Taxonomy and phylogeny of the ascomycetous yeast genus *Zygoascus*, with proposal of *Zygoascus meyeriae* sp. nov. and related anamorphic varieties

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Physiological characters, mating compatibility, PCR-RAPD fingerprints, mol% G + C content, DNA–DNA relatedness, and large-subunit and internal transcribed spacer rRNA gene sequences of strains assigned to the genus *Zygoascus* were re-examined. On the basis of those data, and after phylogenetic analyses, an emendation of *Zygoascus hellenicus* (type material is a cross of CBS 6736^T × CBS 5839^T) is proposed, comprising two novel anamorphic varieties, *Candida steatolytica* var. *steatolytica* (CBS 6736^T) and *C. steatolytica* var. *inositophila* (CBS 5839^T). A novel teleomorphic species, *Zygoascus meyeriae* sp. nov. (type material is a cross of CBS 4099^T × CBS 7521^T) is described, together with two novel anamorphic varieties corresponding to it, *Candida hellenica* var. *hellenica* (CBS 4099^T) and *C. hellenica* var. *acidophila* (CBS 7115^T).

INTRODUCTION

The monotypic genus *Zygoascus* was introduced in 1986 by Smith to accommodate the teleomorph state of three *Candida* species: *Candida hellenica* (Verona & Picci) D. S. King & S.-C. Jong, *Candida inositophila* Nakase and *Candida steatolytica* Yarrow. These species were all found to represent mating types of a single species, with *C. hellenica* and *C. inositophila* representing one mating type, designated **a**, and *C. steatolytica* representing the opposite type, designated **α**. *C. inositophila* and *C. steatolytica* were placed into synonymy with *C. hellenica*, since that name has priority, and the nomenclaturally independent teleomorph name *Zygoascus hellenicus* was modelled after the accepted anamorph name.

In earlier studies, Meyer *et al.* (1984) reported that, of four strains previously identified as being representatives of *C. steatolytica* [Centraalbureau voor Schimmelcultures (CBS catalogues; CBS, 1972)], only one showed a DNA–DNA relatedness of 98 % with the type strain. Therefore, the remaining strains were excluded from this species and remained unclassified. Furthermore, Meyer *et al.* (1984) kept *C. steatolytica* and *C. inositophila* as separate taxa, because the DNA–DNA reassociation value between the

type strains of these species was around 57 %, as determined by the DNA filter reassociation technique (S. A. Meyer, personal communication), a value considered by Meyer to be low enough to keep the species apart. However, the observation of mating among the three unclassified isolates and the type strains of the three aforementioned species, resulting in asci with ascospores, was considered sufficient to conclude that they all belonged to the species *Z. hellenicus* (Smith, 1986). Accepting the presence of mating as an indicator of conspecificity, Meyer *et al.* (1998) excluded these three species from the list of purely anamorphic *Candida* species in a later revision of this genus.

As these species concepts based on sexual compatibility and DNA–DNA relatedness values were discrepant and, as we had obtained some new and difficult-to-place *Z. hellenicus*-like isolates, we undertook a revision of this group. In this study, physiological characters and mating behaviour were studied, and genome-level differences were examined by means of PCR-randomly amplified polymorphic DNA (RAPD) analyses, DNA–DNA reassociation studies and sequencing of the D1/D2 domain of the large-subunit (LSU) rRNA gene, as well as the internal transcribed spacer (ITS) regions ITS1 and ITS2 and the intervening 5·8S rRNA gene.

METHODS

Cultures. In Table 1, 14 *Zygoascus* strains as well as the type strain of *Pichia hangzhouana* (Lu & Li, 1989) are listed, together with their respective origins and original identifications. *P. hangzhouana* was

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Abbreviations: ITS, internal transcribed spacer; LSU, large subunit.

The GenBank/EMBL/DDBJ accession numbers for the 26S and ITS rRNA gene sequences determined in this study are given in Table 1.

Table 1. List of strains of the genus *Zygoascus* examined

Strain*	GenBank/EMBL/DDBJ accession no.		Original denomination	Origin	Mating type	G + C content (mol%)†
	26S	ITS				
CBS 4028	AY447004	AY447020	Type strain of <i>Trichosporon hellenicum</i>	Unknown	a‡	45.1
CBS 4075	AY447005	AY447021		Grape must, Japan	a‡	44.0
CBS 4099 ^T	AY447006	AY447022		Fermenting grape must, Greece	a‡	43.6
CBS 5839 ^T	AY447007	AY447023	Type strain of <i>C. steatolytica</i>	Mastitic udder of cow, South Africa	α‡	43.6
CBS 6173	AY447008	AY447024	Type strain of <i>C. inositophila</i>	Organic acid fermentation, Israel	a‡	45.0
CBS 6360	AY447009	AY447025		Tunnel of <i>Xyleborus ferrugineus</i> , South Africa	a‡	43.7
CBS 6726	AY447010	AY447026		Equipment soft drink factory, Germany	α‡	44.5
CBS 6736 ^T	AY447011	AY447027		Washings of ion-exchange resin, Japan	a‡	44.0
CBS 6779	AY447012	AY447028		Deep-frozen orange juice, Germany	α‡	44.6
CBS 7115 ^T	AY447013	AY447029	Type strain of <i>P. hangzhouana</i>	Synthetic medium pH 1.5–3.5 containing sucrose and ammonia, Israel	a‡	43.7
CBS 7521 ^T	AY447014	AY447030		Rotten pear, China	α§	44.6
CBS 7652	AY447015	AY447031		Skin, Sweden	a§	44.0
CBS 8424	AY447016	AY447032		NCYC 2304	α§	44.1
CBS 8425	AY447017	AY447033		Fruit of <i>Opuntia stricta</i> , Great Inagua Island, Bahamas	α§	44.2
CBS 8426	AY447018	AY447034		<i>Drosophila muelleri</i> on fruit of <i>O. stricta</i>	α§	44.2
CBS 8813 ^T	AF294910	AY518583	Type strain of <i>C. bituminiphila</i>	Bitumen, Canada		

*CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

†G + C values are based on at least two determinations; SD ≤ 0.5 %.

‡Mating types previously assigned (Smith, 1986).

§Mating type assignment in present study.

included because, from the original physiological description and the sequence data of Kurtzman & Robnett (1998), this species resembled *Z. hellenicus*. Strain CBS 8813^T belonging to *Candida bituminiphila* (Robert *et al.*, 2001) was used as an outgroup for phylogenetic analyses.

Phenotypic characterization. Fermentation ability and utilization of carbon and nitrogen compounds were tested according to the methods described by Yarrow (1998). Physiological relationships between the strains of the *Zygoascus* clade were analysed by using the BioloMICS software (Robert & Szoke, 2003). Mating type behaviour was determined by mixing strains in various combinations, as shown in Table 2. Actively growing 2–7 day-old cultures were used. Ascosporeulation medium was composed of 5 % powdered malt extract (Difco) and 2 % agar (Yarrow, 1998), with or without supplemented vitamins (van der Walt & Yarrow, 1984). Mating type assignment was in compliance with the designations of Smith (1986). Mating experiments were repeated twice, and microscopic observations were done at regular intervals from 2 weeks to 3 months of growth.

DNA isolation. For PCR, DNA from 3–7 day-old yeast cultures grown on 4 % glucose/0.5 % peptone/0.5 % yeast autolysate/2 % agar (Yarrow, 1998) was isolated according to the method of Möller *et al.* (1992). To determine the mol% G + C content and DNA–DNA

reassociation values, DNA was isolated from cells grown in 2 l yeast malt broth (Wickerham, 1951) for 2 days, by using hydroxyapatite column chromatography, as described by Smith *et al.* (1995).

PCR-RAPD analysis. For fingerprinting analyses, a decamer primer, OPA13 (Operon Technologies) and the microsatellite primers M13 (Vassart *et al.*, 1987) and (ATG)₅ (Garbelotto *et al.*, 1993) were used in PCRs. The final reaction mixtures of 50 µl containing 50 ng genomic DNA, 10 mM Tris/HCl (pH 9.5), 50 mM KCl, 1.5 mM MgCl₂, 0.01 % gelatin, 200 µM of each of the dNTPs (Roche), 10 pM of primer and 0.5 U of Supertherm polymerase (ITK Diagnostics) were processed in a Biomed thermocycler (model 60; Theres). The programmes of 40 cycles of the thermocycler were: 94 °C for 60 s, 34 °C for 60 s and 72 °C for 120 s for OPA13; 94 °C for 20 s, 60 °C for 60 s and 72 °C for 120 s for M13; and 94 °C for 60 s, 48 °C for 60 s and 72 °C for 120 s for (ATG)₅. The PCR products were separated on 1.7 % agarose gels in 1 × Tris/acetate/EDTA buffer (40 mM Tris/HCl and 2 mM EDTA; pH 8.0), chilled to 14 °C. The PCR-RAPD profiles for each strain obtained with the three primers were combined in a composite fingerprint by using GelCompar 3.1 (Applied Maths). Similarities between combined fingerprints were calculated by using the Pearson product-moment correlation coefficient. A dendrogram was generated by using the UPGMA method.

Table 2. Matrix of mating combinations and DNA–DNA reassociation values among strains of the genus *Zygoascus*

Lower triangle, mating combinations; upper triangle, DNA–DNA reassociation values. –, No ascus production; (+), ascus production without or with immature ascospores; +, ascus production with immature and mature ascospores; mt, mating type.

