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Internal Transcribed Spacer rRNA Gene-Based Phylogenetic Reconstruction Using Algorithms with Local and Global Sequence Alignment for Black Yeasts and Their Relatives

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Sequences of rRNA gene internal transcribed spacer (ITS) of a standard set of black yeast-like fungal pathogens were compared using two methods: local and global alignments. The latter is based on DNA-walk divergence analysis. This method has become recently available as an algorithm (DNAWD program) which converts sequences into three-dimensional walks. The walks are compared with, or fit to, each other generating global alignments. The DNA-walk geometry defines a proper metric used to create a distance matrix appropriated for phylogenetic reconstruction. In this work, the analyses were carried out for species currently classified in *Capronia*, *Cladophialophora*, *Exophiala*, *Fonsecaea*, *Phialophora*, and *Ramichloridium*. Main groups were verified by small-subunit rRNA gene data. DNAWD applied to ITS2 alone enabled species recognition as well as phylogenetic reconstruction reflecting clades discriminated in small-subunit rRNA gene phylogeny, which was not possible with any other algorithm using local alignment for the same data set. It is concluded that DNAWD provides rapid insight into broader relationships between groups using genes that otherwise would be hardly usable for this purpose.

Black yeast-like fungal pathogens are gaining importance because of their increasing incidence in often life-threatening infections in immunocompromised patients, sometimes also in otherwise healthy persons (18). The taxonomy and identification of these fungi is difficult due to a lack of phenetic characters and high degree of morphological plasticity. With the advent of molecular taxonomy, numerous cryptic taxa are being recognized in *Capronia* (24), *Phialophora* (3, 4), *Cladophialophora* (17), *Exophiala* (27, 5), and *Ramichloridium* and *Rhinocladiella* (5).

Several comparative molecular techniques have been applied to display polymorphism among strains of black yeasts. Mitochondrial DNA (13) or mitochondrial cytochrome oxidase have been used for diversity at the population level. Chitin synthase (12), in contrast, has insufficient resolution to recognize species but rather displays phylogenetic relationships between species aggregates. The newly described species mentioned above were invariably supported by sequence data of the internal transcribed spacer (ITS) rRNA gene region. Marked ITS differences can sometimes be found in groups that are otherwise monomorphic, such as *Ochroconis* (G. S. de Hoog and H.-J. Choi, unpublished data). However, the taxonomic value of rRNA gene ITS has been questioned, as it does not provide a sufficient level of resolution in *Trichoderma* (15), *Alternaria* (11, 19), and other genera. It is apparent that ITS divergence rates cannot be used as a gold standard for taxonomic differences all over the fungal kingdom, but particular levels of divergence have been reached in some groups, among which are the black yeasts and their allies. In a number of cases (4, 5, 27) the entities found were found to coincide with phenetic characters that had been neglected or overlooked in earlier taxonomic systems, underlining the taxonomic predictivity of ITS characters.

ITS divergence between black yeast species is marked, species being clearly separated by at least 1% sequence diversity (3). A major drawback of this ITS diversity in black yeasts is the large degree of divergence when the entire ascomycete family *Herpotrichiellaceae*, to which they belong through *Capronia* teleomorphs (24), is compared. Such comparisons may be necessary, because morphology does not provide a priori clues to phylogenetic positions (9). Over larger phylogenetic distances, large parts of the ITS region may not be alignable with confidence (20) and therefore have been excluded from the analysis.

Recently, the algorithm DNA-Walk Divergence (DNAWD) has become available (14). The algorithm's robustness relies on global alignment of complete sequences, which resolves the arbitrariness often associated with the precise identification of *indel* mutation sites, while the integral nature of the DNA-walk still allows for the sensitivity in their occurrence. This allows for comparison of entire ITS sequences including a broad taxonomic distance range. It can be shown that a properly defined DNA-walk is the exact equivalent to the sequence

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TABLE 1. Strains analyzed with collection reference and GenBank accession numbers[a] (SSU numbers of those species that were also analyzed for ITS data are also mentioned)

