conditions for the second round were the same as those for the first round. The second-round PCR products were confirmed as described above. In addition, a serial dilution of fungal DNA of *H. capsulatum* using isolate IFM 5396 stored in our culture collection adjusted from 1.0 ng/μl to 1.0 fg/μl and a negative control using distilled water were examined. The detection limit was 2.5 fg of fungal DNA (Fig. 8).

The PCR-amplified samples were purified using a PCR purification kit (QIAquick®), labeled with BigDye® Terminator Ver. 1.1 (Applied Biosystems) following the manufacturer's protocol. The labeled samples were directly sequenced on an ABI PRISMTM 3100 sequencer (Applied Biosystems) using the primers HcAF, HcCR, and ITS-2 and ITS-3 [36], which are complementary to each other, and which correspond to AB055230 at bp 233–252 and AF322381 at bp 272–291, respectively. DNA sequences were aligned by GENETEX-MAC genetic information processing software (Software Development Co., Ltd.).

In addition, the present study also re-evaluated sequences of paraffin-embedded tissue or pus samples of previously reported canine histoplasmosis in Japan [7–9], which had been stored in a freezer for 2–5 years because some of them were not deposited in GenBank and/or were shorter sequences than the ones generated in the present study.

The nucleotide sequences derived from paraffin-embedded and pus were deposited in the DNA Data Bank of Japan (DDBJ) under the accession numbers shown in Table 2.

Ninety-seven nucleotide sequences from *Ajellomyces capsulatus* isolates or derived from canine samples deposited in GenBank shown in Table 2 were aligned using the computer program CLUSTALX (Version 1.8) [37] followed by manual adjustments with a text editor together with the present nucleotide sequences derived

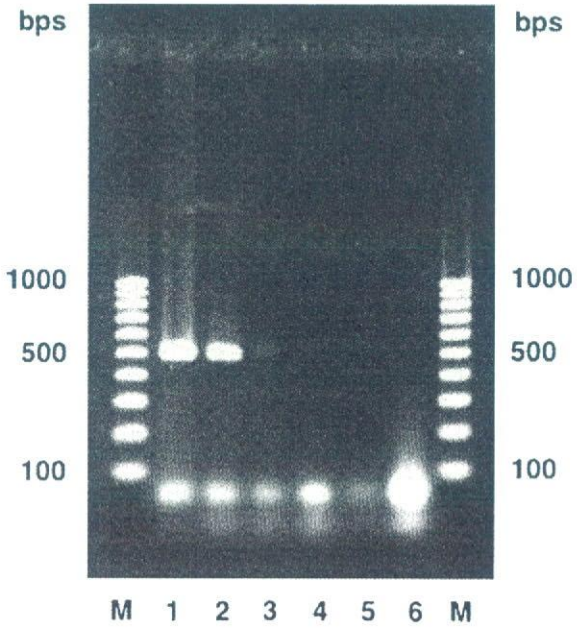


Fig. 8 Detection limit of nested PCR for the ITS1/2 region of *H. capsulatum* using DNA from pus [9]. M, marker; 1, DNA from pus; 2, fungal DNA at 2.5 ng; 3, 250 fg; 4, 25 fg; 5, 2.5 fg; and 6, negative control.

from clinical isolates and canine origins. Phylogenetic analyses were performed with PAUP v4.0b10 [38] using a heuristic search for maximum parsimony trees. Base-pair composition and transition and or transversion patterns of the dataset were estimated by PAUP v4.0b10. Bootstrap values were calculated over 1000 replicates to assess branch topology. A phylogenetic tree was selected from 1,000 un-rooted trees and was drawn by Tree View PPC [39] (Roderic D. M. Page, Glasgow, Scotland, UK, 1998; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Clades were supported by bootstrap values above 50%.

Results

Case 1 was a typical Japanese case of canine histoplasmosis, showing cutaneous lesions without dissemination. Nested PCR and sequencing for detecting a partial sequence of the ITS1/2 region from the pus revealed a 100% sequence identity to GenBank entry AB211551 of *Ajellomyces capsulatus* derived from a clinical isolate of *H. capsulatum* from Thailand. The sequence was deposited as AB220060.

Case 2 was confirmed to be the first case of disseminated canine histoplasmosis following mammalian carcinoma in Japan, as diagnosed by clinical characteristics, histopathological examination, chest X-ray, ocular fundoscopy and molecular biological data. Nested PCR and sequencing for detection of partial sequence of the ITS1/2 region from the paraffin-embedded tissue samples revealed more than 99% identity to the GenBank entry AB220060 derived from Case 1. The sequence was deposited as AB220061.

There were three different sequences of the ITS1/2 region derived from autochthonous canine histoplasmosis in Japan. The revised sequence from a previous case [7] consisted of 516 base pairs, while all other sequences consisted of 518 base pairs. The sequences of the ITS1/2 sequences from the present 2 cases and of 4 previous reported cases were closely related. The sequence from Case 1 (AB220060) and the revised sequences from three previously reported cases (AB220056-59) [7–9] showed 100% identity to the GenBank sequence AB211551 of *Ajellomyces capsulatus* derived from a clinical isolate of *H. capsulatum* from a patient with disseminated histoplasmosis and HIV infection in Thailand. The sequence from Case 2 (AB220061) and the one from a previous case [7] (AB220055) showed more than 99% identity to AB211551. In addition, the identities of the sequences among autochthonous canine histoplasmosis in Japan were more than 98%. The sequence derived from an autochthonous cutaneous [30] and a disseminated human case [31] in Japan deposited in GenBank as AB071841 and AB211529 also showed more than 98% identity to those from canine histoplasmosis. In addition, a sequence derived from a human case in the USA (AF156892) had 95% identity.