Group	CBS no.	6736 ^T mt a	4028 mt a	5839 ^T mt α	6726 mt α	6779 mt α	7652 mt a	8424 mt α	4075 mt a	4099 ^T mt a	6360 mt a	7521 ^T mt α	8426 mt α	6173 mt a	7115 ^T mt a	8425 mt α
Ia	6736 ^T mt a	–		76 %	66 %		70 %			31 %			11 %	23 %		26 %
Ib	4028 mt a			91 %												
	5839 ^T mt α	+	+		94 %	96 %	100 %	96 %	28 %	24 %	30 %		37 %	19 %	28 %	34 %
	6726 mt α	(+)	–	–				96 %		38 %		37 %				
	6779 mt α	+	+	–	–		100 %					20 %			25 %	
	7652 mt a	–		+	–	–										
	8424 mt α	–		–			+							19 %		
IIa	4075 mt a			(+)	(+)	+				93 %			100 %			
	4099 ^T mt a			+	(+)	(+)						100 %	100 %	77 %	65 %	
	6360 mt a			+	(+)	(+)						100 %				
	7521 ^T mt α	–		–		–			+		–		100 %	79 %		
	8426 mt α	–									+					70 %
IIb	6173 mt a	–		+	+	+	–					+	+		100 %	100 %
	7115 ^T mt a			+	(+)	(+)										99 %
	8425 mt α	(+)							–	+				+	–	

Mol% G+C content and DNA–DNA relatedness. Mol% G+C content and DNA–DNA relatedness values were calculated according to previously described procedures (Smith *et al.*, 1995).

rRNA gene sequencing and sequence analysis. The primers NL-1 and NL-4 (O'Donnell, 1993) were used to amplify the D1/D2 region of the LSU rRNA gene, and the primers ITS1 and ITS4 (White *et al.*, 1990) were used to amplify the whole ITS region, including ITS1, ITS2 and the intervening 5·8S rRNA gene. The compositions and final volumes of the reaction mixtures were as for the PCR-RAPD analysis. An initial denaturation step at 94 °C for 5 min was followed by 35 cycles of 94 °C for 45 s, 52 °C for 30 s and 72 °C for 2 min for the D1/D2 amplification, or 35 cycles of 94 °C for 60 s, 55 °C for 60 s and 72 °C for 2 min for the ITS amplification, followed in both cases by final extension at 72 °C for 6 min. The amplified DNA was stored at –20 °C and was purified before use by using GFX columns (27-9602-01; Amersham Biosciences), according to the manufacturer's instructions. For the sequencing of both strands of the D1/D2 and ITS regions, the primers used were the

same as those for the PCRs. The final sequence reaction mixture of 10 µl contained 2 µl Dyanamic (Amersham Biosciences), 1 µl primer (final concentration 0·4 pmol µl^{–1}), 1 µl buffer, 5 µl Milli-Q-water (Millipore) and 1 µl PCR product. Purified sequencing reactions were loaded on an ABI 3700 capillary sequencer (PE Biosystems). Sequences were edited and assembled by using the SeqMan package (DNASTar) and aligned using CLUSTAL W software (Thompson *et al.*, 1994). No manual adjustments were done, except that the ends were trimmed. Phylogenetic relationships were inferred by a combined analysis of the D1/D2 and the ITS regions, by using the maximum-likelihood optimality criterion and the general heuristic search option of PAUP* software, version 4.0 beta 10 (Swofford, 1993). Bootstrap values of less than 50 % were not reported.

RESULTS AND DISCUSSION

Positive mating reactions merely resulting in asci with ascospores are generally accepted as a guideline for

Fig. 1. PCR-RAPD fingerprints of strains of *Z. hellenicus* and *Z. meyeriae* obtained with primers OPA13, (ATG)₅ and M13, and the dendrogram resulting from the combined analysis of the fingerprints using the Pearson product-moment correlation coefficient and the UPGMA clustering method. Ia, Ib, IIa and IIb refer to clusters recognized on the basis of the fingerprints.

conspecificity. However, the ultimate criterion is to confirm the presence of recombinants of parental strains. When such genetic analyses are difficult to perform, alternative approaches must be applied to determine the conspecificity and the taxonomic status of the strains. Initially, DNA–DNA relatedness, by measuring reassociation kinetics of denatured DNA mixtures, was applied; later on, other molecular parameters were introduced. A revision of the genus *Zygoascus* was started by using various approaches to classify recently obtained *Zygoascus*-like isolates.

Molecular analysis

PCR-RAPD results. Individual OPA13, M13 and (ATG)₅ patterns and the dendrogram derived from the combined fingerprints are shown in Fig. 1. At a similarity level of 40%, the 15 strains could be separated into two major groups, Group I and Group II. At a similarity level of 80%, however, each group could be further subdivided. Group Ia was represented by a single strain (CBS 6736^T), Group Ib contained six strains (CBS 4028, CBS 5839^T, CBS 6726, CBS 6779, CBS 7652, CBS 8424), Group IIa included five strains (CBS 4075, CBS 4099^T, CBS 6360, CBS 7521^T, CBS 8426) and Group IIb comprised three strains (CBS 6173, CBS 7115^T, CBS 8425).

DNA base composition and DNA–DNA reassociation. Analysis of the DNA base composition, expressed as mol% G+C content, showed that the range of values obtained was very narrow, spanning only 1.5 mol%. CBS 5839^T was at the lower end of the spectrum with 43.6 mol%, whereas CBS 4028 was at the upper end, with 45.1 mol% (Table 1).

