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Morphological characteristics of sporangiospores of the tempe fungus *Rhizopus oligosporus* differentiate it from other taxa of the *R. microsporus* group

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ABSTRACT

The fungus *Rhizopus oligosporus* (*R. microsporus* var. *oligosporus*) is traditionally used to make tempe, a fermented food based on soybeans. Interest in the fungus has steadily increased, as it can also ferment other substrates, produce enzymes, and treat waste material. *R. oligosporus* belongs to the *R. microsporus* group consisting of morphologically similar taxa, which are associated with food fermentation, pathogenesis, or unwanted metabolite production (rhizonins and rhizoxins). The ornamentation pattern, shape, and size of sporangiospores of 26 *R. microsporus* group strains and two *R. oryzae* strains were studied using low-temperature SEM (LT-SEM) and LM. This study has shown that: (1) LT-SEM generates images from well-conserved sporangiophores, sporangia, and spores. (2) Robust spore ornamentation patterns can be linked to all different taxa of the *R. microsporus* group, some previously incorrectly characterized as smooth. Ornamentation included valleys and ridges running in parallel, granular plateaus, or smooth polar areas. Distribution of ornamentation patterns was related to spore shape, which either was regular, ranging from globose to ellipsoidal, or irregular. Specific differences in spore shape, size, and ornamentation were observed between *Rhizopus* taxa, and sometimes between strains. (3) *R. oligosporus* has a defect in the spore formation process, which may be related to the domesticated nature of this taxon. It had a high proportion, 10–31 %, of large and irregular spores, and was significantly differentiated from other, natural *Rhizopus* taxa as evaluated with partial least squares discriminant analysis. It is remarkable that the vehicle of distribution, the sporangiospore, is affected in the strains that are distributed by human activity. This provides information about the specificity and speed of changes that occur in fungal strains because of their use in (food) industry.

Introduction

The *Rhizopus microsporus* group (*Zygomycota, Mucorales, Mucoraceae*) (Schipper & Stalpers 1984) is a complex group of taxa, which are hard to differentiate morphologically even for a trained observer. The group consists of five species, of which *R. microsporus* has six varieties (Prakash & Sarbhoy 1993; Schipper & Samson 1994; Schipper & Stalpers 1984; Weitzman

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Table 1 – Strains of Rhizopus taxa investigated in the present study

Scientific name	Strain no.[a]	Other strain no.[a]	Source
Rhizopus azygosporus	CBS 357.92	ATCC 200615, MUMDU 444790, NCPF 2840	Peritoneal fluid of premature baby, Australia
R. azygosporus	CBS 357.93^{T}	ATCC 48108^{T}, BCRC 31158^{T}, NRRL 13165^{T}	Tempe, Indonesia
R. caespitosus	CBS 427.87^{T}	BCRC 33515^{T}	Unknown, India
R. chinensis[b]	CBS 261.28[c]	ATCC 52811, DSM 2193, NRRL 13476, VKM F-1361, Scholer M 213	Unknown, USA
R. chinensis[b]	CBS 631.82^{T} [c]	ATCC 52807^{T}, BCRC 33519^{T}, IMI 130842^{T}	Bread, China
R. homothallicus	CBS 336.62^{T} [c]	ATCC 42221^{T}, BCRC 31146^{T}, IMI 089714^{T}, NRRL 2538^{T}	Tropical desert soil, Guatemala
R. homothallicus	CBS 111232	NRRL 2935, NRRL A-10901	Unknown, India
R. microsporus[b]	CBS 699.68[c]	ATCC 52813, BCRC 31140, NRRL 13479, VKM F-773	Soil, Ukraine
R. microsporus[b]	CBS 700.68[c]	ATCC 52814, BCRC 31141, NRRL 13478, VKM F-774	Forest soil, Georgia
R. microsporus[b]	CBS 308.87	NRRL 28628	Human tissue, Australia
R. microsporus[b]	CBS 112285	MRC 303	Groundnuts, Mozambique
R. oligosporus[b]	CBS 337.62[c]	ATCC 46348, BCRC 31997, NRRL 514, NRRL A-9734, Scholer M 225	Probably tempe, Indonesia
R. oligosporus[b]	CBS 338.62[c]	ATCC 22959, BCRC 31996, IMI 174457, FRR 2710, MUCL 28419, NBRC 8631, NRRL 2710, NRRL A-7982, VTT D-82192, Scholer M 140	Tempe, Indonesia
R. oligosporus[b]	CBS 339.62[c,d]	ATCC 48011, NRRL 6205, NRRL A-9867, UMIP 1126.75, VKM F-1415, Scholer M 226	Tempe, Indonesia
R. oligosporus[b]	CBS 228.95		Tempe, Indonesia
R. oligosporus[b]	CBS 112586	ATCC 60826, NRRL 5905, NRRL A-11129	Tempe, Indonesia
R. oligosporus[b]	CBS 112587	ACM 145F, ACM 4668, ATCC 76011, ATCC 96528, FRR 2458	Tempe, Indonesia
R. oligosporus[b]	CBS 112588	ATCC 48012, NRRL 6495, NRRL A-9868	Tempe, Indonesia
R. oligosporus[b]	CBS 112589[d]	CBS 339.62[d]	Tempe, Indonesia
R. oligosporus[b]	ATCC 48109	BCRC 32600	Tempe, The Netherlands
R. oligosporus[b]	ATCC 64063		Tempe, Indonesia
R. oryzae	CBS 112.07^{T} [c]	ATCC 22957^{T}, ATCC 56536^{T}, BCRC 31145^{T}, MUCL 9668^{T}, NRRL 3133^{T}, NRRL A-13164^{T}, TISTR 3246^{T}, VKM F-1414^{T}	Human tissue, The Netherlands
R. oryzae	CBS 329.47[c]	ATCC 10260, ATCC 12732, BCRC 31629, DSM 905, MUCL 30523, NRRL 1526, Thom 1034	Tempe starter
R. pseudochinensis[b]	CBS 264.28^{T} [c]	ATCC 44169^{T}, BCRC 31152^{T}	Chinese yeast, China
R. rhizopodiformis[b]	CBS 343.29^{T} [c,e]	BCRC 31994^{T}, Scholer M 208^{T}	Unknown, former USSR
R. rhizopodiformis[b]	CBS 536.80[c]	BCRC 31995, MRC 1954, VKM F-3697	Sorghum malt, South Africa
R. rhizopodiformis[b]	CBS 102277	IMI 381459, FMR 6691	Rhinocerebral human infection, unknown location
R. schipperae	CBS 138.95^{T}	ATCC 96514^{T}, BCRC 33517^{T}, UTHSC 94-538^{T}	Human lung, USA

All strains, except *Rhizopus oryzae* CBS 112.07 and CBS 329.47, belong to the *R. microsporus* group.

a ACM (former UQM), Australian Collection of Microorganisms, Department of Microbiology and Parasitology, The University of Queensland, Brisbane, Australia; ATCC, American Type Culture Collection, Manassas, USA; BCRC (former CCRC), Bioresource Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, ROC; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; FMR, Unitat de Microbiologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain; FRR, Food Science Australia, North Ryde, Australia; MRC, South African Medical Research Council, Tygerberg, South Africa; IMI, IMI Bioscience – UK centre, Egham, UK; MUCL, Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; MUMDU, Microbiological Diagnostic Unit, Public Health Laboratory, The University of Melbourne, Melbourne, Australia; NBRC (former IFO), NITE Biological Resource Center, Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan; NCPF, National Collection of Pathogenic Fungi, PHLS Mycological Reference Laboratory, Bristol, UK; NRRL, ARS Culture Collection, Northern Regional Research Laboratory, National Center for Agricultural Utilization Research, Peoria, USA; TISTR, Thailand Institute of Scientific and Technological Research, Bangkok, Thailand; UMIP, Collection of Fungi, Institut Pasteur, Paris, France; UTHSC, The University of Texas Health Science Center, San Antonio, USA; VKM, All-Russian Collection of Microorganisms, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Russia; VTT, VTT Biotechnology, Culture Collection, Espoo, Finland.

b Taxon usually regarded as a variety of *R. microsporus*.

c Strain studied by Schipper & Stalpers (1984).

d Approximately 40 y since the lines CBS 339.62 and CBS 112589 were separated. (In 1960, the fungus was deposited at NRRL, and in 1962, NRRL deposited the fungus at CBS as CBS 339.62. CBS 112589 originated from NRRL 6205, and was kept at Department of Microbiology, SLU, until deposited at CBS in 2003.)

e Ex-type strain of *R. pusillus*, synonym (nom. inval., Art. 36.1) of *R. microsporus* var. *rhizopodiformis*.

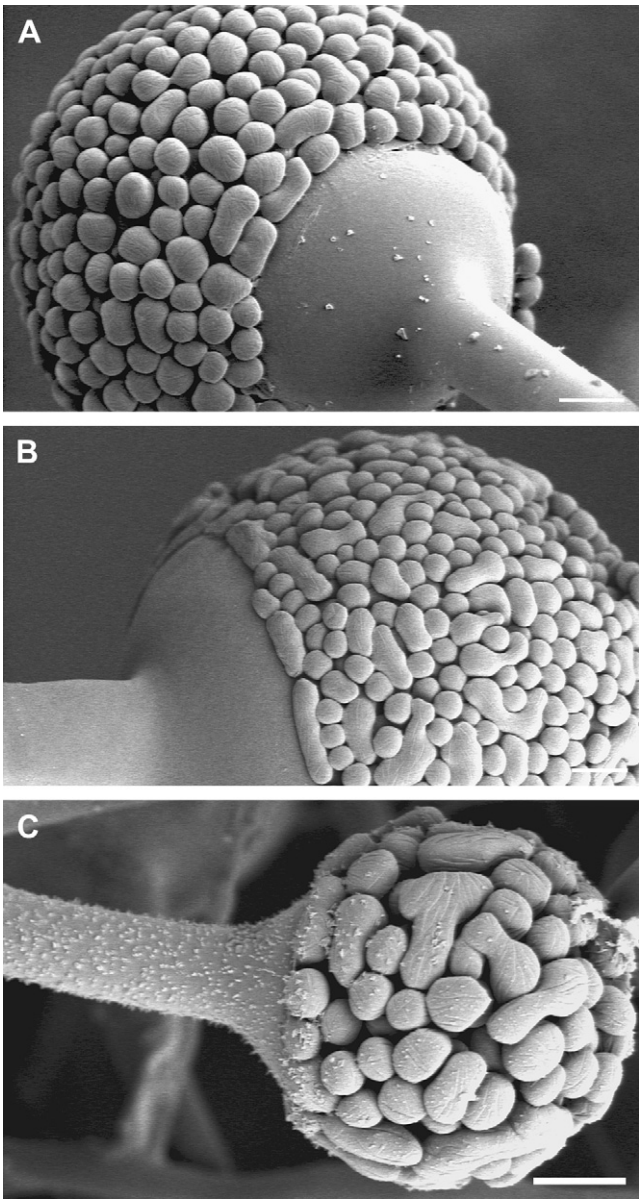


Fig 1 – *Rhizopus oligosporus* sporangiophores with apophyses, and sporangia with sporangiospores as seen under LT-SEM. (A) CBS 338.62; (B) CBS 112588; (C) CBS 112589. Some of the sporangiospores are globose to subglobose, whereas others are large and irregular shaped. (A–B) Three days, MEA; (C) 4 d, MEA. Bars = 10 μm.

