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Molecular Variability of *Pseudallescheria boydii*, a Neurotropic Opportunist

J. RAINER,[1]† G. S. DE HOOG,[1]* M. WEDDE,[2] Y. GRÄSER,[3] AND S. GILGES[4]

Centraalbureau voor Schimmelcultures, Baarn, The Netherlands,[1] and Department of Microbiology, University of Technology,[2] and Institute of Microbiology and Hygiene, Humboldt University,[3] Berlin, and Institute of Hygiene, University of Bonn, Bonn,[4] Germany

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The sequences of the internal transcribed spacer (ITS) ribosomal DNA (rDNA) domain data obtained by restriction fragment length polymorphism analysis with 18S rDNA and fingerprinting (M13) for clinical and environmental strains of *Pseudallescheria boydii* (anamorph, *Scedosporium apiospermum*) were compared to those for related species of *Pseudallescheria*, *Petriella*, and *Scedosporium*. The infraspecific variability of *P. boydii* was considerable. There were five different lengths in the 18S rDNAs within *P. boydii* due to the occurrence of introns. In several cases, strains isolated from a single pond or ditch proved to be genetically very different. Nevertheless, some lineages had a regional distribution. The variability found is unlikely to be explained by meiotic recombination alone. *Pseudallescheria fusoidea*, *Pseudallescheria ellipsoidea*, and *Pseudallescheria angusta* were found to be synonyms for *P. boydii*. *Scedosporium prolificans* was found amid *Petriella* species in the ITS tree and showed no infraspecific variability. The type strain of *Rhinocladium lesnei* proved to be identical to *Graphium putredinis*. *Acladium castellanii*, which is morphologically reminiscent of *S. apiospermum*, was also found to be a separate species, but with an unknown affiliation.

Pseudallescheria boydii (Shear) McGinnis et al. [anamorph, *Scedosporium apiospermum* (Sacc.) Sacc.] is one of the emerging agents of opportunistic mycoses in humans. Twelve case reports for hospitalized patients suffering from myeloid leukemia or subjected to immunosuppressive therapy were published in 1999 alone. Infections are difficult to treat because of its resistance to common antifungal drugs such as amphotericin B. The diagnostic problems with this species are considerable. In tissue *P. boydii* is indistinguishable from other filamentous fungi (20), and *Cryptococcus* antigen testing may lead to false-positive reactions (23). Systemic infections may easily be misidentified as aspergillosis, leading to inappropriate therapy. This is one of the reasons that the mortality rate due to invasive pseudallescheriasis is high.

Judging from the number of patient isolates received by the Centraalbureau voor Schimmelcultures (CBS) Identification Department, there may still be a considerable underdetection of *P. boydii* (9), probably due to the species' clinical and morphological diversity. In the past, etiologic agents were frequently introduced as new taxa, as their identity with existing species was not recognized. Still, the literature contains a number of "ghost taxa" in such divergent genera as *Acremonium*, *Actinomyces*, *Cephalosporium*, *Graphium*, *Madurella*, and *Verticillium* that may be identical to *P. boydii* (8).

The anamorph of the species was introduced more than 100 years ago by Harz and Bezold (see references 27 and 28) as an agent of human otitis. Since the 1920s it became known as one of the major agents of mycetoma (26) and other subcutaneous infections. During the last few decades, rhinopharyngeal and pulmonary colonization was reported in leukemic, cystic fibrosis, and otherwise impaired patients (35). In disseminated cases the species is significant because of its neurotropism, with marked predilection for the cerebrospinal fluid (12). Such infections were sometimes acquired after near-drowning events in polluted waters (24).

Much of the observed morphological variability can be ascribed to variable abundance of (syn)ana- and teleomorphs. This was confirmed by the investigation of characters independent of morphology, such as nutritional physiology (10) and 18S ribosomal DNA (rDNA) sequencing (16). Wedde et al. (36), using rDNA ITS2 sequencing, were able to select primers for species recognition. However, infraspecific molecular variability of the internal transcribed spacer (ITS) rDNA of *P. boydii* seems to be larger than that of species such as *Trichophyton rubrum* (Castell.) Sab. (14), *Hortaea werneckii* (Horta) Nishimura et Miyaji (38), or *Cladophialophora bantiana* (Sacc.) de Hoog et al. (13). Previously, nuclear DNA (nDNA) homology studies had indicated the existence of three infraspecific groups in *P. boydii* (10, 15), and the clinical pictures and environmental sources of isolation of these three groups broadly matched. Bell (2) had found differences in virulence between a strain from patients with subcutaneous infections and one from the environment. Thus, *P. boydii* may comprise entities with different pathogenicities.