Species	Synopsis	Strain no.	Accession no.		ITS (bp) position	NCBI size (bp)
			18S EMBL	ITS EMBL		
Capronia pilosella	Capr 0264	CBS 125.88	AJ232940	AF050264	3–543	1461
Cladophialophora bantiana	C ban 7182	WC 2907	AF397190	AF397182	24–578	632
Cladophialophora bantiana		CBS 173.52	AY554284			
Cladophialophora bantiana	C ban Hc6	DH 11331 = HC6			1–555	557
Cladophialophora bantiana	C ban 1079	UTHSC 94–986		AF131079	24–578	632
Cladophialophora carrionii	C car 7181	FMC 248	AF397188	AF397181	23–566	620
Cladophialophora carrionii		CBS 260.83	AY554285			
Cladophialophora carrionii	C car 0262	CBS 160.54		AF050262	3–547	1466
Cladophialophora carrionii	C car 7180	ITMSP 690		AF397180	24–568	622
Cladophialophora sp.	Cl sp 0265	CBS 552.79		AF050265	3–543	664
Exophiala dermatitidis	E der 7493	IFM 4829		AF147493	43–602	1,604
Exophiala dermatitidis	E der 7494	IFM 4827	X79312	AF147494	48–607	666
Exophiala dermatitidis		CBS 525.76	X79317			
Exophiala dermatitidis		CBS 292.49	AY554286			
Exophiala dermatitidis	E der 0268	CBS 149.90		AF050268	3–582	1,505
Exophiala dermatitidis	E der 0270	CBS 748.88		AF050270	3–581	1,504
Exophiala dermatitidis	E der 7495	IFM 4826 = CBS 100338		AF147495	48–618	671
Exophiala dermatitidis	E der 0269	CBS 207.35 (T)	X79312	AF050269	3–582	1,505
Exophiala dermatitidis	E der 7492	IFM 4960		AF147492	47–622	676
Exophiala dermatitidis		KU A0052 = CBS 709.95 = CDC B-4541	X80702			
Exophiala salmonis	E sal 0274	CBS 157.67 (T)	X80890	AF050274	123–679	1,479
Exophiala jeanselmei	E jea 0271	CBS 507.90 (T) = ATCC 34123	X80705	AF050271	3–552	1,478
Exophiala jeanselmei	E jea 9447	DH 12305 = UTMB 2670		AF549447	1–550	552
Exophiala oligosperma		CBS 725.88 (T)	AY554287			
Exophiala oligosperma	E oli 0289	CBS 265.49 = MUCL 9905		AF050289	3–556	1,483
Exophiala pisciphila	E pis 0272	CBS 537.73 (T)	X89615	AF050272	3–559	1,500
Exophiala aff. *pisciphila*	E pis 0273	CBS 464.81		AF050273	3–575	1,528
Fonsecaea monophora	F mon lacz	DH 12978		AF050262	1–568	
Fonsecaea monophora	F mon 6937	CBS 269.37 (T)	AY554288		1–568	544
Fonsecaea monophora		CBS 289.93	AY554289			
Fonsecaea monophora	F mon 2238	CBS 102238 = DH 11602			1–567	570
Fonsecaea monophora	F mon 2246	CBS 102246 = DH 11611			1–568	569
Fonsecaea pedrosoi	F ped 0131	CBS 201.31			1–567	570
Fonsecaea pedrosoi		CBS 272.37	AY554290			569
Fonsecaea pedrosoi	F ped 2245	CBS 102245 = DH 11610			1–565	567
Fonsecaea pedrosoi	F ped 0276	CBS 271.37 (NT)	AJ232949	AF050276	3–569	1,494
Fonsecaea pedrosoi	F ped 7134	IMTSP 877		AF397134	23–588	642
Phialophora americana	P ame 0283	MUCL 15537 = CBS84069	AY554291	AF050283	3–546	1,465
Phialophora americana	P ame 7136	FMC 2214		AF397136	24–567	621
Phialophora americana	P ame 7133	IMTSP 373		AF397133	24–571	626
Phialophora verrucosa	P ver 0282	MUCL 9768 = CBS 286.47		AF050282	3–550	1,469
Phialophora verrucosa	P ver 1848	NIH 8701 = CBS 224.97		PVU31848	48–595	649
Phialophora verrucosa	P ver 1846	NYS 303 = CBS 226.97		PVU31846	48–592	646
Phialophora verrucosa	P ver 7135	ITMSP 800	AF397187	AF397135	24–568	628
Phialophora verrucosa	P ver 0281	MUCL 9760 = CBS 273.37		AF050281	3–545	1,466
Phialophora verrucosa	P ver 1847	CDC B-2152 = CBS 225.97		PVU31847	48–594	648
Ramichloridium anceps	R anc 0284	CBS 181.65 (NT) = MUCL 8233	AY554292	AF050284	3–549	1,472
Ramichloridium aff. *anceps*	R anc 0285	CBS 157.54 = MUCL 7792		AF050285	3–552	1,475

[a] Abbreviations used: ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CDC, Centers for Disease Control and Prevention, Atlanta; DH, G. S. de Hoog working collection at CBS; FMC, Faculdade de Medicina, Caracas, Venezuela; IFM, Research Institute for Pathogenic Fungi, Chiba, Japan; IMTSP, Instituto de Medicina Tropical, São Paulo, Brazil; KU, Kyushu University, Fukuoka, Japan; MUCL, Mycotheque de l'Université de Louvain, Louvain-la-Neuve, Belgium; NCMH, North Carolina Memorial Hospital, Chapel Hill; NIH, National Institutes of Health, Bethesda; NYS, New York State Dept of Health, New York; UTHSC, Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center, San Antonio; WC, Wadsworth Center for Laboratory and Research, New York. T, ex-type culture; NT, ex-neotype culture; AUT, authentic culture; Aff., with affinity to; sp., unidentified species.

composition, and therefore mutations and dislocations produce divergences in the walk geometry which can be measured. In this paper the DNAWD program is applied to the phylogenetic reconstruction of black yeast strains, exploring total ITS domains of the rRNA gene. A small-subunit (SSU) rRNA gene tree is used to confirm grouping as given by ITS trees.

