High-Frequency Intragenomic Heterogeneity of the Ribosomal DNA Intergenic Spacer Region in *Trichophyton violaceum*\(^\dagger\)

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The intergenic spacer (IGS) of the rRNA genes was analyzed from the dermatophyte *Trichophyton violaceum* isolated from cases of tinea capitis in Taiwan and Iran. *T. violaceum* strains were cultured from different colonies, from single conidial colonies derived by dilution plating, and from micromanipulation of single conidia from clinical samples. A ribosomal DNA probe hybridizing to multiple EcoRI fragments was used to compare restriction fragment length polymorphisms in different *T. violaceum* isolates. The arthroconidia of *T. violaceum* that form in vivo during infection were shown to contain a single nucleus by 4',6-diamidino-2-phenylindole staining. IGS regions from an isolate cultured from a single conidium were amplified, cloned, and sequenced. The results identified that heterogeneity exists between IGS regions within a single *T. violaceum* genome due to different copy numbers of a 171-bp tandem repeat. This suggests that the IGS of *T. violaceum* is partially excluded from the concerted evolution of the rRNA gene locus. The heterogeneous character of the IGS regions in *T. violaceum* contrasts with the closely related dermatophyte *Trichophyton rubrum*, posing further questions on the phylogeny and the evolution of dermatophyte fungi.

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Dermatophytes are a group of fungi specializing in the infection of keratinous tissues such as skin, hair, and nails. *Trichophyton violaceum* is an anthropophilic dermatophyte (transmitted between humans) and is the major causal agent of tinea capitis (scalp ringworm) in many parts of Africa (2, 21, 29) and Asia (12, 18, 22).

A lack of adequate methods for strain differentiation in *T. violaceum* has prevented efforts to address epidemiological questions in *T. violaceum* infections. DNA-based strain typing methods have long been recognized as the best approach to differentiating individual strains of pathogenic fungi, but a strain typing method for *T. violaceum* has not been described. Molecular strain differentiation making use of polymorphisms in the rDNA gene (rDNA) repeat region of the closely related species *Trichophyton rubrum* has been reported previously (13, 14). In *T. rubrum*, there are two different types of subrepeat element (TrS1 and TrS2) in the rDNA intergenic spacer (IGS) region. Analysis of variations in the copy number of the two subrepeats was successfully applied in the strain differentiation of *T. rubrum*. The homogeneous nature of IGS sequences within genomes of any one strain made PCR-based typing amenable. The aim of this study was to identify and characterize subrepeats in the IGS of *T. violaceum* and to determine whether they would provide a useful method of molecular strain differentiation in this species. The actual outcome of this study was the revelation of a high degree of heterogeneity of repeat sequences in the IGS region of *T. violaceum*, raising questions about the evolution of rRNA in fungi.

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randomly selected strains (S1, JC1, JC7, JC16, and JC32) were sequenced using primers ITS1 and ITS4 (13) and compared to sequences at the NCBI; they were similar to sequences labeled as being derived from Trichophyton violaceum, Trichophyton glabrum, Trichophyton gourvilii, or Trichophyton soudanense, all at 99% or greater similarity (the latter four species are considered by many authors as being conspecific with T. violaceum). Differences were seen only in the number of TA repeats in a region of the ITSII.

**Genomic DNA extraction.** The method for extraction of fungal genomic DNA was as described previously (13) with modification to the preparation of fungal biomass. T. violaceum from a SAB agar plate was inoculated into 100 ml of SAB broth in a 250- or 300-ml Erlemeyer flask and incubated with shaking (180 to 200 rpm/min) for 1 to 2 weeks at 30°C. When there was sufficient growth by visual inspection, hyphal biomass was harvested by negative pressure filtration and washed twice with 100 ml of sterile distilled water through a Whatman no. 1 filter paper (Brentford, Middlesex, United Kingdom) in a Buchner funnel. This biomass was stored at −75°C overnight or longer before extraction of nucleic acids. Small amounts of the hyphae grown in broth were subcultured onto a SAB agar slant before filtration and incubated at 25°C for 1 week to assess purity. The concentration of the extracted DNA was adjusted to 1 mg/ml, and the extract was stored at −20°C.

