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Polyphasic taxonomy of the heat resistant ascomycete genus *Byssochlamys* and its *Paecilomyces* anamorphs

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Key words

Byssochlamys
emodin
Eurotiales
extrolites
heat resistance
mycophenolic acid
Paecilomyces
patulin

Abstract *Byssochlamys* and related *Paecilomyces* strains are often heat resistant and may produce mycotoxins in contaminated pasteurised foodstuffs. A comparative study of all *Byssochlamys* species was carried out using a polyphasic approach to find characters that differentiate species and to establish accurate data on potential mycotoxin production by each species. Phylogenetic analysis of the ITS region, parts of the β-tubulin and calmodulin genes, macro- and micromorphological examinations and analysis of extrolite profiles were applied. Phylogenetic analyses revealed that the genus *Byssochlamys* includes nine species, five of which form a teleomorph, i.e. *B. fulva*, *B. lagunculariae*, *B. nivea*, *B. spectabilis* and *B. zollerniae*, while four are asexual, namely *P. brunneolus*, *P. divaricatus*, *P. formosus* and *P. saturatus*. Among these, *B. nivea* produces the mycotoxins patulin and byssochlamic acid and the immunosuppressant mycophenolic acid. *Byssochlamys lagunculariae* produces byssochlamic acid and mycophenolic acid and thus chemically resembles *B. nivea*. Some strains of *P. saturatus* produce patulin and brefeldin A, while *B. spectabilis* (anamorph *P. variotii* s.s.) produces viriditoxin. Some micro- and macromorphological characters are valuable for identification purposes, including the shape and size of conidia and ascospores, presence and ornamentation of chlamydospores, growth rates on MEA and CYA and acid production on CREA. A dichotomous key is provided for species identification based on phenotypical characters.

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INTRODUCTION

Byssochlamys species produce ascospores which are heat-resistant, and survive considerable periods of heat above 85 °C (Beuchat & Rice 1979, Splittstoesser 1987). In addition to their heat resistance, *Byssochlamys* species can grow under very low oxygen tensions (Taniwaki 1995) and can form pectinolytic enzymes. The combination of these three physiological characteristics makes *Byssochlamys* species very important spoilage fungi in pasteurised and canned fruit. *Byssochlamys* has a *Paecilomyces* anamorph and the genus was revised by Stolk & Samson (1971). Samson (1974) accepted three *Byssochlamys* species: *B. fulva*, *B. nivea* and *B. zollerniae* with similar *Paecilomyces* anamorphs. Since then only *B. verrucosa* has been added to this genus (Samson & Tansey 1975).

Paecilomyces was erected by Bainier (1907) to accommodate a single species, *P. variotii*, but many species were added (Brown & Smith 1957, Samson 1974). Luangsa-ard et al. (2004) presented a phylogenetic analysis of the 18S rDNA, demonstrating that *Paecilomyces* is polyphyletic across the *Sordariomycetidae* and *Eurotiomycetidae*. The type species *P. variotii* is a morphologically variable taxon, and has been redescribed under a variety of names broadening its circumscription. Thom (1930) and Samson (1974) mentioned the diversity in conidial shape and size, and Thom made a tentative division based on conidial size.

Paecilomyces variotii and anamorphs of *Byssochlamys* species share several micromorphological characters, including phialides with cylindrical bases that taper abruptly into long cylindrical necks and produce catenate conidia. Some characters are constant at the species level, but vary among species. Houbraken et al. (2006) demonstrated that *Byssochlamys* and its associated anamorph species can be separated into at least nine taxa by investigating the micro- and macroscopical characteristics of *Byssochlamys* and *Paecilomyces variotii*-like isolates. In this study, we have extended this to a polyphasic approach by adding molecular and extrolite data and present a revised taxonomy and nomenclature of the accepted taxa.

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MATERIALS AND METHODS

Strains of *Byssochlamys* and *Paecilomyces* used in this study are listed in Table 1, and are preserved in the Fungal Biodiversity Centre (CBS), Utrecht, the Netherlands.

DNA extraction, sequencing and analysis

Total fungal genomic DNA was isolated using FastDNA® Kit (Bio 101, Carlsbad, USA) according to the manufacturer's instructions. Amplification and sequencing of the ITS region (including internal transcribed spacer regions 1 and 2, and the 5.8S rRNA regions of the nuclear ribosomal RNA gene cluster), and parts of the β-tubulin and calmodulin genes were performed as described by Houbraken et al. (2007). Contigs were assembled from the forward and reverse sequences with the software package SeqMan from the Lasergene package (DNASTAR Inc., Madison, WI). The alignments of the sequence datasets were performed using Clustal W in MEGA 3.1 (Thompson et al. 1994, Kumar et al. 2004) and were, when necessary, adjusted by eye. Phylogenetic analyses of alignments were done using PAUP v. 4.0b10 (Swofford 2000). Alignment gaps were treated as fifth character state, missing data were identified by '?', uninformative characters were excluded and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all datasets using the heuristic search option. The

Table 1 *Byssochlamys* and *Paecilomyces* isolates examined in this study.

Species	Accession No.	Source and notes
B. fulva	CBS 132.33	Bottled fruit, UK; ex-type of *Paecilomyces fulvus*
	CBS 146.48^T	Bottled fruit, UK
	CBS 135.62	Fruit juice, Switzerland; ex-type of *Paecilomyces todicus*
	CBS 604.71	Unknown source
	CBS 113954	Unknown source, patulin producer acc. to Rice et al. (1977)
B. laguncularіae	CBS 373.70^T	Wood of *Laguncularia racemosa* (Mangue), Brazil
	CBS 696.95	Pasteurized strawberries, the Netherlands
	CBS 110378	Unknown source, France
B. nivea	CBS 100.11^T	Unknown source
	CBS 133.37	Milk of cow, USA; ex-type of *Arachniotus trisporus*
	CBS 271.95	Mushroom bed, China
	CBS 102192	Pasteurized drink yoghurt, Belgium
	CBS 113245	Pasteurized fruit juice, Switzerland
B. spectabilis	CBS 338.51	Fruit juice, Switzerland
	CBS 102.74	Unknown source; ex-type of *Paecilomyces variotii*
	CBS 101075^T	Heat processed fruit beverage, Japan
	CBS 121581	Spoiled sweetened tea, USA
B. verrucosa	CBS 605.74^T	Nesting material of *Leipoa ocellata*, Australia
B. zollerniae	CBS 374.70^T	Wood of *Zollernia ilicifolia* and *Protium heptaphyllum*, Brazil
P. brunneolus	CBS 370.70^T	Non fat dry milk, Canada
P. divaricatus	CBS 284.48^T	Mucilage bottle with library paste, USA
	CBS 110429	Pectin, Mexico
P. formosus	CBS 628.66	Quebracho-tanned sheep leather, France
	CBS 371.70	*Annona squamosa*, Brazil; ex-type of *Paecilomyces maximus*
	CBS 990.73B^T	Unknown source
	CBS 296.93	Man, bone marrow of patient, Uzbekistan
	CBS 113247	Soil, Thailand
	CBS 372.70	*Lecythis unsitata* (*Lecythidaceae*), wood, Brazil; ex-type strain of *P. lecythidis*
P. saturatus	CBS 323.34^T	Unknown source; ex-type of *Paecilomyces mandshuricus* var. *saturatus*
	CBS 368.70	Medicine containing quinine, UK
	CBS 251.55^T	Acetic acid, Brazil; ex-type of *P. dactylomorphys*
	CBS 990.73A	Unknown source; ex-type of *Penicillium viniferum*
	CBS 492.84	*Lepidium sativum*, Denmark
Talaromyces byssochlamydoides	CBS 413.71^T	Dry soil under *Pseudotsuga menziesii*, USA
Talaromyces emersonii	CBS 393.64^T	Compost, Italy
Thermoascus crustaceus	CBS 181.67^T	*Parthenium argentatum* (*Compositae*), decaying plant, USA

robustness of the most parsimonious trees was evaluated with 1 000 bootstrap replications (Hillis & Bull 1993). Other statistics, including tree length, consistency index, retention index and rescaled consistency index (CI, RI and RC) were calculated. *Monascus pilosus* (GenBank accession AY629427) was used as an outgroup in the analyses of the ITS dataset; *Thermoascus crustaceus* was used as an outgroup for the β-tubulin and calmodulin data. Newly generated sequences were deposited in GenBank with accession numbers FJ389920–FJ390009. The alignments generated and the most parsimonious trees were deposited in TreeBase under accession numbers S2199 and M4168–M4170.