In the study described in the present paper, the molecular variability of *P. boydii* was evaluated. This should help to establish a clear species concept which is required for reliable molecular identification of this opportunist in the routine laboratory. To this aim we compared the published nDNA-DNA homology data with results of small subunit (SSU) restriction analysis and sequencing and of fingerprinting by PCR (primer M13). The strains of *P. boydii* compared included isolates that originated from a single source (patient or environmental), as well as a worldwide selection of strains collected over a period of 70 years (Table 1). Some closely related taxa are included for comparison.

* Corresponding author. Mailing address: Centraalbureau voor Schimmelcultures, P.O. Box 273, NL-3740 AG Baarn, The Netherlands. Phone: 31-35-5481253. Fax: 31-35-5416142. E-mail: de.hoog@cbs.knaw.nl.

† Present address: Institute of Microbiology (N.F.), Leopold Franzens University, Innsbruck, Austria.

TABLE 1. Strains examined and sources of isolation

Species	Strain no.	Status	Source of isolation	Origin
P. boydii 1	CBS 101.22	Type strain of *Allescheria boydii* Shear	Mycetoma	Texas
P. boydii 2	IP 1411.82		Mycetoma	
P. boydii	CBS 100.870		Mycetoma	
P. boydii	RKI 2782/95		Trauma and sepsis	Hamburg, Germany
P. boydii	RKID 386		Subcutaneous mycosis, leukemic patient	Hungary
P. boydii	CBS 330.93		Bronchial secretion	Alkmaar, The Netherlands
P. boydii	CBS 329.93		Bronchial secretion	Alkmaar, The Netherlands
P. boydii	CBS 101718		Encephalitis of a child after near drowning	Germany
P. boydii 1	IP 1698.87		Lung of a leukemic patient	France
P. boydii 1	IP 1945.90		Sputum of a cystic fibrosis patient	France
P. boydii	RKI 866/94		Sputum of a patient after heart transplantation	Berlin, Germany
P. boydii	RKI 2956/93		BAL fluid[a] of a patient after heart transplantation	Berlin, Germany
P. boydii 2	CBS 695.70	Type strain of *Acremonium suis* Bakai	Sinus of a pig	Kiev, Ukraine
P. boydii 2	IP 1946.90		Sinus	France
P. boydii 2	CBS 987.73		Otitis	CSSR
P. boydii	CBS 100.26	Type strain of *Acladium castellanii* Pinoy in Castellani	Human	
P. boydii	CBS 591.90	Type strain of *Pseudallescheria shearii* Negroni & Fischer	Mycetoma of the knee	Buenos Aires, Argentina
P. boydii 3	CBS 108.54		Soil	Zaire
P. boydii 3	IP 1742.88		Soil	France
P. boydii	CBS 101717		Soil	Brazil
P. boydii 2	CBS 499.90		Mud of a pond	Groningen, The Netherlands
P. boydii	CBS 101719		Mud of a pond	Groningen, The Netherlands
P. boydii	CBS 101720		Sandy soil of a polluted ditch	Site of a car accident (compare CBS 330.93 and CBS 329.93)
P. boydii	CBS 101721		Mud	s'Graveland, The Netherlands
P. boydii	CBS 101722		Mud	Gooimeer, The Netherlands
P. boydii	CBS 101723		Mud	Eempolder, The Netherlands
P. boydii	CBS 101724		Mud	Wasmeer, The Netherlands
P. boydii	CBS 101725		Mud	Lapersveld, The Netherlands
P. boydii	CBS 101726		Mud	Lapersveld, The Netherlands
P. africana	CBS 311.72	Type strain of *Pseudallescheria africana* (von Arx et G. Franz) McGinnis et al.	Brown sandy soil	25 km west of Tsintsabis, Namibia
P. angusta	CBS 254.72	Type strain of *Pseudallescheria angusta* (Malloch et Cain) McGinnis et al.	Half-digested sewage tank	Ohio
P. desertorum	CBS 489.72	Type strain of *Pseudallescheria desertorum* (von Arx et Moustafa) McGinnis et al.	Salt marsh soil	Kuwait
P. ellipsoidea	CBS 418.73	Type strain of *Pseudallescheria ellipsoidea* (von Arx et Fassatiová) McGinnis et al.	Soil	Tadzjikistan
P. fimeti	CBS 129.78	Type strain of *Pseudallescheria fimeti* (von Arx et al.) McGinnis et al.	Dung of a goat	Aligarh, India
P. fusoidea	CBS 106.53	Type strain of *Pseudallescheria fusoidea* (von Arx) McGinnis et al.	Soil	Guipo, Panama
G. calicioides	CBS 102084		Decayed wood	Aomori Prefecture, Japan
G. calicioides	CBS 102080		Decayed wood	Yamanashi Prefecture, Japan
G. penicillioides	CBS 320.72	Type strain of *Stilbum basitruncatum* Matsushima	Forest soil	Honara, Solomon Islands
G. putredinis	CBS 102083		*Chrysalidiocarpus lutescens*	Tokyo, Japan
G. tectonae	CBS 127.84	Type strain of *Graphium tectonae* C. Booth	Seed of *Tectona grandis*	Jamaica
Petriella guttulata	CBS 362.61	Synonymous type strain of *Petriella guttulata* Barron et Cain	Dung of partridge	Germany
Petriella lindforsii	CBS 352.59		Woodland soil	
Petriella musispora	CBS 745.69	Type strain of *Petriella musispora* Malloch	Decayed wood of *Populus tremuloidesetriella*	Ontario, Canada
Petriella setifera	CBS 559.80			Ocho Rios, Jamaica
S. prolificans	CBS 114.90	Type strain of *Scedosporium inflatum* Malloch et Salkin	Bone biopsy specimen of a 6-year old male	United States
S. prolificans	RKID 85		Drain of the sinus maxillaris of a patient with AML[b]	Rostock, Germany
S. prolificans	RKI 1482/95		Human	
S. prolificans	RKI 2399/94		Human	
S. prolificans	CBS 467.74	Type strain of *Lomentospora prolificans* Hennebert et Desai	Greenhouse soil, from mixed forest litter	Heverlee, Belgium

