The Variability in the Fungal Ribosomal DNA (ITS1, ITS2, and 5.8 S rRNA Gene): Its Biological Meaning and Application in Medical Mycology

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The PCR amplification and subsequent restriction analysis of the ribosomal region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5,8S rRNA is accepted as a powerful method for species identification and epidemiological tracing in medical mycology. The extent of detected intraspecies variability varies among different studies. Theoretically, molecular drive may be considered as the main mechanisms contributing to the sequence unification of ribosomal gene arrays. With regards to potential interactions between molecular drive, natural selection and population dynamics, the problem of development and maintenance of intraspecies variability in rDNA region is very complex. The simple laboratory method for detection of inter- and intraspecies variability in the above mentioned region of rDNA is demonstrated on clinical isolates of fungi, the applications in clinical microbiology and environmental studies are discussed together with problems concerning the evolution of this DNA regions.

Keywords medical mycology; epidemiology; species identification; PCR, restriction analysis; ITS1.

1. Introduction

In eukaryotes, the genes encoding ribosomal RNAs are organized in arrays which contain repetetive transcriptional units involving 16 – 18S, 5.5S, and 23 – 28S rRNAs, two transcribed intergenic spacers ITS1 and ITS2 and two external spacer sequences (5´and 3´ ETS). These units are transcribed by RNA polymerase I and separated by non-transcribed intergenic spacers (IGS) as represented on Figure 1.

The product of RNA polymerase I is processed in the nucleolus, where ITS1 and ITS2 are excised and three types of rRNAs produced. In eukaryotic genomes the ITS regions vary greatly in size and sequence. In mice, ITS1 and ITS2 have lengths 999 bp and 1089 bp, respectively. In the parasitic protzoan Giardia lamblia the lengths of these regions are only 41 bp and 55bp [1]. The longest ITSs were found in Coleoptera - the lengths vary between 791 and 2572 kb [2]. In fungi, ITS1 and ITS2 were studied not only with regard to phylogenetics and taxonomy, but also in connection to development of diagnostic strategies for species identification in medicine and ecology [3, 4, 5]. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are widely used as eukaryotic model organisms, therefore the lengths and structures of their ITSs are well described. In *S.cerevisiae* the ITS1 spans 361 bp and ITS2 is 232 bp long, In *S.pombe* the corresponding lengths are 412-420 bp and 300 bp [6].

The polymerase chain reaction (PCR) and subsequent analysis of amplified rDNA using restriction endonucleases were employed in different studies to achieve efficient interspecies discrimination in medical [7] and food [8] mycology. Some of these studies revealed also intraspecies variability in the examined region [9].

The ITS regions have important biological meaning in rRNA processing. They form specific secondary structures they are needed for correct recognition of cleavage sites and provide the binding sites for nucleolar proteins and RNAs during ribosome maturation [1].The available data on rRNA

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structures show that rRNA processing is conserved process. The structures of analyzed ITS2 and ITS2 contain four or three helical arms [1, 10]. The changes in size and sequence of these regions are then biologically permissible as long as they do not disturb the formation of secondary structures which facilitate the rRNA processing.

We present simple method for determination of inter- and intraspecies variability in fungal isolates useful for demonstration of this phenomenon in the laboratory lessons at the university. Simultaneously, we discuss the mechanisms involved in the evolution of rDNA regions and contributing to their variability.

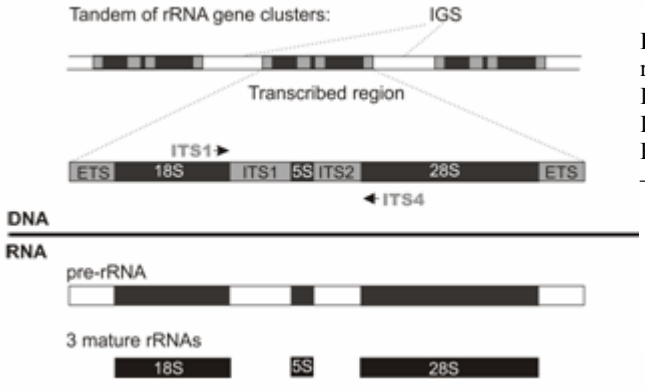

Fig. 1 Schematic representation of rDNA region with primers ITS1 and ITS4 localization (arrows). ITS=internal transcribed spacers, ETS=external transcribed spacers, IGS – non-transcribed intergenic spacers

2. Material and Method

2.1 Strains

We used the pannel of different strains from different species of five genera obtained from the Culture Collection of the Czech National Reference Laboratory for Medical Mycology [9] The species were determined routinely using biochemical, morphological and serological markers. For pedagogical purpose, it is highly recommended to use the isolates or strains belonging to different species and genera to achieve the best demonstration of variability in rDNA region.

