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# The genetic similarity of different generations of *Neocallimastix frontalis* SK

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**Abstract**

The genetic similarity of different generations of *Neocallimastix frontalis* SK was examined by random amplified polymorphic DNA (RAPD) profiling and internal transcribed spacer 1 (ITS1) sequence analysis. *N. frontalis* SK was subcultured every 2–4 days, and SK-1, SK-3M, and SK-1Y represented *N. frontalis* SK cultures after one subculture, 50 subcultures, and 150 subcultures. The DNA polymorphisms of the different *N. frontalis* SK generations were compared by RAPD profiling. The RAPD results gave the same patterns for SK-1, SK-3M and SK-1Y using 12 selected random primers. The partial 18S rDNA, 5.8S rDNA, and ITS1 regions of different generations of *N. frontalis* SK were amplified and sequenced. The results of alignment and pairwise similarity indicated that the analyzed rRNA sequences of SK-1, SK-3M and SK-1Y were totally identical. This study thus demonstrated genetically identical DNA polymorphisms by RAPD profiling and an unvaried ITS1 region for *N. frontalis* SK when the strain is subcultured frequently. This suggests that this strain is homokaryotic and grows via an asexual life cycle in vitro.


*Keywords:* Anaerobic rumen fungus; Genetic similarity; RAPD profiling; *Neocallimastix frontalis* SK

## 1. Introduction

Anaerobic fungi, which commonly colonize the digestive tract of ruminants, are known for exhibiting a high efficiency degradation of plant cell-wall structural polysaccharides. The classification or identification of rumen fungi traditionally relies on morphological examination [1]; however, it is difficult to carry out continuous observation of their life cycle due to their extremely small cell size and their obligately anaerobic growth conditions. Lowe et al. reported the life cycle of *Neocallimastix* sp. R1. They found that there was an alternation between a mobile multi-flagellate zoospore stage and an extensive vegetative stage. The duration of the life cycle of *Neocallimastix* sp. R1 varied from 26 to 32 h [2]. Unfortunately, this work provided little information on the sexual and asexual life cycle of rumen fungi.

*Neocallimastix frontalis* has attracted tremendous attention since the first report by Orpin [3]. Researchers believe that *N. frontalis* contributes significantly to xylan and cellulose hydrolysis. Fujino et al. isolated an endo-1,4-β-glucanase gene from *N. frontalis* MCH-3. The recombinant enzyme was expressed in *Escherichia coli* and demonstrated a high activity against carboxymethylcellulose [4]. Xylanase genes were also isolated from *N. frontalis* and expressed high activity in yeast and filamentous fungus [5]. Although *N. frontalis* isolates have important industrial applications, there is no feasible method for long-term preservation of rumen fungal cultures. Anaerobic fungal isolates have to be transferred every 2–6 days in order to maintain their activity. Their genetic makeup might vary after frequent subculturing, and genetic changes including mutation and recombination might lead to loss of useful gene functions in important isolates. Thus, it is necessary to examine the genetic stability of anaerobic fungi over a significant number of generations.

Higher fungi possess distinctive morphologies that vary between the sexual and asexual parts of their life cycle. The genetic variation and phenotype of offspring reproduced by meiosis or mitosis has been widely studied and are easily compared. Previous studies have suggested that

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variations in the genome after the sexual cycle can be measured in a representative manner by analysis of the internal transcribed spacer (ITS) sequence and of DNA polymorphisms. Muthumeenakshi et al. used the ITS and DNA fingerprints to identify genetic variation among isolates of *Trichoderma harzianum*[6]. Overmeyer et al. showed genetic diversity among the offspring of *Calonectria morganii* by random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) analysis [7]. Brookman et al. found that the ITS1 was the most variable domain of rDNA of *Neocallimastix* spp. and calculated their phylogeny using this region by various algorithms. Although the cladistic trees showed that two groups, the *N. frontalis* group and the *N. patriciarum* group, existed within *Neocallimastix* spp., little is known about genetic variation in their rDNA [8].