Morphological characterisation

The media used for macro-morphological examination included Czapek yeast autolysate (CYA) agar, malt extract autolysate (MEA) agar, yeast extract sucrose (YES) agar and creatine-sucrose (CREA) agar (media compositions were according to Samson et al. 2004). Isolates were incubated at 25, 30 and 37 °C. Micromorphological characterisation of the asexual *Paecilomyces* state was carried out on MEA, hay agar (HAY) and YES agars. The latter was exclusively used to determine the presence of chlamydospores. For the analyses of the features of the sexual *Byssochlamys* state, the media oatmeal agar (OA) and potato-dextrose agar (PDA) were used (for media formulations, see Samson et al. 2004). The surfaces of conidia, chlamydospores and ascospores were examined after prolonged incubation (up to 70 d). Conditions and media for measuring the microaerophily, growth on 0.5 % acetic acid and the resistance to propionic acid are described by Frisvad & Samson (2004).

Extrolite analysis

Strains studied (Table 1) were three-point inoculated on MEA, YES, PDA, OA and CYA agars. All isolates were analysed for extrolite metabolites after 2 wk growth at 30 °C. The cultures were extracted according the method of Smedsgaard (1997) and analysed with high performance liquid chromatography (HPLC) with diode array detection (DAD) (Frisvad & Thrane 1987, 1993). The metabolites found were compared with a spectral UV library derived from authentic standards, including patulin, viriditoxin, mycophenolic acid, byssochlamic acid and physcion, using the same conditions (the maximum similarity is a match of 1 000). The retention indices were compared with those of standards.

RESULTS

DNA sequencing

The trees constructed by maximum parsimony analysis of the datasets of the protein coding genes β-tubulin and calmodulin and the ITS region exhibited similar topologies (Fig. 1–3). Molecular analyses revealed that in the genus *Byssochlamys* nine taxa can be recognised. Five taxa form a teleomorph, namely *B. fulva*, *B. laguncularіae*, *B. nivea*, *B. spectabilis* and *B. zollerniae*, while four are strictly anamorphic, i.e. *P. brunneolus*, *P. divaricatus*, *P. formosus* and *P. saturatus*. Analyses of the ITS showed that *B. verrucosa* is not a member of the genus *Byssochlamys* and is related to *Thermoascus* (Fig. 1). This was also confirmed by analyses of the partial β-tubulin and calmodulin sequence data (data not shown).

The basal nodes were also similar for all three parsimony trees (Fig. 1–3). *Byssochlamys nivea* and *B. fulva* were sister

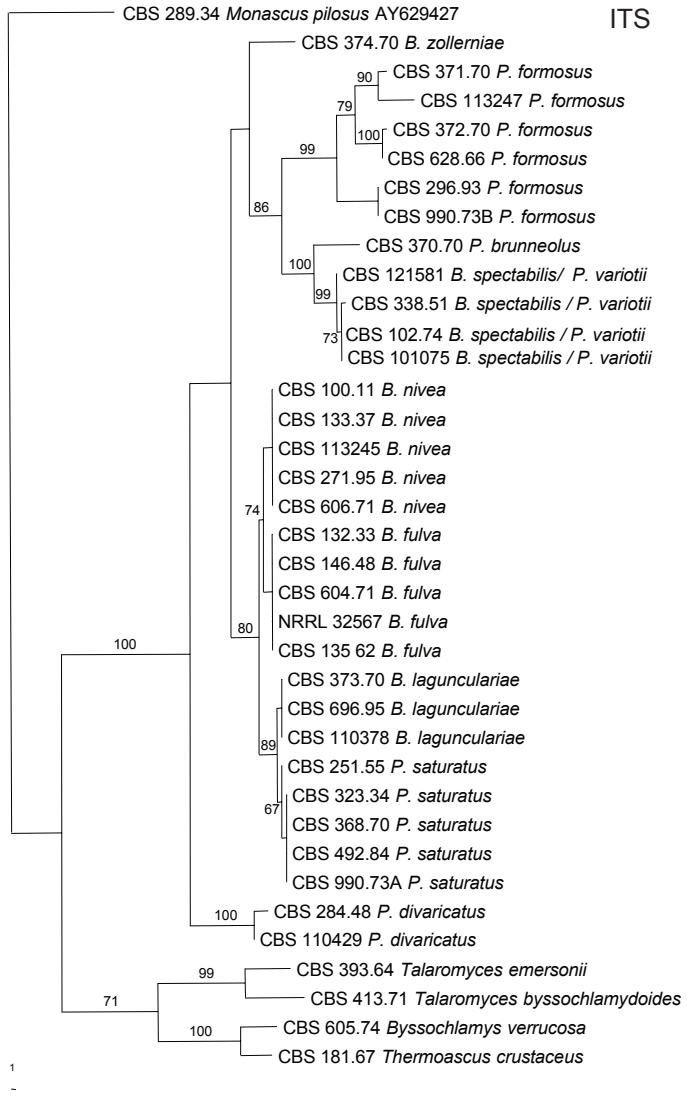

Fig. 1 One of three equally parsimonious trees of the analysed ITS region (55 of the 629 characters were parsimony informative; tree length = 294, CI = 0.738, RI = 0.855, RC = 0.631, HI = 0.262).

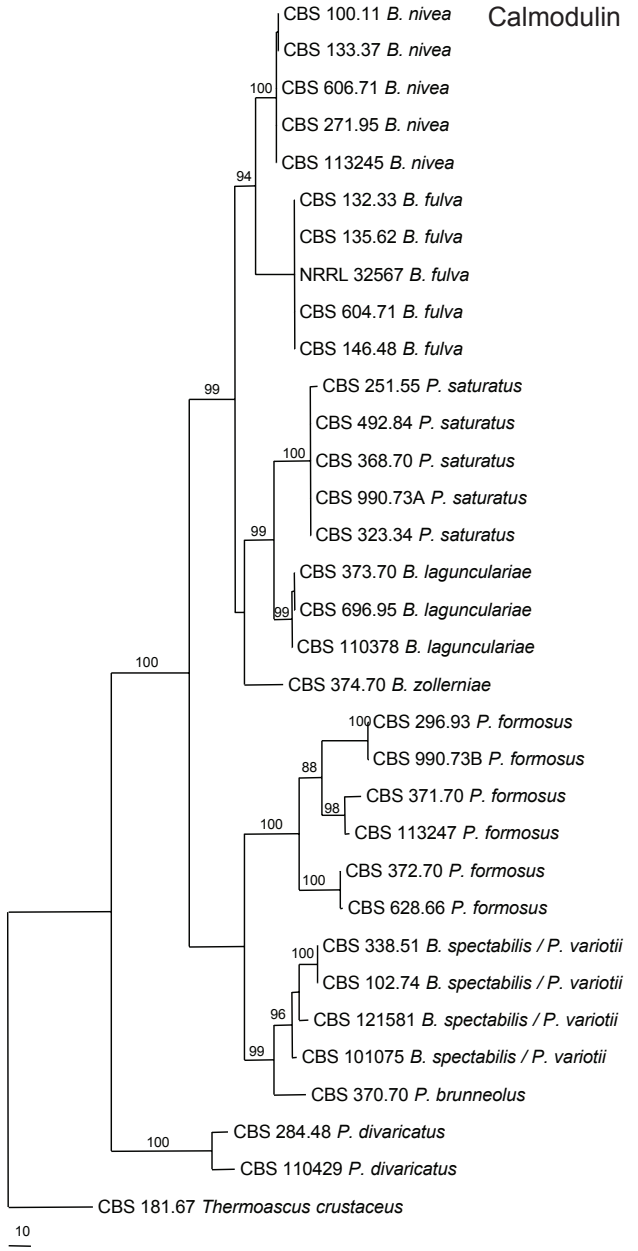

Fig. 2 One of eight equally parsimonious trees of the analysed partial calmodulin gene sequences (204 of the 607 characters were parsimony informative; tree length = 328, CI = 0.708, RI = 0.889, RC = 0.630, HI = 0.292).

species, as were *B. laguncullariae* and *P. saturatus*. These four species form a distinct clade (group I). *Byssochlamys spectabilis* and *P. brunneolus* also clustered together and form, together with *P. formosus*, another distinct clade in *Byssochlamys* (group II). *Paecilomyces divaricatus* was basal to these groups in all analyses, while the position of the single strain of *B. zollerniae* varied. Partial β-tubulin data showed that *B. zollerniae* is related to group I with a high bootstrap support (92 %; Fig. 3). The partial calmodulin data also showed that *B. zollerniae* is related to group I (Fig. 2), while analyses of the ITS regions revealed that it is more related to group II (Fig. 1), but its placement was supported only by low bootstrap values on these trees (Fig. 1, 2).

