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特集

人と動物の共通感染症最前線 7

輸入シマリス（*Tamias sibiricus*）における サルモネラ症の集団発生

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要　約

中国から輸入した若齢シマリスの10～20%が到着直後より呼吸異常を示して死亡したが，下痢等の消化器症状を示す個体はいなかった。発症個体62匹の病性鑑定を行った結果，脾腫，化膿性肺炎が高率にみられ，肺に菌塊や壊死が目立った。また，*Salmonella* Enteritidis（SE）が36/62匹（58.1%），*Bordetella bronchiseptica*（BB）が29/62匹（46.8%）の割合で検出された。主病変は化膿性肺炎で，病変部で確認された細菌の多くがSEだったこと，および多臓器からSEが分離されたことから，本事例を輸入シマリスにおけるサルモネラによる敗血症の集団発生とした。SEはモルモットに対して病原性が高く，マウスは長期間不顕性に保菌するとされているが，リスでの報告はない。本事例はリスにおける致死性サルモネラ症の初の報告で，肺病変を主徴とする特異な病態を示した。

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本研究を行うにあたり，終始熱心にご指導頂きました宇根先生をはじめ，研究に協力して下さいました岡谷先生，病理学研究室および公衆衛生学第二研究室の皆様に，深く感謝申し上げます。今後，SEとBBの関連性についてさらに研究を進めていきたいと考えています。
2人が着ているTシャツは，病理学研究室オリジナルです。

はじめに

げっ歯類のサルモネラ症の病型は，急性敗血症，慢性胃腸炎，チフス症および不顕性感染に分類される。原因としては *Salmonella enterica* serovar Typhimurium や serovar Enteritidis（SE）が最も多い。SEは家畜をはじめ，野生動物，鳥類，爬虫類および両生類と，多数の動物を宿主とするが，特にラットやマウスで感受性が高いとされている[3]。

日本には年間約2万匹のリスが輸入されており[4]，そのうち，シマリス（*Tamias sibiricus*）はペットとしてポピュラーで，輸入数も多い。今回，輸入直後の若齢シマリスの10～20%が到着直後より呼吸異常を示して死亡する例が相次いだため，病性鑑定を行った。

材料と方法（臨床経過を含む）

2009年春，中国天津市よりシマリスが輸入されたが，到着時より健康状態不良の個体が多く，うち10～20%が呼吸異常を示し，削痩，衰弱が進行し死亡した。下痢等の消化器症状を示す個体はいなかった。その後も，天津から輸入された個体群に同様の症状がみられたため，同年4月1日に輸入されたシマリス200匹のうち41匹（A群）と，同月21日に輸入されたシマリス250匹のうち21匹（B群），計62匹の発症個体を対象として検索した。このうち，到着時に生存していたものは，安楽死し，死亡個体とともに病理学的検索に供し，肺，脾臓，肝臓および盲腸内容物を微生物学的に検索した。A群のうち13匹には，係留中に治療試験としてトリメトプリム・サルファ剤（ST合剤），およびトラネキサム酸，リゾチームが1日1回1週間経口投与された（治療群）。また，病態の程度を臨床症状と病変により，軽度：症状または病変がほとんど無い～軽度，中程度：症状または病変が中程度～高度，重度：死亡個体で，かつ病変が高度，の3段階にグレード分けした（表1）。

表1　病態分類

	個体数
軽度 症状または病変がほとんど無い～軽度	37
中程度 症状または病変が中程度～高度	6
重度 死亡個体で，かつ病変が高度	6

＊62頭のうち治療を行った13頭除く49頭が対象

なお，治療試験を行った13匹は対象外とした。

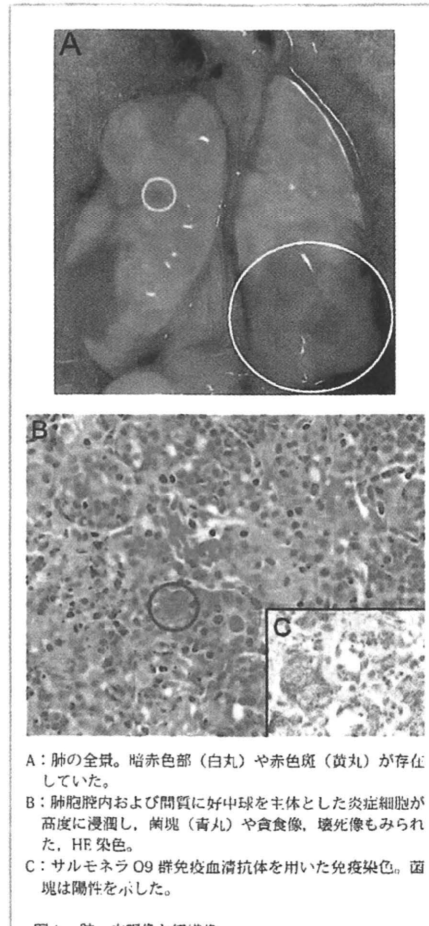
結　果

主病変は肺に観察され，その程度は臨床症状の重篤さと関連していた。肺は様々な程度で暗赤色を呈し，一部の個体では出血や肉変化が認められた。また，脾腫が高率に観察された。病理組織学的には，肺の病変部に一致して，肺胞腔内および間質に好中球を主体とした炎症細胞が高度に浸潤し，菌塊や貪食像，壊死像もみられた（図1）。気管支上皮細胞表面および気管支内腔には，グラム陰性短桿菌が多数存在し，腔内には好中球を主体とした壊死した細胞が高度に充満していたが，上皮細胞の障害は軽微であった（化膿性肺炎）。菌はサルモネラO9群免疫血清抗体に陽性を示した（図1）。脾臓では濾胞形成が目立ったが，菌塊や炎症細胞はほとんど認められなかった。肝臓では類洞の拡張や肝細胞の空胞変性，好中球やマクロファージの集簇が一部の個体で認められた。消化管に著変はみられなかった。また，2個体の大脳に細菌性脳炎がみられた。

病理学的および微生物学的検索で，SEが36/62匹（58.1%），*Bordetella bronchiseptica*（BB）が29/62匹（46.8%）の割合で検出された。SEは肺，脾臓，肝臓から高率に分離され，BBは主に肺から分離された。また，病態別SE検出率は，重度群100%（6/6匹），中程度群83.3%（5/6匹），軽度群54.1%（20/37匹），治療群38.5%（5/13匹）で，BB検出率は，重度群100%（6/6匹），中程度群33.3%（2/6匹），軽度群56.8%（21/37匹），治療群0%（0/13匹）であった。

考　察

主病変として化膿性肺炎がみられ，病変部で確認された細菌の多くがO9群免疫血清抗体を用いた免疫染色で陽性になったこと，複数の臓器からSEが分離されたことから，本事例を輸入シマリスにみられたサルモネラによる敗血症の集団発生とした。サルモネラ属菌は自然界に広く分布し，多くの脊椎動物に感染するが，ペット用の哺乳類での発症はまれである。SEはモルモットに対して病原性が高く，マウスは長期間不顕性に保菌するとされているが，リスでの報告はない。本事例はシマリスだけでなく，リスにおける致死性サルモネラ症としても初の報告となる。



A：肺の全景。暗赤色部（白丸）や赤色斑（黄丸）が存在していた。
B：肺胞腔内および間質に好中球を主体とした炎症細胞が高度に浸潤し，菌塊（青丸）や貪食像，壊死像もみられた，HE染色。
C：サルモネラO9群免疫血清抗体を用いた免疫染色。菌塊は陽性を示した。

図1　肺　肉眼像と組織像

今回の事例では肺病変が重篤であったが，一般的なサル

モネラ症で肺病変を主徴とする病型は見当たらない。本事例では，病態が重度の肺から高率にSEが検出され，組織学的にもSEの肺への起病性が示唆されること，BBのみが検出された個体の多くが軽度であること，BBが検出されていない治療群にも化膿性肺炎病巣がみられたことなどから，肺病変の形成にはSEが主体となっているものの，少なからずBBの関与があるものと考えた。SEの感染時期に関しては，日本到着時にすでに発症個体が確認されていることから，中国現地および輸送中にすでにSEに感染していたものと推察した。また，感染経路に関しては，消化管に著変が認められなかったこと，主病変が肺にあったこと，盲腸内容からのSE検出率が低かったことから，経鼻感染の可能性が高いと考えた。

SEは食中毒菌として[3]，BBは人に百日咳様の気管支炎や鼻炎を起こすことが知られている[2]。また，BBは高感受性のモルモットでは化膿性気管支炎あるいは気管支肺炎を生じ，幼若動物では死亡することもある[1]一方，犬やウサギ，ラットなどには日和見感染的な病原体である。シマリスのBBへの感受性や感染症に関する報告は見当たらない。