[a] BAL, bronchoalveolar lavage.
[b] AML, amyelocytic leukemia.

TABLE 2. Summary of RFLP analysis of the 18S rDNA region with seven restriction enzymes[a]

Species	Strain no.	Length (bp) of SSU rDNA	Pattern obtained with the following enzyme:						
			*Hae*III	*Hin*fI	*Dde*I	*Rsa*I	*Msp*I	*Hha*I	*Taq*I
P. boydii 1	IP 1698.87	1,800	A	A	A	A	A	A	A
P. boydii 1	CBS 101.22	1,800	A	A	A	A	A	A	A
P. boydii 1	IP 1945.90	1,800	A	A		A	A	A	A
P. boydii 2	CBS 499.90	1,800	A	A	A	A	A	A	A
P. boydii 3	CBS 108.54	1,800	A	A	A	A	A	A	A
P. boydii 3	IP 1742.88	1,800	A	A	A	A	A	A	A
P. boydii	CBS 591.90	1,800	A	A	A	A	A	A	A
P. boydii	RKI 866/94	1,800	A	A	A	A	B	A	A
P. boydii	CBS 101720	1,800	A	A	A	A	A	A	A
P. boydii	CBS 101717	1,800	A	A	A	A	A	A	A
P. africana	CBS 311.72	1,800	A	A	A	A	A	A	A
P. angusta	CBS 254.72	1,800	A	A	A	A		A	A
P. ellipsoidea	CBS 418.73	1,800	A	A	A	A	A	A	A
P. fusoidea	CBS 106.53	1,800	A	A	A	A	A	A	A
P. fimeti	CBS 129.78	1,800	D	A	A	A	A	A	A
G. tectonae	CBS 127.84	1,800	E	A	A	A	A	A	A
S. prolificans	RKI 1482/95	1,800	C	J	D	C	A	C	A
S. prolificans	RKI 2399/94	1,800	C	J	D	C	A	C	A
S. prolificans	CBS 467.74	1,800	C	D	D	C	A	C	A
S. prolificans	CBS 114.90	1,800	C	J	D		A		A
P. guttulata	CBS 362.61	1,800	D	A	A	L	H	E	A
P. lindforsii	CBS 352.59	1,800	F	F	F	E	E	A	D
P. boydii	CBS 100.26	1,800	M	K	G	H	F	K	C
P. boydii	CBS 330.93	2,180	B	B	B	B	D	B	F
P. boydii	CBS 329.93	2,180	B	B	B	B	D	B	F
P. boydii 2	IP 1946.90	2,180	B	B	B	B	D	B	B
P. boydii	RKI 2956/93	2,180	B	B	B	B	D	B	B
P. boydii	RKID 386	2,180	B	B	B	B	D	B	B
P. boydii 2	IP 1411.82	2,180	B	B	B	B	D	B	
P. boydii 2	CBS 987.73	2,180	H	H	H	J	M	H	F
P. boydii	CBS 101719	2,180	H	H	J	J	M	H	F
P. boydii	CBS 101718	2,270	I	I	I	I	I	I	I
P. desertorum	CBS 489.72	2,720	G	E	E	D		D	D
P. boydii	RKI 2782/95	2,720	H	G	L	G	C	G	B
P. boydii 2	CBS 695.70	3,300	B	C	C	F	G	F	

[a] The different lengths of the corresponding SSU rDNAs are mentioned. For *P. boydii* the numbers following the species name indicate the nDNA-DNA reassociation groups (15). Banding patterns are characterized as letters.