Morphological characterisation

The genus *Byssochlamys* is morphologically well-defined and characterised by almost naked ascomata in which croziers and globose asci are formed with ellipsoidal ascospores. The ascomatal initials consist of swollen antheridia and coiled ascogonia. All *Byssochlamys* species have a *Paecilomyces* anamorph. The *Byssochlamys* anamorphs belong to *Paecilomyces* sect. *Paecilomyces*, containing mesophilic, thermotolerant and thermophilic species (Samson 1974). As discussed by Luangsa-ard et al. (2005), other species previously classified in *Paecilomyces* sect. *Isarioidea* are now mainly accommodated in *Isaria* or newly described genera.

Various microscopic characters are valuable for identification purposes, including the shape and sizes of conidia and ascospores, and presence and ornamentation of chlamydospores. Useful macroscopical features are growth rates on MEA and CYA, and acid production on CREA. An overview of various macro- and microscopical features is presented in Table 2. The ratio between the colony diameters on MEA incubated at 30 °C or 37 °C is also diagnostic.

Extrolite analysis

The extrolite profiles of the taxa are listed in Table 3. Most strains of *B. fulva* produced byssochlamic acid, but *B. fulva* could be subdivided into two chemically different groups: one group of isolates produced meriditin (a compound produced by *Eupenicillium meridianum*) and a peptide with a cycloaspeptide

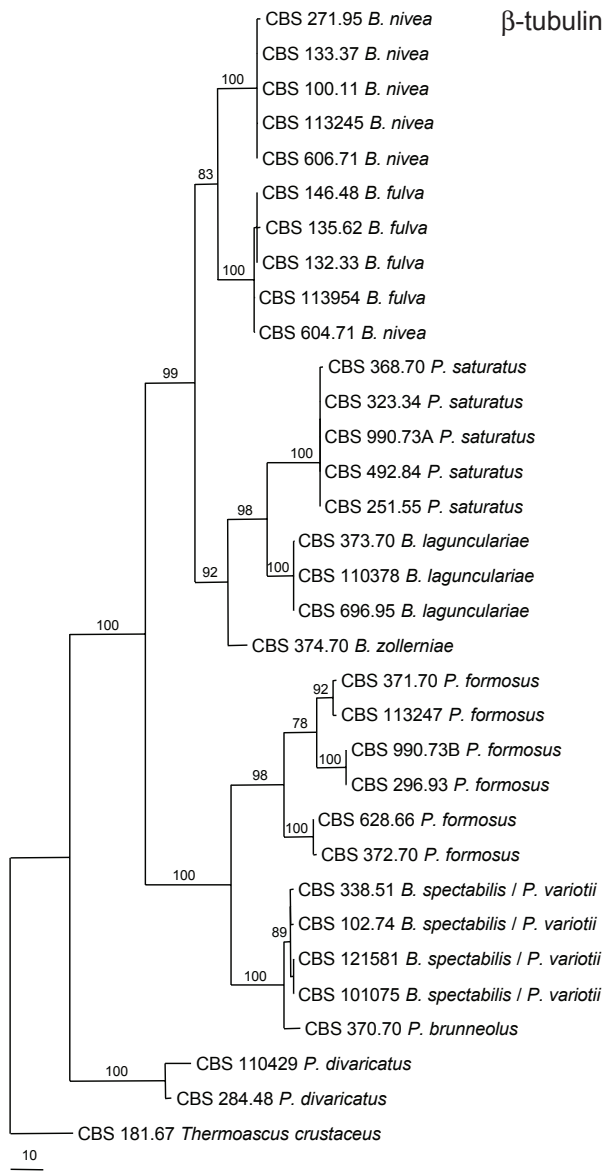

Fig. 3 One of 36 equally parsimonious trees of the analysed partial β-tubulin gene sequences (156 of the 494 characters were parsimony informative; tree length = 304, CI = 0.704, RI = 0.913, RC = 0.643, HI = 0.296).

chromophore (CBS 132.33, CBS 135.62 and CBS 604.71), while the other group produced a series of alkaloids, some with UV spectra similar to that of paspaline (named 'OLK' in Table 2) and some with verrucologen-like UV spectra (called 'URT' in Table 2) (CBS 113225, CBS 113246, CBS 113954 and TM 03.048). The ex-type culture of *B. fulva* was deteriorated and only produced byssochlamic acid, and none of the other mentioned extrolites.

Some strains of *B. nivea* produced the full profile of extrolites: patulin, mycophenolic acid, byssochlamic acid and metabolite 'OLK' (CBS 900.70, CBS 271.95 and CBS 102192). Mycophenolic acid, patulin and byssochlamic acid and some of their precursors were also found by Puel et al. (2005). *Byssochlamys laguncullariae* produces the same overall extrolite profile as *B. nivea*, except that patulin has not been detected in the former species. *Byssochlamys spectabilis* and isolates with only anamorphs (*P. variotii*) consistently produced viriditoxin, which was earlier reported from an isolate identified as *Spicaria divaricata* (Jiu & Mizuba 1974). Other unique extrolites were also produced but the structures of these have not yet been elucidated.

Table 2 Production of extrolites by *Byssochlamys* and *Paecilomyces* species[1].

Species	Extrolites
B. fulva	Group I: byssochlamic acid, meriditin, 'cycloaspeptide-like compound'
	Group II: byssochlamic acid, 'OLK', 'URT'
B. laguncullariae	Byssochlamic acid, mycophenolic acid, 'OLK'
B. nivea	Byssochlamic acid, mycophenolic acid, patulin, 'OLK'
B. spectabilis	Viriditoxin and other compounds with characteristic UV spectra
B. verrucosa	Cornexistin and/or byssochlamic acid
B. zollerniae	No known extrolites, though several compounds characterized by a characteristic UV spectrum are present
P. brunneolus	'Ascofuranone-like compound', 'tetracycline-like compounds'
P. divaricatus	Cornexistin and/or byssochlamic acid, ascofuranone, emodin and other anthraquinones
P. formosus	Variotin
P. saturatus	Group I: patulin, mycophenolic acid or 'aspergillic acid-like compound'
	Group II: brefeldin A

[1] Byssochlamic acid, mycophenolic acid, patulin, and viriditoxin were available as authentic standards. Evidence for production of ascofuranone, cornexistin, meriditin and variotin is based on similar UV spectra as those reported in the literature, and their occurrence in species already known to produce them. Metabolites in inverted commas have UV spectra that indicate a chemical relationship to the compounds mentioned. For example the 'tetracycline-like compounds' in *P. brunneolus* have UV spectra similar to those of tetracycline and viridicatumtoxin. 'OLK' and 'URT' are apolar indole-terpene compounds, but the chemical structure is as yet unknown. 'OLK' has a paspaline UV spectrum.

All strains of *P. divaricatus* produced anthraquinones including emodin. However CBS 110429 only produced another type of anthraquinone, CBS 110428 and CBS 110430 produced ascofuranone. Only the ex-type culture of *B. zollerniae* was available for study and some unique unknown extrolites were found in this isolate. In this study, we could not confirm viriditoxin production in *P. divaricatus* and assume that the strain studied by Jiu & Mizuba (1974) was probably a *B. spectabilis* isolate.

Our phylogenetic analysis showed that *B. verrucosa* is related to *Thermoascus*. *Byssochlamys verrucosa* produced cornexistin and/or byssochlamic acid, and also some specific unidentified extrolites only seen in this species. *Thermoascus aurantiacus* also produces compounds with chromophores (UV spectra) consistent with cornexistin, byssochlamic acid or similar tropolones (Frisvad unpubl. data). The production of cornexistin and byssochlamic acid demonstrates that the species chemically resemble *B. fulva*, *B. laguncularia*, *B. nivea* and *P. divaricatus*.

Paecilomyces saturatus is chemically somewhat diverse. Group I produced at least one of the following extrolites: patulin, mycophenolic acid and a compound which chemically resembles aspergillic acid (CBS 251.55^T, CBS 223.52, CBS 492.84 and IBT 21716), while group II produced brefeldin A (CBS 323.34^T and CBS 990.73A) and may fit with *P. mandshuricus* var. *saturatus*. *Paecilomyces formosus* consistently produced variotin and related compounds, except CBS 296.93, which may be chemically close to *P. saturatus* (group I).

DISCUSSION

Byssochlamys isolates were included in this study to verify the connection between the anamorphic *P. variotii* complex and holomorphic *Byssochlamys* species. The genus *Paecilomyces* was monographed by Samson (1974) who recognised 31 species divided into two sections, *Paecilomyces* and *Isarioidea*. However, the phylogenetic analysis of the 18S rDNA demonstrates that *Paecilomyces* is polyphyletic across two subclasses, *Sordariomycetidae* and *Eurotiomycetidae* (Luangsa-ard et al. 2004).

Table 3 Macro-and microscopical features of *Byssochlamys* and *Paecilomyces* isolates.

