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Taxonomic and diagnostic markers for identification of *Coccidioides immitis* and *Coccidioides posadasii*

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The ribosomal Internal Transcribed Spacer (ITS) regions of the two recognized species of *Coccidioides* were studied using a reference set of strains that had been previously identified with species defining microsatellite polymorphisms. Unambiguous identification of the two species proved to be possible by amplifying and sequencing the ITS region. PCR-reactions are sensitive to amplification conditions requiring their careful optimization. Stable amplification and sequencing was achieved with primers ITS3 and 4, enabling species diagnosis. Alternatively, Restriction Fragment Length Polymorphism (RFLP) of the entire ITS region using an annealing temperature of 52°C with the restriction enzymes *Bsr*I and *Xcm*I can also distinguish the species. Three strains typifying the species, *Glenospora meteuropaea*, *G. metamericana* and *Geotrichum louisianoideum*, were analyzed and found to be conspecific with *C. posadasii*. Although these species have nomenclatural priority over *C. posadasii,* the latter will be proposed for conservation as it has been included in the US select agent list. In addition, *Coccidioides immitis* is neotypified in this report. Results of antifungal susceptibility testing did not reveal differences between the two species.

Keywords *Coccidioides immitis*, *Coccidioides posadasii*, species identification, Internal Transcribed Spacer sequencing, Restriction Fragment Length Polymorphism

Introduction

Over the last decade several epidemiological studies of *Coccidioides* species have been carried out, demonstrating the existence of a wide diversity of genotypes. Zimmermann *et al*. [1] performed genomic Restriction Fragment Length Polymorphism (RFLP) and showed that two groups could be distinguished, i.e., *Coccidioides immitis* types I and II and Burt *et al*. [2] identified a similar subdivision using RFLPs of several independent markers. Kofoupanou *et al*. [3,4] used a sequencing study of five housekeeping genes to show that the two entities had a broadly defined Californian *versus* a non-Californian distribution, and were reproductively isolated. Subsequently, microsatellites were shown to be useful as phylogenetic and population genetic markers in studies with *Coccidioides* [5]. Two major clades were identified by microsatellite analyses [6] and proved to be consistent with previously analyzed loci. Analyses of single nucleotide polymorphisms have shown that internal branches exhibit extensive reshuffling indicating the occurrence of recombination [7,8]. By fulfilling criteria for Genealogical Concordance Phylogenetic Species Recognition (GCPSR), the two entities were proposed and generally

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recognized as two separate species, *Coccidioides immitis* and the novel species *C. posadasii* [6].

The species differ in their geographic distribution with *Coccidioides posadasii* found throughout suitable climatic zones of the southwestern United States, Central and South America, while *C. immitis* exhibited a more constrained geographic range within the San Joaquin Valley area in California. However, low frequencies of occurrence in patients were found south of the Tehachapi Mountains and here infections of the two species were found in sympatry. Californian strain RMSCC Silveira = ATCC 28868 = CBS 113859, which is the focus of a genome project of *Coccidioides,* was selected as the type isolate for *C. posadasii*.

Coccidioides is morphologically characterized by enteroarthric conidia. A selective Small SubUnit (SSU)-based PCR for recognition at the generic level has been established [9] in order to discriminate the genus from morphologically similar members of the order *Onygenales* [10]. However, routine diagnostics of the two species remains problematic, despite their unambiguous genetic separation. The main set of diagnostic criteria is found by discriminating the two species based on microsatellite length polymorphisms [6]. The technique uses polyacrylamide gels run on an automatic sequencer to separate the polymorphisms. Amplicons generated with microsatellite primer GAC are 206 bp in length in *C. posadasii* and 216 bp or more in *C. immitis*. A similar result is obtained with primer 621, generating amplicons of up to 401 bp and over 414 bp, respectively, and these two loci are diagnostic for the two species. Bialek *et al*. [11] discussed recognition of *C. posadasii* antigen 2/proline-rich antigen (PRA) as a vaccine target, but did not provide evidence whether or not *C. immitis* may show a similar antigenicity. Conversely, Greene *et al*. [9] provided an ITS-based *C. immitis* primer, for which the sequence in *C. posadasii* is unknown. Recently Umeyama *et al*. [12] developed specific primers for the two species based on an unspecified DNA region. Phenetic markers for rapid recognition are still lacking; micromorphologies are identical [6]. Colonies of *C. immitis* on average exhibit significantly faster growth on salt-containing media relative to *C. posadasii*, however this character is not diagnostic for the two species [6].

