

ORIGINAL ARTICLE

A significant number of reported *Absidia corymbifera* (*Lichtheimia corymbifera*) infections are caused by *Lichtheimia ramosa* (syn. *Lichtheimia hongkongensis*): an emerging cause of mucormycosis

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Recently, we and others reported the discovery of *Lichtheimia ramosa* (syn. *Lichtheimia hongkongensis*). We also hypothesized that a proportion of '*Absidia corymbifera* (*Lichtheimia corymbifera*)' reported in the literature could be *L. ramosa*. In this study, we characterized 13 strains that had been reported as '*A. corymbifera* (*L. corymbifera*)' in the literature over an 11-year period. Microscopic examination of agar block smear preparations of all 13 strains showed abundant circinate side branches and pleomorphic giant cells with finger-like projections of *L. ramosa*. ITS1–5.8S–ITS2 rRNA gene cluster (internal transcribed spacer (ITS)) and partial elongation factor-1 α (EF1 α) gene sequencing showed that all 13 strains were clustered with *L. ramosa*; partial β -actin gene sequencing showed that most of the 13 strains were clustered with *L. ramosa*; and partial 28S rRNA gene sequencing showed that all 13 strains were clustered with *L. ramosa*, but one strain of *L. corymbifera* (HKU25) was also clustered with other strains of *L. ramosa*. A significant number of reported *A. corymbifera* (*L. corymbifera*) infections are *L. ramosa* infections which are of global distribution. In clinical microbiology laboratories, *L. ramosa* should be suspected if an *Absidia*-like mold that possesses abundant circinate side branches on the sporangiophores and pleomorphic giant cells with finger-like projections is observed. ITS and partial EF1 α gene sequencing are more reliable than partial β -actin and 28S rRNA gene sequencing for identification of the *Lichtheimia* species. *Emerging Microbes and Infections* (2012) 1, e15; doi:10.1038/emi.2012.11; published online 22 August 2012

Keywords: fungus; identification; infection; ITS sequencing

INTRODUCTION

Mucormycosis is an opportunistic infection caused by ubiquitous molds of the order *Mucorales*. Important immunosuppressive conditions associated with mucormycosis include diabetes mellitus, hematological malignancies, use of corticosteroids and other immunosuppressive agents in transplant recipients and severe burn patients.^{1,2} Due to the increasing use of immunosuppressive agents and prolonged survival of these patients, mucormycosis is becoming increasingly common, but mortality remains very high, often above 50%.³ The common clinical manifestations of mucormycosis are rhinocerebral, pulmonary, gastrointestinal and cutaneous, depending on the routes of infection. The most common species of *Mucorales* associated with mucormycosis belong to the genera *Rhizopus*, *Rhizomucor*, *Mucor* and *Absidia*, which all belong to the family *Mucoraceae*.⁴ Among the *Absidia* species, the most important species associated with mucormycosis is *A. corymbifera*.^{2,5–7} According to physiological, phylogenetic and morphological data, it was proposed that three *Absidia* species, *A. corymbifera*, *A. blakesleeana* and *A. hyalospora*,

should be reclassified as a separate family, *Lichtheimiaceae* fam. nov., and the three species renamed as *Lichtheimia corymbifera*, *Lichtheimia blakesleeana* and *Lichtheimia hyalospora*.^{8,9} *L. blakesleeana* was subsequently reduced to a synonym of *L. hyalospora*.¹⁰

Recently, we isolated two fungal strains from two patients with rhinocerebral and gastrointestinal mucormycosis.¹¹ These two strains possessed distinct morphological characteristics that do not fit into patterns of any known fungal species. Moreover, the morphological characteristics of the two strains were similar to another quality control (QC) strain isolated from a patient with cutaneous mucormycosis and sent to our clinical microbiological laboratory from the UK National External Quality Assessment Scheme.¹¹ Phenotypic and genotypic characterization of these three strains revealed that they constitute a novel *Lichtheimia* species, which we named *Lichtheimia hongkongensis*.¹¹ A similar observation was also independently reported by another group, by which the novel *Lichtheimia* species was named *Lichtheimia ramosa*.¹² Evidence from phylogenetic analysis in both studies suggested that *L. ramosa* (syn. *L. hongkongensis*) was

