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Methodology article

Identification of cultured isolates of clinically important yeast species using fluorescent fragment length analysis of the amplified internally transcribed rRNA spacer 2 region

Thierry De Baere[1], Geert Claeys[1], Danielle Swinne[2], Caroline Massonet[3], Gerda Verschraegen[1], An Muylaert[1] and Mario Vaneechoutte*[1]

Address: [1]Department Clinical Chemistry, Microbiology & Immunology, Blok A, Ghent University Hospital, De Pintelaan 185, B9000 Ghent, Belgium, [2]Scientific Institute of Public Health, Mycology Section, Juliette Wytsmanstraat 14, B1050 Brussels, Belgium and [3]Laboratorium Experimentele Microbiologie, U.Z. Herestraat 49-CDG 8ste, B3000 Leuven, Belgium

E-mail: Thierry De Baere - Thierry.DeBaere@rug.ac.be; Geert Claeys - Geert.Claeys@rug.ac.be; Danielle Swinne - DSwinne@iph.fgov.be; Caroline Massonet - Caroline.Massonet@med.kuleuven.ac.be; Gerda Verschraegen - Gerda.Verschraegen@rug.ac.be; An Muylaert - An.Muylaert@azstvdeinze.be; Mario Vaneechoutte* - Mario.Vaneechoutte@rug.ac.be

*Corresponding author

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Abstract

Background: The number of patients with yeast infection has increased during the last years. Also the variety of species of clinical importance has increased. Correct species identification is often important for efficient therapy, but is currently mostly based on phenotypic features and is sometimes time-consuming and depends largely on the expertise of technicians. Therefore, we evaluated the feasibility of PCR-based amplification of the internally transcribed spacer region 2 (ITS2), followed by fragment size analysis on the ABI Prism 310 for the identification of clinically important yeasts.

Results: A rapid DNA-extraction method, based on simple boiling-freezing was introduced. Of the 26 species tested, 22 could be identified unambiguously by scoring the length of the ITS2-region. No distinction could be made between the species *Trichosporon asteroides* and *T. inkin* or between *T. mucoides* and *T. ovoides*. The two varieties of *Cryptococcus neoformans* (var. *neoformans* and var. *gattii*) could be differentiated from each other due to a one bp length difference of the ITS2 fragment. The three *Cryptococcus laurentii* isolates were split into two groups according to their ITS2-fragment lengths, in correspondence with the phylogenetic groups described previously. Since the obtained fragment lengths compare well to those described previously and could be exchanged between two laboratories, an internationally usable library of ITS2 fragment lengths can be constructed.

Conclusions: The existing ITS2 size based library enables identification of most of the clinically important yeast species within 6 hours starting from a single colony and can be easily updated when new species are described. Data can be exchanged between laboratories.

Background

Rapid and correct identification of the different clinically relevant yeast species has become more important because of several reasons. During the last decade, the impact and frequency of yeast infections has gained importance mainly due to an increased number of immunocompromised patients [1]. Furthermore, an increasing number of non-*C. albicans* yeast species are considered as potential agents of clinical infections [2]. Finally, the differences in susceptibility towards antifungal agents between the different species make that rapid yeast identification can be used as a first indication for efficient treatment.

Phenotypic identification relies on cell and colony morphology and on biochemical characteristics, but this approach is not always fully discriminative, may be time-consuming and requires specific expertise of the technicians [3]. Therefore several groups have explored the possibilities of PCR-based techniques for the differentiation between different yeast species. The use of species specific probes [4,5] or molecular beacons [6] resulted in very sensitive and very specific techniques but remains restricted to the species for which the probes are designed for. A more broadly applicable approach is based on PCR with universal fungal primers (directed towards conserved regions in the ribosomal region) and followed by either restriction analysis [7], sequencing [8] or size determination of the amplified fragment(s) [9,10].