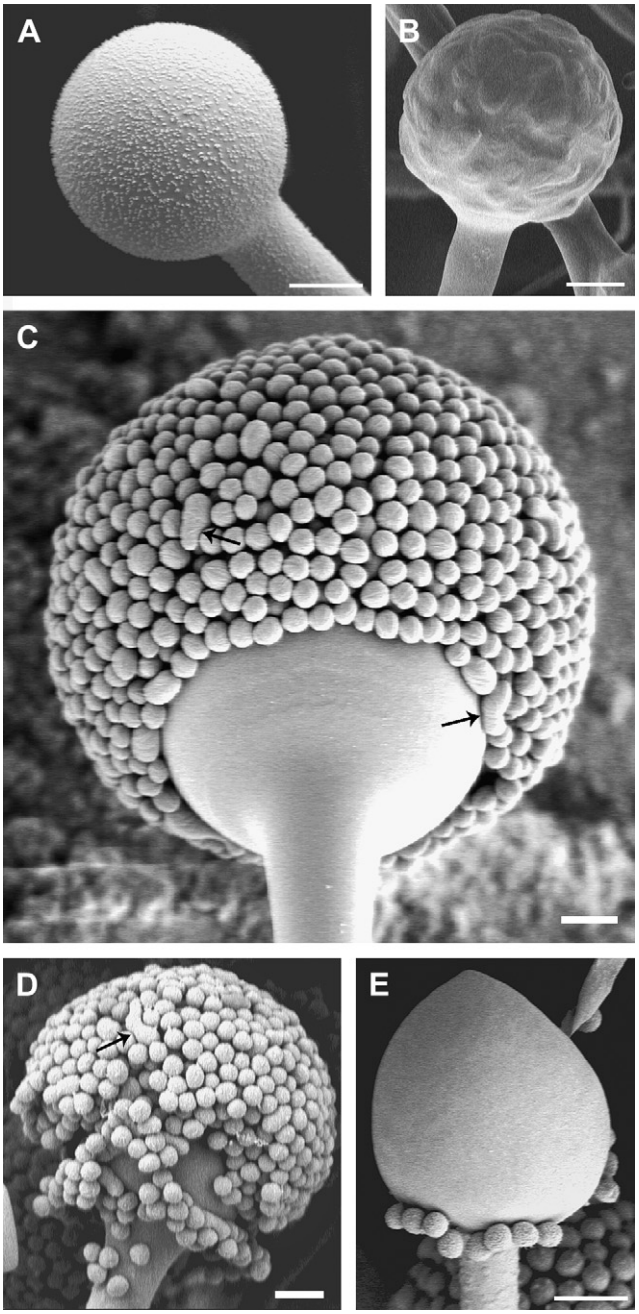


Fig 2 – Developmental stages of *Rhizopus* sporangiophores and sporangia as seen under LT-SEM. (A) Immature sporangiophore; (B) young sporangiophore with developing spores in sporangia; (C) mature sporangium with sporangiospores; (D) sporangiospores starting to be released from an old sporangium; (E) columella with only a few spores left. Arrows indicate examples of anomalies in sporangiospore size and shape. (A) *R. rhizopodiformis* CBS 102277; 3 d, MEA; (B) *R. oligosporus* CBS 339.62; 3 d, MEA; (C) *R. homothallicus* CBS 111232; 4 d, OMA; (D–E) *R. rhizopodiformis* CBS 536.80, 12 d, MEA. Bars = 10 μm.

et al. 1996; Yuan & Jong 1984; Zheng & Chen 1998). For simplicity, the *R. microsporus* varieties are designated, e.g. *R. oligosporus* and not as *R. microsporus* var. *oligosporus*.

Some taxa in the *Rhizopus microsporus* group are used in food fermentations (Beuchat 1987) or in industrial applications (Pandey *et al.* 1999), whereas others are important degraders in soil and on vegetable material (Ribes *et al.* 2000). Some taxa are also known as human opportunistic pathogens (Ribes *et al.* 2000), or associated with production of the highly toxic rhizonins or the pharmaceutically active rhizoxins (Jennessen *et al.*

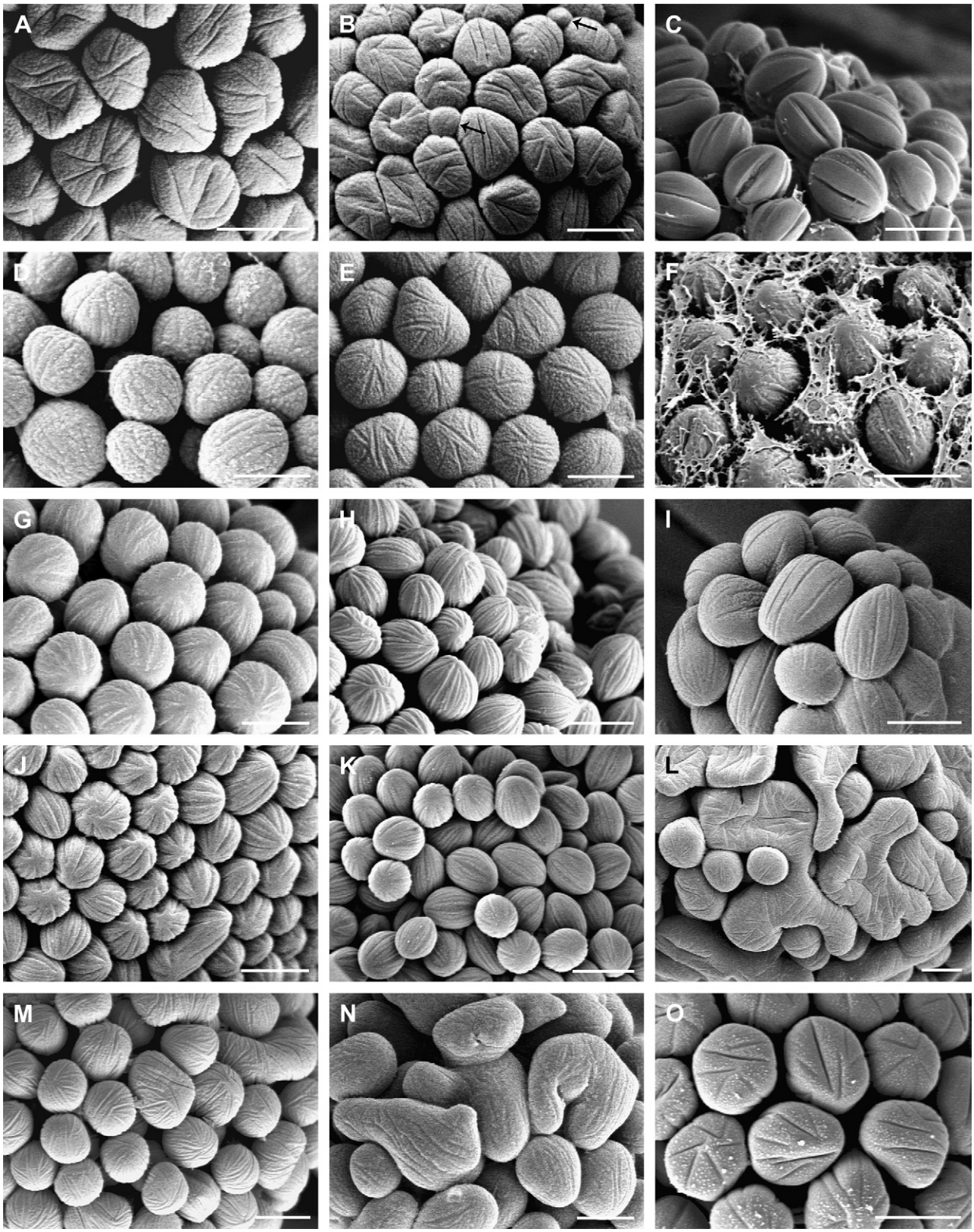


Fig 3 – Sporangiospores of investigated *Rhizopus* strains as seen under LT-SEM. (A) *R. azygosporus* CBS 357.92; (B) *R. azygosporus* CBS 357.93^T; (C) *R. caespitosus* CBS 427.87^T; (D) *R. chinensis* CBS 261.28; (E) *R. chinensis* CBS 631.82^T; (F) *R. homothallicus* CBS 336.62^T; (G) *R. homothallicus* CBS 111232; (H) *R. microsporus* CBS 699.68; (I) *R. microsporus* CBS 700.68; (J) *R. microsporus* CBS 308.87; (K) *R. microsporus* CBS 112285; (L) *R. oligosporus* CBS 337.62; (M) *R. oligosporus* CBS 338.62; (N) *R. oligosporus* CBS 339.62; (O) *R. oligosporus* 228.95; (P) *R. oligosporus* CBS 112586; (Q) *R. oligosporus* CBS 112587; (R) *R. oligosporus* CBS 112588; (S) *R. oligosporus* CBS 112589; (T) *R. oligosporus* ATCC 48109; (U) *R. oligosporus* ATCC 64063;