**Detection of rDNA polymorphisms in T. violaceum.** The restriction fragment length polymorphism (RFLP) Southern blotting methods were as described previously (13) with minor modifications. Ten micrograms of each DNA sample was digested with 25 U of restriction endonuclease EcoRI (Takara Biotechnolog, Japan) for 24 h in a total volume of 20 μl. The rDNA probe was amplified from template DNA of T. violaceum JC16 by using universal primers ITS1 and ITS4 (28). The 700-bp probe consisted of a 30-bp fragment from the 3′ end of the small-subunit (SSU) rDNA plus the adjacent ITS1, 5.8S rDNA, and ITSII regions (see Fig. 2).

**DAPI staining of arthroconidia.** One black-dot sample from a patient with T. violaceum tinea capitis was suspended in 20 μl of sterile distilled water and was teased apart with inoculating needles into small pieces. The suspension was then mounted in 2 μl of sterile distilled water and was viewed with a Leica DMR light microscope equipped with a Leitz Epica 3 camera (Leica Microsystems, Wetzlar, Germany).

**Sequence determination of the IGS region.** The method of sequence determination was adapted from a previous method with slight changes (14). Genomic DNAs extracted from T. violaceum strains S1, S6, S8, and S10 were selected as templates from which to amplify the IGS regions using primers 2SSCON2 and NS1-R (14). The commercial Expand Long Template PCR System kit was used (Roche Applied Science, Mannheim, Germany). Master Mix 1 contained 250 nmol of each deoxynucleoside triphosphate and 2.5 μmol of each primer (2SSCON2) and downstream (NS1-R) primer and was made up in a total volume of 24.0 μl. It was used to amplify the IGS region (13) on an Applied Biosystems 3730 automated sequencer by Mission Biotech, Taipei, Taiwan. A nucleotide sequence accession number can be found in the GenBank/EMBL/DDBJ databases with the accession number EF363337.

**RESULTS**

**RFLPs in the rDNA of T. violaceum isolates.** Hybridization of an rDNA probe to immobilized EcoRI restriction fragments of genomic DNA identified extensive RFLPs in the rDNA of 32 Taiwanese isolates of T. violaceum (Fig. 1). A conserved DNA fragment, approximately 3.2 kb in length, was present in all 32 isolates. In addition to this, each individual strain showed multiple polymorphic bands ranging in size from 4 to 10 kb. In many strains the pattern of bands was characterized by a number of fragments of incrementally increasing sizes from 4 to 10 kb. No two isolates showed identical patterns of RFLPs. The patterns of four Iranian isolates were similar to those of Taiwanese isolates, with the conserved 3.2-kb fragment and abundant higher-molecular-weight bands (data not shown). From the results, a provisional restriction map of the T. violaceum rDNA repeat, similar to that of T. rubrum (13), was constructed (Fig. 2). As with T. rubrum it was proposed that the 3.2-kb constant band represented an EcoRI restriction fragment containing part of the 5.8S rRNA gene, the ITS2 spacer, and most of the large-subunit (LSU) rRNA gene. The variable higher-molecular-weight bands derived from fragments containing the entire IGS region, together with the SSU rRNA gene. The variability seen in these bands was predicted to derive from subrepeat elements within the IGS region (Fig. 2).