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previously classified as *L. corymbifera* (*A. corymbifera*).^{11,12} Therefore, we suspected that a significant number of isolates reported in the literature and identified as '*A. corymbifera* (*L. corymbifera*)' might represent *L. ramosa*. To test this hypothesis, we requested strains that were reported as '*A. corymbifera* (*L. corymbifera*)' in the literature from the corresponding research groups and performed phenotypic and genotypic characterization on them. In addition, we also characterized a *L. ramosa* strain (CBS124198) described by Garcia-Hermoso et al.¹² In this article, we report the results of the characterization and analysis.

MATERIALS AND METHODS

Strains

We sent emails to 55 research groups who had published cases of '*A. corymbifera* (*L. corymbifera*)' infections over an 11-year period (1999–2009), requesting for their strains of '*A. corymbifera* (*L. corymbifera*)'. Among these 55 groups, replies were obtained from 18 groups. Three of these 18 groups have kept and have kindly provided us with their 13 strains of '*A. corymbifera* (*L. corymbifera*)' for the present study (Table 1).^{5–7} *L. ramosa* CBS124198^{10,12} was purchased from The Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre. *L. ramosa* HKU21 and *L. corymbifera* HKU25¹¹ were used as the control strains.

Phenotypic characterization

All strains were inoculated onto Sabouraud dextrose agar (SDA) for fungal culture. Slides for microscopic examination were prepared using the agar block smear method.¹³ Semiquantification of enzymatic activities was performed using the API-ZYM test (bioMerieux Vitek, Hazelwood, MO, USA). All tests were performed in triplicate. Requirement of supplemental thiamine for growth was performed according to published protocols.^{14,15} The effect of different temperatures on growth on SDA and comparison of growth rate on SDA and potato dextrose agar (PDA) at 30 °C were studied using published protocols, with slight modifications.^{6,16} Sporangiospores were harvested in distilled water from 4-day cultures on SDA. Concentrations of sporangiospores were determined using a hemocytometer. A circular cavity was made at the center of each SDA and PDA plate using a Pasteur pipette. Respective plates were centrally inoculated with 5×10^2 spores. SDA plates were incubated at temperatures from

23 °C to 50 °C and PDA plates at 30 °C. Radii of circular colonies were measured in four directions after 2 days. All assays were performed in triplicate.

Scanning electron microscopy

Scanning electron microscopy was performed according to our previous publication, with slight modifications.¹⁷ Briefly, fungal cells or sporangiospores of *L. ramosa* CBS124198 were fixed in 2.5% glutaraldehyde (w/v) for 1 h and washed once in 0.1 M sodium cacodylate buffer. Fixed materials were dehydrated through a 20% increment of ethanol concentration every 15 min from 30% to 90%, followed by two subsequent dehydration steps of 15 min each in absolute ethanol. Dehydrated materials in absolute ethanol were critical point dried in a BAL-TEC CPD O30 Critical Point Drier using carbon dioxide as the drying agent. Critical dried materials were mounted on to an aluminum stub and coated with palladium in BAL-TEC SCD 005 scanning electron microscopy (SEM) coating system. Coated materials were examined in Leica Cambridge Stereoscan 440 SEM operating at 12 kV and the specimen stage was tilted at zero degree.

DNA extraction

Fungal DNA extraction was performed as described in our previous publications.^{18–20} Briefly, DNA was extracted from 1 g of fungal cells in 10 ml of distilled water using the DNeasy plant mini kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). The extracted DNA was eluted in 50 µl of buffer AE, the resultant mixture was diluted 10 times and 1 µl of the diluted extract was used for polymerase chain reaction (PCR).