厚生労働省の「動物の輸入届出制度」導入により（2005年9月1日～），海外から生体を輸入する場合，日本の衛生基準をクリアした繁殖施設からの輸入のみが許可されているが，過去に本研究室で行った『愛玩用輸入げっ歯類の病原体保有調査』では，シマリスからSEが20%検出され，業者別の検出率では，70%と高率に検出された業者もあった。シマリスがこれらの細菌を保菌している可能性を念頭においた飼育管理が必要であり，動物衛生上のみならず，公衆衛生上の十分な配慮は欠かせないであろう。

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特集 人と動物の共通感染症最前線 7

Special Issue

マーラ（*Dolichotis patagonum*）に集団発生した致死性 *Salmonella* Enteritidis 感染症

吉村遥子[*1]　岩田剛敏[*2]　中村進一[*1]　林谷秀樹[*2]　宇根有美[*1]

要　約

マーラ 38 頭を飼育する施設で，7 頭が突然，類似の臨床症状を呈し，うち 6 頭が死亡した。この致死事例の原因究明を目的とし，病理学的，微生物学的に検索した。その結果，6 頭を *Salmonella* Enteritidis（以下 SE）症と診断し，発症率 18.4％，死亡率 85.7％の流行が起こったものと判断した。その病態は，チフス様と形容されるほど激烈で，血清型 Enteritidis による致死例は，野生動物では非常に珍しい。

はじめに

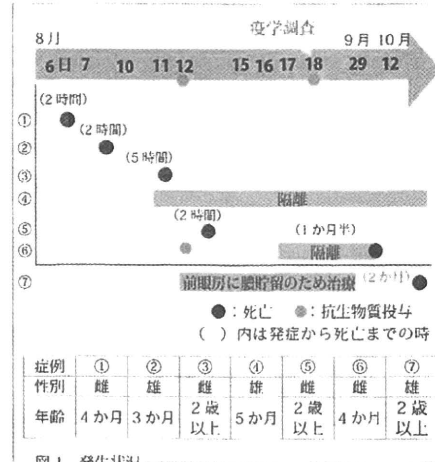
マーラ（*Dolichotis patagonum*）は，げっ歯類テンジクネズミ科に属し，体長 60 ～ 73cm，体重は 9 ～ 16 kg で，国内では，29 施設で 303 頭が飼育されていて（2005 年度），性格が穏やかなため，多くの施設で放し飼いされている。今回，国内のマーラ飼育施設（1 か所）で集団致死事例が発生したため，その原因究明を行った。

材料と方法（臨床経過を含む）

マーラ 38 頭を飼育する施設で，8 月上旬から中旬にかけ，7 頭が突然，食欲低下，起立困難，痙攣，後肢伸張など類似の臨床症状を呈し，10 月中旬までに 6 頭が死亡した（図 1）。下痢はみられなかった。6 頭中 4 頭は 1 週間以内に相次いで死亡した。いずれも異常発見後，数時間以内に死亡する甚急性経過であった。最初の死亡例から 7 日後に雌と子に，13 日後に全頭に抗生物質を投与した。死亡した 6 頭中 2 頭は抗生物質投与により回復傾向がみられたものの，1 頭が 9 月下旬に死亡，さらに 1 頭が 10 月中旬に死亡した。

最初の死亡例から 13 日後，疫学調査として，生存個体全頭（34 頭）の直腸スワブ，獣舎内で捕獲したネズミ，マーラの餌（野菜，チモシー等），同エリア内の動物の餌（鶏頭）を微生物学的に検索した。

死亡個体 6 頭（成獣 3 頭，1 歳未満 3 頭）のうち，最初に死亡した 2 頭は病理解剖検査のみで病理組織検査と



症例	①	②	③	④	⑤	⑥	⑦
性別	雌	雄	雌	雄	雌	雌	雄
年齢	4 か月	3 か月	2 歳以上	5 か月	2 歳以上	4 か月	2 歳以上

図 1　発生状況

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検索にあたっては至らぬ点も多々ありましたが，貴重な症例に遭遇し，数多くのことを学ぶことが出来ました。ご指導頂きました宇根先生，微生物学的検査をして下さいました先生方，剖検等でたくさんの協力をしてくださった病理学研究室の方々に，深く感謝しております。

表1　各症例の検査結果

		症例					
		①	②	③	⑤	⑥	⑦
培養	SE	ND	ND	＋	＋	－	＋
免疫染色	O9	ND	ND	＋	＋	＋（脳）	＋
肉眼	全身性出血	＋	＋	＋	＋	＋（肺）	＋（皮下，肺）
	血様腹水	＋	＋	＋	＋	－	－
	脾腫	＋	＋	＋	＋	＋	－
組織	細菌性脳炎	ND	ND	－	－	＋	＋

→抗生物質投与

致死性 *Salmonella* Enteritidis 症と診断
（発症率 18.4%，死亡率 85.7%）

微生物検査は行わなかったが，その後死亡した 4 頭は病理学的および微生物学的に検索した（表 1）。

組織検査には，全身諸臓器のホルマリン固定材料を供し，これらを定法に従ってパラフィン切片にして，HE 染色，サルモネラ属 O9 群免疫血清抗体を用い免疫染色を行った。

結　果

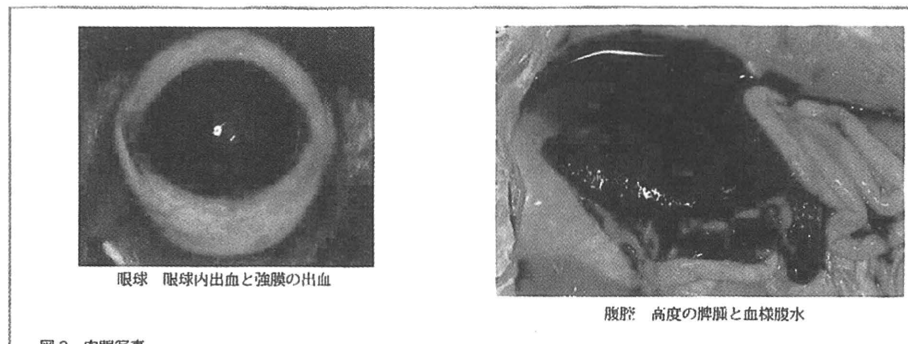
死亡個体のうち，抗生物質投与前に死亡した 4 頭すべてに顕著な脾腫と高度な全身性出血，血様腹水がみられた。ときに眼球内出血があったが下痢はなかった（図 2）。

組織検査では，症例によって病変に程度の差はあるものの，諸臓器に壊死性炎が観察され，治療後に死亡した 2 頭に脳炎が認められた。各所で炎症細胞反応に乏しい細菌塊がみられ，それらはサルモネラ O9 群免疫血清抗体で陽性を示した（図 3）。

微生物検査では，4 頭中 3 頭の臓器より SE が分離され，残り 1 頭は抗生物質が投与されていた（表 1）。直腸スワブ 34 頭中 3 頭からサルモネラ属 O9 群が，鶏頭からサルモネラ属 O7 群が分離されたが，ネズミとマーラの餌からは分離されなかった。

考　察

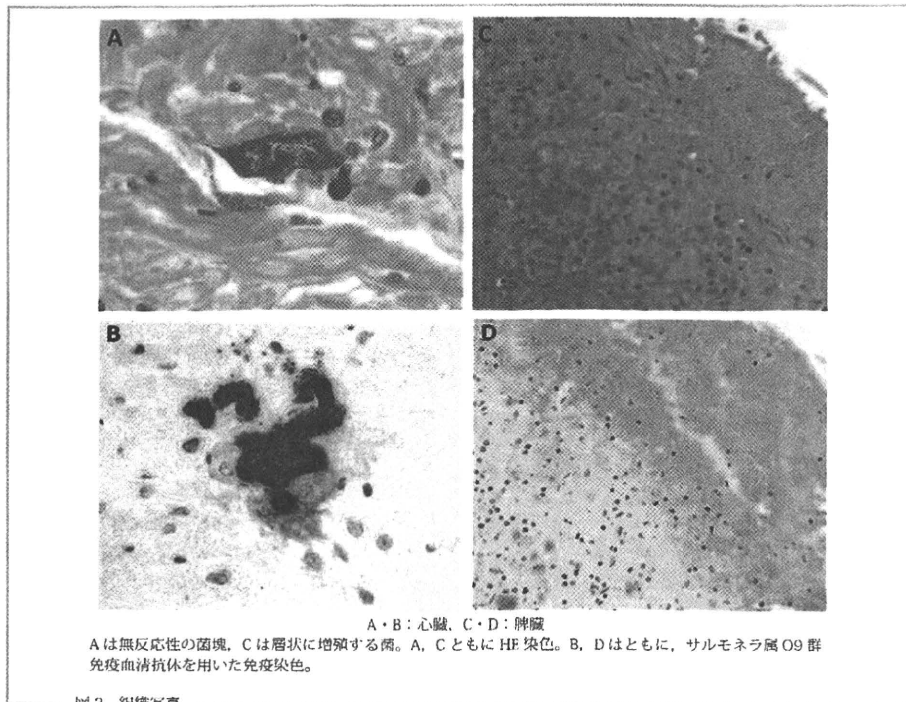
以上の所見より，死亡した 6 頭を致死性 SE 症と診断，生き残った 1 頭も，発症時期や症状から SE 症と判断した。よって，発症率 18.4 %，死亡率 85.7 % の SE 症の流行が起きたものと推察した。サルモネラ症には，下痢を主徴とした下痢症型と，チフス様病態ともいわれる急性敗血症型があり，甚急性例では，目立った病理所見を示さず，急死するのが特徴である。さらに，血清型 Enteritidis は，O9 群に属し，人の食中毒の原因菌として最も多く分離され，Typhimurium に比べ，病原性が弱いとされている。今回，甚急性経過をとった 4 頭で観察された病態は，いわゆるチフスと表現される激烈なものであった。SE による本病



