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ORIGINAL ARTICLE

New Molecular Markers Distinguishing *Fonsecaea* Agents of Chromoblastomycosis

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Abstract The species belonging to the genus *Fonsecaea* are the main causative agents of chromoblastomycosis. The invasive potential of *Fonsecaea* differs significantly among its various sibling species. Moreover, the lack of clarity on the virulence and availability of precise markers to distinguish and detect *Fonsecaea* species is attributed to the different ways of dissemination and pathogenicity. Therefore, the present study aimed to propose new molecular tools to differentiate between sibling species causing chromoblastomycosis. We used an infection model of chromoblastomycosis in BALB/c to study species-specific molecular markers for the in vivo detection of *Fonsecaea* species in biological samples. Specific primers based on the *CBF5* gene were developed for *Fonsecaea pedrosoi*, *Fonsecaea monophora*, *Fonsecaea nubica*, and *Fonsecaea pugnacius*. In addition, a padlock probe was designed for *F. pugnacius* based on ITS sequences. We also assessed the specificity of *Fonsecaea* species using in silico, in vitro, and in vivo assays. The results showed that markers and probes could effectively discriminate the species in both clinical and environmental samples, enabling

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bioprospecting of agents of chromoblastomycosis, thereby elucidating the infection route of the disease.

Keywords Chromoblastomycosis · Molecular detection · RCA · Padlock probe · *CBF5* gene · *Fonsecaea*

Introduction

Human chromoblastomycosis (CBM) is a skin disease that is exclusively caused by the members of order Chaetothyriales (black yeasts and relatives). The infection occurs through inoculation of the pathogen by a trauma or injury caused by sharp natural materials, such as plant thorns or wooden splinters that carry the respective opportunistic pathogen. The agents of the disease may be morphologically indistinguishable from their environmental counterparts. The disease is one of the most prevalent implantation mycoses in the world and occurs particularly in the (sub)tropical regions of South America, Africa, and China [1]. The genus *Fonsecaea* comprises cryptic species related to the disease, known as *F. pedrosoi*, *F. monophora*, and *F. nubica* [2]. All these species are found in the environment and infect humans, and *F. pugnacius* is a recently described agent of CBM [3].

The invasive potential of *Fonsecaea* differs significantly among the species [3, 4]. Species such as *F. pedrosoi*, *F. nubica*, and *Cladophialophora carrionii* are exclusively associated with skin diseases caused by implantation, whereas *F. monophora* and *Cladophialophora bantiana* may also cause primary brain infection by implantation and inhalation, respectively. The single strain known as *F. pugnacius* behaved differently, as it causes a chronic condition of CBM that finally leads to secondary cerebritis by dissemination to the brain, despite the apparent intact immunity of the patient [3]. This type of dissemination and the apparent conversion to another invasive morphology has not been observed in *F. monophora* and *C. bantiana*, and the question arises whether this virulent ability is characteristic of *F. pugnacius* [2, 3].

The traditional methods for the identification of pathogenic fungi are based on morphological characteristics and antigen detection. However, these methods are time-consuming and have low specificity [5]. In general, rapid and accurate recognition of fungal pathogens to the species or strain level is essential for disease surveillance and implementation of disease management strategies, especially for deep and disseminated infections. The development of direct detection assays is challenging because fungal pathogens and opportunists may be present in human and natural environments at a very low density [6]. In this regard, Abliz et al. [7, 8] and Andrade et al. [9] reported certain specific primers based on the rDNA ITS spacer region of the CBM agents *F. pedrosoi* and *C. carrionii*. Likewise, padlock probes have been used in rolling circle amplification (RCA) to supplement the diagnosis of several mycotic diseases [10]. These methods demonstrated good reproducibility and sensitivity with CBM agents such as *F. monophora*, *F. nubica*, *F. pedrosoi*, *Cladophialophora,* and *Exophiala* [11–13]. Moreover, the method has also been applied for the diagnosis of infections caused by *Candida*, *Penicillium*, *Aspergillus*, *Scedosporium*, *Cryptococcus,* and *Trichophyton* [14–17].

The present study aims to propose new molecular tools for discriminating the *Fonsecaea* sibling species related to chromoblastomycosis. Novel specific primers using variations in the centromere microtubule-binding gene (*CBF5*) were developed for pathogenic members of the genus *Fonsecaea*. In addition, a probe based on the ITS sequence variations was proposed for a rapid and sensitive assay using RCA to detect *F. pugnacius*, a recently described species causing CBM that took a fatal turn.

