Molecular Characterization of New Clinical Isolates of *Candida albicans* and *C. dubliniensis* in Japan: Analysis Reveals a New Genotype of *C. albicans* with Group I Intron

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The genetic diversity of recent clinical isolates of *Candida albicans* in Japan was studied on the basis of amplified DNA band lengths determined with a specific PCR primer reported to have been designed to span a transposable intron region in the 25S rRNA gene. Our analyses of 301 clinical isolates of *C. albicans* showed that they could be classified into five genotypes: genotype A (172 isolates), genotype B (66 isolates), genotype C (56 isolates), genotype D (*C. dubliniensis*; 5 isolates), and a new genotype (designated genotype E; 2 isolates). The new genotype E was characterized to have a group I intron-like sequence, which is longer than hitherto reported ones and which has a nucleotide sequence length of 962 bp. Our analysis of the 962-bp sequence indicated that it is composed of an intron similar to that of *C. dubliniensis* of 621 bp with a 341-bp insertion. Analysis of the sequence of the internal transcribed spacer (ITS) region of the genotype E strain showed that its sequence is identical to those of strains of other genotypes, with only a few base substitution differences. Throughout the study, the possible horizontal transfer of the group I intron between *C. dubliniensis* and *C. albicans* was suggested. A high degree of correlation between the presence of a group I intron in *C. albicans* genotype E and susceptibility to the antifungal agent flucytosine was observed. The five isolates of *C. dubliniensis* examined in the present study showed genetic diversity when they were compared by randomly amplified polymorphic DNA fingerprinting pattern analysis, and this diversity was also confirmed by the analysis of ITS region sequences.

The increasing incidence of AIDS and the recent development of a new treatment strategy for patients with hematologic malignancies and organ transplants have led to steady increases in the number of immunocompromised patients with fungal infections (14). Although the number of fungal species responsible for infection in such patients continues to increase, *Candida* species remain the most frequently encountered fungal pathogens (2, 14). Among the *Candida* species, *Candida albicans* is still considered the most important fungal pathogen. However, an increasing number of reports have described atypical *C. albicans* strains among human clinical isolates, and *C. albicans* strains have been subdivided into some biological groups, including genetic subtypes (8). Recent advances in molecular biology-based technology enable detailed analysis of the genetic diversity of *C. albicans*, and some groups of *C. albicans* strains have been genetically characterized and reported (5, 6).

The usefulness of ribosomal sequences for genetic typing has been demonstrated and widely applied to the identification of several fungal pathogens (7–9). McCullough et al. (8) reported that a PCR primer designed to span the 25S rRNA gene (rDNA) region can classify *C. albicans* strains into four genotypes on the basis of the amplified PCR product length: genotype A (450-bp product), genotype B (840-bp product), genotype C (450- and 840-bp products), and genotype D (1,080-bp

product). In their report, they confirmed that *C. albicans* genotype D belongs to the same taxon as *C. dubliniensis*. We have also confirmed that this genotype analysis method is simple and reproducible when reference *C. albicans* strains are used. Since no systematic study on the genetic subtyping of Japanese *C. albicans* isolates has been reported thus far, we were interested in this method from a molecular epidemiological point of view. Here, we report on the molecular characterization of a new genotype of *C. albicans* and new isolates of *C. dubliniensis* which were found in analyses of 301 isolates phenotypically identified as *C. albicans*. We also discuss the possible origin of the group I intron in *C. albicans* and its association with antifungal susceptibility.

MATERIALS AND METHODS

C. albicans and other Candida sp. strains. The following reference strains of *C. albicans* and *C. dubliniensis* were used: *C. albicans* ATCC 90028, ATCC 90029, and CY1123 and *C. dubliniensis* CBS 7987 and 70-12539 (16). Three hundred one isolates of *C. albicans* obtained from clinical specimens (from July 1999 to March 2000) submitted to the Hiroshima Red Cross-Atomic Bomb Survivors Hospital (Hiroshima, Japan), Kurashiki Central Hospital (Okayama, Japan), Kochi Municipal Hospital (Kochi, Japan), Chiba University Hospital (Chiba, Japan), Kitasato University Hospital (Kanagawa, Japan), and Showa University Hospital (Tokyo, Japan) were used in the present study. These clinical isolates were identified as *C. albicans* in each regional hospital on the basis of their cultural and morphological characteristics such as colony color on CHROMagar *Candida* and chlamydospore formation on cornmeal agar, respectively. The isolates were confirmed to be *C. albicans* and *C. dubliniensis* with the API ID32C system (Biomerieux SA, Marcy l'Etoile, France) following the recommendations of the manufacturer. They were inoculated onto potato dextrose agar (PDA; Difco) slants and were incubated at 37°C for approximately 48 to 72 h before DNA extraction.

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TABLE 1. Genotyping of 301 recent clinical *C. albicans* and *C. dubliniensis* isolates from Japan[a]

Source	No. (%) of strains of the following genotype:					
	Total	A	B	C	D (*C. dubliniensis*)	E
Hiroshima Red Cross-Atomic Bomb Survivors Hospital (western Japan)	46	28	9	9	0	0
Kurashiki Central Hospital (western Japan)	28	11	6	9	2	0
Kouchi Municipal Hospital (Shikoku Island, western Japan)	15	7	5	2	1	0
Chiba University Hospital (central Japan)	152	93	29	26	2	2
Kitasato University Hospital (central Japan)	30	17	10	3	0	0
Showa University Hospital (central Japan)	30	16	7	7	0	0
Total	301	172 (57.1)	66 (21.9)	56 (18.6)	5 (1.7)	2 (0.7)

[a] All strains were isolated in Japan from July 1999 to March 2000.