In this study, we examined the genetic features of the different generations of *N. frontalis* SK using RAPD profiling and ITS1 sequence analysis. These molecular results were compared with *Ganoderma lucidium*, whose life cycle has been extensively studied [9–11], and other *Neocallimastix* spp. with known ITS1 sequences. The results provided useful information and increased understanding of the genetic stability across different generations of *N. frontalis*. They were also useful to identify whether *N. frontalis* reproduces asexually or sexually under artificial culture conditions.

## 2. Materials and methods

### 2.1. Fungal strains and genomic DNA extraction

*N. frontalis* SK was isolated from the rumen fluid of Formosa sika deer (*Cervus nippon taiwanus*). Strains SK-1, SK-3M, and SK-1Y represent *N. frontalis* SK cultures after one subculture, 50 subcultures and 150 subcultures. Isolation methods were performed as described by Ho and Bauchop [12]. All rumen fungal strains were incubated in maintenance medium using rice straw as the sole carbon source at 39°C and transferred every 2–4 days. The biomass of *Neocallimastix* strains for DNA extraction was grown in 500 ml serum bottles containing 250 ml maintenance medium [12] supplemented with 1% glucose as the sole carbon source under anaerobic conditions. All the anaerobic techniques followed the Hungate technique [13]. The biomass was collected by centrifugation (4°C, 5000×*g*, 30 min) after 3–5 days incubation at 39°C without shaking. The *Ganoderma lucidium* RZ-2 and RZ-3 monokaryotic strains were obtained from the basidiospores of *G. lucidium* RZ (heterokaryon) by single spore isolation [11]. Monokaryotic and dikaryotic *Ganoderma* samples were incubated in 100 ml of potato dextrose broth (Difco, Detroit, MI, USA) for 7–14 days at 28°C before harvesting. The harvested biomass and mycelia were stored at −20°C before DNA extraction. Genomic DNA for ITS1 and RAPD analysis was extracted by the phenol/chloroform protocol [10].

### 2.2. RAPD analysis

RAPD analysis was carried out according to the method of Hseu et al. [10]. The sequences of the primers are given in Table 1. Ice-bathed samples were quickly transferred in a thermal cycler (Perkin-Elmer Cetus 480, USA), which was preheated to 96°C for 2 min. Samples were then subjected to 45 cycles of amplification as follows: 94°C for 1 min, 36°C for 45 s, and 72°C for 2 min, followed by 10 min extension at 72°C. Each DNA template was amplified at least three times under the same conditions as described above. The amplified products were analyzed by electrophoresis of 10 μl of the amplification reaction mixture on 2% agarose gels run in Tris–acetate–EDTA buffer and detected using ethidium bromide staining.

### 2.3. ITS1 sequence analysis

Two primers, I-Fw (ctaccgattgaatggcttag) and I-Rev (agatccattgtcaaaagttgt) were designed based on the alignment ITS1 sequences of *Neocallimastix* spp. [8] and the relative positions within the rRNA gene structure are shown in Fig. 1. The polymerase chain reaction (PCR) followed the protocols of Moncalvo et al. [11]. The PCR products were purified using a PCR purification kit (Qiagen, Germany), cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into *E. coli* DH5α. Transformed *E. coli* was incubated in Luria–Bertani (LB) broth containing 100 μM ampicillin at 37°C with vigorous shaking. The resultant plasmids were purified using a mini-prep kit (Qiagen) and the sequence of their ITS1 inserts was determined by automatic sequencing (ABI Prism 337, Perkin Elmer). General DNA manipulation was performed as described in the kit manuals or as found in Sambrook et al. [14]. The computer program Bioedit (version 4.8.9) [15] was used to align the *Neocallimastix* strain sequences and to calculate their sequence similarity.

Table 1
RAPD primers used in this study

| Primer | Sequence | G+C content (%) |
|---|---|---|
| R1 | 5′-TGCCGAGCTG-3′ | 70 |
| R2 | 5′-AGTCAGCCAC-3′ | 60 |
| R3 | 5′-AATCGGGCTG-3′ | 60 |
| R4 | 5′-GAAACGGGTG-3′ | 60 |
| R5 | 5′-GCGATCCCCA-3′ | 70 |
| OPB-14 | 5′-TCCGCTCTGG-3′ | 70 |
| OPB-15 | 5′-GGAGGGTGTT-3′ | 60 |
| OPB-16 | 5′-TTTGCCCGGA-3′ | 60 |
| OPB-17 | 5′-GGAGGGTGTT-3′ | 60 |
| OPB-18 | 5′-CCACAGCAGT-3′ | 60 |
| OPB-19 | 5′-ACCCCCGAAG-3′ | 70 |
| OPB-20 | 5′-GGACCCTTAC-3′ | 60 |

