Diversity, ecology, and evolution in *Phycomyces*

Lola P. CAMINO^a, Alexander IDNURM^{b1}, Enrique CERDÁ-OLMEDO^{*a}

^a Departamento de Genética, Universidad de Sevilla, Apartado 1095, E-41080 Sevilla, Spain.

^b Division of Cell Biology and Biophysics, School of Biological Sciences, University of Missouri-Kansas City, USA.

¹*Present adress:* School of BioSciences, University of Melbourne, Australia,

*Corresponding author: Enrique Cerdá-Olmedo, Tel.: +34-954624107, +34-954557111; fax: +34-954557104.

E-mail addresses: lolapcc@us.es (L. P. Camino), alexander.idnurm@unimelb.edu.au (A. Idnurm), eco@us.es (E. Cerdá-Olmedo)

Abstract The fungal genus *Phycomyces* (*Mucoromycotina*, *Mucorales*) has been revised by examining 96 strains, received from established collections or newly isolated from different environments. Morphology, sexuality, DNA sequences, and population structure clearly identify the genus and set it apart from other *Mucorales*. The size of the spores, the sexual interactions, the sequences of genes *sexM* and *sexP* that determine sexual identity, and the DNA for ribosomal RNAs validate the species *P. blakesleeanus* and *P. nitens* and the family *Phycomycetaceae*. Isolates from the same sample of biomass often differ in spore size, sex, DNA sequences, and restriction-length polymorphisms. The widest diversity was found in similar environments in three of the Canary Islands, implying a failure to disperse rapidly over the sea. All strains have apparently functional *sexM* and *sexP* genes and all but some strains of *P. nitens* complete the sexual cycle in the laboratory. The genetic diversity of *P. blakesleeanus* strains provides evidence for geographical clustering. Various sequence comparisons, including the newly isolated genes *sexM* and *sexP* of *P. nitens* and *Blakeslea trispora*, clarify phylogenetic relationships in the *Mucorales* and recommend the *sex* genes for the study of speciation.

Keywords

Biogeography

Speciation

Phylogeny

Sex allelomorphs

Mucorales

Blakeslea

1. Introduction

Phycomyces is a prominent member of the *Mucoromycotina*, a vast taxon of *Fungi*, part of the old *Zygomycetes* and outside the better known *Ascomycota* and *Basidiomycota*. Originally described as an alga (Agardh 1817), hence the genus name, *Phycomyces* has populated research laboratories for about a century and a half (Carnoy 1870), often pioneering new research fields (Bergman et al. 1969; Cerdá-Olmedo & Lipson 1987; Cerdá-Olmedo 2001). It remains a fruitful subject for photobiology and other aspects of cell physiology and behaviour, carotene biosynthesis and other aspects of metabolism, and sexuality. This contrasts with the dearth of information on its natural diversity, geographical distribution, ecology, evolution, and relations to other genera of the Order *Mucorales*, such as *Blakeslea*, *Mucor*, and *Rhizopus*.

Phycomyces is easily recognized by its giant sporangiophores, which grow out of the vegetative mycelium lifting a large sporangium filled with vegetative spores. The mycelia are composed of branched, non-septate, multinucleate hyphae. They belong to either the (+) or the (–) sex, indistinguishable to our sight, but able to interact with each other in a complex series of morphological and biochemical events that culminate in the production of large zygospores. Upon a long dormancy, the zygospore germinates and produces a germsporangiophore, a germsporangium, and germspores, very similar to the akin structures of the vegetative cycle. The germspores, carrying recombinant nuclei, germinate into vegetative mycelia.

Although all natural isolates of *Phycomyces* look alike, they have been assigned to two species, *P. blakesleeanus* and *P. nitens*, on the basis of spore morphology and sexual interactions (Burgeff 1925; Benjamin & Hesseltine 1959; Zycha et al. 1969). Nearly all laboratory research has been done with *P. blakesleeanus* strains of unknown origin, particularly the standard strain NRRL1555, whose complete genome has been available since 2006 (Joint Genome Institute, US Department of Energy). The genetic analysis of *Phycomyces* differs notably from that of other organisms, but has led to recombination maps of phenotypic (Orejas et al. 1987) and molecular markers (Chaudhary et al. 2013).

The two sexes of *P. blakesleeanus* depend on specific allelomorphs, DNA sequences with little homology to each other that occupy the same place in linkage group V of the genome and behave in crosses as single allelic markers (Idnurm et al. 2008). The (+) and (–) allelomorphs contain a gene, *sexP* and *sexM*, respectively, similar to those found in other

Mucorales: *Mucor circinelloides* (Lee et al. 2008), *Rhizopus oryzae* (Gryganskyi et al. 2010), *Syzygites megalocarpus* (Idnurm 2011a), and *M. mucedo* (Wetzel et al. 2012). Gene *sexM* is necessary for mating, but not for vegetative growth in *M. circinelloides* (Li et al. 2011).

The other *Mucorales* share the morphological structures that have been described for *Phycomyces*, with enormous variations in size and shape. The traditional taxonomy, which reached its apex with the book by Zycha et al. (1969), has been revised by DNA sequence comparisons (O'Donnell et al. 2001; Voigt & Wöstemeyer 2001; Tanabe et al. 2004; White et al. 2006; Walther et al. 2013; Hoffmann et al. 2013), complemented by antibiotic-resistance tests (Vitale et al. 2012). The internal transcribed spacer (ITS) sequences of the genes for rDNA have been recommended by the Consortium for the Barcode of Life to distinguish fungal species (Schoch et al. 2012). The definition of species by the possibility of sexual genetic exchange is of limited use in these fungi, because few complete the sexual cycle in the laboratory, but many initiate unfruitful sexual interactions with distant partners assigned to different genera and families (Blakeslee 1904; Blakeslee & Cartledge 1927). The intraspecific variation of the *Mucorales* and its geographical distribution remain largely unexplored (Takó & Csernetics 2005; Hermet et al. 2012).

Here we consider a broad set of *Phycomyces* strains, most of them new ones, from multiple view points and look for the relations among them, with their environment, and with other *Mucorales*. To widen the comparisons, we have obtained the sexual allelomorphs of *P. nitens* and *B. trispora*.

2. Materials and Methods

2.1 Fungal strains

The natural strains of the genus *Phycomyces* (Kunze 1823) used in this work are listed in Table 1, together with the essential data on their origin. *Blakeslea trispora* (Thaxter 1914) wild-type strains F921 and F986 were obtained from VKM (All-Russian Collection of Microorganisms, Moscow, Russia).