The nucleotide sequence accession numbers of the 15 isolates of *H. capsulatum* and those of seven sequences from six cases of canine histoplasmosis are shown in Table 2.

One of the 1,000 most-parsimonious trees obtained from heuristic searches based on the 97 sequences of the ITS1/2 region is shown in Fig. 9. The tree consists of six clades with two independent sequences derived from clinical isolates of *H. capsulatum* var. *capsulatum* from Argentina (AB071832) and Colombia (AF322381).

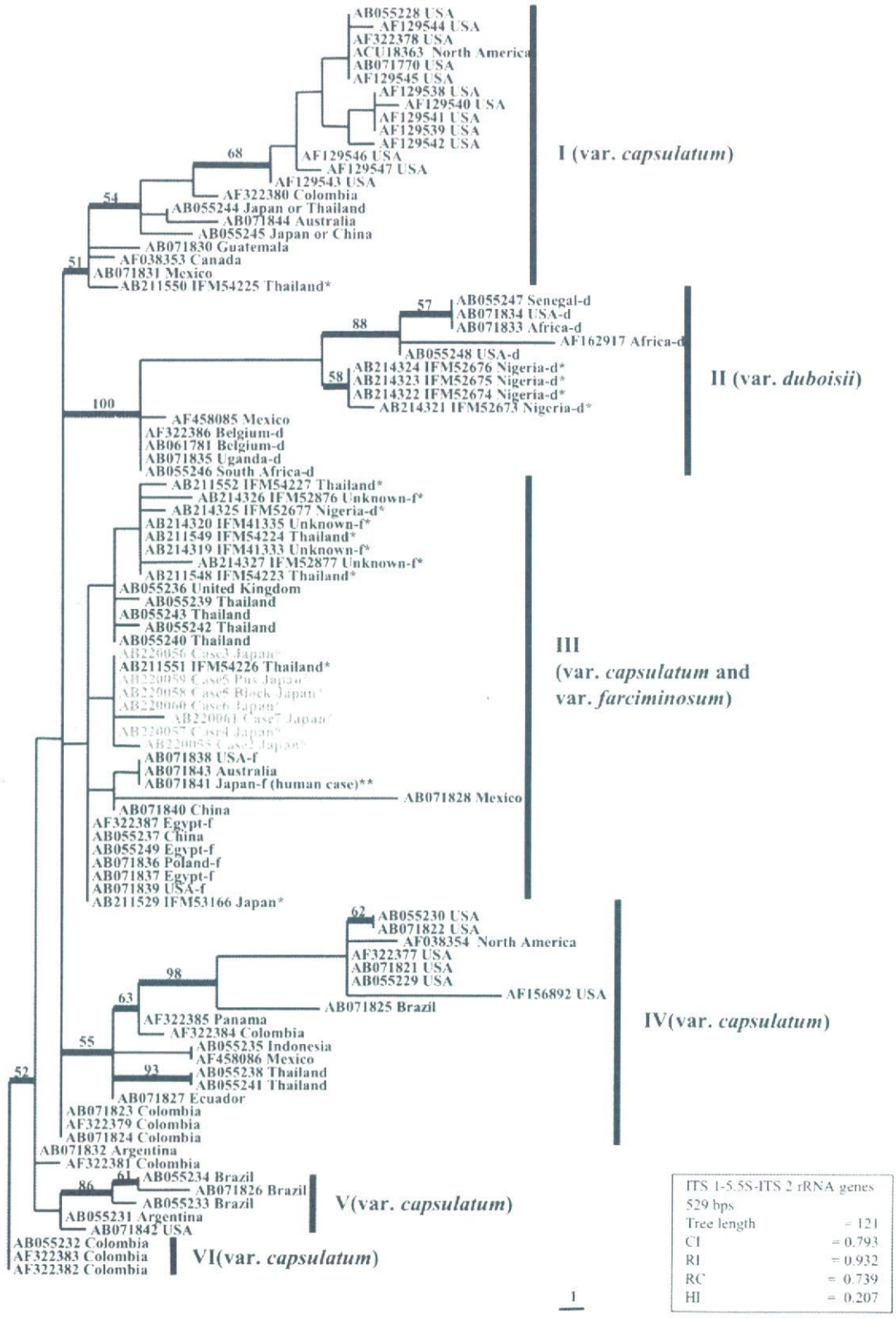
The predominant sequence in clades I, IV, V and VI was *H. capsulatum* var. *capsulatum*, that in the clade II was *H. capsulatum* var. *doboisii*, and that in clade III was a mixture of *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *farciminosum*.

Clade I, containing *H. capsulatum* var. *capsulatum* sequences from the USA, North America, Canada, Colombia, Thailand, Australia, Japan or China, Guatemala and Mexico, was supported by a 51% bootstrap value.

Clade II, containing *H. capsulatum* var. *doboisii* sequences from the USA., Senegal, Nigeria, Belgium, Uganda, South Africa and Africa, and one sequence of *H. capsulatum* var. *capsulatum* from Mexico, had 100% bootstrap support.

