

## Redefinition of *Aureobasidium pullulans* and its varieties

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**Abstract:** Using media with low water activity, a large numbers of aureobasidium-like black yeasts were isolated from glacial and subglacial ice of three polythermal glaciers from the coastal Arctic environment of Kongsfjorden (Svalbard, Spitsbergen), as well as from adjacent sea water, sea ice and glacial meltwaters. To characterise the genetic variability of *Aureobasidium pullulans* strains originating from the Arctic and strains originating pan-globally, a multilocus molecular analysis was performed, through rDNA (internal transcribed spacers, partial 28 S rDNA), and partial introns and exons of genes encoding  $\beta$ -tubulin (*TUB*), translation elongation factor (*EF1 $\alpha$* ) and elongase (*ELO*). Two globally ubiquitous varieties were distinguished: var. *pullulans*, occurring particularly in slightly osmotic substrates and in the phyllosphere; and var. *melanogenum*, mainly isolated from watery habitats. Both varieties were commonly isolated from the sampled Arctic habitats. However, some aureobasidium-like strains from subglacial ice from three different glaciers in Kongsfjorden (Svalbard, Spitsbergen), appeared to represent a new variety of *A. pullulans*. A strain from dolomitic marble in Namibia was found to belong to yet another variety. No molecular support has as yet been found for the previously described var. *aubasidani*. A partial elongase-encoding gene was successfully used as a phylogenetic marker at the (infra-)specific level.

**Key words:** Arctic, *Aureobasidium*, black yeasts, elongase, glacier, ITS, LSU, phylogeny, polar environment, rDNA, sea ice, seawater, taxonomy, translation elongation factor,  $\beta$ -tubulin.

**Taxonomic novelties:** *Aureobasidium pullulans* var. *subglaciale* Zalar, de Hoog & Gunde-Cimerman, var. nov.; *Aureobasidium pullulans* var. *namibiae* Zalar, de Hoog & Gunde-Cimerman, var. nov.

## INTRODUCTION

*Aureobasidium pullulans* (De Bary) G. Arnaud is a black yeast-like species that is particularly known for its biotechnological significance as a producer of the biodegradable extracellular polysaccharide (EPS) pullulan (poly- $\alpha$ -1,6-maltotriose). This component is a promising biomaterial (Rekha & Sharma 2007), and is currently used among others for the packaging of food and drugs (Singh *et al.* 2008). Its biotechnological potential is also seen in the production of a variety of hydrolytic enzymes (Federici 1982, Chi *et al.* 2006, Wang *et al.* 2007, Li *et al.* 2007, Ma *et al.* 2007, Zhiqiang *et al.* 2008).

*Aureobasidium pullulans* was taxonomically characterised by de Hoog & Yurlova (1994) on the basis of its morphology and nutritional physiology. These authors noted some differences in growth with galactitol, glucono- $\delta$ -lactone, creatine and creatinine, and in gelatin liquefaction. Since the species shows considerable variability in its morphological and physiological properties, three varieties have been described during the last decades, viz. *Aureobasidium pullulans* var. *pullulans* (Viala & Boyer 1891), *A. pullulans* var. *melanogenum* Hermanides-Nijhof (1977), and *A. pullulans* var. *aubasidani* Yurlova (Yurlova & de Hoog 1997). The first two of these were distinguishable by culture discolouration, while the latter is unique in its production of aubasidan-like EPS (glucans with  $\alpha$ -1,4-D-,  $\beta$ -1,6-D- and  $\beta$ -1,3-D-glycosidic bonds). Diagnostically, var. *aubasidani* is unique due to the absence of assimilation of methyl- $\alpha$ -D-glucoside and lactose and by N-source assimilation for the production of EPS. In a further study using PCR ribotyping (rDNA RFLP and UP-PCR/hybridisation), Yurlova *et al.* (1996) divided the *Aureobasidium* strains into four groups, which,

however, do not correlate with morphological differences. Yurlova *et al.* (1999) also revealed close relationships between *Kabatiella lini* (Laff.) Karak., the teleomorph species *Discosphaerina (Columnosphaeria) fagi* (H.J. Huds.) M.E. Barr and *Aureobasidium pullulans*.

*Aureobasidium pullulans* is a ubiquitous and widespread oligotrophe that can be found in environments with fluctuating water activities, such as the phyllosphere (Andrews *et al.* 1994), bathrooms, food and feeds (Samson *et al.* 2004). It can also be found in osmotically very stressed environments, such as hypersaline waters in salterns (Gunde-Cimerman *et al.* 2000), and rocks and monuments (Urzı *et al.* 1999). Due to the production of large quantities of yeast-like propagules, this fungus disperses globally, although thus far it has only rarely been reported in cold environments. This may be because most investigations on the occurrence and diversity of fungi in the cold have been limited to frozen Antarctic soils and Siberian permafrost, where basidiomycetous yeasts prevail (Abyzov 1993, Babjeva & Reshetova 1998, Deegenars & Watson 1998, Golubev 1998, Ma *et al.* 1999, 2000, 2005, Margesin *et al.* 2002, Onofri *et al.* 2004, Price 2000, Vishniac 2006, Vishniac & Onofri 2003). Thus far, no investigations of mycobiota in ice had been carried out. We recently investigated ice originating from glacial and subglacial environments of three different polythermal Arctic glaciers in Svalbard (Spitsbergen, Norway) (Butinar *et al.* 2007, 2008, Sonjak *et al.* 2006). During these studies, aureobasidium-like fungi were found among the dominant ascomycetous mycota. Given the known adaptive ability of *A. pullulans* to low water activity ( $a_w$ ) and oligotrophic conditions, it appeared likely that ice from cryoclastic formations and subglacial ice in polythermal glaciers constitute a

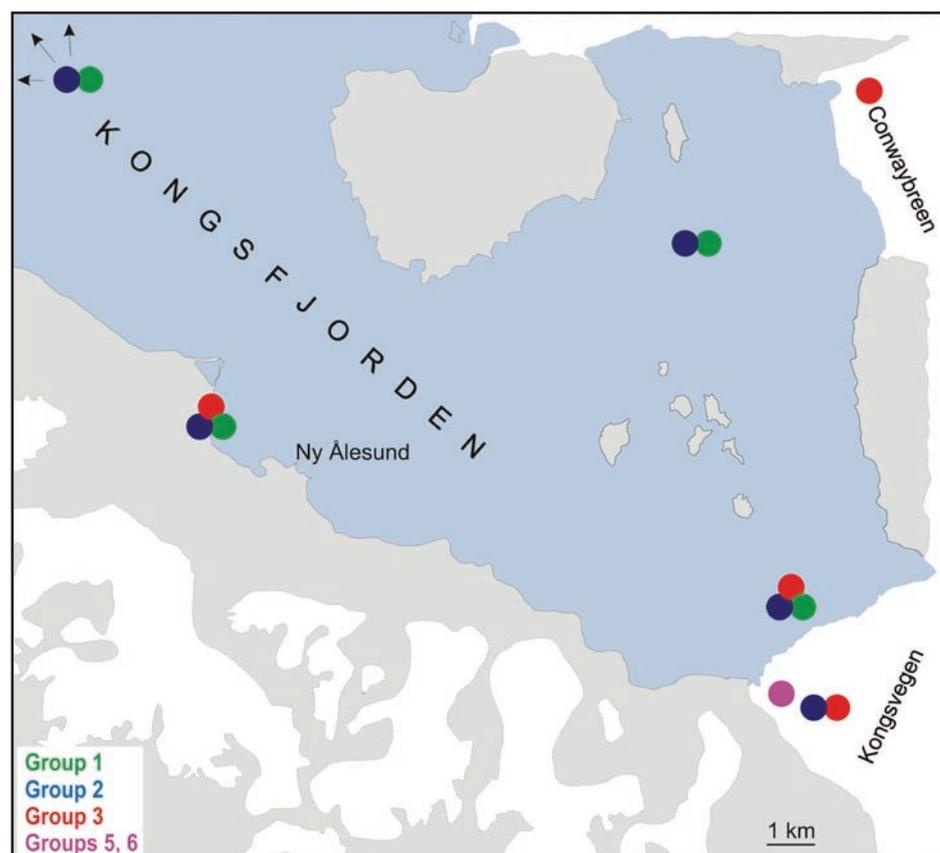


Fig. 1. Detailed map of the sampling area in Svalbard, with sites of retrieved aureobasidium-like isolates marked.

potential natural habitat. Since some of the Arctic aureobasidium-like isolates deviated phenetically from the pan-global population, a taxonomic study into the genus *Aureobasidium* was performed. Isolates obtained from different niches in Arctic, temperate and tropical climates were compared by multilocus analyses of rDNA internal transcribed spacers (ITS), partial large subunit of rDNA (LSU), and partial introns and exons of genes coding  $\beta$ -tubulin (*TUB*), translation elongation factor (*EF1 $\alpha$* ) and elongase (*ELO*). The main aims of the study were to describe the total diversity of *A. pullulans*, to redefine its entities, to describe potentially new varieties, and to correlate these with their ecology, focusing on the Arctic sampling area investigated.

## MATERIALS AND METHODS

### Arctic sampling sites and sample collection

Kongsfjorden is located at 79°N 12°E, and it is one of the larger fjords along the western coast of Spitsbergen, in the Svalbard Archipelago, Norway. The greater part of this drainage basin is covered by glaciers that calve into the fjord. The annual mean temperature is around  $-5^{\circ}\text{C}$ , the mean salinity of the sea water ranges from 34.00 to 35.00 PSU. Twenty-five samples of glacial and subglacial ice were collected aseptically in 2001 and 2003, as described previously (Gunde-Cimerman *et al.* 2003, Butinar *et al.* 2007). These originated from three polythermal glaciers (Copland & Sharp 2001): Conwaybreen, Kongsvegen and austre Lovénbreen. Ice was also collected from a moulin and from a glacial cave at Kongsvegen. The subglacial samples included sediment-rich and overlying clear basal ice. Some ice samples, particularly from Kongsvegen, were rich in gypsum inclusions.

During summer, subglacial meltwaters from Kongsvegen and the austre Lovénbreen glaciers were also sampled directly. The supraglacial samples comprised two samples of snow/ice mixtures from austre Lovénbreen and Kongsvegen, and eight samples of seasonal meltwaters on the glacier surfaces. During the summer season of 2001, samples of seawater and a mixture of snow and ice in the tidal area were collected from six different locations within the fjord.

Physico-chemical parameters (pH,  $\text{Na}^+$ ,  $\text{Mg}^{2+}$  and  $\text{K}^+$  concentrations, and total phosphorus content) were determined for five basal ice samples (originating from Kongsvegen), a sample of subglacial meltwater, and three samples of seawater, as described by Gunde-Cimerman *et al.* (2003).