眼球　眼球内出血と強膜の出血

腹腔　高度の脾腫と血様腹水

図 2　肉眼写真



A・B：心臓，C・D：脾臓
Aは無反応性の菌塊，Cは層状に増殖する菌。A，CともにHE染色。B，Dはともに，サルモネラ属O9群免疫血清抗体を用いた免疫染色。

図3　組織写真

態の報告は，野生動物ではコビトイノシシ，ワシミミズクの報告があるのみである。

マーラと同科のモルモットは，サルモネラ属菌に高感受性とされていることから，この病態は種に関連するかもしれない。また，SE症流行時の餌が保管されていなかったことから，感染源は特定できなかった。しかし，マーラの餌を扱う調理場では，他の動物に与える鶏頭を扱っており，疫学調査時に採材した鶏頭からサルモネラ属菌が分離されたこと，流行時期に，その地域はかなりの猛暑であったことから，調理場を介した食物汚染，経口感染が疑われた。なお，その後，当該施設では，飼育場および調理場の衛生管理を徹底したところ，マーラを含めて他の動物にSE症は発生していない。

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Soluble Components of *Histoplasma Capsulatum* var. *Capsulatum* have Hemagglutinin Activity and Induce Syngeneic Hemophagocytosis In Vitro

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Abstract *Histoplasma capsulatum* var. *capsulatum* is a thermally dimorphic fungus that causes histoplasmosis. Fungal hemagglutination activity and cases of reactive hemophagocytic syndrome (RHS) have been reported in the disseminated form of disease. In the present study, soluble components of *H. capsulatum* var. *capsulatum* have been investigated for hemagglutinin activity and the capacity to induce hemophagocytosis in the mouse system. To analyze hemagglutinating activity, mouse red blood cells (RBC) (1% v/v in PBS) were incubated (37°C, 1 h) with cell-free antigen (CFAg) from *H. capsulatum* var. *capsulatum* (isolate IMT/HC128) (RBC-CFAg) or previously heated CFAg (56°C, 30 min) (RBC-hCFAg) or as control with PBS (RBC-PBS). Hemophagocytosis was analyzed by incubating BALB/c mouse peritoneal phagocytic cells (5×10^6 cells) with syngeneic RBC, sensitized or not with CFAg. In addition, mouse polyclonal antibodies were raised against syngeneic RBC-CFAg (anti-RBC-CFAg) and used to analyze CFAg chromatographic fractions (Sephadex G75/120) by immunoenzymatic assay (ELISA). Hemagglutinin activity was observed with RBC-CFAg, but not with RBC-hCFAg or RBC. Also, hemophagocytosis was observed with RBC-CFAg, but not with RBC. The anti-RBC-CFAg antibodies reacted with CFAg fractions corresponding to a molecular mass (MM) higher than 150 kDa. In conclusion, the yeast form of *H. capsulatum* var. *capsulatum* releases thermolabile soluble components with hemagglutinin activity and it has been demonstrated for the first time that soluble components of the same fungus induce syngeneic hemophagocytosis in the in vitro mouse system. Also, indirect analysis with antibodies suggests that high-MM components (>150 kDa) are responsible for the interaction with RBC.

Keywords Disseminated histoplasmosis · Reactive hemophagocytic syndrome · Hemagglutination · Antibodies · ELISA · Red blood cells

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Introduction

Histoplasma capsulatum var. *capsulatum* is the causative agent of histoplasmosis, a systemic mycosis endemic on the east side of USA and throughout most of Latin America [1–3]. This fungus is thermally

dimorphic (mycelial at 25°C, yeast like at 35°C). The disease is normally caught from the environment by inhaling conidia from the mycelial phase. The initial lung infection is most often asymptomatic or self-limited, but it may lead to a broad spectrum of clinical manifestations, from benign acute pulmonary to fatal disseminated histoplasmosis [4].

Irreversible and fatal disseminated histoplasmosis is classically described as attacking immunosuppressed patients, especially those with AIDS, but it has also been observed in idiopathic $CD4^{+}$ lymphocytopenia and in the very young or very old [5]. The most frequent symptoms in these patients are fever, pulmonary access, anorexia, hepatomegaly, neurological problems, splenomegaly, lymphadenopathy, and meningitis [6].

Some patients with disseminated histoplasmosis have been reported as showing signs of reactive hemophagocytic syndrome (RHS), especially among those suffering from AIDS or immunosuppression [7–9]. RHS is a clinical disorder characterized by an excessive proliferation of non-malignant cells of the monocyte-macrophage-histiocyte line. Symptoms include persistent high fever, cytopenia and swollen liver, and spleen [9–12].

One of the virulence factors described for this fungus is a component with the capacity to induce red-cell agglutination, irrespective of the blood group of the host. According to Taylor et al. [13], this component could be a blood-clotting cofactor mediating the hemagglutinating activity of the yeast forms and hence an important factor in the dissemination of the disease.

Cell-free antigens (CFAg) were introduced by Sá-Nunes et al. [14] as a means of assessing cell immunity to histoplasmosis and to induce protection against experimental histoplasmosis in mice. In view of the possible existence of soluble components with hemagglutinating activity in this fungus, in this study the CFAg of *H. capsulatum* var. *capsulatum* was employed for the analysis and partial characterization of these soluble components and to assess hemophagocytosis induced in mouse cells in vitro.

Methods

Fungal Strain and CFAg Preparation

Histoplasma capsulatum IMT/HC128 (IMT/HC, Lima, Peru) was cultured on Sabouraud agar (Micromed, Rio de Janeiro, RJ, Brazil), and maintained by subculturing at 35°C at 5-day intervals. The CFAg was obtained by the method used for *Paracoccidioides brasiliensis* cell-free antigens [15], modified by adding phenylmethylsulfonyl fluoride (PMSF; Sigma, St. Louis, MO) at 2.5 mM to the supernatant, which was subsequently frozen at −80°C. PMSF was used to reduce the activity of natural fungal proteases, which may be released during antigen preparation. The protein concentration was determined by the Folin method [16], with bovine serum albumin as standard.

Mouse Erythrocytes (1 and 5%)

Erythrocytes (RBC) were obtained from peripheral blood from BALB/c mouse in a heparinized tube. After centrifugation (300×*g*, 5 min) and washing three times in 0.15 M PBS (pH 7.2), also by centrifugation, the cell pellet was adjusted to 1 and 5% (v/v) in PBS and Hank's balanced salt solution (HBSS), respectively.

Hemagglutination Assay

Hemagglutinating activity was determined by the micro-hemagglutination test in 96-well plates with 100 μl/well of yeast form of *H. capsulatum* IMT/HC128 (1×10^5 cells), 100 μl/well of IMT/HC128 CFAg (4.0 mg/ml) and a serial dilution of yeast or CFAg (1/2 to 1/1.024), plus 100 μl/well of 1% mouse erythrocytes. Negative control wells contained only the suspension of erythrocytes (RBC-PBS). A small pellet of erythrocytes at the bottom of the well after 1 h incubation at 37°C were considered negative, and those containing an even sheet of erythrocytes across the well were considered positive. In addition, CFAg heated for 30 min at 56°C (hCFAg) was analyzed by the same assay.

Hemophagocytosis Assay

Peritoneal cells were obtained by injecting ice-cold HBSS (2.5 ml) into BALB/c mice (aged 60 days), gently massaging for 10 min and harvesting by aspiration. The cells were washed, resuspended in RPMI 1640 complete medium supplemented with a final concentration of 10% fetal calf serum, and incubated in Petri dishes at 37°C for 60 min.