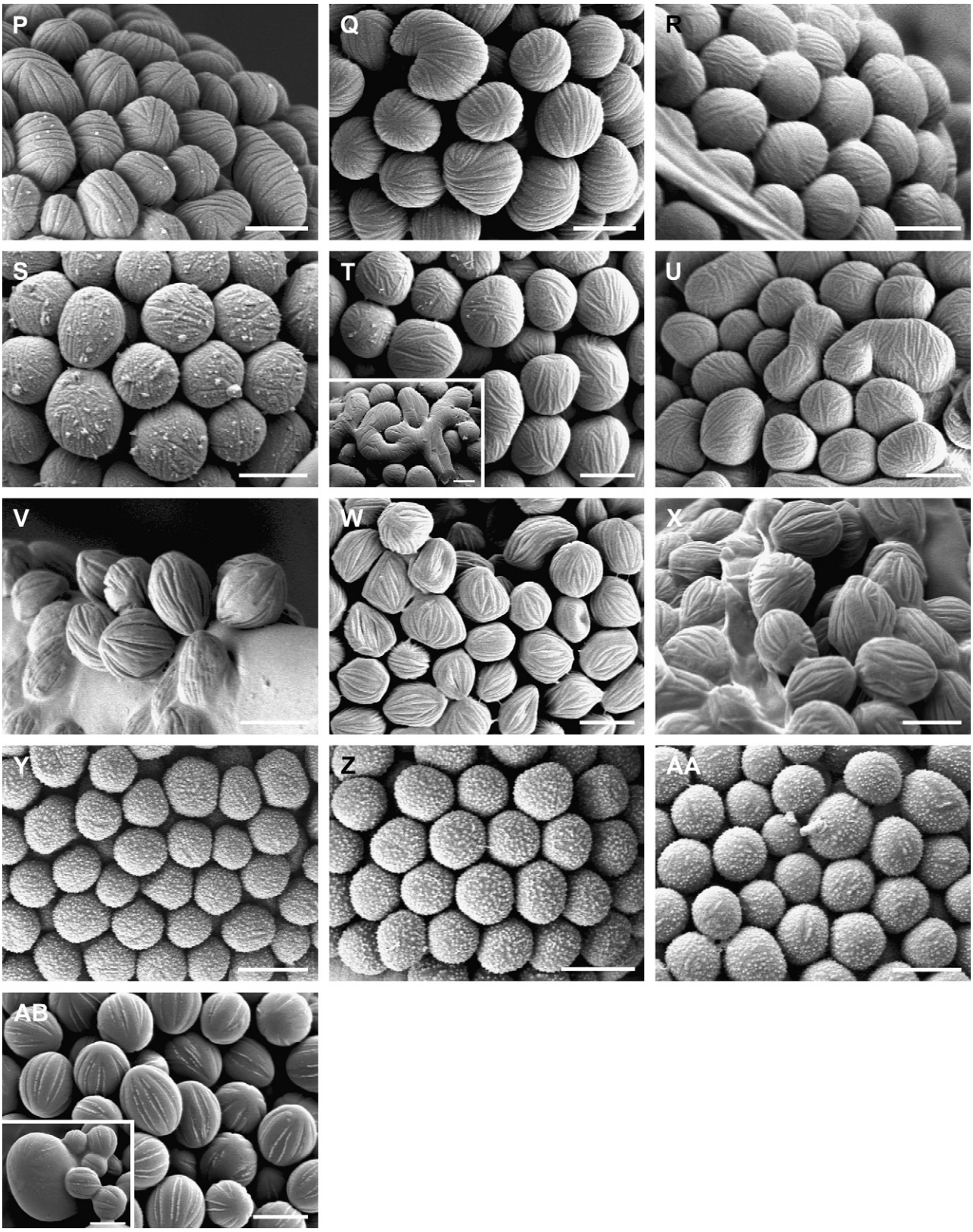


Fig 3 – (*continued*)

(V) *R. oryzae* CBS 112.07^T; (W) *R. oryzae* CBS 329.47; (X) *R. pseudochinensis* CBS 264.28^T; (Y) *R. rhizopodiformis* CBS 343.29^T; (Z) *R. rhizopodiformis* CBS 536.80; (AA) *R. rhizopodiformis* CBS 102277; (AB) *R. schipperae* CBS 138.95^T. Anomalies in sporangiospore size can be seen in (B) (small anomalous spores, indicated by arrows), and (AB) (giant spore, in lower left panel). Examples of extremely irregular sporangiospores can be seen in (L), and (T) (lower left panel). Other examples of irregular sporangiospores, here usually elongated, are shown in (A–B, J, M–N, P–Q, U, W). (A, C, Q, S, U) Four days, MEA; (B, E, H–I, L–P, R, V, Y, AA) 3 d, MEA; (D, K) 6 d, MEA; (F–G) 4 d, OMA; (J) 22 d, MEA; (T, X) 7 d, MEA; (W, Z) 12 d, MEA; (AB) 5 d, Cz. Bars = 5 μm.

2005; Kiyoto et al. 1986; Partida-Martinez & Hertweck 2005; Wilson et al. 1984).

R. oligosporus has been used in Indonesia to produce soybean tempe (tempe kedele) since ancient times, and interest in this food increases worldwide (Nout & Kiers 2005). During incubation with R. oligosporus, the soybeans are bound together by the white mycelium, forming a cake, and enzymes released by the fungus makes the protein-rich product more digestible to humans (Beuchat 1987). Legumes other than soybeans, as well as cereals and process by-products, can also be used as substrates for tempe (Berg et al. 2002; Feng et al. 2005; Nout & Rombouts 1990). Other applications of R. oligosporus include production of industrial enzymes (Casey & Walsh 2004; Ikasari & Mitchell 1994; Jin et al. 1999), and treatment of waste and wastewater (Aloysius et al. 1999; Jin et al. 1999; Vattem & Shetty 2002).

The use of R. oligosporus in food fermentations requires differentiation from related fungi that could be potentially harmful. Rhizopus sporangiospores (spores) are airborne (Ribes et al. 2000) and contamination could thus easily occur, making quality assurance important.

Spore characters (size, shape, and ornamentation patterns) may differentiate the taxa in the R. microsporus group (Schipper & Stalpers 1984). LM has commonly been used to study Rhizopus spore characters (Schipper & Samson 1994; Schipper & Stalpers 1984; Weitzman et al. 1996; Yuan & Jong 1984; Zheng & Chen 1998). However, not all ornamentation patterns on the spore surface can be resolved using this method and SEM provides an alternative, or complement, to LM (Ellis 1981; Liou et al. 1991; Schipper & Stalpers 1984).

The current study presents data on the morphology of spores of 26 strains from all taxa within the R. microsporus group, except R. microsporus var. tuberosus. In addition, two strains of R. oryzae, the species suggested to be the closest to R. microsporus group (Schipper & Stalpers 1984), was included as outgroup. The specific objective was to evaluate the use of spore characters for the taxonomy of the R. microsporus group, using data on spore characters from low-temperature (LT-) SEM and LM investigations. This study is part of a polyphasic approach to elucidate the taxonomy of R. oligosporus and other taxa within the R. microsporus group.

Materials and methods

Fungal strains and culture media

Twenty-eight strains from the Rhizopus microsporus group and R. oryzae were used in this study (Table 1). The strains have previously been identified according to their morphology, temperature requirements, and mating abilities (Schipper & Stalpers 1984). R. oligosporus strains CBS 112586-112589, ATCC 48109 and ATCC 64063 were maintained on silica beads (Pitt & Hocking 1999) at 2 °C. All other strains were stored lyophilised. Cultures were transferred, grown and maintained on 2 % malt extract agar (MEA, Oxoid, Basingstoke, UK) and stored at 4 °C until used for preparing inocula (Samson et al. 2004).

Four culture media were used: Czapek agar (Cz), dichloran 18 % glycerol agar (DG18), 2 % MEA, and oatmeal agar (OA) (Samson et al. 2004). Trace metal solutions were added to all agar media (Samson et al. 2004).

LT-SEM and characterisation of sporangiospores

For LT-SEM of sporangia and spores, fungal strains were inoculated upon small (<0.5 cm^2) excised squares of MEA, which were placed on DG18 to prevent the presence of excess water and to minimise perturbation of the fungal structures. In a number of cases, small squares were cut out directly from fungal colonies with a fresh surgical blade. Due to poor sporulation on MEA, strains of Rhizopus homothallicus and R. schipperae were instead inoculated on OA and Cz, respectively. All cultures were incubated upright in perforated plastic bags at 30 °C. After 3 d incubation, cultures were inspected under a dissecting microscope for developing sporangia. If sporangial walls had not yet ruptured, the incubation time was increased to 4 or 5 d, and in some cases up to three weeks. Agar squares were mounted in specimen holders according to Dijksterhuis et al. (2002). Thereafter, squares were snap-frozen and examined using LT-SEM (Dijksterhuis et al. 1991). Images were acquired as bitmaps using the digitiser system SemAfore version 3.02 (JEOL Skandinaviska AB, Sollentuna, Sweden).

Rhizopus strains were characterised based on spore shape and ornamentation pattern visible on LT-SEM images. Images of each Rhizopus strain were also inspected for anomalies in spore size and shape, and the percentage of anomalous, especially irregular, spores of each strain was determined.

Young sporangia with sporangial walls that covered spores in the sporangia were disregarded in the analysis, as well as spores and sporangia with obvious artefacts. Artefacts were of three main types: (1) collapsed or shrunken spores that affected spore shape and ornamentation; (2) ice formation between and on spores that masked boundaries between spores and ornamental structures; or (3) cracks in spores due to electron beam damage.

LM and sporangiospore size measurements

For LM, strains were inoculated onto MEA and incubated upright in perforated plastic bags at 30 °C for 3–5 d depending on the onset of sporulation. Mycelia and spores were prepared in Shear's mounting fluid (Samson et al. 2004) and examined with DIC microscopy (Olympus BH2-NIC). Spores were photographed at ×1250 magnification, followed by breadth and length measurements of at least 150 spores per strain using the biological manager software BioloMICS version 6.2.17 (BioAware SA, Hannut, Belgium).

Length and breadth were defined as the longest and the shortest side of the spore visible on the image, respectively. For irregular spores with an angular axis, two or more straight distances were measured to acquire the total length. Breadths of irregular spores were measured approximately at the half-length of the spore or at a position with a breadth in-between the widest and the thinnest.

Depending on the spore orientation and the visibility of the pole (see Results section for explanation of polar areas), not all spores were measured. Spores clearly seen in the equatorial view, i.e. with both poles visible, were measured. Spores in polar view, i.e. with only one visible pole that faced upwards, were not

measured as it was only possible to measure the breadth. For *Rhizopus* strains with spores having only indistinct poles, spore orientation could not be determined and, therefore, the influence of the angle of observation could not be accounted for.