MATERIALS AND METHODS

Species. A model set of strains of selected species was compared using ITS regions of the rRNA gene (Table 1). Species included were selected in such a way that both large and small phylogenetic differences, within and between genera, were available in the data set. The identity of the species was verified using a large ITS and phenetic database available at the Centraalbureau voor Schimmelcultures, Utrecht, which contains numerous strains of all existing and recently described species, based on ex-type strains. Each species was also represented in a tree based on SSU rRNA gene sequences.

Sequence verification. The edited sequences all have been deposited in GenBank. The basic structure of the tree based on ITS and generated with conventional methods was verified by near-complete SSU rRNA gene sequencing of a smaller selection of strains of the same species (Table 1) plus 70 additional members of the family *Herpotrichiellaceae*. SSU sequences were aligned and analyzed in an aligned database containing about 3,000 fungal sequences in the ARB package developed by W. Ludwig (www.mikro.biologie.tu-muenchen.de/pub/ARB). The SSU tree was made with the Parsimony/ML algorithm with 100 bootstrap replications; *Phaeomoniella chlamydospora* (CBS 101359) was taken as outgroup.

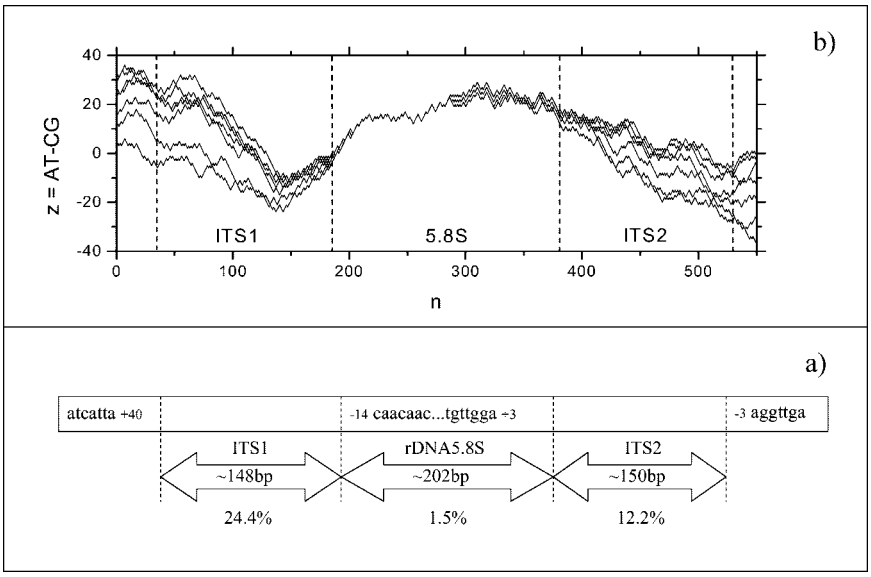

FIG. 1. a. Diagram showing ITS regions defined from strict consensus sequences used in DNAWD analysis. The mean size in base pairs and mean percent variability among species for each region are also noted. b. AT-CG DNA-walk coordinates for seven different species (*P. verrucosa*, *P. americana*, *E. dermatitidis*, *F. pedrosoi*, *E. pisciphila*, *C. carrionii* and *C. bantiana*). The ITS domains used in DNAWD analysis are also shown.

ITS sequences were aligned using the BioNumerics package (Applied Maths, Kortrijk, Belgium). Due to gaps necessary for alignment, the ITS 1 domain spanned 258 positions (real lengths, 197 to 215 bp), the 5.8S gene 157 positions, and the ITS2 domain 224 positions (real lengths, 171 to 211 bp). Positions 97 to 155 and 184 to 208 (ITS1) and 456 to 484 and 541 to 631 (ITS2) could not confidently be aligned and were excluded from most of the comparisons. The same alignment was used for a comparison with different packages applying different algorithms: Phylip package (v.3.572c) (6), according to maximum likelihood; BioNumerics according to Ward's averaging (27), without Indels, using Kimura's two-parameter model, distance estimation through neighbor joining using the Treecon package with Kimura's two-parameter model (25), and Paup (23) according to parsimony. In all comparisons, insertions and deletions (*indels*) were not taken into account. *Exophiala pisciphila* (AF050273) was taken as the outgroup.

Homology analysis by DNA-walk divergence. DNA-walks are defined by incrementing walk steps for each nucleotide in the sequence (for example, a positive step for purines and negative for pyrimidines). The DNA-walk divergence method (14) makes simultaneous three-dimensional walk comparisons (representing three composition skews), AG-TC, AC-TG, and AT-CG for each pair of sequences. One sequence slides against the other until the minimum squared walk difference is found, corresponding to a global alignment. This is then taken as a measure of their distance since statistically independent mutations and *indels* increase the mean square walk differences linearly. The resulting distance matrices are then fed to the Kitsch program of the Phylip package (v. 3.572c) (6), which generates trees with contemporary leaves.

The analyses were carried out for both the ITS1 and ITS2 regions separately, defined within the consensus limits ATCATTA to CAACAAC and TGTTGGA to AGGTTGA, respectively (Fig. 1a). The choice of the intervals was based on apparent homogeneity of sequence dissimilarities among species and can be appreciated in the DNA-walk graph of Fig. 1b. The lengths and average dissimilarities of the sections analyzed are also given in Fig. 1a.