Nonadherent cells were removed by gently rinsing the plates with warm RPMI 1640 medium. Adherent cells were collected by gently rinsing the plates with PBS containing 0.05% ethylenediaminetetraacetic acid (EDTA), then transferred to tubes and washed (3×) by centrifugation in HBSS. The cell concentration was adjusted to 5×10^6 cells/ml in HBSS, and the percentage of viable cells was determined by trypan blue exclusion (>95% viability). Peritoneal macrophages (50 μl) and syngeneic RBC, previously sensitized with CFAg (v/v) (50 μl), were incubated at 37°C for 40 min on glass slides (2.5 × 7.5 cm). For RBC sensitization, samples of CFAg, diluted 1/8, 1/16 and 1/32, were incubated with 1% RBC. RBC-PBS was used as control. After incubation, the samples were fixed with methanol and stained with hematoxylin and eosin (H&E).

Polyclonal Antibodies Against Autologous RBC-CFAg (anti-RBC-CFAg) and Hemagglutination Inhibition

Autologous RBC (5%) were incubated with CFAg from *H. capsulatum* var. *capsulatum* (v/v) for 1 h at 37°C and 1 h at 4°C, washed with ice-cold HBSS and resuspended in HBSS. Sensitized RBC emulsified in complete Freund's adjuvant (v/v) were inoculated subcutaneously into 14 BALB/c mice (60 days, male, 50 μl each). Incomplete Freund's adjuvant was used for second and third inoculations at 2-week intervals. Antiserum (anti-RBC-CFAg) was secured 2 weeks later. Hemagglutination inhibition was assayed by the micro-hemagglutination test: 50 μl/well of anti-RBC-CFAg (1/10, inactivated at 56°C, 30 min) and 50 μl/well of IMT/HC128 CFAg (4.0 mg/ml) were incubated at 37°C for 1 h, then mouse erythrocytes were added (100 μl/well) and, after 1 h incubation at 37°C, hemagglutination was read as described previously.

Gel-Filtration Chromatography and Analysis of Fractions by ELISA

Two milliliters of IMT/HC128 CFAg (4.0 mg/ml) were applied to a Sephadex G75-120 column (1.5 × 22 cm) buffered with 0.15 M PBS, pH 7.2. Fractions of 1.5 ml each were collected in an automatic fraction collector and read in a spectrophotometer (UV/Visible, Ultrospec-2000; Pharmacia Biotech, Auckland, Northland) at 280 nm. For preliminary MM estimation, dextran blue [exclusion volume, more than 150 kDa] was applied to the same column. For ELISA, microplate wells were sensitized with 100 μl of fractions in carbonate–bicarbonate buffer (pH 9.6), for 1 h at 37°C and overnight at 4°C. The plates were washed four times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), blocked with PBS-T-5% skim milk for 1 h at room temperature and incubated with mouse anti-RBC-CFAg serum (1:10), followed by anti-mouse IgG peroxidase conjugate (Sigma A-8924; Sigma Chemical Co., St. Louis, MO), at 37°C for 90 min, washed, and a substrate solution (5 mg *o*-phenylenediamine—Sigma Chemical Co., St. Louis, MO, USA, 10 ml of 0.1 M citrate buffer, pH 4.5 and 10 μl H_2O_2) (100 μl/well) was added. The reaction was halted with 50 μl of 4 N H_2SO_4/well and absorbance was read in a Titertek Multiscan EIA reader (Labsystems, Helsinki, Finland) at 492 nm.

Statistical Analysis

The Tukey–Kramer test was applied to the data using the program GraphPad InStat 3.0. The significance level was set at $P \leq 0.05$.

Results

Hemagglutination Test with CFAg from *H. capsulatum* var. *capsulatum*

Agglutination was observed in this test with mouse erythrocytes previously sensitized with yeast form of *H. capsulatum* or CFAg (RBC-CFAg), pure or diluted 1/2, but not with untreated erythrocytes (RBC-PBS) or those sensitized with higher dilutions of CFAg (from 1/4 to 1/1.024). The hemagglutination reaction was also negative with preheated CFAg (RBC-hCFAg) (Fig. 1).

Hemophagocytosis In Vitro

The results of the hemophagocytosis test demonstrate the phagocytosis of syngeneic red cells sensitized with CFAg in dilutions from 1/8 to 1/64. The percentages of phagocytes showing hemophagocytosis were: 45% with 1/8 CFAg, 50% with 1/32 CFAg, and 40% with 1/64 CFAg. Evidently, the control erythrocytes, which had not been exposed to the

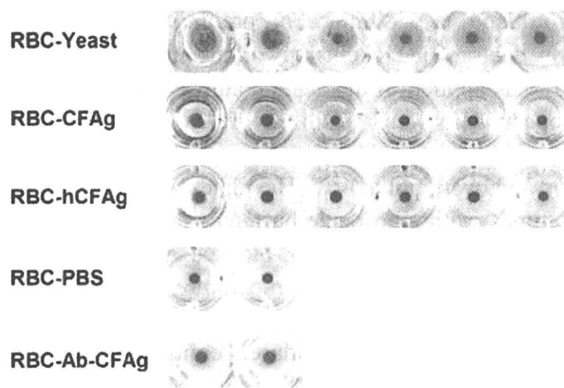

Fig. 1 Hemagglutination with yeast and CFAg from *H. capsulatum* var. *capsulatum* and inhibition of CFAg hemagglutination activity with anti-RBC-CFAg serum. Agglutination results with mouse erythrocytes previously sensitized with: yeast form of *H. capsulatum* var. *capsulatum* IMT/Hc 128 (RBC-Yeast), CFAg of *H. capsulatum* var. *capsulatum* IMT/Hc 128 (RBC-CFAg), preheated CFAg (RBC-hCFAg) and negative control with PBS (RBC-PBS). For *left to right*: RBC-Yeast = pure (10^5 yeast cells), diluted 1/2, 1/4, 1/8, 1/16, and 1/32, RBC-CFAg or RBC-hCFAg = pure (4.0 mg/ml), and diluted as for yeast; and RBC-PBS: without sample (duplicate). Hemagglutination inhibition was assayed by the micro-hemagglutination test by pre-incubation of the anti-RBC-CFAg (1/10, inactivated at 56°C, 30 min) with *H. capsulatum* var. *capsulatum* IMT/Hc 128 CFAg (4.0 mg/ml), v/v. RBC-Ab-CFAg : *right* = CFAg 1.0 mg/ml (final dilution) and *left* = CFAg 0.5 mg/ml

CFAg from strain IMT/Hc 128, did not suffer any phagocytosis, since both erythrocytes and phagocytes came from the syngeneic animal. Figure 2 shows the syngeneic hemophagocytosis.

Inhibition of Hemagglutination Activity of CFAg with Anti-RBC-CFAg Serum

Hemagglutination was inhibited when erythrocytes were sensitized with CFAg that had been pretreated with anti-RBC-CFAg immune serum, at final CFAg dilutions 1/2 (serum/CFAg v/v) (Fig. 1). The positive control, in which the CFAg was not pre-incubated with serum, showed a positive hemagglutination reaction.

Fractionation of the *H. capsulatum* var. *capsulatum* Soluble Antigen by Gel-Filtration Chromatography

The fractions of CFAg emerging from the Sephadex G75/120 column formed a spectrophotometric profile at 280 nm with two absorbance peaks (Fig. 3). The first coincided with the dextran blue peak (exclusion volume) and thus was taken to represent components of molecular weight larger than 150 kDa.

Analysis of CFAg Chromatographic Fractions by ELISA

Each fraction from the Sephadex G75/120 column was tested by ELISA, employing the anti-RBC-CFAg serum, and the first, which corresponded to the first UV absorbance peak (>150 kDa), was highly reactive (Fig. 3).

Discussion

The observation that the yeast form of *H. capsulatum* has the ability to agglutinate human erythrocytes [13] has been corroborated here by experiments with strain IMT/Hc128, which also exhibited a capacity to agglutinate mouse, human and rabbit (data not shown) erythrocytes. The present study demonstrated that, not only the intact yeast, but also some of its soluble components (CFAg) have the capacity to agglutinate mouse erythrocytes. These components lost their hemagglutinating activity when the CFAg was heat-treated before the test, indicating that they are thermolabile.

In disseminated histoplasmosis, some patients develop RHS, a form of hemophagocytosis. According to Fujiwara et al. [17] and Ohga et al. [18], it is commonly associated with hyperactivation of the T lymphocytes, resulting in excessive secretion of cytokines and activation of macrophages.