**rDNA RFLP of T. violaceum single conidial strains.** Thirteen strains were grown from preparations of arthroconidia (95% sin-
gle arthroconidia) from two black-dot samples from one patient with *T. violaceum* tinea capitis. The IGS profiles of these 13 cultures (S1 to S13) are shown in Fig. 3. The pattern of bands in these strains was similar to that seen in RFLP assays of previous strains, with a 3.2-kb constant fragment and multiple high-molecular-weight bands, often increasing incrementally in size. When the profiles were compared by visual inspection, there were nine different patterns in the 13 strains, with strains S2, S3, S7, S9, and S13 appearing identical, while the other eight strains were unique. The results suggested that there were mixed *T. violaceum* strains within a single infection, as multiple patterns were seen from strains isolated from a single patient, but also revealed that different IGS lengths existed within strains derived from a single *T. violaceum* arthroconidium.

In order to determine the source of the variation in the IGS in *T. violaceum*, it was important to analyze strains deriving unequivocally from a single arthroconidium. Strains produced from plating out arthroconidial suspensions may have derived from the small numbers of clumps of more than one conidium present in suspensions. Thus, seven strains were grown from single arthroconidia separated by micromanipulation from a black-dot sample. When digests of genomic DNA were hybridized with the rDNA probe, the RFLP profiles of these seven strains were similar to previous results, and multiple high-molecular-weight fragments were present in all strains (data not shown). The results strongly suggested that different IGS lengths existed within strains derived from a single *T. violaceum* arthroconidium.

In order to determine the source of the variation in the IGS region. PCR across the entire IGS region using primers 25SCON2 and NS1-R on genomic DNA from single conidial cultures produced multiple banding patterns; however, these were smearable and difficult to interpret (data not shown). Therefore, 13 IGS regions derived from PCR products from five different single conidial cultures of *T. violaceum* (S1, S4, S6, S8, and S10) were cloned. Recombinant plasmids from each strain contained inserts of different sizes (Fig. 5). Four of the clones (S1-1, S1-4, S6-5, and S8-1) were sequenced and contained inserts with lengths of 2,601 bp, 2,941 bp, 2,600 bp, and 2,942 bp, respectively. S1-1 and S1-4 were both from the same single conidial strain, while S6-5 and S8-1 were each from different strains. In addition to the entire IGS region, all DNA inserts contained 418 bp from the 3' end of the LSU rDNA and 38 bp from the 5' end of the SSU rDNA.

The IGS maps of clones S1-1, S1-4, S6-5, and S8-1 are shown in Fig. 6. Two repetitive regions were identified in the sequence. The first subrepeat locus was located 451 bp downstream from the predicted 3' end of the LSU rDNA in clone S1-1. Two copies (plasmids S1-1 and S6-5) or four copies (plasmids S1-4 and S8-1) of a tandemly repetitive 171-bp element (*TvS1*) were identified, with an adjacent 67-bp partial

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**Detection of nuclear number in a conidium.** A single nucleus was observed in DAPI-stained arthroconidia examined from a black-dot specimen (Fig. 4). This was also confirmed by transmission electron microscopy (data not shown). This strongly suggested that the different IGS lengths seen by IGS RFLP in single conidial isolates must exist within a nucleus. Furthermore, this suggested that variable copy numbers of subrepeat elements must exist in different copies of the IGS region within a *T. violaceum* genome.
repeat. These results strongly suggested that variable copy numbers of the TvS1 repeat could be present in different copies of the IGS within a single *T. violaceum* genome. A second set of shorter subrepeat elements (TvS2) was located 703 bp upstream from the predicted 5' end of the SSU rDNA. TvS2 was present as one full (77-bp) and one partial tandem copy of a 38-bp repeat sequence, with sequence similarity to the partial S2 repeats of *T. rubrum* and *Trichophyton interdigitale* (14, 15). The TvS2 copy number did not vary among the four IGS sequences. All four clones contained a polydeoxythymidine microsatellite, 17 to 19 bp in length, which was located 135 bp downstream from the predicted 3' end of the LSU rDNA. Overall, the sequences of the four IGSs were very similar except in the copy number of TvS1 and in the length of the poly(T) tract (17 residues in S1-4, 18 in S6-5 and S8-1, and 19 in S1-1). In addition, a number of single nucleotide polymorphisms were identified. Counting from the 3' end of the LSU sequence in S8-1, at positions 771, 1013, and 1369, the bases are T, T, and C, but in the other clones, they are C, C, and T, respectively. In S1-4, at position 1650 there is a G but in the other clones an A. Attempts to design primers from this sequence to amplify TvS1 from genomic DNA again produced smears of bands that were difficult to interpret (data not shown).