### Isolation and preservation

Ice samples were transported to the laboratory, where they were processed. The surface layer of ice was aseptically melted at room temperature and discarded. The remaining ice was transferred to another sterile container and melted. The resulting water, as well as directly sampled glacier meltwater and seawater, were filtered immediately (Millipore membrane filters; 0.22- $\mu\text{m}$  and 0.45- $\mu\text{m}$  pore sizes) in aliquots of up to 100 mL. The membrane filters were placed on general-purpose isolation media [DRBC: Dichloran (2,6-dichloro-4-nitroanilin) Rose Bengal Agar (Oxoid CM729) and Malt Extract Agar (MEA)], as well as on a medium for the detection of moderate xerophiles [18 % dichloran glycerol agar (DG18; Hocking & Pitt 1980)], and on selective media with high concentrations of salt (MEA with addition of 5 % to 15 % NaCl) or sugar (malt extract yeast extract with 20 %, 35 % and 50 % glucose). For prevention of bacterial growth, chloramphenicol (50 mg/L<sup>-1</sup>) was added to all of the media. One drop of the original water sample was applied onto a membrane and was dispersed with a Drigalski spatula. For

each sample and medium, at least four and up to 10 aliquots were filtered in parallel, and average numbers of colony forming units (CFUs) were calculated (Gunde-Cimerman *et al.* 2000). The plates were incubated for up to 14 wk at 4, 10 and 24 °C.

Subcultures were maintained at the Culture Collection of Extremophilic Fungi (EXF, Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia), while a selection have been deposited at the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands). Reference strains were obtained from the CBS, and were selected either on the basis of strain history, name, or on the basis of their ITS rDNA sequence. The strains were maintained on MEA and preserved for long periods in liquid nitrogen or by lyophilisation. The strains studied are listed in Table 1. A detailed map of the sampling area, with the sites of retrieved isolates marked, is shown in Fig. 1.

## Cultivation and microscopy

For growth rate determination and the phenetic description of colonies, the strains were point-inoculated onto potato-dextrose agar (PDA; Oxoid CM139), and Blakeslee's MEA (Samson *et al.* 2004), and then incubated at 25 °C for 7–14 d in darkness. Surface colours were rated using the colour charts of Korerup & Wanscher (1978). For microscopic morphology, MEA blocks of about 1 × 1 cm<sup>2</sup> were cut out aseptically, placed on sterile microscope slides, and inoculated at the upper four edges by means of a conidial suspension (Pitt 1979). Inoculated agar blocks were covered with sterile cover slips and incubated in moist chambers for 2, 4, and 7 d at 25 °C in the dark. The structure and branching pattern of the immersed hyphae were examined under magnifications of 100× and 400× in intact slide cultures under the microscope without removing the cover slips from the agar blocks. For higher magnifications (400×, 1000×) the cover slips were carefully removed and mounted in 60 % lactic acid.

## DNA extraction, sequencing and analysis

For DNA isolation, the strains were grown on MEA for 7 d. Their DNA was extracted according to Gerrits van den Ende & de Hoog (1999), by mechanical lysis of approx. 1 cm<sup>2</sup> of mycelium. A fragment of rDNA including ITS region 1, 5.8S rDNA and ITS 2 (ITS) was amplified using the ITS1 and ITS4 primers (White *et al.* 1990). LSU (partial 28 S rDNA) was amplified and sequenced with the NL1 and NL4 primers (Boekhout *et al.* 1995). For amplification and sequencing of the  $\beta$ -tubulin (*TUB*) gene, primers Bt2a and Bt2b were used (Glass & Donaldson 1995). Translation elongation factor EF-1 $\alpha$  (*EF1 $\alpha$* ) was amplified and sequenced with the primers EF1-728F and EF1-986R (Carbone & Kohn 1999). For amplification and sequencing of the partial elongase gene (*ELO*), the ELO2-F (5'-CAC TCT TGA CCG TCC CTT CGG-3') and ELO2-R (5'-GCG GTG ATG TAC TTC TTC CAC CAG-3') primers were used, designed for *Aureobasidium pullulans*. Reactions were run in a PCR Mastercycler Ep Gradient (Eppendorf) with a profile of initial denaturation of 2 min at 94 °C, followed by 6 cycles of 15 s at 94 °C, 15 s at 58 °C and of 45 s at 72 °C, and 30 cycles of 15 s at 94 °C, 15 s at 56 °C and of 45 s at 72 °C, with a final elongation of 7 min at 72 °C. BigDye terminator cycle sequencing kits were used in sequence reactions (Applied Biosystems, Foster City, CA, U.S.A.). Sequences were obtained with an ABI Prism 3700 (Applied Biosystems). They were assembled and edited using SeqMan 3.61 (DNAStar, Inc., Madison, U.S.A.). Sequences downloaded

from GenBank are indicated in the gene trees by their GenBank accession numbers; newly generated sequences are indicated by their strain numbers (see also Table 1).

## Phylogenetic analyses

Sequences were automatically aligned using ClustalX 1.81 (Jeanmougin *et al.* 1998). Alignments were adjusted manually using MEGA4 (Tamura *et al.* 2007). Gene trees were generated with MrBayes software, applying Bayesian inference (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). Three parallel runs were performed for three million generations with mixed amino-acid models, the default temperature and five chains. The gene trees were sampled every 100 generations. Gene trees sampled before the analysis that reached stationarity of likelihood values, and those sampled before the mean standard deviation of the split frequencies decreased to under 0.5 % were excluded from the final analysis. The stationarity of likelihood values was checked using the Tracer software (Rambaut & Drummond: MCMC Trace Analysis Tool, version 1.4, 2003–2007). In phylogenetic analysis of LSU rDNA the LSU sequence of *Elsinoe veneta* (DQ678060) was selected as an outgroup, according to Schoch *et al.* (2006). Isolates were grouped on the basis of multilocus analyses and representative strains were selected for morphological analyses.

## RESULTS

### Isolates from Arctic samples

The fresh isolates from Arctic samples are listed in Table 1. Subglacial ice samples without and with gypsum inclusions, incubated on MEA with 5 % NaCl at 10 °C, contained aureobasidium-like propagules in the highest CFU range (>30 CFU/mL). Strains were also isolated from gypsum crystals collected from soil bordering subglacial ice with gypsum inclusions. Lower CFU numbers of *Aureobasidium* (2–3 CFU/100 mL) were detected also in other ice samples: in sea ice and moulin ice, and in specimens from an ice cave.

### Phylogenetic analyses

Alignments for the phylogenetic analyses included 599 base pairs for LSU, 488 for ITS, 704 for *ELO*, 323 for *EF1 $\alpha$* , and 425 for *TUB*. Internodes were considered strongly supported if they received posterior probabilities  $\geq 95$  % (Lutzoni *et al.* 2004). Good convergence of the runs was reached when constructing all of the gene trees with MrBayes. The likelihood values reached plateaus after approximately 24,000 (LSU), 4,000 (ITS), 6,000 (*TUB*), 7,000 (*EF1 $\alpha$* ) and 15,000 (*ELO*) generations, while the mean standard deviations of the split frequencies dropped below 1 % after 600,000 (LSU), 300,000 (ITS), 800,000 (*TUB*), 300,000 (*EF1 $\alpha$* ) and 200,000 (*ELO*) generations. The first 6,000 (LSU), 3,000 (ITS), 8,000 (*TUB*), 3,000 (*EF1 $\alpha$* ) and 2,000 (*ELO*) trees were discarded as burn-in.

According to the LSU rDNA analysis (Fig. 2), a high level of support was evident for the clade containing *A. pullulans* (groups 1–4) together with *Selenophoma mahoniae* A.W. Ramaley (CBS 388.92), *Kabatiella caulivora* (Kirchn.) Karak (CBS 242.64) and *Kabatiella microsticta* Bubák (CBS 114.64). Group 7, consisting of *Sydowia polyspora* (Bref. & Tavel) E. Müll., *Pringsheimia smilacis* E. Müll., *Delphinella strobiligena* (Desm.) Sacc. ex E. Müll. & Arx and *Dothichiza pithyophila* (Corda) Petr., formed a well supported,

**Table 1.** List of analysed strains subjected to DNA sequence analyses and morphological studies.

Taxon	Accession no.	dH number	Source	Geography	Collector	GenBank accession no. (LSU, ITS, BTUB, EF, ELO)
Group 1: <i>Aureobasidium pullulans</i> var. <i>pullulans</i>						
( <i>T. Dematioidium nigrescens</i> )	CBS 146.30	dH 15398	Slime flux of <i>Quercus</i> sp.	Germany, Ohlsdorf near Hamburg	–	FJ150916, FJ150902, FJ157871, –, FJ039824
( <i>T. Candida mallicola</i> )	CBS 701.76 = ATCC 11942		<i>Malus sylvestris</i> , fruit	–	–	FJ150951, FJ15090, FJ157865, –, FJ039834
( <b>NT</b> , var. <i>pullulans</i> )	CBS 584.75	dH 16041	<i>Vitis vinifera</i> , fruit	France, Beaujolais, Beaujeu	E. J. Hermandides-Nijthof	FJ150942, FJ150906, FJ157869, FJ157895, FJ039835
	CBS 109810	dH 12237	Chemobil radioactive wall	Ukraine, Kiev region	V. A. Zakharchenko	FJ150953, FJ150901, FJ157868, –, FJ039838
( <b>T</b> , var. <i>aubasidant</i> )	CBS 100524	–	<i>Betula</i> , slime flux	Russia, Leningrad Region	J. M. Voznjakovskaja	FJ150952, FJ150905, FJ157867, FJ157900, FJ039839
	CBS 100280	–	Hypersaline saltern water	Slovenia, Sečovlje saltens	P. Zalar	FJ150941, FJ150910, FJ157864, FJ157906, FJ039831
	EXF-915 = CBS 122385	dH 12636	Glacial ice from sea water	Norway, Svalbard, Conwaybreen, Kongsvegen	N. Gunde-Cimerman	FJ150947, FJ150911, FJ157870, FJ157899, FJ039830
	EXF-88 MZKI B-985	dH 16414	Hypersaline saltern water	Slovenia, Sečovlje saltens	P. Zalar	FJ150957, –, –, FJ157904, FJ039833
	EXF-150	dH 16416	Hypersaline saltern water	Slovenia, Sečovlje saltens	P. Zalar	FJ150915, FJ150908, –, FJ157905, FJ039832
	EXF-1668	dH 13836	Glacial ice from sea water	Norway, Svalbard, Conwaybreen, Kongsvegen	N. Gunde-Cimerman	FJ150949, FJ150900, FJ157875, FJ157898, FJ039827
	EXF-1702B	dH 13844	Glacial ice from sea water	Norway, Svalbard, Conwaybreen, Kongsvegen	N. Gunde-Cimerman	FJ150950, FJ150899, FJ157861, FJ157897, FJ039828
	EXF-2449	dH 13859	Glacial ice from sea water	Norway, Svalbard, Conwaybreen, Kongsvegen	N. Gunde-Cimerman	FJ150955, FJ150898, FJ157866, FJ157907, FJ039837
	MZKI B-700	dH 16413	Hypersaline saltern water	Slovenia, Sečovlje saltens	P. Zalar	FJ150956, FJ150909, –, FJ157903, FJ039826
	–	dH 13843	Subglacial ice from sea water	Norway, Svalbard, Conwaybreen, Kongsvegen	N. Gunde-Cimerman	FJ150954, FJ150904, FJ157876, FJ157896, FJ039829
	–	dH 12637	Glacial ice from sea water	Norway, Svalbard, Conwaybreen, Kongsvegen	N. Gunde-Cimerman	FJ150948, –, FJ157855, FJ157901, FJ039825

Table 1. (Continued).

