Mycoscience (2010) 51:443–455
DOI 10.1007/s10267-010-0059-2

FULL PAPER

# A new acidophilic fungus *Teratosphaeria acidotherma* (Capnodiales, Ascomycota) from a hot spring

**Atsushi Yamazaki · Kyoko Toyama · Akira Nakagiri**

Received: 28 December 2009 / Accepted: 14 May 2010 / Published online: 12 June 2010


**Abstract** A novel acidophilic fungus was isolated by an acidic enrichment culture of microbial mats and biofilms collected at an extremely acidic and high temperature hot spring. In culture studies, this fungus was revealed to produce ascomycetous teleomorph structures. Molecular phylogenetic study and morphological observation showed this fungus is a new species of the genus *Teratosphaeria* (Capnodiales, Dothideomycetes) and is phylogenetically close to *Acidomyces acidophilus* and *Bispora* sp., which were previously reported as acidophilic anamorphic fungi. This new fungus is described here as a new species of *Teratosphaeria*, and its physiological properties adapting to its habitat are demonstrated. This is the first report of a teleomorphic fungus having highly acidophilic and thermophilic properties.

**Keywords** Microbial mat · Teleomorphic acidophile · Thermophilic

## Introduction

In order to investigate fungal diversity and explore fungi with unique and peculiar properties, we have been trying to isolate fungi from various habitats including extreme environments. In our recent survey, we focused on fungi inhabiting extremely acidic and high temperature environments, because these fungi have rarely been investigated so far, especially in Japan.

A. Yamazaki (✉) · K. Toyama · A. Nakagiri
Department of Biotechnology, Biological Resource Center (NBRC), National Institute of Technology and Evaluation, 2-5-8 Kazusakamatari, Kisarazu, Chiba 292-0818, Japan
e-mail: yamazaki-atsushi@nite.go.jp

In acidic water environments, eukaryotes including fungi are known to play an important role in the formation of microbial mats and streamers (Baker and Banfield 2003). Zettler et al. (2002) reported eukaryotic microbes, such as algae, protozoa and fungi, were the principal contributors of biomass in pH 2 and 30°C water at the Tinto River in Spain. In the acid mine drainage, hyphal filaments are the major component of biofilms and provide a surface for the attachment of other microorganisms (Johnson 1998; Baker et al. 2004). These facts suggest that some fungi have succeeded in inhabiting such steadily and highly acidic environments. Though the role of acidophilic fungi in acidic environments is not well known, the microbial mats and biofilms may be an indicator of the existence of fungi.

Acidophilic fungi have been reported from various acidic environments. Hitherto, five fungal species isolated from acidic environments are known to be able to grow in extremely acidic conditions. *Acontium velatum* Morgan was isolated from a solution containing 4% copper sulfate (pH 0.2–0.7) (Starkey and Waksman 1943). *Trichosporon cerebriae* (an invalid name) was reported to grow 2 N sulfuric acid solution containing glucose and peptone (Sletten and Skinner 1948). Capnodialean anamorphic fungi were also isolated from acidic environments. *Acidomyces acidophilus* (Sigler & J.W. Carmich.) Baker et al. ex Selbmann et al. was reported as an acidophilic species and has been isolated from the soil (pH 1.4–3.5) adjacent to a sulphur pilefield from a natural gas purification plant [as *Scytalidium acidophilum* Sigler & J.W. Carmich. (1974)] and acid mine drainage (pH 0.8–1.38) [as '*Acidomyces richmondensis*' (nom. inval.) (Baker et al. 2004)]. *Hortaea acidophila* Hölker et al. was also isolated from brown coal (pH 0.6) containing humic and fulvic acids (Hölker et al. 2004). These latter two species were reported to be able to grow even at pH 1 (Sigler and

Carmichael 1974; Baker et al. 2004; Hölker et al. 2004; Selbmann et al. 2008). Interestingly, these acidophilic fungi mentioned above are all anamorphic fungi, and no teleomorphic species has been reported from such highly acidic environments.

Under these conditions, we tried to isolate fungi from a highly acidic hot spring, the Sainokawara hot spring, where microbial mats and biofilms are abundantly formed, to examine the existence of acidophilic fungi in such an extreme environment. We succeeded in isolating several fungal strains from various materials collected at the hot spring. Taxonomic studies on these isolates revealed they are of a single species of undescribed acidophilic fungus and this species produces ascomata on an agar medium. Phylogenetic studies revealed this fungus belongs to Capnodiales, Dothideomycetes, Ascomycota. This is the first report of acidophilic teleomorphic fungi.

In this paper, we describe the new teleomorphic fungus and its physiological properties adapting to an extremely acidic environment.

## Materials and methods

### Isolation of strains

Samples for isolation were collected at the Sainokawara hot spring (521-3, Ooaza Kusatsu, Kusatsu Town, Agatsuma gun, Gunma Prefecture, Japan) (Fig. 1). Water of the hot spring fountainhead is extremely acidic (pH 1.5) and hot (96°C). Components of the hot spring water are as follows: $Na^{+} = 116$ mg, $K^{+} = 67.7$ mg, $Mg^{++} = 57.2$ mg, $Ca^{+} = 98.3$ mg, $Fe^{++} = 11.1$ mg, $Mn^{++} = 3.09$ mg, $Al^{++} = 55.1$ mg, $H^{++} = 31.6$ mg, $F^{-} = 23.8$ mg, $Cl^{-} = 998$ mg, $SO_4^{-} = 789$ mg, $HCO_3^{-} = 84.1$ mg, $H_2SiO_3 = 537$ mg, $HBO_2 = 27.8$ mg and $H_2SO_4 = 67.2$ mg; Total components are 3,720 mg/l.

Tree branches, stones covered with microbial mats, microbial streamers and microbial mats, all of which were submerged in hot spring water (30–70°C), were collected. The samples were stored in an incubator (40°C) immediately after collecting and during transportation to the laboratory to keep the temperature of the samples at the average water temperature of the collection sites.

The samples were incubated on the PDA-G plates or immersed in YM liquid medium for 1–4 weeks, at 30, 40 and 50°C. The PDA-G plate was prepared by adding 2% Gellan Gum to Potato Dextrose agar (Nissui, Tokyo, Japan) and adjusting to pH 1.0 with HCl after autoclaving. Tetracycline hydrochloride (Wako, Osaka, Japan) 100 ppm was added to the medium for selecting eukaryotes. YM liquid medium contained 0.3% yeast extract (Difco, USA), 0.3% malt extract (Difco), 0.5% peptone (Difco), 1% glucose (Wako) and 100 ppm tetracycline hydrochloride, pH 1.0.

**Fig. 1** Microbial streamers and microbial mats at the Sainokawara hot spring

After incubation on the PDA-G plates and YM liquid medium for 1–4 weeks, mycelia of the same appearance appeared from each of the precultured tree branches, stones, microbial streamers and microbial mats. For isolation, hyphal tips were transferred to the PDA-G plates and YM liquid medium by using a tungsten needle. In total, four strains (KSE, KSI, KSS and KSB) were isolated.

### Morphological observation

Corn meal agar (CMA, Nissui) plates of pH 1–3 (2% gellan gum was added to the pH 1 and 2 media) were used for morphological observation, because we found sexual reproduction was enhanced only on this medium. Ascomata, asci and ascospores were observed using both light and scanning electron microscopes (LM and SEM).

Samples for SEM observations were prepared according to the method described by Nakagiri and Tubaki (1982). Samples were fixed with 1% osmium tetroxide for 12 h at 4°C.

Following complete dehydration in a graded ethanol series (30, 50, 70, 80, 90, 95 and 100%) and substitution by isoamyl acetate (IAA) in a graded IAA-ethanol series (33, 50, 66 and 100%), the materials were critical point dried in a Hitachi Critical Point Drier, coated with platinum palladium in a JEOL Magnetron Sputter, JUC-5000, and observed under a JEOL scanning electron microscope, JSM-6060, operated at 15 kV.

