Specific Oligonucleotide Primers for Identification of *Cladophialophora carrionii*, a Causative Agent of Chromoblastomycosis

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***Cladophialophora carrionii* is one of the relatively common causative agents of chromoblastomycosis. We have developed the specific oligonucleotide primer set based on the internal transcribed spacer regions of ribosomal DNA for the rapid identification of this pathogen. PCR with this primer set amplified a 362-bp amplicon from *C. carrionii* strains. From other relevant dematiaceous species, including medically important dematiaceous fungi, such as *Fonsecaea pedrosoi*, *Phialophora verrucosa*, and *Exophiala dermatitidis*, and eight species of medically important yeasts, such as *Candida albicans* and *Cryptococcus neoformans* var. *neoformans*, the primer set did not produce any amplicon. PCR with this primer set may be a useful tool for the identification of *C. carrionii*.**

Cladophialophora carrionii (synonym, *Cladophialophora ajel-loi*) is one of the relatively common causative agents of chromoblastomycosis, a chronic mycosis of skin and subcutaneous tissue. The most common appearance of such lesions is as verrucous plaques or nodules (13). This fungus is found primarily in arid areas of South America, Africa, and Australia (14) and was first described by Trejos in 1954 under the name *Cladosporium carrionii* (22). Recent taxonomic studies have moved pathogenic species of the genus *Cladosporium*, including *C. carrionii*, to the genus *Cladophialophora* (7). *C. carrionii* is considered the most important pathogenic species in this genus due to the many cases of illness caused by this fungus worldwide (4, 10, 12, 16, 19, 20, 25). Bronchitis in response to the allergenic mechanism of this fungus has also been reported (5). Infections caused by *C. carrionii* are chronic and appear as spreading mycoses of the skin, subcutaneous tissues, and nail (4, 13).

Morphological and physiological characteristics have been used predominantly for the identification of this fungus until recently. However, the similar morphological features of *Cladophialophora* species have sometimes caused misidentification of the fungus and also indicate the difficulties of differentiating species on the basis of these characteristics. In addition, cultivation for 2 or 3 weeks is needed to get a suitable amount of conidia production. The physiological profiles of pathogenic dematiaceous fungi were considered to have limited diagnostic value for characterizing these species (23).

In recent years, PCR-based methods have been used to identify pathogenic fungi, including medically important dematiaceous fungi (1, 2, 15, 18, 21). In the present study, we developed a rapid PCR-based approach for the detection of the medically important dematiaceous species *C. carrionii*.

Eighty-two strains of 54 species (Table 1), including medically relevant dematiaceous species and pathogenic yeasts,

were examined. DNAs were prepared according to the method of Makimura et al. (17). Briefly, approximately 50 mg of fungal elements was suspended in 600 μ l of extraction buffer (200 mM Tris-HCl [pH 7.5], 25 mM EDTA, 0.5% [wt/vol] sodium dodecyl sulfate, 250 mM NaCl) and then incubated at 100°C for 15 min. The solution was extracted with phenol-chloroform-isoamyl alcohol (25:24:1; vol/vol/vol). DNA was precipitated with cold isopropanol (–20°C). The internal transcribed spacer (ITS) regions were amplified with the following universal primers: ITS5, 5'-GGAAGTAAAGTCGTAACAAGG-3', and ITS4, 5'-TCCTCCGCTTATTGATATGC-3' (24). Direct sequencing of PCR products corresponding to the ITS1-5.8S-ITS2 region of the ribosomal DNA (rDNA) was done with an ABI PRISM 3100 sequencer after labeling with BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, Calif.). Two external primers, ITS5 and ITS4, and two internal primers, ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') and ITS3 (5'-GCATCGATGAAGAACGCAGC-3') (24), were used for sequencing. Fourteen strains of *C. carrionii*, three strains of *Fonsecaea pedrosoi*, a strain of *Fonsecaea compacta*, and strains of *Cladophialophora arxii*, *Cladophialophora bantiana*, *Cladophialophora boppi*, *Cladophialophora emmonsii*, *Cladophialophora devriesii*, *Cladophialophora minourae*, *Phialophora verrucosa*, *Exophiala dermatitidis*, *Hortaea werneckii*, *Rhinocladiella aquaspersa*, and *Rhinocladiella atrovirens* were sequenced. The data obtained from the sequencing are shown in Table 1. In addition to our data, the sequences of ITS regions from other dematiaceous fungal species and medically important yeasts were obtained from GenBank and aligned with CLUSTAL W (version 1.6) for primer design (11). We designed a primer set, 5'-TAAACCTCAT GTTGCTTCG-3' (Car-F) and 5'-TCGAGAM(A/C)CACTC GACCAA-3' (Car-R), based on the sequence of the ITS regions to amplify DNAs from *C. carrionii*. The primers were synthesized by and purchased from Sigma Genosys Japan (Tokyo, Japan). Each PCR (25 μ l) consisted of 2.5 μ l of template DNA, 2.5 μ l (2 pmol) of each primer, 2 μ l (2.5 mM) of dNTP mixture (Nippon Gene, Tokyo, Japan), 0.125 μ l (5 U/ μ l) of *Taq* polymerase (Nippon Gene), and 2.5 μ l of 10 $\times$ reaction