The separation into groups as observed by PCR-RAPD

fingerprinting was further examined by determining DNA–DNA relatedness values calculated from reassociation rates (Table 2, upper triangle). Two main reassociation groups could be distinguished, which were identical to the major groups observed following PCR-RAPD fingerprint analysis (Fig. 1). The reassociation values obtained when Group I isolates were paired with Group II isolates ranged from 19 to 38% (Table 2). Detailed examination revealed a further subdivision similar to that obtained in the fingerprint analyses (Fig. 1). Reassociation values of strains of Group Ia, represented by CBS 6736^T (the type strain of *C. inositoliphila*), with strains of Group Ib ranged from 66 to 76%. The values obtained in pairings among the six strains of Group Ib (including the type strain of *C. steatolytica*) ranged from 91 to 100%. Within Group IIa, reassociation values ranging from 93 to 100% were observed. This group included the type strains of *C. hellenica* (CBS 4099^T) and *P. hangzhouana* (CBS 7521^T), which by this way of measuring appeared to share 100% of their DNA. The name *P. hangzhouana*, though pertaining to the teleomorphic genus *Pichia*, was originally given to material that did not contain true teleomorphic elements; ascospores that were ostensibly seen appear to have been oil droplets or artefacts. According to article 59.3 of the *International Code of Botanical Nomenclature* (Greuter *et al.*, 2000), this name is therefore considered to be that of an anamorph. On the basis of the reassociation results obtained, *P. hangzhouana* can be considered to be conspecific with *C. hellenica*. Reassociation values among the three strains of Group IIb, CBS 6173, CBS 7115^T and CBS 8425, originally identified as *Z. hellenicus*, ranged from 99 to 100%. The DNA–DNA reassociation values between strains of groups IIa and IIb ranged from 65 to 79%.

Fig. 2. Phylogenetic tree based on rRNA gene sequences of D1/D2 and ITS (ITS1, 5-8S and ITS2) regions. The tree was constructed using the maximum-likelihood method as optimality criterion and the general heuristic search option of PAUP* software version 4.0 beta 10. The numerals represent 100 replicated bootstrap samplings (values less than 50 % are not reported). Ia, Ib, IIa and IIb refer to clusters recognized on the basis of the PCR-RAPD fingerprints.

Sequence analyses. Analysis of the D1/D2 and ITS regions showed the separation of the strains into two well-supported main clades (100 % bootstrap), which could be further divided into two subclades (Fig. 2). The groupings based on the phylogenetic analysis were identical to those obtained from the PCR-RAPD fingerprints (Fig. 1) and DNA–DNA reassociation analyses (Table 2 and Fig. 3), except for strain CBS 5839^T, which was closer to CBS 6736^T than to the other members of clade Ib. The latter topology was also found in the phylogenetic analysis of the actin gene (Daniel, 2002).

Morphology and physiology

The physiological profiles were compared by using the BioloMICS software based on pairwise similarity (simple matching coefficient), as listed in the CBS yeast database (<http://www.cbs.knaw.nl>). Isolates belonging to the

Zygoascus clade were very similar, with only a few strains being slightly different (see Table 3). The differences seen mainly involved the assimilation of D-ribose, D-arabinose, methyl α -D-glucoside, L-arabinitol, DL-lactate and 1,2-propanediol. The two teleomorphic species newly distinguished below, *Zygoascus meyeriae* sp. nov. and *Z. hellenicus*, could not be distinguished physiologically. Among the four anamorphic varieties newly distinguished below, only *Candida hellenica* var. *hellenica* and *Candida steatolytica* var. *steatolytica* showed different assimilation properties for DL-lactate, whereas *Candida steatolytica* var. *inositophila* differed from *Candida hellenica* var. *acidophila* in the assimilation of methyl α -D-glucoside and 2-keto-D-gluconate.

Results of crosses among strains of the four groups obtained by PCR-RAPD fingerprints and DNA–DNA reassociations are presented in the lower triangle of Table 2. None of the studied strains was self-fertile. Asci with or without (im–)

Fig. 3. Relationships between groups of strains of *Z. hellenicus* and *Z. meyeriae* based on average DNA–DNA reassociation values and distribution of mating types (mt a and mt alpha). Distances are not proportional to levels of relatedness. SD values for levels of reassociation within groups were $\leq 5\%$ and are means of at least two determinations. SD values for levels of reassociation between groups were $\leq 7\%$. Ia, Ib, IIa and IIb refer to clusters recognized on the basis of the PCR–RAPD fingerprints.

mature, hat-shaped ascospores were usually observed after 2–4 weeks of incubation (Fig. 4), when mating was compatible (Table 2). In Group I, ascus formation was not observed for four crosses (CBS 4028 $\times$ CBS 6726^T; CBS 7652 $\times$ CBS 6726; CBS 7652 $\times$ CBS 6779 and CBS 6736^T $\times$ CBS 8424). The same occurred in Group II for the combination of CBS 4099^T and CBS 8425. Asci without or with (im-) mature ascospores were produced when compatible mating types of Group I and Group II were mixed. From the mating tests, it can be concluded that eight strains,

including the type strains of *C. hellenica* and *C. inositolophila*, represent mating type a, and seven strains, including the type strains of *C. steatolytica* and *P. hangzhouana*, represent mating type alpha. The predicted relatedness of *P. hangzhouana* with *Z. hellenicus* on the basis of the D1/D2 rRNA gene sequences (Kurtzman & Robnett, 1998) was confirmed by the observation of ascospore production after mixing of the type strains (Table 2). A summary of the mean reassociation values and the distribution of the mating types between the various groups are given in Fig. 3.