### DNA isolation for phylogenetic studies

The isolated strains (KSE, KSI, KSS and KSB) were incubated on PDA (pH 5.6) plates for 2 weeks at 37°C. Their mycelia were harvested using a spatula and put into

cartridges of a Maxwell 16™ (Promega, Madison, WI). DNA was extracted and purified using the Maxwell 16™ according to the manufacturer's instructions.

### Sequencing of 18S rDNA, rDNA ITS and 28S rDNA D1/D2/D3 regions

Each of the three genes coding 18S rDNA (SSU), rDNA ITS [the first internal transcribed spacer (ITS1), the 5.8S rDNA gene, the second ITS region (ITS2)] and the 5′ end of the 28S rDNA gene (LSU) including D1, D2 and D3 regions was amplified as a single fragment by polymerase chain reaction (PCR) using KOD FX (Toyobo, Tsuruga, Fukui, Japan). The primers employed were the 18S rDNA gene standard primer pairs, 18F (Ueda and Mikata 1999) and ITS2 (White et al. 1990), ITS region standard primer pairs, ITS5 and ITS4 (White et al. 1990), and 28S rDNA gene D1/D2/D3 region standard primer pairs, NL1 (O'Donnell 1993) and LR5 (Vilgalys and Hester 1990). Amplification of the desired fragment was performed in a thermal cycler, Mastercycler ep Gradient S (Eppendorf, Hamburg, Germany) with the following program: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s and extension at 68°C for 2 min (5 min at 72°C for the final extension).

The products were purified using Agencourt AMPure (Beckman Coulter, USA). PCR for sequencing was performed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) in the thermal cycler under the following program: initial denaturation at 96°C for 3 min, followed by 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s and extension at 60°C for 4 min.

The primers employed for sequencing are as follows: 18F, 950F-2 (5′-TCAGTGAACGAAAGTTAGGG-3′) and 950R-2 (5′-TCCCCTAACTTTCGTTCACT-3′), which were originally designed by us, 18R, 1300F, 550F, 550R and 1300R for the 18S rDNA region (Ueda and Mikata 1999), ITS5 and ITS4 for the ITS region (White et al. 1990), and NL1, NL4 and LR5 for the 28S rDNA D1/D2/D3 region (O'Donnell 1993). Sequences were analyzed with an ABI PRISM 3130 or 3730xl Genetic Analyzer (Applied Biosystems).

DNA sequences obtained in this study were deposited at DDBJ/EMBL/GenBank (for accession numbers, see Table 1).

### Phylogenetic analysis

Molecular phylogenetic analyses of our four isolates based on 28S rDNA D1/D2/D3 region sequences (806 bp) were conducted with 86 strains of relatives belonging to the orders Capnodiales and Dothideales by referring Crous et al. (2007a) (Fig. 2). Thirty-seven strains including halophilic, acidophilic or plant-pathogenic fungi were selected for the analysis based on the ITS region sequences (400 bp) (Fig. 3). Alignment data of these sequences were deposited in TreeBASE (http://www.treebase.org/) under the accession number S10548. Clustal X (1.83) (Thompson et al. 1997) and BioEdit software (Hall 1999) were used to generate the evolutionary distances [the $K_{nuc}$ value (Kimura 1980)] and the similarity values.

The neighbor-joining (NJ) analysis (Saitou and Nei 1987) was performed from $K_{nuc}$ values, and the bootstrap resampling method (Felsenstein 1985) with 1,000 replicates was used for evaluation of the topology of the phylogenetic tree. The NJ plot (Perrière and Gouy 1996) was used for plotting the phylogenetic tree.

### Hyphal growth on various media

Hyphal growth was compared by culturing the four strains on the PDA, CMA, malt extract agar (MEA) containing 2% malt extract, 2% glucose, 0.1% peptone and 2% agar (Wako), YM agar, oatmeal agar (OA) containing 2% agar (Wako) in oatmeal extract, which was prepared by decocting oatmeal (4%) in distilled water at 80°C for 1 h and squeezing it through a muslin bag, potato carrot agar (PCA) containing 2% agar (Wako) in potato carrot extract, which was prepared by mashing and straining potato (2%) and carrot (2%) in distilled water after boiling for 20 min, and Czapek Dox Agar (CzA) (Nissui) of neutral pH (pH 5.6–6.0) at 37°C

**Table 1** List of strains examined

| Strain ID | CC no. | Scientific name | Herbarium no. | Source | DDBJ/EMBL/GenBank accession no. (left, 18S; right, ITS-28S) | |
|---|---|---|---|---|---|---|
| KSE | NBRC 106057 | *Teratosphaeria acidotherma* | NBRC H-12769 (Holotype) | Submerged tree branch in hot spring | AB537894 | AB537898 |
| KSB | NBRC 106058 | *Teratosphaeria acidotherma* | NBRC H-12770 | Microbial mats | AB537896 | AB537899 |
| KSS | NBRC 106059 | *Teratosphaeria acidotherma* | NBRC H-12771 | Microbial streamers | AB537897 | AB537900 |
| KSI | NBRC 106060 | *Teratosphaeria acidotherma* | NBRC H-12772 | Submerged stone in hot spring | AB537895 | AB537901 |