RESULTS

All individual species were distinguishable with ITS sequence data with any of the algorithms applied (Fig. 2,3, 4, and 5). Using the complete ITS region, a similar unresolved structure with nine clusters (A to I) was found when neighbor joining, parsimony, and Ward's algorithms were used (Fig. 2). The mutual distances of these groups were inferred from an SSU rRNA gene tree (Fig. 3) of the herpotrichiellaceous black yeasts, containing about 1,600 positions in 70 strains. Cluster numbering (1 to 5) in this tree was derived from a similar SSU tree published by Haase et al. (9), groups having *Exophiala dermatitidis* (in one) *Cladophialophora bantiana* (in two), *Exophiala spinifera* (in three), *Exophiala nigra* (in four), and *Coniosporium perforans* (in five) as core taxa. ITS groups A to C on the one hand and F plus H on the other appeared to be mutually related as they clustered within single SSU groups, while distances between remaining groups were significantly larger.

With DNA-walk, the trees based on the complete ITS region (Fig. 4) as well as that of the ITS2 domain alone (Fig. 5) recognized all species correctly. The analysis of the ITS domain data resulted in slightly different trees when either the complete ITS region, or ITS1 or ITS2 alone were used. In the DNA-walk tree based the complete ITS domain (Fig. 4) all groups, including A to C and F and H, were clearly separated. The structure of the tree could not be linked to the distances as provided in the SSU tree (Fig. 3). In the tree based on ITS2 (Fig. 5) *Fonsecaea* (C), *Phialophora* and *Cladophialophora carrionii* (A), and *C. bantiana* (B) were all found in an area of the tree where mutual distances between taxa were moderate, while groups D (*E. dermatitidis* complex), E, G, and I were more distantly related to other members of the *Herpotrichiellaceae* and mostly also to each other.

DISCUSSION

Sequencing of the rRNA genes is indispensable for species recognition in black yeasts and their allies that are causative agents of important human diseases such as chromoblastomycosis and phaeohyphomycosis (1, 2, 8, 21). Several species are known to have reproducible intraspecific polymorphisms in the ITS domain enabling the recognition of variants differing in virulence that are easily detected in an aligned ITS sequence