As yet no differences have been observed in the human diseases caused by the two species, and no significant differences between their environmental niches have been noted. However, given the proven genetic and geographic separation of the two species, such differences are likely to exist. In view of ongoing clinical and ecological studies that are aimed at revealing evolutionary processes in virulence factors such as the proline-rich antigen (PRA) [13], a wide array of options for diagnostics and screening should be available.

Separation of the two species using molecular methods that are more widely applied than microsatellites, particularly of the ribosomal operon, thus far has received little attention. The Small SubUnit (SSU) or Large SubUnit (LSU) rDNA regions are highly conserved and allow recognition of *Coccidioides* at the genus level only [14]. In general, sequences of the ITS regions of rDNA have become available for nearly all newly described fungi where species concepts are supported by molecular data. rDNA spacers are increasingly viewed as a gold standard for taxonomy of filamentous fungi, and are included in the analysis even where ITS may provide insufficient variability to reflect diversity at the species level, e.g., in recently evolved genera such as *Penicillium* [15], *Thelebolus* [16] or *Trichoderma* [17]. ITS is also used as a routine marker in the taxonomy of other fungi that, like *Coccidioides*, belong to the order *Onygenales*, for example, dermatophytes [18], *Chrysosporium* [19] and *Aphanoascus* [20]. To fulfil the need for a simple and reproducible molecular assay to discriminate between the two species of *Coccidioides*, we verified the distribution of ITS sequence diversity among 42 *Coccidioides* isolates including reference strains of both species. We subsequently reviewed molecular diagnostic options for the routine laboratory, and determined whether differences in susceptibility against antifungals could be observed between the two species.

Materials and methods

Strains

Table 1 lists 14 *C. posadasii* and 8 *C. immitis* reference strains that were previously identified by microsatellite typing, and 20 clinical isolates deposited in the collections of the Centraalbureau voor Schimmelcultures (CBS) and the Robert Koch-Institute (RKI) as '*C. immitis*' according to former taxonomic concepts. Stock cultures were stored in liquid nitrogen or ceramic beads at −70°C (Microbank, MAST-Diagnostica, Reinfelden, Germany) and subcultured on Sabouraud glucose agar.

Biosafety

Procedures of culturing, susceptibility testing and DNA extraction were performed in a biosafety level 3 laboratory within a class II biological safety cabinet.

