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The taxonomic and ecological characterisation of the clinically important heterobasiodiomycete *Fugomyces cyanescens* and its association with bark beetles

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Anamorphic heterobasidiomycete, taxonomically highly related or identical with *Fugomyces cyanescens* (Basidiomycota: *Microstromatales*), formerly known mostly from the clinical material, was frequently found in association with nine phloemophagous bark beetles at eleven localities in Hungary, Bulgaria and in the Mediterranean. The isolates were identified using morphological characteristics, its physiological profile and rDNA sequences and compared with the ex-type strain. The phylogeny was studied based on LSU and ITS-rDNA analysis. The morphology and ecology of the species is discussed in relation to related taxa which occur primarily on plants (phylloplane saprobes, parasitism), but sporadically also on clinical material obtained mostly from immuno-compromised patients.

Key words: *Fugomyces cyanescens*, *Microstromatales*, fungi associated with bark beetles, rDNA phylogeny

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Během studia floémofágních kůrovců na devíti lokalitách v Maďarsku, Bulharsku a ve středomořských státech byla často izolována anamorfní sněť *Fugomyces cyanescens* (Basidiomycota: *Microstromatales*), dříve známá především z klinického materiálu. Získané kmeny byly charakterizovány pomocí morfologických znaků, fyziologických testů, a sekvencí ribosomální DNA (LSU, ITS-rDNA) a srovnány s typovým kmenem. Morfologické a ekologické vlastnosti této houby jsou dále porovnávány s příbuznými taxony. Ty také žijí převážně na rostlinném materiálu, ale příležitostně bývají izolovány z materiálu klinického, získaného převážně z oslabených pacientů.

INTRODUCTION

Fugomyces cyanescens (de Hoog et de Vries) Sigler was described by de Hoog and de Vries (1973) as a new *Sporothrix* species on the basis of six isolates from human skin and air samples. One, of the three isolates from human specimens,

was suspected to be the cause of mycosis. More records originated from blood and various clinical material obtained mostly from immuno-compromised patients without any evidence of active invasion by *Fugomyces cyanescens* (Sigler et al. 1990, Jackson et al. 1990). Tambini et al. (1996) and Grossi et al. (2000) considered *F. cyanescens* a probable etiologic agent of pulmonary lesion and pneumonia in transplant patients. Several clinical cases in which the fungus played a hitherto unclarified role are mentioned by Middelhoven et al. (2000). These findings, together with its thermotolerance, suggest that *F. cyanescens* has a potential to be an opportunistic pathogen in compromised hosts. However, pathogenicity tests using an animal model and skin tests on sporotrichosis patients failed to demonstrate an invasive pathogenic potential (de Hoog and de Vries 1973, Kurata 1981, Sigler et al. 1990, Flores 1991).

F. cyanescens was also isolated nine times from air, soil and various plant material from Australia, India, Israel, the Netherlands, Spain and USA (Sigler et al. 1990, and references therein, Catalogue of Centraalbureau voor Schimmelcultures, Utrecht). The CBS catalogue mentions two isolations of this fungus in Iran from larvae of *Procontarinia matteiana* (Diptera: *Cecidomyiidae*, feeding on *Mangifera indica*) and also from larvae of *Diplolepis rosae* (Hymenoptera: *Cynipidae*, feeding on *Rosa* sp.).

Sporothrix Hektoen et Perkins was a form-genus comprising genera belonging to ascomycetes (*Ophiostomatales* and *Saccharomycetales*) and basidiomycetes. Moore (1987) erected the genus *Cerinosterus* for basidiomycetous *Sporothrix* species, with two species *Cerinosterus cyanescens* (= *Sporothrix cyanescens*) and *C. luteoalba* (de Hoog) R. T. Moore. *C. luteoalba*, the type of the genus, with the teleomorph *Ditiola pezizaeformis* (Lév.) Reid, belongs to the *Dacrymycetales* (Reid 1974). Moore (1987) noticed the fact that the septal pore ultrastructure of *Sporothrix cyanescens* does not show affinity to *Dacrymycetales*, nevertheless he supposed both *Cerinosterus* species to be congeneric, because of low quality and uncertain interpretation of the SEM figures published by Smith and Batenburg-van der Vegte (1985, 1986). However, partial LSU-rDNA sequences and the nutritional profile showed *Cerinosterus cyanescens* to be a close relative of the ustilaginomycetous fungus *Microstroma juglandis* (Ustilaginomycetes: *Microstromatales*) (Weijman and de Hoog 1985, Middelhoven et al. 2000), substantially differing from the type species of the genus *Cerinosterus*. To resolve this problem, Sigler established the new genus *Fugomyces* and named the fungus *Fugomyces cyanescens* (Sigler and Verweij 2003).

The LSU-rDNA sequence obtained by Middelhoven et al. (2000), is not deposited in GenBank and contains the D2 region only. This taxon was therefore omitted from the recent molecular phylogenic studies on heterobasidiomycetes (Begerow and Bauer 2000, Fell et al. 2000, Begerow et al. 2001).

During a survey of fungi associated with bark beetles in Central Europe and in the Mediterranean, a *Sporothrix*-like hyphomycete was found. This fungus was isolated from nine bark beetle species as a relatively frequently occurring fungus. Comparative morphological, molecular and physiological studies indicated identity with *Fugomyces cyanescens*. In this paper, we present our finding that *F. cyanescens* is a relatively abundant and consistent associate of bark beetles. Detailed descriptions of the morphology, physiology and phylogenetical position are given together with a discussion about their relation to other allied genera.