Table 3. Physiological results for the strains belonging to the *Zygoascus* clade

Comp., compiled; d, delayed reaction; v, variable reaction; w, weak reaction; ND, not determined. All tested strains were positive for growth in the presence of D-glucose, D-galactose, L-sorbose, D-glucosamine, D-xylose, L-rhamnose, sucrose, trehalose, cellobiose, salicin, glycerol, xylitol, D-glucitol, D-mannitol, *myo*-inositol, 5-keto-D-gluconate, D-glucuronate, D-galacturonate and ethanol as carbon source; growth on L-lysine, cadaverine and glucosamine as nitrogen source; growth without *myo*-inositol, pantothenate, pyridoxine, niacin, para-aminobenzoic acid and cycloheximide (0.01 and 0.1%); and growth at 25, 30, 35 and 37 °C. All tested strains were negative for growth on melibiose, inulin, erythritol, methanol, 2,3-butanediol, quinic acid, D-glucarate and D-galactonate as carbon source; growth on nitrate, creatine, creatinine and imidazole as nitrogen source; growth without vitamins, thiamine, biotin plus thiamine, and pyridoxine plus thiamine as nitrogen source; growth on 1% acetic acid; starch formation; acetic acid production; urea hydrolysis; Diazonium blue B reaction; and growth at 42 and 45 °C. Strain designations given are CBS numbers.

Characteristic	Z. hellenicus								Comp.	Z. meyerae											Comp.
	C. steatolytica var. steatolytica							C. steatolytica var. inositophila		C. hellenica var. hellenica						C. hellenica var. acidophila					
	4028	5839 ^T	6726	6779	7652	8424	Comp.	6736 ^T		4075	4099 ^T	6360	7521 ^T	8426	Comp.	6173	7115 ^T	8425	Comp.		
Fermentation of:																					
D-Glucose	+	+	+	+	+	+	+	w, d	v	w, d	+	+	+	+	v	+	+	+	+	v	
D-Galactose	w, d	+	w, d	+	+	+	v	—	v	w, d	w, d	w, d	+	+	v	ND	w, d	w, d	v	v	
Maltose	—	w, d	ND	ND	—	—	v	w, d	v	—	—	w, d	w, d	—	v	w, d	—	—	v	v	
Methyl α-D-glucoside	—	—	ND	ND	ND	ND	—	—	—	—	—	ND	ND	ND	—	ND	—	ND	—	—	
Sucrose	+	+	w, d	+	+	+	v	w, d	v	w, d	w, d	+	+	+	v	w, d	w, d	w, d	v	v	
α,α-Trehalose	w, d	+	+	w, d	ND	ND	v	w, d	v	w, d	—	ND	ND	ND	v	ND	w, d	ND	v	v	
Melibiose	—	—	ND	ND	ND	ND	—	—	—	—	—	ND	ND	ND	—	ND	—	ND	—	—	
Lactose	—	—	ND	ND	—	—	—	—	—	—	—	ND	—	—	—	ND	—	—	—	—	
Cellobiose	w, d	w, d	w, d	w, d	ND	ND	v	w, d	v	w, d	—	ND	ND	ND	v	ND	w, d	ND	v	v	
Melezitose	—	—	ND	ND	ND	ND	—	—	—	—	—	ND	ND	ND	—	ND	—	ND	—	—	
Raffinose	w, d	w, d	ND	ND	w, d	+	v	—	v	—	—	w, d	+	+	v	ND	w, d	w, d	v	v	
Inulin	—	—	ND	ND	ND	ND	—	—	—	—	—	ND	ND	ND	—	ND	—	ND	—	—	
Starch	—	—	ND	ND	ND	ND	—	—	—	—	—	ND	ND	ND	—	ND	—	ND	—	—	
D-Xylose	—	—	ND	ND	ND	ND	—	—	—	—	—	ND	ND	ND	—	ND	—	ND	—	—	
Growth on carbon source:																					
D-Ribose	—	d	—	—	—	d	v	w, d, —	v	w, d, —	w, d, —	—	—	w, d, —	v	—	w, d, —	—	v	v	
L-Arabinose	+	+	—	+	+	+	v	+	v	+	+	+	+	+	+	+	+	+	+	+	
D-Arabinose	—	w, d, —	+	—	—	d	v	—	v	—	w, d, —	—	—	—	v	—	—	—	—	v	
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w, d, +	+	+	v	v	
Methyl α-D-glucoside	—	w, d, —	w, d, —	—	—	—	v	+	v	—	w, d, —	d	—	d	v	—	—	—	—	v	
Arbutin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	d	d	+	v	v	
Lactose	—	—, +	—	—	—	—	v	—	v	—	—	—	—	—	—	—	—	—	—	—	
Raffinose	+	+	+	w, d, +	+	+	v	+	v	+	+	+	+	+	+	w, d, +	+	+	v	v	
Melezitose	w, d, —	d	+	w, d, +	d	d	v	+	v	d	w, d, —	+	d	+	v	w, d, —	w, d, —	+	v	v	
Starch	+	+	+	+	+	+	+	+	+	w, d, +	+	+	+	+	v	+	+	+	+	v	
Ribitol	+	+	—	+	+	+	v	+	v	+	+	+	+	+	+	+	+	+	+	+	
L-Arabinitol	—	w, d, +	—	—	d	d	v	+	v	d	d	d	—	d	v	d	—	d	v	v	
Galactitol	d	+	—	w, d, +	+	+	v	w, d, +	v	d	+	w, d, +	+	+	v	+	+	+	+	+	
D-Glucono-1,5 lactone	w, d, +	+	w, d, +	d	d	+	v	+	v	d	+	+	+	+	v	+	+	+	+	v	
2-Keto-D-gluconate	—	—	—	—	—	d	v	w, d, —	v	d	—	—	—	d	v	—	—	—	—	v	
D-Gluconate	+	+	+	+	+	+	+	+	+	d	d	+	+	+	v	+	+	d	v	v	
DL-Lactate	—	w, d, —	—	w, d, —	—	—	v	+	v	—	—	—	—	—	—	w, d, —	—	—	v	v	