Here we report an evaluation and extension of the technique published by Turenne *et al.*[9], whereby the rRNA Internally Transcribed Spacer Region 2 (ITS2), i.e. the region in between the fungal 5.8S and 28S rRNA genes, is amplified and its length is determined by fragment analysis on an ABI Prism 310 capillary electrophoresis system. Turenne *et al.*[9] showed that the length of the ITS2-spacer region was characteristic for most species and that it was identical for all strains within a species, although only a limited number of strains was used per species. By using one fluorescently labeled primer, the size of the amplicon can be determined by electrophoresis on an ABI Prism 310 capillary apparatus, which provides with the one base pair resolution that is needed to differentiate between ITS2-fragment lengths of closely related species. In this study, we also evaluated a simpler and more rapid DNA-extraction method and we applied ITS2-PCR on ABI Prism 310 on more strains and species than in the original publication.

Results

Using DNA-extraction based on boiling/freezing of the yeast colonies, the ITS2-PCR fragment could be amplified easily from all yeast strains. An initial ITS2 size based library was constructed using a panel of reference strains, belonging to most of the clinically important species (Table 1).

Table 1: Reference strains used in this study

Species	Reference strains*
Blastoschizomyces capitatum	IHEM 5091
Candida albicans	IHEM 3242, IHEM 3731, IHEM 3740, IHEM 6440
C. dubliniensis	IHEM 14280
C. glabrata	IHEM 4210, IHEM 4566, IHEM 5573, IHEM 9556
C. guilliermondii	IHEM 1067, IHEM 1879, IHEM 3283
C. humicola	AZB 98-043
C. kefyr	IHEM 4211, IHEM 4593, IHEM 5556
C. krusei	IHEM 4562, IHEM 5579, IHEM 6387
C. lipolytica	HHR A101315
C. lusitaniae	IHEM 4593, IHEM 10293, IHEM 14663
C. norvegensis	HHR A211294
C. parapsilosis	IHEM 1001, IHEM 2052, IHEM 2305, IHEM 3270
C. tropicalis	IHEM 4222, IHEM 4666, IHEM 5609, IHEM 12085
Cryptococcus albidus	IHEM 3267, IHEM 4786, IHEM 6895
C. laurentii	IHEM 895, IHEM 5515
C. neoformans var. *gattii*	IHEM 4170
C. neoformans var. *neoformans*	IHEM 4165, IHEM 4171
Geotrichum candidum	IHEM 6284
Malassezia furfur	IHEM 3697
Saccharomyces cerevisiae	IHEM 3962, IHEM 14402, IHEM 14542, IHEM 17539
Trichosporon asahii	IHEM 9334, IHEM 17910
T. asteroides	IHEM 10214
T. inkin	IHEM 5824
T. mucoides	IHEM 13920
T. ovoides	IHEM 17913

*: IHEM: strains from the BCCM/IHEM Culture Collection, Mycology Section, Scientific Insitute of Public Health, Brussels, Belgium; HHR: Heilig Hart Ziekenhuis, Roeselare, Belgium, AZB: St. Jan Akademisch Ziekenhuis, Bruges, Belgium.

When amplifying the ITS2-PCR fragment, care was taken to label the same primer as used by Turenne *et al.*[9] and to use the same HEX-label, in order not to introduce artefactual length differences. Still, when the obtained fragment lengths were compared with those of Turenne *et al.*[9], differences were observed (Table 2). These could be explained by our use of dTTP, compared to the use of dUTP in the original publication [9]. Unpublished fragment lengths obtained at the laboratory of Turenne when using dTTP were in agreement with our findings (Turenne, pers. comm.).

Table 2: Overview of the different ITS2-fragment lengths observed and of the number of strains identified by means of ITS2-PCR.