Internal transcribed spacer (ITS), partial elongation factor-1alpha (EF1α) gene, partial β-actin gene and partial 28S rRNA gene sequencing

PCR amplification and DNA sequencing of the ITS, partial EF1α genes, partial β-actin genes and partial 28S rRNA genes were performed according to published protocols.^{19,20} Briefly, DNase I-treated distilled water and PCR master mix (which contains deoxynucleoside triphosphates, PCR buffer, and *Taq* polymerase) were used in all PCR reactions by adding 1 U of DNase I (Pharmacia, Uppsala, Sweden) to 40 µl of distilled water or PCR master mix, incubating the mixture at

Table 1 Clinical sources of the 13 strains analyzed in this study

Strain NO (strain NO in original publication)	Sex/age	Predisposing factors	Source	Treatment	Outcome
1 (patient 2) ⁷	M/35	Acute myeloid leukemia	Lung	Surgery, liposomal amphotericin B, itraconazole	Remission
2 (patient 1) ⁵	M/29	Road traffic accident, multiple fractures	Lower limb debrided tissue	Debridement, liposomal amphotericin B	Remission
3 (NA) ⁵	M/27	Left foot trauma, multiple fractures	Swab and tissue	Debridement; conventional amphotericin B, then liposomal amphotericin B, then posaconazole	Remission
4 (03-201) ⁶	NA	NA	Sinus	NA	NA
5 (03-328) ⁶	NA	NA	Brain abscess	NA	NA
6 (04-2314) ⁶	NA	NA	Tracheal	NA	NA
7 (04-3292) ⁶	NA	NA	Pleural fluid	NA	NA
8 (05-641) ⁶	NA	NA	Face biopsy	NA	NA
9 (05-1582) ⁶	NA	NA	Lung	NA	NA
10 (06-26) ⁶	NA	NA	Gingiva	NA	NA
11 (06-1655) ⁶	NA	NA	Blood	NA	NA
12 (06-1670) ⁶	NA	NA	Heart ventricle	NA	NA
13 (06-4282) ⁶	NA	NA	Maxillary sinus	NA	NA

Abbreviation: NA, not available.



Figure 1 Microscopic examination of strain 1 (A and C, lactophenol cotton blue stain) and *L. ramosa* CBS124198 (B and D, acid fuchsin stain) showing branched sporangiophores with characteristic circinate side branches (A and B) and pleomorphic giant cells with finger-like projections (C and D). CBS, Centraalbureau voor Schimmelcultures.

25 °C for 15 min, and subsequently at 95 °C for 10 min to inactivate the DNase I. The fungal DNA extract and controls were amplified with 0.5 µM primers (ITS1 5'-TCC GTA GGT GAA CCT GCG G-3' and ITS4 5'-TCC TCC GCT TAT TGA TAT GC-3' for ITS, M1 5'-GCT GGT ATC TCC AAG GAT GG-3' and M2 5'-CGA CGG ACT TGA CTT CRG TGG-3' for the partial EF1 α gene, LPW8614 5'-CAY ACY TTC TAC AAY GAR CTC C-3' and LPW8615 5'-KGC VAR RAT RGA ACC ACC-3' for the partial β -actin gene and NL-1 5'-GCA TAT CAA TAA GCG GAG GAA AAG-3' and NL-4 5'-GGT CCG TGT TTC AAG ACG G-3' for the partial 28S rRNA gene) (Gibco BRL, Rockville, MD, USA).^{21,22} The PCR mixture (25 µl) contained fungal DNA, PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM MgCl₂ and 0.01% gelatin), 200 µM of each deoxynucleoside triphosphate and 1.0 U *Taq* polymerase (Applied Biosystem, Foster City, CA, USA). The mixtures were amplified in 40 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and a final extension at 72 °C for 10 min in an automated thermal cycler (Applied Biosystem). DNase I-treated distilled water was used as the negative control. Ten microliters of each amplified product was electrophoresed in 1.5% (w/v) agarose gel, with a molecular size marker (ϕ X174 *Hae*III digest; Boehringer Mannheim, Germany) in parallel. Electrophoresis in Tris-borate-ethylenediaminetetraacetic acid buffer was performed at 100 V for 1.5 h. The gel was stained with ethidium bromide (0.5 µg/ml) for 15 min, rinsed and photographed under ultraviolet light illumination.