Soluble antigen (Ag) of *H. capsulatum* has been detected in the blood of patients with histoplasmosis, especially in serious cases of the disseminated disease [19, 20]. Demonstrations that soluble Ag from strain IMT/Hc128 can cause hemagglutination alerted us to the possibility that the RHS observed in disseminated histoplasmosis might occur because these antigens bind to the surface of erythrocytes, where the phagocytes recognize them and thus attack the autologous red cells. Therefore, we sensitized mouse red cells with CFAg at three sub-agglutinating concentrations and incubated them with phagocytes from the syngeneic mouse. This led to hemophagocytosis in 40–50% of the phagocytes, whereas the effect was not seen with red cells not treated with

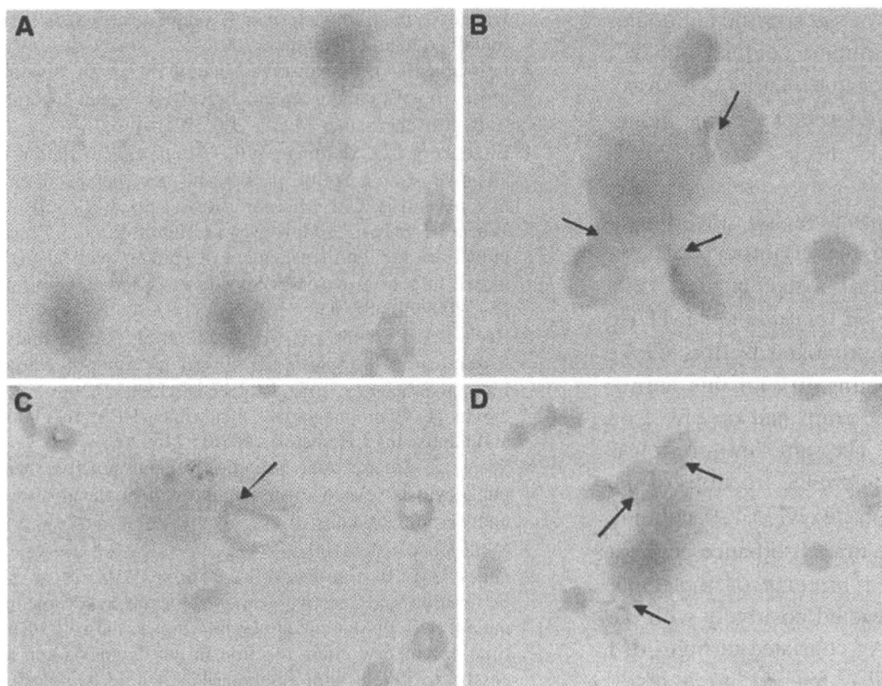

Fig. 2 Syngeneic hemophagocytosis in vitro. Hemophagocytosis of syngeneic red cells sensitized with CFAg from *H. capsulatum* var. *capsulatum* IMT/Hc 128 (diluted 1/32) by BALBc mice peritoneal adherent cells (5×10^6 cells) (**b–d**) and negative control with HBSS (**a**). After the hemophagocytosis, the samples were fixed with methanol and stained with hematoxylin and eosin (H&E; ×400). Hemophagocytosis (*right arrow*)

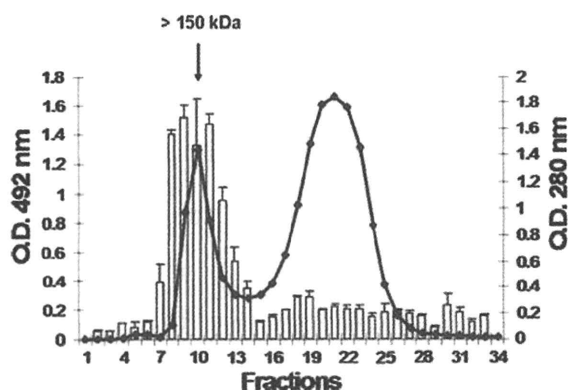

Fig. 3 Fractionation of the *H. capsulatum* var. *capsulatum* CFAg by gel-filtration chromatography and analysis by ELISA. The fractions of CFAg emerging from the Sephadex G75/120 column formed a spectrophotometric profile at 280 nm (−). Each fraction was tested by ELISA ($n = 2$) and the mean results expressed as OD at 492 nm (*bar*), employing the anti-RBC-CFAg serum. The 280 nm first peak: fractions 8–11 were highly reactive by ELISA (492 nm) in relation to other fractions; $P < 0.05$. The first peak coincided with the dextran blue peak elution (exclusion volume) and thus was taken to represent components of molecular weight larger than 150 kDa

CFAg—as expected, given that the red cells and phagocytes were from the genetically identical animals, as the same animal.

Our results suggest that the occurrence of the auto hemophagocytosis with not activated macrophages. Considering that in RHS, the auto hemophagocytosis may be due to excessive secretion of the cytokines by hyper activation of T cells that promote the monocytes-macrophages activation [7, 17, 18] some hemophagocytosis could occur early without activation that could be amplified after macrophages activation, which require further study.

In the complete animal system, besides the binding of the soluble fungal component to the erythrocyte, it is possible that a further interaction occurs with immunoglobulins (Ig), to form the complex RBC-Ag-Ig. This could activate the complement system, so that hemophagocytosis might also occur via the Fc receptor for Ig or complement receptor 1 (CR1), present on the phagocytes. This mechanism could also lead to hemolysis.

The presence of soluble Ag and specific antibodies (Ab) and their circulating immune complexes (CIC) may be the important pathogenic factors. The association between CIC and progress to glomerulonephritis and arthritis has been described in histoplasmosis [21, 22].

To gather further information on the fungal component interacting with the erythrocytes, polyclonal Ab was raised against syngeneic red cells sensitized with CFAg from *H. capsulatum* IMT/Hc 128. The hemagglutination provoked by this CFAg was inhibited by pre-incubating it with this antiserum, while the pre-immune serum had no effect on the CFAg (data not shown). The same antiserum was used to analyze the chromatographic fractions of the CFAg separated in the Sephadex G75/120 column. The fractions making up the first absorbance peak (at 280 nm), which represented proteins of molecular mass exceeding 150 kDa, reacted positively with the Ab, suggesting that the CFAg consisted of high-MM antigens. The antibodies also reacted, to a lesser extent, with another fraction of lower MM.

In conclusion, the yeast form of *H. capsulatum* var. *capsulatum* releases thermolabile soluble components with hemagglutinin activity and it has been demonstrated for the first time that soluble components of the same fungus induce syngeneic hemophagocytosis, therefore as auto hemophagocytosis. Also, the indirect analysis with antibodies suggests that high-MM components (>150 kDa) are responsible for the interaction with red blood cells.

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FULL PAPER

A new species, *Aphanomyces salsuginosus* sp. nov., isolated from ice fish *Salangichthys microdon*

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Abstract A species of *Aphanomyces* was isolated from the ice fish *Salangichithys microdon* living in brackish water in Japan. White cotton-like growth was found on the heads and fins of the fish. Hyphae penetrated into the dermal layers, subcutaneous tissues, muscular layers, and cartilaginous tissue of the mandible and maxilla; these hyphae were associated with cellular debris and lesions in host tissue. White fluffy colonies from subcultures of these growths were isolated on glucose–yeast agar plates with 0.5% sodium chloride (NaCl). These isolates consisted of delicate, slightly wavy, and moderately branched hyphae. Zoosporangia were isodiametric with the vegetative hyphae. Oogonia were abundant and approximately 21–33 μm in diameter, with irregular short papillae. Generally they were spherical or subspherical and only rarely pyriform. Individual oogonia usually contained a single oospore, which was spherical and 19–27 μm in diameter, with a large shiny vesicle. Antheridial branches, when present, were usually androgynous; however, they were sometimes monoclinous or diclinous. The optimal growth temperature of the isolates was 20°C, and cultures grew well at low salinity (0–0.5% NaCl). Phylogenic analysis based on the internal transcribed space 1-5.8S-ITS 2 of the ribosomal RNA gene indicates that these isolates will be an as-yet unidentified species of *Aphanomyces*.

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Introduction

The genus *Aphanomyces* includes saprotrophs, phytopathogens, and animal pathogens (Scott 1961; Johnson et al. 2002). Recently, the phylogenetic relationship of *Aphanomyces* spp. found on plants, animals, and environmental detritus were clearly classified based on internal transcribed spacer (ITS) regions of ribosomal RNA (rRNA) gene sequences (rDNA) (Diéguez-Uribeondo et al. 2009). The main causal agents of fungal diseases in fishes and shellfishes belong to the genus *Aphanomyces* (Hatai 1989) (Table 1). For example, *A. astaci* Schikora has been reported as the most serious pathogen of European and Australian freshwater crayfish (Unestem 1972). Similarly *A. repetans* nom. nud. and *A. frigidophilus* Kitanch. & Hatai were also reported as pathogens of freshwater crayfish (Royo et al. 2004; Ballesteros et al. 2006).