2.2 Collection and isolation of new strains

Dung of mice and other small mammals was collected from many locations of the Iberian Peninsula and the Canary Islands. The samples were either picked up from the field or left by

animals that had been captured with Sherman traps (240 × 80 × 90 mm) in the night and released in the morning. *Phycomyces* was found in less than one-tenth of the sampled locations.

Occasionally *Phycomyces* was found growing on various materials (Table 1), often by volunteers. Particularly successful was a call with a description published in the online forum of the University of British Columbia Botanical Gardens.

Samples were incubated at 22 °C in or on culture medium with sufficient space for the growth of giant sporangiophores and illuminated from above. Single *Phycomyces* sporangia were crushed between wet tweezer tips and suspended in 1 ml water. In some cases the medium was supplemented with chloramphenicol (34 mg/l) to reduce bacterial contamination. Following another round of single-sporangium purification, up to eight strains were isolated from each original sample. In addition, the original cultures were kept in the dark and examined for the production of zygospores, as an indication of the coexistence of both sexes in the sample.

2.3 Morphological and physiological characterization

Standard cultures (Cerdá-Olmedo 1987) were grown from 10⁴ spores in the dark on 25-ml minimal agar in standard Petri dishes, 85 mm in diameter, unless otherwise stated. The standard incubation temperatures were 22 °C for *Phycomyces* and 30 °C for *Blakeslea*. Colonies for viability tests were obtained on minimal agar adjusted to pH 3.3 with HCl.

For mating, mycelial pieces (about 4 mm²) of two strains were set 50 mm apart on potato dextrose agar (prepared from fresh potatoes) and incubated at 16 °C. Alternatively the agar contained 50 ml/l “V8 juice” (Campbell Soup Company, Camden, NJ, USA).

Zygospores were transferred to damp filter paper in Petri dishes and incubated at 22 °C.

Spore dimensions (20 spores per strain) were measured with a microscope equipped with an eyepiece micrometer or by microphotography. Spore nuclei (at least 100 spores per strain) were counted with a fluorescence microscope after staining with 4',6-diamidino-2-phenylindole (DAPI) (Mehta et al. 2003).

Mycelial growth was described by the radial expansion of the mycelia (the average of three colony diameters measured at 24-h intervals) and by the dry mass of lyophilized mycelia from six-days cultures.

The β -carotene content of lyophilized mycelia (Govind & Cerdá-Olmedo 1986) was calculated with the absorption coefficient (1 g/l , 1 cm , $\lambda = 453 \text{ nm}$) = 259.2.

2.4 Molecular methods

The genomic DNA from freeze-dried mycelia was extracted either with GenElute Plant Genomic DNA Miniprep (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions (Sambrook & Russell 2001) or the cetyltrimethyl ammonium bromide protocol (Pitkin et al. 1996).

ITS/5.8S DNA was obtained by PCR with Expand High Fidelity DNA polymerase (Roche Diagnostics, Mannheim, Germany) and primers ITS1 and ITS4 (White et al. 1990) and sequenced at Sistemas Genómicos (Valencia, Spain) by Sanger's dideoxy method.

The restriction-fragment length polymorphisms described by Chaudhary et al. (2013) were tested with the primers listed in Table S1.

The *sex* allelomorphs of *P. nitens* strains S608 and S609 were obtained by a combination of direct and inverse PCR. An internal segment of gene *tptA* was obtained with primers 5'-AT(C/T)AACAT(C/T)TGCATGTGGTA-3' and 5'-CA(A/G)GT(C/T)CA(C/T)TA(C/T)AC(C/T)CC(C/T)AA-3', designed for the conserved oligopeptides INICMW and QVHYTP of TptA. A segment including parts of genes *sexM* and *rnhA* and the intermediate sequence of strain S609 was obtained with primers 5'-GACCAAAGAACGCATTCATGC-3' and 5'-GTATTTGCCAGACCAACCATG-3', which are conserved sequences of the respective genes of *P. blakeleeanus*. The polymerase cited above was used for 2 min at 94° C, followed by 35 cycles of 30 s at 94°, 30 s at 52 or 50°, and 1 min at 72°, and a final extension of 7 min at 72°.

Circularized segments of genomic DNA were obtained by digestion in parallel experiments with 12 restriction enzymes (*Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Hind*III, *Kpn*I, *Nco*I, *Pst*I, *Sal*I, *Spe*I, *Xba*I and *Xho*I) and self-ligation with T4 DNA ligase (New England BioLabs Ipswich, MA, USA). The ligation products were subjected to inverse PCR with primers from sequences of the *P. nitens* genes *tptA*, *rnhA* and *sexM* by using ExTaq DNA polymerase (Takara Bio, Shiga, Japan) for 2 min at 94 °C, 32 cycles of 15 s at 94°, 20 s at 52 to 55° and 3 min at 72°, and a final extension of 5 min at 72°. Sequencing the DNA products provided new primers for additional rounds of inverse PCR. The final sequences were assembled with the Sequencher software (Gene Codes, Ann Arbor, MI, USA).

The same inverse PCR procedure was used for the *sex* allelomorphs of *B. trispora*, strains F921 and F986, starting from a fragment of gene *rnhA* of strain F921, which was obtained as described for *Syzygites* (Idnurm 2011a).

2.5 Bioinformatics

BLAST programs were used to search genome databases. DNA and protein sequences were aligned with ClustalW (Thompson et al. 1994), but those used to obtain cladograms were obtained with Muscle (Edgar 2004), and improved by eye. BLASTn (Altschul et al. 1990) helped localize putative genes, domains, and motifs DNA Strider 1.4 (Marck 1988) produced putative protein sequences, which were inspected by eye. Table S2 has the accession numbers of DNA and protein sequences taken from GenBank and those generated in this work.

Maximum-likelihood cladograms of nucleotide sequences were obtained with Mega5.2.2 (Tamura et al. 2011). The bootstrap consensus trees were inferred from 1000 replicates.