Extraction of DNA. Two or three loopfuls of fungal yeast cells from PDA slants were suspended in 200 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) in an Eppendorf tube (1.5 ml). DNA extraction was carried out by the procedure described by Imai et al. (3) and Tamura et al. (16). Briefly, 250 μl of GPT reagent (6 M guanidine thiocyanate in 50 mM Tris [pH 8.3]) and 450 μl of Tris (pH 8.0)-buffered phenol were added to a suspension of washed yeast cells in an Eppendorf tube, and the mixture was boiled for 15 min to kill the fungal cells and extract the DNA. Chloroform-isoamyl alcohol (250 μl) was then added; and the aqueous phase was separated by centrifugation at $12,000 \times g$, mixed with an equal amount of 100% isopropanol and a 1/10 volume of 3 M sodium acetate, and placed at $-20°C$ for 1 h. Samples were centrifuged at $12,000 \times g$ for 20 min; and the nucleic acid pellet obtained was washed with ice-cold 70% ethanol, dried, and resuspended in sterile TE buffer at a concentration of 5 μg/ml.

Genotype determination by PCR. The primer pairs whose sequences span the site of the transposable intron in the 25S rDNA were those described by Mc-Cullough et al. (8). The PCR primer pairs used were CA-INT-L (5′-ATAAGG GAAGTCGGCAAAATAGATCCGTAA-3′) and CA-INT-R (5′-CCTTGGC TGTGGTTTCGCTAGATAGTAGAT-3′). Amplification reactions were performed in 25 μl of distilled water containing 2.5 μl of each primer (20 pm), 2.5 μl of genomic DNA (5 μg/ml), and one PCR bead (Ready-to-Go PCR beads; Amersham Pharmacia Biotech, Piscataway, N.J.). The PCR conditions used were as follows: denaturation by incubation for 3 min at 94°C prior to 30 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2.5 min and a final extension at 72°C for 10 min in a thermoreactor. All reaction products were characterized by electrophoresis on 1.5% agarose gels in 1× TBE (Tris-borate-EDTA) buffer at 70 V for 100 min and were then stained in a solution of 0.5 μg of ethidium bromide per ml.

DNA sequencing of ITS1-5.8S-ITS2 region of rDNA and introns of *C. albicans* strains. The PCR primers used for amplification and sequencing of the internal transcribed spacer (ITS) regions were the same as those described by White et al. (20). The PCR primers used for ITS region DNA sequencing were ITS5 (5′-G GAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGA TATGC-3′). Primers CA-INT-L, CA-INT-R, Car1F (5′- AAACGGCGG-GAGTAACTAT-3′), Cae2F (5′-TACTTTATGACGACAAC-3′), and Cae1R (5′-TGGCTACCTTAAGC-3′) were used as the primers for sequencing of the intron region sequences. Amplification reactions were performed in 25 μl of distilled water containing 2.5 μl of each primer (20 pm), 2.5 μl of genomic DNA (5 μg/ml), and one PCR bead. PCR was performed by initial denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 2 min, 55°C for 2 min, and 72°C for 2 min and a final extension at 72°C for 10 min (14). The PCR products were purified with a PCR product presequencing kit (U.S. Biochemical Corp., Cleveland, Ohio) and were then sequenced directly with a Big Dye terminator reagent kit with *Taq* polymerase and by the protocol recommended by the manufacturer of the model 310 automated DNA sequencer; Perkin-Elmer/Applied Biosystems, Chiba, Japan). The DNA sequences were aligned with the Clustal W program (version 1.74) (17), and the alignment was visually corrected.

RAPD PCR analysis of new *C. dubliniensis* isolates. Two primers were used for randomly amplified polymorphic DNA (RAPD) analysis: primers R-1 (5′-ATG GATCGGC-3′) and R-2 (5′-ATTGCGTCCA-3′). These were prepared on the basis of the reports of Poonwan et al. (13) and Aoki et al. (1). Amplification reactions were performed in 30 μl of distilled water containing 2.5 μl of each primer (20 pm), 2.5 μl of genomic DNA, and one PCR bead. The PCR was performed under the same conditions as those described previously (1, 3).

***Candida* species identity confirmation by slide agglutination tests, with a yeast identification system, and by phenotypic characterization tests.** After two trans-fers on PDA slants, the identities of the new isolates of *Candida* species were confirmed by slide agglutination tests (*Candida* Check; Iatron Co., Tokyo, Japan). The API ID32C yeast identification system (Biomerieux SA) was also used for confirmation of the identities of the *Candida* species. CHROMagar *Candida* (Kanto Kagaku Co., Tokyo, Japan) (11) was used for observation of colony color, and Tween 80-Oxgall-caffeic acid agar medium (Remel, Lenexa, Kans.) was used for the chlamydospore formation test.

Drug susceptibility testing. Amphotericin B, fluconazole, flucytosine, and itraconazole were gifts from Bristol-Myers Squibb Japan (Tokyo, Japan), Pfizer Pharmaceuticals (Nagoya, Japan), Nihon Roche (Tokyo, Japan), and Janssen Pharmaceutica (Beerse, Belgium), respectively. Susceptibilities to the antifungal agents were determined by a modified NCCLS M27-A broth microdilution method by use of yeast nitrogen base with 1% glucose (Difco, Detroit, Mich.) as the assay medium (10). *C. albicans* ATCC 90028, *C. albicans* ATCC 90029 (which is resistant to flucytosine), and *C. albicans* CY1123 (which is resistant to flucytosine and the azoles) were used as reference strains.

Nucleotide sequence accession number. The whole sequence of the group I intron of the genotype E strains was deposited in the DDBJ database under accession number AB049125. The sequences of the ITS1-5.8S-ITS2 regions of *C. albicans* ATCC 90028 (genotype A), ATCC 90029 (genotype B), CY1123 (genotype C), and IFM 49826 (genotype E) and *C. dubliniensis* CBS 7987 and IFM 49833 are deposited in the DDBJ database with accession numbers AB049119 to AB049124, respectively.