Species	Number of strains tested (number used for calculation of the average length)	ITS2-fragment length (bp), calculated by capillary electrophoresis			Previously published lengths (bp)*[9]	Fragment lengths (bp) derived from Genbank sequences	Genbank accession number of sequences used
		Average	Range	Standard deviation			
Blastoschizomyces capitatum	1	250.7			248	251	AF455443
Candida albicans	116 (19)	281.6	281.0 – 282.6	0.37	279	284	AF217609
C. dubliniensis	1	286.3				289	AF218993
C. glabrata	94 (12)	362.6	362.4 – 362.8	0.15	360	365	AF218994
C. guilliermondii	8	323.2	322.7 – 324.2	0.46	321	325	AF218996
C. humicola	1	295.8					
C. kefyr	7	375.5	375.4 – 375.8	0.17	372	378	AF218997
C. krusei	10 (2)	285.3	285.2 – 285.4	0.13	282	294	L47113
C. lipolytica	3 (2)	180.6	179.9 – 181.1	0.62		190	AF218983
C. lusitaniae	9 (4)	200.3	200.2 – 200.4	0.10	199	204	AF218970
C. norvegensis	3 (2)	267.1	266.6 – 267.1	0.62		318	AF333096
C. parapsilosis	36 (12)	254.1	253.5 – 255.4	0.60	251	259	U10989
C. rugosa	1	215.0	214.8 – 215.1	0.10		223	AF218971
C. tropicalis	34 (28)	270.6	269.3 – 271.2	0.61	269	272 273 274	AF219000 AF219001 AF218992
Cryptococcus albidus	3	353.3	352.4 – 354.7	1.26	350	353	AF218972
C. laurentii (environmental)	2	272.1	271.9 – 272.3	0.26		275	AF218974
C. laurentii (clinical)	1	307.9			306	310	AJ421006
C. neoformans var. *neoformans*	8 (4)	318.3	318.0 – 318.6	0.29	315	320	AF218975
C. neoformans var. *gattii*	1	320.0				321	AF196312
Dekkera bruxellensis	1	261.8				263	AF043502
Geotrichum candidum	3 (1)	193.4			192	195	AF157596
Malassezia furfur	1	494.4				500	AF246896
Saccharomyces cerevisiae	35 (25)	366.6	365.1 – 368.5	0.89	363	365 366 367	AF219005 AF219006 AF219007
Trichosporon asahii	2	301.0	300.8–301.4	0.50		304	AB018014
T. asteroides	1	298.6				304	AF444416
T. inkin	1	298.7				304	AF444420
T. mucoides	1	297.2				297	AF444423
T. ovoides	1	297.5				303	AF444439

*: Values obtained by using dUTP instead of dTTP for the PCR-based amplification reaction [9].