Table 1 Strains analyzed

Strain number	Alternative	Species		GenBank	Location
Reference strains:					
CBS 113838	RMSCC 2214	*C. posadasii* Fisher, Koenig, White & Taylor			Texas
CBS 113839	RMSCC 3737	*C. posadasii*			Brazil
CBS 113840	RMSCC 2233	*C. posadasii*			Texas
CBS 113841	RMSCC 2346	*C. posadasii*			Mexico
CBS 113842	RMSCC 2377	*C. posadasii*			Argentina
CBS 113843	RMSCC 2129	*C. posadasii*		EF186785	Texas
CBS 113844	RMSCC 2376	*C. posadasii*			Argentina
CBS 113845	RMSCC 2379	*C. posadasii*			Argentina
CBS 113846	RMSCC 1040	*C. posadasii*		EF186786	Arizona
CBS 113847	RMSCC 3704	*C. posadasii*			California
CBS 113848	RMSCC 1044	*C. posadasii*			Arizona
CBS 113849	RMSCC 1042	*C. posadasii*			Arizona
CBS 113858	RMSCC 3696	*C. posadasii*			California
CBS 113859[T*]	Silveira 4				
	ATCC 28868	*C. posadasii*			California
CBS 113850*	RMSCC 3703	*C. immitis* Rixford & Gilchrist			California
CBS 113851	RMSCC 2395	*C. immitis*		EF186787	California
CBS 113852	RMSCC 3505	*C. immitis*		EF186788	Mexico
CBS 113853	RMSCC 2007	*C. immitis*			SJV California
CBS 113854	RMSCC 2281	*C. immitis*			SJV California
CBS 113855	RMSCC 2267	*C. immitis*			SJV California
CBS 113856*	RMSCC 3476	*C. immitis*		EF186789	Mexico
CBS 113857	RMSCC 3377	*C. immitis*		EF186790	California
Additional strains:					
CBS 148.33		Original identification *C. immitis*		Reidentification	
		AUT of *Geotrichum immite*	*C. immitis*		Not available
CBS 149.33		*C. immitis*	*C. immitis*		Missouri
CBS 144.34		*C. immitis*	*C. immitis*		California
CBS 145.34		*C. immitis*			
		AUT of *Geotrichum louisianoideum*	*C. posadasii*		Not available
CBS 146.34		*C. immitis*			
		AUT of *Glenospora meteuropaea*	*C. posadasii*		Not available
CBS 196.34		*C. immitis*			
		AUT of *Glenospora metamericana*	*C. posadasii*	EF186784	Not available
CBS 166.51		*C. immitis*	*C. immitis*	EF186783	USA
CBS 146.56		*C. immitis*	*C. posadasii*		Hungary
CBS 711.73		*C. immitis*	*C. posadasii*		Finland
RKI 95-2795*		*C. immitis*	*C. immitis*		California
RKI 98-0204*		*C. immitis*	*C. posadasii*		Arizona
RKI 98-0275*		*C. immitis*	*C. immitis*		California
RKI 98-0528*		*C. immitis*	*C. posadasii*		Arizona
RKI 02-0393*		*C. immitis*	*C. immitis*		USA
RKI 04-0058*		*C. immitis*	*C. posadasii*		Arizona
RKI 04-0092*		*C. immitis*	*C. posadasii*		Arizona
RKI 04-0353*		*C. immitis*	*C. posadasii*		California or Arizona[†]
RKI 04-0477*		*C. immitis*	*C. posadasii*		USA
RKI 05-0603*		*Coccidioides* species	*C. posadasii*		USA
RKI 05-0713*		*Coccidioides* species	*C. posadasii*		Arizona
Selected neotype strain:					
CBS 120936[NT]	RMSCC 2394	*C. immitis*			California

Acronyms of strain collections: CBS: Centraalbureau voor Schimmelcultures, RMSCC: Roche Molecular Systems Cultures Collection, RKI: Robert Koch-Institute. AUT: authentical material, SJV: San Joaquin Valley. *Strain included in study for antifungal susceptibility. [†]The patient travelled in both states.

DNA extraction

Mycelia from cultures of the test strains (approximately 50 mg, wet weight) were homogenized in 300 µl cetyltrimethylammoniumbromide (CTAB) buffer containing glass beads of 0.45–0.50 mm in diameter (B. Braun, Melsungen, Germany) as previously described [21]. After 15 min of sonication in a water bath, followed by an incubation step at 65°C for 10 min, 300 µl ethanol were added and homogenized by vortexing. Total DNA was purified by silicagel columns (Qiamp Mini Kit, Qiagen, Hilden, Germany), according to the manufacturer's instructions and stored at −20°C.

Amplification

Fragments of rDNA were amplified with the primer pairs NS7-ITS2, ITS1-ITS4, ITS2-ITS5, ITS3-ITS4, ITS4-ITS5 [22], ITS3-R635 and F63-R635 [23] in a reaction mixture containing 25 µl distilled water, 5 µl of PCR buffer ($MgCl_2$, 10 ×), 8 µl 0.25 mM dNTP (Roche Diagnostics GmbH, Germany), 1 µl of each primer and 1.25 U of Taq BioTherm^{TM} DNA polymerase (Rapidozym, Germany). Following a 4 minute denaturation step at 94°C, the reaction mixtures were thermally cycled 38 times at 94°C for 30 sec., 55°C for 30 sec. and 72°C for 90 sec. and at least once for 10 min at 72°C. This PCR protocol will be entitled 'original' in the manuscript.