RESULTS

Genotypes of newly isolated *C. albicans* strains from clinical specimens in Japan. All the *C. albicans* strains were inoculated into CHROMagar *Candida*, and the resultant colony color was used to exclude possible contamination with non-*C. albicans* isolates. The genotypes of all 301 isolates of *C. albicans* were analyzed by the PCR method, and the results are shown in Table 1. The lengths of the PCR products of reference strains *C. albicans* ATCC 90028 (serotype A) and ATCC 90029 (serotype B) were about 450 and 840 bp, respectively. *C. albicans* CY1123, which produced two PCR products of about 450 and 840 bp, respectively, was used as the genotype C reference strain. *C. dubliniensis* CBS 7987 exhibited a band of approximately 1,080 bp and was used as the genotype D reference strain (Fig. 1).

Results showed that of the 301 isolates tested, 172 were classified as genotype A, 66 were classified as genotype B, 56 were classified as genotype C, and 5 were classified as genotype D (*C. dubliniensis*). In addition, we observed two new strains that produced a different PCR product of about 1,400 bp (Table 2; Fig. 1), which is longer than that of *C. dubliniensis* (1,080 bp). Therefore, we considered these two strains to belong to a new genotype, designated genotype E. *C. albicans* genotype E strains were isolates from non-AIDS patients in
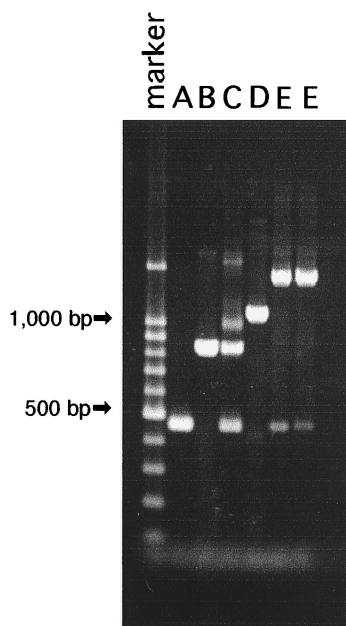
FIG. 1. Genotyping DNA band pattern profiles of reference strains of *C. albicans* genotypes A, B, and C; *C. dubliniensis* genotype D; and two *C. albicans* genotype E strains (lanes A to E, respectively).

Chiba, which is in the central part of Japan, while *C. dubliniensis* strains were distributed among all geographical regions of Japan. The *C. dubliniensis* strains were also found not to be associated with AIDS patients, although the human immunodeficiency virus (HIV) infection status of one patient was not clear (Table 2).

Predicted structure of *C. albicans* genotype E group I intron. The 1,400-bp intron-like sequence of genotype E strains suggested the existence of an intron within the 25S rDNA, which could be removed during maturation of rRNA transcripts. The sequence of the intron-like structure was determined, and the sequence was compared with those of the reported sequences of *C. dubliniensis* CBS 7987 (EMBL database accession number Z70663) and *C. albicans* genotype B (EMBL database accession number X74272) (Fig. 2). The length of *C. albicans* genotype E intron-like sequence was 962 bp, and subsequent base analysis indicated that it belonged to group I introns, as it contained conserved elements of group I introns, such as P, Q, R, and S regions, identical to those that commonly exist in the group I introns of *C. albicans* genotype B and *C. dubliniensis*. The sequence was most similar to that of the *C. dubliniensis* group I intron, with an insertion of a 341-bp fragment at what appeared to be position 255 bp of the internal guide sequence (IGS) site (Fig. 2). Our search with the BLAST program did not reveal a DNA sequence similar to the 341-bp insertion sequence, and the origin of the insertion sequence could not determined.

Analysis of ITS regions of *C. albicans* and *C. dubliniensis*. For the establishment of the phylogenetic position of *C. albicans* genotype E, the sequences of the ITS1-5.8S-ITS2 regions of *C. albicans* ATCC 90028 (genotype A), ATCC 90029 (genotype B), CY1123 (genotype C), and IFM 49826 and IFM 49827 (genotype E) and *C. dubliniensis* CBS 7987 and IFM 49833 were determined. The results showed that the sequences of the ITS1-5.8S-ITS2 regions of *C. albicans* genotypes C and E were identical and that only a 1-bp difference in the sequences between genotypes B and C was observed. The sequence difference between genotypes A and E was minor, with a 1-bp

TABLE 2. History and useful differential features between *C. albicans* genotype E and *C. dubliniensis* strains isolated in the present study

Strain number	Source	Patient characteristic (age [yr])	Chlamydo-spore pattern	API ID32C profile identification	Growth at 45°C	Approximate PCR product length (bp)	RAPD group	
							R-1 primer	R-2 primer
C. albicans genotype A ATCC 90028			Singlet	*C. albicans*	+	450		
C. albicans genotype B ATCC 90029			Singlet	*C. albicans*	+	840		
C. albicans genotype C CY1123			Singlet	*C. albicans*	+	450, 840		
C. dubliniensis CBS 7987			Doublet	*C. dubliniensis*	−	1,080	1	A
C. dubliniensis								
IFM 49829	Feces	Female (70), roentgen ulcer	Chain	*C. dubliniensis*	−	1,080	1	A
IFM 49830	Feces	Male (75), leukemia	Doublet	No ID[a]	−	1,080	2	A
IFM 49831	Sputum	Female (85), anemia	Chain	*C. albicans*	−	1,080	1	C
IFM 49832	Sputum	Female (79), diabetes	Doublet	No ID	−	1,080	1	A
IFM 49833	Unknown	Unknown	Singlet and triplet	*C. dubliniensis*	−	1,080	1	B
C. albicans genotype E								
IFM 49826	Sputum	Female (56), subarachnoid hemorrhage	Singlets	*C. albicans*	+	1,400		
IFM 49827	Urine	Male (80), emphysema of lungs, intensive care unit	Singlet	*C. albicans*	+	1,400		