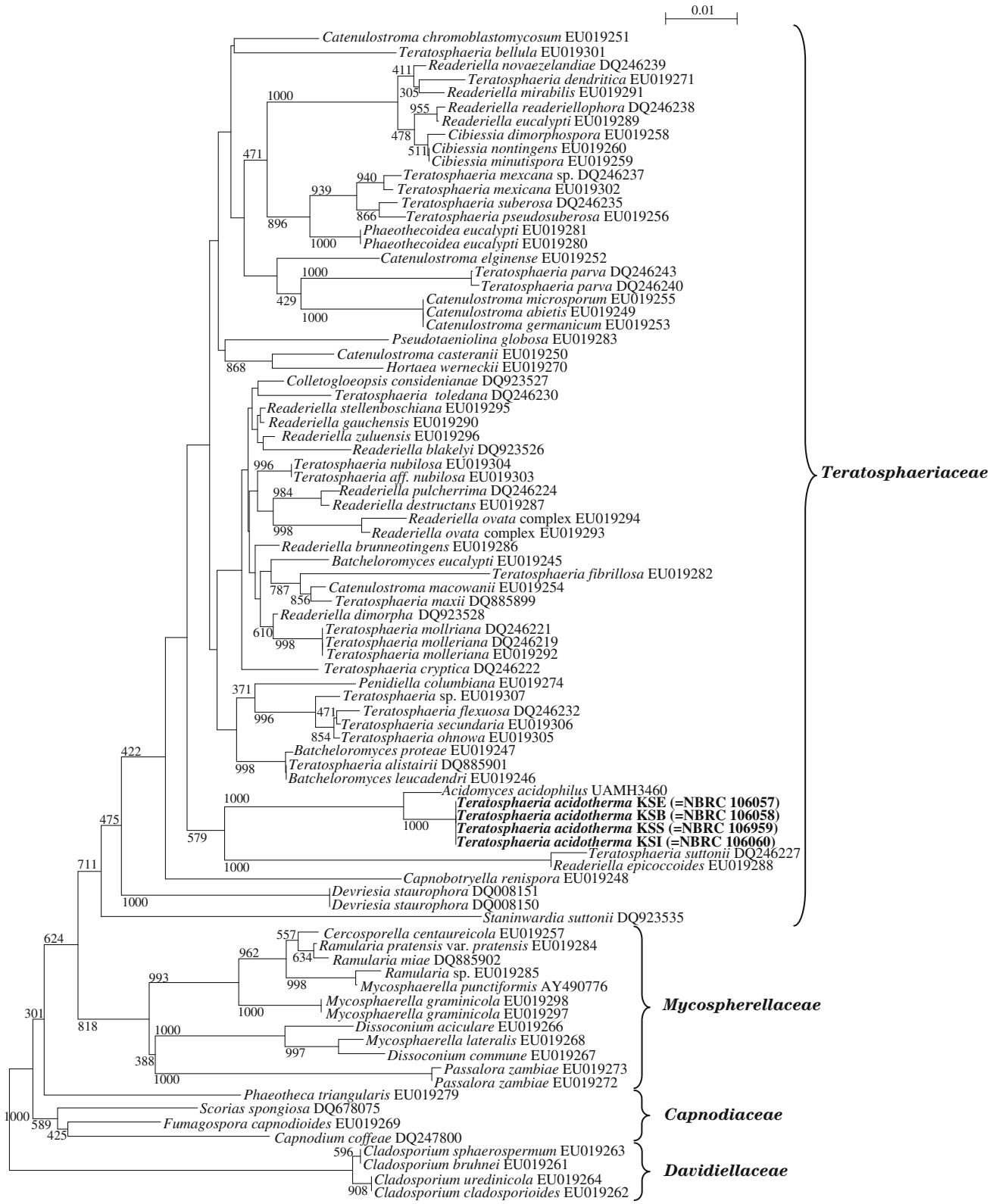


**Fig. 2** Molecular phylogeny tree inferred from 28S rDNA D1/D2/D3 sequences of Capnodiales, Dothidomycetes. Bootstrap support values from 1,000 replicates are shown at the nodes. *Bar* 0.01 $K_{nuc}$ in nucleotide sequence

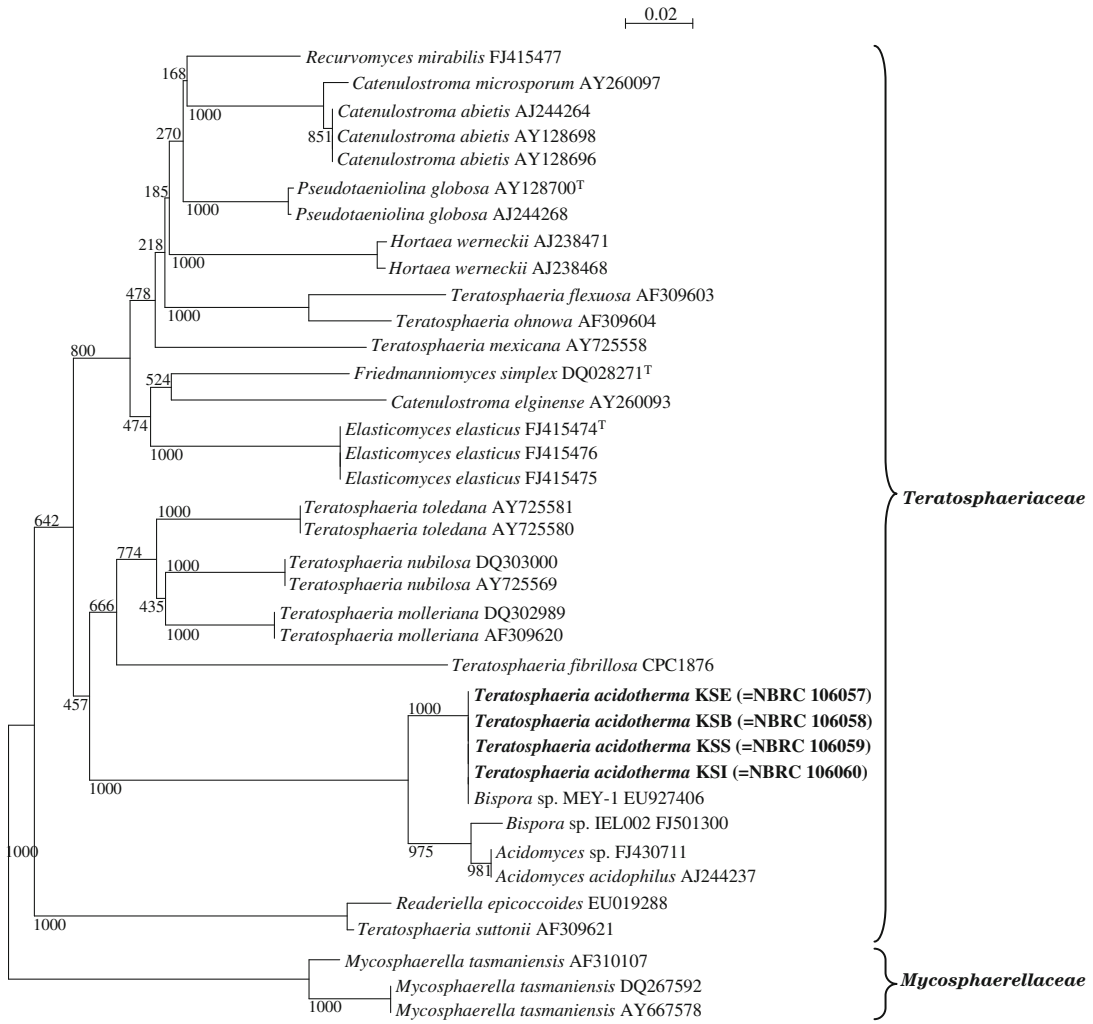


**Fig. 3** Molecular phylogeny tree based on ITS region sequences of Capnodiales, Dothidomycetes. Bootstrap support values from 1,000 replicates are shown at the nodes. Superscript "T" on the accession numbers means the data were obtained from the ex-type strain. *Bar* 0.02 $K_{nuc}$ in nucleotide sequence

for 2 weeks. The strains were inoculated in one spot on three plates of each medium and incubated at 37°C for 2 weeks. Colony diameters were measured regularly. The inocula were prepared by culturing on PDA plates and cutting out agar discs (7 mm diameter) containing mycelium.

## Growth at different pHs

The linear hyphal growth at different pH values was tested using CMA medium at pH 1, 2, 3, 4, 5, 6, 7 and 9. Two percent gellan gum was added for the pH 1 and pH 2 media. Strains were inoculated in two spots on each plate and incubated at 37°C for 1 month by monitoring the colony diameter. These tests were duplicated.

The growth rates at different pH values were also tested by comparing mycelial weight after incubation in YM liquid medium at pH 1, 2, 3, 4, 5, 6 and 7. One disc of inoculum was cultured in 100 ml of medium each. The culture was shaken in 200-ml conical flasks with a rotary shaker at the shaking speed of 115 rpm at 37°C for 3 days. Cultures were filtered, and the biomass of the dried mycelia was weighted. These tests were triplicated.

## Growth at different temperatures

The linear hyphal growth at different temperatures was compared in hyphal elongation using CMA plates (pH 6.0) at 15, 25, 30, 35, 37, 40 and 45°C. Strains were inoculated in two spots on each plate and incubated for 1 month, then the colony diameters were measured.

## Growth at different NaCl concentrations

The ability to grow at different salinities was tested in hyphal elongation on MEA plates (pH 6.0) containing 1.2, 1.5, 3, 5, 7 and 10% NaCl. Strains were inoculated in one

spot on each plate and incubated at 37°C for 2 weeks. Colony diameters more than 2 mm were considered positive (Kane and Summerbell 1987) and measured for comparison. The test was triplicated.

### Formation of ascomata at different conditions

In order to know the optimum condition for ascomata formation, all four strains were cultured on the following agar media, MEA, PDA, CzA, PCA, YMA, CMA and OA.

Because only CMA showed the positive results, ascomata formation was examined by culturing for 2 weeks on CMA at different pHs (1, 2, 3, 4, 5, 6, 7 and 9) at 37°C or at different temperatures (15, 25, 30, 35, 37, 40 and 45°C) at pH 6.0. These tests were duplicated.

## Results

### Strains isolated

Four strains (KSE, KSI, KSS and KSB) were obtained from various materials collected at the Sainokawara hot spring (Table 1). All the strains were later found of a single species. The strain KSE was isolated from a tree branch incubated in YM liquid medium of pH 1 at 40°C for 1 week. The strains KSI, KSS and KSB were isolated from stones, streamers and microbial mats, respectively, by incubating them on PDA-G plates of pH 1 at 40°C for 1 month.