Species	Conidial length (µm)	Conidial shape (predominant)	Chlamydospores	Ascospore length (µm)	Ascospore ornamentation	Colony diam. (mm) on CYA 7 d, 30 °C	Degree of growth on CYA 7 d, 30 °C	Acid production on CREA 7 d, 30 °C
B. fulva	3.7–7.5 × 1.4–2.5	Cylindrical with truncate ends	Absent; in some isolates after prolonged incubation present (40 d)	5.3–7.1 × 3.3–4.3	Smooth	50–90	Good	+
B. laguncullariae	2.7–4.5 × 2.2–3.3	Globose with flattened base	Present, smooth	3.8–5 × 3–3.9	Smooth	45–55	Good	–
B. nivea	3–4.7 × 2.3–4	Globose to ellipsoidal with flattened base	Present, smooth to finely rough	4.1–5.5 × 2.9–3.9	Smooth	(8–)28–50	Weak	– (+)
B. spectabilis	3.3–6.1 × 1.5–4.4	Predominantly ellipsoidal and ellipsoidal with truncate ends	Present, smooth to finely rough	5.2–6.8 × 3.5–4.5	Almost smooth, slightly roughened	30–45 (–55)	Good	–
B. verrucosa	6.3–13.1 × 1.6–4.7	Cylindrical with truncate ends	Absent	6.6–8.4 × 4–6.1	Rough	25–40	Good	–
B. zollerniae	2.5–4 × 1.5–3	Globose to ellipsoidal, apiculate	Present, warted	3–4.5 × 2.5–3	Smooth	30–35	Weak	–
P. brunneolus	3.7–5.5 × 1.8–3.4	Ellipsoid to broadly cylindrical with truncate ends	Present, smooth	No ascospores detected	Not relevant	20–30	Good	–
P. divaricatus	3.2–4.6 × 1.6–2.5	Ellipsoidal to cylindrical with truncate ends	Absent; in some isolates after prolonged incubation present (40 d)	5.3–7 × 3.8–4.9, rarely produced	smooth	10–17	Moderate	–
P. formosus	3–10 × 1.8–3.5	Ellipsoidal to cylindrical with truncate ends	Present, smooth and often pigmented	No ascospores detected	Not relevant	18–90	Good	+
P. saturatus	2.3–7 × 1.7–3.4	Predominantly cylindrical and ellipsoidal without truncate ends	Present, smooth	No ascospores detected	No ascospores detected	22–55	Good	–

Therefore, *Paecilomyces* is only monophyletic within the order *Eurotiales* and characterised by a *Byssochlamys* teleomorph. In the present study the *P. variotii* complex could be divided into four species, *P. divaricatus*, *P. formosus*, *P. saturatus* and *P. variotii*.

Udagawa & Suzuki (1994) described *Talaromyces spectabilis*, but phylogenetic analysis of the 18S rDNA clarified that this species belongs to the genus *Byssochlamys* (Luangsa-ard et al. 2004). Recently, Houbraken et al. (2008) showed that this species is heterothallic and that it is linked to the anamorphic species *P. variotii*. This was confirmed in the present study by morphological features and extrolite data. The presence of a teleomorph with heat-resistant ascospores for *P. variotii* explains the ability of this fungus to spoil heat treated fruit juices (Piecková & Samson 2000).

Short descriptions of *B. laguncullariae*, *B. spectabilis*, *P. brunneolus*, *P. divaricatus*, *P. formosus* and *P. saturatus* are presented. The concepts of *B. fulva*, *B. nivea* and *B. zollerniae* are unchanged since the descriptions by Stolk & Samson (1971), with the remark that *B. nivea* var. *languncullariae* is elevated to species level. *Byssochlamys verrucosa* is not discussed here, because the taxon probably belongs to *Thermoascus*; it was fully described by Samson & Tansey (1975). Microscopic dimensions and cultural characters are summarised in Table 3.

Discussion of accepted taxa

Byssochlamys laguncullariae (C. Ram) Samson, Houbraken & Frisvad, *comb. nov*. — MycoBank MB512557; Fig. 4a–f

Basionym. *Byssochlamys nivea* Westling var. *laguncullariae* C. Ram, Nova Hedwigia 16: 311. 1968.

Byssochlamys laguncullariae strains grow fast on MEA, covering the dish within 7 d at 30 °C. Depending on the isolate, it predominantly forms conidia (CBS 373.70^{T}) or ascomata (CBS 696.95). Colonies 25–55 mm on MEA at 37 °C after 7 d of incubation. Growth occurs under microaerophilic conditions, in the presence of 0.5 % acetic acid or 1 000 ppm propionic acid (pH 3.8). No growth is observed on CYA with 5 % NaCl. Poor growth and no acid production on CREA.

Morphologically, *B. laguncullariae* is similar to *B. nivea* and shares various characters such as fast growth rate on MEA at 30 °C and globose (to ellipsoidal) conidia with a flattened base. Chlamydospores are present, uncoloured and smooth-walled. Though similar in shape to those of *B. nivea*, the conidia and ascospores of *B. laguncullariae* are generally smaller in size. Another difference is that *B. laguncullariae* grows well on CYA while *B. nivea* grows rather poorly.

Our molecular studies and morphological examinations both revealed that *B. laguncullariae* is clearly distinct from *B. nivea*. In the original description by Ram (1968), *B. laguncullariae* was described as a variety of *B. nivea*, distinguished by its smaller conidia and ascospores. Stolk & Samson (1971) synonymised it with *B. nivea*, but the present study showed that the growth rate on CYA, and smaller conidial and ascospore sizes are constant characters that can be used to differentiate between these species.

This species produces a similar range of extrolites to *B. nivea*, but patulin has not been detected in *B. laguncullariae*. Differentiation between *B. nivea* and *B. laguncullariae*, based on extrolite profiles, is not possible.

The ex-type culture was isolated from wood of *Laguncularia racemosa* (mangue) in Brazil and other strains identified as this species were found in soil, and pasteurised strawberries and aloe juice. The occurrence of this species in heat treated products makes it an important food spoilage organism.

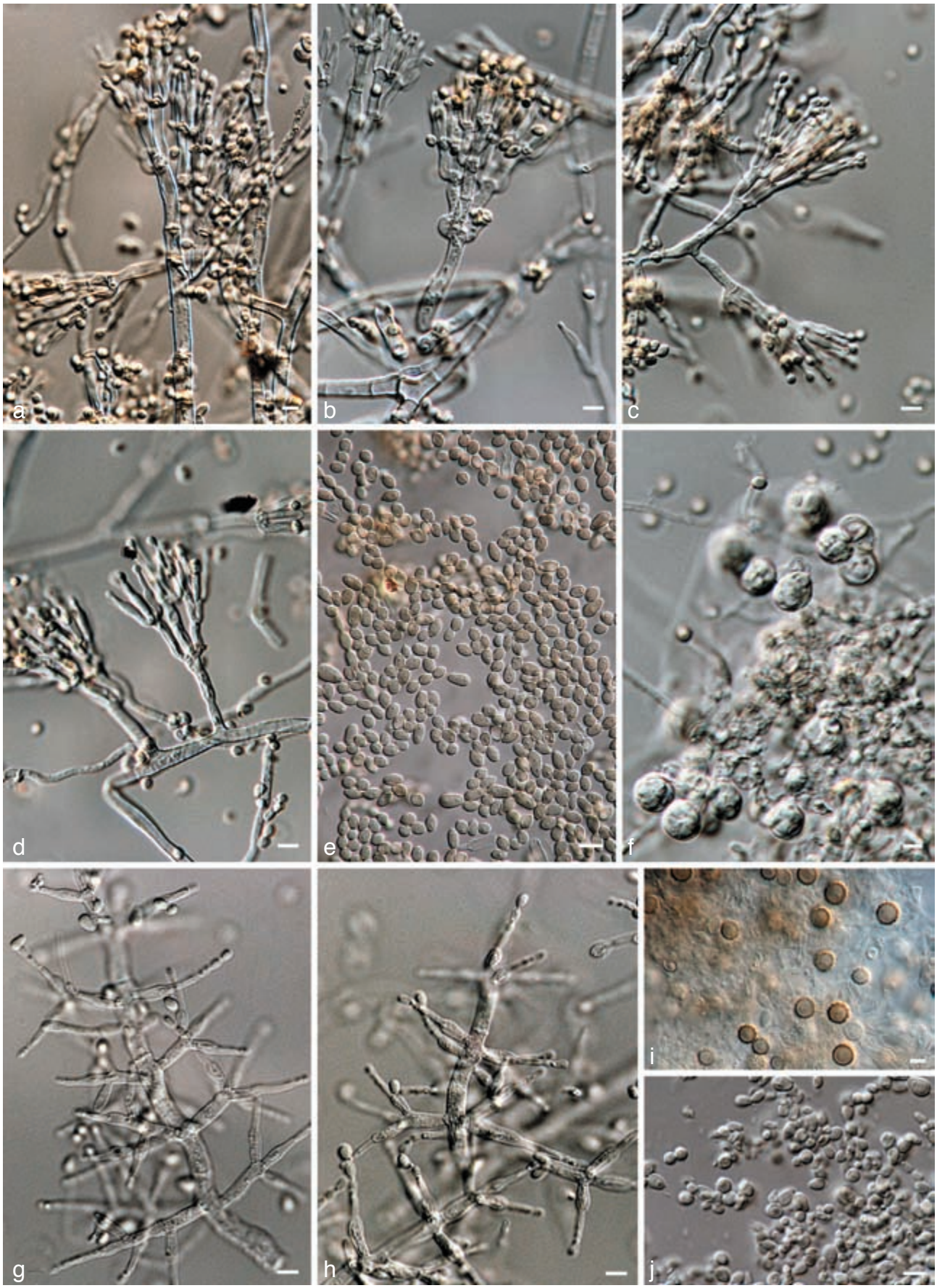

Fig. 4 *Byssochlamys lagunculariae*. a–d. Conidiophores; e. conidia; f. asci and ascospores. — *Byssochlamys zollerniae*. g, h. Phialides; i. chlamydospores; j. conidia. — Scale bars = 10 µm.