Clade III had no significant bootstrap support, and contains sequences derived from *H. capsulatum* var. *farciminosum* from the USA, Egypt, and Poland, and from *H. capsulatum* var. *capsulatum* from Thailand, United Kingdom, Japanese autochthonous human [30,31] and canine cases [7–10], Australia, China, as well as from *H. capsulatum* var. *doboisii* from Nigeria.

Fig. 9 One of the 1,000 most-parsimonious trees obtained from heuristic searches based on ITS1/2 region sequences. Bootstrap support values above 50% are indicated at the nodes. The bar indicates 1 base difference. CI, consistency index; RI, retention index; RC, rescaled consistency index; and HI, homoplasy index. Data are shown with accession numbers, country, and remarks; d, identified as *H. capsulatum* var. *duboisii*; f, *H. capsulatum* var. *farciminosum*; no remark, *H. capsulatum* var. *capsulatum*. *, sequence analyzed in the present study. Case numbers as stated in Table 1. Letters in red were sequences derived from canine histoplasmosis and those in blue were from human clinical isolates at the present study. **, sequence derived from a Japanese autochthonous case determined by molecular biological data. The predominant varieties are parenthesized.


AB055228 USA
AF129544 USA
AF322378 USA
ACU18363 North America
AB071770 USA
AF129545 USA
AF129538 USA
AF129540 USA
AF129541 USA
AF129539 USA
AF129542 USA
68
AF129546 USA
AF129547 USA
AF129543 USA
54
AF322380 Colombia
AB055244 Japan or Thailand
AB071844 Australia
AB055245 Japan or China
AB071830 Guatemala
51
AF038353 Canada
AB071831 Mexico
AB211550 IFM54225 Thailand*
I (var. capsulatum)
57
AB055247 Senegal-d
AB071834 USA-d
88
AB071833 Africa-d
AF162917 Africa-d
AB055248 USA-d
58
AB214324 IFM52676 Nigeria-d*
AB214323 IFM52675 Nigeria-d*
AB214322 IFM52674 Nigeria-d*
AB214321 IFM52673 Nigeria-d*
II (var. duboisii)
100
AF458085 Mexico
AF322386 Belgium-d
AB061781 Belgium-d
AB071835 Uganda-d
AB055246 South Africa-d
AB211552 IFM54227 Thailand*
AB214326 IFM52876 Unknown-f*
AB214325 IFM52677 Nigeria-d*
AB214320 IFM41335 Unknown-f*
AB211549 IFM54224 Thailand*
AB214319 IFM41333 Unknown-f*
AB214327 IFM52877 Unknown-f*
AB211548 IFM54223 Thailand*
AB055236 United Kingdom
AB055239 Thailand
AB055243 Thailand
AB055242 Thailand
AB055240 Thailand
AB220056 Case3 Japan*
AB211551 IFM54226 Thailand*
AB220059 Case5 Pus Japan*
AB220058 Case8 Blood Japan*
AB220060 Case6 Japan*
AB220061 Case7 Japan*
AB220057 Case4 Japan*
AB220055 Case2 Japan*
AB071838 USA-f
AB071843 Australia
AB071841 Japan-f (human case)**
AB071828 Mexico
AB071840 China
AF322387 Egypt-f
AB055237 China
AB055249 Egypt-f
AB071836 Poland-f
AB071837 Egypt-f
AB071839 USA-f
AB211529 IFM53166 Japan*
III
(var. capsulatum and
var. farciminosum)
62
AB055230 USA
AB071822 USA
AF038354 North America
AF322377 USA
AB071821 USA
AB055229 USA
98
AF156892 USA
63
AB071825 Brazil
AF322385 Panama
AF322384 Colombia
55
AB055235 Indonesia
AF458086 Mexico
93
AB055238 Thailand
AB055241 Thailand
52
AB071827 Ecuador
AB071823 Colombia
AF322379 Colombia
AB071824 Colombia
AB071832 Argentina
AF322381 Colombia
IV(var. capsulatum)
61
AB055234 Brazil
86
AB071826 Brazil
AB055233 Brazil
AB055231 Argentina
AB071842 USA
V(var. capsulatum)
AB055232 Colombia
AF322383 Colombia
AF322382 Colombia
VI(var. capsulatum)
1
ITS 1-5.5S-ITS 2 rRNA genes
529 bps
Tree length = 121
CI = 0.793
RI = 0.932
RC = 0.739
HI = 0.207

Clade IV, containing *H. capsulatum* var. *capsulatum* sequences from the USA., North America, Brazil, Panama, Colombia, Mexico, Thailand and Ecuador, was supported by a 55% bootstrap value.

Clade V, containing *H. capsulatum* var. *capsulatum* sequences from the USA, Brazil and Argentina, had no significant bootstrap support.

Clade VI, containing *H. capsulatum* var. *capsulatum* sequences from Colombia, was supported by a 52% bootstrap value.

Discussion

There are two clinical types of canine histoplasmosis reported by Rhoades [40]: the first may involve the pulmonary system and related lymph nodes and tends to be more benign, and the second is a disseminated form that affects not only these structures but also the liver, spleen, intestine and bone marrow. The characteristics of the former type shows small nodes in the lungs and chronic cough, while the latter type has a poor prognosis, accompanied by severe respiratory disorders, swollen lymph nodes, hepatosplenmomegaly, and dissemination to the small intestine, heart, kidney and skin, because of the extended intracellular invasiveness of the yeast cells. On the other hand, a classification proposed by Nielsen is as follows: (i) an acute, fatal, and disseminating disease; (ii) an advanced chronic form; and (iii) a non-fatal form [41]. Most cases of canine histoplasmosis in endemic areas have been disseminated, acute, have had severe symptoms with a poor prognosis, and have always involved pulmonary lesions [14–19,24], except for one cutaneous case from Australia [28]. In contrast, cases from Japan have been reported as a localized disease of the skin or gingiva without pulmonary involvement [6–10].