Statistical analyses were performed in R (R Core Team 2013). A Jaccard distance matrix of dissimilarity of 56 strains of *P. blakesleeanus* was calculated from the presence or absence of 27 restriction-fragment length markers in their genomes and subjected to non-metric multidimensional scaling (NMDS) analysis. Whether the missing data were included or not in the analyses made very little difference (Mantel $r = 0.986$, $p < 0.001$). Computations were conducted using the R ‘vegan’ package (Oksanen et al. 2012) and graphed with the ‘ggplot2’ package (Wickham 2009). The data for different geographical groups of strains in the Jaccard matrix were subjected to analysis of molecular variance (AMOVA; Excoffier et al. 1992) and the significance of their differences was calculated after 5000 permutations. The metric distances required by AMOVA were obtained by setting ‘distance method’ to ‘euclidean’ in the ‘amova’ function of the ‘pegas’ package (Paradis 2010).

3. Results

3.1 Old and new *Phycomyces* strains

We considered 99 *Phycomyces* strains (Table 1), including 45 received from various collections, 21 new strains from Spain, and 33 new strains from the USA. The new strains

were isolated from materials provided by external volunteers, except 12 that were isolated by members of the Spanish laboratory. The new strains were found growing on various substrates, except six that were grown from dung of small animals collected in Andalusia and the Canaries and incubated in the laboratory. The strains isolated from the same biomass were considered a local population; thus, a single sample from Houston, Texas, USA, provided 6 strains that could be distinguished by their sex and other alleles.

All *Phycomyces* strains could be classified unambiguously as *P. nitens* or *P. blakesleeana* by traditional criteria (Burgeff 1925; Benjamin & Hesseltine 1959; Zycha et al. 1969). Both are cosmopolitan species (Fig. 1), although all the new strains from Spain were *P. nitens*, and all but two of the new strains from the USA were *P. blakesleeana*. Our collection is unbiased, because collectors could not distinguish the species by visual inspection. Both species were found in a sample of seeds from an animal feeder in Howell, Michigan, USA, but they could have been brought together accidentally by man or animals.

Both sexes of *P. nitens* were found in a sample from the Higueral cave in Andalusia, Spain (Baena et al. 2012) and both sexes of *P. blakesleeana* were found in three samples, from Texas, Tennessee, and Virginia. The new *Phycomyces* strains were accompanied in the original biomass by many other microorganisms, including various species of *Mucor* and *Rhizopus*.

3.2 Spore size and number of nuclei

The spores of *P. blakesleeana* were smaller, rounder, and more uniform than those of *P. nitens*. The average length of the spores taken from a single sporangium was sufficient to distinguish the species, but a single spore was not, because of some overlap in the spores of extreme size. The length vs. eccentricity plot (Fig. 2) offered a clearer distinction than the length vs. width plot.

The spore size did not correlate with the geographical origin. The largest spores were those of *P. nitens* strains isolated from Japan, England (Avon County), Germany, and La Palma (one of the Canary Islands). The smallest *P. nitens* spores were often found in the vicinity of the largest, in England (Surrey County) and La Gomera (another Canary island).

The average number of nuclei per spore, measured in 18 strains, was another excellent criterion for species determination, being >8 in *P. nitens* and <4 in *P. blakesleeana* (Fig. 2 and Table S3). The number of nuclei per spore in each strain varied widely, along with the spore size, and more so in the former than in the latter. Thus, the variation coefficient of the

distribution of nuclei per spore was 0.34 in *P. nitens* S644 and 0.20 in *P. blakesleeanus* NRRL1555.

The extreme values were the few uninucleate spores found in some *P. blakesleeanus* strains and the spores with over 30 nuclei seen in some *P. nitens* strains. A spore with more than 8 nuclei should be assigned to *P. nitens*, but lower values would be ambiguous. The standard strain NRRL1555 is noteworthy for the low number of nuclei and the low eccentricity of its spores.

3.3 Mycelial growth and color

At a glance, the growth of the strains was rather uniform, and it was measured in only a few strains from different locations. The temperature preferences for growth did not match the geographical origins of the strains tested (Fig. 3). In terms of dry mass, all the strains grew at 30 °C, but not better than at lower temperatures; strains from the Canary Islands grew worst. About half of strains were mesophilic (they preferred 22 °C to 16 °C) and the others relatively psychrophilic (the other way around). At 4 °C, strains from the Iberian Peninsula, Canary Islands and Canada (*P. nitens* S257, S609, S642, S645, and UBC39) produced thick mycelia that took five days to reach 5 mm in diameter; the spores of the other strains tested either did not germinate or produced germlings only.

All strains expanded faster at 22 °C than at 16 °C. The maximal daily expansion at 22 °C was close to 17 mm/day in all strains, with a variation coefficient of only 0.10, the lowest found in our quantitative measurements. The dry biomass per colony surface was also similar for all strains, 43 g/m². *P. nitens* S642 was noted by its rapid expansion and abundant sporulation from its thin and largely submerged mycelium.

A high β -carotene content would offer an attractive start for the breeding of industrial strains. We paid particular attention to our new isolates of *P. nitens*. Some of them had about 90 μ g β -carotene/g dry mass four days after spore germination, and were therefore similar to NRRL1555, the standard strain of *P. blakesleeanus*. Many exceeded this value and *P. nitens* S642 more than doubled it (Table S4).

3.4 DNA sequences for ribosomal RNA in *Phycomyces*

Tandem DNA sequence repeats (Fig. 4), located in linkage group VII, code for the four rRNAs (18S, 5.8S, 28S, and 5S) and contain the internal transcribed spacers (ITS1 and ITS2) and the intergenic spacers (IGS1 and IGS2). The structure in Fig. 4 was assembled from

sequences located in the *P. blakesleeanus* genome. IGS2 varies in sequence and in length (4723, 6096, 4704 bp), but the other seven segments of the structure have the same length (7051 bp) and nearly the same sequence. The 5S rRNA and the other rRNAs are coded in opposite DNA strands.

The DNA for the 5.8S rRNA was identical in the ninety-three strains of *P. nitens* and *P. blakesleeanus* that were sequenced. The ITS/5.8S sequences, composed of ITS1 (195 bp), 5.8S (157 bp), and ITS2 (262 bp), fell in two clusters that corresponded to the biological species. Strains from different species varied in 14 to 15 % of the 614 bp and those from the same species varied much less (Fig. 5). In *P. blakesleeanus* 50 strains, including NRRL1555, shared the ITS/5.8S sequence; three other strains diverged in one to five nucleotides. In *P. nitens* 38 strains shared the ITS/5.8S sequence or differed from it in only one nucleotide. The largest divergence was that of strain NRRL2444, from England, which differed from the predominant sequence in 14 nucleotides.