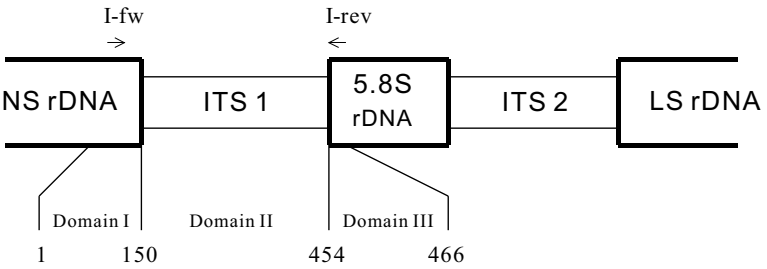


Fig. 1. The locations of the ITS1 PCR primers given in the text and the three domains of the PCR product sequences. NS rDNA represents the nuclear small rDNA and LS rDNA represents nuclear large rDNA. The regions of nuclear small rDNA and nuclear large rDNA are truncated in this figure.

## 3. Results and discussion

### 3.1. RAPD analysis

The 12 RAPD primers given in Table 1 were used to examine the DNA polymorphisms of *N. frontalis* SK-1, SK-3M and SK-1Y as well as heterokaryotic and homokaryotic *Ganoderma* strains, and their RAPD patterns are shown in Fig. 2. Distinct RAPD profiles were obtained from the *Ganoderma* strains using the primers OPB-15 (Fig. 2a) and OPB-19 (Fig. 2b), and the other RAPD primers gave similar results for each of the *Ganoderma* strains (data not shown). In OPB-15 profiles, the *Ganoderma* dikaryotic strain, RZ, showed seven fragments between 300 bp and 1.6 kb in size, while one monokaryon, RZ-2, showed several bands ranging from 200 bp to 1.6 kb, and another monokaryon strain, RZ-3, showed three products on agarose gel (Fig. 2a). OPB-19 also gave different patterns for RZ and its two basidiospor-derived strains (Fig. 2b). The difference in the DNA polymorphisms between the *Ganoderma* strain and two monokaryotic strains suggests that genetic similarity is not maintained unless the derived strain reproduced asexually.

The OPB-15 amplification patterns of the *N. frontalis* strains SK-1, SK-3M and SK-1Y are identical and consist of three bands between 200 and 650 bp in size (Fig. 2a). Using OPB-19, more than five fragments, ranging from 200 to 1000 bp, were obtained from the three *N. frontalis* strains and the three profiles are very similar (Fig. 2b). The other primers listed in Table 1, when profiled, gave the same patterns for all three different generations of strains as well. These results indicated that *N. frontalis* SK did not show any changes in pattern of DNA polymorphism that could be detected by RAPD profiling after one subculture, 50 subcultures or 150 subcultures. These results indicated that no significant genetic variation occurred during frequent subculture.