Case 1 was typical canine histoplasmosis from Japan, having cutaneous lesions without dissemination. A similar disease process showing multiple dermal nodules without visceral dissemination was found in an Australian case [28]. The common point is that both countries are located on the Pacific basin rim. However, any epidemiological relationship is unclear.

On the other hand, case 2 was confirmed to be the first case of disseminated canine histoplasmosis following metastatic mammary carcinoma. This case was diagnosed by clinical manifestations, histopathological examination, chest radiography, ocular fundoscopy images similar to canine histoplasmosis reported from endemic areas [15,29], and molecular biological data. These findings led us to conclude that both cutaneous and disseminated forms of canine histoplasmosis exist in Japan.

The infectious routes of the canine histoplasmosis in Japan were thought to be *via* external injuries such as trauma, mange and surgical operation for castrations [7], which is in contrast to the fact that inhalation is thought to be general route for the infection in endemic areas [5]. It was thought that case 1 and previously reported cutaneous cases might have been caused by external injuries, while case 2 might be suspected to be caused by both external injury and inhalation. Either the primary skin ulcer was caused by metastases of carcinoma and the fungus later arose in the lesion and disseminated, or inhalation of the fungus was followed by dissemination. There was no marked change in the chest X-ray image at the onset of cutaneous histoplasmosis, which indicated that dissemination in case 2 followed the infection of cutaneous neoplastic ulcers.

In general, a definite diagnosis for histoplasmosis should be accompanied by the isolation of the causative agent *H. capsulatum* from clinical materials. However, the isolation rates from human and animal cases in Japan are extremely low. Only about 30% of diagnosed cases in humans were accompanied by isolation of the causative agent [5], while no isolation was obtained in multiple animal cases [6–10,12]. The present canine cases were also culture negative. Therefore, clinical signals and other supportive examinations such as histopathological findings from smears and tissues, complement binding reaction, hemagglutination tests, immunodiffusion technique, latex agglutination and skin tests [42], and molecular biological techniques [7–9,43] have to be used for diagnoses of histoplasmosis. In addition the Histoplasma Immunodiffusion Reagents kit from Immuno-Mycologics, Inc., Norman, OK, USA, was not suitable for the diagnosis of Japanese canine histoplasmosis case. Differences in antigenic profiles between *H. capsulatum* in the USA and Japan, and/or differences in host-dependent reactions between humans and dogs could possibly explain this result.

Treatment of canine histoplasmosis in Japan has not been standardized yet. Oral administration of itraconazole was effective in a previously reported skin case [7]. However, antifungal agents were not effective in either of the present cases, even amphotericin B, which was effective in an American case [42], failed. According to Li *et al.*, voriconazole was recommended for treatment of histoplasmosis [44]. However, the medicine could not be commercially provided for dogs infected with *Histoplasma* spp. in Japan. Although a surgical excision was effective on canine cases [7,14] and two human cases [45,46], the procedure was not suitable for case 2 and simultaneously produced a severe aggravation of both carcinoma and histoplas-

mosis. A background of immunosupression was suspected in case 2. However, we did not evaluate the dog's CD4 and CD8 levels. Taking the clinical state of the dog into account euthanasia might be the inevitable option, which should be considered in moribund cases [6,8,14].

There were 3 sequence types of the ITS1/2 sequences derived from canine histoplasmosis cases in Japan, showing more than 99% identity to each other and located in the same clade. The species variation of the ITS1/2 region among *H. capsulatum* isolates is below 5% [8], permitting the identification of the sequences as belonging to *H. capsulatum*. The sequences from 4 out of 6 canine cases showed 100% identity to AB211551, derived from a clinical isolate of *H. capsulatum* from an HIV-infected patient in Thailand. The results indicated that both human and dogs could be infected with the same genotype of *H. capsulatum*. In addition, the detection of the gene sequence from the pus sample of case 1 and the coincidence of the sequences between the tissue and the pus samples of the previously reported case by Nishifuji *et al*. [9] indicate that a pus sample obtained non-invasively can be useful and adequate for the molecular biological diagnosis of histoplasmosis.

The homology of the sequences among canine cases in Japan, among Japanese autochthonous cutaneous (AB071841) [30] and a disseminated (AB211529) [31] human and canine histoplasmosis case were more than 98%, and that of the sequence derived from the human case in the USA (AF156892) was 95%, indicating that the sequences derived from autochthonous histoplasmosis from both humans and canines in Japan might have a close phylogenetic relationship.

The varieties of *H. capsulatum* based on ITS1/2 sequences are controversial. According to Kasuga *et al*. [47,48] and Tamuta *et al*. [32], the varieties of *H. capsulatum* categorized by hosts and geographical distribution might be meaningless or may cause confusion; they recognized that there were several geographically dependent genotypes of *H. capsulatum* based on the ITS1/2 sequences or multiple gene sequence analysis.