The PCR product was purified using the QIAquick Gel Extraction kit (QIAGEN). Both strands of the PCR product were sequenced twice

with an ABI Prism 3700 DNA Analyzer (Applied Biosystems), using the PCR primers. The sequences of the PCR products were compared with sequences of closely related species in GenBank by multiple sequence alignment using ClustalX 1.83.²³

Phylogenetic characterization

Phylogenetic tree construction was performed using the neighbor-joining method with MEGA4.0.2. Six hundred and four nucleotide positions of ITS, 399 nucleotide positions of EF1 α , 428 nucleotide positions of β -actin and 609 nucleotide positions of 28S rRNA gene were included in the analysis.

RESULTS

Morphological characteristics

On SDA, all the 13 strains identified as '*A. corymbifera* (*L. corymbifera*)' and *L. ramosa* CBS124198 grew rapidly as cottony colonies and attained diameters of 60–73 mm after incubation at 37 °C for 48 h. Microscopic examination of all the 13 strains and *L. ramosa* CBS124198 showed non-septate broad hyphae. The sporangiophores were highly branched, with the diameters of the main sporangiophores being 8–10 µm and those of the side branches being 3–5 µm. The sporangiophores terminated in sporangia or the columellar remnants. Similar to *L. ramosa* HKU21, HKU22 and HKU23,¹¹ most of the side branches of the 13 strains and *L. ramosa* CBS124198 were characteristically circinate (Figure 1A and 1B), although sometimes they were arranged in umbel, usually at the terminals. Rhizoids

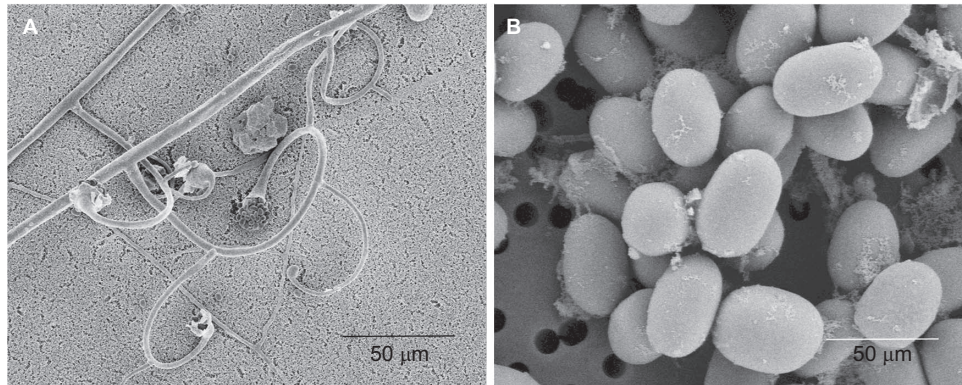


Figure 2 Scanning electron micrograph of *L. ramosa* CBS124198. The characteristic sporangiophores with circinate side branches (A) and ellipsoid sporangiospores (B) were observed.

were not observed. The sporangia (25–35 µm) were hyaline, multi-spored and pyriform in shape and the columella (15–21 µm) was dome-shaped with a prominent flask-shaped apophysis. Hyaline sporangiospores ((2–3) × (3–4) µm) were formed inside sporangia. Most of them were smooth and ellipsoidal in shape. Similar to *L. ramosa* HKU21, HKU22 and HKU23,¹¹ abundant pleomorphic giant cells with finger-like projections, usually submerged in agar, were observed in all the 13 strains and *L. ramosa* CBS124198 (Figure 1C and 1D).

Enzyme production and requirement of supplemental thiamine for growth

The API-ZYM test for all the 13 strains and *L. ramosa* CBS124198 showed positive results for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucosidase and *N*-acetyl-β-glucosaminidase. Thiamine supplementation was not required for the growth of all the 13 strains and *L. ramosa* CBS124198.

Effect of temperature on growth

All the 13 strains and *L. ramosa* CBS124198 grew at 23–44 °C but not at 47 °C and 50 °C. Their median rates of growth at 30 °C on SDA and PDA were 43 (range: 38–56) mm/48 h and 27 (range: 25–30) mm/48 h respectively.

Scanning electron microscopy

Scanning electron microscopic examination of *L. ramosa* CBS124198 revealed the characteristic sporangiophores with circinate side branches and ellipsoid sporangiospores, consistent with the results observed on light microscopy (Figure 2).