### Phylogeny

The four strains, KSE, KSI, KSS and KSB, were found to have completely identical DNA sequences of 18S rDNA, ITS regions and 28S rDNA D1/D2/D3 regions. A NJ tree inferred from the 28S rDNA rDNA D1/D2/D3 sequences is shown in Fig. 2. The outgroup was represented by *Cladosporium sphaerospermum* Penz., *C. bruhnei* Linder, *C. uredinicola* Speg. and *C. cladosporioides* (Fresen.) G.A. de Vries. The four strains are nested within a large cluster of *Teratosphaeria* Syd. & P. Syd., in which many plant-associated species are included (Taylor et al. 2003; Crous et al. 2004, 2006, 2007a, b). The four strains form an isolated clade clustering with *Acidomyces acidophilus* as the sister clade. *Teratosphaeria suttonii* (Crous & M.J. Wingf.) Crous & U. Braun and its anamorphic species *Readeriella epicoccoides* (Cooke & Massee) Crous & U. Braun are linked closely to this clade. This result was sustained with the bootstrap value 57.9%. Their sequence similarities in the 28S rDNA D1/D2/D3 region to the present fungus were 91.9% (*Teratosphaeria suttonii*) and 92.0% (*Readeriella epicoccoides*).

In the ITS tree (Fig. 3), three strains of *Mycosphaerella tasmaniensis* Crous & M.J. Wingf. were selected as an outgroup. The tree contains a number of *Teratosphaeria* found from plant materials, and also anamorphic taxa such as *Acidomyces* species and *Bispora* sp., which were reported from acidic environments. Our four strains are clustering with *Acidomyces acidophilus*, *Acidomyces* sp. and *Bispora* sp., which are all nested in the *Teratosphaeria* clade, including *Teratosphaeria fibrillosa* Syd. & P. Syd. (type species of the genus *Teratosphaeria*), *T. toledana* (Crous & Bills) Crous & U. Braun, *T. nubilosa* (Cooke) Crous & U. Braun, *T. molleriana* (Thüm.) Crous & U. Braun and *T. suttonii*.

We found the strain *Bispora* sp. MEY-1 has the identical sequence of 547 base pairs in the ITS region to those of our four isolates, though no description about the teleomorph was given for MEY-1 by Luo et al. (2008). Because there is a possibility MEY-1 is in the same taxon as our strains, we tried to obtain the MEY-1 (CGMCC 2500) from the Chinese Academy of Sciences. However, the strain was deposited as a patent strain and not available for our comparative study. There has been no taxonomically valid description about *Bispora* sp. MEY-1, so we describe here our strains as a new species of *Teratosphaeria*, based on our morphological and phylogenetical studies.

### Taxonomy

***Teratosphaeria acidotherma*** A. Yamaz., K. Toyama & Nakagiri sp. nov. Figs. 4, 5

Coloniae lente crescentes in CMA (pH 6.0), 38–40 mm in diametro post 2 hebdomates ad 37°C, celeriter 57–63 mm in acido agaro CMA (pH 2–5). Hyphae pallide fuscae in CMA; fuscae vel vitridifuscae in PDA (pH 5.6); subhyalinae vel pallide fuscae in MEA (pH 6.0). Ascomata pseudothecia, semiimersus vel immersas in CMA, globosa vel subglobosa, 43–73 μm lata, 55–80 μm alta, brunneola, papillata 16–30 μm in diametro ostiolata cum periphysibus. Asci fasciculati, bitunicati, sessiles, obovati vel anguste ellipsoidi, recti vel parum incurvati, octospori, 24–36 × 8–10.5 μm. Pseudoparaphyses nullae. Ascosporae fusoideae vel ellipsoideae basi et apice obtusae, latissimae ad medium cellulae apicalis, mediano 1-septatae, ad septum subconstrictae, laeves, cum vagina mucosa, 8.5–11.5 × 2.7–3.5 μm. Anamorphosis absens, sed proferens cellulas meristemoidea simulans *Acidomyces acidophilus*, et hyphas disarticulatus.

Holotypus: NBRC H-12769, colonia exsiccata in cultura, ex ramulo immersus in aqua therma, Sainokawara, Kusatsu, Agatsuma-gun, Gunma Pref., Japonia, 5 Sep. 2008, coll. & isol. A. Yamazaki, in Herbario NBRC conservata. Cultura viva: NBRC 106057 (=KSE).

Etymology: *acido* (= acidic) + *therma* (= hot spring), referring to the habitat of this fungus.

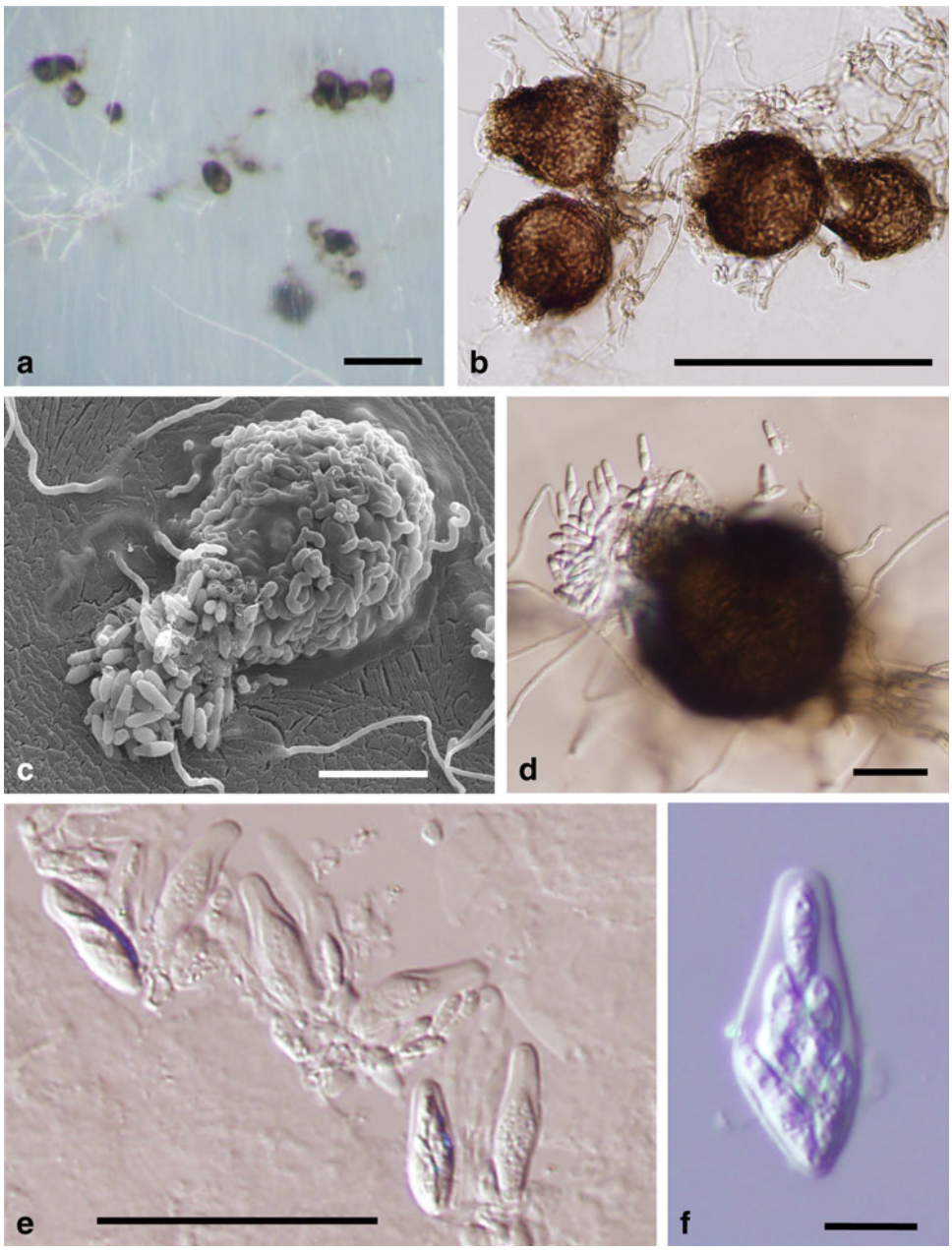


**Fig. 4** *Teratosphaeria acidotherma*. **a** Ascomata on CMA plate. **b** Ascomata (LM). **c** Ascoma releasing ascospores from ostiole (SEM). **d** Ascoma and ascospores squeezed out (LM). **e**, **f** Bitunicate asci (LM). *Bars* **a** 200 μm; **b** 100 μm; **c**, **d** 20 μm; **e** 50 μm; **f** 10 μm