Geographically dependent genotypes might be improbable except for the *H. capsulatum* var. *duboisii* isolates of African origin. In fact, the genotypes of *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *farciminosum* are spreading worldwide [32,47,48]. More people travel around today internationally. Infections also became global after the Crusades, the Age of Great Voyages, the Colonial and Slave Importation Periods, and especially after World War II. A certain genotype of *H. capsulatum* might localize in special regions or be spread following travel, migration, war and trade.

The unrooted tree by Kasuga *et al*. [47] showing a star-like phylogeny without correlation between the genotypes and neighboring areas might be the most obvious evidence of transferring causative agents for infections accompanied with human activities. The representative examples are scattered clades with sequences originating in Latin American countries, which have been known in recent decades for emigration. The involvement of the Eurasia clade in a Latin American clade was also incompatible with the existence of geographically dependent genotypes. An excellent example for the importation of a new genotype of fungal clones accompanied with human activities is the cryptococcosis outbreak on Vancouver Island [49], which backed up the disavowal of geographically dependent genotypes of *H. capsulatum*. However, both Kasuga *et al*., and Tamura *et al*. [32,47,48] showed an independent clade or cluster consisting of *H. capsulatum* var. *duboisii*-derived sequences indicating that the existence of *H. capsulatum* variety *duboisii* was obvious. We also undoubtedly recognized that *H. capsulatum* var. *duboisii* had a correlation between genotypes and varieties identified by geographical and morphological means. In fact, *H. capsulatum* var. *duboisii* was predominantly located in the clade II. In addition, the fact that other clades such as clades I, IV, V, and VI included *H. capsulatum* var. *capsulatum*, suggested that there was no objection to identifying the variety as *capsulatum*.

On the other hand, the mixture of varieties in clade III consisting of *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *farciminosum* proposed a delicate question: why were both varieties located in the same clade and some of their sequences coincident? The bootstrap support value was not significantly different from the other clades. However, the fact that others were at least 50% could prove that the clade consists of particular genotypes. The genotypes derived from *H. capsulatum* var. *farciminosum* were limited to clade III, suggesting the correlation between the genotypes and the old-fashioned classification of the variety. Furthermore, some of the sequences located in the Eurasia clade proposed by Kasuga *et al*. [47], and Tamura *et al*. [32] corresponded to clade III, although they proposed that DNA polymorphism in the ITS1/2 region was not prevalent enough to resolve the varieties of *H. capsulatum*.

However, Japanese autochthonous histoplasmosis in both humans and dogs was caused by *H. capsulatum* var. *farciminosum*-related clones suggested that *H. capsulatum* var. *farciminosum* can cause infections

not only to horses but also to humans and dogs, and does not only cause cutaneous but also systemic infection regardless of the host species. This had already been proposed previously on the basis of its clinical manifestation and the historical background of equine epizootic lymphangitis in our country [7–9].

In conclusion, autochthonous human and canine histoplasmosis in Japan emphasise the current status of the three varieties of *Histoplasma capsulatum* according to the host species and this topic remains a fertile topic for further investigation.

Acknowledgements

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Lecythophora hoffmannii isolated from a case of canine osteomyelitis in Japan

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A 2-year-old spayed female mongrel dog showed claudication with abnormal ossification containing fungal cells detected by biopsy. The dog was treated with ketoconazole and itraconazole perorally for 5 months; however, the osteomyelitis became aggravated, and an amputation from the scapula was performed. The right superficial cervical lymph node became swollen 5 months after the operation. The lymph node contained PAS positive fungal elements and a portion of tissue produced mycelial fungal growth on potato dextrose agar supplemented with chloramphenicol. The culture was identified as *Lecythophora hoffmannii* based on morphology, physiology and 100% identity in the sequence of the D1/D2 domain of the large subunit ribosomal RNA gene of the fungal species in the GenBank database (accession number AB100627). In addition, the sequence from the present isolate was submitted as AB189164. The isolate showed resistance to antifungal agents, i.e., amphotericin B, 5-FC, fluconazole, itraconazole, miconazole and micafungin. The dog developed cachexia 2 months after the onset of lymphadenopathy, and was euthanized on the 459th day after onset of clinical symptoms. This was the first disseminated case of *L. hoffmannii* infection in Japan.

Keywords dog, *Lecythophora hoffmannii*, osteomyelitis

Introduction

Lecythophora hoffmannii can be a causative agent for emerging fungal infection. The fungal species exists as a saprophyte in the environment [1–3] and has been isolated from soybean curd (tofu) [4]. *L. hoffmannii* infections have been reported as resulting in human subcutaneous abscess [5], keratitis [6], and sinusitis [7], and abortion of cattle [8]. In addition, a closely related species; *L. mutabilis*, has been described as the causative agent for human peritonitis [9], endocarditis [10,11], endophthalmitis [12,13], keratomycosis [14], phlegmone (sited from the origin of UAMH 9863, http://www.devonian.ualberta.ca/uamh/). In addition, it has been reported associated with an outbreak in a large-scale zebrafish colony (*Brachydanio rerio*) [15] and isolated from a fruit-eating bat (*Eidolon helvum*) [16]. However, there have been no reports of fungal infections caused by *Lecythophora* spp. in Japan.

The present study introduces the first case of *L. hoffmannii* infection in Japan and involved osteomyelitis in a household dog.