The arithmetic mean, standard deviation (S.D.), and coefficient of variation (CV) of spore breadth, length, breadth to length ratio, and volume were calculated for each strain. The spore volumes (V) were approximated using the equation for prolate spheroids:

$$V = \frac{\pi}{6} * length * (breadth)^2$$

Multivariate data analysis

Rhizopus taxa were compared by analysis of data on the percentage of irregular spores and spore size measurements using the multivariate data analysis software SIMCA P + 10.0 (Umetrics AB, Umeå, Sweden). Partial least squares discriminant analysis (PLS-DA) was used to distinguish classes by creating a discriminant variable used in the *y*-space (e.g. *R. oligosporus* strains = −1, other taxa = 1) of the PLS-DA model (Sjöström *et al.* 1986; Ståhle & Wold 1987). As *x*-variables, data on spore characters based on percentage irregular spores and spore sizes were used. To allow all variables equal influence on data analysis, variables were scaled to unit variance before analysis. The numbers of principal components were determined significant by cross-validation.

Results

LT-SEM visualisation of sporangia and sporangiospores

LT-SEM preserved the delicate structure of the sporangia (Fig 1), which otherwise appears distorted using conventional SEM. LT-SEM revealed many well-preserved sporangia at different stages of development (Figs 1 and 2). The technique also revealed specific differences in ornamentation and shape of the spores of the 28 different *Rhizopus* strains (Fig 3). Some of the spores differed in size and shape by being larger and irregular, compared with the majority of spores in the same sporangium.

Sporangial size differed between strains and within a single specimen, as illustrated by three strains of *R. oligosporus* (Fig 1) and one *R. homothallicus* strain (Fig 2C). The sporangia of *R. oligosporus* in Fig 1 had diameters of 80, 120, and 40 μm, whereas the sporangium of *R. homothallicus* in Fig 2C measured 100 μm. Sporangial diameters of all the 28 *Rhizopus* strains studied ranged from 20–140 μm.

The number of spores per sporangia also differed between strains and even within a single specimen. The image of the small sporangium of *R. oligosporus* (Fig 1C) shows about 33 spores, indicating that the sporangium had approximately 100 spores. Conversely, the image of the large sporangium in Fig 2C shows over 350 spores in one image only, indicating that this sporangium carried far over 1000 spores, especially as Fig 2D shows that more layers of spores are carried by the sporangium.

Different developmental stages of sporangia were often observed within the same sample. Fig 2 summarises the different stages of sporangium development in the *R. microsporus* group and *R. oryzae*. The immature and young stages have sporangial walls visible on the sporangia (Fig 2A). This is clearly not observed during the subsequent stages. Young sporangia had developing spores starting to be visible inside the sporangial wall (Fig 2B). After spore release, the columella was exposed (Fig 2D–E). No differences, with respect to spore shape and ornamentation, were observed between mature and older sporangia.

Fig 4 shows a phenomenon occurring in only one of the strains we studied, namely *R. oligosporus* CBS 112586, where so-called 'double sporangia' were observed. In all cases, one sporangium was smaller than the other. The phenomenon was observed in five out of the 18 sporangiophores of CBS 112586 observed (*ca* 30 %).

Ornamentation patterns of Rhizopus *sporangiospores*

The spores of the different fungal taxa exhibited a wide variety of ornamentation patterns on the spore wall. To characterise surface ornamentation differences between the taxa we used a 'topological' terminology, illustrated by surface characteristics of the spores of *Rhizopus schipperae* CBS 138.95^T, *R. microsporus* CBS 699.68, *R. chinensis* CBS 631.82^T, and *R. oligosporus* CBS 338.62 (Fig 5).

'Valleys', defined as the lowest areas of the spore wall, occurred in all the spores of the studied *Rhizopus* strains (Figs 3 and 5: region V). In the case of *R. chinensis* and *R. rhizopodiformis*, the valleys were inconspicuous, but visible as indicated by the absence of granules (see below). In Fig 5, the valleys of the strains of *R. schipperae* and *R. microsporus* are especially

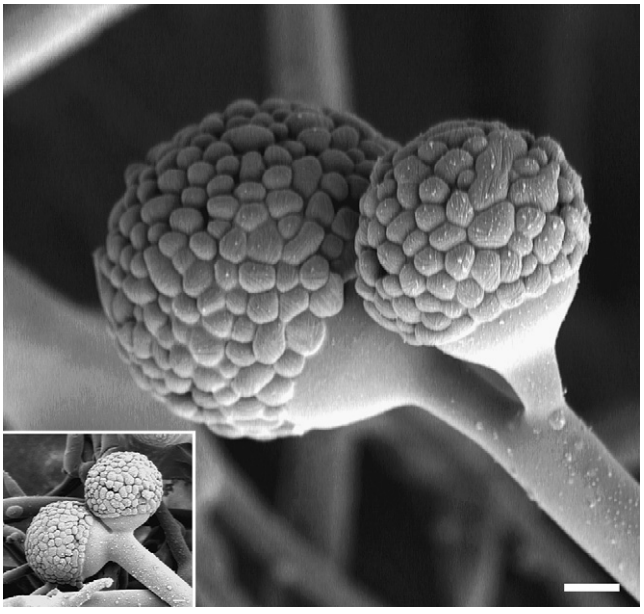


Fig 4 – Two examples of sporangiophores and sporangia of *Rhizopus oligosporus* CBS 112586 that deviates from other *Rhizopus* sporangiophores by bearing double sporangia as seen under LT-SEM. Note that some sporangiospores are elongated and irregular in shape and large compared with the globose to subglobose spores. Three days, MEA. Bars = 10 μm.