Other specimens examined: NBRC H-12770 (ex NBRC 106058 = KSB), NBRC H-12771 (ex NBRC 106059 = KSS) and NBRC H-12772 (ex NBRC 106060 = KSI), all isolated from submerged twigs in hot spring water, Sainokawara, Kusatsu, Agatsuma-gun, Gunma Pref., Japan, 5 Sep 2008, coll. & isol. A. Yamazaki.

Colonies grow slowly, 38–40 mm in diameter, on CMA (pH 6.0) at 37°C in 2 weeks, and grow fast on acidic agar medium CMA (pH 2–5), 57–63 mm in diameter. Hyphae are pale brown on CMA, dark brown to dark green on PDA (pH 5.6), and subhyaline to pale brown on MEA (pH 6.0). Only on CMA plates, ascomata formation was initiated after 3–5 days incubation at 20–40°C.

Ascomata pseudothecial, semi-immersed to immersed in CMA, globose to subglobose, 43–73 μm wide, 55–80 μm high, brownish, with a papilla of 16–30 μm diameter, ostiolate, periphysate. Asci fasciculate, bitunicate, sessile, obovoid to narrowly ellipsoid, straight or slightly curved, 8-spored, 24–36 × 8–10.5 μm. Pseudoparaphyses absent. Ascospores fusoid to ellipsoidal with obtuse ends, medianly 1-septate, widest in the middle of the upper cell, slightly constricted at the septum, smooth, with a mucous sheath, 8.5–11.5 × 2.7–3.5 μm.

Anamorph absent, but forming a meristematic cell-like body with brown thick-walled cells like *Acidomyces acidophilus*, and also disarticulating hyphae, which are

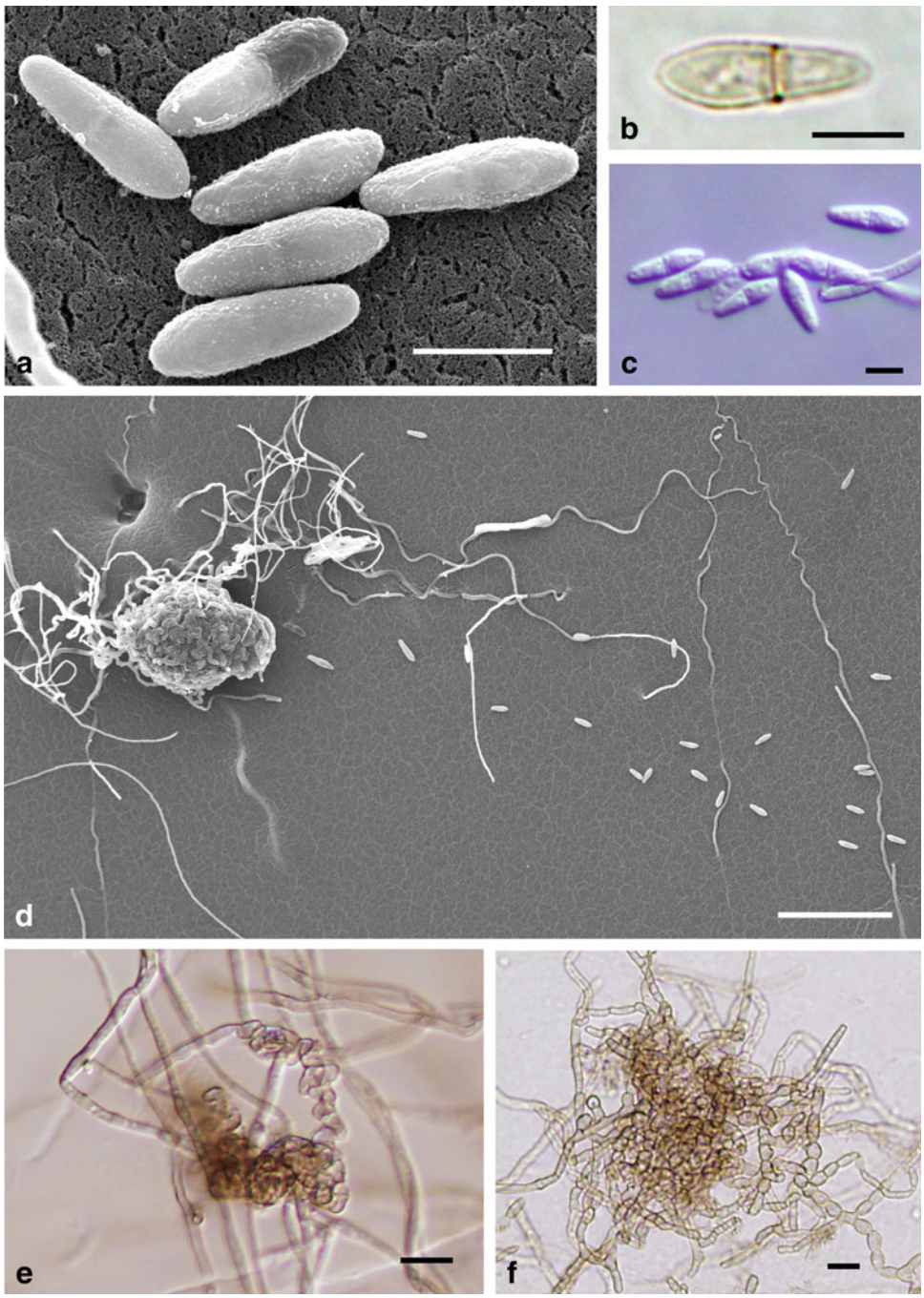


**Fig. 5** *Teratosphaeria acidotherma*: **a** Ascospores (SEM). **b**, **c** Ascospores (LM). **d** Ascoma and forcibly ejected ascospores from a beak (SEM). **e** Meristematic cell-like body (LM). **f** Disarticulating hyphae (LM). *Bars* **a**, **b**, **c** 5 μm; **d** 50 μm; **e**, **f** 10 μm

simple or irregularly branching, terminal or intercalary, fragmenting into 1–5 or more cells, 7–108 × 2.5–7 μm.

## Physiology

Cultural properties of the four strains of *Teratosphaeria acidotherma* at different media, pH, temperature and salinities were examined. The growth test on various media of neutral pH showed that all strains were able to grow on the all of the media tested, and the best hyphal growth was on MEA plates. Growth reduction was clearly shown when seeded on CzA plates (Fig. 6).