MATERIALS AND METHODS

DNA extraction. The methods applied for DNA extraction were described previously (13).

Restriction analysis. Amplification of the 18S rDNA was performed with primers Oli4 and NS24 (31). The resulting amplicons were digested with the restriction endonucleases *Hae*III, *Hin*fI, *Dde*I, *Rsa*I, *Taq*I, *Msp*I, and *Hha*I (Amersham/Pharmacia Biotech) under the conditions recommended by the manufacturer. The corresponding products were electrophoresed in 1.5% agarose gels at 150 V for 2 to 3 h. The fragments were analyzed by computer-aided image analysis (ImageMaster; Pharmacia Inc.) and were verified by comparison with the expected patterns on the basis of the sequences of the same or related strains.

Sequence determination and analysis. The ribosomal ITS region was amplified with primers V9G and LS266 (13). Both strands were sequenced with the internal primers ITS2, ITS3, ITS4, and ITS5 and the Big Dye terminator cycle sequencing kit (PE Applied Biosystems, Warrington, United Kingdom), as recommended by the manufacturer, combined with an ABI automatic DNA sequencer. Sequences were sampled with the Seqman package (DNAStar Inc., Madison, Wis.) and were aligned with BioNumerics software (Applied Maths, Kortrijk, Belgium). The distance tree was constructed with the neighbor-joining algorithm with the Kimura correction in the Treecon package (32). The robustness of the branches was assessed by bootstrap analysis with 100 replicates. The topology of the tree was verified with several algorithms including the parsimony algorithm.

PCR fingerprinting. The core sequence of phage M13 was used as a single primer in the PCR experiments. Amplification reactions were performed as described by Weising et al. (37) in an Amplitron II thermocycler. For each reaction 10 ng of DNA was used. Forty PCR cycles were programmed as follows: 20 s at 93°C, 60 s at 50°C, and 20 s at 72°C, with elongation at 72°C for 6 min and chilling to 4°C. The PCR products were electrophoresed on 1.5% agarose gels at 100 V for 4 to 6 h. The gels were stained with ethidium bromide and photographed under UV light. The DNA fragment profiles were analyzed with the help of Gelcompar software (Applied Maths).

RESULTS

Restriction analysis. Data obtained by restriction fragment length polymorphism (RFLP) analysis of the 18S rDNA gene are summarized in Table 2. Amplicons varied in length because of the occurrence of inserts of about 380, 470, 920, or 1,500 bp. Only restriction patterns that resulted from the digestion of 18S rDNA genes of the same length are comparable. Twenty-two strains had no inserts. Ten such strains, identified as *P. boydii*, yielded identical patterns when the same enzymes were used; the exception was RKI 866/94 digested with *Msp*I. The patterns of strains of nDNA reassociation groups 1 to 3 were identical. The patterns of type strains of *Pseudallescheria africana* (von Arx et G. Franz) McGinnis et al., *Pseudallescheria angusta* (Malloch et Cain) McGinnis et al., *Pseudallescheria fusoidea* (von Arx et al.) McGinnis et al., and *Pseudallescheria ellipsoidea* (von Arx et Fassatiová) McGinnis et al. were iden-

FIG. 1. Phylogenetic tree of species of the family *Microascaceae* studied. The tree was constructed on the basis of confidently aligned positions of the rDNA ITS domain. The tree was generated with the Treecon package with the neighbor-joining algorithm and Kimura correction. *G. calicioides* was used as the outgroup. The tree was subjected to 100 bootstrap replications; only values >90 are shown. G, *Graphium*; P, *Pseudallescheria*; Pe, *Petriella*; R, *Rhinocladium*; S, *Scedosporium*; (T), ex type strain; 1 to 3, nDNA homology groups (15).

tical to those of *P. boydii*. In contrast, strain CBS 100.26, maintained in the CBS culture collection as *P. boydii*, was deviant with all enzymes used. *Graphium tectonae* C. Booth and *Pseudallescheria fimeti* (von Arx et al.) McGinnis et al. deviated from *P. boydii* in *Hae*III restriction patterns. Four strains of *Scedosporium prolificans* (Hennebert et Desai) Guého et de Hoog had the same profiles only with *Msp*I and *Taq*I; they were identical to each other except for the *Hin*fI pattern for CBS 467.74. *Petriella guttulata* Barron et Cain and *Petriella lindforsii* Curzi showed significant differences with most enzymes.