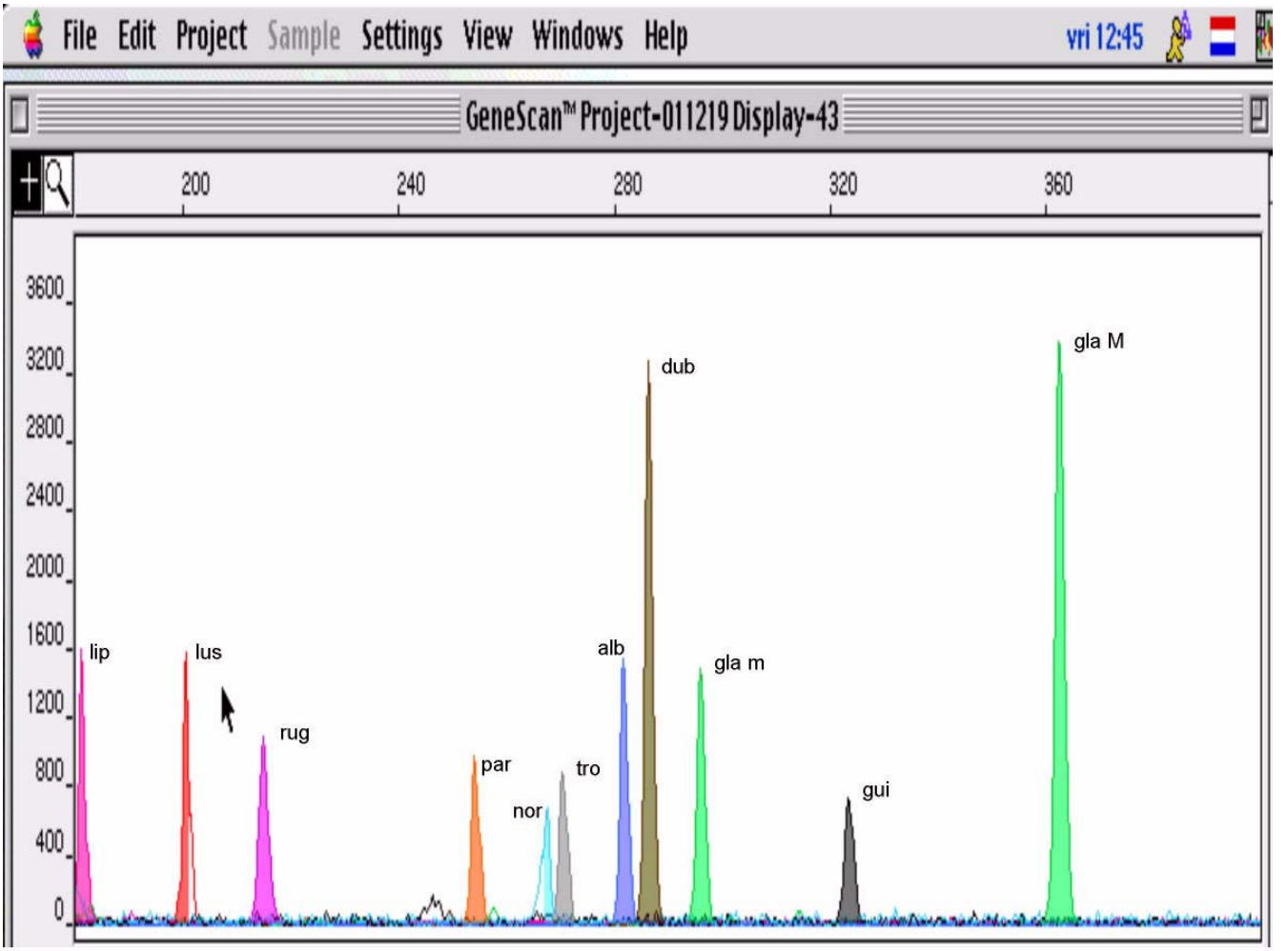

Figure 1
The ITS2-PCR fragment lengths observed for 10 *Candida* species (GeneScan Analysis screen, ABI Prism 310, Applied Biosystems).

The reproducibility of ITS2-PCR is apparent from Table 2. For example, for 19 *C. albicans* strains tested, the observed fragment sizes ranged between 281.0 bp and 282.6 bp (with an average value of 281.6 bp and a SD of 0.4 bp). Table 2 presents the ITS2-lengths observed for the different species and reference strains tested.

In one laboratory, we observed in a reproducible manner a smaller sized peak with lower intensity for half of the strains and for more than half of the species. An example is shown in Figure 1 for *C. glabrata*. However, using the same DNA-extracts, primer batch, commercially prepared PCR mixture and thermal cycling protocol, this additional fragment could not be observed in a second laboratory. When the primers of the second laboratory were used in the first laboratory, the additional fragments were observed again. The only technical difference noticed between both laboratories were the thermal cyclers.

Of the 26 species, 22 could be identified unambiguously by scoring the length of the ITS2-region. In the genus *Trichosporon* however, no distinction could be made between the species *T. asteroides* and *T. inkin*, since the ITS2-fragment length is approximately 298.6 bp for both species, and between the species *T. mucoides* and *T. ovoides*, which both have an ITS2-fragment of approximately 297.5 bp.

Also the two varieties of *Cryptococcus neoformans* (var. *neoformans* and var. *gattii*) could be differentiated from each other due to a one bp length difference of the ITS2 fragment, although these findings should be further confirmed by inclusion of more strains.