The multicopy ITS/5.8S sequences were uniform in most strains, with a few cases of heterogeneity detected in a single nucleotide of the ITS1 sequence. S609 and eight other *P. nitens* strains had A/G in position 17 and *P. blakesleeanus* CBS188.27 had A/C in position 15.

3.5 Intraspecific diversity in *P. blakesleeanus*

The strains of *P. blakesleeanus* were tested for twenty-seven single nucleotide polymorphisms (SNP). Strains NRRL1555 and UBC21, the only wild types whose genomes have been sequenced, differ in these markers localized throughout the genome (Chaudhary et al. 2013). The same analyses could not be repeated with *P. nitens* because of insufficient conservation of the DNA sequences.

Isolates with identical alleles were discarded from subsequent analysis if they had the same sex and came from the same site (Table 1). Strains CBS189.27 and CBS282.35, suspected to be identical (Ootaki & Miyazaki 1993), are in fact different.

A simple analysis of the alleles that each strain shares with UBC21 (Table S6) unveils the close relationships within each local population and the wide spread of the strains from North America.

The powerful NMDS analysis represents the relationships of the genome sequences as a two-dimensional graph of the strains (Fig. 6). Local populations form tight clusters. The strains from Europe, Eastern North America, and Western North America form significantly

different groups according to the analysis of molecular variance ($\sigma^2 = 0.05$, $p < 0.001$).

Some strains were aberrant in morphology and in conduct. Two European strains in the lower left are natural *piloboloides* mutants, with sporangiophores bulged just below the sporangium (Burgeff 1915, 1928; Benjamin & Hesseltine 1959; Yoshida et al. 1980; Ootaki & Tsuru 1993). UBC1, a strain from British Columbia that groups with other Western North American strains, may be called *nanus* because of its very short sporangiophores and its incomplete sexual interaction (Burgeff 1928).

3.6 Sexual behavior of *Phycomyces*

All the *P. blakesleeanus* strains interacted sexually under the laboratory conditions and could be classified as (+) or (–). Along the line of contact between mycelia of opposite sex appeared a bright yellow band with modified hyphae (“sexual tissue”) and abundant, fertile zygospores. Only UBC1 failed to produce normal zygospores.

Under the same conditions most of the *P. nitens* strains reacted weakly, with only a slight accentuation of the yellow color and few or no zygospores; sixteen strains could not be clearly assigned to one of the sexes, except by analysis of the *sex* genes. The (+) strains S256 and S608 and the (–) strains S605, S606, S609, and S642 completed the sexual cycle. After a dormancy period similar to that of *P. blakesleeanus*, most of their zygospores germinated to produce viable germ spores (Fig. S1).

Crosses of the *P. blakesleeanus* strains NRRL1555 and UBC21 with many of the new strains of *P. nitens* produced the bright yellow band and “sexual tissue”, but no mature zygospores.

3.7 Sex allelomorphs of *P. nitens* and *B. trispora*

The sex allelomorphs of *P. blakesleeanus* and *M. circinelloides* are flanked by genes *tptA*, for a putative triose phosphate transporter, and *rnhA*, for a putative RNA helicase, and contain each a gene, *sexM* or *sexP* (Idnurm et al. 2008; Lee et al. 2008). The sequences of these genes were used to obtain the sex allelomorphs of *P. nitens* and *B. trispora* (Fig. 7). No sequences related to the sex allelomorphs were found around gene *tptA* of *P. nitens*.

The sex allelomorphs of *B. trispora* are the shortest known in the *Mucorales*, with 1145 and 993 bp for (+) and (–), respectively. The (–) allelomorph of *P. nitens*, with 2084 bp, is the second longest, after that of *P. blakesleeanus*. The (+) allelomorph, with 3716 bp, is the third longest, after those of *R. oryzae* and *P. blakesleeanus*.

The *sex* genes proper are short and very similar in length: 958 ± 24 bp for *sexP* and 668 ± 69 bp for *sexM* (mean and standard deviation for the species shown in Fig. 7). They are all transcribed in the same direction as gene *rnhA*, with the exception of *sexP* of *Phycomyces*; thus, in this genus the *sex* genes are inverted with respect to each other.

The *sexP* gene of *P. nitens* is preceded by the sequence GATCgtccAcCCAAT, a transcription-factor binding motif (Jones et al. 1987). The capitalized nucleotides are conserved in *P. blakesleeanus*, *M. mucedo*, *M. circinelloides*, and *R. oryzae* (Wetzel et al. 2012). This motif was not found in *B. trispora*.

Of the *P. nitens* strains whose sex had to be determined by sequencing, nine had gene *sexP* and seven had gene *sexM*, all identical to those of the sexually active strains S609 and S608. The only exception was strain S607, whose predicted SexP protein sequence differs from the others in eight amino acids that do not affect the HMG domain. The 573-bp non-coding sequence between *sexM* and *rnhA* is highly conserved in the eight strains of *P. nitens* that were sequenced, with variations in less than 0.1 % of the nucleotides. This sequence presumably contains the *rnhA* promoter.

4. Discussion

4.1 Species of *Phycomyces*

The species *P. nitens* and *P. blakesleeanus* are clearly set apart by spore morphology, number of nuclei per spore, sexual behavior, *sex* loci, ITS/5.8S sequences, and the failure to amplify *P. nitens* DNA with PCR primers from *P. blakesleeanus*. The length and width of a few spores suffice to assign isolates to one or the other species.

We do not know the selective forces that separated the two species of *Phycomyces* and maintain each as a tight cluster of strains. So tight, for example, that gene *sexM* has the same sequence in all the strains of *P. nitens* that we have analyzed, irrespective of their geographical origin all over the Earth. No strains, old or new, justify the recognition of new species.

NRRL1555, the standard and most used strain of *P. blakesleeanus*, resembles the majority of the strains of this species in all respects, but for its few nuclei per spore, a convenient trait for mutational analysis. Strain NRRL2444, from England, used in sexuality tests (Benjamin & Hesseltine 1959), is the most outlying, and thus the least representative, of all the strains of *P. nitens*, as seen in their spores and their ITS/5.8S sequences.

4.2 Geographical distribution and ecology

Phycomyces has been found in the Old and the New Worlds and in Oceania at latitudes ranging from Finland and Canada to the tropics. Nearly all the strains look the same and the small differences in growth demands are unrelated to their geographical origins. Growth is thus likely to be limited to seasons and other circumstances that meet the demands in each geographical location, with the adverse intervals straddled by the durability of vegetative spores and zygospores.