Taxon	Accession no.	dH number	Source	Geography	Collector	GenBank accession no. (LSU, ITS, BTUB, EF, ELO)
<b>Group 2: <i>Aureobasidium pullulans</i> var. <i>melanogenum</i></b>						
( <i>T.</i> var. <i>melanogenum</i> )	CBS 105.22	dH 15197	–	–	–	FJ150926, FJ150886, FJ157858, FJ157887, FJ039812
( <i>AUT.</i> <i>Torula schoenii</i> )	CBS 123.37	dH 15346	–	–	–	FJ150917, FJ150881, FJ157852, –, FJ039818
	CBS 621.80	dH 16090	Deteriorated army supplies	Russia	–	FJ150921, FJ150885, FJ157859, FJ157890, FJ039813
	CBS 109800	dH 11797	Endoperitoneal fluid	Greece, Athens	–	FJ150925, FJ150880, FJ157851, –, FJ039814
	CBS 100225	dH 15131	Bathroom glass	Netherlands, Hilversum	G.S. de Hoog	FJ150923, FJ150890, FJ157854, –, –
	CBS 110373		Soil	Thailand	M. Sudhadham	FJ150928, FJ150887, –, FJ157888, FJ039810
	CBS 110374		Public fountain	Thailand, Bangkok	M. Sudhadham	FJ150929, FJ150888, –, FJ157886, FJ039808
	EXF-924	dH 13831	Ponds on sea ice	Norway, Svalbard, Kongsfjorden	N. Gunde-Cimerman	FJ150918, FJ150883, FJ157850, FJ157885, FJ039817
	EXF-926	dH 13840 =dH12625	Surface glacier ice	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150922, FJ150884, FJ157853, FJ157894, FJ039816
	EXF-3382	275-1-1	Deep sea (4500 m depth)	Japan	F. Abe	FJ150930, FJ150876, –, –, FJ039807
	EXF-3383	N11	Deep sea (4500 m depth)	Japan	F. Abe	FJ150931, FJ150877, –, FJ157884, FJ039806
	EXF-3384	671-3-PI	Deep sea (4500 m depth)	Japan	F. Abe	FJ150932, FJ150879, –, –, FJ039820
	EXF-3385	671-3-MI	Deep sea (4500 m depth)	Japan	F. Abe	FJ150933, FJ150878, –, –, FJ039821
	–	dH 12640	Fountain	Thailand	M. Sudhadham	FJ150919, FJ150889, FJ157857, FJ157889, FJ039809
	–	dH 12643	Air	Thailand	M. Sudhadham	FJ150924, FJ150882, FJ157856, FJ157892, FJ039815
	–	dH 12676	Soil	Thailand	M. Sudhadham	FJ150927, –, FJ157860, FJ157893, FJ039811
	–	dH 12740	–	Thailand	M. Sudhadham	FJ150920, FJ150874, FJ157862, FJ157891, FJ039819
<b>Group 3: <i>Aureobasidium pullulans</i> var. <i>subglaciale</i></b>						
	EXF-2479	dH 13860	Glacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150935, FJ150893, FJ157877, FJ157910, FJ039846
	= CBS 123388					
	EXF-2480	dH 13880	Subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150934, FJ150891, FJ157879, FJ157909, FJ039841
	EXF-2481	dH 13862	Subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150913, FJ150895, FJ157878, FJ157911, FJ039845
	= CBS 123387					
( <i>T.</i> var. <i>subglaciale</i> )	EXF-2491	dH 13865	Subglacial ice	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150936, FJ150894, FJ157880, FJ157902, FJ039844
	EXF-2510	dH 13868	Moulin	Norway, Svalbard, Conwaybreen	N. Gunde-Cimerman	FJ150938, –, –, –
	= CBS 123386					

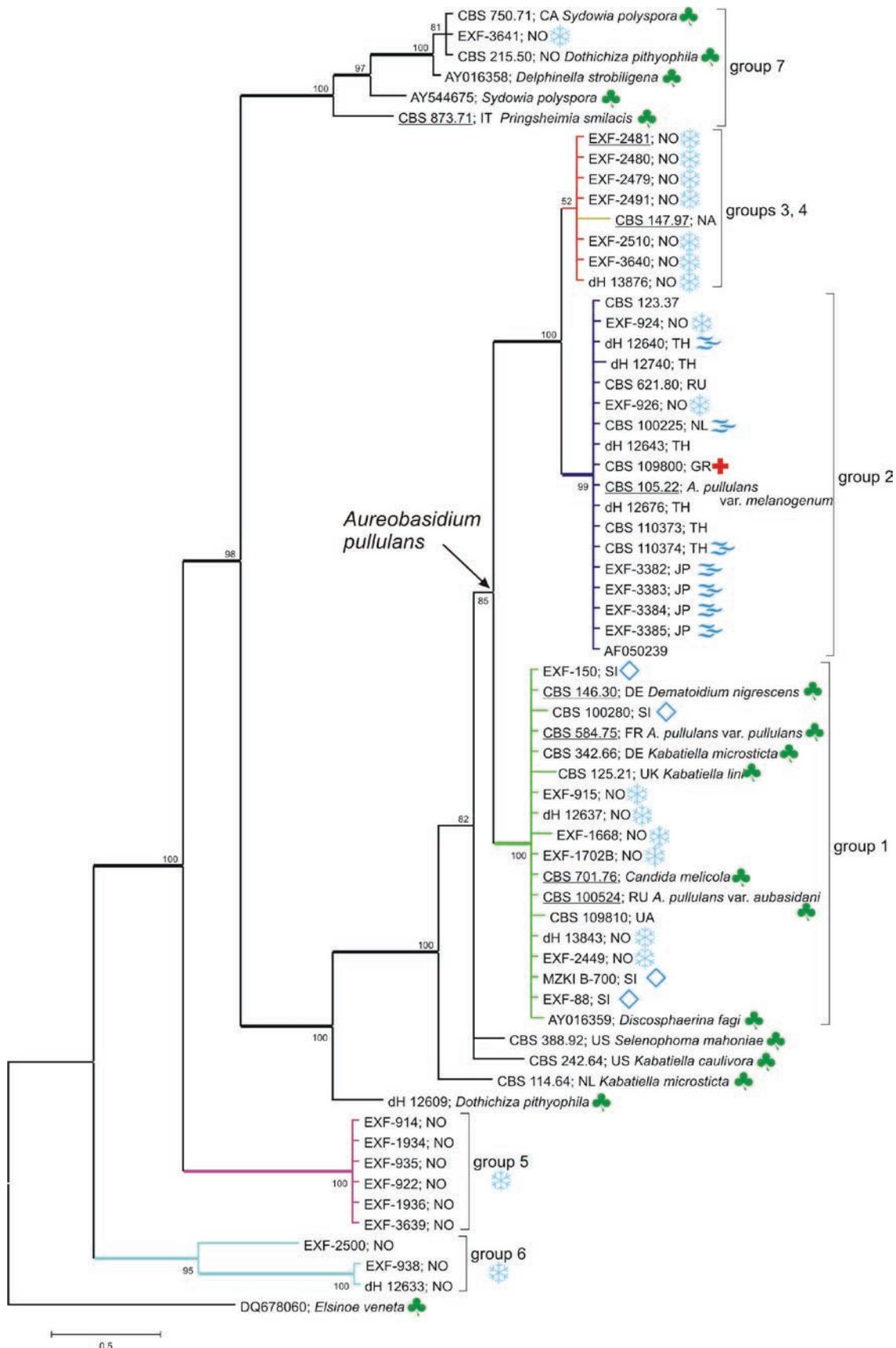
Table 1. (Continued).

Taxon	Accession no.	dH number	Source	Geography	Collector	GenBank accession no. (LSU, ITS, BTUB, EF, ELO)
	EXF-3640	dH 13864	subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150939, FJ150896, FJ157882, FJ157913, FJ039842
	–	dH 13876	coastal ponds of melted snow and ice	Norway, Svalbard, Kongsfjorden, Ny Alesund	N. Gunde-Cimerman	FJ150958, FJ150892, FJ157881, FJ157912, FJ039843
<b>Group 4: <i>Aureobasidium pullulans</i> var. <i>namibiae</i></b>						
( <i>T.</i> var. <i>namibiae</i> )	CBS 147.97	–	dolomitic marble	Namibia, Namib Desert	U. Wollenzien	FJ150937, FJ150875, FJ157863, –, FJ039822
<b>Group 5, Unnamed</b>						
	EXF-914 = CBS 122350	dH 12626 = 13830	subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150961, –, –, –, –
	EXF-3639 = CBS 122359	dH 13832	subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150966, –, –, –, –
	EXF-935 = CBS 122362	dH 12635	subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150963, –, –, –, –
	EXF-922	dH 12623	subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150964, –, –, –, –
	EXF-1934	dH 13842	subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150962, –, –, –, –
	EXF-1936	dH 13839	subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150965, –, –, –, –
<b>Group 6, Unnamed</b>						
	EXF-2500 = CBS 123390	dH 13881	glacial ice	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150914, –, –, –, –
	–	dH 12633			N. Gunde-Cimerman	FJ150960, –, –, –, –
	EXF-938 = CBS 123389	dH 12634	subglacial ice from sea water	Norway, Svalbard, Conwaybreen	N. Gunde-Cimerman	FJ150959, –, –, –, –
<b>Species included for comparison.</b>						
<i>Dothichiza pithyophila</i>	CBS 215.50	–	<i>Abies concolor</i> , dead bark	Norway	–	FJ150968, –, FJ157883, –, –
<i>Dothichiza pithyophila</i>	–	dH 12609	Vishniac Y-31	–	–	FJ150969, –, –, –, –

Table 1. (Continued).

