

Short Report

Reexamination of *Coccidioides* spp. Reserved in the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Based on a Multiple Gene Analysis

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Abstract

The Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University is the only organization in Japan to possess a series isolates of *Coccidioides* spp., which are the most virulent pathogenic fungi and which are treated as biosafety level 3 microorganisms. Recently, the genus *Coccidioides* has been classified into two species, *C. immitis* and *C. posadasii*, based on their endemic areas and genotyping; the former species is endemic to the state of California, and the latter is endemic to other parts of North and South America. We reevaluated 19 isolates of *Coccidioides immitis* stored in our center using a multiple gene analysis. Five isolates were identified as *C. immitis* and 14 as *C. posadasii*. Their sequence information in GenBank will help to identify the two genospecies of *Coccidioides* spp.

Key words: *Coccidioides immitis*, *Coccidioides posadasii*, multiple gene analysis

Introduction

Coccidioides spp., the causative agents of coccidioidomycosis are the most virulent among pathogenic fungi. Coccidioidomycosis is treated as an imported fungal infection in Japan because almost all the patients were assumed to have gotten the infection in the United States or Mexico. It causes pneumonia and sometimes fatal systemic infection in healthy subjects, and is considered as a category-4 infection by the Ministry of Health, Labor, and Welfare of Japan. Clinicians who see patients with category-4 infections are required to report them to a local public health center¹⁾.

The habitats of *Coccidioides* spp. are desert areas in North and South America. In Arizona, an endemic area, more than 2,000 new cases per year are reported and more than 100,000 individuals are infected with the fungus without

symptoms²⁾. In Japan, more than 45 cases had been recorded as of November 2005 (<http://www.pf.chiba-u.ac.jp/>). Most of the Japanese patients were infected in endemic areas to which they had traveled or resided, except for 1 case caused by contact with crude cotton imported from the United States. We could not determine the place of infection for some patients who had traveled to several endemic areas in The United States and Mexico.

The disease is caused by inhalation of arthroconidia of the fungi. The fungi grow as mycelia in spring and produce arthroconidia. They are easily spread into the air by the wind, by new construction activities, and by excavations. The clinical isolates of *Coccidioides* spp. on slants or plates are also easily diffused into the air and are apt to cause infections in laboratories. The culturing and identification of the fungi by mycological examination require at least 2 weeks for experts with special training¹⁾.

C. immitis has been treated as a single species as the causative agent of coccidioidomycosis. Since 2002, *C. immitis* has been classified into

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two species, *C. immitis* and *C. posadasii*, based on the endemic areas and genotypes; the former species is endemic to the state of California, while the latter is endemic to other parts of North and South America³⁾. In 1997, Koufopanou *et al.* tried to reclassify the species into two geographic types, a California type and an outside-California type, based on the combination of five gene analogues: chitin synthase, dioxygenase (DO), orotidine decarboxylase, serine proteinase (SP), and chitinase (CT)⁴⁾. Fisher *et al.* created the two genospecies taxa based on the multiple gene analysis of microsatellites³⁾. They also suggested a convenient method for the differentiation of *Coccidioides* spp. by a multiple gene analysis using a combination of three gene sequences: DO, SP, and CT⁵⁾. The sequence data obtained from their investigations were not released to GenBank completely, because of patenting and bioterrorism crisis-management programs.

The present study aimed to reevaluate 19 strains formerly identified as *C. immitis* in our center following the criteria for genospecies proposed by Fisher *et al.* in 2002, to release their sequence data for public convenience, and to clarify the place of infection for Japanese patients. In addition to the genes used by Fisher *et al.*³⁾, we analyzed the internal transcribed spacer (ITS) 1-5.8S-ITS 2 and the D1/D2 regions of ribosomal RNA genes that are generally used as phylogenetic markers^{6, 7)} as well as a partial sequence of the urease gene (URE), which codes the virulence factor of pathogenic fungi⁸⁾. The present study also tried

to find specific genes to classify *Coccidioides* spp. from the genes named above.