Eight strains identified as *P. boydii* had amplicon lengths of about 2,180 bp, corresponding to the presence of an intron of about 380 bp that was demonstrated in two *P. boydii* strains by Issakainen et al. (16). Identical RFLP patterns were generated for four strains; however, some deviations were found with *Taq*I. The *Taq*I patterns of strains CBS 330.93 and CBS 329.93 of *P. boydii* deviated. Two strains (strains CBS 987.73 and CBS 101719) were different in tests with all enzymes except *Taq*I, which suggests that another intron of a similar length is present in *P. boydii*. The few strains with longer 18S rDNA amplicons yielded incomparable restriction patterns.

Sequencing. For the distance trees presented in Fig. 1 and 2 we included ITS1 and ITS2 sequences published by Wedde et al. (36) and Lennon et al. (18). Sequences could not always be aligned with confidence, particularly the sequence of *P. boydii* CBS 100.26 (*Acladium castellanii* Pinoy), which was excluded from further analysis, and a group around *Petriella setifera*, which could be only partially aligned, as indicated in Fig. 1. Separate partial and complete ITS domain analyses were performed with and without this group. Table 3 summarizes the numbers of base substitutions in the ITS domains by taking *P. boydii* CBS 330.93 as a reference. Within the entire group compared, a maximum of 58 positions in ITS1 were variable, 79 positions in ITS2 were variable, and 1 position in the 5.8S rDNA gene was variable.

In the general tree (Fig. 1), *P. setifera* ATCC 26490 was taken as the outgroup. All strains morphologically identified as *P. boydii* composed a main group. The 10 strains that had deviating 18S rDNA amplicon lengths are now confirmed to fall

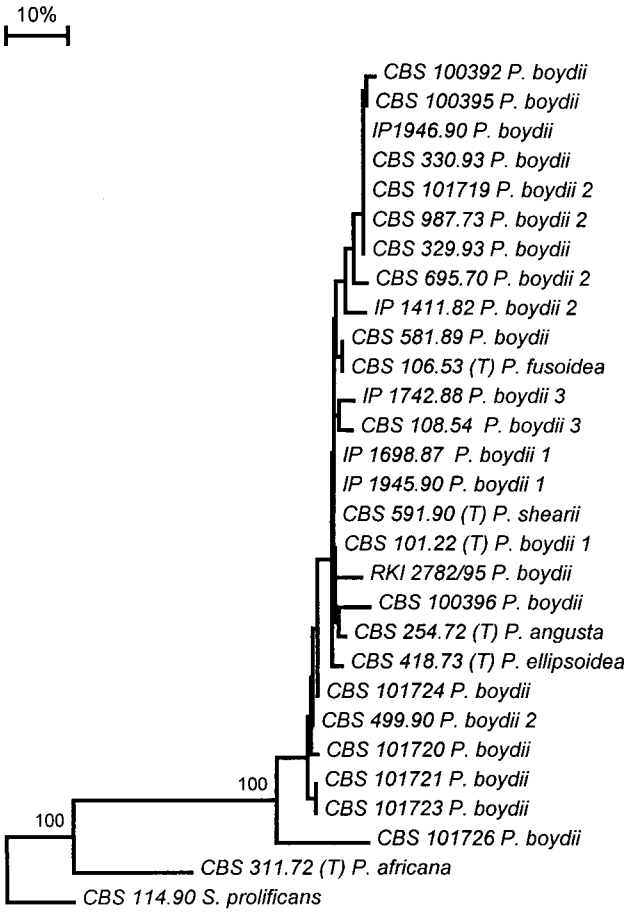

FIG. 2. Phylogenetic tree of *P. boydii*, constructed as described for the tree in Fig. 1. *S. prolificans* CBS 114.90 was used as the outgroup.

within the range of variability of *P. boydii*. Among these were the type strains of *P. angusta* (CBS 254.72), *P. ellipsoidea* (CBS 418.73), and *P. fusoidea* (CBS 106.53). *P. africana* (CBS 311.72) and *Pseudallescheria desertorum* (v. Arx et Moustafa) McGinnis et al. (CBS 489.72) were located outside the *P. boydii* clade. CBS 101721 had considerable deviations in ITS1, while the ITS2 domain was nearly identical (Table 3).

P. guttulata-P. setifera, *Pseudallescheria fimeti*, *G. tectonae*, *Graphium calicioides* (Fr.) Cooke et Massee, and *Graphium putredinis* (Corda) S. Hughes took rather isolated positions. The type strain of *Rhinocladium lesnei* Vuill., CBS 108.10, was nearly identical to *G. putredinis*. Five strains of *S. prolificans* were strictly identical to each other. They did not match any of the teleomorphs included in the study.