The three *C. laurentii* isolates were split into two groups according to the ITS2-fragment length. The clinical isolate (IHEM, 1296, human mouth) had a fragment of 307.9 bp, while the two environmental isolates (from filter board of humidifier system (IHEM 0895) and from indoor air (IHEM 5515)), had an ITS2 length of 272.1 bp. The ATCC 18803 type strain, an isolate from palm wine, was found to yield a fragment of 306 base pairs, using dUTPs [9]. Sequencing of the ITS2 of the three strains identified them all as *C. laurentii*. Cluster analysis with Genbank sequences, published recently [11], revealed that the clinical isolate (IHEM 1296, Genbank AJ421004) clustered most closely to the type strain in Sugita's phylogenetic group I [11], in correspondence with the ITS2-PCR results, whereas the environmental strains (IHEM 0895: Genbank AJ421005 and IHEM 5515: Genbank AJ421006) clustered in phylogenetic group II, which contained mostly environmental strains and one isolate from the bronchi of a lung patient (Figure 1).

After construction of the library, a large collection of clinical isolates was identified both with ITS2-PCR and by phenotypic means, using Albicans ID plate (BioMérieux, Marcy-l'Etoile, France) and/or Auxacolor (Sanofi-Pasteur, Marnes-la-Coquette, France) and/or cellular and colony morphology. The number of additional strains tested for each species is listed in Table 2. Two isolates could initially not be identified by ITS2-PCR, because the observed ITS2-fragment lengths were not present in the library. According to biochemical and ITS2-sequencing data, the first isolate belonged to the species *Candida rugosa*, a species initially lacking in the library. The ITS2 fragment length value of 215.0 bp for *C. rugosa* was added to the database. Another isolate from a hematology patient and from syrup present in his room, had a previously not observed ITS-length of 261.8 bp and sequencing revealed an identity as *Dekkera (Brettanomyces) bruxellensis*, a species that had not been included in the library initially. The ITS2 fragment lengths obtained for 25 *S. cerevisiae* strains ranged between 365.1 and 368.5 bp (average 366.7 bp., SD 0.89 bp.), but no misidentifications occurred.

Clear differences could be observed between the length of the ITS2-region as derived from published sequences (theoretical length) and as calculated after capillary electrophoresis (estimated length) (Table 2). Although the estimated length is highly reproducible, it is consistently shorter than the theoretical length (except for *Blastoschizomyces capitatum*, *C. albidus*, *T. mucoides* and *S. cerevisiae*, where it is identical). A maximal difference of 9 bp was observed for *C. krusei*. For *S. cerevisiae*, the range of 3 bp observed for the estimated ITS2 lengths was also present in the theoretical lengths derived from the Genbank sequences.

Discussion

ITS2-PCR followed by size determination of the fragment length by capillary electrophoresis has been described as a possible tool for the identification of yeasts by Turenne *et al*. [9] and by Chen *et al*. [10]. The latter authors amplified a 50 bp longer stretch of the ITS2 region than did the former. Since ITS2-PCR fragments of different species may differ by only one bp, highly reproducible high resolution electrophoresis as provided by capillary electrophoresis on ABI Prism 310 is necessary.

In order to increase the speed of this approach we used a simple and rapid boiling/freezing based DNA extraction protocol, which proved to be highly efficient. Together, DNA-extraction, PCR and capillary electrophoresis took 3.5 hours. Thus, the total time to identification is 3.5 hours for the first sample, added with 45 minutes for each following sample, when using a single capillary apparatus like the ABI Prism 310. When a 16 capillary apparatus is used (ABI Prism 3100), the first 16 strains can be identified within 3.5 hrs. Besides speed and high discriminatory power, another advantage of this approach is that newly recognized species or species not yet present in the database can be added after appropriate initial identification, whereafter their identification by means of ITS2-PCR is straightforward. Given the identical values we obtained with unpublished ones by Turenne *et al.* [pers. comm.], it can be assumed that the data are exchangeable between laboratories, as also has been shown for other DNA-fingerprints obtained on ABI Prism 310 machines [12,13]. The lengths obtained for the amplified ITS2 fragments in two different laboratories during this study were indeed perfectly comparable. The only difference was an apparently artefactual additional peak of lower size and lower intensity in one laboratory, never observed in the other laboratory.