Materials and Methods

Coccidioides spp. strains examined are shown in Table 1. They were formerly identified as *C. immitis* by the morphology observed through the test tubes, by the patient's history, or by identifications in the original country.

The fungi were cultured at 37°C for 2 weeks on potato dextrose agar (Difco, Franklin Lakes, NJ, USA) slants with a silicon plug. Then, a needle with a syringe containing 85% ethanol was pierced the plug and ethanol was slowly injected until the plug was immersed. The point of needle insertion into the plug was covered with a mass of cotton soaked with 70% ethanol. The fungal mass was fixed by a final concentration of approximately 70% ethanol for 48 hours at room temperature. A loop of fungal mass was spread on a PDA plate and cultured at 37°C for 7 days to check its survival. The next step was started after we confirmed that the fungus had no sprouts. The above procedures were done in a special laboratory for level 3 biohazard pathogens.

DNA was extracted with a DEXPAT[®] Kit (TaKaRa, Ohtsu, Japan) with a modification of the manufacturer's protocol. Approximately 100 μ l of the fungal mass was transferred to a sterilized microtube (1.5 ml), homogenized with 0.5 ml of DEXPAT[®] solution by a plastic pestle. The mixture was incubated at 100°C for 10 min and centrifuged at 12,000 rpm (13,201 g) for another 10 min. The supernatant was used

Table 1. *Coccidioides* spp. isolates and their accession numbers

Isolate		Gene (length)							
IFM No.	Strain	Identification	Origin	Country	Deoxygenase (636)	Serine proteinase (646)	Chitinase (487)	Urease (536)	rRNA (1251)
4935	Nagoya	<i>C. posadasii</i>	Human case	Japan	AB232864	AB232726	AB232745	AB232707	AB232883
4945	Ohashi	<i>C. posadasii</i>	Human case	Japan	AB232865	AB232727	AB232746	AB232708	AB232884
45809	Silveira (SAP2)	<i>C. posadasii</i>	Animal passage 11	USA	AB232866	AB232728	AB232747	AB232709	AB232885
45810	Silveira (SAP3)	<i>C. posadasii</i>	Animal passage 111	USA	AB232867	AB232729	AB232748	AB232710	AB232886
45811	Arizona	<i>C. posadasii</i>	Human case	USA	AB232868	AB232730	AB232749	AB232711	AB232887
45812	San Antonio	<i>C. posadasii</i>	Human case	USA	AB232869	AB232731	AB232750	AB232712	AB232888
45813	New York	<i>C. posadasii</i>	Human case	USA	AB232870	AB232732	AB232751	AB232713	AB232889
45815	91-48	<i>C. immitis</i>	Human case	USA	AB232871	AB232733	AB232752	AB232714	AB232890
45816	91-153	<i>C. immitis</i>	Human case	USA	AB232872	AB232734	AB232753	AB232715	AB232891
45817	Nicols	<i>C. posadasii</i>	Human case	USA	AB232873	AB232735	AB232754	AB232716	AB232892
46868	Yokohama	<i>C. immitis</i>	Human case	Japan	AB232874	AB232736	AB232755	AB232717	AB232893
50992	90-242	<i>C. immitis</i>	Human case	USA	AB232875	AB232737	AB232756	AB232718	AB232894
50993	Kanazawa	<i>C. posadasii</i>	Human case	Japan	AB232876	AB232738	AB232757	AB232719	AB232895
50994	Toranomon	<i>C. posadasii</i>	Human case	Japan	AB232877	AB232739	AB232758	AB232720	AB232896
50995	Handai	<i>C. immitis</i>	Human case	Japan	AB232878	AB232740	AB232759	AB232721	AB232897
51112	Himeji	<i>C. posadasii</i>	Human case	Japan	AB232879	AB232741	AB232760	AB232722	AB232898
54194	Nagano	<i>C. posadasii</i>	Human case	Japan	AB232880	AB232742	AB232761	AB232723	AB232899
54195	Fukunaga	<i>C. posadasii</i>	Human case	Japan	AB232881	AB232743	AB232762	AB232724	AB232900
54196	Chiba	<i>C. posadasii</i>	Human case	Japan	AB232882	AB232744	AB232763	AB232725	AB232901