MATERIAL AND METHODS

Cultivation and isolation. Media: 2 % malt agar modified after Fassatiová (1986) (MA2; brewery malt 2° Balling, 1 l, agar 15 g,); malt extract agar (MEA; Pitt 1979b); potato carrot agar (PCA; Fassatiová 1986); corn meal agar (CMA; Difco corn meal agar 20 g, plain agar 10 g, H_2O 1 l); meat peptone agar (MPA; meat extract 10 g, peptone 4 g, NaCl 5 g, agar 20 g, H_2O 1 l); and a semi-natural medium containing 20 g of inner bark obtained from branches of *Prunus domestica* and 20 g of agar in 1 l of H_20. All strains were cultivated on PCA, CMA and on semi-natural medium for 1 month either in pure or cultures mixed with *Penicillium* species or *Cladosporium herbarum* to stimulate formation of the sexual morph. Plant samples containing galleries, larvae and adults of various bark beetles species were collected during the years 1997-2005 in Central Europe and the Mediterranean area. An single sample consisted of a population of one bark beetle species in larval feeding stage sampled at one time at one sampling site from one host plant. Five adults, larvae, detritus or wash solution from one gallery system (= larval galleries belonging to one parental gallery) were placed directly onto one Petri dish (MA2). Fifteen gallery systems were processed from each sample. The samples containing *Fugomyces cyanescens* are listed in Tab. 1. Representative strains were freeze-dried in skimmed milk and are deposited in the Culture Collection of Fungi (CCF), Department of Botany, Faculty of Science, Charles University, Prague or in the collection of M. Kolařík (MK) maintained at the Institute of Microbiology, ASCR, Prague.

Cultural and morphological characteristics. Micromorphology was studied on 7 days and 14 days old colonies grown on MEA at 24 °C, 37 °C and 41 °C. All cultures were examined microscopically during germination to detect the yeast stage. Mounts were prepared in Melzer´s reagent. Intact conidiophores were observed in slide cultures according to Cole et al. (1969). Measurements are reported as the minimum, mean and maximum values of 40 measurements of conidia and 10 of other structures from each strain. Conidiophores and conidia were photographed and measured using differential interference contrast or phase contrast with an Olympus BX51 microscope equipped with a digital camera (Camedia) and image-processing software QuickPhoto Micro 2.0 (Olympus, Hamburg, Germany).

DNA analysis and phylogenetic analysis. DNA isolation and ITS-rDNA analysis were performed as in Kolařík et al. (2005). The ITS-rDNA sequence of the *F. cyanescens* ex-type strain CBS357.73 and strains isolated from bark beetles (CCF3526, CCF3527, MK617, MK742, MK808, MK1710) were both strand sequenced using the primers ITS4S and ITS5 and are deposited in GenBank (see Fig. 2 for accession numbers). Amplification of the D1/D2 region of the nuclear large subunit of the ribosomal RNA gene was conducted using the primers NL1 and NL4 as described by Begerow et al. (1997). Partial LSU-rDNA genes of the strains CBS357.73, CCF3526, CCF3527, MK742 were sequenced and the GenBank accession numbers are given in Fig. 1.

Two separate data sets containing 16 ITS-rDNA and 46 LSU-rDNA sequences of ustilaginomycetous fungi were created (GenBank accession numbers are given in Figs. 1 and 2). Sequences were edited in BioEdit 5.0.9 (Hall 1999) and aligned using ClustalX 1.81 (Thompson et al. 1997). Phylogenetic trees were constructed using the maximum parsimony, and Fitch-Margoliash algorithms with Logdet distance meth-

ods implemented in PAUP* 4.0b10 (Swofford 1998), and by the Bayesian method implemented in the program MrBayes 3.0 (Huelsenbeck and Ronquist 2001). For the maximum parsimony and distance methods, full heuristic searches with 10 replicates and random taxa addition were used. Bootstrapping was performed with 1000 replicates. In order to lower the violation of the rate homogeneity across sites assumptions, constant positions were excluded from the alignment before performing the Logdet distance analysis (Waddell and Steel 1997). In MrBayes, base frequencies, rates for six different types of substitutions, number of invariant sites, and shape parameter of the gamma correction for rate heterogeneity with four discrete categories were allowed to vary. 3×10^6 generations in the LSU data set and 1×10^6 in the ITS dataset of the Markov Chain Monte Carlo were run with four simultaneous chains and heating temperature 0.2. The first 1000 (in LSU dataset) and 2500 (in ITS dataset) trees were discarded as burn-in.

Physiological tests. The physiological characteristics of isolates were examined using the methods described by Yarrow (1998).