Phycomyces requires a relatively high humidity. We sampled many environments in the Canary Islands, from deserts to wet forests, from coasts to high mountains. All the strains were found at around one km over sea level in humid, often foggy laurisilva.

The natural microenvironments of *Phycomyces* are relatively uniform. Eight of the Spanish strains were found in three deep limestone caves and most of the others came from volcanic and karstic terrains where caves abound. *Phycomyces* does not shun human proximity; many of the samples sent by volunteers were picked up near, or even inside houses.

Our most diverse strains were collected less than 150 km from each other in very similar environments of three Canary Islands. This is an extension to the fungal world of many observations of island biology, for example, on the finches of the Galápagos by C. Darwin and on the *Gallotia* lizards of the Canaries (Cox et al. 2010). The divergence of island strains indicates that *Phycomyces* does not disseminate rapidly to large distances. Nor was this expected, because the vegetative spores and the zygospores are hardly dispersible in air or water and do not seem to associate with birds; on the contrary, they are sticky and easily carried by mammals.

4.3 Local populations

Knowledge of the diversity of local populations, scarce in the non-Dikarya Fungi, is needed to understand ecology and evolution and to manage emerging problems, such as the world-wide decline of amphibians caused by *Batrachochytrium dendrobatidis* (Rosenblum et al. 2013) and the outbreak of mucormycosis caused by *Apophysomyces trapeziformis* among victims of a very localized tornado (Weddle et al. 2012). Moreover, screening the natural diversity should be the first step for practical applications.

Our local populations were genetically heterogeneous, as shown in *P. blakesleeanus* by restriction-length polymorphisms and in *P. nitens* by differences in sex, spores, and DNA sequences. Notable is the case of the three strains found in a single biomass sample from the Higueral cave, which differed in sex, sexual vigor, ten nucleotides of the ITS/5.8S sequences, and fifteen nucleotides of the *sexP* gene.

The quantitative analysis of restriction-length differences in *P. blakesleeanus* allowed us to distinguish local populations and to group them in larger geographical areas (Fig. 6). This analysis was limited to differences between the reference strains NRRL1555 and UBC21, for which the needed sequence information was available. Unfortunately, the origin of these is not documented.

Strain UBC21 comes from the collection of R. J. Bandoni, University of British Columbia, and NRRL1555 may well come from A. F. Blakeslee (Benjamin & Hesseltine 1959), who worked mostly at Cold Spring Harbor, near New York. Our results are understandable if both strains are autochthonous American. NRRL1555 is likely to come from southeastern USA. If this is the case, our analysis forces the strains from Europe and Eastern Asia to be placed between the reference strains, even if differences in other restriction sites would have placed them far away from both. Our results make it unlikely that NRRL1555 and UBC21 come from Europe and even more so that they come from different continents.

4.4 Sex determination

The SexP and SexM proteins of the *Mucorales* (Fig. S2) are putative sex-specific transcription factors, since they contain a sequence similar to the HMG domain found in genes responsible for sex determination in many organisms (Idnurm et al. 2008; Czaja et al. 2014). The DNA-binding amino acids in HMG have been identified in the tridimensional structures of proteins from *Saccharomyces cerevisiae* (Bell et al. 1977) and *Mus musculus* (Love et al. 1995). The HMG domains of the *sex* genes of the *Mucorales* evolve under general constraints, evidenced by the absolute conservation of seven amino acids, and sex-specific constraints, evidenced by the conservation of twelve amino acids in SexP and one in SexM. The generally-constrained amino acids and many sex-constrained ones coincide with or are contiguous to the DNA-binding amino acids.

Genes *sexP* and *sexM* are related to each other and have diverged to the same extent in different *Mucorales*, with 39.1 ± 1.4 % coincident nucleotides (mean and standard deviation

for the four species represented in Fig. 7). This suggests that these organisms have arrived to a similar equilibrium between the opposite selective pressures to maintain the common function as transcription factors and to carry out sex-specific functions.

The transcript level of gene *sexM* in *M. mucedo* increases in mated cultures of strains of opposite sex and upon exposure to a mixture of trisporoids; the same occurs to a lesser extent with *sexP* (Wetzel et al. 2012). Sexual development is accompanied by modified levels of the transcripts of several hundred genes in mated cultures (Kuzina et al. 2008).

The high conservation of the sex and species-specific segments on both sides of *sexP* in *B. trispora* and between *sexM* and *rnhA* in *P. nitens* makes them useless for strain identification and implies a selective pressure. This comes, at least in part, from the regulation of *rnhA*, *tptA*, *sexM*, and *sexP*, whose promoters are presumably in those regions, and in fact a target for transcription factors is present upstream of *sexP* in *Phycomyces* and other *Mucorales*.

The sex allelomorphs have four anti-recombination features: the *sex* genes are small, their sequences are only partially homologous, they are surrounded by unique sex-specific sequences, and, in some cases, they run in opposite direction. These features are particularly strong in *Phycomyces* and all the tested strains of this fungus can be assigned unequivocally to the (+) or the (−) sex and have a complete and apparently functional *sexP* or *sexM* gene. The situation in *Blakeslea* appears more prone to internal recombination and it would be worth knowing the frequency in this fungus of natural strains with a dysfunctional, recombinant *sex* gene.

In *Phycomyces* we found no indications of intraspecific barriers to sexual reproduction, such as the vegetative incompatibilities common in many fungi and plants.

4.5 rRNA

There are three types of tandem DNA repeats for rRNA in the fungi, each widely distributed. In one of the types, found in species of all major fungal groups, the 5S gene is not part of the tandem repeats that include the other rRNA genes (Bergeron & Drouin 2008). When it is part of the repeat, it can be transcribed in the opposite direction to the other genes, as it occurs in the *Mucorales* *P. blakesleeanus* (Fig. 4), *M. racemosus* (Cihlar & Sypherd 1980), *M. (now Rhizomucor) miehei* (Maicas et al. 2000), and *Absidia glauca*, and in many other fungi, including *Saccharomyces cerevisiae* (Aarstad & Oyen 1975) and most of the *Ascomycota*. In

R. oryzae and in most of the *Basidiomycota* the 5S gene is transcribed in the same direction as the other genes.

A low level of sequence heterogeneity within each strain, as found in our ITS/5.8S sequences of *Phycomyces*, is probably common in the Fungi. Other examples were found in the *P. blakesleanus* genome sequence, in *R. microsporus* (Woo et al. 2010), and outside the *Mucorales* (Ganley & Kobayashi 2007).