as the DNA sample⁹). DNA extract (2.5 μ l), Ready-to-Go beads (Amersham Pharmacia, Tokyo, Japan), 20 μ l of distilled water, and 2.5 μ l of 10 pM of each primer for DO, SP, CT, and rRNA genes⁴⁻⁷ were mixed, and polymerase chain reactions (PCR) were processed. PCR products were separated by electrophoresis on 1.0% agarose gels in 1 \times TBE buffer (0.04 M Tris-boric acid, 0.001 M EDTA pH 8.0) and visualized by ethidium bromide staining. PCR products were purified with a PCR purification kit (QIAquick[®], Qiagen) and labeled using BigDye[®] terminator Ver. 1.1 (Applied Biosystems, Foster City, CA, USA) by the manufacturer's protocol.

The partial sequence of the urease gene (URE) was amplified with a primer set (CIS2410-2434: CGG GTA TTT ACA AGG CTG ATA TTG G and CIAS2945-2922: GAA GCC AGA TTC GTT CAG GGT GTC) designed from the *C. immitis* urease gene sequence deposited in GenBank under accession number U81509⁸). The PCR conditions for URE were as follows: the reaction mixture was subjected to 1 cycle of denaturation at 95°C for 4 min, 30 cycles of amplification at 94°C for 1 min, at 50°C for 1 min, and at 72°C for 2 min, and a final extension cycle at 72°C for 10 min with a PCR Thermal Cycler MP (TaKaRa).

The combined data set for phylogenetic study

was performed in *Fusarium* spp.¹⁰. The present study applied above analysis for alignment of combined sequences consisted of at least 1,769 base pairs obtained from the DO, SP, and CT genes using CLUSTAL X (Version 1.8)¹¹. An unrooted tree was constructed using Njplot (<http://pbil.univ-lyon1.fr/software/njplot.html>)¹². The trees were also constructed based on each gene alone. Consistencies in clade formation between the unrooted trees based on each gene and the combined one were compared.

The genospecies of *Coccidioides* were determined based on the location of the clade. Strains located in the clade involving IFM 50995 derived from a Japanese patient whose infection was suspected of having occurred in Bakersfield, California¹³ was identified as *Coccidioides immitis*, and those in the clade with IFM 45811 and IFM 45812 originating in the state of Arizona were identified as *Coccidioides posadasii*, respectively.

Results and Discussion

The accession numbers of the genes, the lengths of the sequences, and the identification based on the cluster analysis with the three-gene combination are shown in Table 1. The unrooted tree based on a combination of three genes is shown in Fig. 1. Five isolates of *Coccidioides* spp. in our center were identified as