The growth test at different pHs revealed all the strains grow better at pH 2–5 and well even in extremely acidic conditions (pH 1), but their growth is depressed at pH 6–7 and greatly retarded at pH 9 (Figs. 7, 8). These phenomena were observed almost equally except at pH 1 in the two different tests, measuring the colony size on the solid

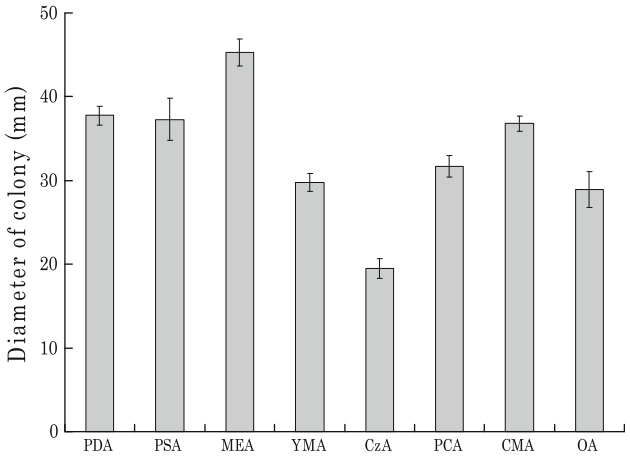


**Fig. 6** Hyphal growth of *Teratosphaeria acidotherma* (4 strains) on different media (37°C, pH neutral not adjusted, 2 weeks)

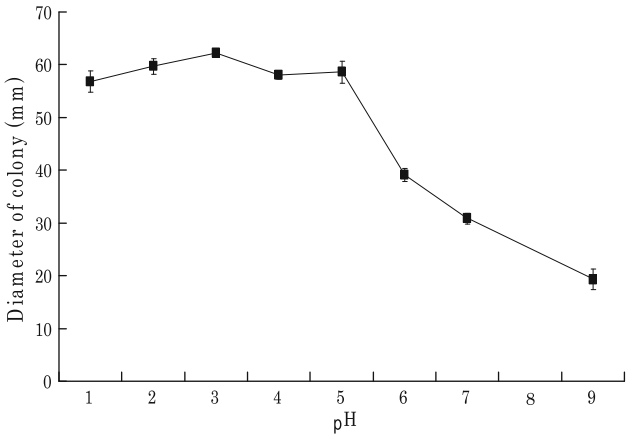


**Fig. 7** Hyphal growth of *Teratosphaeria acidotherma* (4 strains) on a CMA plate at different pHs after 2 weeks of incubation at 37°C

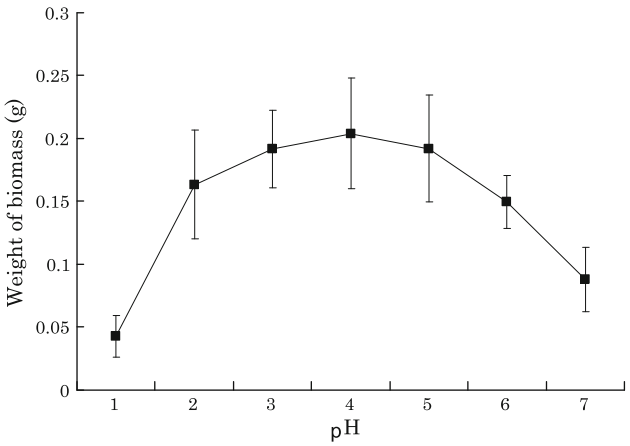


**Fig. 8** Biomass growth of *Teratosphaeria acidotherma* (4 strains) in YM liquid medium at different pHs after 3 days of incubation at 37°C

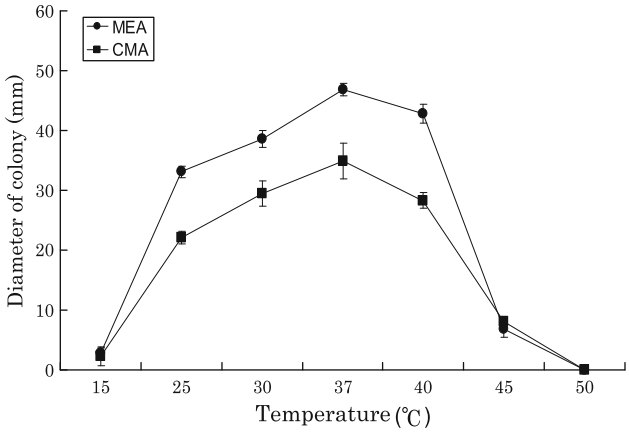


**Fig. 9** Hyphal growth of *Teratosphaeria acidotherma* (4 strains) on MEA and CMA (pH 6.0) at different temperatures after 2 weeks

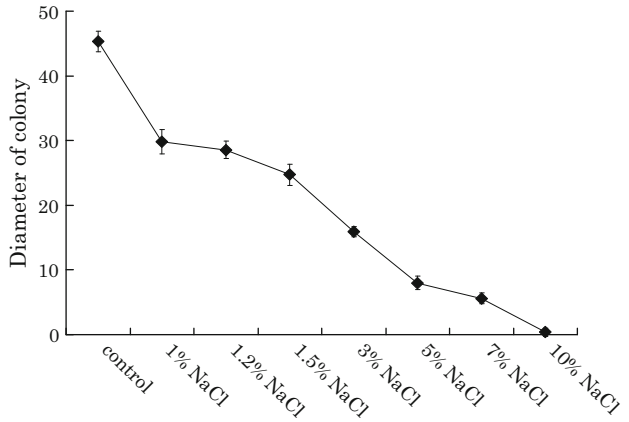


**Fig. 10** Growth of *Teratosphaeria acidotherma* (4 strains) on MEA at different NaCl concentrations (pH 6.0)

medium and weighing the biomass cultivated in liquid medium. A slight growth decline at pH 1 was observed on the solid medium by comparing with pH 2 (Fig. 7), but this phenomenon was more pronounced in the liquid medium.

The growth tests at different temperatures showed all the strains are able to grow in the range of 15–45°C, with better growth at 30–37°C and optimum at 37°C. No visible hyphal growth was detected at 50°C (Fig. 9).

The growth tests at different salinities revealed that the four strains are moderately halotolerant, because they are able to grow up to 7% NaCl but not at 10% (Fig. 10).

In the growth tests for the optimum medium, temperature and pH, we found that the four strains formed ascomata only on CMA plates at the wide range of pHs (pH 1–7) among the media we examined. The maximum formation of ascomata was obtained by culturing on CMA of pH 1–2 (Table 2).

**Table 2** Ascomata formation on CMA at different pHs

| | Incubation period | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 10 | 14 |
| pH | | | | | | | | | | |
| 1 | − | − | w | w | + | + | + | + | ++ | +++ |
| 2 | − | − | w | ++ | ++ | ++ | +++ | +++ | +++ | +++ |
| 3 | − | − | − | w | w | + | + | + | + | + |
| 4 | − | − | − | w | w | + | + | + | + | + |
| 5 | − | − | − | w | w | w | w | w | w | w |
| 6 | − | − | − | − | − | w | w | w | w | w |
| 7 | − | − | − | − | − | − | w | w | w | w |
| 9 | − | − | − | − | − | − | − | − | − | − |

The degree of ascomata formation is indicated with "+++", "++", "+" and "w" in accordance with the number of ascomata on the CMA plate. "+++" means the largest number of ascomata in this study, and "w" means more than one ascomata were detected. "−" means no ascomata were observed

## Discussion

### Morphology

The morphology of the present fungus, i.e., ascomata with periphysate ostioles, bitunicate asci and one-septate ascospores with sheaths, which are forcibly ejected, indicates affinity to Capnodialean Dothideomycetes, especially to the genus *Teratosphaeria*.

The genus *Teratosphaeria* was established by finding *Teratosphaeria fibrillosa* was different from *Mycosphaerella* species in its ascomatal arrangement and periphysate ostioles (Müller and Oehrens 1982). It was later synonimized within the genus *Mycosphaerella* by ITS sequence data (Taylor et al. 2003). But later, Crous et al. (2007a) re-examined *Mycosphaerella* and *Mycosphaerella*-like genera in the Teratosphaeriaceae 28S rDNA D1/D2/D3 sequences and morphologies, and concluded that Teratosphaeriaceae should be separated from Mycosphaerellaceae. These taxonomic arrangements were probably caused because the genus *Mycosphaerella* and related genera are polyphyletic and difficult to distinguish clearly by phenotypic characters. Crous et al. (2007a) suggested the morphological characters of *Teratosphaeria* species, such as superficial stroma linking ascomata together, brown color ascospores, pseudoparaphyses (uncommon), ascospores covered with mucous sheath, multi-layered endotunica of asci (uncommon) and ostiolar periphyses, which are not observed in many *Mycosphaerella* species. Though pseudoparaphyses were not observed, the present fungus showed the morphology of *Teratosphaeria* such as brown color ascospores covered with a mucous sheath and ostiolar periphyses.