A. invadans Willoughby, R.J. Roberts & Chinabut (also called *A. piscicida* Hatai) was first reported as the pathogen causing mycotic granulomatosis in ayu *Plecoglossus altivelis* in Japan (Hatai 1980). Later, *A. invadans* was identified as the cause of epizootic ulcerative syndrome (eEUS) in some fishes from Southeast Asian countries (Willoughby and Roberts 1994; Lilley et al. 1998) and in sand whiting *Sillago ciliate* from South Africa (Andrew et al. 2008). Similarly, *A. invadans* was found to cause ulcerative mycosis in Atlantic menhaden *Brevoortia tyrannus* from the USA (Dykstra et al. 1986) and red spot disease (RSD) in sea mullet *Mugil cephalus* and yellowfin bream *Acanthopagrus australis* from Australia (Fraser et al. 1992). In 2002 a group of experts on fungal diseases

Table 1 *Aphanomyces* species isolated from fish and shellfish

Species	Host	Reference
Aphanomyces helicoides	Common carp	Srivastava and Srivastava (1976)
A. laevis	Guppy	Shanor and Saslow (1944)
A. stellatus	Eel	Hoshina et al. (1960)
	Common carp	Shah et al. (1977)
A. astaci	Crayfish	Unestem (1972)
A. repetans	Crayfish	Royo et al. (2004)
A. frigidophilus	Japanese char egg	Kitancharoen and Hatai (1997)
	Crayfish	Ballesteros et al. (2006)
A. invadans (=*A. piscicida*)	Ayu	Hatai (1980)
	Atlantic menharden	Dykstra et al. (1986)
	Sea mullet	Fraser et al. (1992)
	Yellowfin bream	Fraser et al. (1992)
	Sand whiting	Fraser et al. (1992)
Aphanomyces sp.	Soft-shelled turtle	Sinmuk et al. (1996)

of fish from Japan, Australia, USA, India, Philippines, and Thailand concluded that mycotic granulomatosis (MG), RSD, ulcerative mycosis (UM), and eEUS are all the same disease and proposed a new name: epizootic granulomatous aphanomycosis (EGA) (Baldoc et al. 2005). This effort helped bring continuity to the variety of reports on fungal fish disease from around the world in which *A. invadans* had been isolated from the fish suffering from disease. Reports also indicate that *A. laevis* de Bary has been repeatedly isolated from the guppy *Lebistes reticulates* (Shanor and Saslow 1944). Similarly, *A. stellatus* de Bary was isolated from the common carp *Cyprinus carpio* (Shah et al. 1977) and the Japanese eel *Anguilla japonica* (Hoshina et al. 1960). All of these *Aphanomyces* spp. were isolated from fish suffering from pathogenic fungal growth.

In February 2008, we encountered an outbreak of a fungal infection in the ice fish *Salangichtys microdon* in an aquarium at Lake Shinji, Shimane Prefecture, Japan. Ice fish live in blackish water, but water mold infection usually occurs in fresh water. We were interested in the infection because it occurred in blackish water fish, then tried to isolate the water mold. As a result, *Aphanomyces* spp. were only isolated from the fish. In this paper, we describe the causal agent of the infection caused by *Aphanomyces* sp., and compare it to the other animal parasitic *Aphanomyces* spp. according to morphological, physiological, and phylogenetic criteria.

Materials and methods

Isolation

Typically, an infected fish showed small whitish masses with cotton-like appearance at the mouth and/or fins (Fig. 1). Small pieces from these masses were rinsed in sterilized tap water with 0.5% sodium chloride (NaCl). The rinsed pieces were then used to inoculate the center of 90 × 20 mm disposable Petri dishes containing 1% glucose–0.25% yeast agar (GY agar) supplemented with 0.5% NaCl and 500 mg/ml of each ampicillin and streptomycin, Inoculated plates were incubated at 15°C for 4–6 days. The resulting fungal colonies were subsequently cultured on GY agar plates supplemented with 0.5% NaCl. Five fungal colonies were isolated from the specimens: NJM 0801, NJM 0802, NJM 0803, NJM 0804, and NJM 0805 (Table 2).

Histopathological observation

All fish bodies were fixed in 10% phosphate-buffered formalin solution, decalcified in ethylenediaminetetraacetic acid (EDTA) with 5% formalin, and processed for routine histology, as described in Hendrickson (1985). All paraffin sections from the specimens were stained with hematoxylin and eosin (H & E), Gomori's methenamine silver-nitrate, Grocott's variation counterstained with H & E (Grocott-H & E), and Gram stains.

Identification

To grow mycelia, an agar block was cut from the edge of a colony, put into GY broth with 0.5% NaCl, and incubated at 20°C for 2 days. Growing mycelia were rinsed twice in sterilized tap water with 0.5% NaCl and resuspended in sterilized tap water with 0.5% NaCl to induce development

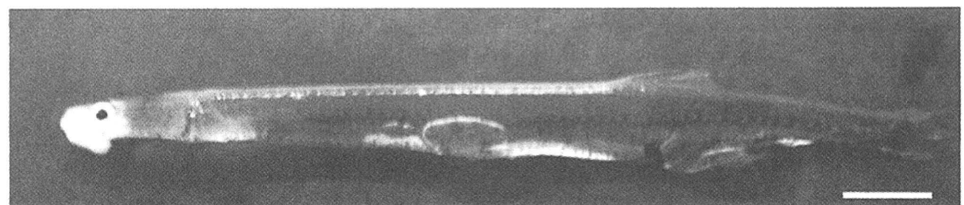

Fig. 1 Ice fish affected with water mold. Lesions of white, cotton-like masses of mycelia on the head, mouth, and/or fins of ice fish. *Bar* 1 cm

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Table 2 Isolates from ice fish

Strains	Fish no.	Site of isolation	Accession no.
NJM 0801	1	Rostrum	AB510348
NJM 0802	2	Rostrum	AB510349
NJM 0803	3	Rostrum	AB510350
NJM 0804	4	Natsi fin	AB510351
NJM 0805	5	Rostrum	AB510352

of zoospores and oogonia. The isolated fungi were identified according to Sparrow (1960) and Scott (1961) based on the asexual and sexual structures in the water culture.

Physiological studies

The effect of temperature on vegetative growth was determined using isolates NJM 0801 and NJM 0803. The advancing edge of 6-day-old colonies cultured on GY agar with 0.5% NaCl was cut out with a No. 2 cork borer (approximately 5.5 mm in diameter). Individual agar cores were placed on the center of 90 × 20-mm Petri dishes containing 20 ml of GY agar with 0.5% NaCl, and the plates were incubated at 5, 10, 15, 20, 25, 30, or 35°C. Colony diameters were estimated as follows: The widest and narrowest diameter of radial growth from the center of each core was measured. Colony diameter was estimated as the average of these two measurements minus the 5.5 mm of the original agar core. Estimates of colony diameter were determined 3, 5, and 7 days after inoculation. The effect of NaCl concentration on vegetative growth was determined using isolates NJM 0801 and NJM 0803. The advancing edge of 7-day-old colonies cultured on GY agar with 0.5% NaCl were cut out with a No. 2 cork borer. Individual agar cores were placed on the center of 90 × 20-mm Petri dishes containing 20 ml of GY agar and 0, 0.5, 1, 2, 3, or 5% NaCl. All plates were incubated at 20°C. The diameter of each colony was determined using the same method described for studies on the effects of temperature on vegetative growth. These colonies were measured at days 3, 5 and 7. These tests were carried out one time.