[a] No ID, not identified; the profile does not resemble any of those stored in the database.

```
                                                        50
G.E.    CAACCTATAA GGGAGGCAAA AGTAGGGACG CCATGGTTTC CAGAAATGGG
C.D.    .......... .......... .......... .T........ ..........
C.A.    .......... .......... .......... .......... ..........

                                                       100
G.E.    CCGCGGTGTT TTTGACCTGC TAGTCGATCT GGTTAATTAG GTATTTTGTA
C.D.    ...A...... .......... .......... .......... ..........
C.A.    .......... .......... .......... ..CCAGACGT A.C.G.G.GT

                                                       150
G.E.    TATTACTTAT CAGAGTATTC TCCTGGTATT ATACATTTTA CTTTATGACG
C.D.    .......... .......... .......... .......... ..........
C.A.    GG-------- ---------- ---------- ---------- ----------

                                                       200
G.E.    ACAACTATTA CCCGCGGGAC AACCATTTCT TGAATTTATT TACTGCAAGT
C.D.    .......... .......... .......... ..... ..... ..........
C.A.    ---------- ---------- ---------- ---------- ----------

                                                       250
G.E.    G-ATTCTAGA ATATGGTGAT TCCAGTTATA ACACCAACTG TTATGACACA
C.D.    .T........ .......... .......... .......... ..........
C.A.    ---------- ---------- ---------- ---------- ----------

                                                       300
G.E.    AGTGTGATAC AGTCATGGTA TTATCATAGA AACCTTTTTC TTGTAGGAAA
C.D.    ......-... ..------- ---------- ---------- ----------
C.A.    ---------- ---------- ---------- ---------- ----------

                                                       350
G.E.    GGAATGCCGG TAAGCTAAAG GCCGAAATCT TTTTTTACTG AAGAGAATTA
C.D.    ---------- ---------- ---------- ---------- ----------
C.A.    ---------- ---------- ---------- ---------- ----------

                                                       400
G.E.    GGATCTGTGA AGTTATACTG GTATTTGTTT TCGCGGGTTT TAGTAAACCC
C.D.    ---------- ---------- ---------- ---------- ----------
C.A.    ---------- ---------- ---------- ---------- ----------

                                                       450
G.E.    TATTGGTAGT TTTGATGGAT TTTTTTGCAA CACAATTTTA ATTCTGGATT
C.D.    ---------- ---------- ---------- ---------- ----------
C.A.    ---------- ---------- ---------- ---------- ----------

                                                       500
G.E.    GAACCAGGTT AATTTCGTGA TAGTACAATT TATCCATTTT TTTTCTAATT
C.D.    ---------- ---------- ---------- ---------- ----------
C.A.    ---------- ---------- ---------- ---------- ----------

                                                       550
G.E.    CGTTTTTGTA ATTAGCAATA ATTTCTAATC TAGTATTAAA GTGATTTTTG
C.D.    ---------- ---------- ---------- ---------- ----------
C.A.    ---------- ---------- ---------- ---------- ----------

                                                       600
G.E.    TCTTTTCTTT ACAGAAACTT TAGAATCAAT CTCCAAATTT GGTGTGGATG
C.D.    ---------- ---------- ---------- ---------- ----------
C.A.    ---------- ---------- ---------- ---------- ----------

                                               P    650
G.E.    TAGTCATAAG CTGTGGGTAA CCAGCGGCGA CATAACCTGG TACGGGGAAG
C.D.    ----...... ---------- .......... .......... ..........
C.A.    ---------- ---------- .......... .......... ..........

                                                       700
G.E.    GCCTCGAAGC AGTATATATT TTTGGGATTG AAAATCGGGT TGCAAAAACT
C.D.    .......... .......... .......... .......... ..........
C.A.    .......... ...G.TC.CC ..-...---- ---------- ----------

                                                       750
G.E.    TTTGTTTTTG GAAACACGGT TGGTGAGGAA AAAAAA-TAT TTTT--TCCC
C.D.    .......... .......... .......... ......A... ....--...-
C.A.    ---------- ---------- ---------- ---------- ----AG.G.G

                                               Q    800
G.E.    CGCACTTGAA GAAATATATG TTGTATGGGG TTAATCCCGT GGCGAGCCGT
C.D.    .......... .......... .......... .......... ..........
C.A.    .AAG.ACA.. ..GG.GAG.. .......... .......... ..........

                                               R    850
G.E.    CAGGGCGCGA GTTCTGGCAG TGGCCGTCGT AGAGCACGGA AAGGTATGGG
C.D.    .......... .......... .......... .......... ..........
C.A.    .......... .......... .......... .......... ..........

                                               S    900
G.E.    CTGGCTCTCT GAGTTGGCTT AAGGTACGTG CCGTCCCACA CGATGAAAAG
C.D.    .......... .......... .......... .......... ..........