Materials and methods

Case

A 2-year-old female mongrel dog from Tokyo had a history of bacterial external otitis at 8 months and pyoderma at 1 year of age. The dog was kept in the house and took occasional walks with a young 8-month-old female companion animal which was

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housed with it. The dog received vaccinations for rabies, canine distemper, parvovirus, adenovirus type 2, parainfluenza virus, coronavirus and leptospire on a routine schedule, and was fed commercial dry chow.

The main symptom was claudication, with the middle part of the right brachium swollen which caused compression pain. There were no superficially swollen lymph nodes on the first visit. The roentgenogram image showed an abnormal ossification at the right brachium (Fig. 1a). The biopsy sample contained mycelial cells colored slightly brown by hematoxylin and eosin staining and were positive in periodic acid Schiff's reaction (Fig. 2a and b). The physiological and biochemical studies of the blood were within normal limits.

Five months' oral administration of an antifungal regimen consisting of 6 mg/kg of ketoconazole twice a day for 3 months, and then 5 mg/kg of itraconazole once a day, did not improve the dog's condition. The abnormal ossification showed aggravation and intense enlargement was detected in the middle part of the right brachium both in microscopy and a roentgenogram image at the 86th day from the onset (Fig. 1b). Claudication and pain were moderated by administration of an anti-inflammatory drug. The brachium became intensely swollen on the 170th day (Fig. 1c). The owners were informed that it was impossible to treat the dog by medication alone, and surgery was recommended. An amputation from the scapula was performed and the bone marrow was found to have become severely purulent (Fig. 1d). The dog continued to receive 5 mg/kg of itraconazole once a day for 2 months.

The right superficial cervical node became swollen 5 months after the operation. The organ contained PAS-positive fungal elements surrounded by giant cells or free in the necrotic legion (Fig. 2c–e). Portions of the lymph nodes were cultured on potato dextrose agar (PDA) supplemented with 100 mg/l of chloramphenicol at 25°C. After 3 days of incubation, numerous

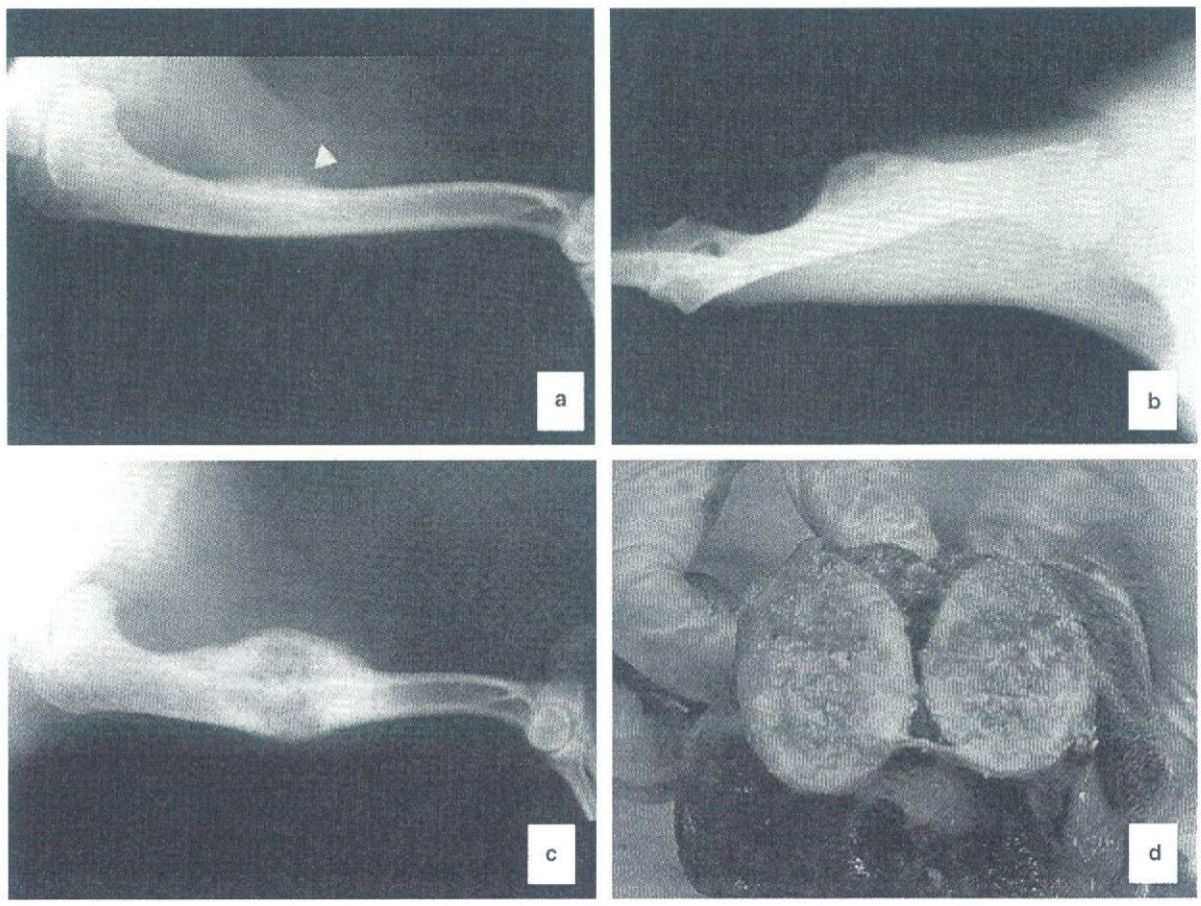

Fig. 1 An abnormal ossification at the right brachium (a), the ossification showed aggravation 2 months after the onset (b) and at the 170th day (c), and the section of the region showing necrosis at the amputation (d).