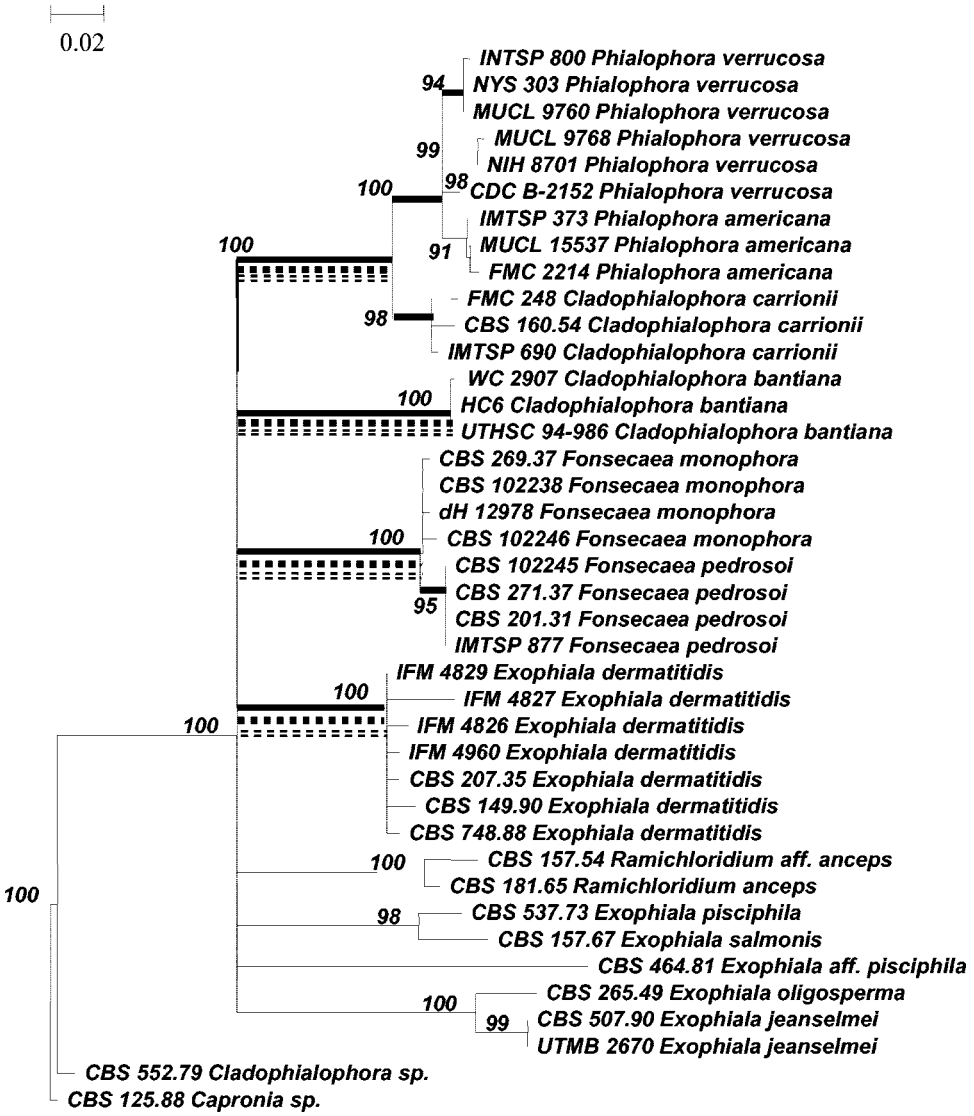

FIG. 2. Consensus tree of 40 strains of black yeasts and related fungi based on confidently aligned rRNA gene ITS sequences, i.e., excluding positions 97 to 155 and 184 to 208 (ITS1), and 456 to 484 and 541 to 631 (ITS2), using the neighbor joining algorithm in the Treecon package with Kimura-2 correction. Bootstrap values of >90 from 100 resampled data sets are shown: solid bar, branches also recognized with Ward's averaging; dotted bar, branches also recognized with UPGMA; dashed bars, branches also recognized with parsimony.

base (16). On the other hand, considerable interspecific polymorphisms may occur between morphologically identical species (5), as phenetically similar structures can be found all over the phylogenetic tree of the *Herpotrichiellaceae* (9). Sequences may show too much divergence to allow reliable alignment comparison, limiting identification of clinical samples to local BLAST searches. nBlast in GenBank for most species is insufficient, since the taxonomic representation is as yet fragmentary. As yet no molecular marker other than ITS is available with optimal display of differences between species. For this reason the best approach is application of a comparative method that is based on ITS but is independent of alignment bias.

Our selection of species and strains analyzed is more or less representative for the hyper-variability of the ITS domains of black yeasts and allies. With growing distances between strains, alignments quickly can no longer be done with confidence. Meaningful comparison to reveal evolutionary relationships between some of the species is then only possible when small selections of the ITS domains are taken into account, as mentioned in Materials and Methods. Phylogenetic interpretation is strongly hampered, as trees lack any resolved substructure, being based on too low number of mutations in the small portions that are still comparable. Due to the large distance between most of the groups, the clusters were robust and remained strictly identical with any algorithm applied, particularly in the lack of a hierarchical structure of main branches (Fig. 2).

A substructure, i.e., with separate species in a more or less hierarchical ordering, was revealed with ITS in the *Phialophora*/*Cladophialophora carrionii* cluster (group A) only. When the neighbor joining algorithm was applied to the complete ITS