C.A.    .......... .......... .......... .......... ..........

                                                       950
G.E.    TGTGCGGTGC AGAATAGTTC CCACAGAACG AAGCTGCGCC GGAGAAAGCG
C.D.    .......... .......... .......... ..-....... ..........
C.A.    .......... .......... .......... .......... ..........

                966
G.E.    ATTTCTTGGA GCAATG
C.D.    .......... ......
C.A.    .......... ......
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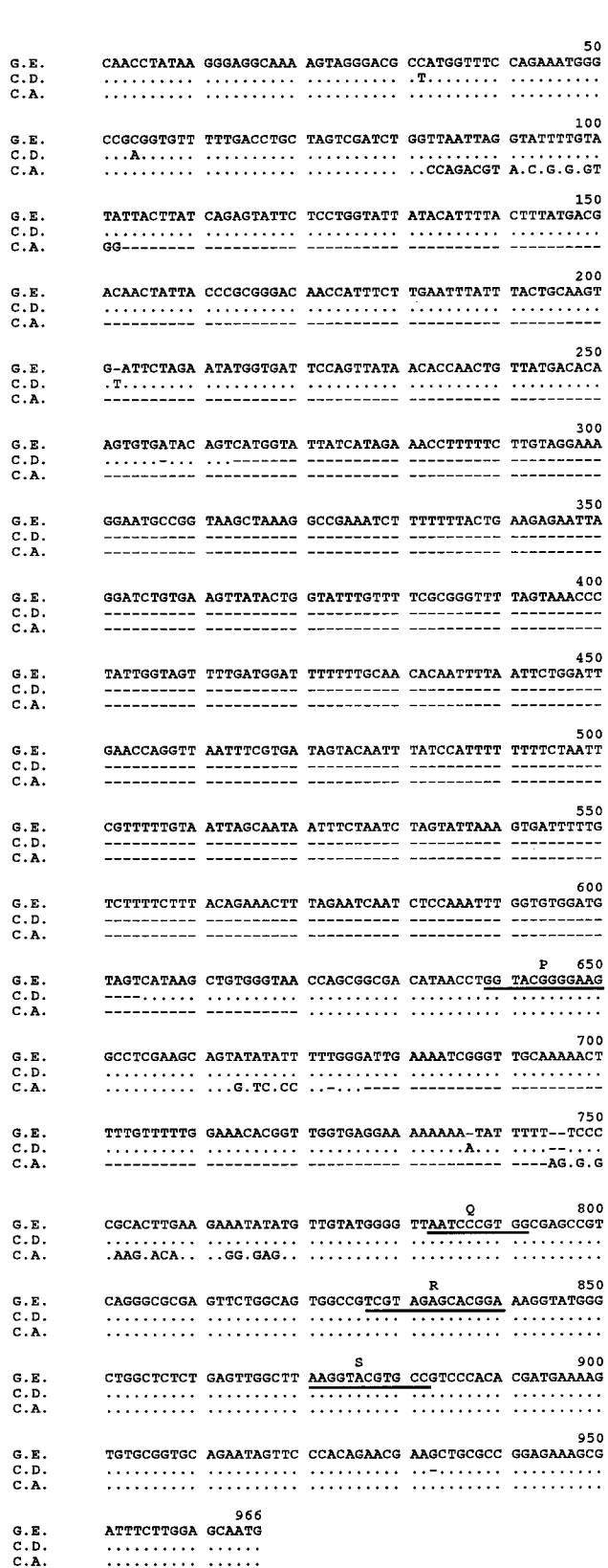
FIG. 2. Alignment of group I introns of *C. albicans* genotype E (G.E.), *C. dubliniensis* (C.D.), and *C. albicans* genotype B (C.A.). The underlining indicates the group 1 intron conserved sequence elements.

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        ITS1->                                          50
G.A.    CTGATTTGCT TAATTGCACC ACATGTGTTT TTCTTTGAAA CAAACTTGCT
G.B.    .......... .......... .......... .......... ..........
G.C.    .......... .......... .......... .......... ..........
G.E.    .......... .......... .......... .......... ..........
C.D.1   .......... .......... .......... .GT.C..G.- ..........
C.D.2   .......... .......... .......... .GT....G.- ..........

                                                       100
G.A.    TTGGCGGTGG GCC-CAGCCT GCCGCCAGAG GTC-TAAACT TACAACCAA-
G.B.    .......... ...-...... .......... ...-...... .G.......-
G.C.    .......... ...-...... .......... ...-...... .........-
G.E.    .......... ...-...... .......... ...-...... ..........
C.D.1   .......... ...C.T.... .......... .A.A...... .........A
C.D.2   .......... ...T.TA... .......... .A.A...... .........A

                                               141
G.A.    TTTTTTATCA ACTTGTCACA CCAGATTATT ACTAATAGTC A
G.B.    .......... .......... .......... .......... .
G.C.    .......... .......... .......... .......... .
G.E.    .......... .......... .......... TT........ .
C.D.1   ........A. ..........G --........ TT........ .
C.D.2   ........A. ..........G --........ TT........ .

        ITS2->                                          50
G.A.    CTCCCTCAAA CCGCT-GGGT TTGGTGTTGA GCAATACGAC TTGGGTTTGC
G.B.    .......... .....-.... .......... .......... ..........
G.C.    .......... .....-.... .......... .......... ..........
G.E.    .......... .....-.... .......... .......... ..........
C.D.1   .......... ..C..A.... .......... .......... ..........
C.D.2   .......... ..C..A.... .......... .......... ..........

                                                       100
G.A.    TTGAAAGACG GTAGTGGTA- -AGGCGGGAT CGCTTTGACA ATGGCTTAGG
G.B.    .......... .........- -......... .......... ..........
G.C.    .......... .........- -......... .......... ..........
G.E.    .......... .........- -......... .......... ..........
C.D.1   ........T. A.........T A......AGA T....-.... ..........
C.D.2   ........T. A.........T A......AGA T....-.... ..........

                                                       150
G.A.    TCTAACCAAA AACATTGCTT --GCGG-CGG -AACGTCCAC CACGTATATC
G.B.    .......... .......... --....-... T......T.. ..........
G.C.    .......... .......... --....-... T......T.. ..........
G.E.    .......... .......... --....-... T......... ..........
C.D.1   .G........ .........A AG....T.TC TGG....GC. ..TT.TAT..
C.D.2   .G........ .........A AG....T.TC TGG....GC. ..TT.TAT..

                160
G.A.    TTCAAACTTT
G.B.    ..........
G.C.    ..........
G.E.    ..........
C.D.1   ..........
C.D.2   ..........
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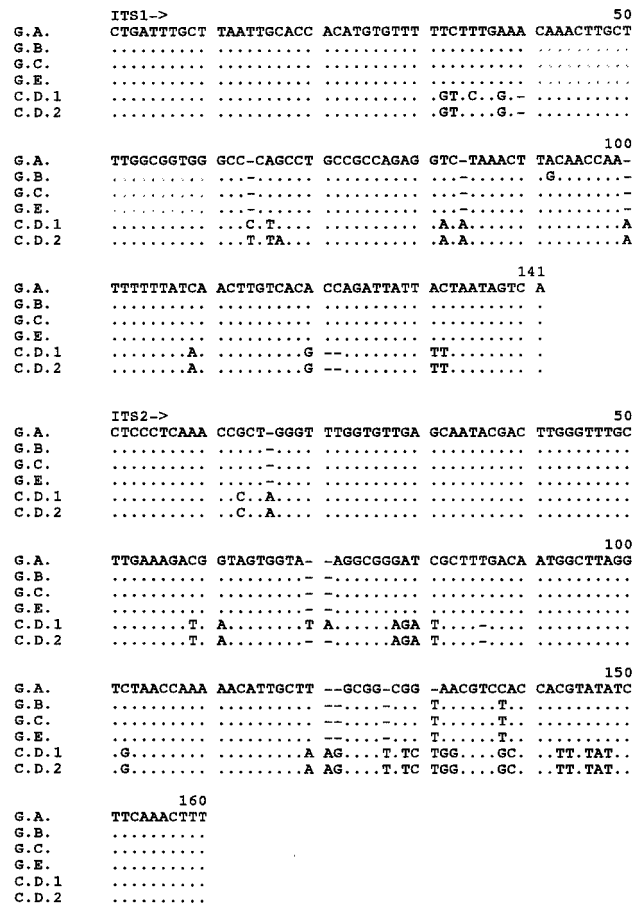
FIG. 3. Alignment of ITS1 and ITS2 region sequences of *C. albicans* genotype E strains and *C. dubliniensis* CBS 7987 and IFM 49833 in comparison with those of *C. albicans* genotype A, B, and C isolates. G.A., *C. albicans* genotype A, ATCC 90028; G.B., *C. albicans* genotype B, ATCC 90029; G.C., *C. albicans* genotype C, CY1123; G.E., *C. albicans* genotype E, IFM 49826; C.D.1, *C. dubliniensis* CBS 7987; C.D.2, *C. dubliniensis* IFM 49833.

difference and a 1-bp deletion observed in *C. albicans* genotype A compared with the sequence of genotype E (Fig. 3).