Taxon	Accession no.	dH number	Source	Geography	Collector	GenBank accession no. (LSU, ITS, BTUB, EF, ELO)
<i>Dothichiza</i> sp.	EXF-3641 = CBS 122355	dH 13858	sea ice with sediment	Norway, Svalbard	N. Gunde-Cimerman	FJ150967, -, -, -, -
<i>Kabatella caulivora</i>	CBS 242.64	dH 15602	<i>Trifolium incarnatum</i>	U.S.A., Oregon	-	FJ150944, FJ150871, -, -, FJ039836
<i>Kabatella microsticta</i>	CBS 114.64 = MUCL 18713	dH 15299	<i>Hemerocallis</i> sp.	Netherlands, Wageningen	-	FJ150940, FJ150873, -, FJ157914, FJ039848
<i>Kabatella microsticta</i>	CBS 342.66	dH 15774	<i>Convallaria majalis</i> , dying leaf	Germany	W. Gams	FJ150945, FJ150903, FJ157872, -, FJ039823
<i>Kabatella lini</i>	CBS 125.21 T = MUCL 8712	dH 15350	<i>Linum usitatissimum</i>	U.K.	-	FJ150946, FJ150897, FJ157873, FJ157908, FJ039840
<i>Pringsheimia smilacis</i>	CBS 873.71 T	-	<i>Smilax aspera</i> , twig	Italy, Napoli	L. Froidevaux	FJ150970, -, -, -, -
<i>Selenophoma mahoniae</i>	CBS 388.92 T	dH 15823	<i>Mahonia repens</i> , leaf	U.S.A., Colorado	A.W. Ramaley	FJ150943, FJ150872, FJ157874, FJ157915, FJ039847
<i>Sydowia polyspora</i>	CBS 750.71	-	<i>Pinus strobus</i> , twig	Canada, Quebec; Normand Lac	E. Müller	FJ150912, -, -, -, -

\* Abbreviations used: ATCC = American Type Culture Collection; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; dH = de Hoog Culture Collection, CBS, Utrecht, The Netherlands; EXF = Culture Collection of Extremophilic Fungi, Ljubljana, Slovenia; MUCL = Mycothèque de l'Université catholique de Louvain; MZKI = Microbiological Culture Collection of the National Institute of Chemistry, Ljubljana, Slovenia; NT = ex-neotype strain; T = ex-type strain.



**Fig. 2.** Consensus phylogram (50 % majority rule) of 24000 trees resulting from a Bayesian analysis of the LSU sequence alignments using MrBayes v. 3.1.2. Bayesian posterior probabilities are indicated at the nodes, branches with posterior probabilities >95 in bold. The tree was rooted to the sequence of *Elsinoe veneta* (DQ678060). Ex-type and ex-neotype strains are underlined; when known origin two digit country codes are listed after strain numbers. The colour marks stand for:

- Green clover: plant associated;
- Blue snowflake: originating from Arctic ice;
- Light blue diamond: originating from hyperosmotic environment;
- Red cross: clinical strain.
- Light blue wave: water.

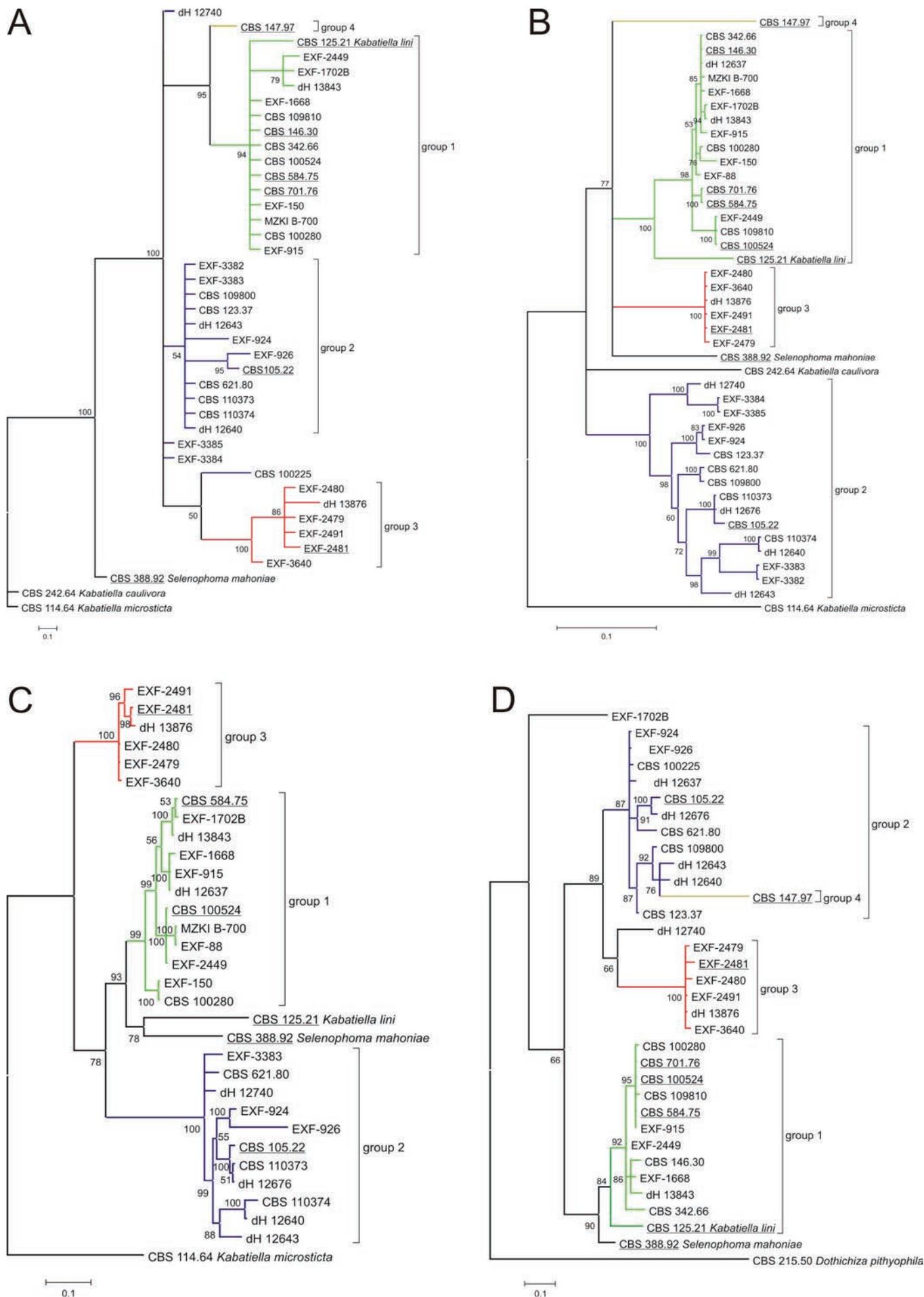
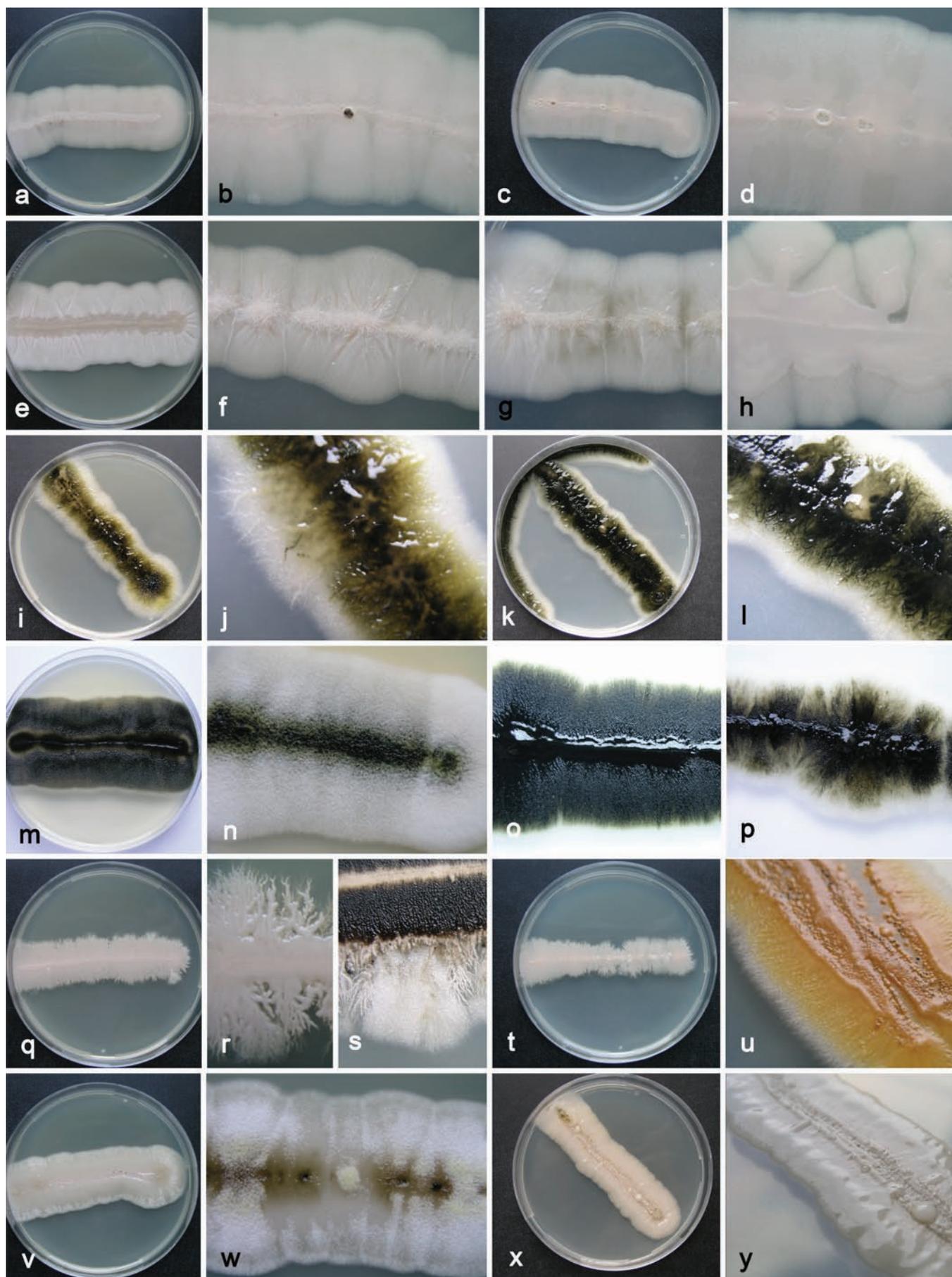


Fig. 3. Consensus phylogenetic trees (50 % majority rule) resulting from a Bayesian analysis of: A. ITS rDNA; B. elongase gene; C. translation elongation factor EF-1 $\alpha$  gene; D.  $\beta$ -tubulin gene. Bayesian posterior probabilities are indicated at the nodes. The trees are not rooted. Ex-type and ex-neotype strains are underlined.