Variation of polymerase and annealing temperature, selection of specific primers

To optimize the amplification of *Coccidioides* DNA, an alternative PCR protocol was used. The PCR reaction started with a initial denaturation at 96°C, 5 min. followed by 30 cycles 96°C, 30 sec.; 52°C, 30 sec.; 72°C, 75 sec. The last elongation step at 72°C was prolonged by 3.5 min. Different primer combinations, polymerases and annealing temperatures were tested. An alternative primer ITSv3 (5′-CAGTCTGAGCATCATAGC-3′) was chosen at position 155 in ITS1. The sequence of the ITS4 primer was not consistent with the known sequences of the LSU. Therefore a shorter primer was designed, CocBW (5′-TCCGCTTATTGATATGC-3′). Subsequently four specific PCR primers were selected: *C. immitis* – specific primers, Ci1FW (5′-TTACTGAACTATTGTTGC-3′) and Ci2FW (5′-CACCCGTGTTTACTGAACT-3′), and *C. posadasii* – specific primers, Cp1Fw (5′-TTACTGAACCATTGTTGC-3′) and Cp2Fw (5′-CACCCGTGTTTACTGAACC-3′). The specific primers were tested with CocBW as a reverse primer and a thermostable polymerase with proofreading activity (Accuzyme, Gentaur, Belgium) was used at a magnesium concentration of 1.5 mM. For the specific PCR eight different annealing temperatures were tested, 60, 59, 57.2, 54.5, 50.7, 48, 46.1 and 45°C. The primer combinations ITS1-ITS4 or ITS1-CocBW were used for RFLP. The recently published primer pair Coi9-1F and Coi9-1R of a randomly selected region of the *Coccidioides* genome [12] has been evaluated with all strains in this study.

Restriction analysis

Amplification of the entire ITS region was performed with primer ITS1 in the terminal region of SSU and primer ITS4 in the LSU. A virtual analysis of more than 270 endonucleases predicted *C. immitis* and *C. posadasii* to have a species-specific restriction site when cut with either *Bsr*I or *Xcm*I. RFLP was performed with *Bsr*I (ACTGGn) at 65°C for 3 h, and with *Xcm*I (CCAnnnnnnnnnTGG) at 37°C for 3 h.

Susceptibility testing

A total of 15 isolates of *Coccidioides* species were tested, 5 of them were *C. immitis* and 10 *C. posadasii*. Isolates were subcultured on potato dextrose agar and incubated for 10 days at 30°C in neck-styled cell culture flasks with filter caps (NUNC, Wiesbaden, Germany). Thereafter the surface of the agar was rinsed with 5 ml sterile 0.85% [w/v] NaCl and the suspension was adjusted to a McFarland 2 standard followed by dilution of 1:20 with the test medium (0.165 M MOPS-buffered RPMI 1640 medium, pH 7.0). This inoculum proved to be superior to other dilutions for an optimal reading of the inhibition after 96 h of incubation.

Microdilution testing was performed in microtiter plates adapted to the guidelines of the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards). The microtiter plates contained a twofold concentrated dilution series of antifungal substances at 100 µl per well. The wells were inoculated with the suspension and incubated at 30°C. Fungal drug concentrations tested were: for itraconazole, posaconazole, voriconazole and terbinafine 0.016–16 µg/ml; ketoconazole 0.016–8 µg/ml; fluconazole 0.06–64 µg/ml; caspofungin 0.06–32 µg/ml; amphotericin B 0.03–16 µg/ml. Amphotericin B was tested with RPMI medium as well as with Antibiotic Medium 3; for the latter medium a lower inoculum was used (dilution 1:200). MICs were defined for the azoles and terbinafine as the lowest concentrations showing a prominent decrease of growth compared to the growth control and for

amphotericin B as the lowest concentration inhibiting growth completely. For caspofungin the minimal effective concentration (MEC) was determined microscopically [24]. A reading mirror was used for visual reading by two independently operating persons. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as quality control strains.