Phenotypic characterization of *C. albicans* genotype E. All *C. albicans* genotypes, including the five *C. dubliniensis* strains, grew well at 45°C on culture media such as PDA and Sabouraud dextrose agar. However, reference isolate *C. dubliniensis* CBS 7987 did not grow at the restricted temperature. Genotype E isolates produced chlamydospores on TOC agar medium, and most of them were terminal singlets, similar to those of *C. albicans*. The number of chlamydospores produced by *C. albicans* genotype E was lower than the number produced by *C. dubliniensis* CBS 7987. On the basis of this information, some useful differential techniques enabled us to separate genotype E strains from strains of the other genotypes of *C. albicans* and *C. dubliniensis* (Table 2). Additionally, *C. albicans* genotype E also produced a green color on CHROMagar, similar to genotype A to D strains, appeared to be serotype A, and was biochemically confirmed to be *C. albicans* with the API ID32C system.

RAPD analysis and comparison of ITS region sequences of Japanese *C. dubliniensis* isolates. Five strains of *C. dubliniensis*
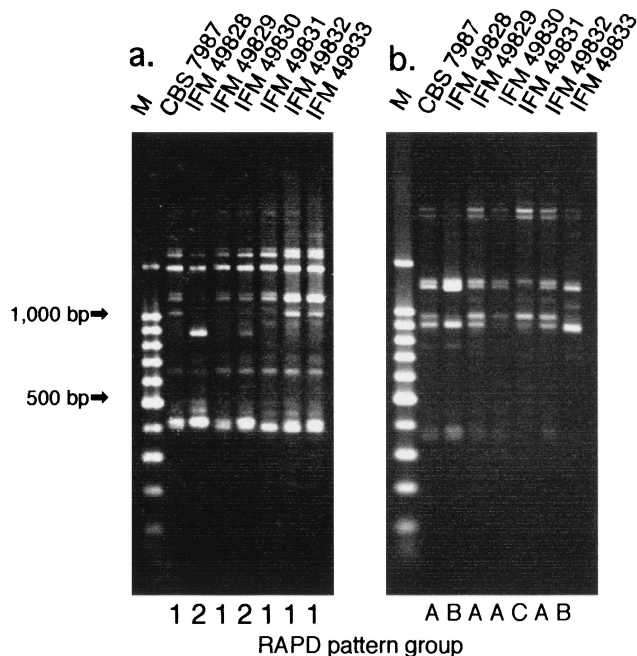
FIG. 4. RAPD band patterns of five *C. dubliniensis* isolates in comparison with those of the *C. dubliniensis* type strain and strain IFM 49828 from our culture collection. (a) The R-1 primer was used. (b) The R-2 primer was used. Lanes M, molecular size markers.

were compared to the type strain (strain CBS 7987) and the other reference strain (strain IFM 49828 [which is the same as strain 70–12539]) (16) by their fingerprinting patterns obtained by RAPD analysis with two 10-mer primers. Genetic typing of the five *C. dubliniensis* strains revealed heterogeneous RAPD fingerprinting patterns. Typing with primer R-2 showed a more characteristic RAPD fingerprinting pattern for each strain than typing with primer R-1, with primer R-2 being able to classify the six strains of *C. dubliniensis* into three groups, while primer R-1 classified them into two groups (Fig. 4). These results suggested the existence of at least three different genotypic groups of *C. dubliniensis* in Japan. The sequences of the ITS regions of the remaining four strains were identical to that of the types strain of *C. dubliniensis* (strain CBS 7987).

The results of the analyses of the sequences of the ITS regions of five epidemiologically unrelated *C. dubliniensis* strains from different hospitals in Japan are shown in Fig. 3 and are compared with those for *C. dubliniensis* strains isolated from different geographic regions. The sequence data show the heterogeneity of the sequences of the ITS regions of *C. dubliniensis* strains. Among the five strains of *C. dubliniensis* tested, the sequence of the ITS region of *C. dubliniensis* IFM 49833, from Kochi Hospital, was clearly different from those of the type strain of *C. dubliniensis* (strain CBS 7987) and the four other Japanese clinical isolates of *C. dubliniensis*.

The API ID32C identification kit gave phenotypic profiles compatible with that expected for two strains of *C. dubliniensis*, while the remaining three strains had unidentifiable profiles.

Susceptibilities of *C. albicans* genotype E and *C. dubliniensis* isolates to four antifungal drugs in comparison with those of other reference strains of *C. albicans*. The two *C. albicans* genotype E strains had susceptibilities to amphotericin B, flu-

conazole, and itraconazole almost identical those of the genotype A, B, and C reference strains. On the other hand, the MICs of flucytosine for the two strains of *C. albicans* genotype E were the lowest of those for all groups. All strains of *C. dubliniensis* had similar susceptibilities to amphotericin B, itraconazole, fluconazole, and flucytosine. Although the susceptibilities of five strains of *C. dubliniensis* to amphotericin B, itraconazole, and fluconazole were almost identical to those of the reference *C. albicans* strains, the MICs were lower than the MIC for *C. albicans* (data not shown).

DISCUSSION

C. albicans is a ubiquitous commensal organism and has been considered a major pathogen for immunocompetent patients as well as immunocompromised patients. Different methods have been developed to differentiate isolates of *C. albicans* (11, 14) and to determine the relationships of these subtypes to human disease. Moreover, advances in molecular biology have enabled the use of various new molecular biology-based genetic methods to answer a variety of epidemiological questions regarding infection with this organism (11, 14).