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Materials and Methods

Fungal Strains and DNA Extraction

Fonsecaea and related species associated with CBM that were used as references are presented in Table 1. The following clinical strains were used for in vitro assay: *F. monophora* (CBS 269.37), *F. nubica* (CBS 269.64), *F. pedrosoi* (CBS 271.37), *F. pugnacius* (CBS 139214), *F. brasiliensis* (CMRP 2382), *F. multimorphosa* (CBS 980.96), *C. carrionii* (CBS 160.54), *Exophiala dermatitidis* (CMRP 2768), *Cyphellophora ludoviensis* (CMRP 1317), *Sporothrix brasiliensis* (CMRP 1171), *Histoplasma capsulatum* [18], *Candida albicans* (CMRP 3416); environmental strains: *Cladophialophora immunda* (CMRP 2693), *F. erecta* (CMRP 1635), and *Penicillium* sp. (CMRP 2968), *Cladosporium* sp. (CMRP 2959). For in vivo assay *F. pugnacius* (CBS 139214) was used. The strains were maintained at the Microbiological Cultures Collections of Paranaense Network/Taxonline (http://taxonline.bio.br/) and Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands (CBS). DNA was extracted as described previously [19].

GenBank Accession Numbers for the *CBF5* Gene and Species-Specific PCR Primers Design for *Fonsecaea* Species

In order to design specific primers, the gene for centromere/microtubule-binding protein cbf5 (CBF5) of the species was used. The sequences were deposited in GenBank (NCBI) as follows: *F. erecta* (XM_018834995), *F. nubica* (XM_022646888), *F. monophora* (XM_022652626), *F. pedrosoi* (XM_013424470), *F. pugnacius* (in progress), and *C. carrionii* (KB822705) (Table 2). The sequences were aligned using MAFFT v7 [20] (a multiple sequence alignment program) and were used for in silico screening. Based on the nucleotide polymorphisms, short informative regions with variations of 18–25 nucleotides were selected manually. The software Primer3 [21] was used to evaluate melting temperatures, %GC contents, dimer sequences, and mismatches in the sequences. Subsequently, the primers were evaluated using the Mfold software [22] for potential secondary structures, which could reduce the amplification efficiency. The primers that could generate products of different sizes for each species to facilitate visualization via electrophoresis were selected.

Table 1 Strains used as the template for in vitro and in vivo primers and padlock probe assays

Strain code	CBS code	Species	Source	Geograph
CMRP 3416	–	*C. albicans*	Environmental	Brazil/PR
CMRP2383	CBS 160.54^T	*C. carrionii*	Human	Australia
CMRP 2959	–	*C. halotolerans*	Environmental	Brazil/PR
CMRP1724	CBS 126869^T	*C. immunda*	Environmental	Brazil/MA
CMRP1317^T	–	*C. ludoviensis*	Human	Brazil/MA
CMRP2768	CBS 207.35^T	*E. dermatitidis*	Human	Japan
CMRP2382	CBS 119710^T	*F. brasiliensis*	Animal	Brazil/SE
CMRP1635	CBS 125763^T	*F. erecta*	Environmental	Brazil/MA
CMRP1655	CBS 269.37^T	*F. monophora*	Human	South Africa
CMRP1640	CBS 980.96^T	*F. multimorphosa*	Animal	Australia
CMRP1657	CBS 269.64^T	*F. nubica*	Human	West Cameroon
CMRP1654	CBS 271.37^T	*F. pedrosoi*	Human	South America
CMRP1343	CBS 139214^T	*F. pugnacius*	Human	Brazil/MA
24A	–	*H. capsulatum*	Human	Brazil/PR
CMRP1171	–	*S. brasiliensis*	Animal	Brazil/PR

T—type strain

Table 2 Primer species-specific FOMO, FONU, FOPE, and FOPU sequences and PCR conditions

	Sequences (5′–3′)	[ ]DNA	[ ]$MgCl_2$	Annealing	Amplicons	Access number
FOMO_F	TAAGCGCATATTGCGGGTAGAGAA	30 ng/µL	2 mM	60 °C	970pb	XM_022652626
FOMO_R	AAAGCGGATGAGAGGAAGTGG					
FONU_F	AAGTCCCCAAACGGAACACT	30 ng/µL	2,5 mM	58 °C	363pb	XM_022646888
FONU_R	CAGCAGCAATCCCAGGTGTA					
FOPE_F	AGCTCCAATTAACGTGACAATTC	15 ng/µL	2 mM	62 °C	500pb	XM_013424470
FOPE_R	TGACCCAGAAAACGCCAAGA					
FOPU_F	CGATTCCTGTTGGTTGCAC	30 ng/µL	2 mM	58 °C	351pb	In progress
FOPU_R	TGAGAGGAGGTCGTTGAAATAGT					

Nomenclature primers: FOMO—*F. monophora*, FONU—*F. nubica*, FOPE—*F. pedrosoi*, FOPU—*F. pugnacius*; the symbol [ ] represents the concentration of the indicated compound, F—forward, and R—reverse