Results

The terminal region of the SSU and the D1/D2 domain of LSU rDNA proved to be monomorphic for all strains analyzed, except for some phylogenetically non-informative single nucleotide changes. Ribosomal PCR products remained short and were often generated only after repeated amplification with general fungal primers NS7-ITS2, ITS1-ITS4, ITS2-ITS5 and ITS4-ITS5, when the original PCR protocol was used. We found that only the primer pair ITS3-ITS4 stably amplified the ITS2-region in all isolates. The primer pair ITSv3-ITS4 could be used to connect the ITS1 and ITS2 regions. ITS1 had three and ITS2 had two informative polymorphisms, which allowed differentiation of *C. immitis* from *C. posadasii* (Table 2). However, these regions also contained indels that were located in a T-rich region and were therefore not ideally suited as primer-binding sites for a species specific PCR. Nevertheless four specific primers were selected and tested (see above). When species-specific amplification of DNA was tested with the primer pairs Cp1FW-CocBW and Ci1FW-CocBW using the original PCR protocol, amplification was successful only in 13/15 *C. immitis* isolates and 17/27 *C. posadasii* isolates. When DNA amplification was successful, the species were correctly identified for all tested *C. posadasii* isolates. However, false positive results were obtained in *C. immitis* isolates, making this method inappropriate for use as a diagnostic technique.

The efficiency of the primers Cp1FW and Cp2FW for *C. posadasii* and Ci1FW and Ci2FW for *C. immitis* combined with CocBW were studied in a gradient PCR (BioRad) with DNA of the strains *C. immitis* CBS 144.34 and CBS 166.51, and *C. posadasii* CBS 113838 and CBS 113844. Decreasing the annealing temperature increased the rate at which DNA was successfully amplified for ITS1-4 and ITS4-5, but lowered the specificity of the specific primer sets (data not shown).

Table 2 Phylogenetically informative sites in ITS1 and ITS2

Length	ITS1 (221 bp)			ITS2 (169 bp)			
Position	37	74	125	34	37	148	157-161
C. posadasii	T	C	C	C	–/A	C	ATT-A ATT-T
C. immitis	–	T	T	T/C	–	T	T---A TTWAA TT-AA TTTAA

Amplification was successfully performed for all but one (RKI 02-393) strains using the primer set Coi9-1F and Coi9-1R [12]. Nearly all products had the lengths expected for *C. posadasii* and *C. immitis,* respectively, with one exception, strain CBS 113850 = RMSCC 3703. Microsatellites and ITS-sequencing indicated that this strain should be *C. immitis*, but had Coi9-amplicon lengths matching that of *C. posadasii* (Fig. 1).

Restriction analysis of the amplicon created by ITS1-ITS4 with *Bsr*I generated a fragment of 180 bp in *C. immitis* which remained absent from *C. posadasii* and *Xcm*I generated a fragment of 80 bp in *C. posadasii* and not in *C. immitis* (Fig. 2).

In vitro susceptibility testing of a limited number of strains (Table 3) against amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, ketoconazole, caspofungin and terbinafine resulted in narrow MIC ranges for each antifungal agent with no difference between *C. immitis* and *C. posadasii* isolates calculated statistically. The MIC ranges are comparable with published data on *Coccidioides* [24,25].

Discussion

The present ITS sequencing study applied a set of reference strains verified to be *Coccidioides immitis* and *C. posadasii*, respectively, with microsatellite primers GAC and 621 by Fisher *et al*. [6]. The results confirmed differences found in other genomic regions [1–3,5,6]. Variability was detected both in ITS1 and ITS2 that was diagnostic for the two species. Having established ITS-based species discrimination, additional strains from RKI and CBS were subsequently reidentified resulting in the identification of seven as *C. immitis*, and 13 as *C. posadasii*. The finding of consistent ITS differences between *C. immitis* and *C. posadasii* makes possible their identification by established parameters and routine methods. Amplification and sequencing of the characteristic ITS2 region is simple when primers ITS3 and ITS4 are used, allowing unambiguous identification of the two species. The method is robust and is not sensitive to the PCR protocol used. In contrast, amplification of ITS1 and the entire ITS region using primers ITS1 or 5 and ITS4 was found to be problematic using the original *Onygenales* amplification protocol. This protocol is routinely used at the RKI for the identification of other members of the