Mercure et al. (9) reported that *C. albicans* produces a well-characterized *Eco*RI restriction fragment length polymorphism pattern whose bands are intensively stained by ethidium bromide. Of these bands, the dimorphic (3.7- and 4.2-kbp) fragment, which was shown to have originated from the rRNA-encoding regions (rDNA), has been used to classify *C. albicans* into two types (genotypes A and B). Their further studies confirmed that the presence or absence of group I introns among *C. albicans* strains accounts for the difference in the DNA band lengths of the rDNA fragment (3.7 or 4.2 kbp) observed (12). Those studies led to the preparation by McCullough et al. of a PCR primer pair that can demonstrate the presence of group I introns in the 25S rDNA (8). Our present studies confirmed the usefulness of the PCR primer pair for the genotyping of *C. albicans*. An additional advantage of using this primer pair is that it can detect *C. dubliniensis* as well as determine the genotypes of *C. albicans*.

Analysis of 301 *C. albicans* isolates obtained from 1999 to 2000 in Japan revealed that they could be classified into the four previously recognized genotypes: genotypes A (172 isolates), B (66 isolates), C (56 isolates), and D (*C. dubliniensis*; 5 isolates). Interestingly, two isolates that had unclassifiable PCR band patterns were also found. Since the API ID32C identification system and slide agglutination tests indicated that both strains were phenotypically *C. albicans*, we designated them as a new genotype of *C. albicans*, genotype E. The sequences of the ITS regions of the new genotype E strains also confirmed their identities as *C. albicans*. However, the genotype E strain was different, in that a 341-bp insertion occurred within the group I intron. Thus, the most significant observation in the present study concerns analysis of this insertion fragment within the group I intron. However, this genotype E strain showed a high degree of similarity to *C. dubliniensis* compared to the degree of similarity of strains of other *C. albicans* genotypes when the similarity was determined on the basis of the group I intron sequence. These data suggest a possible horizontal transfer of the group I intron from *C. dubliniensis* to *C. albicans* genotype E, or vice versa (Fig. 5). In
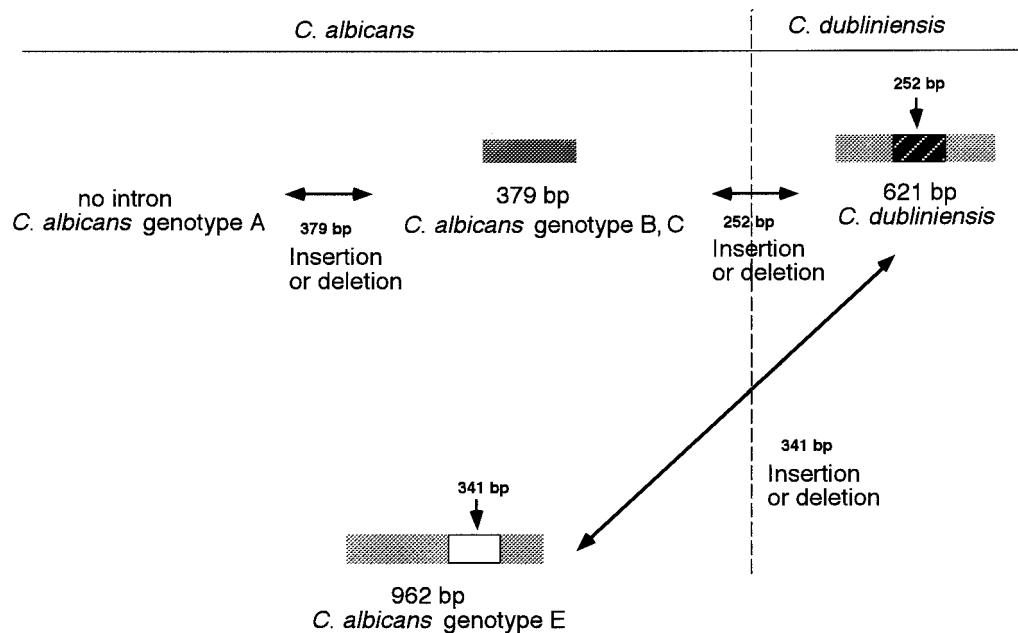
FIG. 5. Proposed horizontal transfer of group I intron between *C. albicans* and *C. dubliniensis*.

the present experiment, no group I intron was observed in other *Candida* species tested such as *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* (data not shown). Therefore, this intron transfer might be possible only between microorganisms of highly related taxons, such as between *C. albicans* and *C. dubliniensis*. This observation that the *C. albicans* group I intron is confined to *C. albicans* genotypes B, C, and E, including *C. dubliniensis*, is consistent with the fact that sexual reproduction has yet to be reported for *C. albicans* (18). In addition, the lack of a group I intron in *C. albicans* genotype A suggests an active mechanism for the prevention of intron acquisition by this taxon. Therefore, the present results seem to support the usefulness of this intron-based PCR genotyping method for epidemiological and taxonomic studies.

The first strains identified as *C. dubliniensis* were recovered from the oral cavities of Irish HIV-infected individuals (15). However, since then there have been a number of reports on the isolation of *C. dubliniensis* in laboratories throughout the world, including Europe, North America, and Australia (15, 16). Kamei et al. reported the first case of *C. dubliniensis* infection in Asia (4). We have reported 1 *C. dubliniensis* isolate among 100 isolates of *C. albicans* being maintained in our laboratory (16). We became interested in determining the number of *C. dubliniensis* isolates among recent clinical isolates of *C. albicans* in Japan because no previous systematic study had been done to determine the frequency of this yeast over a large geographical area. Herein we have reported on the isolation of five additional strains of *C. dubliniensis* among the 301 clinical yeast isolates surveyed in the present study, confirming the existence of the taxon in Japan. Interestingly, these *C. dubliniensis* strains were found to be genetically heterogeneous. When their genetic patterns were analyzed by the RAPD method, these strains of *C. dubliniensis* could be classified into at least three groups. In addition, we found sequence differences at three positions in the ITS1 region and a