Species-Specific PCR and Assay Sensitivity

The total DNA of *Fonsecaea* and the related species associated with CBM and other pathogens described to date in the literature was used as templates (Table 1) in the PCRs for each pair of specific oligonucleotides for the *CBF5* gene. The reactions were performed in a final volume of 25 µL, including 10 µL of Master Mix PCR buffer containing 2.0–2.5 mM $MgCl_2$, 400 mM dNTPs each, and 50 U/mL Taq polymerase, 5.6 µL of water, 1 µL of each of the forward and reverse oligonucleotides (20 pmol/µL), and 2 µL of target DNA. PCR was conducted as described by Rodrigues et al. [19]. The PCR conditions were as follows: an initial denaturing step of 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at the annealing temperature, and 1 min at 72 °C, followed by a final step of 10 min at 72 °C. The sensitivity of primers was established using 10-fold serial dilutions of PCR products, starting with 30 ng/µL and ending with 0.03 fg/µL [23].

Padlock Probe Design for *Fonsecaea pugnacius*

The design of padlock probes for *F. pugnacius* (FPgP) was based on the ITS sequence variations among *Fonsecaea* species (Table 3). ITS sequences of closely related species in *Capronia*, *Cladophialophora*, *Cyphellophora*, *Exophiala*, *Fonsecaea*, *Phialophora*, and *Rhinocladiella* were used as references (Table S1). The sequences of ITS regions were accessed from NCBI, aligned by MAFFT online [20], and adjusted manually using MEGA7 software [24] to identify single-nucleotide polymorphisms (SNPs). To optimize the joining efficiency to target DNAs, padlock probes were designed with minimal secondary structure and with T_m of the 5′ end probe binding arm near to or above the ligation temperature

Table 3 ITS sequences of *Fonsecaea* species used as reference for padlock probe design

Strain code	Species	SNPs region	Access number
CBS 119710	*Fonsecaea brasiliensis*	3′GGACGCGGCACG**A**TGGCGGTCCTCCGGCG5′	JN173784
CBS 125763	*Fonsecaea erecta*	3′GGACGCCGAG**G**TAGGCGATCCTCCGGCG5′	KC886414
CBS 269.37	*Fonsecaea monophora*	3′GGCAACGCCCGCA**T**TGAGCGGTCCTCCAGCG5′	AY857511
CBS 980.96	*Fonsecaea multimorphosa*	3′GGACGCCGGG**T**AGGCGGTCCTCCGGCG5′	JF267657
CBS 269.64	*Fonsecaea nubica*	3′GGCAACACCCATA**T**TAGGCGGTCCTCCGGCG5′	EU93859
CBS 271.37	*Fonsecaea pedrosoi*	3′GGTAACACCCGCA**T**TAGGCGGTCCTCCAGCG5′	AY366914
CBS 139214	*Fonsecaea pugnacius*	3′GAGAACACCCGTG**A**CAGGCGGTCCTCCAGCG5′	KR706553

Bold highlighted nucleotide indicates *Fonsecaea* ITS variation (SNPs) for all sequences used for padlock probe designing

(63 °C). Further, to improve their discriminative specificity, the 3′ end binding arm was designed with a T_m of 10–15 °C under ligation temperature. In order to evaluate FPgP specificity, in silico assays were performed using Primer-BLAST platform [25] and different databases implemented in the online tool (Ref-Seq mRNA, genome, non-redundant database) and a validated in-house reference database for filamentous fungi available at the Westerdijk Institute [10].

ITS Amplification and Padlock Probe Ligation

ITS amplicons of *Fonsecaea* and related species associated with CBM (Table 1) were produced with primers ITS1 (5′TCCGTAGGTGAACCTTGCGG3′) and ITS4 (5′TCCTCCGCTTATTGATATGC3′), with the following PCR conditions: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Amplification products were recognized by electrophoresis on 1.6% agarose gels. One microliter of ITS amplicon was mixed with 2 U of pfu DNA ligase (Epicentre Biotechnologies; Madison, WI, USA) and 0.1 μmol L^{-1} padlock probe in 20 mmol L^{-1} Tris–HCl (pH 7.5), 20 mmol L^{-1} KCl, 10 mmol L^{-1} $MgCl_2$, 0.1% IGEPAL, 0.01 mmol L^{-1} rATP, and 1 mmol L^{-1} DTT, with a total reaction volume of 10 μL. Ligation conditions were as follows: 94 °C for 5 min, followed by five cycles of 94 °C for 30 s, and 4 min of ligation at 63 °C. The ligation was visualized by electrophoresis on a 1% agarose gel. Exonucleolysis was used to remove the unligated padlock probes and template PCR products to reduce ligation-independent amplification events. This was performed in a 10 μL reaction mixture containing 10 U each of exonucleases I and III (New England Biolabs; Hitchin, UK) with incubation at 37 °C for 30 min, followed by 94 °C for 3 min to inactivate the exonuclease reaction [12].