The molecular weights of bands in the 5- to 10-kb range in Fig. 3 were estimated by ONE-Dscan 1-D gel analysis software, and the differences between adjacent band sizes were calculated. The results showed that there is a good correlation between the estimated length differences and the size of the 171-bp repeat or multiples of this size (data not shown).

**DISCUSSION**

When 32 randomly selected Taiwanese *T. violaceum* isolates were analyzed by hybridization of EcoRI-digested genomic DNA with an rDNA probe, all 36 isolates showed abundant bands and every individual pattern was distinct (Fig. 1). This high level of variation in the rRNA IGS in *T. violaceum* isolates is unique in the genus *Trichophyton*. Analysis of a small number of Iranian isolates suggested that this variation was independent of the geographical site of isolation of the *T. violaceum* strains. Although *T. violaceum* and *T. rubrum* are very closely related (9), most *T. rubrum* strains (41 of 50, 82%) when analyzed in the same way produced a single high-molecular-weight band, which varied in size between strains (13). In this report and similar studies of the IGS of *Trichophyton tonsurans* (4) and *Trichophyton interdigitale* (15, 20), the majority of strains studied shared a small number of simple pattern types. The question of why there are so many polymorphic bands and unique pattern types in *T. violaceum* was therefore an important one to address. The presence of mixed-strain infections in onychomycosis was demonstrated using rDNA IGS analysis (10, 30). Our results show that mixed *T. violaceum* strain infection in tinea capitis can also occur, though a clear picture of the extent of mixed infection will be difficult to obtain due to the complexity of the banding pattern produced and the lack of knowledge of the frequency changes in this DNA region during infection. The IGS sequences of *T. rubrum* (14) and *T. violaceum* are very similar (>85% identity), and both contain large (TrS1 and TvS1) and small (TrS2 and TvS2) subrepeats. The large subrepeat in *T. violaceum* is 171 bp, and that in *T. rubrum* is 200 bp. The difference of 29 bp in TvS1 compared to the *T. rubrum* equivalent repeat TrS1 is the loss of a palindromic sequence seen only in the *T. rubrum* large subrepeat (14). The results of a sequence similarity search using the 5S rRNA gene of *Aspergillus nidulans* revealed that the 5S RNA gene of *T. violaceum* is not located in the IGS. This is similar to the situation in *T. rubrum* (14), and as with most eukaryotes (25) the 5S rRNA genes are probably dispersed throughout the genome in dermatophytes.

The number of copies of the rDNA repeats in *T. violaceum* or other dermatophytes has not been defined, but in *Candida albicans* there are approximately 120 copies (16) and in other fungi the copy number varies between 45 and 180 (5).

We propose that the size variation seen in DNA fragments
that include the IGS region can be accounted for at least in part by differences in the copy number of the TvS1 repeat unit within different IGS regions within the genome of a strain. However, we cannot rule out the presence of other genetic variations within the IGS or elsewhere in the rDNA that may account for some of the variation seen, particularly in strains where there are high-molecular-weight fragments observed.