2.2 DNA isolation from cultures

Cells were directly sampled from a fresh colony, washed in sterile water, incubated in 100 µl lyticase (Sigma–Aldrich) solution (15 mg/ml water) at 30°C for 1 hour. Then 20 µl proteinase K (Sigma–Aldrich, 20 mg/ml water) were added and the suspension was incubated at 55°C for 90 min. After incubation, the suspension was boiled for 8 min. 10 µl these samples were used for amplification reactions.

2.3 PCR reactions, DNA digestions and fragment analysis

PCR reaction and digestion of amplified fragments were performed according to the conditions described in literature [8]. Briefly, for the 100 µl PCR reactions, the primers ITS1 (5´TCC GTA GGT GAA CCT TGC GG 3´) and ITS4 (5´TCC TCC GCT TAT TGA TAT GC 3´) were used [11]. The PCR reaction mix contained 0.5 µM of each primer, 10 µM deoxynucleotides, 1.5 mM $MgCl_2$ and 1 x buffer (Promega). The suspension was heated at 95°C for 15 min in a PE 2400 (Perkin Elmer) thermocycler. One unit of the Taq Polymerase (Promega) was then added to each tube. PCR conditions were as follows: 35 cycles of denaturing at 94°C for 1 min; annealing at 55.5°C for 2 min and extension at 72°C for 2 min; and final extension at 72°for 10 min. PCR products (10µl) were digested without further

purification with the restriction endonucleases CfoI, HaeIII and HinfI (New England BioLabs). PCR products and their restriction fragments were separated on agarose gels, stained with ethidium bromide and visualized under UV light. Sizes were determined by comparison against the DNA length standard (100 bp ladder, Promega) using LabWorks 3.0.2. software (UVP).

3. Results

3.1 Results of PCR with ITS1 and ITS4 primers and sizes of amplicons

We received following lengths of amplified fragments using the primers ITS1 and ITS4: 550 bp for *Candida tropicalis* and *C.parapsilosis*, 550 or 625 bp for *Candida guillermondii*, 550, 650 or 900 bp for *C.glabrata*, 530 or 870 bp for *C. colliculosa,* 480 bp for *C. inconspicua*, 880 bp for *Saccharomyces cerevisiae*, 380 bp for *Geotrichium candidum*, 380 bp for *Trichophyton beherendi* and 560 bp for *Cryptococcus neoformans* [9].

3.2 Results of restriction analysis of amplicons

Figures 2, 3 and 4 show examples of the results obtained after digestions of these amplicons with enzymes Hinf I, Hae III, and CfoI in clinical isolates of above mentioned species.

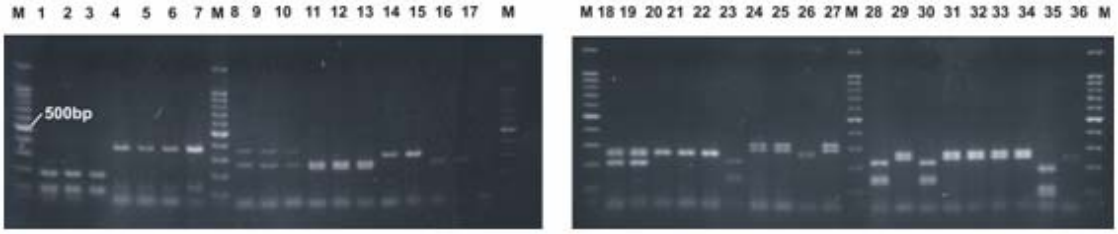

Fig. 2 Restricticton analysis with HinfI. M – 100 bp molecular marker, lines 1 to 3 - *Geotrichium candidum*, lines 4 to 7 – *Saccharomyces cerevisiae*, lines 8 to 15 - *C.glabrata,* lines 16 and 17 - *C. colliculosa,* lines 18 and 19 - *C. inconspicua,* lines 20 to 23 - *Candida tropicalis,*. lines 24 to 27- *Candida guillermondii*, lines 28 to 34 - *C.parapsilosis*, line 35 - *Trichophyton beherendi,* line 36 - *Cryptococcus neoformans.*