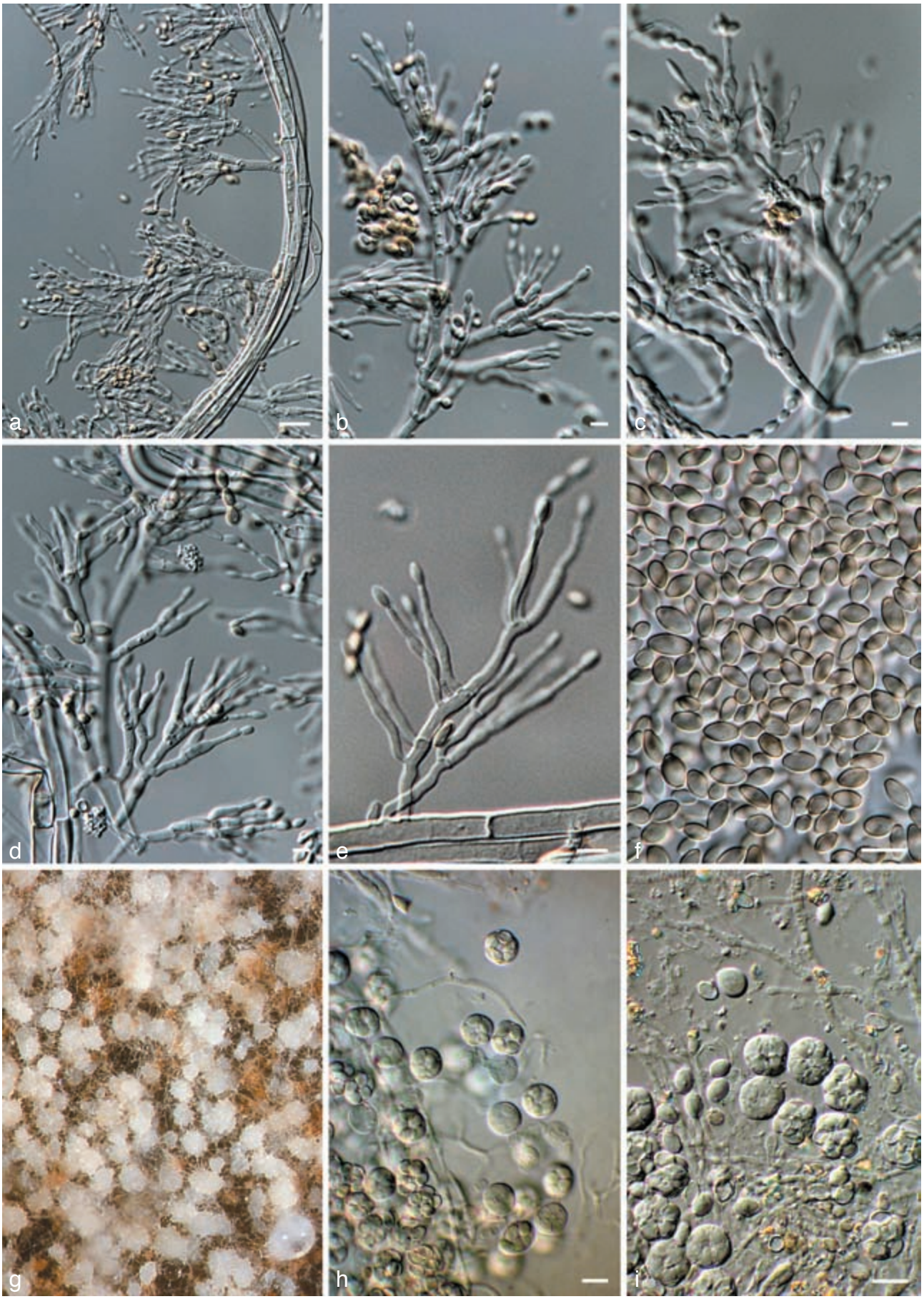

Fig. 5 *Byssochlamys spectabilis*. a–e. Conidiophores; f. conidia; g. ascomata; h, i. asci and ascospores. — Scale bars = 10 µm.

Byssochlamys spectabilis (Udagawa & Shoji Suzuki) Houbraken & Samson, Appl. Environm. Microbiol. 74: 1618. 2008. — Fig. 5

Basionym. *Talaromyces spectabilis* Udagawa & Shoji Suzuki, Mycotaxon 50: 82. 1994.

Anamorph. *Paecilomyces variotii* Bainier, Bull. Trimestriel Soc. Mycol. France 23: 26. 1907.

≡ *Penicillium variotii* (Bainier) Sacc., Syll. Fung. 22: 1273. 1913.

Colonies spreading rapidly on MEA at 30 °C and covering the Petri dish within 7 d. Similar or higher growth rate at 37 °C than at 30 °C. Good growth under microaerophilic conditions, on MEA with 0.5 % acetic acid and on CYA with 1 000 ppm propionic acid (pH 3.8). No or weak growth on CYA with 5 % NaCl (0–10 mm). Since *B. spectabilis* forms its ascospores in a heterothallic manner, only the anamorph is usually produced. The conidiophores are irregularly branched and ellipsoidal and/or cylindrical; truncate conidia are formed, which are often pale yellow brown. Chlamydospores present, smooth-walled, in some isolates finely roughened. Often broad, thick-walled hyphae are present in fresh isolates. Ascospores are formed when strains of opposite mating types are grown together. Our recent study (Houbraken et al. 2008) showed that strains originating from heat treated products are mostly frequently capable of producing fertile progeny. The ascospores are ellipsoidal, smooth to finely roughened, 5.5–6.5 × 3.5–4.5 µm. Colonies on MEA agar growing rapidly, attaining a diameter of 7 cm within 7 d at 30 °C. Poor growth and no acid production on CREA.

The extrolite viriditoxin was produced by all investigated isolates. Whether this extrolite is also produced in foodstuffs is unknown.

Byssochlamys spectabilis commonly occurs in air, compost, infected humans and various foodstuffs (including pasteurised fruit juices, rye bread). It is frequently found in heat treated products, although after isolation only the anamorph is produced.

Paecilomyces brunneolus (N. Inagaki) Samson & Houbraken, *comb. nov.* — MycoBank MB512559; Fig. 6g–i

Basionym. *Paecilomyces variotii* Bainier var. *brunneolus* N. Inagaki, Trans. Mycol. Soc. Japan 4: 4. 1962.

The type strain of *P. brunneolus* on MEA in 7 d forms colonies of 35–45 mm diam (30 °C) with well-defined margins. Colonies on MEA 20–30 mm diam in 7 d at 37 °C. Growth occurs under microaerophilic conditions and in the presence of 0.5 % acetic acid or 1 000 ppm propionic acid (pH 3.8). No growth is observed on CYA with 5 % NaCl. Poor growth and no acid production on CREA. Microscopical examination showed short, irregular branched conidiophores (2–3 × 15–25 µm). Conidia ellipsoidal to broadly cylindrical with truncate ends (1.5–)2–3(–3.5) × (3.5–)4–5(–5.5) µm. Chlamydospores present, smooth and hyaline.

Paecilomyces brunneolus is only known from its type culture, CBS 370.70. Analyses of a part of the β-tubulin, calmodulin gene and the ITS regions showed that this strain is closely related to *B. spectabilis* (*P. variotii)*. However, extrolites and morphological data support that this is a distinct species. Viriditoxin is consistently produced by *B. spectabilis*, while *P. brunneolus* produces an ascofuranone like compound and tetracyclic compounds. *Paecilomyces brunneolus* colonies are more restricted than strains of *B. spectabilis* (*P. variotii*) on MEA. Another difference is that the growth rate of *P. brunneolus* at 37 °C is slower than at 30 °C, while this is the opposite for *B. spectabilis*.