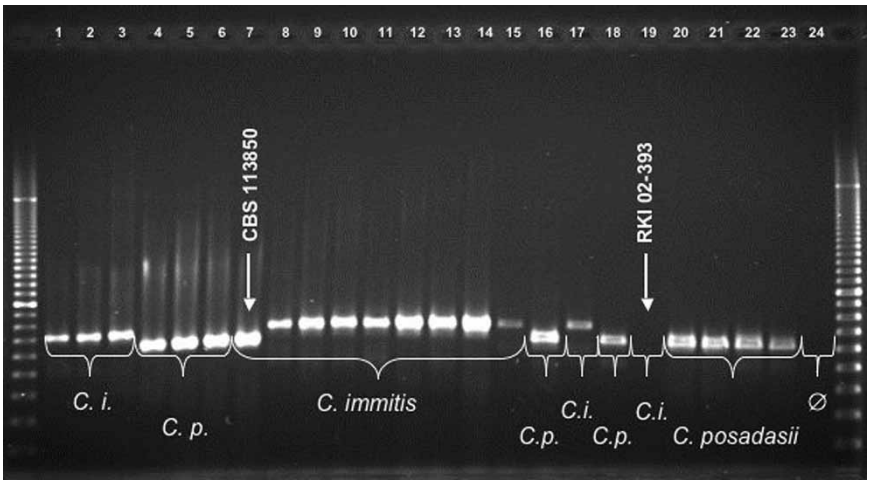

Fig. 1 Amplification of *Coccidioides* DNA with primers Coi9-1F and Coi9-1R according to Umeyama [12]. 1 CBS 148.33, 2 CBS 149.33, 3 CBS144.34, 4 CBS 146.34, 5 CBS 113838, 6 CBS 113839, 7 CBS 113850, 8 CBS 113851, 9 CBS 113852, 10 CBS 113853, 11 CBS 113854, 12 CBS 113855, 13 CBS 113856, 14 CBS 113857, 15 RKI 95-2795, 16 RKI 98-0204, 17 RKI 98-0275, 18 RKI 98-0528, 19 RKI 02-0393, 20 RKI 05-0603, 21 RKI 05-0686, 22 RKI 05-0713, 23 RKI 04-0477. *C.i.*: *Coccidioides immitis*, *C.p.*: *Coccidioides posadasii*.

order *Onygenales,* such as *Histoplasma* and dermatophytes. Modification of the amplification protocol to include a denaturation temperature of 96°C and lowering the annealing temperature from 55°C to 52°C allowed efficient and reliable amplification of a larger part of the ITS domain using primers ITS1–4. Remarkably, Umeyama *et al*. [12] used ITS1-4 amplification as a positive control for the presence of suitable DNA for their development of anonymous diagnostic primers, although the annealing temperature in their study was higher (60°C) than that in the original RKI protocol. We were unable to reproduce these results.

The development of specific primers for the two species proved to be problematic. Greene *et al*. [9] applied ITS primers specific for the environmental detection of *C. immitis* in the San Joaquin Valley, California. Target regions were located at the 3' end of ITS1 and in the middle of ITS2, both in areas that are strictly monomorphic for the two species. From a large number of soil samples screened, a recovery rate of less than 1% was achieved. As no further typing was done, their strains may just as well have been *C. posadasii*. The region with the largest differences between *C. immitis* and *C. posadasii* is located at the end of ITS2 (Table 2). Here, the sequence contains 60–80% GC, has relatively long repeats and is prone to the formation of palindromes. Specific amplification using several primers based on these regions proved to be insufficiently predictive to be used as a reliable diagnostic test for the two species.