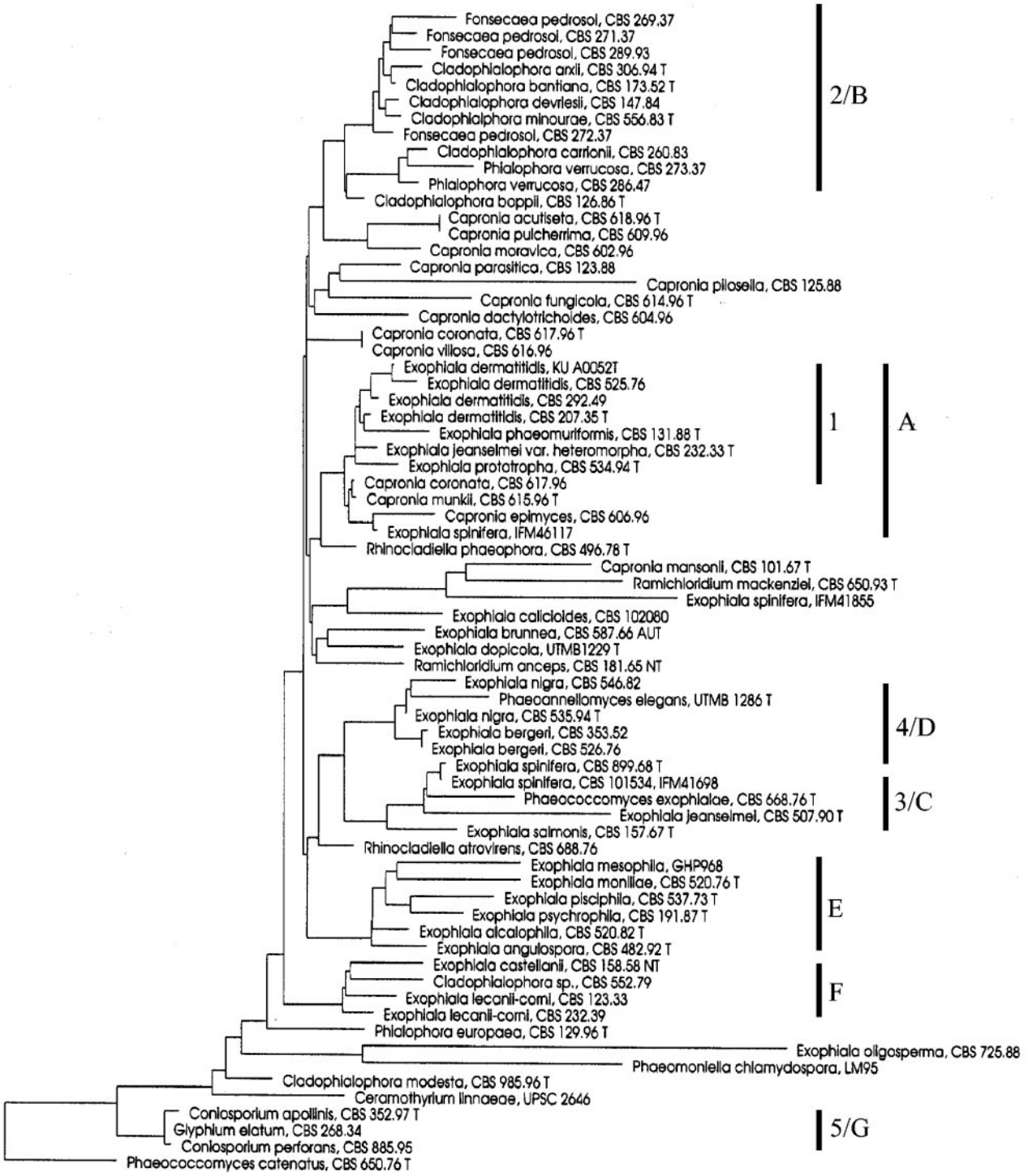

FIG. 3. Neighbor-joining tree based on 1,660 positions of 70 SSU rRNA gene sequences generated with the ARB package. *Phaeomoniella chlamydospora* CBS 101359 was used as the outgroup. Strains with numbers 1 to 5 are proven to belong to SSU groups with the same numbering indicated by vertical bars, the cluster delimitation derived from groups recognized by Haase et al. ITS groups (A to G) are superimposed, indicated with arrows.

domain, which could be confidently aligned in the mentioned cluster, *C. carrionii* was found paraphyletically to *Phialophora*. This matches phenetic data because members of the clade share potential production of phialides and are agents of human chromoblastomycosis. These characters are also found in *Fonsecaea* (group C). In the SSU tree of the *Herpotrichiellaceae* published by Haase et al. (9) *Fonsecaea* has members of *Cladophialophora* other than *C. carrionii* as sister group species