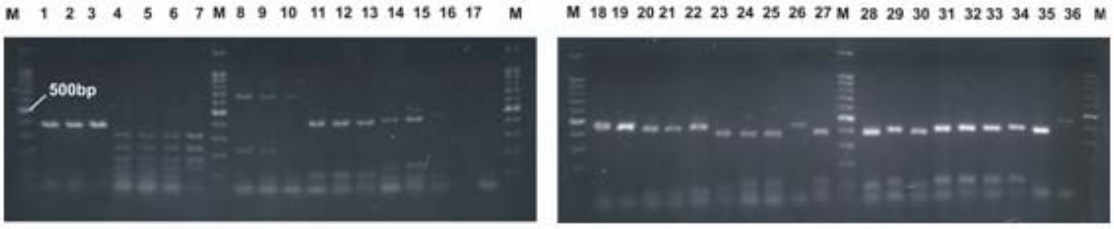

Fig. 3 Restricticton analysis with HaeIII. M – 100 bp molecular marker, lines 1 to 3 - *Geotrichium candidum*, lines 4 to 7 – *Saccharomyces cerevisiae*, lines 8 to 15 - *C.glabrata,* lines 16 and 17 - *C. colliculosa,* lines 18 and 19 - *C. inconspicua,* lines 20 to 23 - *Candida tropicalis,*. lines 24 to 27- *Candida guillermondii*, lines 28 to 34 - *C.parapsilosis*, line 35 - *Trichophyton beherendi,* line 36 - *Cryptococcus neoformans.*

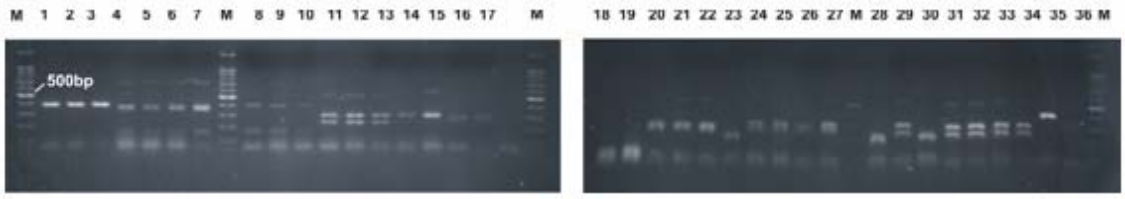

Fig. 4 Restricticton analysis with CfoI. M – 100 bp molecular marker, lines 1 to 3 - *Geotrichium candidum*, lines 4 to 7 – *Saccharomyces cerevisiae*, lines 8 to 15 - *C.glabrata,* lines 16 and 17 - *C. colliculosa,* lines 18 and 19 - *C. inconspicua,* lines 20 to 23 - *Candida tropicalis,*. lines 24 to 27- *Candida guillermondii*, lines 28 to 34 - *C.parapsilosis*, line 35 - *Trichophyton beherendi,* line 36 - *Cryptococcus neoformans.*

4. Discussion

The application of the above described method allows the demonstration of inter - , and - in some cases - also intraspecies variability in the ITS-5.8S rDNA region in fungi. When a suitable panel of restriction endonucleases is used, the obtained restriction profiles may serve for species determination [3, 4, 5, 7, 8, 9].

Theoretically, each copy of such multiple genes organized in arrays (see Fig.1) could accumulate ist own set of mutations. However, the results of restriction analysis of our amplicons show that there must be a great deal of similarity among sequence units within an array. The term conserted evolution is used to describe the unusual evolutionary behavior of multigene family members. This term is coined for the process in which the individual repeats in a multigene family evolve in concert rather than independently. The concertrd evolution leads to homogenization of all repeats in an array. Without this homogenization, we could observe much more complex patterns on our gels after restriction analysis of rDNA regions. Homogenizing mechanisms are still poor unerstood. Two main mechanisms were proposed to be responsible for conserted evolution: gene conversion and unequal crossing over [12]. In the process of molecular drive the sequence of a single repeat within an array is selected, fixed, and spread both through a multigene family and through a sexual population. The extent to which natural selection affects molecularly driven mutation depends on the ecology and reproductive behavior of the population [13].

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