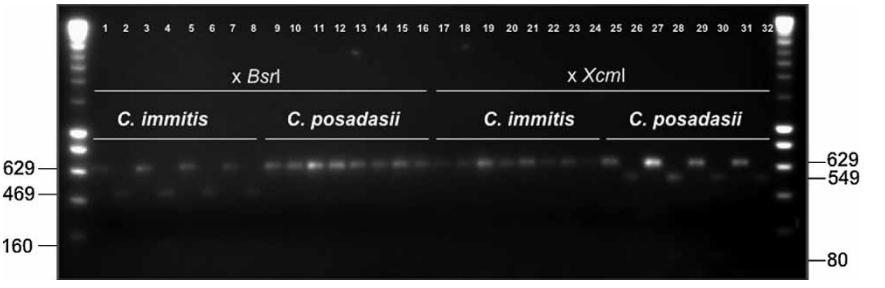

Fig. 2 ITS-RFLP of *Coccidioides immitis* and *Coccidioides posadasii* with *Bsr*I and *Xcm*I. Original amplicon/digestion. 1, 2. CBS 149.33, *C. immitis*-/ x *Bsr*I; 3, 4. CBS 144.34, *C. immitis*-/ x *Bsr*I; 5, 6. CBS 166.51, *C. immitis*-/ x *Bsr*I; 7, 8. CBS 113850, *C. immitis*-/ x *Bsr*I; 9, 10. CBS 146.34, *C. posadasii* -/x *Bsr*I; 11, 12. CBS 113838, *C. posadasii*-/ x *Bsr*I; 13, 14. CBS 113839, *C. posadasii* -/ x *Bsr*I; 15, 16. CBS 113849, *C. posadasii*-/ x *Bsr*I; 17, 18. CBS 149.33, *C. immitis*-/ x *Xcm*I; 19, 20. CBS 144.34, *C. immitis*-/ x *Xcm*I; 21, 22. CBS 166.51, *C. immitis*-/ x *Xcm*I; 23, 24. CBS 113850, *C. immitis*-/ x *Xcm*I; 25, 26. CBS 146.34, *C. posadasii*-/ x *Xcm*I; 27, 28. CBS 113838, *C. posadasii*-/ x *Xcm*I; 29, 30. CBS 113839, *C. posadasii*-/ x *Xcm*I; 31, 32. CBS 113849, *C. posadasii*-/ x *Xcm*I.

Table 3 *In vitro* susceptibility of *Coccidioides immitis* and *Coccidioides posadasii*

		C. immitis ($n=5$)		*C. posadasii* ($n=10$)	
	All isolates MIC range (μg/ml)	MIC_{50} (MIC_{90}) (μg/ml)	GM MIC (μg/ml)	MIC_{50} (MIC_{90}) (μg/ml)	GM MIC (μg/ml)
Fluconazole	2.0–16.0	4 (16)	4.00	4 (4)	3.48
Itraconazole	0.25–1.0	0.25 (1)	0.38	0.5 (1)	0.57
Ketoconazole	0.06–0.25	0.13 (0.25)	0.11	0.06 (0.25)	0.09
Posaconazole	0.06–0.13	0.13 (0.13)	0.09	0.13 (0.13)	0.09
Voriconazole	0.03–0.13	0.06 (0.13)	0.06	0.06 (0.06)	0.05
Amphotericin B/RPMI	0.5–2.0	1 (2)	1.00	1 (2)	1.05
Amphotericin B/AM3	0.06–0.5	0.25 (0.5)	0.25	0.25 (0.5)	0.29
Caspofungin (MEC)	0.13–0.25	0.13 (0.25)	0.14	0.13 (0.13)	0.13
Terbinafine	0.06–0.25	0.13 (0.25)	0.14	0.12 (0.12)	0.11

MIC, minimal inhibitory concentration; MEC, minimal effective concentration; GM, geometric mean; MIC_{50} (MIC_{90}), MIC at which 50 and 90% of the isolates tested are inhibited respectively; RPMI, RPMI 1640 medium according to the NCCLS (CLSI) guidelines; AM3, Antibiotic Medium 3.

The nucleotide differences show that the phylogenetic distance between *C. immitis* and *C. posadasii* is reflected in a 1.5% sequence divergence at the ITS region. This is a degree of diversity expected between neighbouring species in the order *Onygenales*. If we assume strict neutrality of ribosomal markers that mutate at a molecular clock speed of 10^{-9}, genetic distances are broadly indicative of time of phylogenetic differentiation. The time of divergence of the two *Coccidioides* species has been estimated to be 11.10^6 years [6].

Subsequent restriction analysis of amplicon ITS1-4 with *Bsr*I and *Xcm*I allowed rapid and confident discrimination of *C. immitis* and *C. posadasii*: the fragments generated by these enzymes are 180 bp and 80 bp, respectively, in length and thus easily visible on agarose gels.