Rolling Circle Amplification

Rolling circle amplification (RCA) was executed for all strains tested (Table 1) in a 12 μL reaction mixture containing 8 U Bst DNA polymerase (New England Biolabs), 400 μmol L^{-1} deoxynucleoside triphosphate mix, and 10 pmol of every RCA primer in distilled water with 2 μL ligation product as the template. RCA conditions were as follows: 65 °C for 60 min, followed by 85 °C for 2 min to inactivate the enzyme and stop the amplification. The results of RCA reactions were visualized by electrophoresis in a 2% agarose gel to validate the specificity of the probe–template binding. Positive responses revealed a ladderlike structure, whereas negative responses showed a clean background. The sensitivity of padlock probe was evaluated to ensure reliable amplification at low levels of target DNA by 10-fold serial dilutions of PCR products, starting with 2 ng/μL and ending with 0.02 pg/μL [7].

Fonsecaea pugnacius in Animal Infection Model

An infection model was developed in immunocompetent 6–8-week-old male BALB/c mice infected with *F. pugnacius* (CBS 139214). The animals were maintained under standard laboratory conditions with controlled temperature (23 ± 2 °C) and ad libitum access to food and water. This model was used to evaluate the efficiency of padlock probe in vivo to validate the padlock probe (FPgP) specificity in biological samples. The mice were divided into four groups of six animals each; three groups were inoculated with *F. pugnacius*, and one negative control group was inoculated with sterile phosphate-buffered saline (PBS). Subsequently, the animals were infected via intradermal (ID) and intraperitoneal (IP) routes with 100 μL of 1×10^6 *F. pugnacius* propagules or sterile PBS. The mice were monitored weekly for up to 21 days post-inoculation and were killed with CO_2 anesthesia in an appropriate chamber at 7, 14, and 21 days post-infection [8]. The samples of the brain, lungs, liver, kidneys, footpad, and blood were aseptically collected from the infected mice and evaluated with in vitro hybridization using *F. pugnacius* specific primers (FOPU) and the padlock probe (FPgP).

Results

The oligonucleotide primers used for *F. monophora* (FOMO), *F. nubica* (FONU), *F. pedrosoi* (FOPE), and *F. pugnacius* (FOPU) were specific to the target species studied. The primers amplified fragments of different sizes under varying reaction conditions of temperature and $MgCl_2$ concentration (Table 2).

PCRs of the specific primers generated amplicons of different sizes for each pathogenic species of *Fonsecaea*, i.e., 970 bp for *F. monophora*, 363 bp for *F. nubica*, 500 bp for *F. pedrosoi*, and 351 bp for *F. pugnacius*. Sensitivity assays demonstrated that the FOPU primers could detect *F. pugnacius* up to a DNA concentration of 30 ng/μL, whereas FOMO, FONU, and FOPE primers could detect concentrations only up to 3 ng/μL (Fig. 1).

The RCA padlock probe for *F. pugnacius* (FPgP) was designed based on the rDNA ITS region, as described by Najafzadeh et al. [21] for *F. pedrosoi*, *F. monophora*, and *F. nubica*. The padlock probe designed had 94 bp as follows: 5′P-cagggcggtcctccagcggatca**TGCTTCTTCGGTGCCCAT**tacgaggtgcggatagctac**CGCGCAGACACGATA**gtctagagaa cacccgtga3′, with annealing and melting temperatures of 47.4 °C and 62.4 °C, respectively (Table 4). The in silico assays demonstrated that the RCA padlock probe designed was specific to *F. pugnacius* (Fig. 2). Optimal results for RCA padlock probe specificity were obtained at a concentration of 10^{-5} μM using