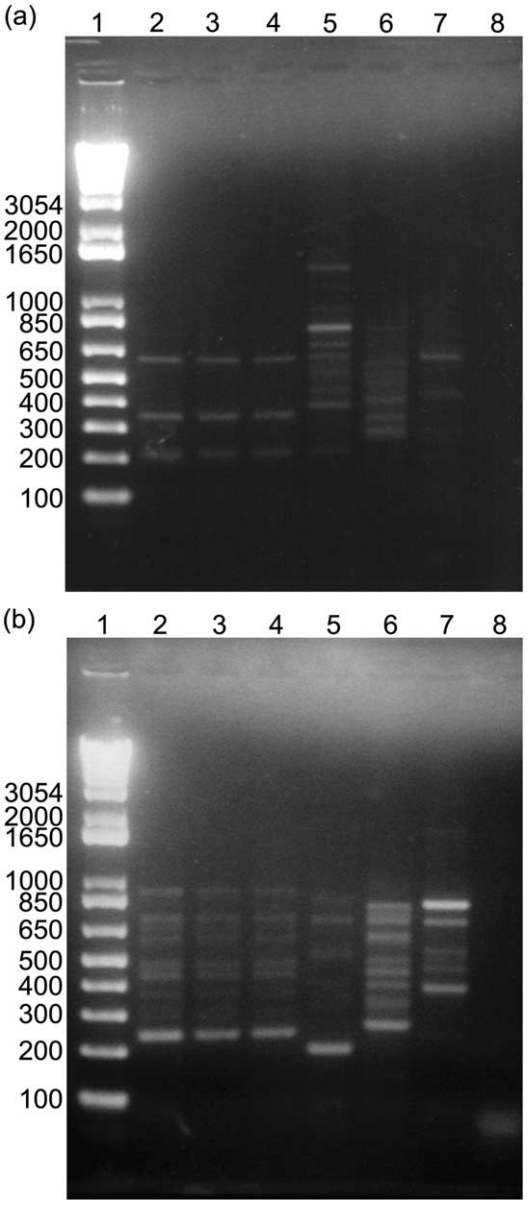


Fig. 2. The RAPD patterns of the *Neocallimastix* strains and the *G. lucidium* strains produced by the OPB-15 (a) and OPB-19 (b) primers; lane 1, 1 kb plus ladder marker (Invitrogen); lane 2, *Neocallimastix* sp. W-1; lane3, *N. frontalis* SK; lane 4, *N. frontalis* SK-3M; lane 5, *N. frontalis* SK-1Y; lane 6, *G. lucidium* RZ; lane 7, *G. lucidium* RZ-2; lane 8, *G. lucidium* RZ-3.

### 3.2. rDNA sequence analysis

The ITS1 and partial rDNA sequences of *N. frontalis* SK were analyzed and registered in GenBank under the accession number AY070440. The partial 18S rDNA and 5.8S rDNA and ITS1 sequences of different generations of


Fig. 3. A comparison of the partial ITS1 sequences from various *N. frontalis* SK generations and a series of *Neocallimastix* spp. accessed from GenBank. The access numbers of the sequences are shown in Table 2. The shaded regions indicate identical residues.

*N. frontalis* SK were divided into three domains after alignment [8] and this is presented in Fig. 1. Domain I (bases 1–150) represents the 3′ end of the 18S rDNA; domain II (bases 151–454) represents the ITS1 region; and domain III (bases 455–466) represents the 5′ end of the 5.8S rDNA. The results of the alignment show that domains I and III are highly conserved between *N. frontalis* SK and other *Neocallimastix* species (AF170192, AF170193, AF170194, AF170196, AF170197, AF170198, AF170199, AF170200, AF170201, and AY070440), while domain II is the most diverse one.

The sequence alignments shown in Fig. 3 illustrate that while there is a great deal of genetic variation between *Neocallimastix* spp., there is full conservation between different generations of *N. frontalis* SK. Although domain II sequences of *Neocallimastix* spp. are the most diverse among the three domains, the domain II sequences of *N. frontalis* SK-1, SK-3M and SK-1Y are identical and thus totally conserved (Fig. 3). Table 2 lists the identity matrix of three domains from various *Neocallimastix* spp. The similarity of the pairwise sequence comparison of *Neocallimastix* spp. accessed from GenBank ranges from 77.3 to 94.0% with a mean value of 82.1%. The minimum level of variation between two *Neocallimastix* isolates is 6%, and this contrasts with the ITS1 sequences of all three SK generations, which are identical. These results indicate that the ITS1 sequences of *N. frontalis* SK do not vary after 50 or 150 artificial subcultures.

Many researchers have suggested that variation should occur in the genome of offspring reproduced by a sexual cycle, and that the DNA polymorphism profiles of such an organism will not be the same as their parents or their siblings. These changes can be easily detected by RAPD profiling or RFLP analysis[7,10]. In this study, RZ-2 and RZ-3 isolated from *G. lucidium* RZ basidiospores showed significant variation in their RAPD profiles (Fig. 2). These results are consistent with the concept that sexual offspring have different DNA profiles. SK-1, SK-3M, and SK-1Y were subcultured from *N. frontalis* SK one, 50, and 150 times, respectively, and their ITS1 sequence and RAPD patterns remained identical. These results strongly suggest that SK-1, SK-3M and SK-1Y have retained the identical genetic characteristics.