This species has only been isolated from non-fat, dry milk from Canada in the 1960s.

Paecilomyces divaricatus (Thom) Samson, Houbraken & Frisvad, *comb. nov.* — MycoBank MB512561; Fig. 6a–f

Basiomym. *Penicillium divaricatum* Thom, Bull. Bur. Anim. Ind. U.S. Dep. Agric. 118: 92. 1910.

≡ *Spicaria divaricata* (Thom) J.C. Gilman & E.C. Abbott, Iowa State Coll. J. Sci. 1: 301. 1929.

≡ *Spicaria divaricata* (Thom) R.M. Ma, Lingnan Sci. J. (Suppl.) 12: 115. 1933.

The growth rate of *P. divaricatus* on MEA is restricted, compared with other members of the investigated *Byssochlamys* clade, and colonies of 30–40 mm are attained after 7 d of incubation at 30 °C. Only *P. brunneolus* has colony diameters of comparable size. Grows at 37 °C, although slower at 30 °C. Weak growth and no acid production on CREA. *Paecilomyces divaricatus* is characterised by its ellipsoidal to cylindrical, truncate conidia, measuring 3.5–4.5 × 1.5–2 µm and the absence of chlamydospores. Ascomata absent on agar media, though ascomatal initials, arising as coils, can be observed. Smooth ellipsoidal ascospores were once observed in a fresh isolate (5.3–7 × 3.8–4.9 µm) but have not been seen since.

Anthraquinones including emodin are produced by all investigated isolates. The production and presence of emodin, a genotoxic and diarrheagenic mycotoxin, in foods and feeds is unknown.

Thom (1910) described the species *Penicillium divaricatum* but placed this species later in synonymy with *P. variotii* (Thom 1930). We examined the ex-type isolate and conclude that *P. divaricatus* is a distinct species. Microscopical analyses of the original type isolate (CBS 284.48) showed a few structures resembling ascoma initials. Using a heat treatment, strains of *P. divaricatus* could be isolated from various food products. The survival of a heat treatment suggests the presence of a *Byssochlamys* teleomorph (ascospores), and many *Byssochlamys* initials were present (croziers) in these strains; however, no ascomata were detected even after prolonged incubation.

This species was isolated from a bottle with mucilage library paste, heat treated pectin and fruit concentrates. Its presence in heat treated products and the absence of thick-walled chlamydospores, suggests that this species is able to form heat resistant ascospores.

Paecilomyces formosus (Sakag., May. Inoue & Tada) Houbraken & Samson, *comb. nov.* — MycoBank MB512562; Fig. 7g–i

Basionym. *Monilia formosa* Sakag., May. Inoue & Tada, Zentralbl. Bakteriol., 2. Abt. 100: 302. 1939.

= *Paecilomyces maximus* C. Ram, Nova Hedwigia 16: 306. 1968.

= *Paecilomyces lecythidis* C. Ram (as *lecythisii*), Nova Hedwigia 16: 307. 1968.

Fast growth on MEA at 30 °C and covering the dish within 7 d. Ratio between growth rates at 30 and 37 °C variable; most strains have slower or similar growth rates (< 1), though CBS 371.70 (ex-type strain of *P. maximus*) and CBS 113247 are exceptions and have a faster growth rate at 37 °C. Growth on MEA with 0.5 % acetic acid varying from absence of growth to more than 80 mm after 1 wk of incubation. Variable growth patterns on CYA with 5 % NaCl, 0–25 mm. All investigated strains have poor growth on CREA and acid production under colonies. Conidiophores irregularly branched, with olive-brown conidia. Chlamydospores present (often on small stalks), smooth walled, globose and (weakly) pigmented. The conidia of this species are variable, varying from ellipsoidal to cylindrical; all with truncate ends. In some isolates conidial shape might vary from ellipsoidal to cylindrical.

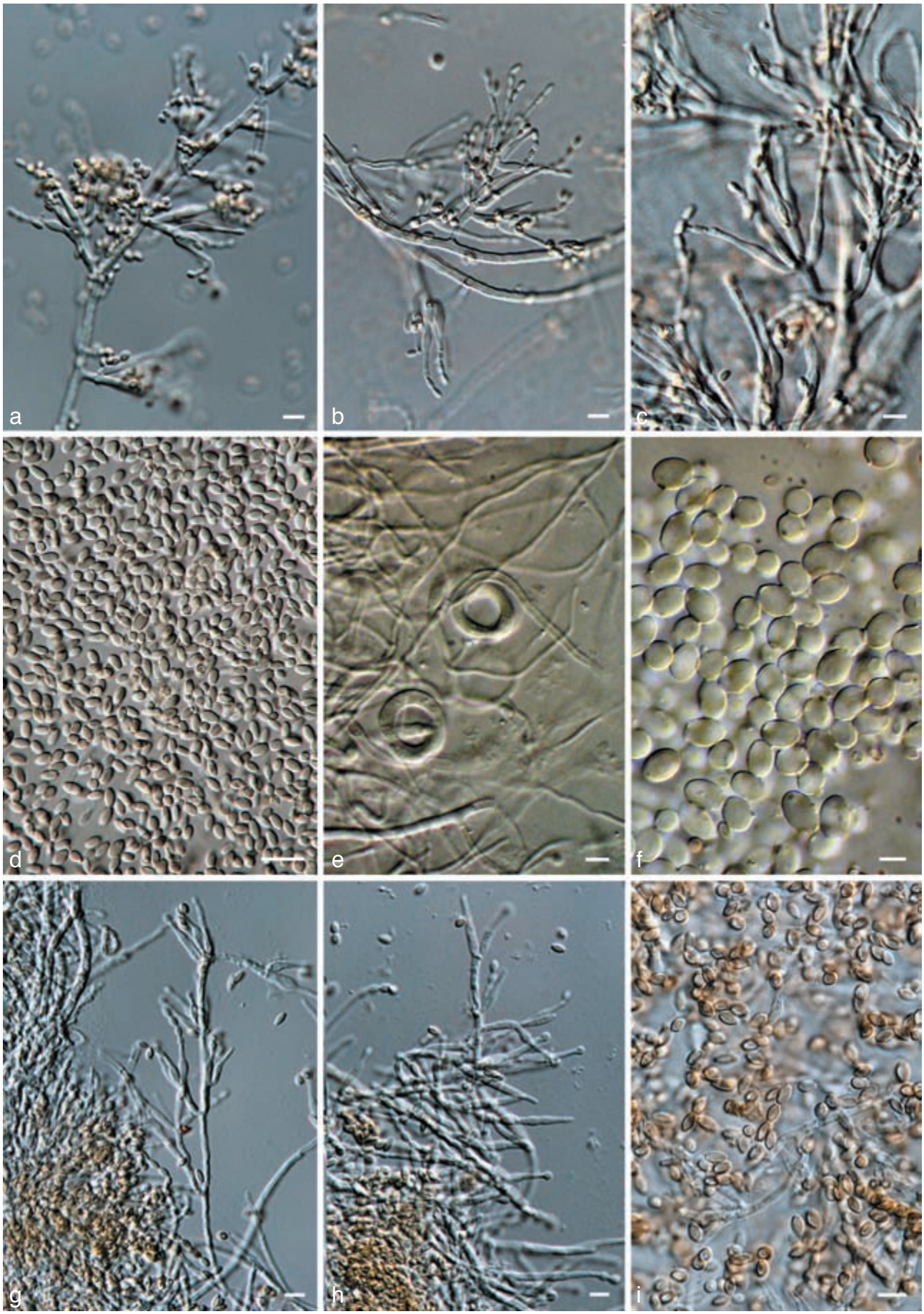

Fig. 6 *Paecilomyces divaricatus.* a–c. Conidiophores; d. conidia; e. ascoma initials; f. ascospores. — *Paecilomyces brunneolus.* g, h. Conidiophores; i. conidia. — Scale bars = 10 µm.