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TABLE 1. Strains examined in this study and PCR results

Species	Strain	Source ^a	Accession no.	PCR product ^b
<i>Cladophialophora carrionii</i>	IFM 4808 ^{T,c}	ATCC 16264	AB109177	+
	IFM 4805 ^c	ATCC 44535	AB087204	+
	IFM 4810 ^c	DCU 300	AB109170	+
	IFM 4811 ^c	DCU 301	AB109178	+
	IFM 4812 ^c	DCU 302	AB109168	+
	IFM 4813	DCU 303		+
	IFM 4814	DCU 692		+
	IFM 4815	DCU 693		+
	IFM 4985 ^c	patient, China	AB109179	+
	IFM 4986 ^c	patient, China	AB109180	+
	IFM 41444 ^c	UNEFM/SR3	AB109169	+
	IFM 41446 ^c	DCU 606	AB109171	+
	IFM 41641 ^c	BMU 237	AB109172	+
	IFM 41644	BMU 2127		+
	IFM 41645	BMU 2484		+
	IFM 41646	BMU 2557		+
	IFM 41650 ^c	BMU 5302	AB109173	+
	IFM 41651 ^c	BMU 5438	AB109174	+
	IFM 41807 ^c	Venezuela	AB109175	+
	IFM 41814 ^c	Venezuela	AB109176	+
<i>Cladophialophora arxii</i>	IFM 52022 ^{T,c}	CBS 306.94	AB109181	—
<i>Cladophialophora bantiana</i>	IFM 46165 ^c	CBS 173.52	AB091211	—
	IFM 41433	DCU 607		—
	IFM 41434	DCU 611		—
<i>Cladophialophora boppii</i>	IFM 52024 ^{T,c}	CBS 126.86	AB109182	—
<i>Cladophialophora emmonsii</i>	IFM 52025 ^{T,c}	CBS 979.96	AB109184	—
<i>Cladophialophora devriesii</i>	IFM 51369 ^{T,c}	CBS 147.84	AB091212	—
<i>Cladophialophora minourae</i>	IFM 4818 ^c	DCU 428	AB091213	—
<i>Cladosporium cladosporioides</i>	IFM 41447	IFO 6368		—
<i>Cladosporium colocasiae</i>	IFM 51371	CBS 386.64		—
<i>Cladosporium coralloides</i>	IFM 41451	IFO 6536		—
<i>Cladosporium elatum</i>	IFM 41452	IFO 6372		—
<i>Cladosporium fulvum</i>	IFM 40703	IAM 5006		—
<i>Cladosporium herbarum</i>	IFM 41454	TMI		—
<i>Cladosporium minusculum</i>	IFM 51370	URM 721		—
<i>Cladosporium sphaerospermum</i>	IFM 41453	IFO 4458		—
<i>Cladosporium variabile</i>	IFM 41458	IFO 6378		—
<i>Fonsecaea pedrosoi</i>	IFM 4856 ^c	DCU 677	AB091203	—
	IFM 4887 ^{T,c}	CBS 271.37	AB087202	—
	IFM 4889 ^c	ATCC 44356	AB091204	—
<i>Fonsecaea compacta</i>	IFM 4886 ^c	KUM 911	AB091208	—
<i>Phialophora verrucosa</i>	IFM 4928 ^c	ATCC 38561	AB087203	—
	IFM 41710	corn, China		—
	IFM 41871	soil, Colombia		—
<i>Phialophora alba</i>	IFM 51363	IFO 31973		—
<i>Phialophora americana</i>	IFM 51361	CBS 273.37		—
<i>Phialophora atrovirens</i>	IFM 51364	IFO 6793		—
<i>Phialophora bubakii</i>	IFM 51365	IFO 6794		—
<i>Phialophora cinerescens</i>	IFM 51366	IFO 6849		—
<i>Phialophora fastigiata</i>	IFM 41577	IFO 6856		—
<i>Phialophora heteromorpha</i>	IFM 41578	IFO 6878		—
<i>Phialophora lagerbergii</i>	IFM 51367	IFO 8576		—
<i>Phialophora melinii</i>	IFM 51362	CBS 268.33		—
<i>Phialophora oxyspora</i>	IFM 51368	URM 2904		—
<i>Phialophora repens</i>	IFM 4925	CBS 423.73		—
<i>Phialophora richardsiae</i>	IFM 4926	KUM 1681		—
<i>Phaeoacremonium parasiticum</i>	IFM 4924	KUM 1827		—
<i>Lecythophora hoffmannii</i>	IFM 4922	CBS 245.38		—
<i>Lecythophora mutabilis</i>	IFM 4923	ATCC 26223		—
<i>Exophiala alcalophila</i>	IFM 41654	bark, China		—
<i>Exophiala dermatitidis</i>	IFM 41479 ^{T,c}	CBS 207.35	AB087205	—
	IFM 41818	Venezuela		—
	IFM 41828	soil, Brazil		—
<i>Exophiala jeanselmei</i>	IFM 41691	BMU 2756		—
	IFM 45989	patient		—
<i>Exophiala moniliae</i>	IFM 41500 ^T	CBS 520.76		—
<i>Exophiala spinifera</i>	IFM 4883 ^T	ATCC 18218		—
<i>Hortaea werneckii</i>	IFM 51373 ^c	URM 704	AB087201	—