Molecular studies

The ITS rDNA was sequenced. DNA was extracted from hyphae cultured at 20°C in GY broth with 0.5% NaCl. Mycelia were dried on tissue paper, and each mycelium was transferred to a separate 1.5-ml microcentrifuge tube. The samples were frozen at −85°C prior to DNA extraction as described by Abliz et al. (2004). ITS rDNA sequences were determined as follows: We used a nested polymerase chain reaction (PCR) system to amplify ITS rDNA using universal primers (rDNA primer sequences for fungal sequencing can be found at http://www.biology.duke.edu/fungi/mycolab/primers.htm, and Kurtzman and Robnett 1997). PCR reaction mixtures included 2.5 μl of DNA extracted with a Ready-to-Go bead (Amersham Pharmacia Tokyo, Japan), 2.5 μl of 10 pM primers solution (NS-7: 5′-GAG GCA ATA ACA GGT CTG TGA TGC-3′ and NL-2: 5′-CTT GTT CGC TAT CGG TCT C-3′), and 17.5 μl of distilled water. The reaction mixtures were subjected to one cycle of denaturation at 95°C for 4 min, 30 cycles of amplification (94°C for 1 min, 54–55°C for 1 min, and 72°C for 2 min), and a final extension cycle at 72°C for 10 min in a PCR Thermal Cycler MP (TaKaRa, Ohtsu, Japan). First-round PCR products were purified with a commercial kit (QIAquick®, Qiagen Co. Ltd., Tokyo, Japan) and then diluted 10- to 100-fold. The second round of PCR amplification used one of the following combinations of internal primer pairs: NS-7 and ITS-2: 5′-GCT GCG TTC TTC ATC GAT GC-3′, ITS-5: 5′-GGA AGT AAA AGT CGT AAC AAGG-3′, ITS-4; 5′-TCC TCC GCT TAT TGA TAT GC-3′, and ITS-3; 5′-GCA TCG ATG AAG AAC GCA GC-3′. The condition of second-round PCR was performed under the same condition as the first. The second-round PCR products were visualized by electrophoresis in 1.0% agarose in 1 × TBE buffer [0.04 M Tris-boric acid, 0.001 M EDTA (pH 8.0)] followed by ethidium bromide staining. The PCR products were purified directly from PCR reaction mixtures using a PCR purification kit (QIAquick®) and then 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) using the same primers used in the second round of PCR amplification.

Phylogenetic analysis

DNA sequences were aligned using GENETEX-WIN genetic information processing software (Software Development, Tokyo, Japan). Sequences were analyzed using the BLAST search of GeneBank (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi), and closely related sequences were identified through analysis of the tree generated by the BLAST program. *Aphanomyces* species with sequence data deposited in GenBank were selected as relatives of the *Aphanomyces* sp. isolated in our studies based on a distance tree view available via a BLAST search (Table 3). Thirty nucleotide sequences selected from the GenBank database and this study were aligned using the computer program Clustal X (Version 1.8) (Jeanmougin et al. 1998), followed by manual adjustments with a text editor.

The aligned data set used in the analysis has been deposited with the TreeBASE under the accession number

Table 3 List of accession numbers of the internal transcribed spacer (ITS) gene of *Aphanomyces* spp. at the GenBank database

Species	Host	GenBank accession no.	Reference
Aphanomyces spp.			
A. frigidophilus	Japanese char egg	AY647192	Unpublished
A. piscicida	Ayu	AY283643	Unpublished
A. piscicida	Striped snakehead	AY455773	Unpublished
A. invadans	No date	AY082908	Unpublished
A. invadans	No date	AY082907	Unpublished
A. astaci	Crayfish	AY310499	Oidtmann et al. (2004)
A. astaci	Crayfish	AY683893	Royo et al. (2004)
A. astac	Crayfish	AY683894	Royo et al. (2004)
A. euteiches	Alfalfa	AY353908	Levenfors and Fatehi (2004)
A. euteiches	Green bean	AY353910	Levenfors and Fatehi (2004)
A. euteiches	Green bean	AY353909	Levenfors and Fatehi (2004)
A. euteiches	Garden pea	AY353902	Levenfors and Fatehi (2004)
A. laevis	No date	AY683885	Royo et al. (2004)
A. laevis	No date	AM947028	Vralstad et al. (2009)
A. laevis	No date	AY310497	Oidtmann et al. (2004)
A. stellatus	Water	AY455774	Unpublished
A. stellatus	Unknown	AM947029	Vralstad et al. (2009)
A. stellatus	Soil	AY283647	Unpublished
A. cladogamus	Spinach	AY353918	Levenfors and Fatehi (2004)
A. cladogamus	Alfalfa	AY353913	Levenfors and Fatehi (2004)
A. cladogamus	Red clover	AY353915	Levenfors and Fatehi (2004)
A. repetans	Crayfish	AY683889	Royo et al. (2004)
A. repetans	Crayfish	AY683892	Royo et al. (2004)
A. repetans	Crayfish	AY683890	Royo et al. (2004)
A. repetans	Crayfish	AY683891	Royo et al. (2004)
Out group			
Pythium ultimum	No date	EU253560	Unpublished

S2676. Phylogenetic analyses were performed with PAUP v4. 0 (beta 10) (Swofford 2001) using a heuristic search for maximum parsimony (MP) trees. Base-pair composition and transition and/or transversion patterns of the data set were estimated by PAUP v4.0 (beta 10). Bootstrap values were calculated over 1,000 replicates to assess branch topology. A phylogenetic tree was selected from 1,000 unrooted trees and was drawn by Tree View PPC (Roderic D. M. Page, Glasgow, Scotland, UK, 1998; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Clades were supported by bootstrap values >80%.

Results

Case

The ice fish, *S. microdon*, were captured from a wild population in Lake Shinji in Shimane Prefecture, Japan, located at northern latitude of 35°25′ and longitude 132°57′ east. These fish belong to the family Salangidae, which have a transparent body with few dark pigments and very few scales, even as adults. The digestive tract is straight, has no gastric gland, and retains larval features, not only in appearance but also in internal organs. Therefore, this ice fish is considered neotenic (Harada et al. 2005). These fish were transferred to the aquarium to be exhibited at the end of January 2008. They were housed in a rearing tank with a closed circulating system, and the water was aerated and heated to maintain a temperature of 15°C. The fish were fed brine shrimp *Artemia* sp. Fish colonies were maintained in exhibits of 20 fish per 300-l tank. Lesions began to appear on some fish at the beginning of February 2008. The affected fish were approximately 10 months posthatching and had developed white cotton-like lesions on the fins and heads. Three moribund and one dead fish with body weights ranging from 1.3 to 2.4 g were collected from the rearing tank.

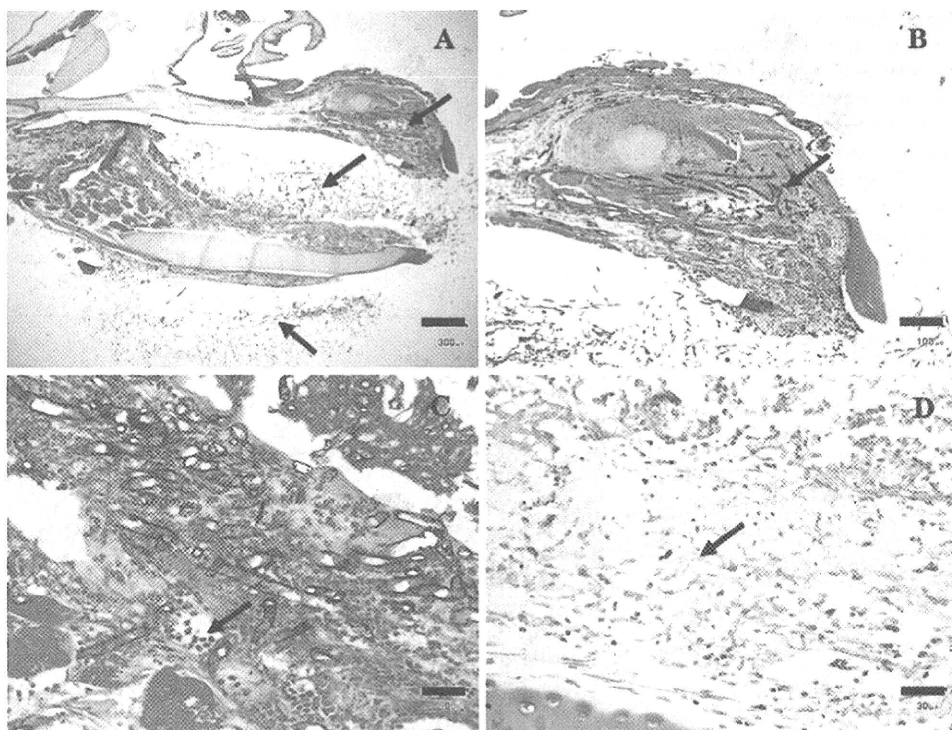

Fig. 2 Histopathological characteristics of diseased fish. **a** Mycelial growth in the rostrum, lower jaw, and oral cavity (*arrow*). Grocott-hematoxylin and eosin (H&E). **b** Hyphae penetrating deep into tissue are associated with necrosis (*arrow*). Grocott-H&E. **c** Inflammatory cells surround hyphae (*arrow*). Grocott-H&E. **d** Gram-negative short-rod bacteria in the lesion (*arrow*). Gram stains. *Bars* **a** 300 μm; **b** 100 μm; **c**, **d** 30 μm

Histopathological observation

Lesions in the affected ice fish contained massive amounts of aseptate fungal hyphae that filled the oral cavity and covered wide areas of the mandible and maxilla (Fig. 2a). Many hyphae penetrated into dermal layers, subcutaneous tissues, and muscular layers and were associated with cellular debris. Hyphae often infiltrated into the cartilaginous tissue of the mandible and maxilla (Fig. 2b). The hyphae that had infiltrated into the host tissue were surrounded by a thin layer of the mononuclear rounded macrophage-like cells (Fig. 2c). Often, numerous Gram-negative short-rod bacteria were associated with the mycotic lesions on the affected fish (Fig. 2d). Multinucleated giant cells were not observed in any of the lesions.