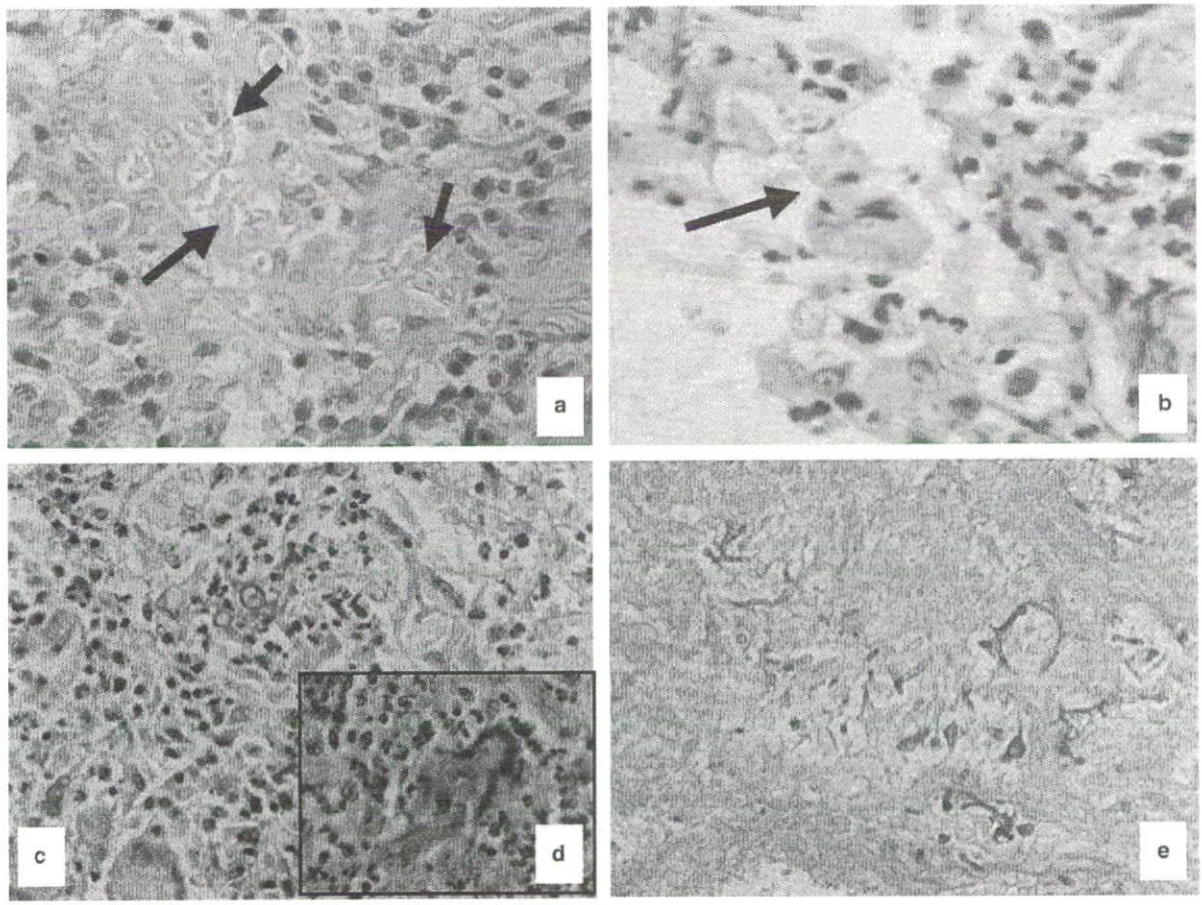

Fig. 2 The biopsy sample stained by haematoxylin and eosin contains hyaline mycelial cells colored with slightly brown (a, arrows, ×400), those positive in periodic acid Schiff's reaction (b, ×400), the right superficial cervical node contained PAS positive fungal element (c, ×200) surrounded by giant cells (d, ×200) or free in the necrotic legion (e, ×200).

yellowish mycelial colonies were found in the cultures. The causative agent was identified as *Lecythophora hoffmannii* based on mycological and molecular biological studies. The dog developed cachexia 2 months after the onset of lymphadenopathy with tonic convulsion, and was euthanized at the 459th day.

Isolation

Abundant homogenous yellowish small mycelial colonies were noted 3 days after inoculation at 25°C on PDA supplemented with 100 mg/l of chloramphenicol. The fungal isolate was subcultured (1) on a Sabouraud dextrose agar (SDA) and PDA plates at 25°C for 1 month, (2) on a PDA slant at 25°C for 2 months, and (3) on a PDA agar block at 25°C for 3 weeks in a micro-culture system. The isolate was deposited as IFM 53859 in the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan.

In addition, the maximum growth temperature was evaluated on PDA slants at 37, 39, 42 and 45°C.