RESULTS

Fugomyces cyanescens (de Hoog et de Vries) Sigler

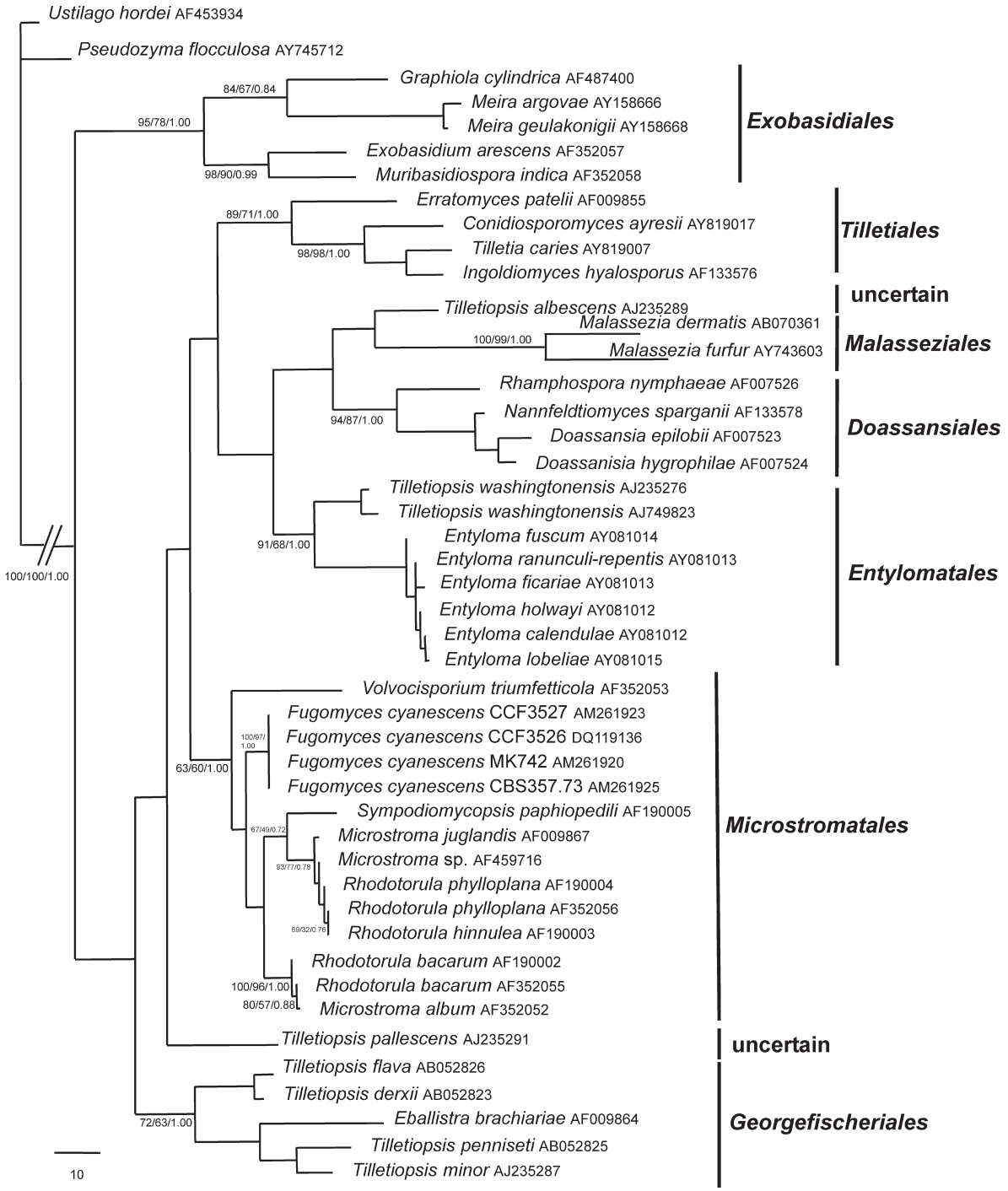
Isolation

Phloem feeding bark beetles were collected from several locations in Central Europe and the Mediterranean during the last seven years (for a list see Kolařík et al. 2005) and *F. cyanescens* was isolated from 13 samples infested with 9 bark beetle species spread over 11 localities in Bulgaria, Croatia, Hungary, Spain, Syria and Turkey (Tab. 1). *F. cyanescens* was common especially in association with *Phleotribus scarabeoides* (in 5 from 7 samples studied) and was typically found as most frequent after two species of the genus *Geosmithia* Pitt. From the bark beetle *Thamnurgus characiae* and from one of the *Phleotribus scarabeoides* samples from Spain, where *Geosmithia* spp. were absent, *Fugomyces cyanescens* was found as the principal fungal associate.

Phylogeny

All four studied D1/D2 region LSU-rDNA sequences of *F. cyanescens* (549 bp) were identical and were placed into *Microstromatales* (Fig. 1). Other sequences used in this comparison were grouped into well-supported clusters, which were congruent to the orders discussed by Bauer et al. (2001, 2006). Sequences of

Fig. 1. 50 % majority-rule consensus tree of members of Exobasidiomycetidae based on LSU region sequences. The tree was constructed by the maximum parsimony method. Heuristic search found 14 equally parsimonious trees. The topology was rooted with *Ustilago hordei*. The alignment contained 569 positions, of which 207 were parsimony informative. Bootstrap values from LogDet, maximum parsimony, and Bayesian posterior probabilities, respectively, are shown at the nodes. Statistical supports of internal branches in the *Doassansiales*, *Entylomatales*, *Exobasidiales*, *Georgefischeriales*, *Malasseziales* and *Tilletiales* clades are not shown, nor supports with the bootstrap or posterior probability below the 50 or 0.5 respectively. The scale bar represents one change per 100 positions.


Ustilago hordei AF453934
Pseudozyma flocculosa AY745712
Graphiola cylindrica AF487400
Meira argovae AY158666
Meira geulakonigii AY158668
Exobasidium arescens AF352057
Muribasidiospora indica AF352058
Exobasidiales
84/67/0.84
95/78/1.00
98/90/0.99
Erratomyces patelii AF009855
Conidiosporomyces ayresii AY819017
Tilletia caries AY819007
Ingoldiomyces hyalosporus AF133576
Tilletiales
89/71/1.00
98/98/1.00
uncertain
Tilletiopsis albescens AJ235289
Malassezia dermatis AB070361
Malassezia furfur AY743603
Malasseziales
100/99/1.00
Rhamphospora nymphaeae AF007526
Nannfeldtiomyces sparganii AF133578
Doassansia epilobii AF007523
Doassanisia hygrophilae AF007524
Doassansiales
94/87/1.00
Tilletiopsis washingtonensis AJ235276
Tilletiopsis washingtonensis AJ749823
Entyloma fuscum AY081014
Entyloma ranunculi-repentis AY081013
Entyloma ficariae AY081013
Entyloma holwayi AY081012
Entyloma calendulae AY081012
Entyloma lobeliae AY081015
Entylomatales
91/68/1.00
100/100/1.00
Volvocisporium triumfetticola AF352053
Fugomyces cyanescens CCF3527 AM261923
Fugomyces cyanescens CCF3526 DQ119136
Fugomyces cyanescens MK742 AM261920
Fugomyces cyanescens CBS357.73 AM261925
Sympodiomycopsis paphiopedili AF190005
Microstroma juglandis AF009867
Microstroma sp. AF459716
Rhodotorula phylloplana AF190004
Rhodotorula phylloplana AF352056
Rhodotorula hinnulea AF190003
Rhodotorula bacarum AF190002
Rhodotorula bacarum AF352055
Microstroma album AF352052
Microstromatales
100/97/1.00
63/60/1.00
67/49/0.72
93/77/0.78
69/32/0.76
100/96/1.00
80/57/0.88
Tilletiopsis pallescens AJ235291
uncertain
Tilletiopsis flava AB052826
Tilletiopsis derxii AB052823
Eballistra brachiariae AF009864
Tilletiopsis penniseti AB052825
Tilletiopsis minor AJ235287
Georgefischeriales
72/63/1.00
10