4.6 Cladograms of Mucorales

For comparisons with other *Mucorales* we chose strains NRRL1555 and UBC21 of *P. blakesleanus* and strains S608 and S609 of *P. nitens* because they carry the most common sequence variants that we have studied. The cladograms of these sequences (Fig. 8) coincide in topology and overall size, although the sequences have very different functions and are presumably subject to very different selection pressures. The seven species are repeatedly distributed in three groups: *Phycomyces*, *Blakeslea-Mucor*, and *Rhizopus-Syzygites*. The detection of separate *sexM* and *sexP* genes in all the *Mucorales* under study indicates that these organisms diverged after the two genes had diverged in a common ancestor.

The internodal distances indicate variations in the divergence rate of the sequences. The ITS/5.8S sequences of *Blakeslea* and *Mucor* are tightly clustered, suggesting that these genera of different families form a young clade. Their *sex* genes have evolved very rapidly. Even more divergent are the non-transcribed sequences in the sexual allelomorphs, where the promoters should be located. The sexual allelomorphs would be relevant for speciation (Idnurm 2011b) by making the function and the regulation of the *sex* genes dependent on the external environment and on the molecular composition of the cells. The barriers to genetic exchange would facilitate the rapid evolution of the traits subject to weak selective pressures, including the morphology used to define genera and species. We recommend comparisons of the *sex* genes and the whole allelomorphs to study the genera that are poorly resolved by other sequences (Hoffmann et al. 2013; Walther et al. 2013).

The *sex* genes of the *R. oryzae* and *S. megalocarpus* (Fig. 8) have diverged slower than their ITS/5.8S sequences, a surprising observation since the first is heterothallic and the second homothallic. In the cladogram of the *Rhizopodaceae*, homothallic species of *Syzygites* and *Rhizopus* are interspersed with heterothallic species of *Rhizopus* and other genera (Walter et al. 2013). The presence of both *sexP* and *sexM* in homothallic species may be due to asymmetric recombination or translocation during the sexual cycle (Idnurm 2011a). Single

colonies of intersexual partial diploids (Mehta & Cerdá-Olmedo 2001) and intersexual heterokaryons (Murillo et al. 1978) of *Phycomyces*, which carry both *sex* genes, exhibit partial sexual interactions and novel structures, the pseudophores, but are not homothallic. The production of zygosporangia would require additional mutations, but these should not be very numerous, given the interspersed nature of both life styles in the *Rhizopodaceae*.

According to the ITS/5.8S sequences (Fig. S3), the distance between the two species of *Phycomyces*, which look nearly identical, is larger than the distances between the genera *Blakeslea*, *Choanephora*, and *Poitrasia* of the family *Choanephoraceae*. The morphology of *Phycomyces* is presumably maintained by its adaptation to a rather constant environment, found all over the world.

4.7 Life cycles of the Mucorales

The results on the diversity of local populations and on *sex* gene sequences suggest that natural populations of *Phycomyces* are much more active sexually than usually assumed by laboratory researchers. Active sexuality explains the rarity of heterokaryosis in natural isolates. The sexual cycle favors homokaryosis because the nuclei in each germ-spore derive normally from a single haploid genome produced by recombination of a single pair of parental nuclei (Cerdá-Olmedo 1975). On the contrary, the multinucleate mycelia and spores of the vegetative cycle facilitate the maintenance and the transmission of spontaneous mutations (Heisenberg & Cerdá-Olmedo 1968).

There are clear parallelisms between the life cycles of *Phycomyces* and other *Mucorales* and *Homo sapiens*. The vast majority of the genome replications occur in the respective vegetative cycles, leading to the formation of large colonies or individuals, where most of the life processes take place. The sexual cycle plays an essential role in population structure, but is spaced in time by powerful clocks that in one case keep the zygosporangia dormant for months and in the other delay sexual maturity for years. The genetic diversity introduced during the vegetative cycle by mutations, even deleterious ones, is largely kept under cover by multinucleatedness in one case and diploidy in the other. Sex purifies most of this variation in both cases. At the same time, the sexual processes introduce new variation in the form of recombinants and new mutations.

The life cycles of the *Mucorales* are more flexible than ours, as they allow unlimited vegetative growth and clonal subcultures, and are more strict in the elimination of deleterious

mutations, which do not survive the haploidy of the germ spores, but are maintained by our diploidy.

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Data Accessibility

The accession numbers in Table S2 refer to DNA sequences available in EMBL.

Figure captions

Fig. 1 Global distribution of *P. blakesleeanus* (stars) and *P. nitens* (circles). Isolation sites of new strains (filled symbols) and old strains (empty symbols). The Iberian Peninsula and the Canary Islands are enlarged in the boxes.

Fig. 2 The spores of *Phycomyces*. For strain identification see Table 1; empty symbols are used for *P. blakesleeanus* and filled symbols for *P. nitens*. (Above) Micrographs of spore samples from four representative strains; phase contrast (left) and DAPI nuclear stain (right). (Below left) Mean length (major axis) and mean eccentricity of the microscopic image of the spores from 42 strains. The eccentricity of each spore was calculated as $\sqrt{1 - b^2/a^2}$, where “a” and “b” are the major and minor axes. (Below right) Cumulative frequencies of the number of nuclei per spore in 18 strains.

Fig. 3 Growth of *P. nitens* at different temperatures. Dry mass after six-days' growth and radial colony expansion during the third day of growth. For strain identification see Table 1; empty circles are used for *P. blakesleeanus* and filled symbols for *P. nitens*.

Fig. 4 Tandem DNA repeat for rRNAs of *P. blakesleeanus* strain NRRL1555. Filled bars, sequences for the four rRNAs; empty bars, intergenic spacers (IGS) and internal transcribed spacers (ITS).

Fig. 5 Parsimony cladogram of the ITS/5.8S sequences of 93 strains of *Phycomyces*. The tree is drawn to scale with the number of nucleotide changes in each branch.