2-bp deletions in the ITS2 region between IFM 49833 (an isolate from Kochi Hospital) and the remaining four *C. dubliniensis* strains (Fig. 3). To our knowledge, this is the first report of heterogeneity among sequences of the ITS region of *C. dubliniensis*. The present study also suggests that *C. dubliniensis* is more widespread in the western part of Japan than in the eastern part because of 30 *C. albicans* isolates obtained from Kurashiki Hospital and 15 isolates obtained from Kochi Hospital, 2 and 1 isolates, respectively, were identified as *C. dubliniensis*.

The prevalence of *C. dubliniensis* in the oral cavities of HIV-infected individuals and AIDS patients has been reported by many researchers (5, 15). However, those investigations have been hampered by the lack of a simple and reliable method capable of unequivocally differentiating *C. dubliniensis* from *C. albicans* in a clinical laboratory. Although we have reported on a new PCR primer pair that is useful for differentiating *C. dubliniensis* from *C. albicans* (16), the present PCR system is designed to detect genotypes A, B, C, and E and *C. dubliniensis* (genotype D) and was considered to be more useful for these studies because all *C. albicans* genotypes, including *C. dubliniensis*, could be determined by only a single PCR run.

Doonelly et al. reported that 1.8% of their isolates recovered from asymptomatic healthy individuals and 16.5% of their isolates recovered from HIV-infected individuals are *C. dubliniensis* (2). In a similar study, Odds and Bernaerts reported that approximately 2% of the 2,500 yeast isolates stored in an archival culture collection and originally identified as *C. albicans* were reclassified as *C. dubliniensis* (11). The present experimental data that indicate a 1.6% rate of isolation of *C. dubliniensis* isolates among newly isolated *C. albicans* isolates in Japan is similar to the rates obtained by other investigators (2, 15) and suggests that *C. dubliniensis* is distributed all over the world. Moreover, of the five isolates of *C. dubliniensis* detected

in the present study, four were found not to be associated with AIDS, indicating that this fungal infection may not necessarily be associated with AIDS in Japan, although further epidemiological studies are necessary.

We also wanted to confirm whether common phenotypic tests could be used to confirm the species identities of the five strains as *C. dubliniensis*, i.e., growth at 45°C and colony color on CHROMagar *Candida*. None of the five isolates grew at 45°C, but reference *C. albicans* strains including the *C. albicans* genotype E strains grew well at this temperature. These results confirm that the temperature susceptibility pattern test is useful for the differentiation of *C. dubliniensis* from *C. albicans*. In contrast, the dark blue color of colonies on CHROMagar *Candida*, which has been considered one of the more useful features for the identification of *C. dubliniensis*, was not confirmed for four of the five isolates. Therefore, differentiation of the two taxa on the basis of colony color on CHROMagar *Candida* may not be as reliable as was previously thought. We did confirm that the ITS region sequence information is highly useful and reliable for the identification of *C. dubliniensis* species, particularly for the differentiation of *C. dubliniensis* from *C. albicans*.

Mercure et al. (9) reported that there may be some correlation between the presence of a self-splicing intron in the 25S rDNA of *C. albicans* strains and susceptibility to flucytosine. They also indicated that *C. albicans* strains that have the group I intron are more susceptible to flucytosine (9). We found that the two *C. albicans* genotype E strains with the group I intron are more susceptible to flucytosine than other reference *C. albicans* strains, supporting the hypothesis described above. However, recently, we found that some *C. albicans* genotype A strains without the group I intron as well as genotype B and C strains with the group I intron are susceptible to flucytosine. Therefore, the contribution of the group I intron to the susceptibilities of *Candida* spp. to flucytosine may be only one factor, and other factors (18, 19) may play more important roles in the flucytosine susceptibilities of *Candida* species. Detailed studies are in progress in our laboratory in order to more clearly define the role of the group I intron in antifungal susceptibility or resistance among the various *C. albicans* genotypes and *C. dubliniensis*.

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