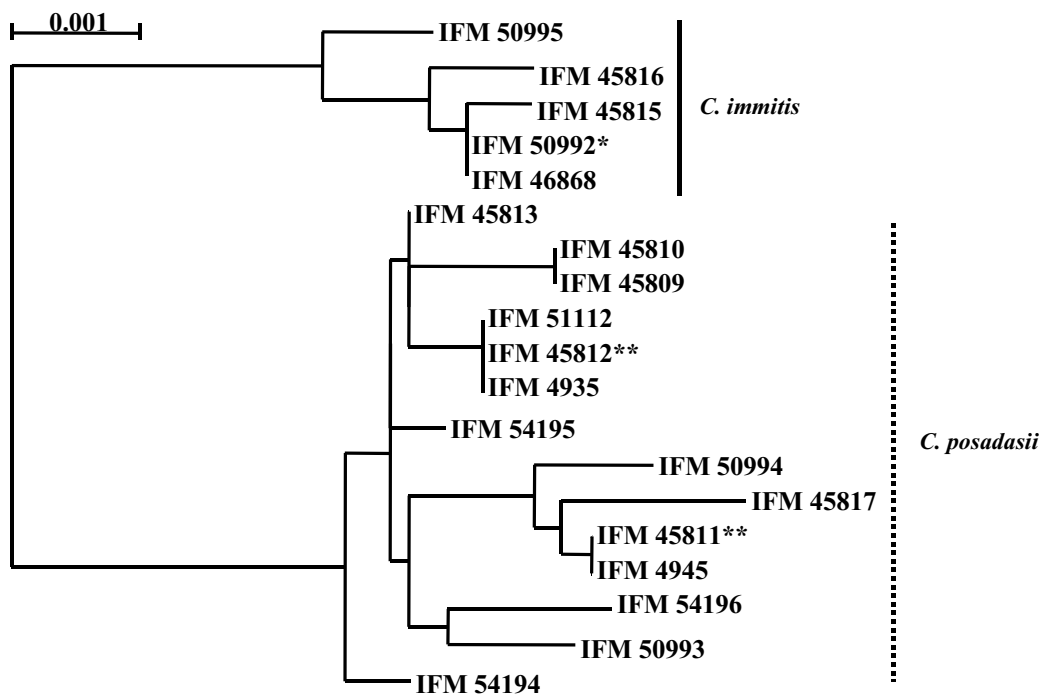


Fig. 1. Unrooted tree based on a combination of three gene sequences—dioxygenase (DO), serine proteinase (SP), and chitinase (CT) consisting of 1769 base pairs constructed by the neighbor-joining method. The scale bar represents a difference corresponding to 0.001 (0.1%). Isolates identified as *C. immitis* are in the upper clade and those as *C. posadasii* are in the lower one. *: isolate used as *C. immitis* standard; **: isolate used as *C. posadasii* standard.

C. immitis and 14 as *C. posadasii*. The data confirmed that 2 of the Japanese cases were caused by *C. immitis*.

Unrooted trees based on individual DO, SP, and URE genes were consistent with the combination of three genes (DO, SP, and CT) and could separate two clades, while those of CT and rRNA genes could not (data not shown). The homology among isolates was more than 99% in all genes evaluated.

The GenBank database was not sufficient for genetic identification of *Coccidioides* spp. There was one sequence of DO on *C. posadasii* (L38493), four sequences of SP on *C. immitis* (S77562, AJ408857, AJ408861, and M81863) and four sequences on *C. posadasii* (AJ408858, AJ408859, AJ408860, and X63114), three sequences of CT on *C. posadasii* (L41663, U51271, and U60806), one sequence of URE on *C. posadasii* (U81509), two sequences of the ITS region of rRNA on *C. posadasii* (CIU18360, X94142), and two sequences of the D1/D2 region of rRNA on *C. immitis* (AY176713, AB040702). The gene sequences of the species used for the present study are helpful for genetic identification. Cluster analysis in the present study with a combination of these data found an inconsistency on the SP gene. The sequence AJ408857, derived from *C. immitis* in the GenBank database, was located on the cluster of *C. posadasii*.

Thus far, two genes, DO and URE, might be useful for identifying genospecies of *Coccidioides* spp. alone. Such an analysis will allow speculation on where infection occurred, while both the ITS and D1/D2 regions of rRNA genes, which are representative genetic markers for classifying and identifying fungal species^{6, 7)}, were incompatible with the unrooted tree based on the cluster analysis by combination of the three genes. Identification based on ribosomal RNA genes could not identify the species of *Coccidioides* because of strongly similar identity among strains according to the criteria proposed by Kurtzman and Robnett⁷⁾.

The inter species differences between *C. immitis* and *C. posadasii* linked both geographic distribution and virulence⁵⁾. However, clinical isolates of both *C. immitis* and *C. posadasii* in Japan should be regarded as the most virulent fungal species. We should keep in mind that the clinical isolates of *Coccidioides* spp. should be handled in accordance with biohazard regulations at a bio-safety level 3 laboratory.

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