Table 3. cont.

Characteristic	Z. hellenicus										Z. meyerae									
	C. steatolytica var. steatolytica					C. steatolytica var. inositophila					C. hellenica var. hellenica					C. hellenica var. acidophila				
	4028	5839 ^T	6726	6779	7652	8424	Comp.	6736 ^T	Comp.	4075	4099 ^T	6360	7521 ^T	8426	Comp.	6173	7115 ^T	8425	Comp.	
Succinate	+	+	+	+	+	+	+	+	+	d	+	+	+	+	V	+	+	+	V	
Citrate	+	+	+	+	+	+	+	+	+	- w, d, -	d	+	+	+	V	+	+	+	V	
1,2-Propanediol	-	-	-	-	-	-	-	-	-	d	-	d	d	d	V	d	-	d	V	
Growth on nitrogen source:																				
Nitrite	-	-	+	-	-	-	V	-	V	-	-	-	-	-	-	-	-	-	-	
Ethylamine	+	+	+	+	+	+	+	+	+	+	+	w, d, +	w	w	V	+	+	w	V	
D-Tryptophan	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	V	
Growth without biotin	w	-	-	-	-	ND	-	ND	-	-	ND	-	ND	ND	-	-	-	ND	-	
Growth on D-glucose:																				
50 %	-	+	w, d	+	ND	+	V	+	V	+	+	w, d	+	+	V	-	+	+	V	
60 %	ND	-	-	-	ND	-	-	-	-	+	-	ND	ND	w, d	V	ND	-	-	V	
Growth at 40 °C	-	-	-	-	-	-	-	-	-	-	-	+	+	-	V	-	-	-	V	
Coenzyme Q system	ND	9	ND	ND	ND	ND	9	9	9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	

The occurrence of mating between Group I and Group II isolates with low DNA–DNA relatedness indicates that they possess the same mating type system and are closely related (belonging to the same genus). Whether recombination can occur between these two groups should be investigated by genetic analysis, but this would be an onerous and difficult procedure because of the extreme hyphal state of the teleomorph. However, the latter genetic analysis has been performed for the yeast genus *Arthroascus* (Naumova *et al.*, 2003), where mating occurred between taxa with low (19–31 %), as well as intermediate (65 %), nuclear DNA–DNA relatedness. The progeny of crosses of taxa with low DNA–DNA relatedness showed an absence of recombinants, indicating that the crosses represented a poorly compatible type of interspecies hybridization (Naumov *et al.*, 2003). Analyses of crosses between taxa sharing intermediate DNA–DNA reassociation values of 65–70 % has revealed some genetic exchange (G. I. Naumov & M. Th. Smith, unpublished data). The progeny of crosses between strains within the same group with high reassociation values gave fertile recombinants (Naumov *et al.*, 2003). A similar example was demonstrated for the teleomorphic genus *Stephanoascus*. In this genus, mating occurred among taxa with low DNA–DNA relatedness, as well as differences in 18S rRNA gene sequences (Ueda-Nishimura & Mikata, 2002). These authors therefore concluded that mating indicates close relatedness, not conspecificity.

In addition, the division of the *Zygoascus* isolates into four clusters is supported by the actin gene-sequencing study of Daniel (2002; and unpublished data). Each of the four groups could be distinguished by differences of more than 20 nucleotides. H.-M. Daniel (personal communication) considered this value to be high enough to support the hypothesis that these groups represented separate taxa.