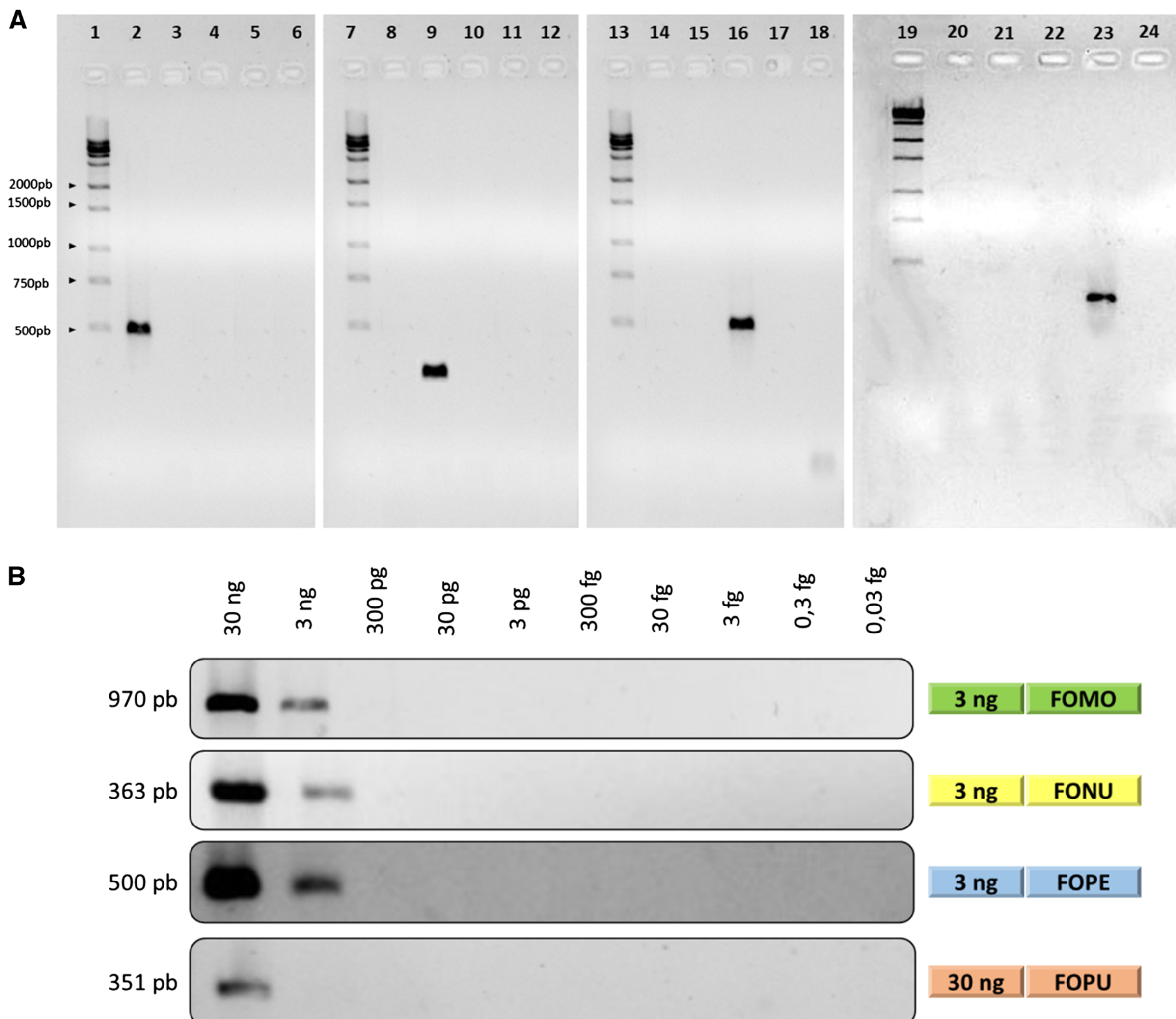

Fig. 1 Electrophoresis of the DNA samples amplified with specific primer sets. **a** PCRs with specific primers. Ladder (M), *F. monophora*, *F. nubica*, *F. pedrosoi*, *F. pugnacius* and negative controls shown in lanes 1–6, 7–12, 13–18, and 19–24, respectively, with the following specific positive reactions: *F. monophora*/FOMO (lane 2), *F. nubica*/FONU (lane 9), *F. pedrosoi*/FOPE (lane 16), and *F. pugnacius*/FOPU (lane 23). **b** Sensitivity assays for primers FOMO, FONU, FOPE, and FOPU using 10-fold serial dilutions from 30 ng to 0.03 fg. Nomenclature primers used: FOMO = *F. monophora*, FONU = *F. nubica*, FOPE = *F. pedrosoi*; FOPU = *F. pugnacius*

Table 4 Rolling circle amplification padlock probes used in this study

	Sequences (5′–3′)
RCA1	5′ ATGGGCACCGAAGAAGCA 3′
RCA2	5′ CGCGCAGACACGATA 3′
Probe FPgP	5′P—99CAGGGCGGTCCTCCAGCG83 gatcaTGCTTCTTCGGTGCCCATtacgaggtgcggatagctac CGCGCAGACACGATAgtcta 113GAGAACACCCGTGA100 3′

Nomenclature probe: FPgP—*F. pugnacious* padlock probe; the underlined sequences are the binding arm of padlock probe