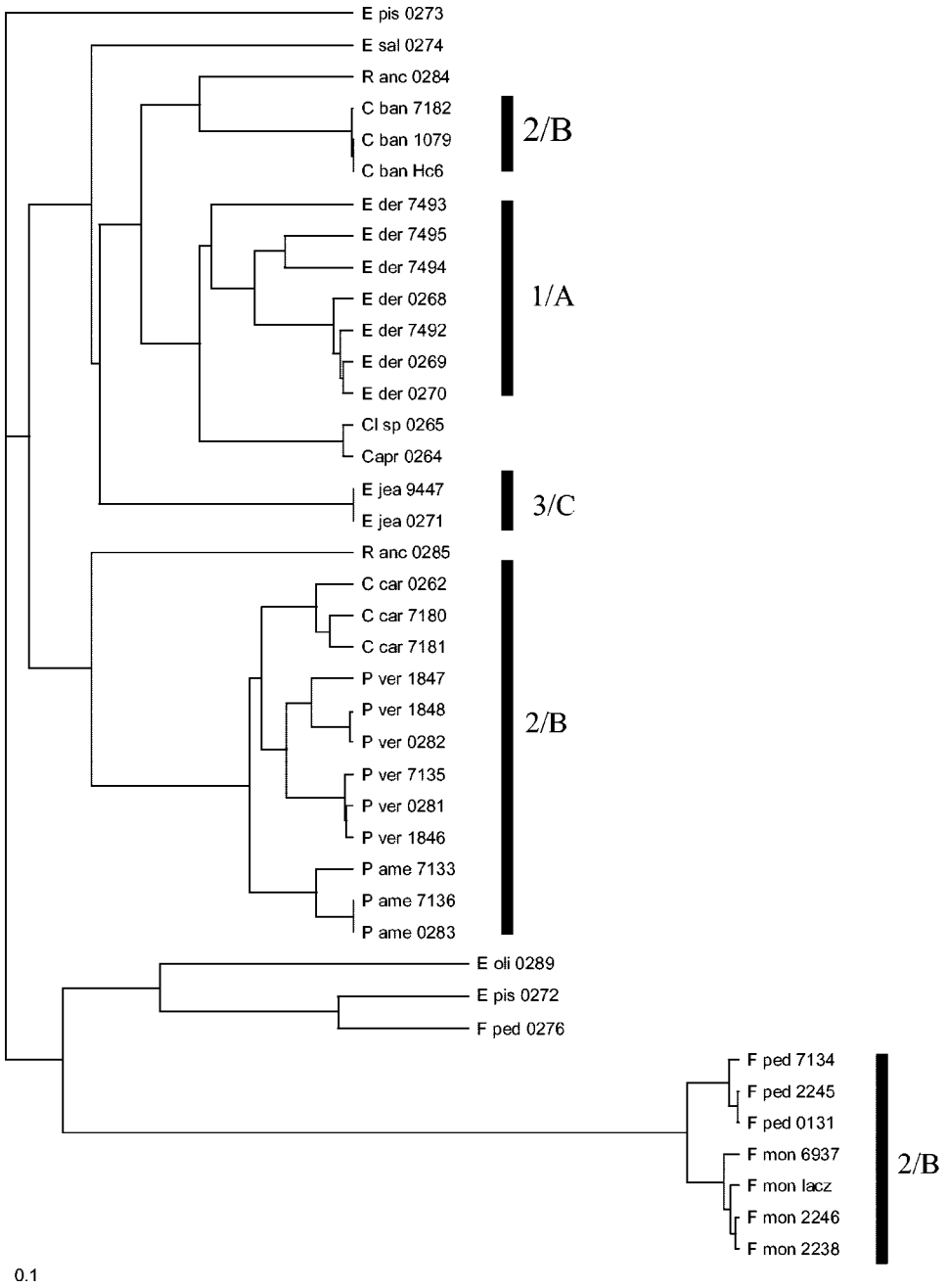

FIG. 4. Unrooted tree made with the Kitsch program in the Phylip package for construction of the DNAWD distance matrix of the entire ITS region of 40 black yeasts and related fungi. Group indications are those of the rRNA gene ITS in Fig. 2 (letters) and the SSU rRNA gene recognized by Haase et al. in Fig. 3 (numbers).

(cluster 2 of Haase et al.) (9). *Cladophialophora* species other than *C. carrionii* cause phaeohyphomycoses rather than chromoblastomycoses.

In our updated SSU tree of the same family (Fig. 3) members of *Fonsecaea*, *Phialophora*, and *Cladophialophora* (A to C) all are united in a single clade (2), showing phylogenetic coherence of the *Herpotrichiellaceae* with high degrees of virulence. In the DNA-walk tree based on ITS2 (Fig. 5) *Fonsecaea*, *Phialophora*, and *Cladophialophora* are all found in a main subdivision of the tree (II) where distances between species are smaller than the isolated, more distantly related taxa opposite of the dotted line (I) in Fig. 5. The radial scale measures twice the percent dissimilarity in composition. Branches crossing the dotted circle (phenon circle) group about 13 species-aggregates with dissimilarities below 6.3%.

With DNA-walking, the analysis of ITS domain data resulted in slightly different trees when either the complete ITS region (Fig. 4) or ITS2 alone (Fig. 5) was used. All trees recognized all species correctly. The question of whether the structure is phylogenetically interpretable can be verified by a