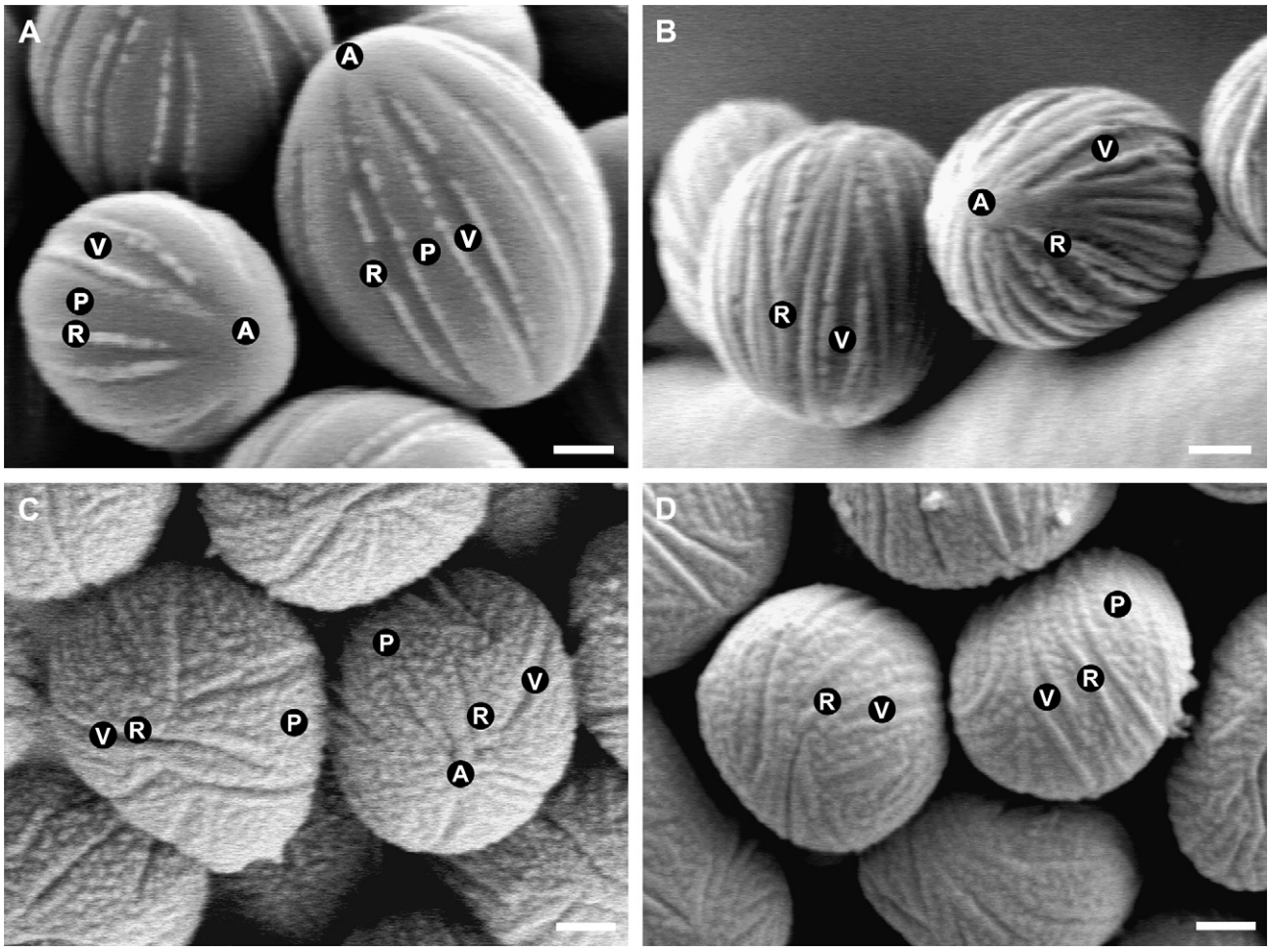


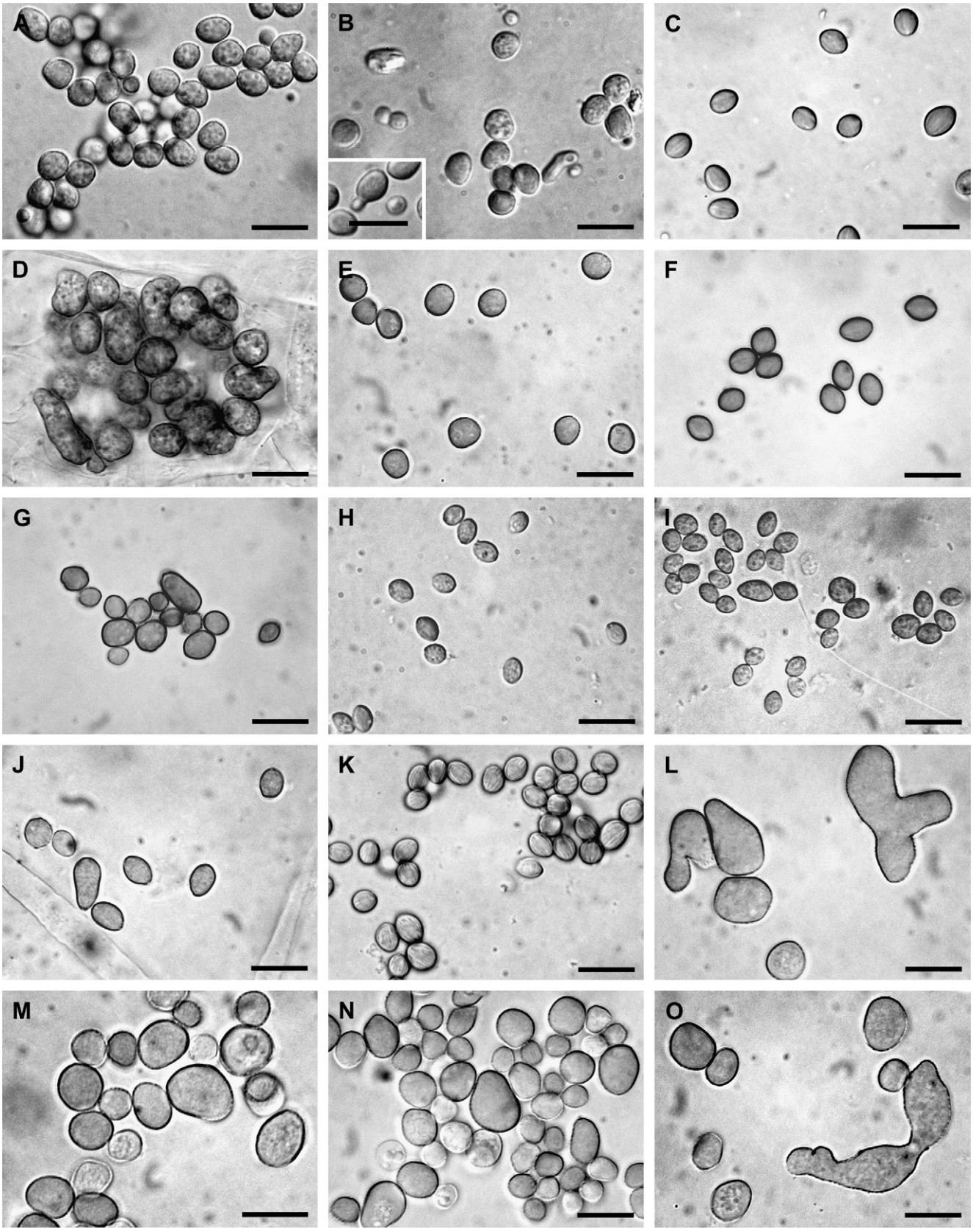
Fig 5 – 'Topological' description and definition of terms used to describe ornamentation patterns of *Rhizopus* sporangiospores, exemplified by (A) *R. schipperae* CBS 138.95^T, (B) *R. microsporus* CBS 699.68, (C) *R. chinensis* CBS 631.82^T, and (D) *R. oligosporus* CBS 338.62. In the panels, examples of the distribution of four regions are indicated: valleys (V), ridges (R), plateaus (P), and polar areas (A). Some regions are only present in some strains. (A) Five days, Cz; (B–D) 3 d, MEA. Bars = 1 μm.

notable, running in parallel. For strains of *R. chinensis* and *R. oligosporus*, the valleys were less pronounced and only some valleys ran in parallel (Fig 5C–D).

'Ridges', defined as higher areas composed of linear structures, were seen in most of the spores of the *Rhizopus* strains, often delineating the valleys (Figs 3 and 5: region R). In common with valleys, ridges either ran parallel (Fig 5A–B) or not parallel (Fig 5C–D). Ridges were separated from other ridges by a wider valley (Fig 5A), or ran together with other ridges very closely (Fig 5B). Some ridges branched, crossed each other, or appeared to fuse (Fig 5B–D). In Fig 5A, the ridge is of an intermittent type, not continuous, but rather a linear array of spore wall structures. However, most ridges on spores of *Rhizopus* strains were continuous.

'Plateaus' were defined as higher areas on the spore wall that exhibited non-linear structures (Figs 3 and 5: region P). Plateaus

Fig 6 – Sporangiospores of investigated *Rhizopus* strains as seen with LM DIC. (A) *R. azygosporus* CBS 357.92; (B) *R. azygosporus* CBS 357.93^T; (C) *R. caespitosus* CBS 427.87^T; (D) *R. chinensis* CBS 261.28; (E) *R. chinensis* CBS 631.82^T; (F) *R. homothallicus* CBS 336.62^T; (G) *R. homothallicus* CBS 111232; (H) *R. microsporus* CBS 699.68; (I) *R. microsporus* CBS 700.68; (J) *R. microsporus* CBS 308.87; (K) *R. microsporus* CBS 112285; (L) *R. oligosporus* CBS 337.62; (M) *R. oligosporus* CBS 338.62; (N) *R. oligosporus* CBS 339.62; (O) *R. oligosporus* 228.95; (P) *R. oligosporus* CBS 112586; (Q) *R. oligosporus* CBS 112587; (R) *R. oligosporus* CBS 112588; (S) *R. oligosporus* CBS 112589; (T) *R. oligosporus* ATCC 48109; (U) *R. oligosporus* ATCC 64063; (V) *R. oryzae* CBS 112.07^T; (W) *R. oryzae* CBS 329.47; (X) *R. pseudochinensis* CBS 264.28^T; (Y) *R. rhizopodiformis* CBS 343.29^T; (Z) *R. rhizopodiformis* CBS 536.80; (AA) *R. rhizopodiformis* CBS 102277; (AB) *R. schipperae* CBS 138.95^T. (A–C, I, K, M–N, Q–S, V–W, AA) Four days, MEA; (D–E, H) 5 d, MEA; (F–G, J, L, O–P, T, U, X–Z, AB) 3 d, MEA. Bars = 10 μm.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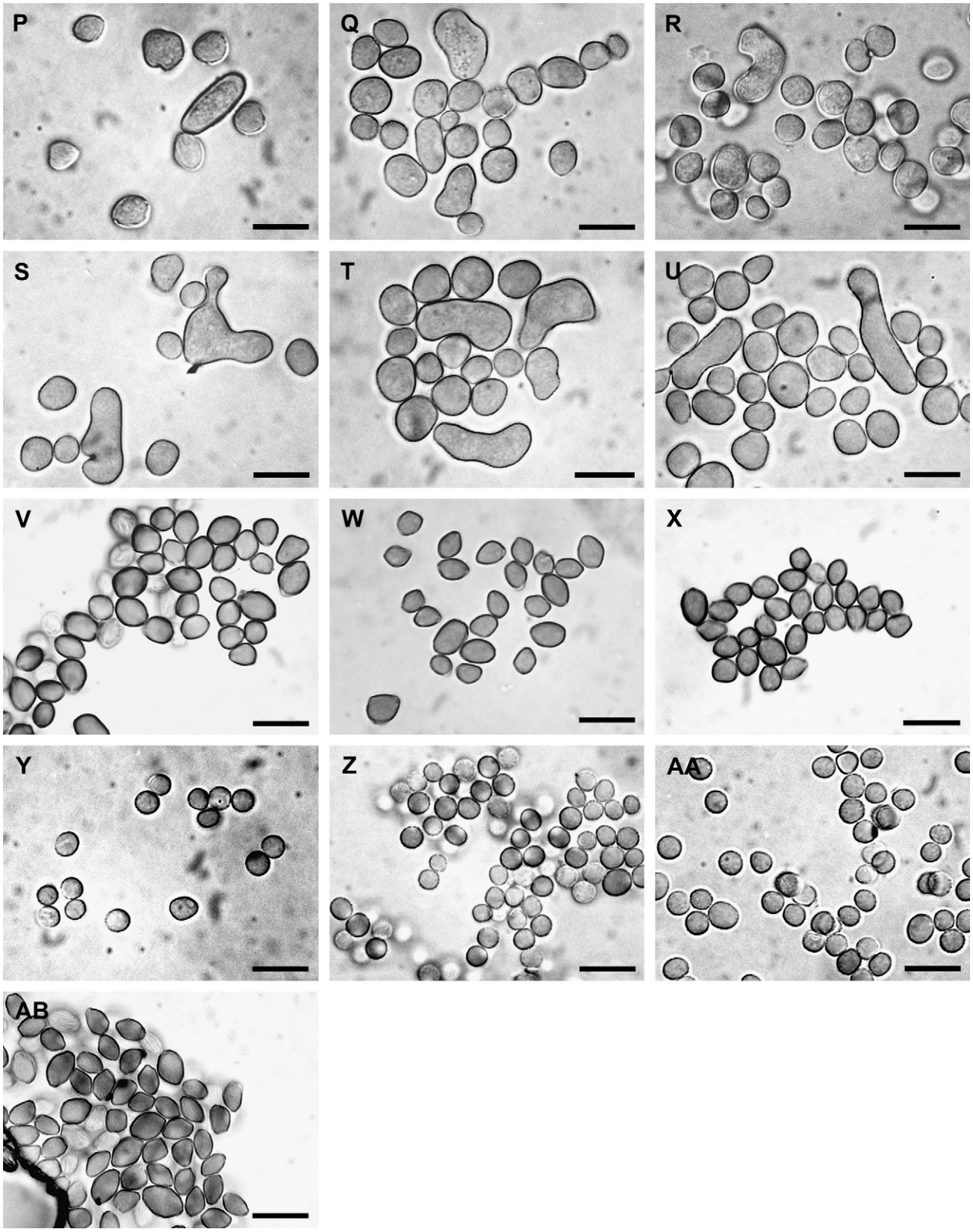

Fig 6 – (*continued*)

could appear smooth (Fig 5A) or granular. Small granules at a low number per spore profile were noticeable on *R. chinensis* (Fig 5C), and less obvious on the spores of *R. oligosporus* (Fig 5D). Larger granules in a higher number per spore profile were seen in *R. rhizopodiformis* (Fig 3Y–AA).