Fig. 6 Genetic relationships of 56 strains of *P. blakesleeanus*. NMDS distances between the strains according to a Jaccard matrix of the dissimilarity of 27 restriction-length polymorphisms. For strain identification see Table 1. Squares represent strains from Europe; circles, from North America; triangle, from Asia; and lozenges, unknown origin. Seven local populations isolated from the same biomass sample are color-marked: Florida, pink; South Carolina, red; Houston, Texas, blue; Dallas, Texas, yellow; Tennessee, green; Virginia, orange; Michigan, light green.

Fig. 7 The sex allelomorphs of four *Mucorales*. Comparison of those of *P. nitens* S608 and S609 and *B. trispora* F986 and F921, obtained in this work, and those of *P. blakesleeana* (Idnurm et al. 2008) and *M. circinelloides* (Lee et al. 2008). Filled bars, coding sequences of genes *tptA*, *rnhA*, *sexP*, and *sexM*. Empty bars, homologous non-coding sequences. Hatched bars, unique, sex- and species-specific sequences.

Fig. 8 Evolutionary relationships of seven *Mucorales*. Maximum-likelihood cladograms of *sexM*, *sexP*, *rnhA*, and ITS/5.8S sequences, with branch lengths proportional to the number of substitutions per site. Dotted lines represent branches with bootstrap values under 0.5.

Table 1 Strains of *Phycomyces* used in this work.

Strain ^a	Origin	Collector	Isolator, year	Sex	Ref. ^b	Comments
<i>Phycomyces blakesleeanus</i>						
1 C168	Santa Cruz, CA, USA		Burke, 1971	(+)	(1)	Thick sporangiophores
2 Carmichael666	Alberta, Canada		Carmichael, 1959	(−)	(1)	Dung of mouse
Carmichael667	Alberta, Canada		Carmichael, 1959	(−)	(1)	Dung of deer mice
3 CBS142.22	Utrecht, Holland		Wassink, 1922	(−)	(1)	
4 CBS189.27	USA		Blakeslee, 1927	(−)	(1)	
5 CBS282.35	Utrecht, Holland		Wassink, 1922	(−)	(1)	
6 G5	Worcester, MA, USA		Lougee, 1952	(+)	(1)	Over leaf
7 K1	Germany			(+)	(4)	
8 KACC46094	South Korea			(−)	(3)	From fermented soybean
KACC46095	South Korea			(−)	(3)	From fermented soybean
9 KC1	Charleston, SC, USA	M. Powell	Idnurm, 2010	(+)		Vomit of dog
10 KC2	Charleston, SC, USA	M. Powell	Idnurm, 2010	(+)		Vomit of dog
11 KC3	Charleston, SC, USA	M. Powell	Idnurm, 2010	(+)		Vomit of dog
12 KC4	Charleston, SC, USA	M. Powell	Idnurm, 2010	(+)		Vomit of dog
13 KC5	Charleston, SC, USA	M. Powell	Idnurm, 2010	(+)		Vomit of dog
14 KC6	Charleston, SC, USA	M. Powell	Idnurm, 2010	(+)		Vomit of dog
15 KC7	Houston, TX, USA	B.W. Wallace	Idnurm, 2010	(−)		Organic material under oak tree
16 KC8	Houston, TX, USA	B.W. Wallace	Idnurm, 2010	(+)		Organic material under oak tree
17 KC9	Houston, TX, USA	B.W. Wallace	Idnurm, 2010	(+)		Organic material under oak tree
18 KC10	Houston, TX, USA	B.W. Wallace	Idnurm, 2011	(+)		Organic material under oak tree
19 KC11	Houston, TX, USA	B.W. Wallace	Idnurm, 2011	(+)		Organic material under oak tree
20 KC12	Houston, TX, USA	B.W. Wallace	Idnurm, 2011	(−)		Organic material under oak tree
21 KC13	Dallas, TX, USA	E. Goodale	Idnurm, 2012	(−)		Dung of cat
22 KC14	Dallas, TX, USA	E. Goodale	Idnurm, 2012	(−)		Dung of cat
23 KC15	Dallas, TX, USA	E. Goodale	Idnurm, 2012	(−)		Dung of cat
24 KC16	Fairfax, VA, USA	D. Johnston	Idnurm, 2012	(−)		Dung of raccoon in attic
25 KC17	Fairfax, VA, USA	D. Johnston	Idnurm, 2012	(+)		Dung of raccoon in attic
26 KC18	Fairfax, VA, USA	D. Johnston	Idnurm, 2012	(−)		Dung of raccoon in attic

27	KC19	Fairfax, VA, USA	D. Johnston	Idnurm, 2012	(-)		Dung of raccoon in attic
28	KC20	Fairfax, VA, USA	D. Johnston	Idnurm, 2012	(+)		Dung of raccoon in attic
29	KC21	Fairfax, VA, USA	D. Johnston	Idnurm, 2012	(-)		Dung of raccoon in attic
30	KC22	Howell, MI, USA	M. and S. Bowen	Idnurm, 2013	(+)		Seeds in bird feeder
31	KC23	Howell, MI, USA	M. and S. Bowen	Idnurm, 2013	(+)		Seeds in bird feeder
32	KC24	Nashville,TN, USA	C. McAlister	Idnurm, 2013	(+)		Seeds
33	KC25	Nashville,TN, USA	C. McAlister	Idnurm, 2013	(-)		Seeds
34	KC26	Sarasota, FL, USA	Wall family	Idnurm, 2013	(-)		Vomit of cat
35	KC27	Sarasota, FL, USA	Wall family	Idnurm, 2013	(-)		Vomit of cat
36	KC28	Sarasota, FL, USA	Wall family	Idnurm, 2013	(-)		Vomit of cat
37	KC29	Sarasota, FL, USA	Wall family	Idnurm, 2013	(-)		Vomit of cat
38	KC30	Sarasota, FL, USA	Wall family	Idnurm, 2013	(-)		Vomit of cat
39	NRRL1464				(-)	(1, 2)	
40	NRRL1465			1903	(+)	(1, 2)	Dung of rabbit
41	NRRL1554				(+)	(1, 2)	
42	NRRL1555				(-)	(1, 2)	
43	NRRL2565	Germany		Burgeff, 1920	(+)	(1, 2)	<i>Piloboloides</i>
44	NRRL2566	Germany		Zycha, 1935	(-)	(1, 2)	<i>Piloboloides</i>
45	NRRL6737				(-)	(1)	
46	NRRL6740				(-)	(1)	
47	RSA499	Peterborough, NH, USA		Benjamin, 1956	(-)	(1)	Dung of rodent
48	RSA531	Claremont, CA, USA		Benjamin, 1957	(-)	(1)	Dung of pack rat
	RSA537	Claremont, CA, USA		Benjamin, 1957	(-)	(1)	Dung of mouse.
49	RSA1739	Montreal, Canada		Lewis, 1961	(+)	(1)	Dung of chipmunk
50	S271	British Columbia, Canada		Bandoni	(+)		Dung of cat
51	UBC1	British Columbia, Canada		Bandoni, 1970s	(+)	(1)	<i>Nanus</i>
52	UBC21				(+)	(1)	
53	UBC24				(-)	(1)	
54	UBC25				(+)	(1)	
55	UBC31				(+)	(1)	
56	UBC33				(-)	(1)	