ITS, partial EF1α gene, partial β-actin gene and partial 28S rRNA gene sequencing and phylogenetic characterization

PCR of the ITS region of the 13 strains and *L. ramosa* CBS124198 showed bands at about 800 bp. Phylogenetic analysis showed that they were clustered with *L. ramosa* strains (GenBank accession numbers HQ285686, HQ285700, HM104215 and HM104212), *L. ramosa* HKU21 (GenBank accession number EU852633), *L. ramosa* HKU22 (GenBank accession number EU852634) and *L. ramosa* HKU23 (GenBank accession number EU852632) as well as four other strains named *A. corymbifera* (GenBank accession numbers AY944896, EF136359, AY944897 and AB305110) (Figure 3).

PCR of the partial EF1α genes of the 13 strains and *L. ramosa* CBS124198 showed bands at about 450 bp. Phylogenetic analysis

showed that they were clustered with *L. ramosa* strains (GenBank accession numbers FJ719479 and FJ719476), *L. ramosa* HKU21 (GenBank accession number EU852637), *L. ramosa* HKU22 (GenBank accession number EU852636) and *L. ramosa* HKU23 (GenBank accession number EU852635) (Figure 3).

PCR of the partial β-actin genes of the 13 strains and *L. ramosa* CBS124198 showed bands at about 900 bp. Phylogenetic analysis showed that 10 of the 13 strains and *L. ramosa* CBS124198 were clustered with *L. ramosa* HKU21 (GenBank accession number FJ444938), *L. ramosa* HKU22 (GenBank accession number FJ444939) and *L. ramosa* HKU23 (GenBank accession number FJ444939) (Figure 3). Two strains (Nos. 3 and 11) were clustered with *L. ramosa* (GenBank accession numbers GQ342746 and GQ342747) and another strain (NO. 13) was clustered with *L. ramosa* (GenBank accession number GQ342845) (Figure 3).

PCR of the partial 28S rRNA genes of the 13 strains and *L. ramosa* CBS124198 showed bands at about 500 bp. Phylogenetic analysis showed that they were clustered with *L. ramosa* HKU21, *L. ramosa* HKU22 and *L. ramosa* HKU 23 (Figure 3). Notably, one strain of *L. corymbifera* (HKU25), confirmed by phenotypic tests and ITS, partial EF1α gene and partial β-actin gene sequencing, was also clustered with other strains of *L. ramosa* (Figure 3).

DISCUSSION

A significant number of reported *A. corymbifera* (*L. corymbifera*) infections are *L. ramosa* infections which are of global distribution. Recently, we reported the discovery of *L. ramosa* from three patients with mucormycosis.¹¹ The first patient was a liver transplant recipient with rhinocerebral mucormycosis, the second a renal transplant recipient with gastrointestinal mucormycosis and the third a burn patient with cutaneous mucormycosis.¹¹ Since phylogenetic analysis inferred by ITS sequences showed that these three strains of *L. ramosa* were clustered with at least four sequences named ‘*A. corymbifera* (*L. corymbifera*)’ deposited in the GenBank database,¹¹ we suspected that a significant proportion of *A. corymbifera* (*L. corymbifera*) infections were actually *L. ramosa* infections. In this study, among the 13 published strains of *A. corymbifera* (*L. corymbifera*) from Spain, France and Qatar we collected and re-characterized, all were unambiguously identified as *L. ramosa* using both phenotypic and genotypic methods. These 13 strains were obtained from diverse sites, including the respiratory tract, paranasal sinuses, brain, heart, blood and wound (Table 1), implying that *L. ramosa* could be the cause of different clinical forms of mucormycosis. Therefore, we concluded that a