Polar areas (poles, Figs 3 and 5: region A) were a type of plateau, generally seen at the ends of the spores where ridges and/or plateaus from different sides of the spore fused. As for the plateaus, the polar areas could appear smooth or granular.

Sporangiospore shape can be regular or irregular

The distribution of spore ornamentation patterns was related to the general shape of the spores. The shape of the spores was either regular or irregular. Regular spores generally were ellipsoidal to broadly ellipsoidal, or subglobose to globose (Kirk *et al.* 2001), whereas irregular spores were anomalous in size and shape compared with the regular spores.

Ellipsoidal to broadly ellipsoidal spores were prolate spheroids, with parallel valleys and ridges running from pole to pole. Close to the poles, ridges fused, usually resulting in smooth and pronounced polar areas. The ratio between breadth and length as indicated by DIC LM (see below, Fig 6), varied from 0.73–0.83 in these spores. Ellipsoidal to broadly ellipsoidal spores were particularly prominent in *Rhizopus caespitosus* CBS 427.87^{T}, *R. homothallicus* CBS 336.62^{T}, *R. schipperae* CBS 138.95^{T}, *R. microsporus* strains, and *R. chinensis* CBS 261.28 (Figs 3, 5 and 6). The first three strains were characterised by regular, broad valleys that ran from pole to pole. Ridges could be intermittent (*R. schipperae* CBS 138.95^{T}, and *R. homothallicus* CBS 336.62^{T}), or continuous. However, the ridges of *R. caespitosus* were indistinct. Plateaus were either granular (*R. homothallicus* CBS 336.62^{T}, and *R. chinensis* CBS 261.28) or smooth. The *R. microsporus* strains were characterized by valleys that did not run from pole to pole, but along part of the length of the spore. Ridges of the spores were distinct and could fuse, branch or cross.

Subglobose to globose spores had polar areas that seldom were pronounced, and valleys and ridges that usually did not run in parallel. The distribution of ridges over the spore wall was thus less ordered, compared with (broadly) ellipsoidal spores. The ratio between breadth and length in these spores ranged between 0.84 and 0.92. Subglobose to globose spores were seen especially in *R. rhizopodiformis* strains (ratio >0.88), *R. azygosporus* strains, some *R. oligosporus* strains, *R. homothallicus* CBS 111232, and *R. chinensis* CBS 631.82^{T} (Figs 3, 5 and 6). The plateaus of the subglobose to globose spores were granular and sometimes smooth. *R. rhizopodiformis* strains had a high density of granules on the plateaus, whereas the other (sub)globose strains had fewer and smaller (or no) granules. *R. rhizopodiformis* lacked ridges. *R. rhizopodiformis* and *R. chinensis* CBS 631.82^{T} both had inconspicuous valleys running between or underneath the granules.

Irregular spores were elongated, angular, furcated, boot-shaped, kidney-shaped, or peanut-shaped, and were usually larger than regular spores (clearly seen in Fig 3L, N, T). In many cases, these spores looked as if two or more spores had not been totally separated from each other. Irregular spores were by far the most frequent in the *R. oligosporus* strains. Some irregular spores were found also in *R. azygosporus*, *R. microsporus*, *R. rhizopodiformis*, and *R. chinensis* CBS 631.82^{T} (Figs 3 and 6). In common with the regular (sub)globose spores, irregular spores normally had indistinct polar areas. In addition, valleys and ridges did not run in parallel, at least not along the whole spore breadth or length. Many ridges appeared branched or fused, and sometimes, two ridges ran along each other without any visible valleys.

R. oryzae and *R. pseudochinensis* differed from other taxa in both shape and ornamentation pattern. They had spores of irregular shape compared with the (sub)globose and (broadly) ellipsoidal spores of the other taxa. The irregularity was different from that observed in, for instance, *R. oligosporus* spores. Figs 3V–X and 6V–X clearly illustrate the nature of the shape of these spores. The valleys themselves were characterised by a variable breadth within the same valley and that they followed the irregular boundaries of the spores. This resulted sometimes in the demarcation of triangular and squared areas. Between the valleys, plateaus fused and formed pole-like areas that were irregular in size and number.

In three taxa, some regular spores with anomalous size were seen. 'Dwarf' spores were observed in *R. azygosporus* strains (Fig 3B, Table 2), whereas enlarged globose spores were found in *R. schipperae* CBS 138.95^{T} (Fig 3AB), and *R. chinensis* CBS 261.28 (Table 2).

Large and irregular sporangiospores of Rhizopus oligosporus *strains*

To compare the distribution of spores of irregular shape between strains, LT-SEM images of up to 425 spores from up to six sporangia per *Rhizopus* strain were analysed for the proportion of irregular spores (Table 2). Only *R. homothallicus* CBS 336.62^{T} sporulated too sparsely to be fully analysed. Most *Rhizopus* taxa had less than 5 % irregular spores, whereas *R. oligosporus* strains had between 10 and 31 % irregular spores (Table 2).

DIC LM revealed specific differences in size and shape of the spores of the 28 different *Rhizopus* strains (Fig 6). Data on spore sizes (mean, S.D., and CV of breadth, length, breadth to length ratio, and volume), based on breadth and length measurements of up to 201 spores per strain, were used to compare the spore size distribution between strains (data partly shown in Table 2). The spore shapes observed with DIC LM and with LT-SEM were in good agreement for all taxa.

R. oligosporus strains differed from other strains by having the largest (up to 43 μm) and most irregular spores with the most variable sizes (Fig 6L–U). This was, for instance, reflected as high values in the spore volume (96–223 μm^3 $spore^{-1}$), and in the S.D. values of the ratios, lengths, and volumes. Conversely *R. rhizopodiformis* strains had spores that were generally the smallest and most globose with the smallest size variation (Fig 6Y–AA). This was, for instance, reflected as low values in the spore volume (26–35 μm^3 $spore^{-1}$), and in the S.D. values of breadth, length, and volume. The other taxa fell in between *R. oligosporus* and *R. rhizopodiformis* with regard to spore size data. The very small spore type of *R. azygosporus*, observed using LT-SEM, was also seen using DIC LM (Fig 6A–B). These spores were much smaller than the average size of the spores of this species (Table 2). Some of the irregular *R. azygosporus* spores looked as if they were 'budding' (Fig 6B, compare with Fig 3A).

Table 2 – Distribution of irregular spores counted from low-temperature (LT)-SEM images, and mean and standard deviations of breadth and length from LM DIC images of sporangiospores of *Rhizopus* strains

Rhizopus strain	LT-SEM images			LM images		
	Sporangia counted	Spores counted	Percent irregular spores	Spores counted	Mean breadth in μm (S.D.)	Mean length in μm (S.D.)
R. azygosporus CBS 357.92	5	425	2.1[a]	180	4.1 (0.78)[a]	4.9 (1)[a]
R. azygosporus CBS 357.93^T	3	133	5.3[b]	170	4.3 (0.85)[b]	5.2 (1)[b]
R. caespitosus CBS 427.87^T	≥1	100	0	160	4.4 (0.55)	5.3 (0.76)
R. chinensis CBS 261.28	2	121	0[c]	151	5 (1)	6.1 (1.6)
R. chinensis CBS 631.82^T	6	385	2.9	161	4.5 (0.3)	5.1 (0.39)
R. homothallicus CBS 336.62^T	NA[d]	NA	NA	156	4.2 (0.35)	5.3 (0.54)
R. homothallicus CBS 111232	2	231	1.7	157	4.4 (0.75)	5.2 (1.2)
R. microsporus CBS 699.68	4	316	6.3	173	4 (0.56)	5 (0.7)
R. microsporus CBS 700.68	2	21	9.5	154	3.6 (0.41)	4.6 (0.66)
R. microsporus CBS 308.87	4	473	4.4	152	4.1 (0.63)	5.3 (0.91)
R. microsporus CBS 112285	≥2	312	0.6	162	3.9 (0.35)	5.0 (0.45)
R. oligosporus CBS 337.62	2	79	24.1	164	6.3 (1.3)	8.9 (4)
R. oligosporus CBS 338.62	4	288	20.5	153	5.6 (0.87)	7.0 (2.3)
R. oligosporus CBS 339.62	6	153	30.7	151	5.5 (1)	6.7 (1.7)
R. oligosporus CBS 228.95	5	231	10	151	6.5 (1.4)	8.5 (4.4)
R. oligosporus CBS 112586	6	221	18.1	159	5.3 (0.66)	7.7 (3)
R. oligosporus CBS 112587	4	97	23.7	161	5.6 (0.94)	8 (3.8)
R. oligosporus CBS 112588	6	410	14.6	172	5.2 (0.65)	6.6 (1.7)
R. oligosporus CBS 112589	6	173	23.1	159	5.4 (1.1)	7.4 (3.1)
R. oligosporus ATCC 48109	4	177	26	201	6.1 (1.2)	8.3 (4.1)
R. oligosporus ATCC 64063	1	51	15.7	160	6.1 (1.1)	7.7 (2.6)
R. oryzae CBS 112.07^T	3	234	2.6	186	4.3 (0.51)	5.3 (0.67)
R. oryzae CBS 329.47	4	292	3.4	187	3.9 (0.6)	5.3 (0.79)
R. pseudochinensis CBS 264.28^T	3	253	4	152	4.0 (0.62)	5.3 (0.81)
R. rhizopodiformis CBS 343.29^T	3	185	5.9	195	3.7 (0.3)	4.1 (0.31)
R. rhizopodiformis CBS 536.80	4	134	1.5	150	3.6 (0.33)	3.9 (0.33)
R. rhizopodiformis CBS 102277	1	42	0	161	3.9 (0.29)	4.4 (0.5)
R. schipperae CBS 138.95^T	3	304	0[e]	163	4.2 (0.52)	5.7 (0.8)

a In addition, 0.7 % small regular ('dwarf') spores were observed with LT-SEM. With LM, 12 % of the measured spores were small, having a mean breadth and S.D. of 2.2 μm (0.34), and a mean length and S.D. of 2.5 μm (0.38), whereas 88 % had a mean breadth and S.D. of 4.3 μm (0.41), and a mean length and S.D. of 5.2 (0.49) μm.
b In addition, 6 % small regular ('dwarf') spores were observed with LT-SEM. With LM, 12 % of the measured spores were small, having a mean breadth and S.D. of 2.5 μm (0.26), and a mean length and S.D. of 2.8 μm (0.3), whereas 88 % had a mean breadth and S.D. of 4.6 μm (0.56), and mean length and S.D. of 5.6 μm (0.58).
c Instead of irregular spores, 5 % enlarged regular spores were observed.
d NA, not available.
e Instead of irregular spores, 1.3 % enlarged ('giant') spores were observed.