In conclusion, stable amplification and sequencing is achieved with primers ITS3 and 4. Alternatively, RFLP of the entire ITS region using an annealing temperature of 52°C with the restriction enzymes *Bsr*I and *Xcm*I can also distinguish between the two species.

Interestingly, the isolate CBS 113850 (RMSCC 3703) was shown by microsatellite and ITS RFLP analysis to be *C. immitis*, however clustered with *C. posadasii* after amplification with Coi9 primers [12]. There are three possible reasons that can account for this result. First, a mutation may have occurred in RMSCC 3703 resulting in the occurrence of an indel that matches those seen in *C. posadasii* (known as size homoplasy). Second, RMSCC 3703 may be a hybrid genotype between *C. immitis* and *C. posadasii*, and may therefore share polymorphisms that are characteristic of both species. Of note, RMSCC 3703 is one of the strains used for genome sequencing of *C. immitis* (www.broad.mit.edu). Third, the indel found at Coi9 may not have drifted to fixation in *C. immitis* and may still be polymorphic within this species. Given that the evolution of an indel of exactly the same size is probably a rare event, we believe that homoplasy does not explain our result. Further, hybrids between the two *Coccidioides* species have never been observed, and it appears likely that if it had occurred here, then more of the loci within this isolate would share characteristics of both species but this was not the case. Therefore, we believe that the Coi9 marker is polymorphic, albeit at a low frequency, within *C. immitis*, and more work needs to be done on this marker to assess its utility as a diagnostic marker.

Results of antifungal susceptibility testing did not yield significant differences between the two *Coccidioides* species showing that the two species have not evolved significantly different metabolic profiles with respect to their *in vitro* drug resistance.

Coccidioides posadasii was recently typified by strain ATCC 28868 = CBS 113859 [6], but the authentic material of *Coccidioides immitis* is not known to be preserved (E. Hornsby and W. G. Merz, pers. comm.). In accordance with a suggestion of C. W. Stiles, Rixford & Gilchrist [26] introduced in 1893 the species for a mold recovered from a specimen recovered from a fatal disseminated case in a 40-year-old agricultural labourer who had been admitted to the municipal hospital of San Francisco. In the same article, Rixford & Gilchrist [26] introduced *Coccidioides pyogenes* Rixford & Gilchrist, type material of which is probably lost as well. The specimen came from a 33-year-old worker who had been engaged in manual labour, chiefly on farms in the San Joaquin Valley, California. Strain RMSCC 2394 (CBS 120936) matches the original geographical location, and is therefore indicated here

as **neotype** of *Coccidioides immitis*. Dried material is deposited in the herbarium of the Centraalbureau voor Schimmelcultures, under No. CBS H-19842. Further possible synonyms [27] are *Coccidium neoplasicum* Cantón [28], *Posadasia esferiformis* Cantón [in 29], *Pseudococcidioides mazzae* da Fonseca [in 30], *Oidium protozoides* Ophüls [31] and *Trichosporon proteolyticum* Negroni & de Villafañe Lastra [32]. Most of these species have never been cultured, and authentic materials are not known to be preserved. Hence it is impossible to establish whether *C. immitis* or *C. posadasii* was the causative agent in each of these cases and the names are regarded to be of doubtful identity.

Authentic materials of the species introduced by Aldo Castellani and coworkers [33] are still available as living isolates in the CBS culture collection. *Geotrichum lousianoideum* Castell. (CBS 145.34, from a patient living in Louisiana, USA), *Glenospora meteuropaea* Castell. (CBS 146.34, from an English patient that was reported to be infected in the Balkan) and *Glenospora metamericana* Castell. (CBS 196.34, origin unknown) all proved to be identical to *C. posadasii*. As these taxa fulfil the requirements for novel species descriptions under the International Code of Botanical Nomenclature, these names can be regarded to be older synonyms of *C. posadasii,* having priority over *C. posadasii* by more than 60 years. The name *C. posadasii* has been included in the US' select agent list and thus has become widely used. Since we believe that renaming the species might cause confusion, we are preparing a formal proposal for conservation of the name *C. posadasii*.

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