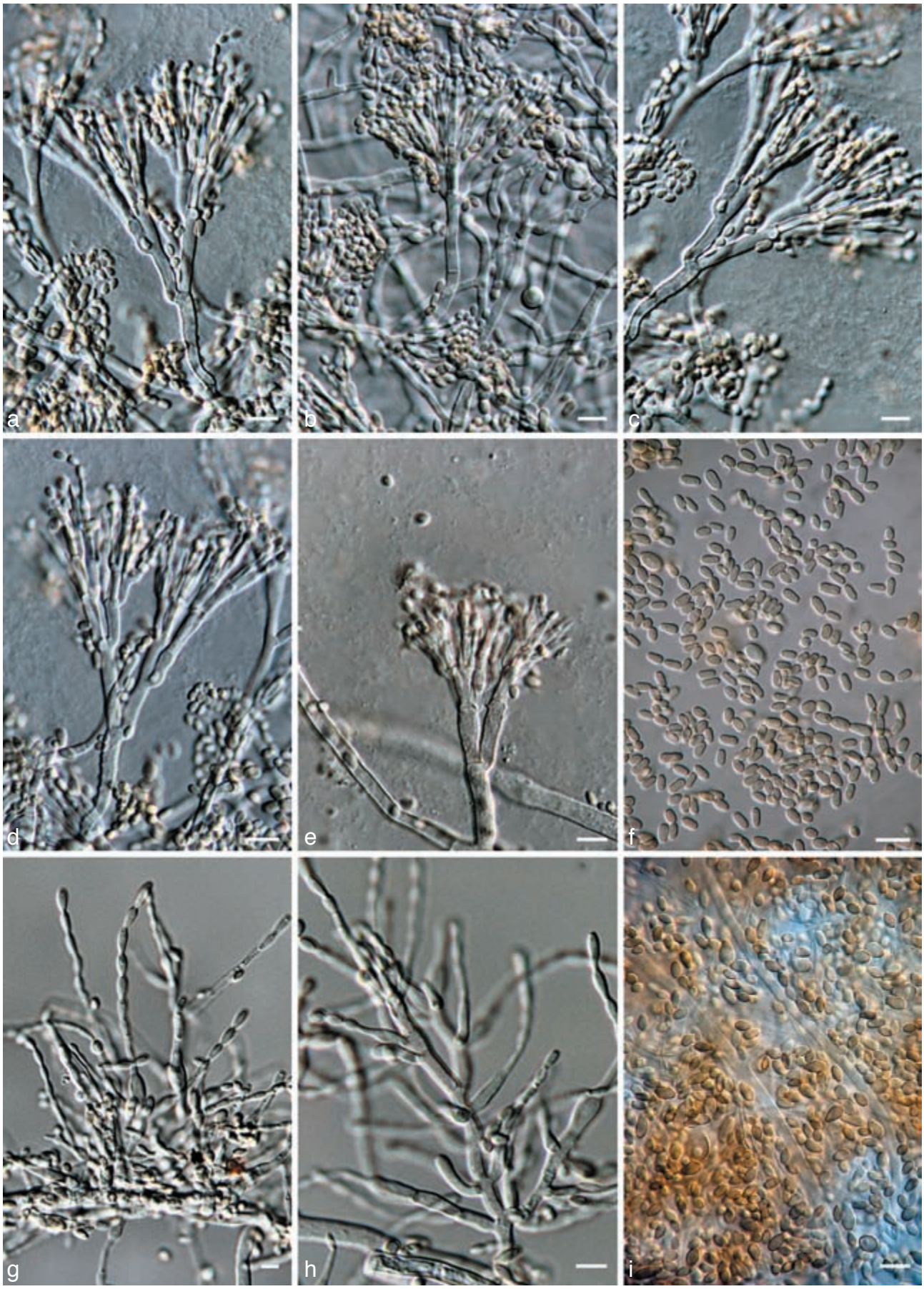

Fig. 7 *Paecilomyces saturatus.* a–e. Conidiophores; f. conidia. — *Paecilomyces formosus.* g, h. Conidiophores; i. conidia. — Scale bars = 10 µm.

Ram (1968) described two *Paecilomyces* species, *P. lecythidis* and *P. maximus*, based on their cultural characters and their large-sized conidia.

The results of the sequencing of the ITS region, and parts of the protein coding genes β-tubulin and calmodulin, showed that *P. formosus* may consist of three taxa, *P. formosus*, *P. lecythidis* and *P. maximus*. However, the three species could not be distinguished by microscopical examination. One difference between strains belonging to the '*P. maximus*-clade' and the other members of this diverse group, is the faster growth rate of this species at 37 °C than at 30 °C. The sequence and morphological diversity was not detected by the extrolite analyses: the ex-type cultures of *P. lecythidis* and *P. maximus* produced similar extrolite profiles, while the ex-type culture of *P. formosus* is degenerated and is a weak producer of extrolites.

For a more detailed conclusion and delimitation of these three groups more strains should be studied, particularly emphasising conidial shape and extrolites production. For the time being we propose to place *P. lecythidis* and *P. maximus* in synonymy with *P. formosus.*

This species is morphologically similar to *P. variotii* and the main difference is the consistent acid production on CREA.

Paecilomyces formosus has been isolated from tropical and subtropical soils, wood, sponge, man (bone marrow, blood), air in a bedroom (Denmark) and pot plant soil of *Senseviera trifasciata* (Denmark).

Paecilomyces saturatus (Nakaz., Y. Takeda & Suematsu) Samson & Houbraken, *comb. nov*. — MycoBank MB512560; Fig. 7a–f

Basionym. *Paecilomyces mandshuricus* (Saito) Thom var. *saturatus* Nakaz., Y. Takeda & Suematsu, J. Agric. Chem. Soc. Japan 10: 102. 1934.
= *Penicillium viniferum* Sakag., May. Inoue & Tada, Zentralbl. Bakteriol., 2. Abt. 100: 303. 1939.
= *Paecilomyces dactylethromorphus* Bat. & H. Maia, Anais Soc. Biol. Pernambuco 15: 152. 1957.

The oldest basionym available for this taxon is *P. mandshuricus* var. *saturatus* and therefore the name of this taxon is derived from this varietal name.

Isolates growing on MEA at 30 °C cover the Petri dish within 7 d, with strong olive-brown sporulation. Good growth at 37 °C, though slower at 30 °C. Good growth on MEA with 0.5 % acetic acid and CYA with 1 000 ppm propionic acid (pH 3.8). No growth observed on CYA with 5 % NaCl. Poor growth and no acid production on CREA.

Morphological examination of various strains showed that this species forms fairly regularly branched, penicillium-like conidiophores, with ellipsoidal and/or cylindrical conidia without a distinct truncation. Chlamydospores present, hyaline and smooth walled. No teleomorph observed.

The production of extrolites depends very much on the growth medium; patulin or brefeldin A can be produced.

Paecilomyces saturatus is an easily recognisable species with its ellipsoidal and/or cylindrical conidia and its fairy regularly branched, penicillium-like conidiophores. Sakaguchi et al. (1939) described *Penicillium viniferum* and this species was subsequently placed in *Paecilomyces* by Raper & Thom (1949). In retrospect, the placement in *Paecilomyces* was correct, although this species could be interpreted, with its penicillium-like conidiophores and the presence of chlamydospores, as an intermediate form between *Penicillium* and *Paecilomyces*. Molecular studies now show that this species belongs to the *Byssochlamys* clade and is different from other olive-brown coloured species such as *Hamigera avellanea* (Luangsa-ard et al. 2004), *Penicillium digitatum* and *P. cylindrosporum* (unpubl. data).

This species has been isolated from a variety of substrates, e.g. acetic acid, leather, medicine containing quinine, a dispersion of fenylacetate and dibutylmaleinate and *Lepidium sativum*.

Notes on the ecology and extrolites production

Byssochlamys and *Paecilomyces* species are often found in acidic habitats such as silage (Scurti et al. 1973, Escoula 1975a, b, Anderson et al. 1979), and in common with *Penicillium* series *Roqueforti* species (Frisvad & Samson 2004) can also tolerate microaerophilic conditions (Escoula 1975a, b, Taniwaki 1995). *Byssochlamys nivea* was initially reported to produce patulin under the name *Gymnoascus* sp. (Karow & Forster 1944, Kuehn 1958), later confirmed by Kis et al. (1969), Scurti et al. (1973), Rice et al. (1977) and Draughon & Ayres (1980). *Byssochlamys fulva* was also reported to produce patulin, albeit by few strains (Escoula 1975a, b, Percebois et al. 1975, Rice et al. 1977). Besides their presence in pasteurised fruit, *B. fulva* and *B. nivea* also form toxic extrolites, such as byssotoxin A and byssochlamic acid (Kramer et al. 1976, Rice et al. 1977). Besides mycotoxins, also an antitumor metabolite, byssochlamysol, a steroid against IGF-1 dependent cancer cells, is produced by *B. nivea* (Mori et al. 2003).