Multivariate differentiation of R. oligosporus *sporangiospores from those of other* Rhizopus *taxa*

The multivariate data analytical method PLS-DA was used to investigate whether data on the percentage of irregular spores and spore size (Table 2) could distinguish *Rhizopus oligosporus* from other *Rhizopus* taxa. The PLS-DA calculations yielded a significant two-principal-component model, which explained a large percentage of the variation of both the y-matrix ($R^2y_{cum} = 0.93$, $Q^2_{cum} = 0.90$) and the x-matrix ($R^2x_{cum} = 0.84$).

The scores scatter plot for the PLS-DA model, where t_1 is plotted against t_2 (Fig 7A), shows that *R. oligosporus* strains were differentiated from all other investigated *Rhizopus* strains. *R. oligosporus* CBS 228.95 constituted a potential outlier within the cluster of *R. oligosporus* strains. To evaluate the robustness of the model (Fig 7A), data for this strain was excluded from the analysis, but this did not markedly affect the model (data not shown).

Fig 7B shows the regression coefficients plot of the PLS-DA model, in which the x-variables that best described *R. oligosporus* and other taxa, respectively, can be interpreted. All variables, except the CV of breadth and volume, were more important for *R. oligosporus* compared with other taxa. The variable percentage of irregular spores was found to be of especially high importance in *R. oligosporus*. All variables, except the S.D. of breadth and volume, and mean ratio, were statistically significant ($P < 0.05$).

Discussion

The results of this study have shown that: (1) intact sporangia of *Rhizopus* species could be observed with LT-SEM; (2) all *Rhizopus* taxa had distinct spore ornamentation, sometimes previously incorrectly characterised as smooth; (3) robust spore ornamentation patterns were linked to the different taxa of

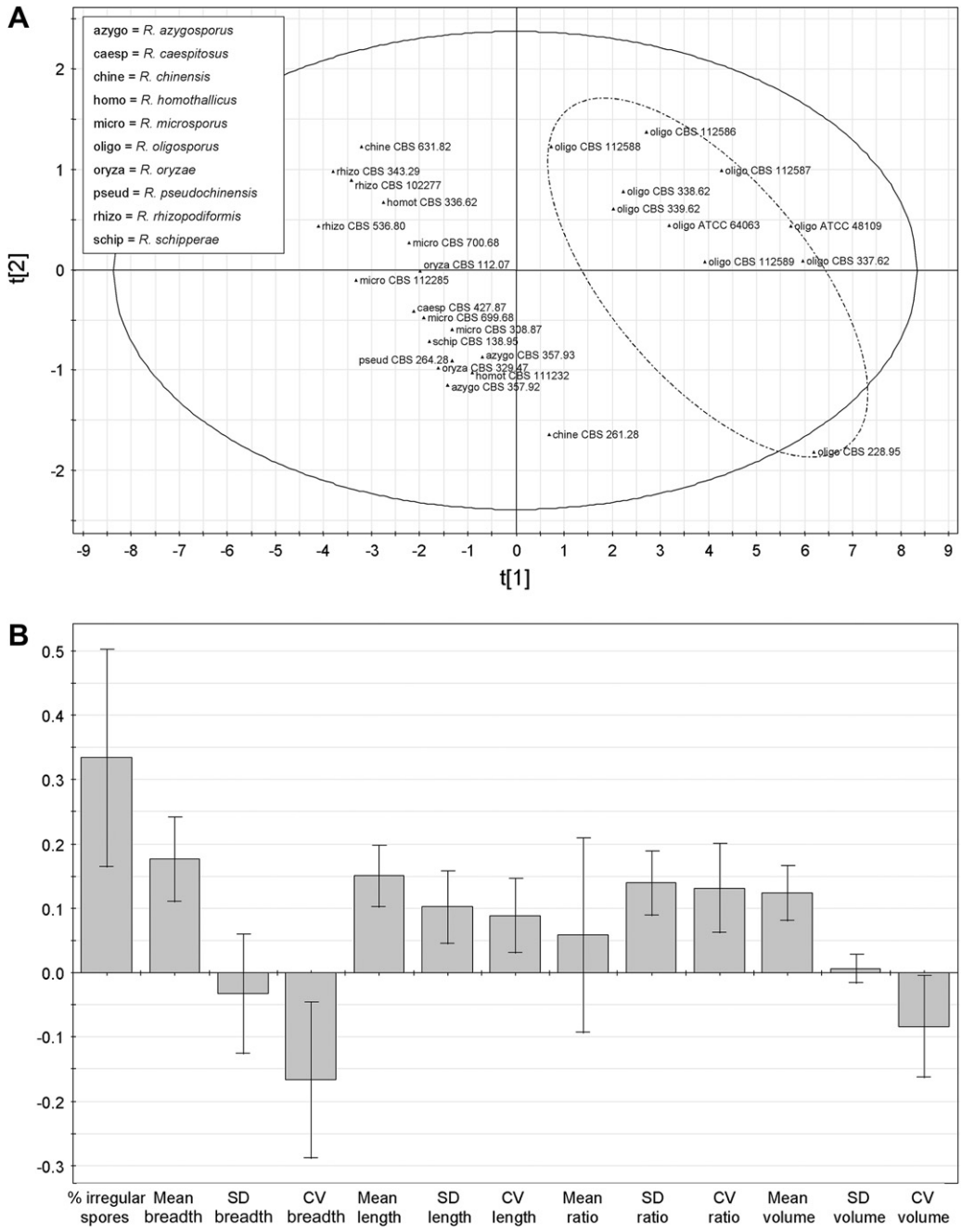

Fig 7 – PLS discriminant analysis of sporangiospore characters (percentage irregular spores, and spore size) where *Rhizopus oligosporus* strains are compared with strains of other *Rhizopus* taxa. (A) Scores scatter plot (t_1/t_2), showing separation of *R. oligosporus* strains from other *Rhizopus* strains. Each plot mark corresponds to a strain. A dashed line encloses plot marks corresponding to *R. oligosporus*. The solid line indicates a 95 % confidence ellipse based on Hotelling's T^2-test. (b) Regression coefficients plot of the mean of each of the spore variables. Bars indicate standard deviations. Columns above the line indicate that *R. oligosporus* strains generally have higher means of the variables than other *Rhizopus* taxa. The percentage irregular spores were found to be of higher importance than other variables. (b) CV, coefficient of variation; ratio, breadth to length ratio.

the *R. microsporus* group; (4) compared with other *Rhizopus* taxa, *R. oligosporus* had a defect in the spore formation process, which may be related to the domesticated nature of this taxon; (5) based on spore morphology (shape, size, and ornamentation), three subgroups within the *R. microsporus* group were identified.

Preparation of sporangiospores and sporangia for LT-SEM

Rapid freezing for LT-SEM enabled successful preparation not only of spores, but also of intact sporangiophores and sporangia. Specific differences in spore morphology were found between the ten *Rhizopus* taxa studied, including *R. caespitosus*

and *R. pseudochinensis*, which never have been reported to be investigated with SEM.

Earlier SEM studies of *Rhizopus* spores have mainly used chemical fixation and dehydration, followed by critical point drying in carbon dioxide (Ellis 1981; Liou *et al.* 1991; Lusta *et al.* 2003; Samson *et al.* 1979; Schipper *et al.* 1985; Schipper *et al.* 1996; Schipper & Stalpers 1984; Schwertz *et al.* 1997). In some studies, the spores were instead air-dried without fixation (Ellis *et al.* 1970). Compared with LT-SEM, these techniques make it more difficult to acquire images of intact sporangiophores. Common effects of chemical fixation, critical point drying and air-drying are collapsed or shrunken spores and sporangia, as well as difficulties to prepare intact sporangia due to that spores are washed away from the structure.

LM studies of *Rhizopus* spores are common in the literature, which has lead to frequent reports about taxa having smooth or faintly striated spores (Hesseltine & Ellis 1961; Schipper & Samson 1994; Scholer *et al.* 1983; Yuan & Jong 1984). The high resolution of LT-SEM revealed that all *Rhizopus* taxa had ridges, valleys, or some other kind of ornamentation patterns. Upon germination of fungal spores, ornamentation may disappear. This has been shown for *R. oryzae* (*R. arrhizus*), where material in the ridges was stretched more than the wall material between the ridges, leading to almost completely lost ridges upon germination (Hess & Weber 1973).

Are irregular sporangiospores and double sporangia an effect of strain domestication?

With both LM and SEM, we observed a high degree of irregular spores in *Rhizopus oligosporus* compared with the other taxa of the *R. microsporus* group and to *R. oryzae*. In addition, the spores of *R. oligosporus* were larger than spores of other taxa.

Saito was the first to observe that the spores of *R. oligosporus* were 'sometimes growing together' (Saito 1905). Several authors, among them Hesseltine (1965) and Schipper & Stalpers (1984), have made similar observations; mainly as notes in species descriptions that *R. oligosporus* has irregular spores. Observations that irregular spores also occur among other *Rhizopus* taxa are rare (Zheng & Chen 1998), and as far as we know, quantification of the percentage of irregular spores among *Rhizopus* taxa has not been made before.

R. oligosporus has rarely been isolated from sources other than tempe, and, through repeated sub-culturing of the fungus since ancient times, it has been domesticated (Pitt & Hocking 1999; Samson 1985). It is tantalising to speculate that as *R. oligosporus* has been cultivated by humans, it shows spore defects that otherwise would be potentially disadvantageous in natural selection, but that can be harboured without any obvious negative consequences for the fungus.

Other features, aside from irregular spores, may be a result of strain domestication. The double sporangia seen in *R. oligosporus* CBS 112586 (NRRL 5905) could be another degeneration effect. Together with CBS 338.62 (NRRL 2710), this strain is one of the most well-documented *Rhizopus* strains and it is commonly used in various solid-state fermentations (Nout & Rombouts 1990).