Among *Teratosphaeria* species, *T. acidotherma* resembles *T. toledana* and *T. suttonii* in having fusoid-ellipsoid, one-septate ascospores with obtuse ends. The ascospore size of *T. acidotherma* (8.5–11.5 × 2.7–3.5 μm) is nearer to *T. suttonii* (11–12 × 3–3.5 μm) than *T. toledana* (8–10 × 3 μm) in these species. The phylogenetic tree indicates that *T. acidotherma* and *T. suttonii* made a sister clade (Fig. 2), suggesting that *T. suttonii* is one of the *Teratosphaeria* species most related to *T. acidotherma*. However, *T. suttonii* has morphologically and physiologically different characters from *T. acidotherma*. *Teratosphaeria suttonii* has an anamorph (*Readeriella epicoccioides*) producing 3- to 5-celled conidia from conidiogenous cells (Crous and Wingfield 1997). On the other hand, no anamorph of *T. acidotherma* was observed in this study.

In the genus *Teratospheria* (Teratosphaeriaceae), 13 anamorphic genera have been described: *Pseudotaeniolina*, *Cibiessia*, *Phaeothecoidea*, *Devriesia*, *Capnobotryella*, *Hortaea*, *Nothostrasseria*, *Readeriella*, *Stainwardia*, *Penidiella*, *Batcheloromyces*, *Catenulostroma* and *Acidomyces*. These genera were distinguished by their morphology of conidia, conidiomata, conidiophores and disarticulating hyphae (Crous et al. 2007a, b; Selbmann et al. 2008). Though an anamorph of *T. acidotherma* was not observed, the meristematic cell-like body was observed (Fig. 5e) in this study. We also found 1- to 5-septated hyphae (Fig. 5f) in liquid culture, which looked like disarticulating hyphae of *Acidomyces acidophilus* (Selbmann et al. 2008). These characters of *T. acidotherma* suggest that this fungus is probably able to disperse such disarticulated cells as propagules, in addition to the ascospore formation.

*Teratosphaeria suttonii* was isolated from leaves of *Eucalyptus* sp. and has no acidophilic character growing well on neutral MEA (pH 6.0). The optimum growth temperature of *T. suttonii* is 20–25°C, and it can grow up to 35°C (Crous and Wingfield 1997), which is lower than *T. acidotherma* (optimum 37°C, up to 45°C).

These morphological and physiological characters of *T. suttonii* and *T. acidotherma* suggest clearly that these fungi are different species.

## Phylogeny

For phylogenetic study based on the 28S rDNA D1/D2/D3 region, we analyzed with capnodiales fungi by referring to the study by Crous et al. (2007a) (Fig. 2), because teleomorphic characters of the present fungus are of the Dothideomycetes, and the BLAST search of the 28S rDNA sequence showed a close affinity with the capnodialean fungi. The phylogenetic tree (Fig. 2) indicates *Teratosphaeria acidotherma* is nested in the Teratosphaeriaceae clade and forms an isolated clade with *Acidomyces acidophilus.* The tree also shows that *Pseudotaeniolina globosa* De Leo et al., *Hortaea acidophila* and *Hortaea werneckii* (Horta) Nishim. & Miyaji are far apart from the present fungus, though they had been considered as genetically related with *Acidomyces* (Selbmann et al. 2008). We also analyzed ITS region sequences of *T. acidotherma* with Mycosphaerellaceous and Teratosphaeriaceous fungi (Fig. 3). The ITS tree shows that *Acidomyces* spp. and *Bispora* sp. are anamorphic fungi of Teratosphaeriaceae and that *T. acidotherma* forms a sister clade of these anamorphic fungi. *Bispora* and *Acidomyces* strains are similar to the present fungus in having acidophilic properties (Sigler and Carmichael 1974; Baker et al. 2004; Luo et al. 2008; Selbmann et al. 2008). We found that the *Bispora* sp. MEY-1 (CGMCC 2500) strain has a completely identical ITS sequence with that of *T. acidotherma*. *Bispora* sp. MEY-1 was isolated from acidic waste water of the "721" uranium mine in China, and its optimum pH for growth was reported as pH 2.5–3 (Luo et al. 2008). *Bispora* sp. MEY-1 is close to *T. acidotherma* in habitats and optimum pH conditions. From the description about *Bispora* sp. MEY-1 strain (Luo et al. 2008) and our phylogenetic analysis, we consider the strain *Bispora* sp. MEY-1 is possibly identical with *T. acidotherma*. There was no detailed description about the *Bispora* sp. MEY-1 strain given by Luo et al. (2008), and the strain is not available, so that we could not compare the morphological characters between *Bispora* sp. MEY-1 and *T. acidotherma*. We suspect, however, that Luo et al. (2008) gave the name of the *Bispora* sp. MEY-1 strain by observing the morphology of disarticulating hyphae, which might look like the conidia of *Bispora*. Among validly described species, *Acidomyces acidophilus* (≡*Scytalidium acidophilum*, '*A. richmondensis*') is the most closely related (but distant) species to *T. acidotherma* in this phylogenetic analysis. *Acidomyces acidophilus* is, however, known to produce one- to three-celled conidia by arthric disarticulation of hyphae (Selbmann et al. 2008), which is absent in *T. acidotherma*. In our DNA sequence data, we found base differences between *T. acidotherma* and *A. acidophilus* as follows: 1–2 bp in 18S rDNA, 10 bp in the 28S rDNA D1D2D3 region and 29 bp in the ITS region. These data indicate that the two species are different fungi. *Acidomyces acidophilus* was previously suggested to be similar in morphology to species of *Friedmanniomyces* Onofri and *Pseudotaeniolina* J.L.Crane & Schokn. (Crous et al. 2007a). But our phylogenetic analysis here (Figs. 2, 3) showed that *Friedmanniomyces simplex* Selbmann et al. and *Pseudotaeniolina globosa* De Leo et al. were far separated from the *A. acidophilus* and *T. acidotherma* clade. This result is concordant with the report of Selbmann et al. (2008). Based on the phylogenetic analysis and morphology of teleomorphic characters, we assigned the present fungus to the genus *Teratosphaeria. Teratosphaeria acidotherma* is a unique species of *Teratosphaeria* in its acidophilic character, because the species of *Teratosphaeria* have been described and known as plant parasitic fungi.

## Physiology

Vegetative growth and sexual reproduction at various conditions of pH, temperature, osmotic pressure and media were examined in our four strains (KSE, KSI, KSS and KSB) of *Teratosphaeria acidotherma*. The test at various pH conditions showed that optimum pH for growth was pH 2–5. The four strains grow fast even at the pH 1. Delay of growth was observed at high pH (pH 6–9). This result indicates that *T. acidotherma* has an acidophilic property like *Acidomyces* spp. and *Hortaea acidophila*, which have been reported from acidic environments (Sigler and Carmichael 1974; Baker et al. 2004; Hölker et al. 2004; Selbmann et al. 2008). In the examination at various temperatures, we found that 37°C is optimum for growth of *T. acidotherma*, and it can grow up to 45°C. The optimum and maximum growth temperatures of *T. acidotherma* are higher than those of other species of *Teratosphaeria*, *Hortaea* and *Acidomyces*, whose optimum and maximum temperatures are 18–25 and 30°C, respectively (Hölker et al. 2004; Selbmann et al. 2008). This suggests that *T. acidotherma* with a moderately thermophilic character is apparently different from these species. We examined the salinity tolerance of *T. acidotherma* because *Hortaea werneckii*, which was known to be genetically close to *Teratosphaeria* species, was reported to be resistant to high osmotic pressure (27% salinity) (Sterflinger 1998; Zalar et al. 1999). *Teratosphaeria acidotherma* grew on the medium containing up to