Straatsma and Samson supported the ideal that strains are made up of one single variable species, and the different strains may be produced by a single species after the sexual part of the life cycles. A strain genotype will be changed during meiosis to give a monokaryon and after mating, when a dikaryon is formed [16]. The genetic invariability of the three *N. frontalis* SK strains in this study is consistent with the hypothesis that this strain is homokaryotic and grows via an asexual life cycle in vitro.

Table 2
ITS1 sequence identity matrix of *Neocallimastix* spp. and the various *N. frontalis* SK generations

| | SK-1 | SK3M | SK1Y | NUC1 | NMW2 | NMW3 | NMW4 | NMW5 | NMG2 | NCS1 | NCS2 | Nhur | MCH3 | Npat | NPW1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SK-1 | 1 | 1 | 1 | 0.91 | 0.851 | 0.892 | 0.874 | 0.878 | 0.888 | 0.914 | 0.859 | 0.89 | 0.902 | 0.804 | 0.768 |
| SK3M | – | 1 | 1 | 0.91 | 0.851 | 0.892 | 0.874 | 0.878 | 0.888 | 0.914 | 0.859 | 0.89 | 0.902 | 0.804 | 0.768 |
| SK1Y | – | – | 1 | 0.91 | 0.851 | 0.892 | 0.874 | 0.878 | 0.888 | 0.914 | 0.859 | 0.89 | 0.902 | 0.804 | 0.768 |
| NUC1 | – | – | – | 1 | 0.871 | 0.868 | 0.867 | 0.86 | 0.895 | 0.906 | 0.856 | 0.888 | 0.906 | 0.835 | 0.784 |
| NMW2 | – | – | – | – | 1 | 0.916 | 0.92 | 0.907 | 0.842 | 0.87 | 0.884 | 0.847 | 0.852 | 0.894 | 0.754 |
| NMW3 | – | – | – | – | – | 1 | 0.949 | 0.933 | 0.855 | 0.879 | 0.919 | 0.843 | 0.865 | 0.873 | 0.76 |
| NMW4 | – | – | – | – | – | – | 1 | 0.93 | 0.834 | 0.856 | 0.904 | 0.827 | 0.84 | 0.899 | 0.765 |
| NMW5 | – | – | – | – | – | – | – | 1 | 0.839 | 0.862 | 0.888 | 0.832 | 0.845 | 0.856 | 0.761 |
| NMG2 | – | – | – | – | – | – | – | – | 1 | 0.931 | 0.835 | 0.902 | 0.936 | 0.805 | 0.761 |
| NCS1 | – | – | – | – | – | – | – | – | – | 1 | 0.847 | 0.927 | 0.95 | 0.82 | 0.781 |
| NCS2 | – | – | – | – | – | – | – | – | – | – | 1 | 0.821 | 0.848 | 0.843 | 0.75 |
| Nhur | – | – | – | – | – | – | – | – | – | – | – | 1 | 0.916 | 0.801 | 0.75 |
| MCH3 | – | – | – | – | – | – | – | – | – | – | – | – | 1 | 0.812 | 0.772 |
| Npat | – | – | – | – | – | – | – | – | – | – | – | – | – | 1 | 0.75 |
| NPW1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 1 |

Note: The sequences shown in this table are *N. frontalis* SK-1 (SK, AY070440), *N. frontalis* SK-3M (SK3M), *N. frontalis* SK-1Y (SK1Y), *Neocallimastix* sp. NMW2 (NMW2, AF170198), *Neocallimastix* sp. NMW3 (NMW3, AF170199, *Neocallimastix* sp. NMW4 (NMW4, AF170200), *Neocallimastix* sp. NMW5, (NMW5, AF170201), *N. frontalis* NCS1 (NCS1, AF170194), *N. frontalis* NCS2 (NCS2, AF170196), *N. hurleyensis* Nhur, AF170193), *N. frontalis* MCH-3 (MCH3, AF170192), *N. patriciarum* (Npat, AF170197) and *Neocallimastix* sp. W-1 (NPW1, AY070440).

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