**Fig. 4.** Macromorphology of different *Aureobasidium pullulans* varieties incubated for 7 d at 25 °C in the dark on MEA (left 2 columns) and on PDA (right two columns). **a–h.** *A. pullulans* var. *pullulans*: a, b. CBS 584.75 (MEA); c, d. CBS 584.75 (PDA); e. CBS 109810 (MEA); f, g. CBS 701.76 (MEA, PDA); h. MZKI B-700 (PDA). **i–p.** *A. pullulans* var. *melanogenum*: i, j. CBS 105.22 (MEA); k, l. CBS 105.22 (PDA); m. EXF-3382 (MEA); n. CBS 621.80 (MEA); o. EXF-924 (PDA); p. CBS 100225 (PDA). **q–u.** *A. pullulans* var. *subglaciale*: q, r. EXF-2481 (MEA); s. EXF-2481 after 14 d incubation (MEA); t. EXF-2481 (PDA); u. EXF-2479 (PDA). **v–y.** *A. pullulans* var. *namibiae*. v, w. CBS 147.97 (MEA); x, y. CBS 147.97 (PDA).

but separate, clade. Separate well-supported clades (groups 5 and 6) joined arctic strains of no affinity to any of the known taxa. Clade *Aureobasidium pullulans* was badly supported (85 posterior probability). Groups 1 and 2 within this clade were statistically supported, while groups 3 and 4 reached a poor posterior probability value. Group 1 contained the ex-neotype strain of *A. pullulans* var. *pullulans* (CBS 584.75), its supposed teleomorph *Discosphaerina* (*Columnosphaeria*) *fagi*, the ex-type strain of *Kabatiella lini* (CBS 125.21), the ex-type strain of *Dematoidium nigrescens* Stautz (CBS 146.30), the ex-type strain of *A. pullulans* var. *aubasidani* (CBS 100524), and a strain of *Kabatiella microsticta* (CBS 342.66). Another strain of *K. microsticta* was placed on the basal branch as the sister taxon of *K. caulivora* (CBS 242.64) and *Selenophoma mahoniae* (CBS 388.92). Group 2 contained the ex-type strain of *A. pullulans* var. *melanogenum*. Group 3 contained exclusively Arctic strains, while group 4 consisted of one strain only (CBS 147.97). Analyses of the more variable ITS spacers (Fig. 3A), and *ELO* (Fig. 3B), *EF1 $\alpha$*  (Fig. 3C), and *TUB* (Fig. 3D) introns and exons almost consistently supported the first three groups, with only a few exceptions. For example, several strains of group 2 were dispersed outside the clade of group 2 in ITS analysis, while in other analyses they formed a monophyletic group. In analyses of ITS and *ELO*, group 4 was supported, whereas based on *TUB* it was grouped together with group 2, but on a separate and long branch. The amplification of the *EF1 $\alpha$*  gene failed in the only strain of group 4; therefore, its phylogenetic position concerning this gene is unknown.

## Morphology

The main difference observed among isolates was pigmentation of cultures (Fig. 4). Strains belonging to groups 1 and 3 remained pinkish for at least 1 w. The majority of strains from group 1 became pigmented only after 3 wk, or even later. The only exception among this group was the ex-type strain of *Dematoidium nigrescens* (CBS 146.30), which was darkly pigmented already after 1 wk of incubation due to melanised septated hyphae. This was also the only strain that was exclusively filamentous and formed no conidia. Strains of group 3 became melanised only at the margin, where dark pigmented, heavily branched hyphae developed, while the colony centre remained pinkish at least for 3 wk. All strains of group 2 were green or black after 7 d of incubation, due to the production of melanised hyphae and conidia. Structures of cultures differed within different varieties, from almost entirely yeast-like to entirely filamentous, and also yeast-like with marginal or central aerial mycelium. Marginal areas of colonies were significantly different in at least two pink pigmented groups, consisting of arachnoid mycelium in group 1 and of thick and undulating hyphae in group 3. Conidiogenesis seen in the groups of *A. pullulans* (groups 1–4) studied was synchronous, either on rather undifferentiated short denticles, intercalary or terminally on hyaline (groups 1, 3, 4) or melanised (group 4) hyphae. This kind of conidiogenesis was also seen on enlarged globose conidiogenous areas that developed laterally on hyphae, giving rise to multiple conidia, or on enlarged yeast cells, which were synchronously budding from both poles. Synchronous conidiogenesis was sometimes difficult to observe due to heavy yeast proliferation. More complex conidiophore-like structures were noted in group 3. Another mode of conidiation seen in all varieties was percurrent conidiogenesis alongside the hyphae. Conidia formed either synchronously or percurrently, and were budding secondarily in all groups; therefore, the size and shape

of conidia in *Aureobasidium* in general was very variable. Conidia in groups 1 and 4 were almost exclusively non-pigmented, while in group 2, as well as 1-celled non-pigmented conidia, also melanised 1–2-celled conidia were also abundant. Pigmented conidia were also seen with the strain CBS 100524, the ex-neotype strain of *A. pullulans* var. *aubasidani*. Endoconidia were seen in only some strains of groups 1 and 3.

## TAXONOMY

***Aureobasidium pullulans*** (de Bary) G. Arnaud var. ***pullulans*** – Annales École Nat. Agric. Montpellier 16: 39, 1918. MycoBank MB101771). Fig. 5.

*Synonyms:* *Dematium pullulans* de Bary 1884 (MB 219317; NT = CBS 584.75)

*Aureobasidium pullulans* (de Bary) Arn. var. *aubasidani* Yurlova in Yurlova & de Hoog 1997 (MB 442903; T = CBS 100524)

*Candida malicola* D.S. Clark & R.H. Wallace 1955 (MB 294033; T = CBS 701.76)

*Dematoidium nigrescens* Stautz 1931 (MB 272259; T = CBS 146.30)

*Cultural characteristics:* Colonies on MEA/PDA at 25 °C attaining about 40/30 mm diam after 7 d, appearing smooth and slimy due to abundant sporulation, pinkish (pinkish white, 7A2) to yellowish (light yellow, 3A4), reverse yellowish (pale yellow (4A3) to light yellow (4A4)). Black sectors composed of dark pigmented hyphae or conidia develop in some isolates after 14 d. Margin composed of arachnoid mycelium, sometimes in sectors. No aerial mycelium.

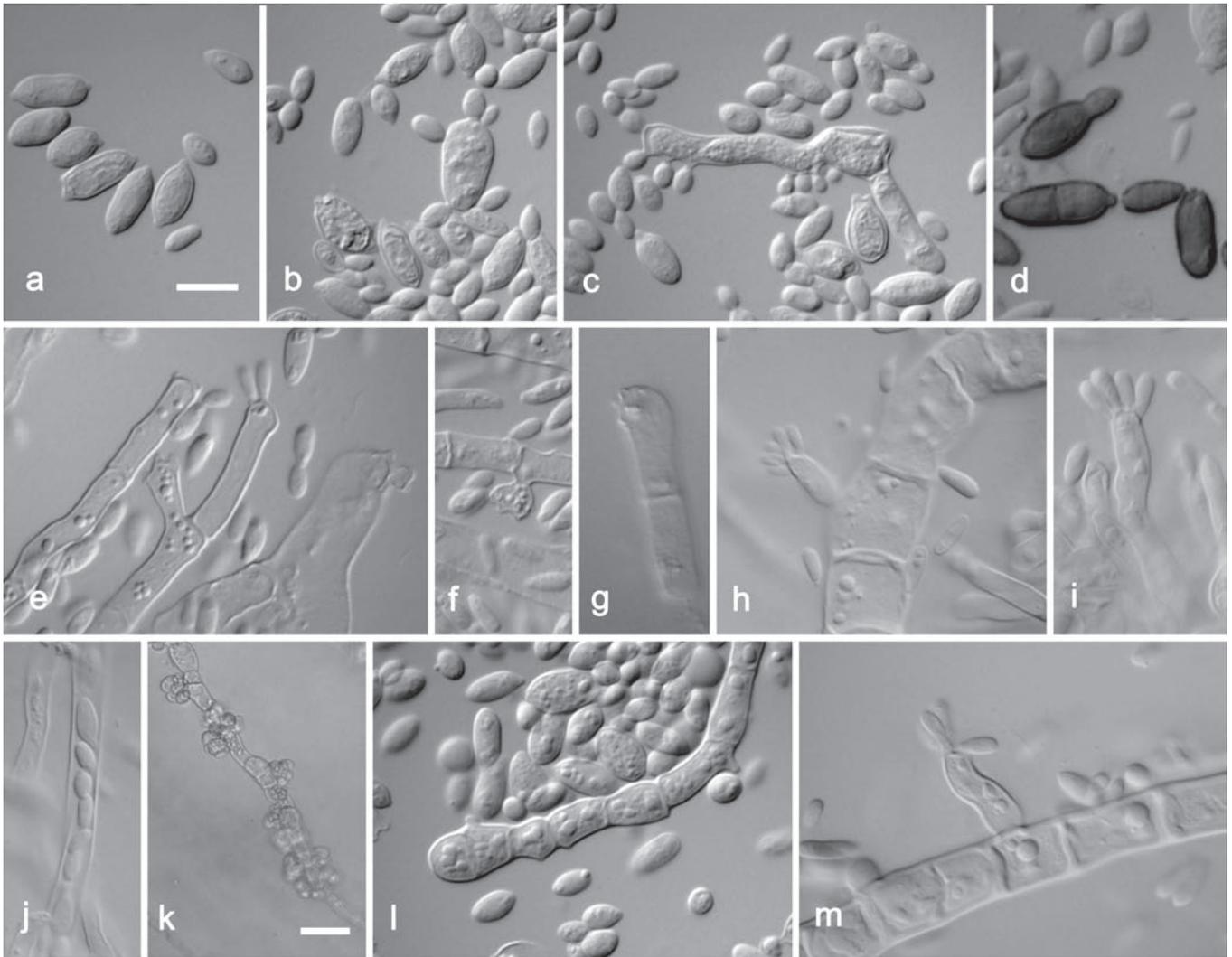
*Deviations:* White aerial mycelium at the edge of cultures present in some strains (CBS 109800, EXF-915), some strains entirely filamentous (dH 12637), some develop white, setae-like mycelial formations in colony centre and marginal leathery mycelium (CBS 701.76). Strain CBS 146.30 was black and filamentous already after 1 wk of incubation.

*Microscopy:* Vegetative hyphae hyaline, smooth, thin-walled, 4–12  $\mu$ m wide, transversely septate, in older cultures sometimes locally converted to dark-brown hyphae. Conidiogenous cells undifferentiated, intercalary or terminal on hyaline hyphae. Conidia produced synchronously in dense groups from small denticles, and also formed percurrently on short lateral denticles. Conidia hyaline to dark brown. Hyaline conidia one-celled, smooth, ellipsoidal, very variable in shape and size, 7.5–16  $\times$  3.5–7  $\mu$ m, often with an indistinct hilum. Dark brown conidia (measured in strain CBS 100524, developed after 2 wk) 1–2 celled, one celled 10–17  $\times$  5–7  $\mu$ m, two celled slightly constricted at septum, 14–25  $\times$  5–11  $\mu$ m. Budding of hyaline and dark brown conidia frequently seen, with the secondary conidia being smaller than the primary ones. Conidia in old cultures transfer to globose, brownish structures of 10–15  $\mu$ m diam. Endoconidia, about 6  $\times$  3  $\mu$ m occasionally seen in intercalary cells.