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57	Anastasiou538	Claremont, CA, USA		Benjamin, 1960	(+)	(1)	Dung of mouse
58	ATCC16327	Japan		Indoh	(−)	(1)	Dung of pig
59	CBS149.24	Germany		Burgeff, 1924	(+)	(2)	<i>P. theobromatus</i> Burgeff
60	ICMP10093	Auckland, New Zealand		McKenzie	(+)		Carpet
61	ICMP16924	Christchurch, New Zealand		Cooper, 2007	(−)		Dung in wetlands
62	IMI051077	Avon, England		Parry, 1952	(+)	(1, 2)	Front coconut meal
63	Indoh1521	Gunma, Japan		Takasaki, 1963	(−)	(1)	Dung of pig
64	KC31	Howell, MI, USA	M. and S. Bowen	Idnurm, 2013	(+)		Seeds in bird feeder
65	KC32	Howell, MI, USA	M. and S. Bowen	Idnurm, 2013	(+)		Seeds in bird feeder
66	KC33	San Jose, CA, USA	J. Epler	Idnurm, 2013	(−)		Dung of cat
67	NRRL2444	Surrey, England		Martin	(+)	(2)	Dung of rabbit
68	NRRL2678	Chiba, Japan			(−)	(1)	
69	NRRLA7595	Blue Rapids, KA, USA		C.T. Rogerson	(+)	(2)	Dung in gypsum mine
70	S256	La Laguna, Tenerife, Spain		Cerdá, 1978	(+)		Dung
71	S257	La Laguna, Tenerife, Spain		Cerdá, 1978	(+)		Dung
72	S270	British Columbia, Canada		Bandoni	(+)		Dung of cat
73	S465	Exeter, England		Webster	(−)		Dung of rabbit
74	S466	Exeter, England		Webster	(−)		Dung of rabbit
75	S605	Aracena, Huelva, Spain		Flores, 1999	(−)		Mammal dung
76	S606	Cortes, Málaga, Spain		Cerdá Olmedo, 2003	(−)		Dung of dog
77	S607	Jerez, Cádiz, Spain	J. Ramos Guelfo	Camino, 2003	(+)		Mammal dung in Higueral cave
78	S608	Jerez, Cádiz, Spain	J. Ramos Guelfo	Camino, 2003	(+)		Mammal dung in Higueral cave
79	S609	Jerez, Cádiz, Spain	J. Ramos Guelfo	Camino, 2003	(−)		Mammal dung in Higueral cave
80	S642	El Paso, La Palma, Spain	E. Cerdá Olmedo	Camino, 2003	(−)		Dung of rabbit
81	S643	Hermigua, Gomera, Spain	E. Cerdá Olmedo	Camino, 2007	(+)		Organic matter
82	S644	Hermigua, Gomera, Spain	E. Cerdá Olmedo	Camino, 2007	(+)		Organic matter
83	S645	Navia, Asturias, Spain	E. Rubio Domínguez	Camino, 2007	(+)		Dung of dog
84	S646	Los Barrios, Cádiz, Spain	E. Cerdá Olmedo	Camino, 2008	(+)		Dung of rat
85	S651	El Garrobo, Sevilla, Spain	J. Ramos Guelfo	Camino, 2010	(+)		Dung of mammal
86	S653	Ardales, Málaga, Spain		Jurado, 2011	(−)		Organic matter in Ardales cave
87	S654	Ardales, Málaga, Spain		Domínguez, 2011	(−)		Dung of mammal in Ardales cave

88	S655	Ardales, Málaga, Spain		Domínguez, 2011	(-)	Dung of mammal in Ardales cave
89	S656	Ardales, Málaga, Spain		Domínguez, 2011	(-)	Dung of mammal in Ardales cave
90	S657	Aracena, Huelva, Spain		Domínguez, 2011	(-)	Organic matter in Aracena cave
91	S658	Cortegana, Huelva, Spain	M. Alvarado Cordobés	Camino, 2011	(-)	Dung of mammal
92	S659	Cazalla, Sevilla, Spain	M. Alvarado Cordobés	Camino, 2012	(-)	Dung of mammal
93	S660	C. las Guardas, Sevilla, Spain	I. López Calderón	Camino, 2012	(-)	Dung of cat
94	UBC39	British Columbia, Canada		Spencer, 1963	(-)	Trash of basement
95	UBC67				(+)	
96	UBC149	British Columbia, Canada		Reid, 1972	(-)	Dung of cat

^a The number on the left is the abbreviation used in the Figures; the three unnumbered strains are believed to be identical to the ones preceding them. Strain names refer to the laboratory where they were isolated or the collection where they are deposited: C, Laboratory of M. Delbrück, California Institute of Technology, Pasadena, CA, USA; S, Laboratory of E. Cerdá-Olmedo, Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Spain; KC, A. Idnurm, now at University of Melbourne, Australia; CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; G, Indoh, Anastasiou, and Carmichael: University of Saskatchewan, Saskatoon, Canada; NRRL, Northern Regional Research Laboratory, USDA, Peoria, IL, USA; RSA, R.K. Benjamin, Claremont, CA, USA; K, B. Furch, Universität Kiel, Germany; UBC, R. J. Bandoni, University of British Columbia, Canada; KACC, Korean Agricultural Culture Collection, Swon, South Korea; ATCC, American Type Culture Collection, USA; ICMP, International Collection of Microorganisms from Plants, New Zealand; IMI, International Mycological Institute, Kew, UK.

^b (1) (Ootaki and Miyazaki 1993) (2) (Benjamin and Hesseltine 1959) (3) (Hong et al. 2012) (4) (Van Laere 1983)

Figure(s)

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8