Morphology

Five strains, NJM 0801, 0802, 0803, 0804, and 0805, were isolated from affected ice fish and determined to be identical to one another based on morphological observations. Colonies of all isolated strains developed white flat mycelia when grown on GY agar with 0.5% NaCl. Vegetative mycelia were delicate, about 5–10 μm in width, aseptate, smooth, slightly wavy, and moderately branched (Fig. 3). Zoosporangia were slender with the same diameter as hyphae, namely, isodiametric. Primary zoospores were produced in a single row within zoosporangium and were encysted in a cluster at the top of the zoosporangium.

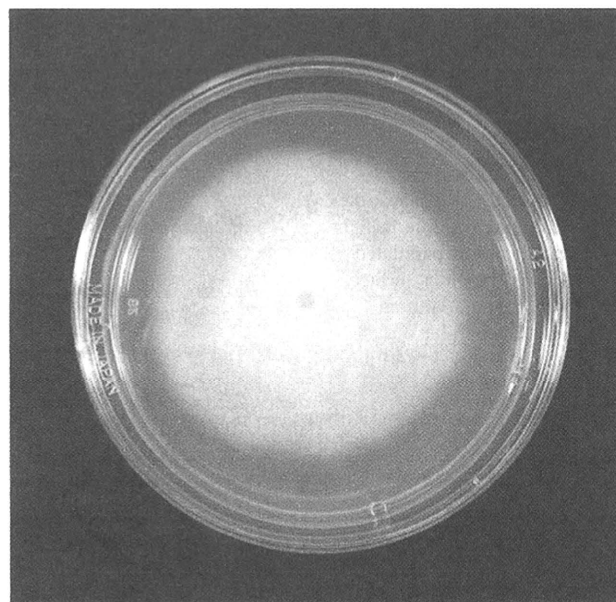

Fig. 3 A colony of the isolate NJM 0801 cultured on glucose–yeast (GY) agar with 0.5% sodium chloride (NaCl) at 20°C

Primary zoospores were about 8–11 μm in diameter. Based on this mode of zoospore formation and other morphological criterion, strains NJM 0801, 0802, 0803, 0804, and 0805 were identified as members of the genus *Aphanomyces* (Figs. 4, 5).

After hyphae were resuspended in sterilized tap water with 0.5% NaCl, sexual reproductive organs were observed

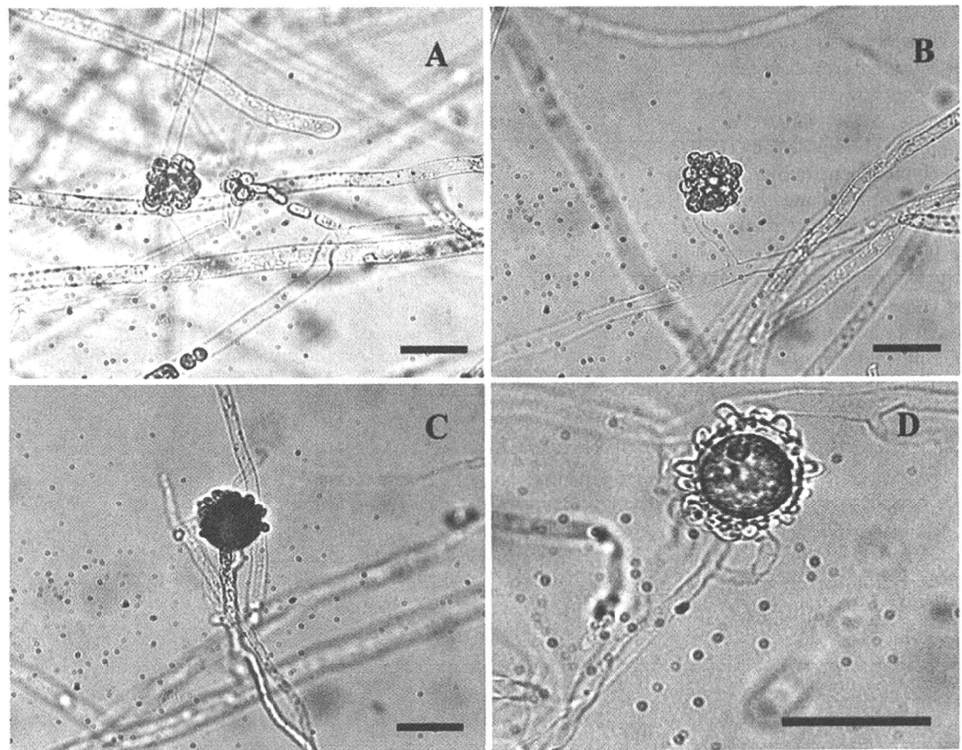

Fig. 4 Morphological characteristics of *Aphanomyces* sp. NJM 0801 isolated from ice fish. **a** Zoospores swimming away from zoosporangium in a row. **b** Primary zoospores, which encysted as cluster at the orifice. **c** A young oogonium, which has irregular short papillated antheridia on the outer surface. **d** A matured oogonium with an antheridium, which has a subcentric oospore. A matured oogonium with irregular short papillate. *Bars* 40 μm

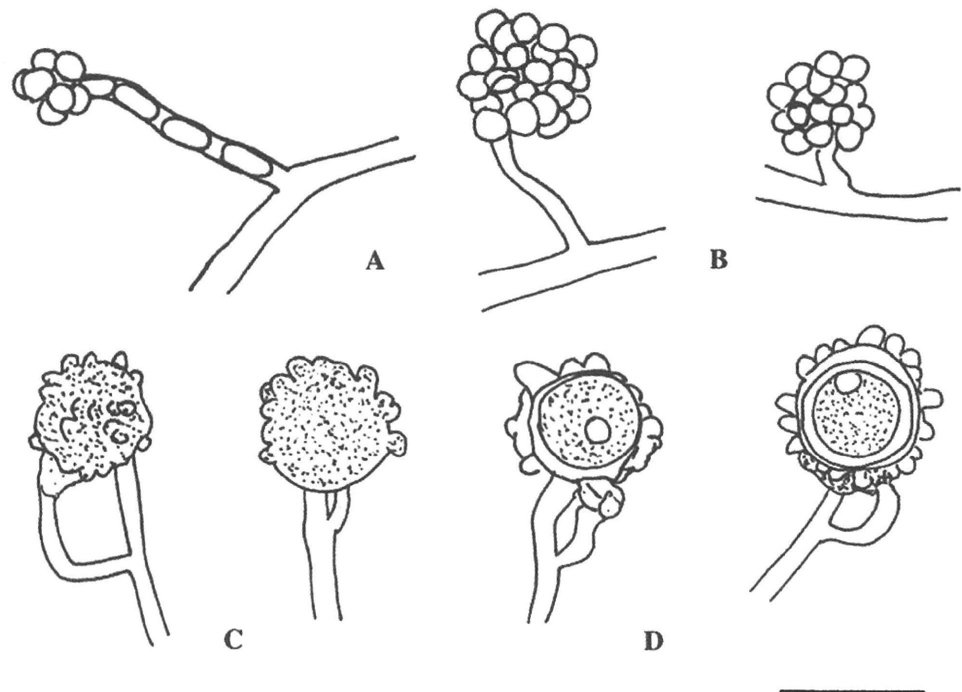

Fig. 5 Morphological characteristics of *Aphanomyces* sp. NJM 0801 isolated from ice fish. **a** Zoospores swimming away from zoosporangium in a row. **b** Primary zoospores, which encysted as cluster at the orifice. **c** Young oogonia, which have irregular short papillated antheridia on the outer surface. **d** Matured oogonia with an antheridium, which have a subcentric oospore. *Bar* 40 μm

at day 3. Oogonia were abundant, usually spherical or subspherical, and only rarely pyriform; they ranged in diameter from 21 to 33 μm, with irregular short papillae, even in young oogonia. Singly sprouted oospores mostly filled oogonium and were generally spherical, ranging from 19 to 27 μm in diameter. Oospores contained a subcentric refractive globule in the cytoplasm. Antheridal branches, when present, were predominantly androgynous; monoclinous and diclinous antheridial branches occurred infrequently. Oogonial stalks were unbranched. Oogonia with single antheridium predominated; however, there were a few oogonia with double or triple antheridia (Figs. 4, 5).