*Maximum tolerated salt concentration:* 15 % NaCl.

*Cardinal temperatures:* Minimum at 4 °C, optimum at 25 °C, maximum at 30 °C.

*Specimens examined:* **France**, fruit of *Vitis vinifera*, 1974, coll. and isol. E.J. Hermandes-Nijhof, ex-neotype culture CBS 584.75; for additional specimens, see Table 1.



**Fig. 5.** *Aureobasidium pullulans* var. *pullulans*. a. Liberated conidia transforming to budding cells. b. Synchronous production of conidia on a transformed conidium – yeast cell. c. Short hypha synchronously producing conidia. d. Dark brown conidia. e–i. Poorly differentiated, terminal and intercalary conidiophors performing synchronous conidiation. k. Immersed hypha with lateral accumulation of conidia. l. Hypha with lateral scars – conidiogenous loci. j. Endoconidia. a–c, e–g, k–m. CBS 584.75 (ex-neotype strain); d. CBS 100524; h–i, j, m. EXF-1702B. Scale bars: a–j, l–m= 10  $\mu$ m; k= 20  $\mu$ m.

***Aureobasidium pullulans*** (de Bary) G. Arnaud var. ***melanogenum*** Hermanides-Nijhof – Stud. Mycol. 15: 161, 1977. MycoBank MB352628. Fig. 6.

Synonyms: *Torula schoenii* Roukhelman 1937 (MB 445735; AUT = CBS 123.37) (Invalid; Art. 37 ICBN)

*Pullularia fermentans* Wynne & Gott var. *schoenii* (Roukhelman) Wynne & Gott 1956 (MB 352450)

*Aureobasidium pullulans* (de Bary) G. Arnaud var. ***melanogenum*** Hermanides-Nijhof 1977 (MB 352628; T = CBS 105.22)

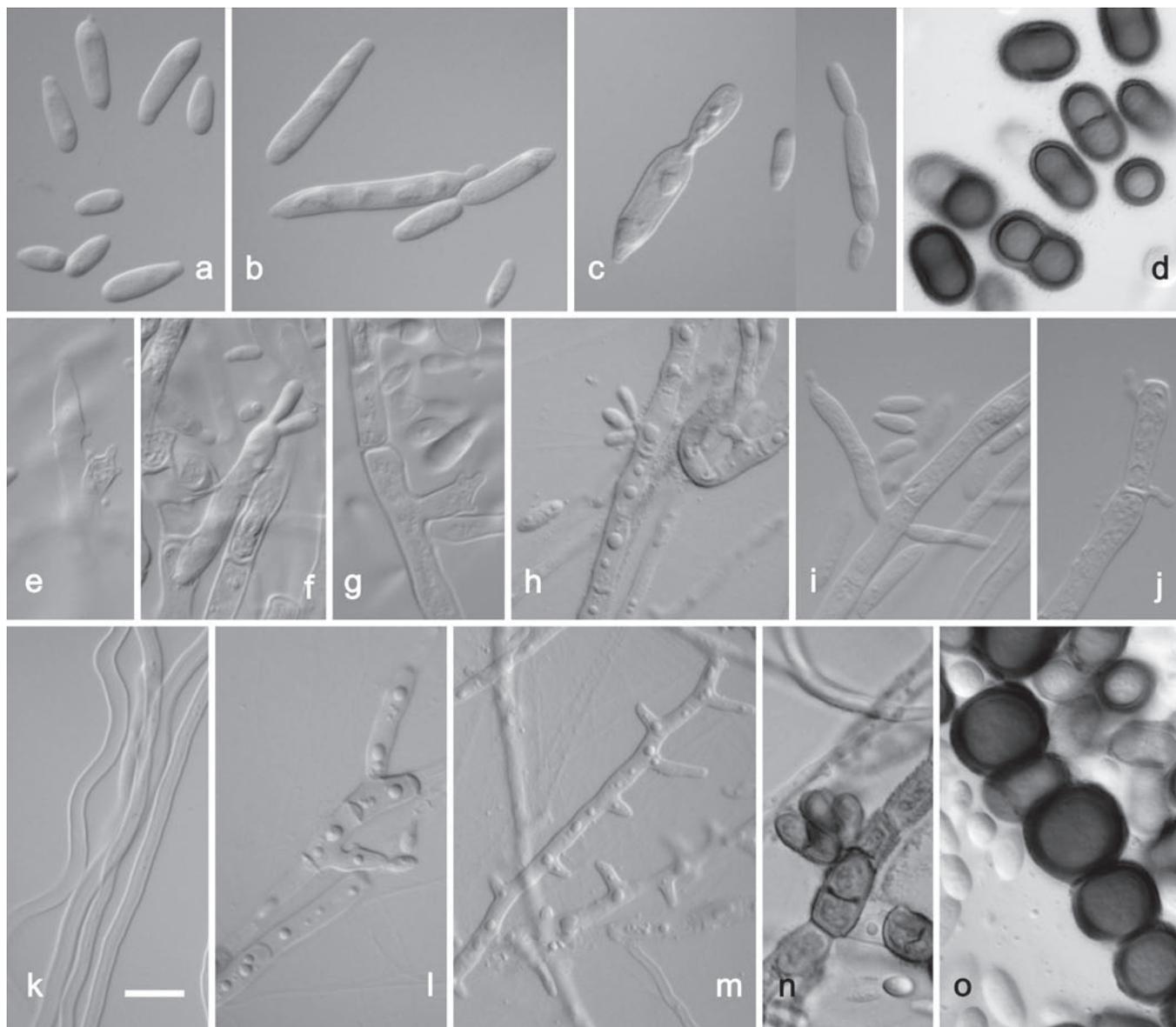
**Cultural characteristics:** Colonies on MEA/PDA at 25 °C attaining 25 mm diam after 7 d, appearing smooth and slimy due to abundant sporulation and EPS formation, olive brown (4F3–4F8) to black in centre, towards margin mustard yellow (3B6), margin yellowish white (3A2); reverse olive-grey (3E2) at the centre, towards margin dull yellow (3B4), at the margin yellowish white (3A2). Margin composed of arachnoid to thick undulating hyphae growing into the agar, sometimes sectorial. After 14 d the entire colonies are green to black. Aerial mycelium develops in some parts of the colonies. Deviations: White aerial mycelium present in strain CBS 621.80.

**Microscopy:** Vegetative hyphae in the central part of colonies, dark brown, smooth to slightly roughened, thick walled, 6–12  $\mu$ m

wide, transversely septate, constricted at septa, embedded in EPS, disarticulating to 1–2-celled, dark brown chlamydospores, one celled 13–16  $\times$  8–12  $\mu$ m, two celled 17–24  $\times$  10–12  $\mu$ m. Vegetative hyphae at colony edge hyaline, smooth, thin-walled, 2–10  $\mu$ m wide, transversely septate, getting thicker and darker with age. Immersed hyphae with multiple lateral pegs. Conidiogenous cells undifferentiated, intercalary or terminal on hyaline hyphae, sometimes grown in the form of an outgrowth with three denticles. Conidia produced synchronously in dense groups from small denticles (1.0–2.5  $\mu$ m long), and also formed percurrently alongside hyphae and on short lateral branches. Conidia hyaline and dark brown. Hyaline conidia one-celled, smooth, ellipsoidal, very variable in shape and size, 8–30  $\times$  3.5–5  $\mu$ m, often with an indistinct hilum. Dark brown conidia 1–2-celled, smooth, ellipsoidal when one celled, 7  $\times$  6  $\mu$ m, slightly constricted at septa when two celled, 12–20  $\times$  4–12  $\mu$ m. Unilateral and bilateral budding of hyaline conidia frequently seen, with the secondary conidia being smaller than the primary ones. Endoconidia not seen.

**Maximum tolerated salt concentration:** 10 % NaCl.

**Cardinal temperatures:** Minimum at 10 °C, optimum at 30 °C, maximum at 35 °C.



**Fig. 6.** *Aureobasidium pullulans* var. *melanogenum*. a–c. Liberated conidia transforming to budding cells. d. Dark brown conidia. e–h. Poorly differentiated, terminal and intercalary conidiophors performing synchronous conidiation. i, l. Hypha with long lateral conidiogenous cells. j. Hypha with prolonged lateral pegs. k. Vegetative hyphae. m. Immersed hyphae with multiple lateral pegs. n. Melanized hyphae with intercalary synchronous conidiogenesis. o. Melanized hyphae / chlamydospores. a–c, e–n. CBS 105.22 (ex-type strain); d, o. EXF-926. Scale bar: as marked on k (a–o) = 10 µm.

*Specimens examined:* **Unknown**, culture ex-type CBS 105.22 = ATCC 12536 = CECT 2658 = IMI 062460 = NRRL Y-7469, isolated by M. Church; additional specimens see Table 1.

***Aureobasidium pullulans* (de Bary) G. Arnaud var. *subglaciale*** Zalar, de Hoog & Gunde-Cimerman, **var. nov.** MycoBank MB512380. Fig. 7.