7% NaCl but did not at 10% NaCl, showing a moderately halophilic character. This suggests that *T. acidotherma* is physiologically different from *Hortaea werneckii*. MEA was the best medium for growth of *T. acidotherma*. This was the same as the other related species such as *Acidomyces acidophilus*. We employed various media in order to examine the ability of the present fungus to produce teleomorphs. Because many acidophilic and acid-tolerant fungi isolated from various kinds of acidic environments have not been documented on their teleomorphs (Starkey and Waksman 1943; Sletten and Skinner 1948; Ivarson and Morita 1982; Schleper et al. 1995; Hölker et al. 2004; Baker et al. 2004; Selbmann et al. 2008), we supposed that extremely acidic environments may inhibit the formation of a teleomorphic character. Contrary to the above expectation, we found *T. acidotherma* produced ascomata on CMA plates of pH 1–4. Because *T. acidotherma* produces ascomata well in acidic conditions (pH 1–2, 25–40°C), it can be speculated that this species forms the ascomata on plant substrates soaking in the acidic hot spring water. When the substrates were exposed to the air, ascomata might eject and disperse ascospores to the air as we observed ascospore ejection on the CMA plate (Fig. 5d). This may suggest that extremely low pH conditions do not prevent the formation of ascomata and that comparatively lower nutrient conditions (as CMA medium) may induce ascomata formation. This is the first report of ascomata formation of Teratosphaeriaceae in extremely low pH conditions. Thus, it is possible that the other acidophilic or acid-tolerant fungi might be induced to form their teleomorphs by the cultural studies at various conditions, especially using a variety of media including low nutrient media like CMA. Because almost all fungi that have been isolated from acidic or hypersaline environments are anamorphic species (Starkey and Waksman 1943; Sletten and Skinner 1948; Sigler and Carmichael 1974; Sterflinger 1998; Zalar et al. 1999; Baker et al. 2004; Hölker et al. 2004; Selbmann et al. 2008), there may be some relationships between the anamorphic state and adaptation to acidic or hyper-saline environments. Investigation of the optimum condition for anamorph or teleomorph formation in various acidophilic fungi may give us the clue to understanding their life cycle in natural habitats and how they adapt to acidic environments. Many of the species in Capnodiales (Dothideomycetes) are known foliicolous fungi on terrestrial plants. It is interesting that *T. acidotherma* and other acidophlic and/or halotolerant species are co-members with plant pathogenic species in Capnodiales clade. It is surely an interesting problem for the future to investigate how and when they evolved acidophilic or halotolerant properties from plant pathogenic characters (or vice versa).

## References


Baker BJ, Banfield JF (2003) Microbial communities in acid mine drainage. FEMS Microbiol Ecol 44:139–152

Baker BJ, Lutz MA, Dawson SC, Bond PL, Banfield JF (2004) Metabolically active eukaryotic communities in extremely acidic mine drainage. Appl Environ Microbiol 70:6264–6271

Crous PW, Wingfield MJ (1997) New species of *Mycosphaerella* occurring on *Eucalyptus* leaves in Indonesia and Africa. Can J Bot 75:781–790

Crous PW, Groenewald JZ, Mansilla JP, Hunter GC, Wingfield MJ (2004) Phylogenetic reassessment of *Mycosphaerella* spp and their anamorphs occurring on *Eucalyptus*. Stud Mycol 50:195–214

Crous PW, Wingfield MJ, Mansilla JP, Alfenas AC, Groenewald JZ (2006) Phylogenetic reassessment of *Mycosphaerella* spp and their anamorphs occurring on *Eucalyptus*. Stud Mycol 55:99–131

Crous PW, Braun U, Groenewald JZ (2007a) *Mycosphaerella* is polyphyletic. Stud Mycol 58:1–32

Crous PW, Summerell BA, Carnegie A, Mohammed C, Himaman W, Groenewald JZ (2007b) Foliicolous *Mycosphaerella* spp and their anamorphs on *Corymbia* and *Eucalyptus*. Fungal Divers 26:143–185

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Hölker U, Bend J, Pracht R, Müller T, Tetsch L, de Hoog GS (2004) *Hortaea acidophila*, a new acidophilic black yeast from lignite. Antonie Van Leeuwenhoek 86:287–294

Ivarson KC, Morita H (1982) Single-cell protein production by the acid-tolerant fungus *Scytalidium acidophilum* from acid hydrolysates of waste paper. Appl Environ Microbiol 43:643–647

Johnson DB (1998) Biodiversity and ecology of acidophilic microorganisms. FEMS Microbiol Ecol 27:307–317

Kane J, Summerbell RC (1987) Sodium chloride as aid in identification of *Phaeoannellomyces werneckii* and other medically important dematiaceous fungi. J Clin Microbiol 25:944–946

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Luo H, Wang Y, Wang H, Yang J, Yang Y, Huang H, Yang P, Bai Y, Shi P, Fan Y, Yao B (2008) A novel highly acidic $\beta$-mannanase from the acidophilic fungus *Bispora* sp. MEY-1: gene cloning and overexpression in *Pichia pastoris*. Appl Microbiol Biotechnol 82:453–461

Müller E, Oehrens E (1982) On the genus *Teratosphaeria* (*Ascomycetes*). Sydowia 35:138–142

Nakagiri A, Tubaki K (1982) A new marine ascomycete and its anamorph from Japan. Trans Mycol Soc Jpn 23:101–110

O'Donnell K (1993) *Fusarium* and its near relatives. In: Reynolds DR, Taylor JW (eds) The fungal holomorph: mitotic meiotic and pleomorphic speciation in fungal systematics. CAB International, Wallingford, pp 225–233

Perrière G, Gouy M (1996) WWW-Query: an on-line retrieval system for biological sequence banks. Biochimie 78:364–369

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Schleper C, Pühler G, Kühlmorgen B, Zillig W (1995) Life at extremely low pH. Nature 375:741–742

Selbmann L, de Hoog GS, Zucconi L, Isola D, Ruisi S, Gerrits van den Ende AHG, Ruibal C, De Leo F, Urzì C, Onofri S (2008) Drought meets acid: three new genera in a dothidealean clade of extremotolerant fungi. Stud Mycol 61:1–20

Sigler L, Carmichael JW (1974) A new acidophilic *Scytalidium*. Can J Microbiol 20:267–268

Sletten O, Skinner CE (1948) Fungi capable of growing in strongly acid media and in concentrated copper sulfate solutions. J Bacteriol 56:679–681

Starkey RL, Waksman SA (1943) Fungi tolerant to extreme acidity and high concentrations of copper sulfate. J Bacteriol 45:509–519

Sterflinger K (1998) Temperature and NaCl-tolerance of rock-inhabiting meristematic fungi. Antonie Van Leeuwenhoek 74:271–281

Taylor JE, Groenewald JZ, Crous PW (2003) A phylogenetic analysis of *Mycosphaerellaceae* leaf spot pathogens of *Proteaceae*. Mycol Res 107:653–658

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Ueda K, Mikata K (1999) A group I intron in the nuclear 18S rRNA gene of the yeast-like fungus, *Arxula terrestris*. Inst Ferment Osaka Res Commun 19:15–21

Vilgalys L, Hester M (1990) Rapid genetic identification and mapping enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Am Soc Microbiol 172:4238–4246

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Inns MA, Gelfaud DH, Sninsky JJ, White TJ (eds) PCR protocols a guide to methods and applications. Academic Press, San Diego, pp 315–322

Zalar P, de Hoog GS, Gunde-Cimerman N (1999) Ecology of halotolerant dothideaceous black yeasts. Stud Mycol 43:38–48

Zettler LA, Gomez F, Zettler E, Keenan B, Amils R, Sogin M (2002) Eukaryotic diversity in Spain's river of fire. Nature 417:137