The variety of species that can be identified can be expanded and this can be achieved in a joint effort between laboratories. Moreover, laboratories not having constructed such a database their selves, can compare the data of their unknowns to a publicly available database, like the one published here. Care should be taken to use the same primer labeled in the same manner and to use the same size markers and size standard files (available upon request) to normalize the capillary electrophoresis runs, since minor differences between these can introduce migration and normalization differences.

The differences between the theoretical length (as obtained after counting the bases of the available ITS2 sequences) and the calculated length (as obtained after capillary electrophoresis) can be explained by the fact that electrophoretic migration is also partially sequence dependent, such that the calculated length will not always

match exactly the theoretical length. However, as long as the calculated length is reproducible from run to run and between laboratories, this observation poses no problem for the purpose of identification. Only for one species (*C. norvegensis*) the obtained length in this study was completely different from the one derived from the published GenBank sequence (Table 2).

Using ITS2-PCR, identification to species level is very efficient for the genera *Candida* and *Cryptococcus*, while four species of the five tested for the genus *Trichosporon* could be split into two groups only. However, the current taxonomy of *Trichosporon* needs further refinement, and our findings should be corroborated on additional, taxonomically well characterized strains.

The two different ITS2 lengths found for the *C. laurentii* strains in this study appear to correspond with a recently described phylogenetic subdivision within this species, that was based on the sequences of the 28S rRNA and of the ITS1 and ITS2 regions [11]. Thus, it appears that both groups can be differentiated simply by determination of the ITS2 length and that a distinction between environmental and clinical isolates might be possible. Although the ITS2-fragment lengths range between 365.1 and 368.5 basepairs for *S. cerevisiae* strains, identification remains possible since these fragment lengths are not encountered in other clinically important yeast species studied thus far.

When methods are used that are suitable for DNA-extraction directly from clinical specimens, it should be possible to detect and identify at once the different species that may be simultaneously present, since the fingerprint will consist of ITS2 fragments of different lengths, corresponding to the different ITS2-lengths for each species. However, when applied for direct detection, the minor peaks, as observed in one of the laboratories, could lead to problems in assessing the number and nature of the species present.

When ITS2-primers are labeled differently from e.g. tRNA-PCR primers [12–14], electrophoresis of bacterial tRNA-PCR and fungal ITS2-PCR can be carried out simultaneously.

Materials and Methods

Strains

A collection of 53 culture collection strains (Table 1) and 90 phenotypically well-identified strains, representing 24 species, was used to evaluate the technique and to set up an initial database. The technique and database were evaluated by identification of 242 clinical isolates from four Belgian hospitals (Ghent University Hospital, Heilig Hart Ziekenhuis Roeselare, Akademisch Ziekenshuis VUB Jette and Akademisch Ziekenhuis St. Jan Bruges). In total, 385 strains belonging to 26 species (Table 2) were included.

DNA extraction

A boiling-freezing protocol was used for DNA-extraction. A 1 µl loopful of growth on Sabouraud agar (Becton Dickinson, Maryland, Ca.) was suspended in 500 µl distilled water and heated for 15 minutes at 95°C, immediately followed by freezing at -70°C for at least 15 minutes. Before adding the DNA-extract to the PCR mix, the samples were thawed at room temperature, and shortly centrifuged to pellet the cell debris still present in the extract.

ITS2-PCR

The ITS2-region was amplified with the following primers: ITS86 (GTG AAT CAT CGA ATC TTT GAA C-HEX) (fluorescently labeled) and ITS4 (TCC TCC GCT TAT TGA TAT GC) [9]. The ITS86 primer was used as a mixture of 10% fluorescently labeled and 90% non-fluorescently labeled primer in order to avoid out of range peak heights. The PCR reaction mix contained 12.5 µl Qiagen Mastermix (Qiagen, Hilden, Germany), 0.5 µM of each primer, 2.5 µl of the DNA extract and 9 µl of sterile distilled water in a final volume of 25 µl. The thermal cycling was carried out according to the following protocol: 5 min at 94°C, 30 cycli of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C and a final extension of 7 min at 72°C, followed by cooling at 4°C.