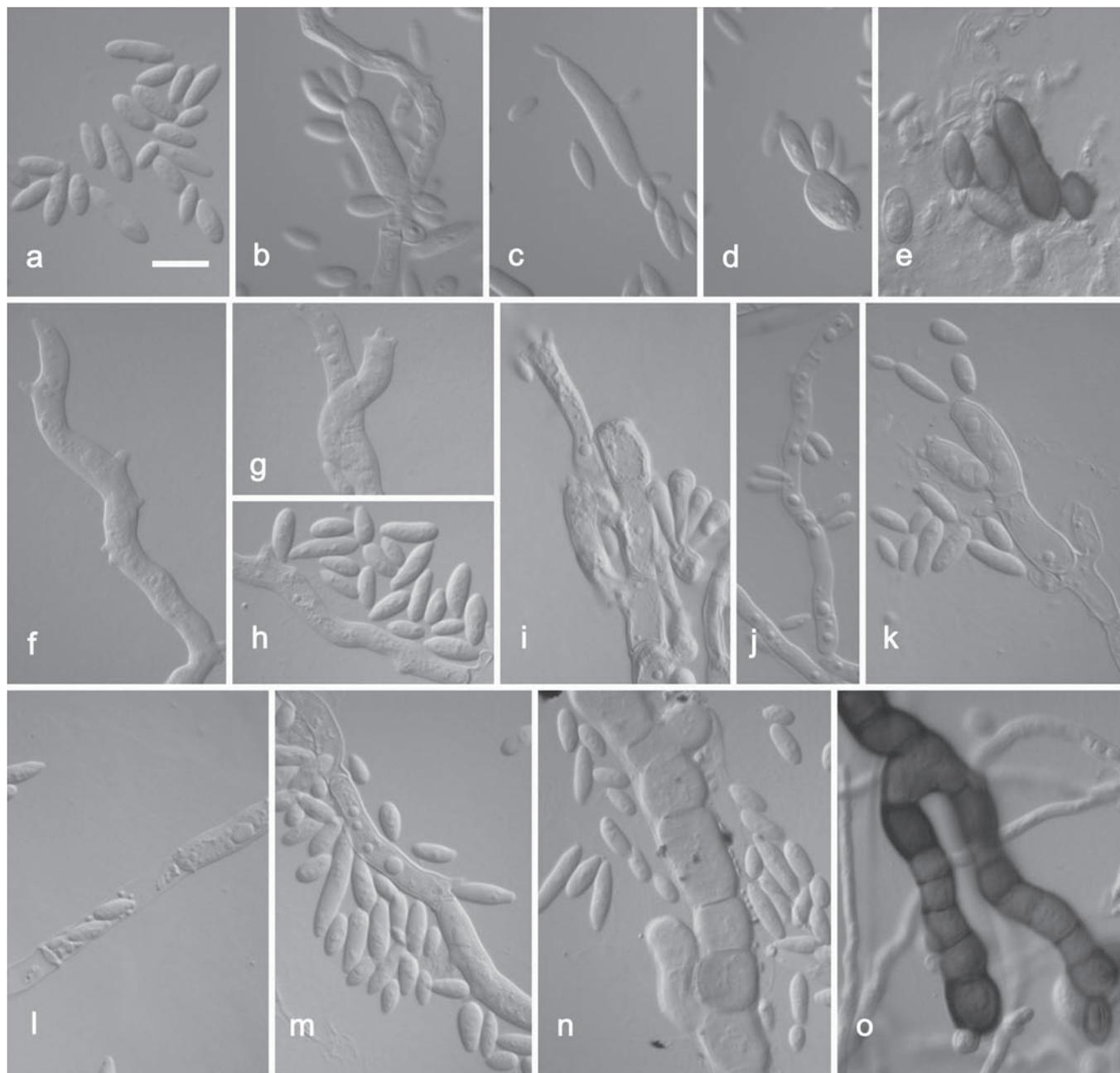
Coloniae in agar MEA vel PDA 25 °C ad 20 mm diam post 7 dies, leves, haud lucidae, copiose sporulantes, roseae, reverso dilute aurantiaco; post 15 dies in medio roseae, marginem versus obscure brunneae; hyphae marginales superficiales latae, undulantes, nonnumquam sectores formantes; hyphae aerae absentes. Hyphae vegetativae hyalinae, leves, tenuitunicatae, 2–10 µm latae, in coloniis vetustis nonnumquam hyphae fuscae, crassitunicatae, 5–9 µm latae. Conidia hyalina vel fusca, hyalina unicellularia, levia, ellipsoidea, forma magnitudineque variabilissima, 5.5–28 × 2–6.5 µm, fusca 1- vel bicellularia, 8–16(–25) × 5–9 µm. Conidia saepe gemmantia, secundaria primariis minora; endoconidia circa 8 × 3 µm nonnumquam in cellulis intercalaribus formata. Temperatura optima et maxima crescentiae 25 °C.

*Holotype:* CBS H-20186

*Cultural characteristics:* Colonies on MEA/PDA at 25 °C attaining 20 mm (10–35 mm) diam after 7 d, appearing smooth and matt due to abundant sporulation, pinkish (pinkish white, 7A2), reverse pale orange (5A3). After 14 d central areas of colonies remain pinkish, towards the margin becoming dark-brown (greyish brown, 5F3). Margin composed of thick undulating superficial and immersed branched hyphae, sometimes with sectors. Aerial mycelium absent.

*Deviations:* Culture EXF-2479 develops more intensively pigmented colonies than others, pink in centre and yellowish orange towards the colony margin on MEA, and golden-yellow on PDA.

*Microscopy:* Vegetative hyphae hyaline, smooth, thin-walled, 2–10 µm wide, transversely septate, in older cultures locally converted to dark brown, thick-walled hyphae of 5–9 µm diam. Conidiogenous cells mostly undifferentiated, intercalary or terminal on hyaline hyphae, sometimes developed in clusters as conidiophore-like structure. Conidia produced synchronously in dense groups from small denticles, and also percurrently on short lateral branches. Conidia hyaline to dark brown. Hyaline conidia one-celled, smooth,



**Fig. 7.** *Aureobasidium pullulans* var. *subglaciale*. a. Conidia. b–d. Budding conidia. e. Dark brown conidia. f, g. Hyphae with multiple lateral pegs, which develop into synchronous conidiation apparatus. h, m. Hyphae with lateral pegs. i, k. Conidiophore-like structure synchronously producing conidia. l. Endoconidia. n. Hyaline vegetative hyphae. o. Melanized hyphae. a–o. EXF-2481 (ex-type strain). Scale bar: as marked on a (a–o) = 10  $\mu$ m.

ellipsoidal, very variable in shape and size, 5.5–28  $\times$  2–6.5  $\mu$ m, often with an indistinct hilum. Dark conidia 1–2-celled, one celled 8–16  $\times$  5–9  $\mu$ m, two celled 9–25  $\times$  5.5–7.5  $\mu$ m. Budding frequently seen, with secondary conidia being smaller than the primary ones. Endoconidia, about 8  $\times$  3  $\mu$ m, sometimes present in intercalary cells.

*Maximum tolerated salt concentration:* 10 % NaCl.

*Cardinal temperatures:* Minimum at 4  $^{\circ}$ C, optimum and maximum at 25  $^{\circ}$ C.

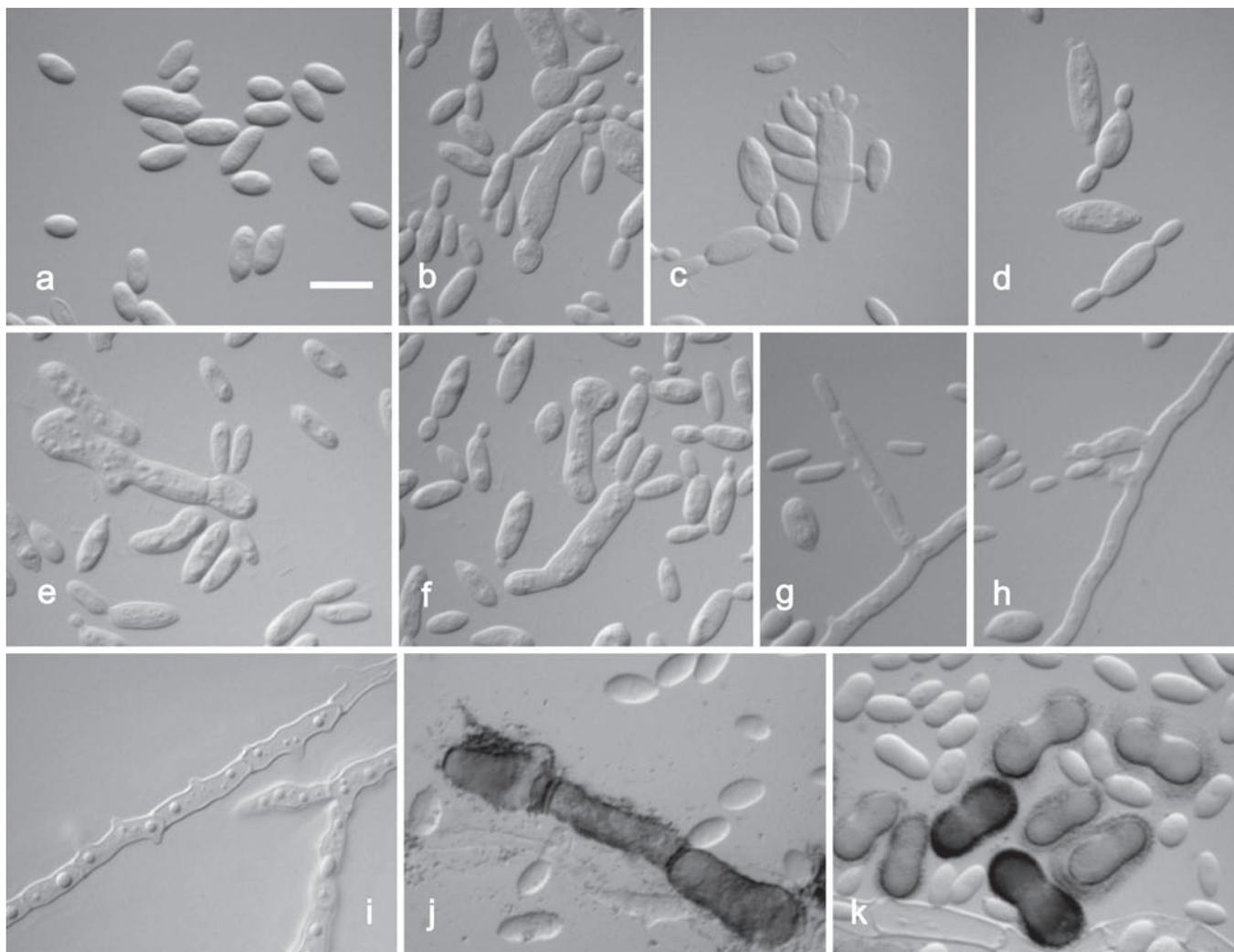
*Specimen examined:* Norway, Spitsbergen, subglacial ice from sea water, 2003, coll. and isol. N. Gunde-Cimerman, Holotype CBS H-20186, culture ex-neotype EXF-2481 = CBS 123387; additional specimens see Table 1.

***Aureobasidium pullulans* (de Bary) Arnaud var. *namibiae* Zalar, de Hoog & Gunde-Cimerman, var. nov.** MycoBank MB512381. Fig. 8.

Coloniae in agaro MEA 25  $^{\circ}$ C ad 25 mm diam post 7 dies, leves, lucidae textura coriacea, roseae, in medio brunneae, margine hyphis aereis albae, reverso griseo-luteo; coloniae in agaro PDA post 7 dies ad 20 mm diam, leves, sporulatione copiosa lucidae, in medio aurantio-albae olivascentes, nonnumquam hyphales et setosae, reverso armeniaco. Hyphae aerae absentes. Hyphae vegetativae hyalinae, leves, tenuitunicatae, 2–13  $\mu$ m latae, in coloniis vetustis nonnumquam hyphae fuscae. Conidia hyalina vel fusca, hyalina unicellularia, levia, ellipsoidea, forma magnitudineque variabilissima, 7–17  $\times$  3.5–7  $\mu$ m, fusca 1- vel bicellularia, 8–13(–24)  $\times$  5–9(–10)  $\mu$ m, saepe granulic ectoplasmaticis circumdata. Conidia saepe gemmantia, secundaria primariis minora; endoconidia haud visa. Temperatura optima crescentiae 25  $^{\circ}$ C, et maxima 30  $^{\circ}$ C.