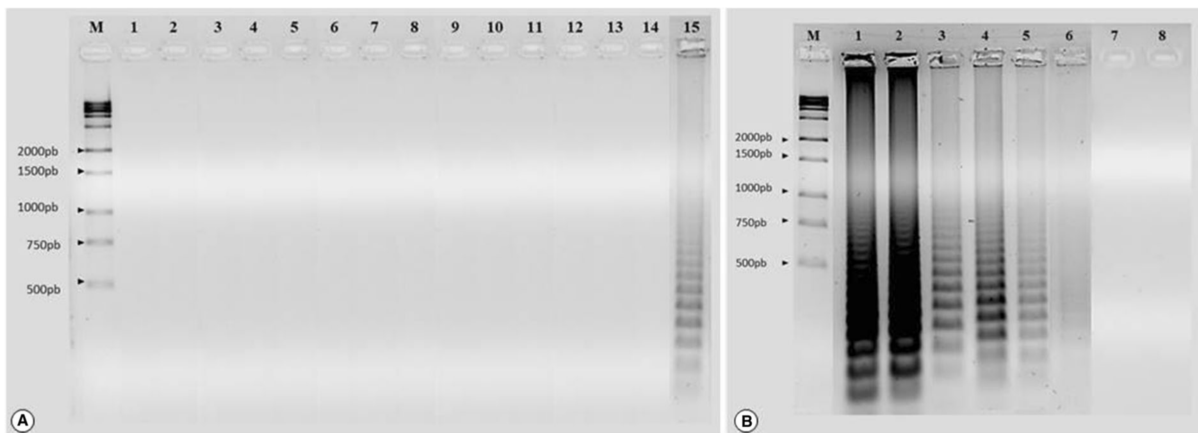

Fig. 2 Amplification profile of *Fonsecaea pugnacius* RCA padlock probe. **a** M. Ladder 1 kb; lanes 1–15 *Fonsecaea* sibling species and reference strains: 1. *F. erecta*; 2. *F. brasiliensis*; 3. *F. monophora*; 4. *F. multimorphosa*; 5. *F. nubica*; 6. *F. pedrosoi*; 7. *Cladophialophora immunda*; 8. *Cladosporium halotolerans*; 9. *Candida albicans*; 10. *Sporothrix brasiliensis*; 11. *Cladophialophora carrionii*; 12. *Cyphellophora ludoviensis*; 13. *Exophiala dermatitidis*; 14. *Histoplasma capsulatum*; 15. *F. pugnacius*. **b** Sensitivity assays of RCA probe FPgP using *F. pugnacius* ITS amplicons as shown in the lanes: M. Ladder 1 Kb; 1. 2.87×10^9; 2. 2.87×10^8; 3. 2.87×10^7; 4. 2.87×10^6; 5. 2.87×10^5; 6. 2.8×10^4; 7. 2.8×10^3; 8. 2.8×10^2

PCR products diluted at 2 ng/µL (Fig. 2a, b). The RCA reaction amplification limit was observed at a dilution of 10^{-5} PCR product (0.03 pg/µL), representing 10^9 DNA copies according to Avogadro's number (Fig. 2c).

Concerning the animal model infection by *F. pugnacius*, none of the infected animals presented neurological symptoms. The BALB/c mice infected via the ID route presented necrosis and swelling in the footpad tissues (Fig. 3a). The fungus was recovered only from the lungs (Fig. 3b). The DNA tissue samples (lungs, kidneys, liver, brain, footpad, and blood) of all animals tested were evaluated in vitro using the RCA probe and *CBF5* primers. All collected samples reported positive results for DNA viability using the β-actin gene amplification (Fig. 3c). In vitro assays with the RCA probe demonstrated positive results for the brain, lungs, and footpad samples from the mice (Fig. 3d). It was not possible to detect the presence of the *F. pugnacius* DNA in biological samples using *CBF5* primers, which can be explained by the low concentration of DNA in the samples.

Discussion

Chromoblastomycosis (CBM) is considered to be the second most prevalent fungal implantation mycosis worldwide. It is characterized by chronic cutaneous and subcutaneous lesions that develop at the site of previous transcutaneous trauma. The causative agents of CBM are found in decaying plant debris, including