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TABLE 1—Continued

Species	Strain	Source ^a	Accession no.	PCR product ^b
<i>Hormoconis resinae</i>	IFM 51372	IFO 8588		—
<i>Alternaria alternata</i>	IFM 41348	TIMM 1289		—
<i>Aureobasidium pullulans</i>	IFM 4802	ATCC 15233		—
<i>Capronia hanliniana</i>	IFM 52023 ^c	CBS 588.93	AB109183	—
<i>Rhinocladiella aquaspersa</i>	IFM 4930 ^c	CBS 313.73	AB091214	—
<i>Rhinocladiella atrovirens</i>	IFM 4931 ^{T,c}	CBS 317.33	AB091215	—
<i>Candida albicans</i>	IFM 40214	ATCC 90029		—
<i>Candida dublinensis</i>	IFM 48313	CBS 7987		—
<i>Candida glabrata</i>	IFM 46888	Vagina		—
<i>Candida parapsilosis</i>	IFM 46863	CUH 2565		—
<i>Candida tropicalis</i>	IFM 46816 ^T	CBS 94		—
<i>Cryptococcus neoformans</i> var. <i>neoformans</i>	IFM 40216	ATCC 90113		—
<i>Malassezia furfur</i>	IFM 48585	CBS 7019		—
<i>Trichosporon asahii</i> var. <i>asahii</i>	IFM 48429 ^T	CBS 2479		—

^a Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; BMU, Department of Dermatology, Beijing Medical University, Beijing, China; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; CUH, Department of Laboratory Medicine, School of Medicine, Chiba University, Chiba, Japan; DCU, Department of Dermatology, School of Medicine, Chiba University, Chiba, Japan; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; IFM, Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan; IFO, Institute for Fermentation, Osaka, Japan; KUM, Department of Dermatology, School of Medicine, Kanazawa University, Kanazawa, Ishikawa, Japan; MTU, Department of Bacteriology, Faculty of Medicine, University of Tokyo, Tokyo, Japan; TIMM, Research Center for Medical Mycology, Teikyo University, Tokyo, Japan; TMI, Tottori Mycological Institute, Tottori, Japan; UNEFM, Universidade Nacional Experimental Francisco de Miranda, Coro, Falcon, Venezuela; URM, Department of Mycology, Federal University of Pernambuco, Recife, PE, Brazil.

^b +, obtained; —, not obtained.

^c Strains newly sequenced in this study. ^T, type strain.

buffer (Nippon Gene). Amplification was performed with a PCR Thermal Cycler MP (TaKaRa Shuzo Co., Ltd., Tokyo, Japan) under the following conditions: 1 cycle at 95°C for 4 min, followed by 30 cycles at 94°C for 1 min, at 62°C for 1.5 min, and at 72°C for 1.5 min, with a final extension at 72°C for 10 min. After thermal cycling, 2 µl of each amplified product was separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and visualized with UV light.