Molecular biological identification based on D1/D2 domain of large subunit ribosomal RNA gene sequence

The D1/D2 domain of large subunit ribosomal RNA gene sequence was compared with the Gen Bank database [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=nucleotide]. Briefly, fungal DNA was extracted with a DEXPAT® Kit (TaKaRa, Ohtsu, Japan) following the manufacturer's protocol with slight modification from cultures incubated on PDA slants at 25°C for 1 to 2 weeks. Approximately 100 μl of fungal mass was transferred to a sterilized microtube (1.5 ml), and homogenized with 0.5 ml of DEXPAT® solution with a plastic pestle. The mixture was incubated at 100°C for 10 min and centrifuged at 12,000 rpm (13,201 *g*) for 10 min. The supernatant was

used as the DNA sample. The sequence of the D1/D2 LSU rRNA gene was processed by a standard method described by Kurtzman and Robnett, 1997 [17], through amplification with the following set of primers. NL-1: 5′- GCA TAT CAA TAA GCG GAG GAA AAG-3′and NL-4: 5′- GGT CGG TGT TTC AAG ACG G-3′.

Then, 2.5 μl of DNA extract, a piece of Ready-to-Go beads (Amersham Pharmacia Tokyo, Japan), 2.5 μl of 10 pM of the above primers, and 17.5 μl of distilled water were mixed. Amplification was performed for an initial denaturing step of 4 minutes at 95°C, 30 cycles of 1 minute at 94°C for DNA denaturation, 90 seconds at 50°C for primer annealing, 2 minutes at 72°C for primer extension, a final extension of 10 minutes at 72°C, and a 4°C soak.

The amplified PCR product was confirmed by electrophoresis on 1.0% agarose in 1 ×TBE buffer (0.04 M Tris-boric acid, 0.001 M EDTA [pH 8.0]) and ethidium bromide staining. The amplified product was purified with a PCR purification kit (QIAquick PCR Purification Kit, QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Cycle sequencing was performed with BigDye fluorescent-labeled DyeDeoxy protocols (BigDye Terminator ver. 1.01; Amersham Pharmacia, Piscataway, NJ, USA) as follows: 0.25 min at 96°C, 0.5 min at 55°C, and 4 min at 60°C for 25 cycles, followed by a 4°C soak. All sequencing reactions were run on an automated DNA sequencer (3100, Applied Biosystems, Foster City, CA, USA) after purification by ethanol precipitation. To determine the sequences, two internal primers, NL-2; 5'-CTT GTT CGC TAT CGG TCT C-3′ and NL-3; 5′-GAG ACC GAT AGC GAA CAA G- 3′, were adopted together with NL-1 and NL-4.

Susceptibility testing

Susceptibility tests were performed according to the broth microdilution modified method of the NCCLS M38-A [18] accepted standard using RPMI 1640 medium (Sigma, Poole, UK) buffered to pH 7.0 with MOPS (Sigma) using serial concentrations amphotericin B, 5-FC, itraconazole, miconazole, fulconazole and micafungin. Note that the latter three antifungal were included even though the method was originally described for use with amphotericin B, 5-FC, and itraconazole. The test was performed in a 96-well round-bottomed plastic plates using 100 μl of RPMI 1640 medium with fungal cells and antifungal substances.

An isolate of *Candida albicans* IFM 40213 originating from ATCC 90028 (American Type Culture Collection, Manassas, VA, USA, strain number 90028) was included as a quality control strain for susceptibility testing. *L. hoffmannii* isolates IFM 4922 and IFM 51330 stored in our center were also evaluated as controls for the fungal species.

Fungal conidia were obtained from 2-week-old cultures on PDA slants at 25°C by adding approximately 10 ml of physiological saline (0.85% NaCl) with 0.01% Tween-80 was onto the surface of the slants, with the resulting suspension collected after gentle scraping with a plastic sterile loop. Large mycelial fragments were removed by passing through a glass filter, after which the samples were washed 3 times with sterile saline and the concentration adjusted to 10^4/ml as demonstrated by quantitative colony counts. Drug-free and cell-free controls were included. The microdilution plates were incubated in air and readings were made after 48 h of incubation at 37°C (the *Candida* control strain was examined at 24 h). The minimal inhibitory concentration (MIC) endpoints for amphotericin B and itraconazole were read visually as the lowest drug concentration that prevented any discernible growth. The MIC endpoints for other antifungal drugs were also read visually and determine as that which reduced growth by 80% compared with the drug-free control.

Results

Colonies on the Sabouraud dextrose agar plates incubated at 25°C for 3 weeks were flat, smooth and orange colored, while colonies on PDA had irregular surfaces with black colored heaping centers and yellowish margins (Fig. 3a and b). The hyaline hyphae on PDA produced abundant hyaline, smooth- and thin-walled, broadly ellipsoidal to cylindrical or allantoid conidia which were 3 to 4 ×1–2 μm. In contrast, colonies on PDA slant at 25°C for 2 months were composed of tan-colored hyphae (Fig. 4a and b). The maximum growth temperature was 42°C.

The sequence of the D1/D2 domain of the large subunit ribosomal RNA gene deposited as AB189164 was 100% identical to that of AB100627 derived from *L. hoffmannii* (IFM 4922) in GenBank.

The MICs of the present isolate were beyond the measuring scale, with values of over 16 μg/ml for amphotericin B, 64 μg/ml for 5-FC, over 8 μg/ml for itraconazole, over 32 μg/ml for miconazole, over 64 μg/ml for fluconazole and over 16 μg/ml for micafungin. The MIC values for isolates IFM 4922 and 51330 were 2 μg/ml in amphotericin B, 2 and 64 μg/ml in 5-FC, 4 and over 8 μg/ml in itraconazole, 8 and 1 μg/ml in miconazole, and over 16 μg/ml in micafungin, respectively.