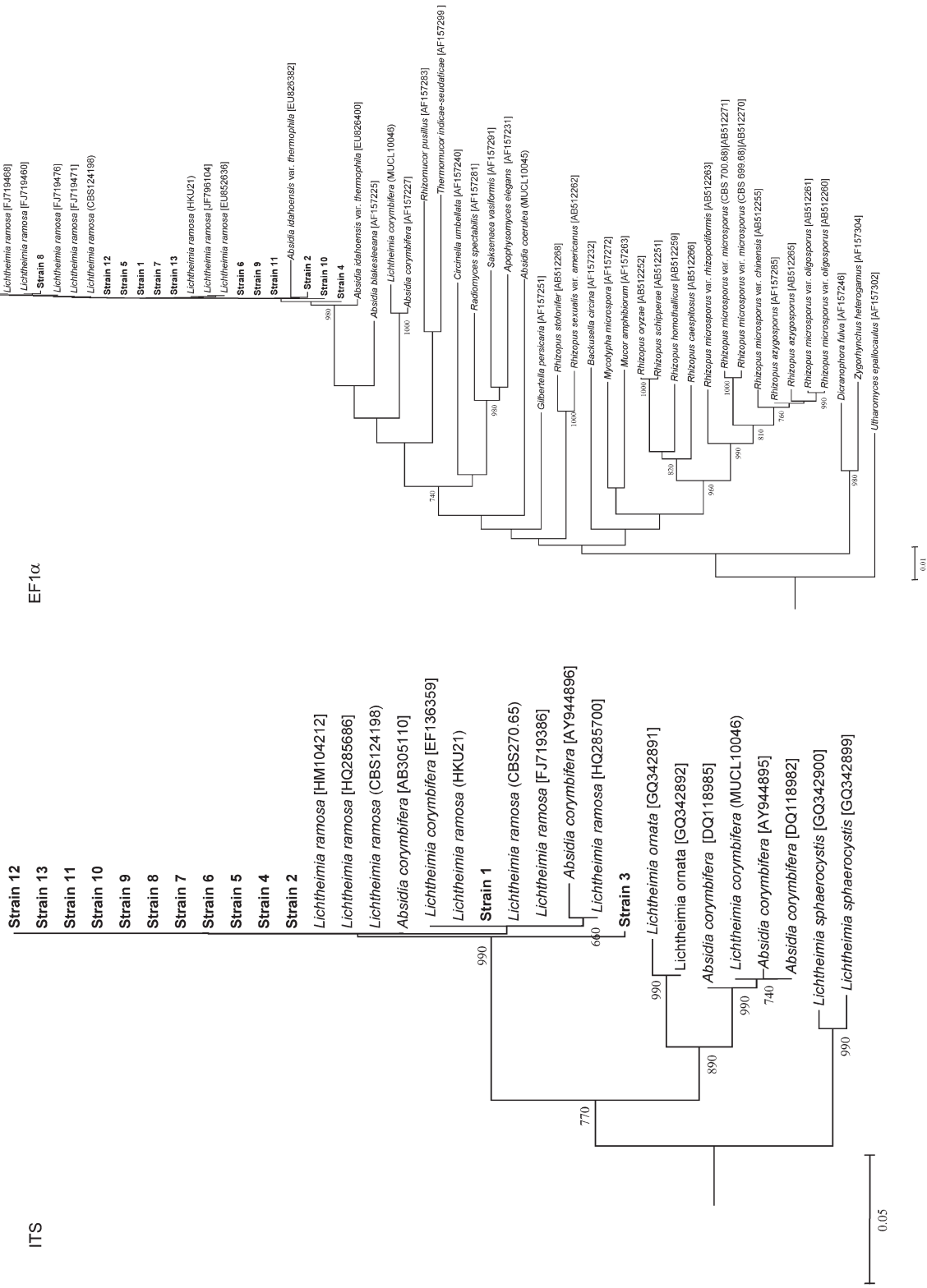


Figure 3 Phylogenetic trees showing the relationship of the 13 strains (highlighted in bold) and *L. ramosa* (639 nucleotide positions), *EF1 α* gene (544 nucleotide positions), β -actin gene (441 nucleotide positions) and 28S rRNA gene (639 nucleotide positions) sequence data by the neighbor-joining method and rooted using *Fennefomyces linderi* (GQ249890), *Piobolus umbonatus* (AF157277), *dichotomocladium robustum* (EU826396) and *Paecilomyces lilacinus* (AY213717), respectively. The scale bars indicate the estimated number of substitutions per 20, 100, 50 and 10 bases, respectively. Numbers at nodes indicated levels of bootstrap support calculated from 1000 trees. All names and accession numbers are given as cited in the GenBank database.