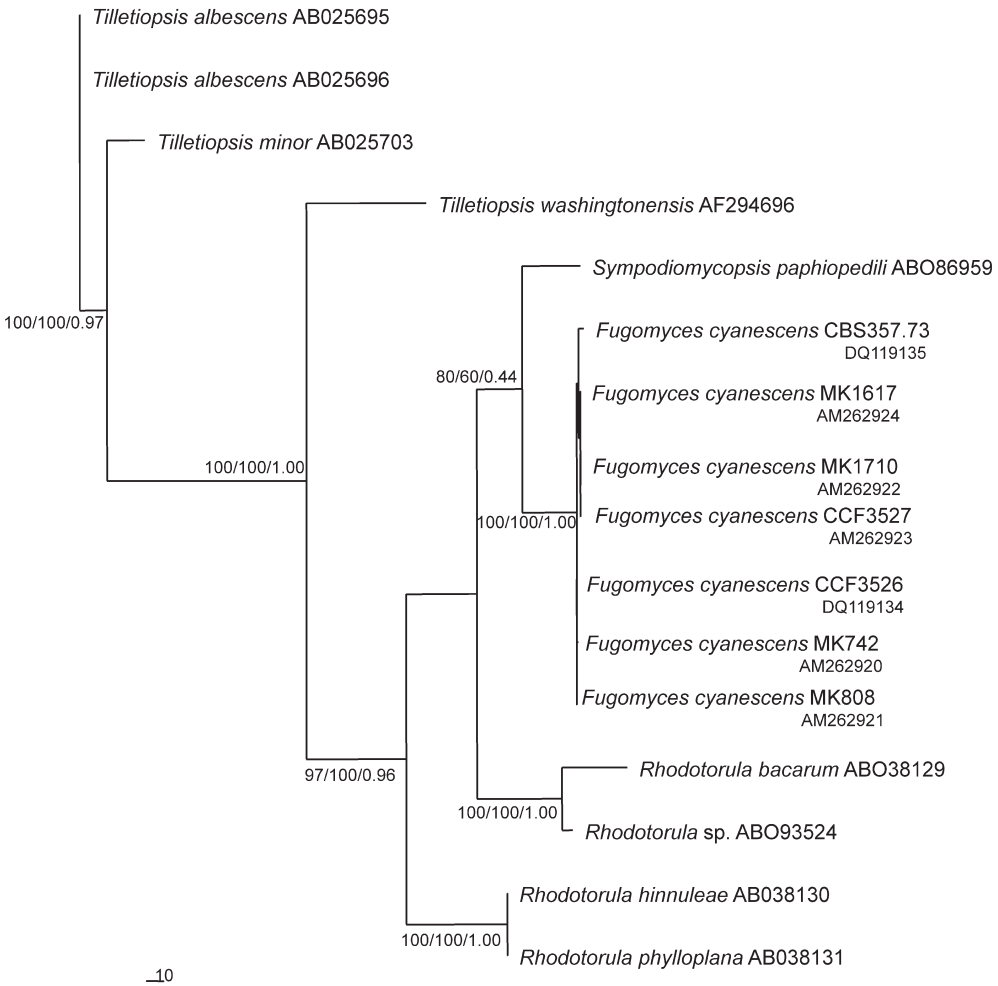


Fig. 2. Unrooted 50 % majority-rule consensus tree of the members of *Exobasidiomycetidae* based on ITS region sequences. The tree was constructed by the maximum parsimony method. Heuristic search found 24 equally parsimonious trees. The alignment contained 631 positions, of which 245 were parsimony informative. The tree was constructed by the maximum parsimony method. Bootstrap values from LogDet, maximum parsimony, and Bayesian posterior probabilities, respectively, are shown at the nodes. Statistical supports with the bootstrap or posterior probability below the 50 or 0.5 are not shown. The scale bar represents one change per 100 positions.

Tilletiopsis albescens and *T. pallescens* did not fit to any known group and their positions remained unresolved. The nodes with significant statistical support were identical in all topologies. There are two distinct evolutionary lineages within *Microstroma* and *Rhodotorula*. *Fugomyces cyanescens*, *Volvocisporium triumfetticola* and *Sympodiomycopsis paphiopedili* are located outside these

Tab. 1. *F. cyanescens* isolates obtained from the bark beetles and frequencies of their transportation.

Bark beetle and host tree	Collection site and date	Representative strain no.	Percentage of samples with *F. cyanescens*[1]
Ernoporicus caucasicus on *Tilia cordata*	Bulgaria, Rodopy Mts., Bačkovo, 41°57'0" N, 24°52'0" E; leg. M. Kolařík; Sept. 2005	MK1750	6 % (15)
Scolytus intricatus on *Quercus* sp.	Bulgaria, Rodopy Mts., Bačkovo, 41°57'0" N, 24°52'0" E; leg. M. Kolařík; Sept. 2005	MK 1710	5 % (20)
Hypoborus ficus on *Ficus carica*	Turkey, Içel Province, Silifke, 36°22'40" N, 33°56'4" E; leg. M. Kolařík; Apr. 2004	CCF 3526 (= MK 842)	3 % (50)
Scolytus carpini on *Carpinus betulus*	Hungary, Bakony range, forest near Vinye, 47°22'0" N, 17°48'0" E; leg. M. Kolařík; Jun. 2003	CCF 3527 (= MK 617)	25 % (4)
Chaetoptelius vestitus on *Pistacia vera*	Turkey, Içel Province, Silifke, Demircili, 36°22'41" N, 33°56'4" E; leg. M. Kolařík; Apr. 2004	MK 742	25 % (4)
Scolytus rugulosus on *Malus domestica*	Turkey, Içel Province, Silifke, Demircili, 36°22'41" N, 33°56'4" E; leg. M. Kolařík; Apr. 2004	MK 809	5 % (20)
Scolytus amygdali, on *Amygdalis communis*	Syria, Baniyas, Al Marquab; leg. M. Kolařík, 35°8'60" N, 35°57'0" E, Apr. 2004	MK 755	50 % (2)
Phleotribus scarabeoides on *Olea europea*	Syria, Krak des Chevaliers, 34°46'0" N, 36°19'0" E; leg. M. Kolařík; Apr. 2004	MK 765, MK808	71 % (7)
	Croatia, Brač island, Sumartin, 43°17'6" N, 16°52'32" E; leg. M. Kolařík; Aug. 2004; together with *Chaetoptelius vestitus*	CCF 3528 (= MK 936)	
	Spain, Andalusia, Huelva province, Galaroza, 37°55'0" N, 6°42'0" W; leg. M. Kolařík; May 2005	MK 1617	
	Spain, Andalusia, Almeria province, Darrical, 36°55'0" N, 3°1'0" W; leg. M. Kolařík; May 2005	MK 1630	
	Spain, Andalusia, Granada province, Yátor, 36°57'0" N, 3°8'60" W; leg. M. Kolařík; May 2005	MK 1616	
Thamnurgus characiae on *Euphorbia charracis*	Croatia, Brač island, near Blaca monastery, 43°19'35" N, 16°27'1" E; leg. M. Kolařík; Aug. 2004	MK 917	50 % (2)

[1] Number and percentage indicate the proportion of samples of particular bark beetles species collected during 2000-2005 in Central Europe and the Mediterranean where the fungus was found, in brackets, is the total number of studied bark beetle samples (see Methods for sample definition).

clades and represent unique lineages. There is only a small number of published sequences of the less conservative ITS-rDNA region in *Microstromatales* and the closest sequence belongs to *Sympodiomycopsis paphiopedili* (Fig. 2).