Comparison of the results seen here in \textit{T. violaceum} to the results of similar analysis in \textit{T. rubrum} highlights an important difference, in that most strains of \textit{T. rubrum} (82\%) were homogeneous in their IGS regions (13), while all \textit{T. violaceum} strains were shown to have heterogeneous IGSs. Although apparent IGS heterogeneity has been suggested for a few strains of \textit{T. rubrum} (10, 13, 14, 30), \textit{Trichophyton mentagrophytes} (19), and \textit{Microsporum canis} (31), prior to this study it had never been proven by cloning and sequencing of multiple IGSs from a strain derived from a single, mononucleotide consensus. Heterogeneity within the rDNA repeats is relatively rare in eukaryotes but has been reported in \textit{Fusarium} (23), \textit{Neotyphodium lolii} (a grass endophyte) (6), \textit{Xanthophyllumyces dendrorhous} (a basidimycete yeast) (3), and \textit{Pythium helicoides} (17). In other eukaryotes intragenomic variation has been observed in organisms as diverse as crayfish (11), ladybird beetles (26), and larches (27), in each case involving ITS heterogeneity. In \textit{Xanthophyllumyces} and \textit{Pythium} intragenomic heterogeneity was ascribed to indel variation in the ITS or the ITS and IGS regions (3, 17). Interestingly in \textit{Neotyphodium lolii} variable numbers of 111- and 119-bp repeats were seen in the IGS regions within individual strains (6), very similar to the situation seen in \textit{T. violaceum}.

In the present study, the rDNA RFLP Southern blotting method was initially evaluated for its ability to differentiate \textit{T. violaceum} strains cultured from patients with tinea capitis in order to address questions of the epidemiology of the disease. However, the unexpected variation and complexity seen in different isolates and strains, together with a lack of knowledge of the details of the infection process and the frequency of changes in the IGS during infection, are likely to limit its use in epidemiologic studies.

It has been suggested that variation in the IGS of rDNA may have a considerable effect on development, evolution, and ecology through the effects on growth rate regulation, resulting from the role of the IGS in production of rRNA (7). Although the \textit{T. violaceum} IGS has been completely sequenced in this study, the precise locations of the external transcribed spacer or equivalent and any promoters, enhancers, or terminators of rDNA transcription have not been determined. The chromosomal location or locations of the tandemly repeated rDNAs in \textit{T. violaceum} or \textit{T. rubrum} are not known, though in many fungi they are on a single chromosome. If the rDNA was distributed on multiple chromosomes in \textit{T. violaceum}, this may account for the IGS heterogeneity seen. In most eukaryotic organisms, the ribosomal genes are tandemly arrayed and undergo concerted evolution (5). This is a universal biological phenomenon and is responsible for the homogenization of rDNA repeats within a repeat array, among arrays dispersed in an individual genome, and throughout a recombining population (1). It has been shown that the rRNA repeats within the genomes of several fungi are highly conserved including the IGS regions (5). In this study, it appears that the IGS of \textit{T. violaceum} is partially excluded from the concerted evolution of the rRNA gene locus, especially in the TvS1 region. Recently, the relaxation of concerted evolution in the different ITS regions within the genome of the fungus \textit{Glomus etunicatum} was demonstrated (24). Furthermore, variation seen in the SS rDNA multigene family of four filamentous fungi, \textit{Aspergillus nidulans}, \textit{Fusarium graminearum}, \textit{Magnaporthe grisea}, and \textit{Neurospora crassa}, indicates “birth-and-death” evolution (25). The formation of IGS heterogeneity in \textit{T. violaceum} may be similar or may reflect differences in, for example, the rates of mitotic recombination in contrast with the relative homogeneity of the closely related \textit{T. rubrum}. Recently, Graser et al. (8) have analyzed differences in microsatellite markers in isolates of \textit{T. rubrum} from around the world and shown that there are two broad population groups, one of which derived predominantly from Africa and was largely associated with tinea capitis and corporis. Isolates of \textit{T. violaceum} had similar marker loci (unlike the other dermatophytes examined) but were clearly distinct as a species. It would be of interest to examine the IGS regions of these “population 2” isolates of \textit{T. rubrum}, which may shed light on how IGS heterogeneity evolved in \textit{T. violaceum}.

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