On the basis of the results summarized in Fig. 3 and the above cited data, it seems warranted to propose two novel teleomorphic species within the genus *Zygoascus*, namely, *Z. hellenicus* and *Z. meyeriae* sp. nov. These species correspond to Group I and Group II, respectively. To deal with the two molecular genetic subgroups found within each of these *Zygoascus* species, we propose *C. steatolytica* var. *steatolytica* and *C. steatolytica* var. *inositophila* as two novel anamorphic varieties connected to *Z. hellenicus*, and *C. hellenica* var. *hellenica* and *C. hellenica* var. *acidophila* as two novel anamorphic varieties linked to *Z. meyeriae*. The varieties are proposed only at the anamorphic level, because the level of DNA–DNA relatedness (71 %) between varietal subgroups within each species appears sufficient to predict fully compatible mating; thus, the organisms at the teleomorphic level represent (at least potentially) unified biological species. This matter needs to be further investigated with studies on actual introgression levels among the groups and subgroups within the genus *Zygoascus*. In the meantime, because strong consistency was observed among all the molecular characters studied, we feel confident to propose the two teleomorphic species. Because all the

Fig. 4. Ascus and one hat-shaped ascospore of *Z. meyeriae* CBS 4099^T × CBS 7521^T (arrow). Bar, 5 µm.

observations (DNA–DNA reassociation, D1/D2, ITS and actin gene sequences, and PCR-RAPD fingerprints) consistently suggest that two subgroups can be distinguished within each teleomorphic species, but not to the point where we could describe them as separate species, we propose to consider them as four anamorphic varieties.

Taxonomy

The following species and varieties within the *Zygoascus* clade are proposed.

I. *Zygoascus hellenicus* M. Th. Smith.

Antonie van Leeuwenhoek (1986) 52, 27.

Teleomorph of *C. steatolytica* var. *steatolytica* and *C. steatolytica* var. *inositophila*.

Type specimen: a dried specimen of mixed sporulating culture of CBS 5839^T × CBS 6736^T is preserved at the CBS.

Ia. *Candida steatolytica* Yarrow var. *inositophila* (Nakase) M. Th. Smith & V. Robert comb. nov., stat. nov.

Basionym: *C. inositophila* Nakase

Antonie van Leeuwenhoek (1975) 41, 206.

Anamorph of *Z. hellenicus*.

Type strain: ex-type isolate CBS 6736^T (= IFO 1575^T) is a living strain in the CBS yeast collection.

Ib. *Candida steatolytica* Yarrow var. *steatolytica*.

Antonie van Leeuwenhoek (1969) 35, 24.

Anamorph of *Z. hellenicus*.

Type strain: ex-type isolate CBS 5839^T (= IFO 10184^T) is a living strain in the CBS yeast collection.

II. *Zygoascus meyerae* M. Th. Smith & V. Robert sp. nov.

Etymology: the epithet *meyerae* is chosen in honour of S. A. Meyer, who played a great role in the establishment of DNA–DNA reassociation methods in yeast taxonomy.

Teleomorph of *C. hellenica* var. *hellenica* and *C. hellenica* var. *acidophila*.

Type specimen: a dried specimen of a mating culture of CBS 4099^T × CBS 7521^T with ascospores is preserved at the CBS as Herb. CBS 6599^T.

Latin diagnosis of *Zygoascus meyerae* M. Th. Smith et V. Robert sp. nov.

Species heterothallica. Coloniae in agar maltoso post 7 dies 25 °C 7–10 mm diametro, elevatae, restrictae, tenaces, rugosae vel crispatae, pubescentes vel floccosae, albae vel cremeae, margine fimbriate circumdatae. Cellulae gemmantes ovoideae, elongatae vel cylindricae, (2.2–5.4) × (5.4–10.2) µm, saepe catenulatae et pseudohyphae formantes. Hyphae septatae, ramosae, 2.0–3.5 µm latae. Microsporus singulus in centro septum. Blastosporae ellipsoideae, ovoideae vel elongatae, cylindricae. Asci ovoidei vel globosi, parietibus persistentibus, plerumque unispori, sed etiam 4-spori (4.1–6.2) × (5.2–7.4) µm. Ascosporaehemisphaericae ad galeatae, ad basim margine prominente praeditae, (1.5–3.5) × (2.5–4.5) µm. Glucosum, galactosum, maltosum, sucrosum, trehalosum, cellobiosum, raffinsum (variabile) fermentur, at non methyl α-D-glucosidum, melibiosum, lactosum, melezitium, inulinum, amyllum nec xylosum. Glucosum, galactosum, sorbosum, glucosaminum, xylosum, L-arabinosum, rhamnosum, sucrosum, trehalosum, cellobiosum, salicinum, glycerolum, ribitolum, xylitolum, glucitolum, mannitolum, galactitolum, inositolum, 5-ketogluconatum, glucuronatum, galacturonatum et ethanolum assimilantur, at non melibiosum, lactosum, inulinum, erythritolum, methanolum, butane-2,3-diolum, acidum quinicum, glucaratum nec galactonatum. Ribosum, D-arabinosum, maltosum, methyl α-D-glucosidum, arbutinum, raffinsum, melezitium, amyllum, L-arabinitolum, glucono-1,5-lactonium, 2-ketogluconatum, gluconatum, acidum DL-lacticum, acidum succinicum, acidum citricum, propane-1,2-diolum variabile assimilantur. Lysinum, cadaverinum, glucosaminum assimilantur at non kalium nitricum, kalium nitrosum, creatinum, ceatininum nec imidazolum. Ad crescentiam vitaminarum necessariae sunt. Diazonium caeruleum B negativum. Augmentum ad 37 °C. Holotypus cultura conjugata ascigera CBS 4099^T × CBS 7521^T, exsiccate in collectione CBS, Utrecht, praeservatur.