Paecilomyces variotii s.l. also produces mycotoxins (Scott 1965), such as patulin (Escoula 1975a, b), sphingofungin E and F (Frommer et al. 1992) and viriditoxin, reported originally from an isolate named *Spicaria divaricata* (Jiu & Mizuba 1974). Apart from being reported as being a potential mycotoxin, viriditoxin has also been reported to be a candidate for treatment of antibiotic resistant bacteria (Wang et al. 2003). Among the known extrolites are the antifungal drug variotin (Takeuchi et al. 1959, 1964, Suzuki et al. 1990, Omolo et al. 2000), and other drug candidates such as cornexistins (Nakajima et al. 1991, Fields et al. 1996), SCH 643432 (Hegde et al. 2003) and a penicillin-like compound (Burton 1949). The sideramins ferrirubrin and fusigen (Diekmann 1967, Domsch et al. 1980) and the organic acids 3-indole-acetic acid (Bakalinerov 1968, Voinova-Raikova et al. 1969), citric acid (Loesecke 1945), ethyleneoxide-α, β-dicarboxylic acid (Sakaguchi et al. 1939), (3Z,5E)-octa-3,5-diene -1,3,4-tricarboxylic acid 3,4-anhydride (Aldridge et al. 1980) have also been reported. The possible mycotoxins and/or potential drugs byssotoxin A, sphingofungin E and F, SCH 643432 and byssochlamysol were not available to us as standards. Given the taxonomic revision presented here, it remains to be seen which species produce these extrolites. Some connections between species and bioactive extrolites were confirmed or established here and several species had a high consistent extrolite profile. However, the chemotaxonomy of *P. saturatus* is unresolved, because there appear to be two chemotypes, which may or may not indicate that there are two species rather than one. Likewise, *B. fulva* appears to have two chemotypes, with only byssochlamic acid as a common extrolite in all isolates examined.

KEY TO *BYSSOCHLAMYS* AND RELATED *PAECILOMYCES* ANAMORPHS*

1. Conidia with conspicuously truncate ends or a flattened base 2
1. Conidia ellipsoidal or cylindrical with inconspicuously truncate ends . . . . . *P. saturatus*
2. Conidia predominantly ellipsoidal and/or cylindrical chlamydospores absent or present; *Byssochlamys* teleomorph absent or present 3
2. Conidia predominantly globose to subglobose, chlamydospores present; *Byssochlamys* teleomorph present 8

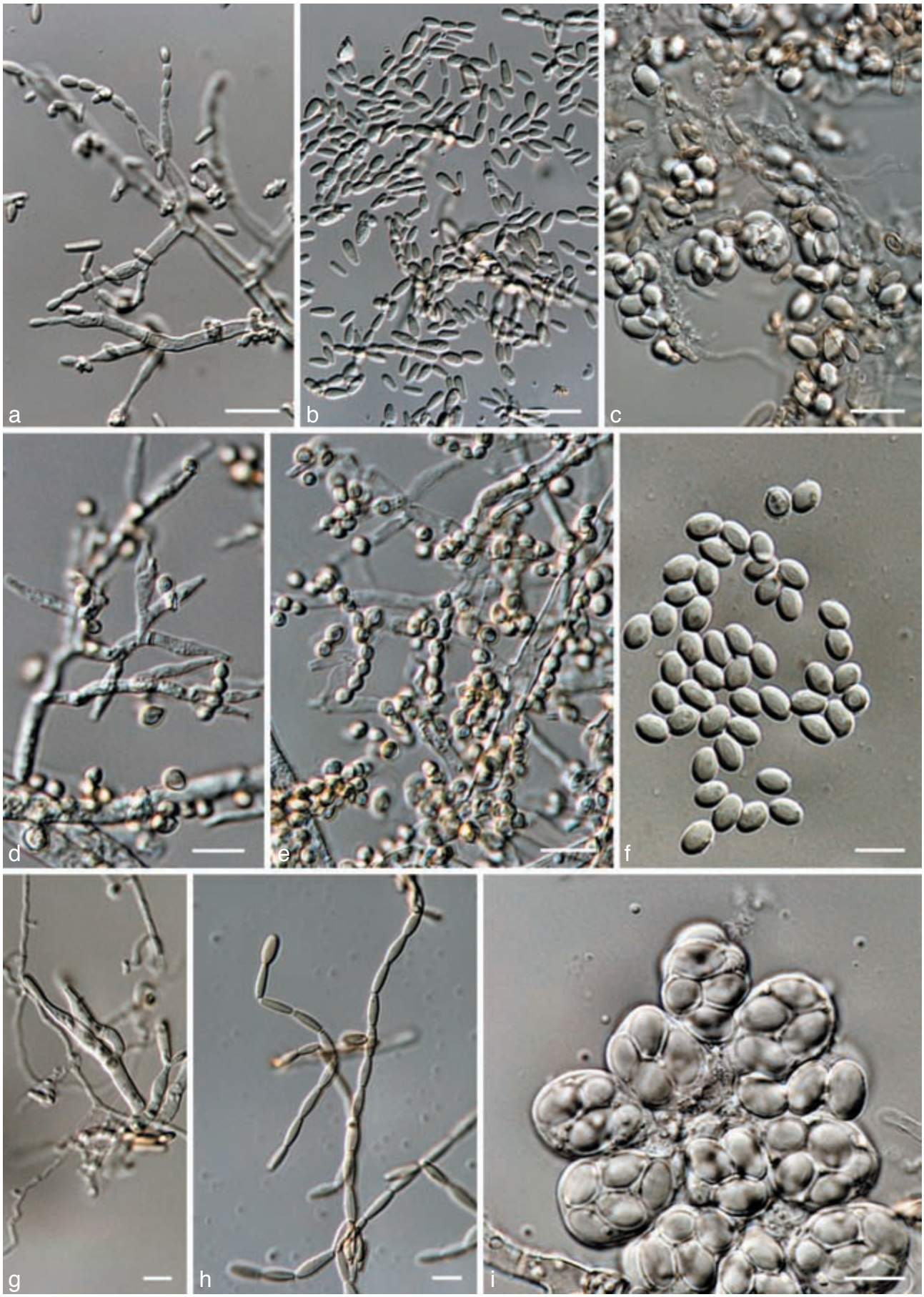

Fig. 8 *Byssochlamys fulva.* a. Conidiophores; b. conidia; c. asci and ascospores. — *Byssochlamys nivea.* d. Conidiophores; e. conidia; f. ascospores. — *Byssochlamys verrucosa.* g, h. Conidiophores and conidia; i. asci and ascospores. — Scale bars = 10 µm.

3. Conidia cylindrical and/or ellipsoidal; measuring 3.4–4.2 × 1.7–2.1 µm, colonies on MEA restricted, attaining a diameter of less than 45 mm in 7 d at 30 °C 4
3. Conidia larger 2.3–8(–13) × 1.5–4.5 µm, cylindrical or ellipsoidal, colonies on MEA larger than 45 mm after 7 d at 30 °C 5
4. Chlamydospores present, ratio between diameters on MEA at 37:30 °C between 0.25 and 0.45, colonies with well-defined margins . . . . . *P. brunneolus*
4. Chlamydospores absent, ratio between diameters on MEA at 37:30 °C between 0.60 and 0.75, colonies with more or less feathery margins . . . . . *P. divaricatus***
5. Conidia predominantly ellipsoidal with truncate ends, chlamydospores present 6
5. Conidia predominantly cylindrical, chlamydospores absent 7
6. Conidia measuring 3.7–5.6 × 2.4–3.6 µm, no acid production on CREA; *Byssochlamys* teleomorph produced in a heterothallic manner . . . . . *B. spectabilis*
6. Conidia measuring 3.2–5.7(–10) × 2–2.9(–3.4) µm, acid production under colony on CREA; *Byssochlamys* teleomorph absent . . . . . *P. formosus*
7. Conidia measuring 4.5–6 × 1.7–2.2 µm; ascospores, smooth-walled, 5.5–6.5 × 3.5–4.1 µm, acid production under colony on CREA . . . . . *B. fulva* (Fig. 8a–c)
7. Conidia measuring 9–11.8 × 2.5–4 µm; ascospores, verrucose and large, 7.1–8.1 × 5–5.7 µm, no acid production on CREA . . . . . *B. verrucosa* (Fig. 8g–i)*
8. Chlamydospores distinctly rough-walled . . . . . *B. zollerniae* (Fig. 4g–j)
8. Chlamydospores smooth walled or finely roughened . . . 9
9. Conidia measuring 3.1–3.8 × 2.5–3.2 µm, good growth on CYA . . . . . *B. laguncularia*e
9. Conidia measuring 3.1–4.3 × 2.6–3.4 µm, moderate growth on CYA . . . . . *B. nivea* (Fig. 8d–f)

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* *Byssochlamys verrucosa* is phylogenetically unrelated to the true *Byssochlamys* spp., but the taxon is included in the key because of its striking morphological similarity to other *Byssochlamys* species.

** Smooth ellipsoidal ascospores once observed in a fresh isolate (5.3–7 × 3.8–4.9 µm).

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