ITS-rDNA sequences (657 bp) of six *Fugomyces* strains from bark beetles showed variability in seven positions including two positions in which both pyrimidine bases (T, C) were presented in the same position and strain. The sequence of *F. cyanescens* ex-type strain differs in four bases from other *Fugomyces* strains (Tab. 3). Sequences from strains originating from European localities are more similar than those from Asia, but no statistically supported clusters were generated in phylogenetical analyses (Fig. 2).

Tab. 2. Nutritional profile of *Fugomyces cyanescens* (isolates CCF 3527, CCF 3526, CCF 3528) compared with *Sympodiomycopsis paphiopedili* (SP, from Sugiyama et al. 1991) and *Microstroma juglandis* (MJ, from Middelhoven et al. 2000). The data from *F. cyanescens* are compared with those recorded by Middelhoven et al. (1992). The activities differing from our results are shown in brackets. Abbrevations used: – negative; + positive; W weakly positive; ND not determined; SS some isolates positive.

Physiological test	Reaction			Physiological test	Reaction		
	FC	SP	MJ		FC	SP	MJ
Fermentation:							
lactose, maltose, sucrose	–	–	–	glucose, galactose, raffinose	–	–	–
Assimilation:							
D-glucose	+	+	+	Inositol	+	+	+
D-galactose	+ (–)	W	–	Ethanol	+	ND	+
Maltose	+	+	+	Glycerol	+	+	+
Sucrose	+	+	+	L-sorbose	–	+	–
Lactose	– (SS)	W	–	L-rhamnose	–	–	–
Raffinose	+	+	+	D-ribose	W	+	+
Melezitose	– (+)	+	+	Ribitol	– (SS)	–	ND
D-xylose	+	+	–	Galactitol	–	–	–
D-arabinose	+	+	–	D-mannitol	+	+	+
L- arabinose	+	+	+	D-glucitol	+	+	+
Cellobiose	+	+	+	Salicin	– (SS)	–	–
Trehalose	+	+	+	D,L-lactate	W	W	–
Soluble starch	+	W	–	Citrate	– (+)	–	–
Inulin	– (SS)	W	–	Succinate	W	+	–
Erythritol	+	+	+	D-galacturonic acid	–	ND	–
Melibiose	+	+	–				
Nitrogen sources:							
Potassium nitrate	+	+	+	Sodium nitrite	+	ND	ND
L-lysine	+	ND	–	Creatine	–	ND	–
Ethylamine	–	ND	–				
Additional growth tests:							
Vitamin-free medium	+	+	+	Starch synthesis	–	–	–
Actidion 0.01% (cycleheximide)	–	ND	–	Formation of carotenoid pigments	–	–	–
Growth at 5 °C	–	ND	ND	Urease test	+	W	ND
Growth at 20 °C	+	+	+	Extracellular DNase test	+	+	ND
Growth at 30 °C	+	+	ND	Arbutin degradation	+	ND	W
Growth at 37 °C	+	–	–	Growth on 50 % (w/w) glucose medium	–	–	ND
Growth at 42 °C	– (SS at 41°C)	–	–				

Tab. 3. Variability in ITS-rDNA sequences of *Fugomyces cyanescens*.

Strain no.	Country of origin	Variable position in ITS-rDNA region										
		22	79	99	105	128	409	426	497	501	512	538
CBS 357.73^{T}	Netherlands	C	G	A	C	C	C	A	–	–	T	G
GCCF 3526	Turkey	C	A	A	T	C	G	A	–	–	T	A
MK 742	Turkey	C	A	A	T	C	G	C	–	–	T	A
MK 808	Syria	–	A	A	T	C	G	A	T	C	Y	A
MK 1710	Bulgaria	–	A	A	T	T	G	A	T	C	Y	A
CCF 3527	Hungary	N	A	–	T	Y	G	A	T	C	Y	A
MK 1617	Spain	N	A	–	T	Y	G	A	T	C	Y	A

Physiological characteristics

All tested strains (CCF 3526, 3527, 3528) of *F. cyanescens* from bark beetles exhibited the same physiological characters and their characteristics are given in Tab. 2.

Morphology

MEA (14 d, 24 °C): colonies 10-18 mm in diameter consisted of dense, hyaline aerial and substrate mycelium, plane or wrinkled, surface velvety or lanose, texture soft, conidia en masse white, lilac to violet with age, reverse dull yellow to violet with age, soluble pigment violet (only in the strains CCF 3526, MK 742, MK 755, MK1710). MEA (14 d, 37 °C) 10 mm, similar to previous, but colonies with a typical brain-like appearance. PCA (14 d, 24 °C): colonies 20 mm in diameter, substrate and aerial mycelium very sparse; reverse and soluble pigment intensely violet coloured. CMA (14 d, 24 °C): Colonies 55 mm in diameter, other characteristics similar to those on MEA. CYA (14 d, 24 °C): Colonies 20 mm (MK 613) to 30 mm in diameter (CCF 3527), sporulation of all isolates lilac with age, reverse dull lilac to dull blue, soluble pigment absent. Other characteristics similar to those on MEA.

No clamp connections, basidia or ballistospores were observed on any of the media screened. The entirely filamentous appearance was maintained upon cultivation at 25, 30, or 37 °C, but solitary yeast cells were rarely observed (see below).