Ila. *Candida hellenica* (Verona & Picci) D. S. King & S.-C. Jong var. *hellenica*.

Mycotaxon (1977) 6, 413.

Basionym: *Trichosporon hellenicum* Verona & Picci, *Ann Microbiol Enzimol* (1958) **8**, 106.

Synonym: *Pichia hangzhouana* X.-H. Lu & M.-X. Li, *Acta Mycol Sin* (1989) **8**, 253.

Anamorph of *Z. meyeriae*.

Type strain: ex-type isolate CBS 4099^T (= IFO 10246^T) is a living strain in the CBS yeast collection.

IIb. *Candida hellenica* var. *acidophila* M. Th. Smith & V. Robert nov. var.

Etymology: the epithet *acidophilus* is chosen because some strains were isolated from acidic source material.

Anamorph of *Z. meyeriae*.

Type strain: ex-type isolate CBS 7115^T (= NCYC 2544^T) is a living strain in the CBS yeast collection.

Latin diagnosis of *Candida hellenica* var. *acidophila* M. Th. Smith et V. Robert nov. var.

Charactera morfologica similes Candida hellenica var. *hellenica*. *Glucosum fermentur et galactosum, maltosum, sucrosum, trehalosum, cellobiosum, raffinotum variabile fermentur, at non methyl α -D-glucosidum, melibiosum, lactosum, melezitotum, inulinum, amyllum nec xylosum. Glucosum, galactosum, sorbosum, glucosaminum, amyllum, glucono-1,5-lactotum, acidum succinicum, acidum citricum, xylosum, L-arabinosum, rhamnolum, sucrosum, trehalosum, cellobiosum, salicinum, glycerolum, ribitolum, xylitolum, glucitolum, mannitolum, galactitolum, inositolum, 5-ketogluconatum, glucuronatum, galacturonatum et ethanolum assimilantur, at non melibiosum, D-arabinosum, methyl α -D-glucosidum, 2-ketogluconatum, lactosum, inulinum, erythritolum, methanolum, butane-2,3-diolum, acidum quinicum, glucaratum nec galactonatum. Ribosum, maltosum, arbutinum, raffinotum, melezitotum, L-arabinotolum, gluconatum, acidum DL-lacticum, propane-1,2-diolum variabile assimilantur. Lysinum, cadaverinum, glucosaminum assimilantur at non kalium nitricum, kalium nitrosum, creatinum, ceatininum nec imidazolium. Diazonium caeruleum B negativum. Ad crescentiam vitaminarum necessariae sunt. Augmentum ad 37 °C. Typus CBS 7115^T lyophilus depositus in collectione zymotica CBS, Utrecht.*

As mentioned above, a dried, sporulated specimen from a cross between the two mating strains, *C. steatolytica* CBS 5839^T $\times$ *C. inositophila* CBS 6736^T was originally deposited by Smith (1986) as the holotype of *Z. hellenicus*. This specimen was selected because asci with ascospores were abundantly present. In crossings with the ex-type isolate of *C. hellenica*, reduced ascospore formation was seen, but Smith considered this isolate to be conspecific with the isolates used in making the holotype. This then made the basionym *T. hellenicum* the oldest available name for the anamorphic species involved, and Smith (1986) selected 'hellenica' as the epithet for the species as revised. The

epithet of the teleomorph name *Z. hellenicus* was adopted as an echo, at another taxonomic level, of the established anamorph epithet, following examples such as the echoing of the anamorph name *Cryptococcus neoformans* in the teleomorph name *Filobasidiella neoformans*. Our current application of molecular criteria has somewhat inconveniently shown that the type strain of *Candida hellenica* differs at the species level from both allotype isolates of *Z. hellenicus*. Therefore, in the present study, we were obliged to redispense *C. hellenica* as the anamorph of a novel teleomorph species, which in this case bears the quite different name *Z. meyeriae*.

It should be noted that the practice of echoic designation of teleomorph epithets automatically carries with it the potential disadvantage that the anamorph and teleomorph epithets may later become separated. Though superficially it may seem sensible to apply a 'one yeast-one epithet' policy in order to simplify nomenclature when a new teleomorph species is being described, the separate rooting of each epithet to its own type material needs to be borne in mind. The potential for later separation of the two identical epithets is, of course, minimized when the ex-type isolate of the anamorph name is a vigorously mating isolate used in preparing the holotype of the teleomorph name. It seems sensible to suggest that reuse of currently valid anamorph epithets for newly obtained teleomorphs should be restricted to cases where this condition holds.

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