Internal branches within the *P. boydii* clade (Fig. 2) were found to correspond partly with the postulated infraspecific groups on the basis of nDNA-DNA reassociation data (15), but this could not be statistically confirmed. Strains CBS 329.90 and CBS 330.93, isolated from a single comatose patient after a near drowning, had identical sequences. In contrast, strain CBS 101720, isolated 2 years later from water at the site of the accident, is significantly different. Strains CBS 499.90 and CBS 101719, both isolated from mud of the same pond, had different ITS sequences. *P. boydii* strains CBS 101725 and CBS 101726, which were isolated from a single sample of polluted pond water, proved to be a significant distance from each other. In contrast, the ITS sequences and M13 fingerprints of pairs of separately isolated strains, strains CBS 101721 and CBS 101723 and strains IP 1698.87 and 1945.90, were (nearly) identical.

Fingerprinting with M13. Examples of the banding patterns resulting from PCR fingerprinting are shown in Table 4. Profiles were found to be highly heterogeneous. Almost every strain had its own pattern; very few bands could be matched with confidence. All tests were repeated several times and proved to be reproducible. The fingerprints of the type strains of *P. africana* (CBS 311.72) and *P. ellipsoidea* (CBS 418.73), having somewhat deviant ITS sequences, proved to be identical to each other. Clinical strains IP 1698.87 and IP 1945.90 were also identical. The two strains from a single patient, CBS 329.93 and CBS 330.93, had the same patterns (data not shown), whereas strain CBS 101720, from the site of the accident but with another ITS sequence, proved to have different patterns.

All *S. prolificans* strains except CBS 467.74 had identical banding patterns. Strain CBS 467.74 deviated in some bands. This strain also had different RFLP patterns.

DISCUSSION

P. boydii is a cleistothecial ascomycete that belongs to the family *Microascaceae*. This corresponds to 18S rDNA sequencing data (16), which also indicated that *Microascus cirrosus* Curzi is a close relative. In a similar study, Okada et al. (21) found three *Graphium* species among the members of the family *Microascaceae*. From our more detailed ITS data, *G. putredinis* was found to be close to *G. tectonae*, while *Graphium penicillioides* Corda took an isolated position amid the *Petriella* species.

Phenetically the genera *Petriella* and *Pseudallescheria* are distinct by having ostiolate versus nonostiolate ascomata, respectively (33). Our ITS1 and ITS2 distance tree (Fig. 1) does not show a clear bipartition. Most of the nonostiolate species are found in the upper part of the tree, and the ostiolate species are found in the lower branch of the tree. Issakainen et al. (16) supposed that *S. prolificans* is close to *P. setifera*, but in our study *S. prolificans* proved to be clearly different. No teleomorph has yet been found for *S. prolificans.*

von Arx et al. (33) distinguished seven species in the genus *Pseudallescheria*. Of these, only *P. fimeti* was found to have clearly different ITS sequences. *P. angusta*, *P. ellipsoidea*, and *P. fusoidea* were found within the *P. boydii* main group and must be regarded as synonymous names, as already supposed on the basis of morphological studies by McGinnis et al. (19)

TABLE 3. ITS sequence diversity in *Pseudallescheria* and related fungi

Strain	No. of base substitutions[a]		
	ITS1	5.8S	ITS2
P. boydii CBS 330.93	0	0	0
P. boydii CBS 100396	7	3	8
P. boydii CBS 101721	17	0	5
P. africana CBS 311.72^T	32	3	35
G. tectonae CBS 127.84^T	43	0	41
R. lesnei CBS 108.10^T	45	0	51
S. prolificans CBS 114.90	46	0	43
P. fimeti CBS 129.78^T	55	0	74
P. guttulata CBS 362.61^T	81	0	69
G. penicillioides CBS 320.72	70	0	78
P. lindforsii CBS 352.59	84	3	100

[a] Numbers of base substitutions with respect to the sequence of *P. boydii* CBS 330.93.