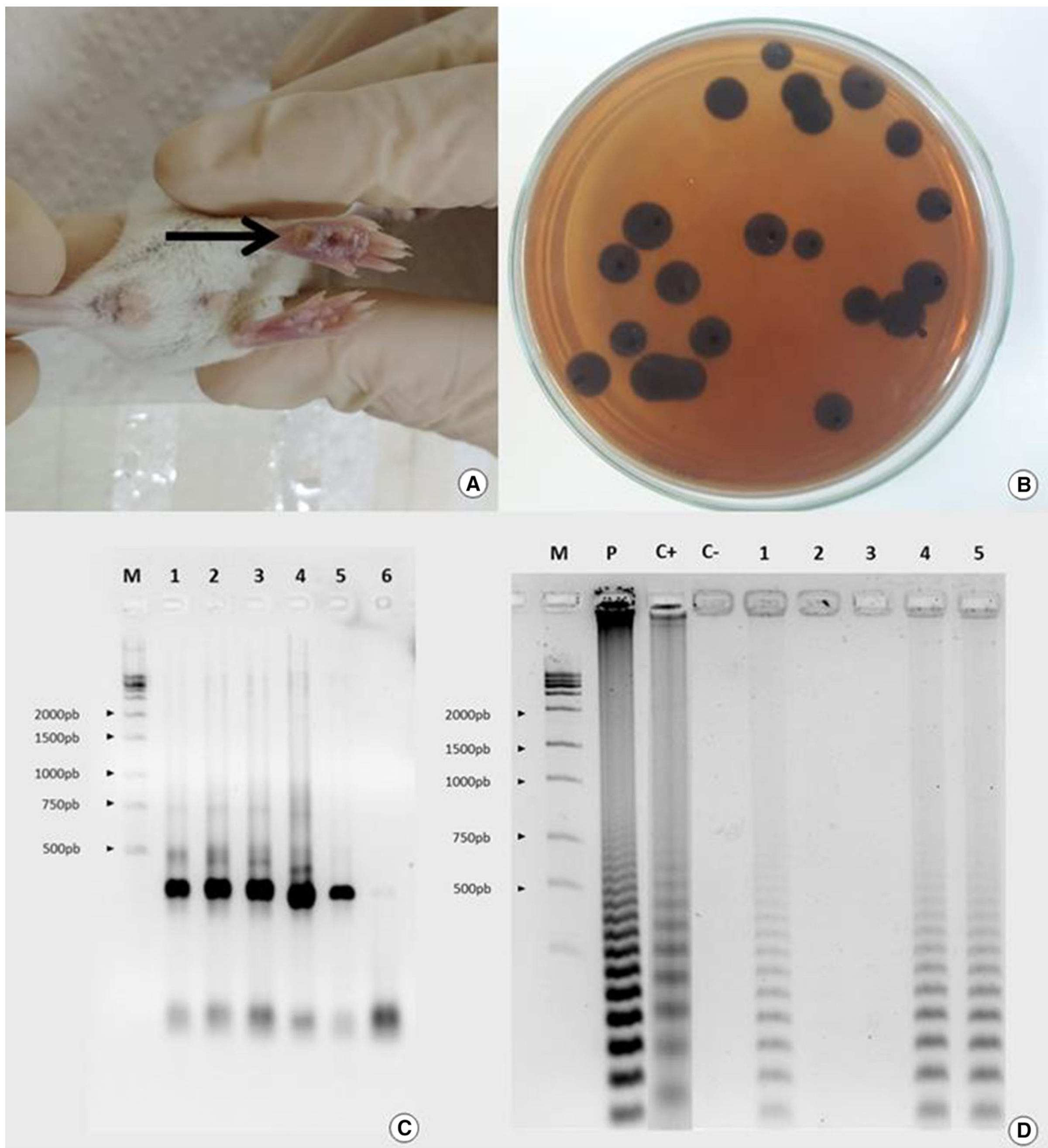

Fig. 3 In vitro assays with the rolling circle amplifications probe to detect *F. pugnacius* in biological samples. **a** Indicator showing footpad necrosis lesion and swelling in intradermal pathway in the infected BALB/c mice; **b** re-isolated *F. pugnacius* colony from BALB/c lung; **c** qualitative evaluation, with β-actin primers, of genomic DNA extracted from mice samples being demonstrated in lanes 1–6: M. Ladder 1 Kb; 1. F8 brain; 2. F8 kidney; 3. F8 liver; 4. F8 lung; 5. F8 footpad; 6. F8 blank; **d** specificity RCA padlock probe FPgP being demonstrated in lanes: M. Ladder 1 Kb; P. *F. pugnacius* DNA; C +. *F. pugnacius* DNA mixed with BALB/c DNA; C-. *F. nubica* DNA mixed with BALB/c DNA; 1. F8 brain; 2. F8 kidney; 3. F8 liver; 4. F8 lung; 5. F8 footpad

wood and soil microbiota. Main causative agents are *F. pedrosoi*, *F. monophora*, and *C. carrionii* [1, 2]. Recently, a new causative agent, *F. pugnacius,* was described that resulted in cerebral dissemination, and thus was a variation to the primary cerebral cases caused by *F. monophora* [4]. However, infection sources and routes, and clinical evolution have not been entirely elucidated, partly owing to the lack of detection markers.

In general, diagnosis of CBM involves clinical and laboratory evaluation of tissue samples by direct microscopy to visualize muriform cells, followed by identification by sequencing [9, 26], usually involving the ITS fungal barcoding region [27, 28]. Moreover, serological tests have been developed to aid in CBM diagnosis although these are not routinely applied because of relatively low sensitivity and specificity, making new techniques more rapid and cost-effective for the diagnosis of CBM and other diseases caused by the black yeast-like fungi [29].