Morphological defects of domestication of sporogenous structures of other fungi have earlier been observed in, for instance, the white cheese fungus *Penicillium camemberti*, which compared to its 'wild' species, *P. commune*, has irregular conidiophore structure and larger conidia (Samson & Frisvad 2004).

Are irregular sporangiospores the result of a defective sporangiospore formation process?

The *Rhizopus* sporangiospore formation, sporangiosporogenesis, consists of ten stages, including pre-cleavage, cleavage, and post-cleavage (Bracker 1968). Spore formation starts when the cytoplasm cleaves and forms spores. At sporangium maturity, the sporangial wall ruptures, followed by liberation and dispersal of spores (Bracker 1968; Cole & Samson 1983; Ho & Chen 1994). Sporangiosporogenesis resembles the process of zoospore formation (zoosporogenesis) in chytridiomycetes (Fisher *et al.* 2000), and in oomycetes (Hardham & Hyde 1997). Irregular spores, found in particular in *R. oligosporus*, suggest a defect in spore formation related to multinucleation and to the cytoskeleton. Regular spores of representatives from the *R. stolonifer* group (Schipper & Stalpers 1984) usually contain several nuclei per spore (Hawker & Abbott 1963). This may be true also for the regular spores from the *R. microsporus* group and from *R. oryzae*. When it comes to irregular *Rhizopus* spores, the larger and more irregular the spore, the more nuclei the spores probably have, as has been shown for multinucleated spores of *Mucor circinelloides* f. *circinelloides* (*Mucoraceae*) (Hammill *et al.* 1983), and for spores of the domesticated fungus *Aspergillus oryzae* (Hara *et al.* 2002; Ishi *et al.* 2005). The large and irregular multinucleated spores may be caused by the formation of a disrupted actin microfilament cytoskeleton, which initially would lead to blocked nuclear migration, disrupted membrane alignment and cytoplasmic delimitation (Lowry *et al.* 2004).

Delineation of species and varieties in the genus Rhizopus

The delineation of species and varieties in the genus *Rhizopus* stems from the latest revision of the genus in 1984, based on spore characters, mating abilities, and temperature requirements (Schipper & Stalpers). Several of the species and varieties included in our study were described after this revision and were claimed to be members of the *R. microsporus* group (Prakash & Sarbhoy 1993; Schipper & Samson 1994; Weitzman *et al.* 1996; Yuan & Jong 1984). Our results indicate that the placement of some *Rhizopus* taxa can be questioned if only spore characteristics are taken into account. The observations of spore shape, size, and ornamentation patterns of the 28 strains of the *R. microsporus* group and *R. oryzae*, and the suggested relatedness of the taxa are summarised in Fig 8. Based on spore morphology, three subgroups were discerned within the *R. microsporus* group: (1) *R. oligosporus, R. azygosporus, R. chinensis*, and *R. rhizopodiformis*; (2) *R. caespitosus, R. homothallicus*, and *R. schipperae*; and (3) *R. microsporus* (as discussed below).

R. oligosporus is often regarded as a variety of *R. microsporus* (Schipper & Stalpers 1984). *R. microsporus* had ellipsoidal to broadly ellipsoidal spores of intermediate size, distinct polar areas, and distinct ridges and valleys that ran along part of the spore length. Conversely, *R. oligosporus* had large, subglobose to globose spores, and high proportion irregular spores

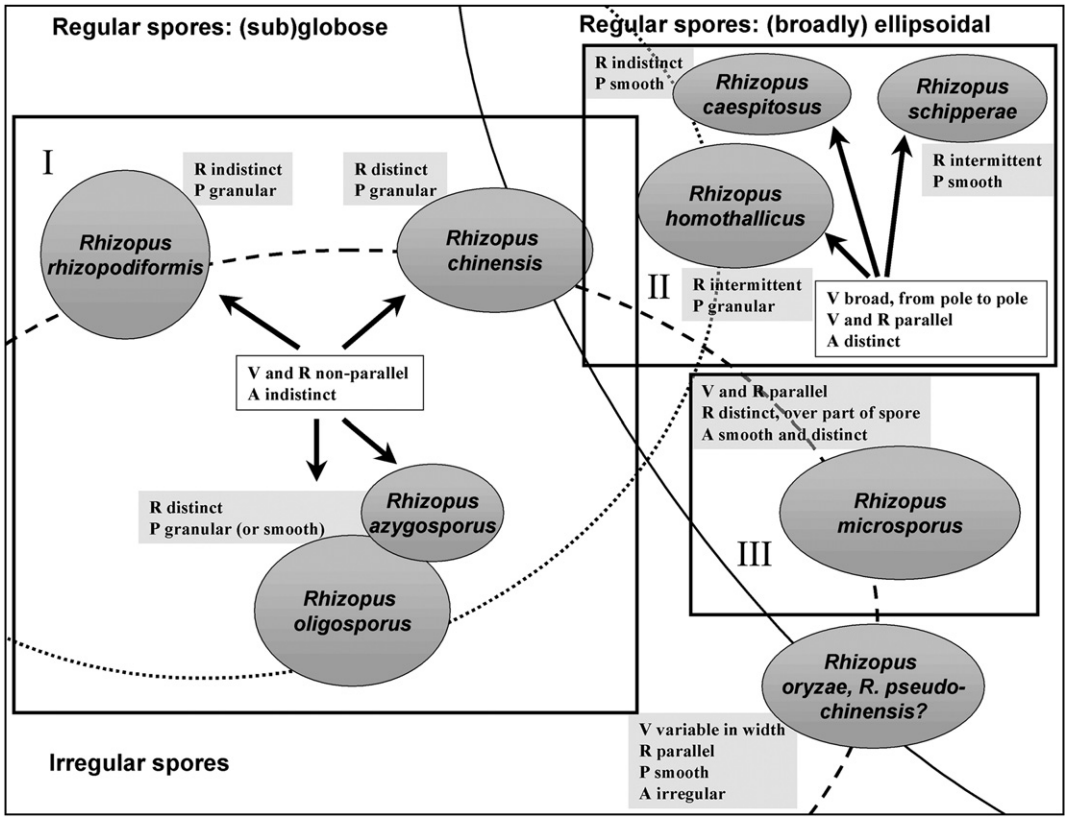

Fig 8 – Overview of relatedness of taxa of the *Rhizopus microsporus* group and *R. oryzae*, based on sporangiospore shape, size, and ornamentation patterns. Within the *R. microsporus* group, three subgroups are indicated (I, II, and III). The dotted ellipse at the left side of the figure encloses strains with regular spores of subglobose to globose shape, and the dashed ellipse encloses strains with irregular spores. To the right of the unbroken curve, strains with regular spores of ellipsoidal to broadly ellipsoidal shape are presented. Each grey circle represents one taxon, and corresponds roughly with the shape of the spores. *R. oryzae* and *R. pseudochinensis* spores were identical, and these taxa are thus placed within the same circle. Note that some areas overlap, as do some of the taxa (*R. oligosporus* and *R. azygosporus*). *R. microsporus* CBS 700.68 was omitted from the figure because the number of observed spores with LT-SEM was too low. V, valleys; R, ridges; P, plateaus; A, polar areas.

(>10 %). *R. oligosporus* also differed by having spores with non-parallel valleys and ridges, and plateaus that sometimes were granular. The distinct spore morphology of *R. oligosporus* shows that it can be clearly differentiated from both *R. microsporus*, as well as from other varieties in the *R. microsporus* group. *R. oligosporus* is frequently used by humans (Nout & Kiers 2005), is not found in nature (Pitt & Hocking 1999), and is not associated with production of any potentially harmful metabolites (Jennessen *et al.* 2005). Conversely, *R. microsporus* is associated with both human infection (Kerr *et al.* 1988) and with production of the metabolites rhizonins and rhizoxins (Jennessen *et al.* 2005; Partida-Martinez & Hertweck 2005); therefore, it is especially important to differentiate *R. oligosporus* from *R. microsporus*.

With respect to the spore morphology, *R. oligosporus* was most similar to the species *R. azygosporus*. The two taxa differed only in that *R. azygosporus* had a lower proportion irregular spores than *R. oligosporus*, and that *R. azygosporus* had a proportion of very small regular spores. The globose, normal-sized spores of these two taxa could, however, not be differentiated. This suggests that *R. oligosporus* could be considered a domesticated *R. azygosporus* with defects in sporangiospore formation. Although *R. azygosporus*, just as *R. oligosporus*, can be used in soybean tempe production (Yuan & Jong 1984), *R. azygosporus* can also be associated with human infections (Schipper *et al.* 1996). This makes a clear understanding of the relations between these two taxa important.

Spores of *R. oligosporus* were similar to *R. chinensis*, which is often regarded as a *R. microsporus* variety (Schipper & Stalpers 1984). Just as *R. oligosporus*, *R. chinensis* is not associated with potentially harmful metabolites (Jennessen *et al.* 2005; Partida-Martinez & Hertweck 2005), thus suggesting that *R. oligosporus* is more closely related to *R. chinensis* than to *R. microsporus* (Jennessen *et al.* 2005).

R. rhizopodiformis, often regarded as a variety of *R. microsporus* (Schipper & Stalpers 1984), had small subglobose to globose spores with granular plateaus. These characteristics made it possible to differentiate it from all other taxa investigated, and therefore, it may be regarded as a species. *R. rhizopodiformis* was most similar to *R. chinensis*, but *R. chinensis* had larger spores with a higher subpopulation of irregular spores, and less granular plateaus.

R. caespitosus, *R. homothallicus*, and *R. schipperae* had ellipsoidal to broadly ellipsoidal and sometimes subglobose spores

(*R. homothallicus* CBS 111232), with pronounced polar areas and broad parallel valleys running in between the poles. These characteristics distinguished them from other taxa.

With respect to spore morphology, the *R. oryzae* strains were identical to *R. pseudochinensis* CBS 264.28^T, which often is regarded as a variety of *R. microsporus* (Prakash & Sarbhoy 1993). Based on the unique irregularity of these spores in combination with a unique pattern of valleys, *R. pseudochinensis* may instead be a misidentified *R. oryzae*.

The genus *Rhizopus* is used in food fermentations and other industrial applications, but some taxa can also be human pathogens. Further studies of the genus *Rhizopus*, based on spore morphology, as well as physiology (e.g. carbon source utilization) and DNA sequence analysis, are required to fully resolve the relatedness and taxonomy of this genus.

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