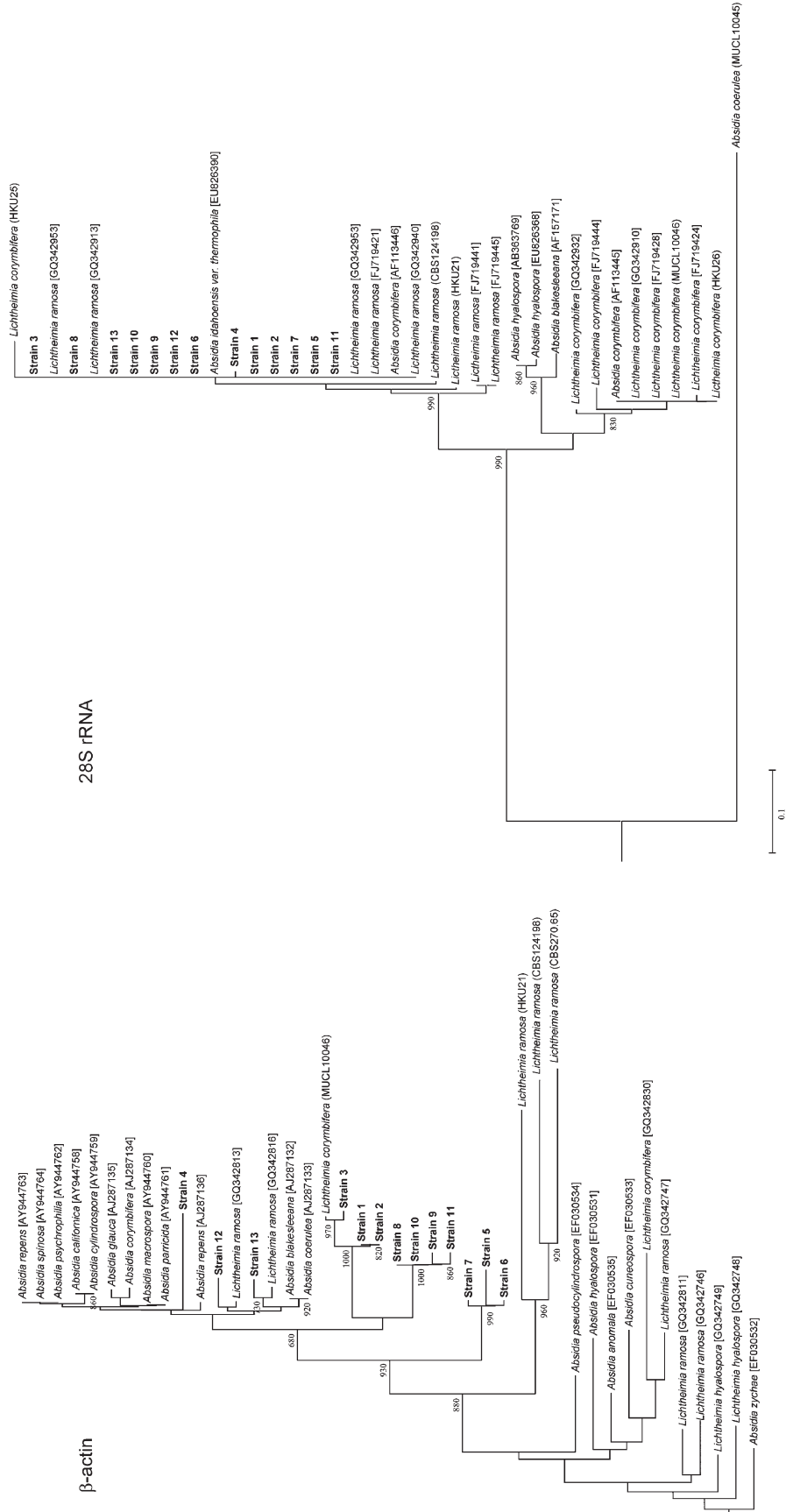


Figure 3

significant proportion of infections caused by *A. corymbifera* (*L. corymbifera*) were in fact *L. ramosa* infections. Further basic and clinical studies on *L. corymbifera* and *L. ramosa* will reveal any difference between the pathogenicity, epidemiology, anti-fungal susceptibility, clinical disease spectrum and outcome of these two *Lichtheimia* species.