Conidiophores on MEA hyaline, 40–60 × 1.5–2.0 µm; mono- or semimacronematous, arising only from aerial mycelium. Vegetative mycelium hyaline, thin-walled, septate, 1.5–3.0 µm (Figs. 3, 4, 7, 8, 10-12); conidiogenous cells polyblastic with holoblastic sympodial proliferation, 2–35 µm long, lateral or intercalar (as a result of subsequent proliferation), mostly developing directly from the undifferentiated hypha, with an apical swelling or rachis bearing numerous denticles (Figs. 3a, 3b). Conidiogenous cells sometimes repeatedly proliferating to other conidiogenous cells (Fig. 12). Proximal (primary) conidia clavate, oblong ellipsoidal, (5.0–)6.0(–8.8) × (2.2–)2.7(–3.5) µm, arranged in whorls of 4–10, imminently

germinating with distal (secondary) conidia (Fig. 11), apical part bearing numerous denticles after distal conidia dehiscence; distal conidia oval, amygdaliform, solitary or rarely in blastic acropetal chains of two, (2.8–)2.9(–3.1) × (1.5–)1.6 (–1.8) µm, 1-8 in one cluster (Figs. 5, 6). Both type of conidia, when placed onto MEA, immediately produced (secondary conidia later) germination tubes. Forming of other conidia (yeast stage) from these solitary conidia was observed at 40 °C on MEA and on MPA in a mucoid plaque, which later converted in the hyphal phase. The yeast stage was detected only in colonies inoculated from old PCA slants, and consequential subculturing gave rise only to the hyphal phase under the same cultivation conditions. Solitary yeast cells were uni– or multipolar, budding with globose to oblong conidia of different size (0.5–1.5 µm) or with phialides or anellides (?) (Figs 9, 13). Conidia arising through enteroblastic budding and successive collarettes resembling percurrent proliferation were observed.

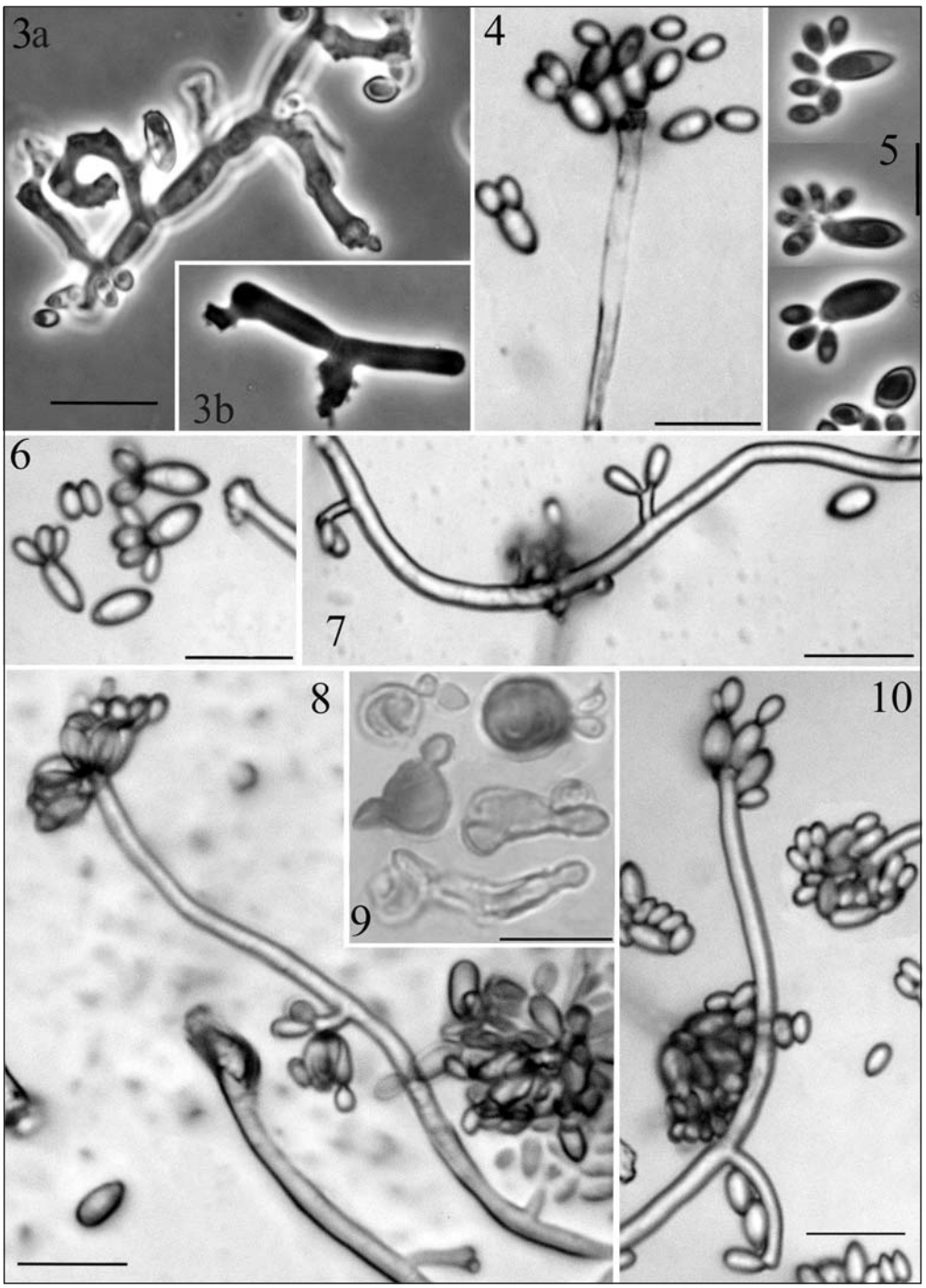
In spite of prolonged incubation on all media used, in pure culture as well as in mixed culture, the sexual stage was not observed. The substrate mycelium of *Fugomyces cyanescens* loosely intertwined with the hyphae of the added fungi. No special hyphal structures typical of mycophagous heterobasidiomycetes like colacosomes or haustoria were observed.

DISCUSSION

The taxonomic identity of *Fugomyces* strains from bark beetles

The morphology of the *Fugomyces* isolates from bark beetles agreed very well with published descriptions of *F. cyanescens* (de Hoog and de Vries 1973, Sigler et al. 1992), which we found very precise. The violet, pH-dependent, soluble pigment was produced on MEA and CMA only by some strains. This variability was observed also in previous morphological descriptions including Middelhoven et al. (2000) on various complex media. However, we found two media (PCA, CYA) on which this pigment was produced by all strains, which suggested their suitability for detection of this pigment.