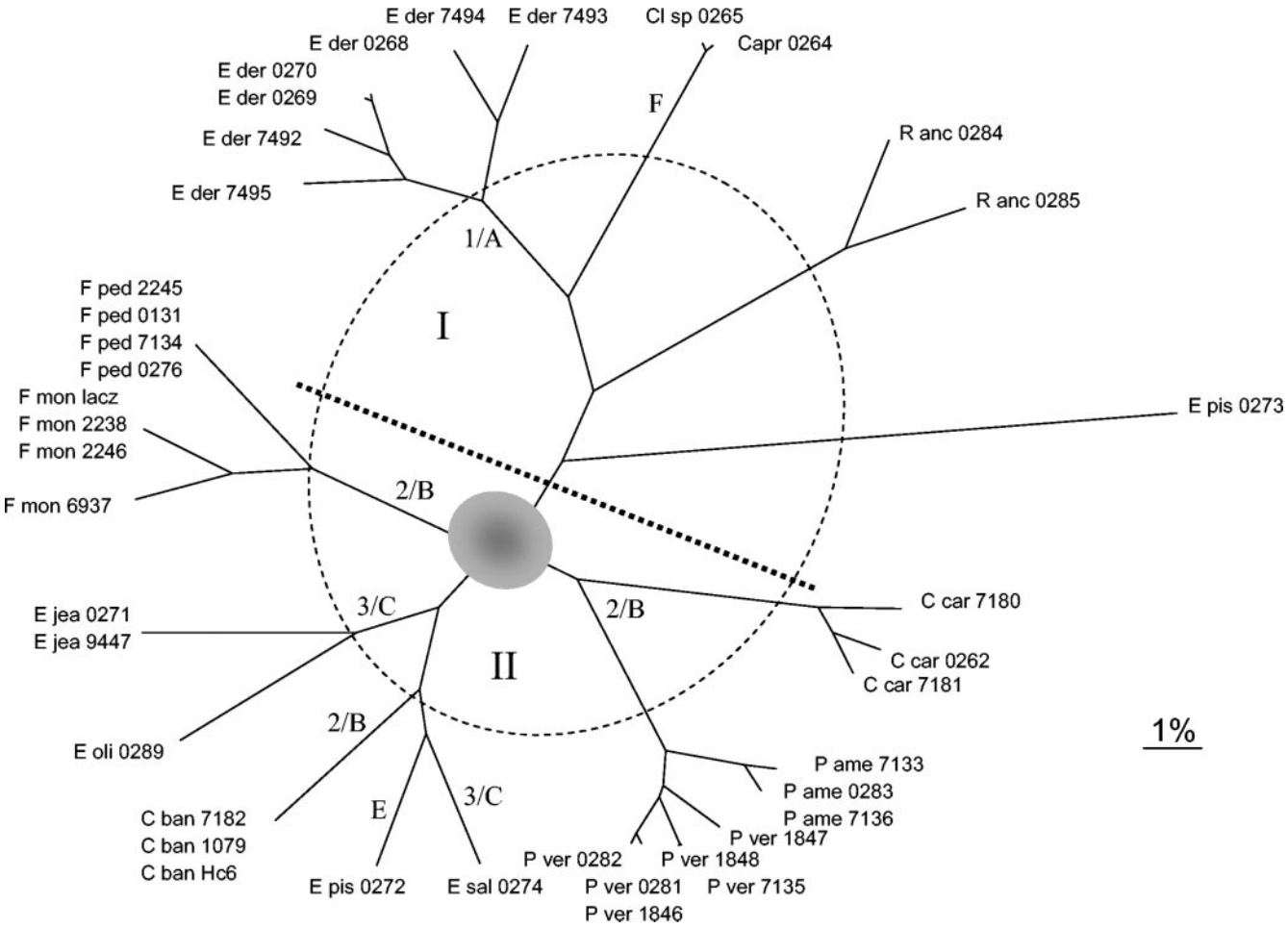

FIG. 5. Unrooted tree made with the Kitsch program in the Phylip package for construction of the DNAWD distance matrix of ITS2 of 40 black yeasts and related fungi. The radial scale measures twice the percent dissimilarity in composition. Branches crossing the dotted circle (phenon circle) group about 13 species-aggregates with dissimilarities below 6.3%. The inner circle estimates large-distance dispersion and covers the most unreliable branching far away from the leaves.

comparison with SSU rRNA gene data of the same fungi. The most comprehensive phylogenetic overview of the family is that of Haase et al. (9). These authors noted that the tree was poorly resolved but recognized five approximate clades, with *Exophiala dermatitidis*, *Cladophialophora bantiana*, *E. spinifera*, *E. nigra*, and *Coniosporium perforans* as core species, as listed above. Ecologically these groups seem to be meaningful. Clade 1 is a thermophilic yeast group around the neurotrope *E. dermatitidis* (10), clade 2 is a group with virulent species with accent on species causing chromoblastomycosis and brain disease (7), clade 3 is the *E. spinifera*/*E. jeanselmei* complex (5), clade 4 is a meso- to psychrophilic group of *Exophiala* species in showers and ocean waters (9), and clade 5 contains meristematic species inhabiting rocks (22). This ecological support for the groups suggests that this reflects the phylogeny of these groups optimally.

In the SSU tree based on our expanded and updated data set (Fig. 3) some further, ecologically defined clades can be located. ITS groups F and G are psychrophilic species similar to the fish pathogen *E. pisciphila*, although unexpectedly *E. salmonis* is found in E, which is a cluster of mutually distantly related environmental species. In the SSU tree these taxa are mutually remote (Fig. 3). *Exophiala oligosperma* is a close relative of *E. jeanselmei* (5) and indeed is found together with this species in ITS cluster C (Fig. 5), matching the *E. spinifera* complex SSU group 3 (Fig. 3). Some dispersed strains previously listed as "*E. spinifera*" (Fig. 3) are now known to represent individual taxa, such as IFM 14855, which has been described as *E. nishimurae* (26).

With the DNA-walk of the entire ITS domain, no coherent substructure was recognizable. SSU-clade 2/ITS-clades A to C were found at the longest mutual distances (Fig. 4). In contrast, a major subdivision (I and II) was consistently recognized based on ITS2 alone, with *E. pisciphila* CBS 464.81, representing a distantly related, undescribed species, in an external position (G). Main branches I and II contained five to nine subclusters representing the individual species analyzed (Fig. 5). *C. carrionii* and *Phialophora* (2/A) were found to be interrelated, with *Fonsecaea* (2/C) branching off at the base; all are agents of chromoblastomycosis. *Cladophialophora bantiana* (2/B) was found as well-individualized clusters paraphyletically to psychrophilic *Exophiala* species (F).

In conclusion, the DNA-walk showed consistent results in the recognition of individual species, whether these consisted of several strains or a single strain. This was achieved with the complete ITS domain as well as with rRNA gene spacers 1 and 2 separately. In addition, ITS2 alone also enabled phylogenetic reconstruction to some extent, which was not possible with any other algorithm using the same data set. DNA-walk divergence proved to be a powerful tool for the analysis of ribosomal genes and their evolutionary interpretation. The gold standard for phylogenetic interpretations is the small or large rRNA gene subunits, which in general contain more information. However, in the case of the black yeasts and their relatives, these genes often contain degrees of variability too small to resolve individual species (9). DNA-walk divergence bridges the gap between current methods of phylogenetic reconstruction. Another straightforward application of this technique could be the molecular identification of the agents of phaeohyphomycosis and chromoblastomycosis isolated in the clinical laboratory.

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