*Holotype:* CBS H-20184

*Cultural characteristics:* Colonies on MEA at 25  $^{\circ}$ C attaining 25 mm



**Fig. 8.** *Aureobasidium pullulans* var. *namibiae*. a. Conidia. b–f. Liberated conidia transforming to budding cells. g–h. Hypha with long lateral conidiogenous cells. i. Immersed hyphae with multiple lateral pegs. j. Melanized hypha surrounded by EPS. k. Hyaline and dark brown conidia. a–k. CBS 147.97 (ex-type strain). Scale bar: as marked on a (a–k) = 10  $\mu$ m.

diam after 7 d, appearing smooth and shiny due to the leathery structure of colonies, pinkish (pinkish white, 7A2) with brownish (greyish brown, 5E3) central part, margin white (5A1), reverse yellowish (greyish yellow, 4B4); margin composed of superficial aerial mycelium. Colonies on PDA at 25 °C attaining 20 mm diam after 7 d, appearing smooth and shiny due to abundant sporulation, orange-white (5A2) with olive-brown (4F3) centre, sometimes hyphal and with setae, reverse apricot (orange white, 5A2). No aerial mycelium.

**Microscopy:** Vegetative hyphae hyaline, smooth, thin-walled, 2–13  $\mu$ m wide, transversely septate, locally converted to dark brown, thick-walled hyphae. Conidiogenous cells undifferentiated, intercalary or terminal on hyaline hyphae and on larger transformed conidia. Conidia produced synchronously in dense groups from small denticles, later formed percurrently on short lateral branches. Conidia hyaline and dark brown. Hyaline conidia one celled, smooth, ellipsoidal, very variable in shape and size, 7–17  $\times$  3.5–7.0  $\mu$ m, often with an indistinct hilum. Dark brown conidia 1–2-celled, one celled 8–13  $\times$  5–9  $\mu$ m, two celled 8–24  $\times$  2–10  $\mu$ m, surrounded by granular EP; if two-celled, constricted at the septum. Budding frequently seen, with secondary conidia smaller than the primary ones. Endoconidia not seen.

**Maximum tolerated salt concentration:** 10 % NaCl.

**Cardinal temperatures:** Minimum at 10 °C, optimum at 25 °C and maximum at 30 °C.

**Specimen examined:** Namibia, dolomitic marble in Namib Desert, 1997, coll. and isol. U. Wollenzien, holotype CBS H-20184, culture ex-type CBS 147.97.

## DISCUSSION

The elongase-encoding gene (*ELO*) was used as phylogenetic marker for the first time. Southern blotting of *A. pullulans* genomic DNA did not suggest the existence of more than one copy of the elongase gene in the genome of *A. pullulans*, while this is the case in other fungi (Gostinčar *et al.* 2008); this would have diminished its value for routine studies. The gene provided excellent resolution of the *Aureobasidium* complex and thus could reliably be used for tree reconstruction.

The anamorph genus *Aureobasidium* phylogenetically belongs to *Ascomycota*, order *Dothideales*, family *Dothideaceae* (Schoch *et al.* 2006). The fungi have been known since the late 19<sup>th</sup> century, when Viala & Boyer (1891) described *A. vitis* as a common coloniser of the sugary surface of grapes (*Vitis vinifera*). Type material is not known to be preserved. In her revision of the genus, Hermanides-Nijhof (1977) neotypified *Dematium pullulans* De Bary (1884) with CBS 584.75, thus establishing *A. pullulans* as the oldest name for

the type species of *Aureobasidium*. The genus was circumscribed using criteria of conidiogenesis, *i.e.*, synchronous holoblastic conidium production. This feature is also known in sporodochial *Kabatiella* species forming defined leaf spots on specific host plants. When these fungi are cultured, the sporodochia fall apart, and the micromorphology becomes very similar to that of *Aureobasidium pullulans*. For this reason, Hermanides-Nijhof (1977) classified all *Kabatiella* species in *Aureobasidium*, even though most *Kabatiella* species have not been cultured and are only known from the sporodochial anamorph on the host plant. LSU sequences of the few species thus far available for study indeed show affinity to *A. pullulans*.

*Kabatiella zae* Narita & Y. Hirats. (Hermanides-Nijhof 1977) is found in isolated positions away from other aureobasidia (de Hoog *et al.* 1999, Yurlova *et al.* 1999). Synchronous conidiation is thus polyphyletic. However, in addition to molecular differences for some species, morphological distinctions may also be possible, since most *Kabatiella* species have sickle-shaped conidia, such as *K. caulivora*, *K. harpospora* (Bres. & Sacc.) Arx, *K. phoradendri* (Darling) Harvey f. *umbellulariae* Harvey, and *K. zae*. In *Kabatiella lini*, a species clustering within *A. pullulans* (Fig. 2), the conidia have similar shape, but are slightly larger than in the var. *pullulans*. *Kabatiella microsticta*, *K. caulivora* and another, related pycnidial fungus, *Selenophoma mahoniae* deviate in all of the genes studied at the variety level. Thus, they might be regarded as separate varieties of *A. pullulans*, but the possibility cannot be excluded that unintentionally the ubiquitous phyllosphere fungus *A. pullulans* was isolated instead of the pathogen. It appears likely that the plant-invading, host-specific pathogens are consistently different from *A. pullulans*, which on host plants colonises surfaces only, but unambiguously identified strains are needed to prove this.

Our multilocus analysis shows that *Aureobasidium pullulans* consists of three robust main groups, two of which have high statistic support in LSU and show the same topology with all of the genes sequenced. The ex-neotype of the species, CBS 584.75, is in group 1, *A. pullulans* var. *pullulans*. This group also contains CBS 146.30, the ex-type strain of *Dematoideum nigrescens* Stautz, CBS 701.76, the ex-type strain of *Candida malicola* D.S. Clark & R.H. Wallace, and CBS 100524, the ex-type strain of *A. pullulans* var. *aubasidani* Yurlova, which should thus be regarded as synonyms. The production of aubasidan rather than pullulan as the main extracellular exopolysaccharide (Yurlova & de Hoog 1997) is apparently strain dependent. Although the production of EPS and other previously described diagnostic characters for this variety were not evaluated in this study, we believe that used multilocus approach as molecular diagnostic tool would show the difference of var. *aubasidani* to other varieties. The ex-type strain of *Dematoideum nigrescens* (CBS 146.30) was the only initially darkly pigmented strain in group 1, which is probably due to its degeneration. The var. *pullulans*, which is newly defined, is phenetically characterised by rapidly expanding, pinkish cultures that can develop radial dark brown sectors due to the local presence of thick-walled, melanised hyphae. Most isolates attributed to this variety originate from sugary or osmotically fluctuating habitats, such as saline water in the salterns, tree slime flux, fruit surfaces and phyllosphere (Table 1). This well supported variety was obtained pan-globally from temperate to tropical habitats, and was also found trapped in Spitsbergen glaciers and in ice released from these glaciers into the sea water. Its distribution is wide, ranging from the Arctic to the Mediterranean coast. Given the small degree of diversity with *TUB*, *ELO* and *EF1 $\alpha$* , the taxon can be regarded as being relatively recent.

Group 2, *A. pullulans* var. *melanogenum* contains CBS 105.22, the ex-type strain of this variety, and an authentic strain, CBS 123.37 with the invalid description of *Torula schoenii* Roukheldman. Earlier data (Yurlova *et al.* 1995) suggested that this taxon cannot be distinguished from var. *pullulans*, but current sequence data show that the groups are strictly concordant. Cultures are characteristically black from the beginning. They produce an abundance of dark, ellipsoidal conidia, which can either originate from disarticulating hyphae (arthroconidia) or transfer from hyaline conidia. The hyaline conidia are ellipsoidal and emerge from inconspicuous scars alongside undifferentiated hyphae; the process of conidiogenesis is synchronous in addition to percurrent, the latter being identical to that in the anamorph genus *Hormonema*. The sources of isolation of the strains of this variety, as far as is known, are low-nutrient, mostly low-strength environments, such as moist metal and glass surfaces, showers, fountains, as well as ocean water. Only one strain of this variety was retrieved from a human patient, but it is also possible that this was a culture contaminant, since it is often reported in air, especially in warmer climates (Punnapayak *et al.* 2003). Strains of this variety have a world-wide distribution, from the Arctic to the tropics. Given its marked diversity with *TUB*, *ELO* and *EF1 $\alpha$* , this may be an ancestral taxon, the introns having accumulated more mutations than var. *pullulans*.

Group 3, *A. pullulans* var. *subglaciale* Zalar *et al.* is exclusively known from Kongfjorden glacial and subglacial ice and sea water. Its psychrotolerant nature is in line with its active metabolism under conditions of permanently cold in Arctic glaciers.

Group 4 consists of a single isolate, CBS 147.97, the ex-type of the monotypic variety *A. pullulans* var. *namibiae*, isolated from marble in Namibia, Africa. The strain takes an isolated position with all sequenced genes, but has not drifted far away from the ancestral variety.

Other related groups are 5 and 6, which are aureobasidium-like but consistently different. Strains of these groups thus far have only been recovered from glacial ice in Spitsbergen. The species occurred with very high densities in subglacial ice in microchannels, and in gypsum-rich ice at high pH. During their travel through the glacier, these cells have been subjected to extreme variations in  $a_w$  due to ice freezing and thawing. These conditions are highly selective, for which reason this entity is likely to be restricted to small endemic areas, such as Kongsfjorden (Skidmore *et al.* 2005). The description as novel species will be the subject of a later paper.

The overall phylogenetic structure of *A. pullulans* suggests that the species is strictly clonal. A possible teleomorph, *Discosphaerina fagi*, has been suggested on the basis of ITS sequence similarity (de Hoog *et al.* 2000), but this finding awaits confirmation with multilocus analysis and re-isolation from single ascospores.

The varieties of *Aureobasidium pullulans* are markedly different for melanin production. This can be of biotechnological interest, since the organism is highly significant for its pullulan and aubasidan production (Yurlova & de Hoog 1997). Melanin contamination leads to low pullulan quality. Attempts have been made to grow non-pigmented yeast cells, *e.g.* by culturing *A. pullulans* in a two-stage fermentation process in media with a special nutrient combination (Shabtai & Mukmenev 1995), or with melanin-deficient mutants (Gniewosz & Duskiewicz-Reinhard 2008). From the present study, it is apparent that the use of strains of the variety *pullulans* is recommended.

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