ITS and partial EF1 α gene sequencing are more reliable than partial β -actin gene and partial 28S rRNA gene sequencing for identification of the *Lichtheimia* species. In our previous study and the present study, all 16 strains of *L. ramosa* were identified correctly using ITS and partial EF1 α gene sequencing (Figure 3).¹¹ For partial β -actin gene sequencing, some strains of *L. ramosa* (e.g., strains 3 and 11) were not as closely related to most strains of *L. ramosa* than the cluster of *A. hyalospora* (EF030531 and PW1264), *L. hyalospora* (GQ342748 and GQ342749) and *A. blakesleeana* (PW1263 and AJ287132) to most strains of *L. ramosa* (Figure 3). As for partial 28S rRNA gene sequencing, although all 16 strains of *L. ramosa* were identified correctly, one strain of *L. corymbifera* (HKU25) showed clustering with other strains of *L. ramosa* (Figure 3). These indicated that the ITS and partial EF1 α genes are better targets for identification of *Lichtheimia* species. It also implied that sequencing multiple genes should be performed for more reliable speciation in the *Lichtheimia* genus.

This polyphasic approach of phenotypic and genotypic characterization should be used for speciation of 'difficult-to-identify' pathogenic fungi. Accurate identification of medically important fungi is the cornerstone to prescription of the correct antifungal drugs to patients with fungal infections. In clinical microbiology laboratories, *L. ramosa* should be suspected if an *Absidia*-like mold that possesses abundant circinate side branches on the sporangiophores and pleomorphic giant cells with finger-like projections is observed. Since the presence of circinate side branches on the sporangiophores and pleomorphic giant cells is not exclusively found in *L. ramosa* though they are more abundantly found in it,^{10,24} these are not absolute microscopic characteristics of this fungus. With the increasing availability of PCR and DNA sequencing, ITS and partial EF1 α gene sequencing should be performed for accurate identification of *L. ramosa*.

TAXONOMY

L. ramosa (syn. *Lichtheimia hongkongensis*)

Mycobank MB515445

In 'SDA' post-dies 2 ad 37 °C, coloniae pilosae, coloniarum 64–85 mm diametros, hyphae latarum aseptatarum. Sporangiophores ramosissimae, sporangiophorerum potissimarum 8–10 μ m diametros, sporangiophorerum ramis lateralibus 3–5 μ m diametros. Ramis lateralibus pleris circinalibus, interdum umbelliformis. Sine structuris rhizoideis similibus. Sporangii (25–35 μ m) hyalinis, pyriformibus cum sporis numerosis. Collumellis (15–21 μ m) tholiformibus cum apophysis ampulliformibus. Sporangiophoris hyalinis, cotidianum teres, ellipticus, et cellulis pleomorphus grandibus, numerosis cum projecturis digitiformibus.

Description of *L. ramosa* (syn. *Lichtheimia hongkongensis*)

On SDA, *L. ramosa* grows rapidly as cottony colonies and attains a diameter of 64–85 mm after incubation at 37 °C for 48 h. Microscopic examination shows non-septate broad hyphae. The sporangiophores are highly branched. The diameters of main sporangiophores are 8–10 μ m and those of the side branches are 3–5 μ m. The sporangiophores terminate in sporangia or the columellar remnants. Most of the side branches are characteristically circinate, but sometimes they are arranged in umbel, usually at the terminals. The circinate sporangio-

phores are also evident when young colonies are directly observed under the microscope using a $\times 10$ objective. Rhizoids are not observed. The sporangia (25–35 μ m) are hyaline, multispored and pyriform in shape. The columella (15–21 μ m) is dome-shaped with a prominent flask-shaped apophysis. Hyaline sporangiospores ((2–3) \times (3–4) μ m) are formed inside sporangia. Most of them were smooth and ellipsoidal in shape. Characteristically, abundant pleomorphic giant cells with finger-like projections, usually submerged in agar, are observed.

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