TABLE 4. Banding patterns from fingerprinting with M13

Strain	Presence or absence of bands at the following positions[a]:																																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
P. boydii RKI D 386	+	−	−	+	+	−	−	−	−	+	−	−	+	−	−	−	−	+	−	−	+	−	−	−	+	−	+	−	−	−	+	−	−	−	−	−	+	+	
P. boydii RKI 2956/93	+	−	−	+	−	−	−	−	−	+	−	−	+	−	+	−	−	+	+	−	+	−	−	−	−	+	−	−	+	−	+	−	−	+	−	+	−	−	−
P. boydii 3 CBS 108.54	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	+	+	−	−	−	−	−	+	−	−	+	−	−	+	−	−	−	+	−	−	−
P. fimeti CBS 129.78	−	−	−	−	+	−	−	−	−	−	+	−	−	+	−	+	−	+	−	+	−	−	−	−	−	−	−	+	−	+	−	−	−	+	−	−	−	−	−
P. boydii CBS 101717	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	+	−	−
P. angusta CBS 254.72	+	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	+	−	−	−	−	+	+	−	−	−	+	−	−
P. boydii CBS 101718	−	−	+	−	+	−	−	−	−	−	+	−	−	−	+	−	−	+	−	+	−	−	−	−	+	−	+	−	−	−	+	−	−	−	+	−	−	−	−
P. boydii 2 CBS 695.70	−	−	−	−	+	−	−	−	−	−	+	−	−	+	+	−	−	+	−	+	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−
P. desertorum CBS 489.72	+	−	−	−	+	−	−	−	−	+	−	−	−	+	−	−	+	+	−	−	−	+	−	−	−	+	−	−	−	−		+	−	−	−	−	−	+	−
P. boydii 2 IP 1946.90	+	−	−	+	+	−	−	−	+	−	+	−	−	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+	−
P. fusoides CBS 106.53	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−
P. boydii 1 IP 1698.87	+	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	+	−	+	−	−	−	−	+	−	+	−	−	−	−	+	−	−	−	−	+	+	−
P. boydii 1 IP 1945.90	+	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	+	−	+	−	−	−	−	+	−	+	−	−	−	−	+	−	−	−	−	+	+	−
P. boydii CBS 591.90	+	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	+	−	+	−	−	+	−	+	−	+	−	−	−	−	−	−	−	+	−	−	+	−
G. tectonae CBS 127.84	−	−	−	−	−	−	−	+	−	+	−	−	−	+	+	−	−	+	−	−	+	−	−	−	−	−	−	−	−	+	−	−	+	−	+	−	−	−	−
P. boydii CBS 100.26	−	−	−	−	−	−	+	−	−	−	−	−	+	−	+	−	−	+	−	−	−	+	−	−	−	+	−	−	+	+	−	+	−	−	+	−	+	−	−
P. lindforsii CBS 352.59	−	−	−	−	−	+	−	+	−	−	−	+	−	−	+	−	−	+	−	+	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
P. boydii CBS 101720	−	−	−	−	−	+	−	+	−	−	+	−	−	−	+	−	−	−	+	−	−	+	−	−	−	−	+	−	+	−	−	+	−	−	+	−	−	−	−
P. boydii 2 CBS 499.90	−	−	−	−	−	+	+	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	+	−	−	+	−	+	−	−	+	−	−	−	−
P. guttulata CBS 362.61	+	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+
P. boydii RKI 866/94	−	−	−	−	+		−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	+

[a] The occurrence (+) or lack of occurrence (−) of bands with a certain molecular weight is indicated. Thirty-nine band positions were taken into account.

and on the basis of the ITS2 sequences by Wedde et al. (36). The species have been distinguished primarily on the basis of sizes of the cleistothecia and ascospores, but ranges of variability were strongly overlapping. The positions of *P. desertorum* and *P. africana* remain ambiguous, since their sequences differed from that of the type strain of *P. boydii*, CBS 101.22, by 12.8 and 6.8%, respectively.

The patterns of the ITSs of strain CBS 100.26, included in the CBS List of Cultures as *P. boydii*, obtained by RFLP analysis with SSU were markedly different from those of the remaining species; the ITSs could not be aligned. Earlier, it was found to have a clearly different mole percent G+C DNA content (15). The strain is of the *Acladium castellanii* type and was described from a patient with subcutaneous, suppurative mycosis (5). The fungus was described as having yellowish cultures which became blackish on some media; conidiogenous cells were aggregated in fascicles and produced apical clusters of truncate conidia (4). Butler (4) supposed that it was related to *Sporothrix schenckii* Hektoen et Perkins, while de Hoog (6) suggested an affinity to *Raffaelea* on the basis of its truncate sympodial conidia. Both suggestions were refuted on the basis of ITS (data not shown) and 18S rDNA sequence data (M. Blackwell, personal communication; compare these suggestions with those in reference 17), respectively. The taxonomic position of *A. castellanii* remains uncertain.

Rhinocladium lesnei (34) was found to be identical to *G. putredinis* and represents an anamorph taxon clearly apart from *P. boydii*. Morphologically and culturally, the two strains analyzed, CBS 108.10 and CBS 102083, were very different. CBS 108.10 was whitish, showing a *Scedosporium* anamorph only. However, in the original publication of Vuillemin (34), fasciculate conidiophores and denticulate conidiogenous cells are depicted. Thus, the identity of the two taxa is highly probable.