Our ITS sequence data for 14 strains of *C. carrionii* revealed that there were five base pairs in intraspecies nucleotide substitutions. The reverse primer (Car-R) was constructed at the location that showed high variation in interspecies and a nucleotide substitution in intraspecies of *C. carrionii*. The specificity of our newly designed primer set is summarized in Table 1. This primer set amplified the expected 362-bp fragment only from DNAs extracted from the 20 strains of *C. carrionii* (Fig. 1). In order to verify the specificity of the primers, we performed PCR analysis of multiple strains of medically important dematiaceous species such as *F. pedrosoi*, *P. verrucosa*, *E. dermatitidis*, *Exophiala jeanselmei*, and *C. bantiana*. We also used this primer set for PCR analysis for species of the genera *Cladophialophora*, *Fonsecaea*, *Phialophora*, *Cladosporium*, *Ex-*

ophiala, *Rhinocladiella*, *Alternaria*, *Aureobasidium*, *Hortaea*, *Hormoconis*, *Capronia*, and eight species of medically important yeasts, including *Candida albicans* and *Cryptococcus neoformans* var. *neoformans*. None of these tested species yielded any amplification product (data not shown). PCR bands were obtained within 7 h from initiating the DNA extractions: 3 h for preparation of the template DNAs, 3 h for PCR, and 1 h for electrophoresis, staining, and detecting the band profiles.

Recent taxonomic studies have shown that the genus *Cladophialophora* consists of eight species: *C. arxii*, *C. bantiana*, *C. boppi*, *C. carrionii*, *C. devriesi*, *C. emmonsii*, *C. minourae*, and *C. modesta* (7, 8). Among these eight species, *C. carrionii* is the most common pathogen in chromoblastomycosis. *C. carrionii* has traditionally been identified according to morphological and physiological characteristics and by antigen detection methods (7, 9); however, these methods are time consuming and require technical experience. *C. bantiana* is also a common pathogenic species that has been isolated as an etiologic agent of brain abscesses and that has been shown to have neurotropic properties. Because *C. carrionii* and *C. bantiana* are morphologically very similar, differentiation according to morphological characteristics is difficult. Analysis of

FIG. 1. Agarose gel electrophoresis of PCR products of *C. carrionii*. Lane 1, 100-bp ladder; lanes 2 to 21, *C. carrionii*.

ribosomal DNAs by restriction fragment length polymorphisms revealed that *C. carrionii* and *C. bantiana* have DNA restriction patterns that are identical to those of most enzymes (6). Our PCR assay offers a reliable method to discriminate these species.

The rapid and accurate identification of causative agents is necessary in medicine. The ITS region located between the 18S and 28S ribosomal DNAs is subdivided into two spacers, ITS1 and ITS2, separated by the 5.8S conserved region. ITS regions are thought to have higher rates of mutation than those of 18S, 5.8S, and 28S rDNAs. Therefore, ITS regions are being used to design specific PCR primers for the rapid identification of human fungal pathogens, including dematiaceous fungi. The ITS sequences of 14 strains of *C. carrionii* revealed that they have intraspecies variation. However, the interspecies variation of *C. carrionii* with other dematiaceous fungi is more remarkable. Both of the primers Car-F and Car-R are constructed from the regions with high interspecies variation among all species examined. Furthermore, we designed the reverse primer (Car-R) to allow the possible substitution of nucleotide A or C at the position of a nucleotide substitution in the region originally used for the primer design. The design may have contributed to our getting the expected results in the present study. This suggests that primers designed with possible alternative nucleotides may be useful for enhancing the specificity as well as the efficiency of PCR assays. Indeed, the assay using our newly designed primer set was enhanced to yield the expected band for *C. carrionii* but not for any of the other species examined. The D1/D2 domain sequence analysis of rDNA has been useful for identifying pathogenic dematiaceous fungi at the species level (3). However, such analysis requires expensive equipment and reagents and takes longer than the PCR identification method; the PCR with our designed primer set gives a result within 7 h. As a matter of course, this assay is a more rapid and accurate method than morphological and physiological identification methods.

In conclusion, we developed a species-specific PCR strategy for identification of a dematiaceous pathogen, *C. carrionii*. The species specificity of our PCR assay was verified. A PCR analysis with this primer set may be a useful tool for the identification of *C. carrionii*, a causative agent of chromoblastomycosis.

Nucleotide sequence accession numbers. The sequences determined as a result of the present study were registered in the DNA Data Bank of Japan under the accession numbers shown in Table 1.

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