Currently, non-culture methods are being used to improve the sensitivity and specificity of mycological diagnosis [29]. Several studies have been published on the identification of specific CBM agents [12, 14, 30]. Although the ITS region efficiently detects specific causative agents, it is not always recommended because molecular identification may be hampered by sequence variability in the ITS domain caused by difficult-to-sequence homopolymeric regions [25]. In the present study, we developed primers to partially amplify the *CBF5* gene encoding a small nucleolar ribonucleoprotein required for the correct processing of rRNA. Kendall et al. [31] reported that *CBF5* gene mutation is relates to RNA-binding and pseudouridine (ψ) synthase activity. This mutation was considered as an evolutionarily conserved mutation in *Saccharomyces cerevisiae* [32].

Pseudouridine (ψ), a C-glycoside isomer of uridine, is the most common single-nucleotide modification found in functional RNA and often appears in highly conserved regions of homologous RNAs [33]. Several lines of evidence suggest an important role for this base modification in ribosome activity [34]. It has been hypothesized that ψ is an essential component of proper RNA folding and function [33]. In addition, this protein has been associated with pathogenicity and virulence in several microorganisms. When incorporated into RNA, ψ alters the RNA structure, increases the base stacking, improves base-pairing, and rigidifies the sugar–phosphate backbone. Earlier studies have also linked ψ, either directly or indirectly, to several human diseases [35].

We used the single mutation observed in this region to develop primers. The specific primers demonstrated specificity for the target variable region among the clinically cryptic *Fonsecaea* species. Moreover, the primers demonstrated high specificity in the regions selected for primer designing. No cross-reactions were observed with any of the 15 reference strains analyzed in the comparison (Fig. 2a). Sensitivity assays reported specific primers to detect low DNA concentrations (Fig. 2b). The novel molecular markers for *Fonsecaea* agents of CBM are very accurate, as demonstrated by 100% specificity of unambiguous profiles using gel electrophoresis.

The advantage of isothermal amplification methods is that these systems do not require a thermal cycler to produce, but just a simple platform, such as a heating block or water bath. Detection by loop-mediated isothermal amplification (LAMP) or RCA has become increasingly popular for SNP detection to rapidly identify the fungus in a variety of samples [6]. RCA has been proven to be useful for ultra-high specificity reactions. In this isothermal enzymatic process, a short DNA or RNA primer is amplified to form a long, single-stranded DNA fragment using a circular DNA template and special DNA polymerases. The method is particularly useful to differentiate closely related species or genotypes within species, which may differ by a single SNP only. The RCA padlock probe detects SNPs by creating a circular form to bind to the target DNA and to efficiently decrease the risk of nonspecific sequence replication. Accordingly, RCA is more potent compared to its linear alternative, yielding 10^9 or more copies of a circular sequence within an hour [36].

In general, applicability of RCA padlock probe can range from bioprospecting, biotechnological potential [37], and biosensing [38] to diagnostic of viral [39] and bacterial [40] infections. Besides, some studies have already distinguished several CBM agents of the genus *F. pedrosoi*, *F. nubica* [10], and *C. carrionii* [13] and associated CBM species such as *Exophiala* [11, 12] and *Cyphellophora* [43]. Several studies have reported the application of the RCA padlock probe to detect species-specific fungal diseases with great public impact, e.g., in opportunistic infections caused by species of *Candida* [16], *Aspergillus* [16], and

Cryptococcus [7, 41]; agents associated with systemic infections such as *Histoplasma* [18], *Fusarium* [42] and *Talaromyces marneffei* [15]; and dermatophytoses caused by species of the genus *Trichophyton* [17].

The RCA padlock probe (FPgP) in the present study demonstrated greater sensitivity than previously reported probes developed for *Fonsecaea* species [13, 15]. Given the specificity of the RCA padlock probes, RCA was judged to be suitable for *Fonsecaea* detection and species differentiation without sequencing in a wide range of biological samples. The establishment of the test is relatively expensive; however, with high-throughput applications, the performance of testing will be rapid and cost-effective. Moreover, the developed specific primers could be useful for routine testing in clinical laboratories that screen a large number of samples.

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Author Contributions GXS, RRG, and VAV performed the experiments. CMPSA and SGM strains offered epidemiology data and substantial contributions to the work. RRG, FFC, AB, MFV, MJN, JS, and ACRL contributed to genomic data and bioinformatics analysis. BJFSL, BSS, AB, IRC, and KZ helped with animal maintenance and histopathology analysis. GSH, RRG, and VAV prepared and critically revised the manuscript, and conceived and revised the paper. GXS, RRG, GZC, KZ, MFV, and VAV contributed to preparation, creation, and/or presentation of the tables and figures. GXS, GSH, and VAV conceived and designed the work and wrote the manuscript.

Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed (Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Parana (CEUA/BIO UFPR) with certified number 1179).

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