The genetic variability within *P. boydii* is considerable. Guého and de Hoog (15) found three infraspecific ecological and clinical groups on the basis of nDNA-DNA reassociation experiments. Reassociation between these groups was consistently about 50%, whereas within the groups the values were >80%. Similar reassociation groups in *Galactomyces geotrichum* Redhead et Malloch have recently been recognized as separate taxonomic entities (29). Contrary to these findings, two groups in *Cladophialophora* were judged to belong to different species, despite nDNA-DNA homology values of >80% (13). In bacteria, homology values are more precise than sequence data (30), but this is particularly due to the use of SSU sequences. In fungi, DNA homology values compared to ITS sequence data indicate a level of diversity which is similar or somewhat lower. The *P. boydii* reassociation groups are not supported statistically in the ITS tree (Fig. 1). It is remarkable, however, that all strains of reassociation group 2 except strain CBS 499.90 contain an intron in the SSU rDNA gene and can be found in a single branch in the ITS tree.

Judging from the data obtained by molecular fingerprinting with M13, the variability within *P. boydii* is considerable. Nearly all strains proved to be different from each other; the exceptions were three pairs (CBS 329.93 and CBS 330.93, CBS 101721 and CBS 101723, and IP 1698.87 and IP 1945.90). The ITS sequences of these pairs were also identical, while for two of the three pairs the sources of isolation were remote from each other. This finding indicates the existence of widespread genotypes. Due to the molecular variability of *P. boydii*, identification based on species-specific primers or RFLP analysis should be interpreted with care in routine diagnostics.

In the case of strains CBS 329.93, CBS 330.93, and CBS 101720, molecular fingerprinting with M13 proved to be an appropriate method for characterization of individual strains, since identical fingerprinting patterns were generated for the strains isolated from a single patient. Data obtained by molecular biological analysis for all strains from the same nonclinical isolation site (strains CBS 499.90 and CBS 101719 and strains CBS 101725 and CBS 101726) were different (Fig. 1 and 2; Tables 2 to 4). For one pair of strains (CBS 101725 and CBS 101726), the strains were different as determined by culture, and their ITS sequences deviated to such an extent that they may be two different species (Fig. 1). These findings are in agreement with those of April et al. (1), who found *P. boydii* strains from closely similar sites to be highly variable in cultural and physiological parameters.

Apparently, environments which are suitable for the growth of *P. boydii* are consistently inhabited by different populations.

The small amount of correspondence between fingerprinting bands indicates that variation is continually generated, probably by meiotic recombination. However, the variability of the ITS sequences of *P. boydii* strains exceeds that of intermating populations. This may be a reason why such a broad spectrum of clinical pictures caused by *P. boydii* occurs, and a tendency to neurotropic colonization has evolved. The distance between CBS 101725 and CBS 101726 from a single sample is particularly illustrative. It is unclear why different populations have been maintained in a single environment and isolates with similar genotypes have been isolated from locations that are separated by significant geographic distances. A population genetic study is required to obtain more insight into speciation and evolution of *P. boydii*. M13 fingerprints, ITS sequences, and RFLP patterns proved to be more conserved in *S. prolificans.* Wedde et al. (36) and San Millán et al. (25) showed that variability in ITS sequences and randomly amplified polymorphic DNA analysis data, respectively, for this anamorphic taxon seem low. These results indicate that the life cycle of this species might be reduced to clonal reproduction. In contrast to *P. boydii*, a teleomorph name that indicates a sexual mode of reproduction is not known for *S. prolificans.*

Clinical strains are distributed over the entire tree. Infections caused by *P. boydii*-related species other than the opportunistic species *P. boydii* and *S. prolificans* are a case of lobomycosis caused by *Petriella setifera* in a dolphin (11) (another case of infection in a dolphin was attributed to *P. boydii* [see also reference 22]) and a human skin infection due to *R. lesnei* (34). Within *P. boydii*, the amount of variability of clinical strains is comparable to that of the environmental strains of that species (Fig. 1). This suggests that no particular selection by the host occurs and, thus, that all environmental strains have equal pathogenic potential. The natural ecological niche of *P. boydii* remains unknown, but nutrient-rich, poorly aerated environments have been described as the ecological niche of *P. boydii* (10). Ecological data should be taken into account in clinical practice, as they differ considerably between *P. boydii* and *Aspergillus fumigatus. P. boydii* has often been isolated from polluted ponds frequented by waterbirds; the fungus possibly occurs as a commensal organism in their intestinal tracts. It may then be expected that *P. boydii* has a significant potential to be an opportunistic pathogen. Given the fact that the species is particularly controlled by unspecific defense systems (3), primary pathogenicity seems unlikely.

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