Capillary electrophoresis

The capillary electrophoresis apparatus used was the ABI Prism 310 Genetic Analyser (Applied Biosystems, Foster City, Ca.). Electrophoresis was done in a single capillary, filled with liquid polymer (POP-4: Performance Optimized Polymer, Applied Biosystems), which was automatically replaced after every electrophoresis run. Preparation of the sample before electrophoresis consisted of adding 1 µl of ITS2-PCR product to 12.5 µl of electrophoresis mixture (0.1 µl HD 400 marker, 0.3 µl of ROX-500 marker and 12.1 µl of deionized formamide, all from Applied Biosystems). The double stranded ITS2-PCR fragments were denatured by heating this mixture for 2 minutes at 95°C, followed by immediate cooling on ice. Electrophoresis of each sample was carried out at 60°C and at 15 kV, during 35 min. The Gene Scan analysis software (Applied Biosystems) was used to derive the fragment length of the HEX-labeled DNA-fragments using the known fragment lengths of the ROX-labeled marker peaks. The results were presented in a table indicating length and intensity of the observed fragments. BaseHopper software, described previously [12,13] and freely available upon request was used to quickly compare the obtained ITS2-fragment lengths with those of the ITS2-database.

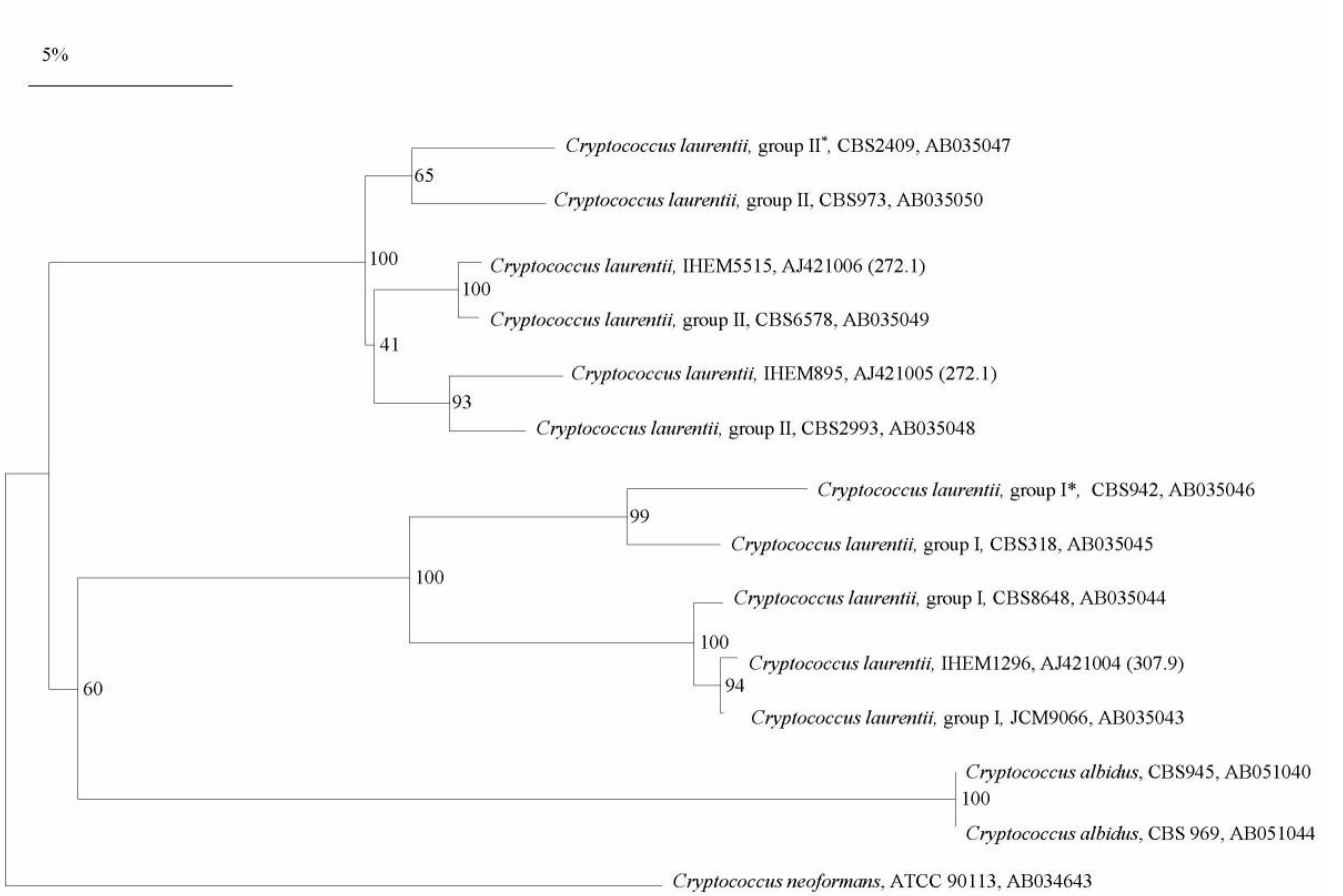

Figure 2
Dendrogram of ITS2 sequences of *Cryptococcus laurentii*, *C. albidus* and *C. neoformans*. Numbers on branches indicate bootstrap value. Species name, phylogenetic group according to Sugita *et al.*[11], strain number, GenBank accession number (and ITS2-length in bp) are indicated.

Sequencing of the ITS2 region

After amplification of the ITS2 region, the amplicon was purified using the Qiaquick PCR purification kit, according to the manufacturer's instructions. Cycle sequencing was performed using the Ready-reaction mix (ABI Prism Bigdye Terminator Cycle Sequencing kit, Applied Biosystems) according to the manufacturer's instructions. Analysis of the sequencing products was done on the