Figs. 3-10. *Fugomyces cyanescens* CCF 3562. **Fig. 3a, 3b.** Sympodially proliferating conidiogenous cells with scars after having released conidia (phase contrast). **Figs. 4, 8, 10.** Conidiophores bearing proximal and distal conidia. **Figs. 5, 6.** Released conidia forming rosette-like formations. **Fig. 7.** Formation of the first two proximal conidia from a conidiogenous cell. **Fig. 9.** Yeast cells. Microphotographs are directly from the Petri dish with MEA after 1 day at 24 °C (Figs. 4, 6-8, 10), from slide mounts after 7 days on YMEA at 40 °C in near anoxic condition (Fig. 9) and after 7 days on MEA at 24 °C. Bar = 10 µm (Figs. 3-8, 10), 1 µm in Fig. 9.


3a
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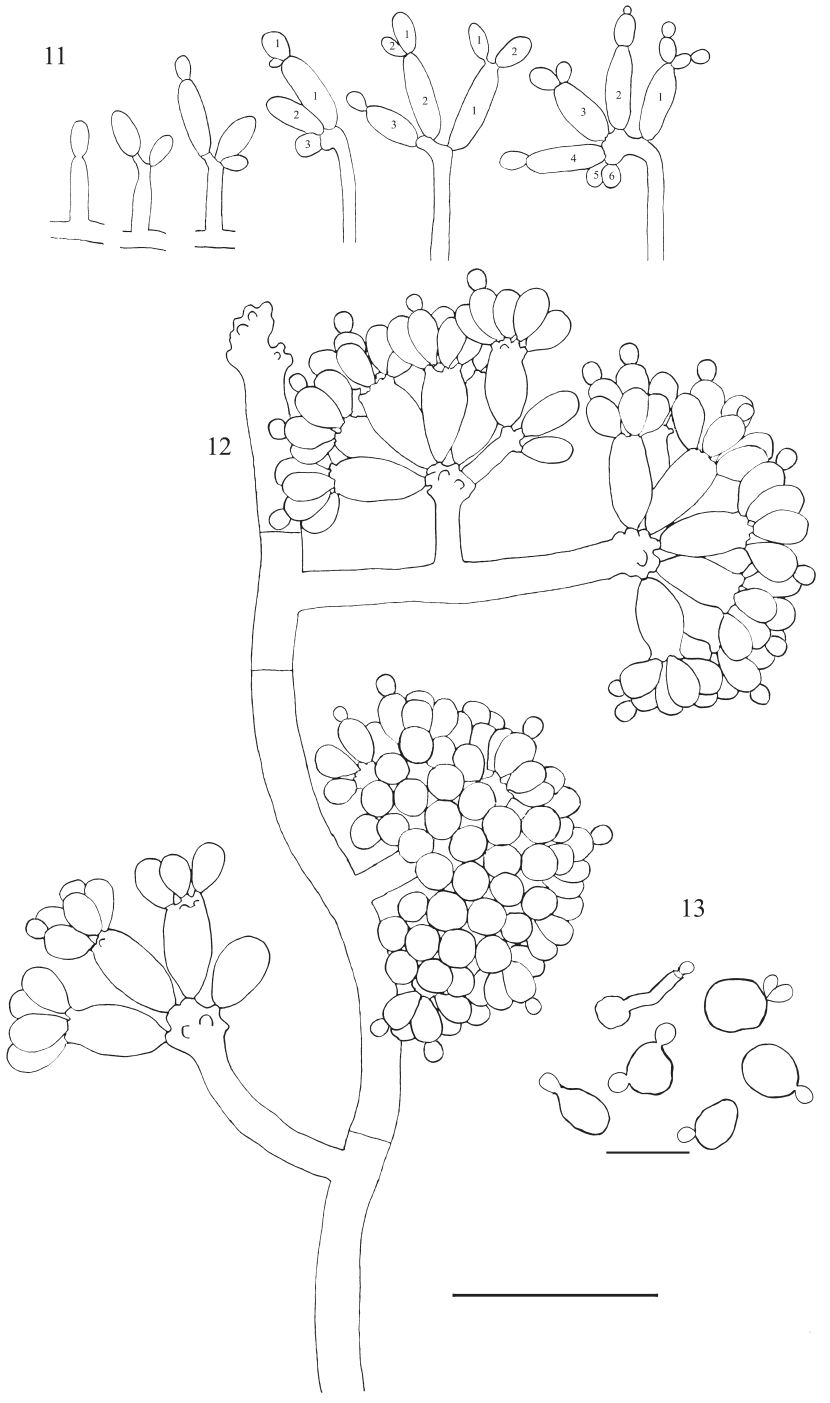
11
12
13

The assimilation profile of *Fugomyces* strains derived from bark beetles were identical to those from *F. cyanescens* strains (Middelhoven et al. 2000), with the exception of citrate and melezitose which were assimilated by all of the strains tested by Middelhoven and colleagues (Tab. 2). Ours strains also differ in D-galactose assimilation, which was together with a positive reaction for urease and DNase and with the inability to assimilate L-sorbose and ethylalamine regarded as a specific character of *F. cyanescens* (Middelhoven et al. 2000).

LSU-rDNA pointed at the identity of our strains with the *F. cyanescens* ex-type strain. The more divergent ITS region detected variability among our *Fugomyces*, apparently ascribable to the infraspecific clinal variation. This pattern, although based on six strains only, suggests the formation of geographically limited populations, which is perhaps due to short range dispersal mediated by bark beetles instead of air dispersal across long distances. The observed ITS region similarity between ex-type strain and strains from bark beetles is higher then 99.3 %, which suggests a high evolutionary proximity.

The standard technique for species identification of ustilaginomycetous anamorphs is traditionally based on phenotypic characteristic. However, a major difficulty in accurately identifying of these fungi is the small number of polymorphic morpho-characters and different within-species strain responses to many physiological tests, such as utilisation of carbon and nitrogen compounds (Boekhout 1987, 1995; Boekhout et al. 1995; Middelhoven 2004). Therefore, a complex analysis on many strains using molecular markers like LSU-rDNA and ITS-rDNA sequences can clarify the taxonomic position, especially in cases of closely related species. Identity in the D1/D2 region of LSU-rDNA is supposed in most cases to be evidence of conspecificity. Nevertheless, constant differences in less conserved rDNA spacers (ITS1, ITS2), supported by additional biological characteristics like mating type, virulence, host range and physiological profile can determine unique biological species within strains with identical D1/D2 regions (Fell at al. 2000). We supposed our strains from bark beetles to be *F. cyanescens*, based on many similarities in phenotype and genotype with the ex-type strain. Nevertheless, detailed comparison with other strains from non-bark beetle habitats can further elucidate the taxonomic position of *Fugomyces* strains from bark beetles.