ABI Prism 310 capillary (Applied Biosystems). Assembly of sequence fragments and editing was done with GeneBase (Applied Maths, St. Martens Latem, Belgium). The obtained ITS2 sequences were compared to all known sequences in the Genbank by use of Blast 2.0 (National Center for Biotechnology Information, Bethesda, Md. [http://www.ncbi.nlm.nih.gov/BLAST/]).

Cluster analysis and dendrogram construction of ITS2 sequences

Comparative sequence analysis was performed by using the GeneBase software package (Applied Maths, St. Martens Latem, Belgium), as described previously [15]. First, pair wise alignment using UPGMA was carried out with a gap penalty of 100 %, a unit gap cost of 20 % and an ambiguity cost of 50 % of the mismatch cost. Subsequently, global alignment – with *C. neoformans* ATCC 90113 (Genbank AB034643) used as the outgroup – was carried out on the region between 26 and 205 bp of the ITS2 region of *C. neoformans*, with costs as above. Finally, a similarity matrix of the aligned sequences was constructed by global alignment homology calculation and a gap penalty of 20 %. The neighbour-joining method was used to construct the dendrogram based on this similarity matrix. Bootstrap (n=100) values were calculated.

Authors' contributions

Authors 1 (TD), 4 (CM) and 6 (AM) carried out the molecular studies and participated in the sequencing work and the interlaboratory comparisons. Authors 2 (GC), 3 (DS) and 5 (GV) provided the clinical and reference strains, Authors 1 (TD), 2 (GC), 5 (GV) and 7 (MV) participated in the design of the study and and author 7 (MV) coordinated the study and drafted the manuscript.

All authors read and approved the final manuscript.

Abbreviations

alb: *Candida albicans*

dub: *C. dubliniensis*

gla m: *C. glabrata,* minor peak

gla M: *C. glabrata,* major peak

gui: *C. guilliermondii*

lip: *C. lipolytica*

lus: *C. lusitaniae*

rug: *C. rugosa*

par: *C. parapsilosis*

nor: *C. norvegensis*

tro: *C. tropicalis*

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