Figs. 11-13. *Fugomyces cyanescens* CCF 3562. **Fig. 11.** Scheme of conidiophore ontogenesis. **Fig. 12.** Mature conidiophore. **Fig. 13.** Yeast cells. Bar = 10 μm (Figs. 11, 12), 1 μm in Fig. 13.

The genus *Fugomyces* and related genera

Five genera are hitherto placed within *Microstromatales*, three of them being anamorphic without known teleomorph: *Fugomyces*, *Sympodiomycopsis* Sugiy., Tokuoka et Komag. and *Rhodotorula* Harrison. A dominant hyphal morph with whorls of holoblastic sympodial conidia bearing other holoblastic conidia occurs only in the genus *Fugomyces*. *Sympodiomycopsis* is characterised by a dominant yeast morph with enteroblastic-annellidic (rarely holoblastic-sympodial) budding cells and a hyphal morph with holoblastic sympodioconidia (Sugiyama et al. 1991). The phylogenetically related species from the genus *Rhodotorula* are yeasts with enteroblastic conidiogenesis. Morphologically, the *Microstromatales* are characteristic by the formation of a yeast stage with non-septate, globose to ellipsoidal yeast cells (Begerow et al. 2001). We observed the same type of yeast cell as that recorded in the original protologue of *Sporothrix cyanescens* (de Hoog and de Vries 1973) and by Sigler (1990).

Sympodiomycopsis is probably the closest known relative to *Fugomyces*, when we compare all taxa from *Microstromatales*. Both genera have a hyphal morph with holoblastic sympodioconidia and their physiological profiles differ only in the utilisation of L-sorbose and citrate and in growth at higher temperatures (Tab. 2). According to Sugiyama et al. (1991), the hyphal morph of *Sympodiomycopsis paphiopedili* bears solitary conidia only. These conidia highly resemble proximal conidia of *Fugomyces cyanescenes* in shape and size. The yeast stage of *Sympodiomycopsis paphiopedili* is represented by yeasts (4-11 × 2-6 µm) which bear many holoblastic conidia apically (3-5 × 1.5-2 µm) on short denticles (Sugiyama et al. 1991). However, *Sympodiomycopsis* with a yeast stage as a dominant morph is known only from orchid nectar and does not form a monophyly with *Fugomyces* in rDNA phylogenetical trees, which suggest a separate position of this genus.

Similarly looking and often pigmented colonies and conidiophores with more or less sympodial and often catenulate blastoconidia, originating on sterigmata, are known in many other mitosporic genera from the class Ustilaginomycetes, such as *Tilletiopsis* Derx (Boekhout 1991), *Pseudozyma* Boekhout (Boekhout 1995), *Meira* Boekhout, Scorzetti, Gerson et Sztejnberg (Boekhout et al. 2003, Yasuda et al. 2006) and *Acaromyces* Boekhout, Scorzetti, Gerson et Sztejnberg (Boekhout et al. 2003). Another common character of the ustilaginomycetous anamorphs are often lysing hyphae, with the cytoplasm retracted in cells surrounding living cells, especially in sporulating regions (Sigler 1999, Boekhout et al. 2003). We observed this also on our *Fugomyces* strains from bark beetles.

Ecology of *Fugomyces cyanescens*

Bark beetles are known to be associated with predominantly ascomycetous fungi. Nevertheless, Kirschner (1998) recently discovered more than 20 different heterobasidiomycetes associated with bark beetles. *Cuniculitrema polymorpha* belongs to Heterobasidiomycetes (*Tremellales*) (Kirschner et al. 2001), *Colacogloea papilionacea*, *Atractocolax pulvinatus*, *Chionosphaera cuniculicola* and *Mycogloea nipponica* to Uredinomycetes (Agaricostilbomycetidae and Microbotryomycetidae) (Fell et al. 2000, Kirschner et al. 1999, Kirschner et al. 2001, Kirschner et al. 2003). *Fugomyces cyanescens* is the first fungus associated with bark beetles belonging to the class of Ustilaginomycetes, known to date (R. Kirschner, pers. comm.).

Bark beetle galleries appear to be a home niche of many heterobasidiomycetes, but the way in which these taxa interact with their insect associates and with other arthropods or fungi in the bark beetle habitat is unknown. Some of them are mycoparasites and may be significant antagonists of more intimate associates such as ambrosia and blue-stain fungi (Kirschner 1998, Six 2000). *F. cyanescens* does not exhibit mycoparasitic ability. There are no data about the entomopathogenicity of this fungus, nevertheless it was isolated from living adults or larvae, but never from cadavers, and it seems to be harmless to bark beetles.

F. cyanescens is regarded as a rare opportunistic pathogen of weakened patients. Sigler (1992) mentioned *F. cyanescens* to be a skin commensal. From all the records of *F. cyanescens*, this fungus seems to be a native saprophyte of various plant materials. We suppose this fungus also to be a regular inhabitant of bark beetles, which is apparent from its widespread distribution and from the host preference to a particular vector (*Phleotribus scarabeoides*). The affinity to plant material was formerly confirmed by its ability to degrade many typical plant metabolites such as ferulic acid or other complex benzene compounds which are constituents of the plant cell wall (Middelhoven et al. 2000). The fungus is also well adapted to living close to bark beetles, which is indicated by its ability to utilise DNA, urea and lipids (mentioned by Middelhoven et al. 2000). This enzymatic equipment together with thermotolerance and the ability to utilise Tween 80 (Middelhoven et al. 2000) facilitate the accidental jump to immunocompromised hosts. The similar ecology is found in many allied (and often ancestral) taxa, which are typically inhabitants of the phyllosphere (*Tilletiopsis*, *Rhodotorula*) and sugar-rich plant parts (*Rhodotorula*, *Sympodiomycopsis* and *Tilletiopsis* species listed in Fig. 1). Some of them, like *T. albescens* and *T. minor*, are also known as opportunistic pathogens of humans (Boekhout 1991, Ramani et al. 1997).

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