

## CHAPTER 6

### Analyses and Comparison of Ribosomal DNA Sequences

#### 6.1 INTRODUCTION

During the last few decades, molecular analyzing techniques have advanced the investigation of fungal phylogeny. Just the same as in other fungal taxa, these techniques have advanced phylogenetic studies of the Kickxellales. In the earliest work, Porter & Smiley (1979) compared molecular weights of 18S and 28S ribosomal RNA (rRNA), and then Walker (1984) analyzed 5S rDNA sequences of some kickxellalean species. In these investigations, the former rejected the affinity between the Kickxellales and the Harpellales (Porter & Smiley 1979), while the latter presented ambiguous, or rather a negative relationship between the orders (Walker 1984); however, both could not reach the final conclusion on the phylogenetic relationship between the orders.

Among the molecular analyzing techniques, DNA sequencing would be the most practical at the present since it can provide us information independent of the morphology of the objective fungi (McLaughlin et al. 1995), and thus a large number of studies using the technique have been conducted in the past decade. The sequencing has also been applied to the Kickxellales. O'Donnell et al. (1998) analyzed 18S rDNA sequences of the seven of eight kickxellalean genera known at that time, and indicated the polyphyly of the Kickxellales. They showed that the Kickxellales contains two clades; the clade of *Spiromyces* and that of the remaining genera, and the sister relationships between them and also between the two clades and the Harpellales clade varied depending on the outgroups employed for the analyses (O'Donnell et al. 1998). More recently, Tanabe et al. (2000) compared 18S rDNA sequences of the kickxellaleans with those of other orders of the Zygomycota, and concluded that the *Spiromyces* made a sister clade with the Zoopagales Bessey ex R. K. Benj. 1979 and the Harpellales, and the remaining kickxellalean genera might be in the sister relationship with the Dimargaritales. As these results show, the accurate phyletic position of the Kickxellales remains undecided up to the present.

Because the sequence is independent to the morphology (McLaughlin et al. 1995), it would be appropriate for the evaluation of the morphological groups of the chapter 4. Therefore, in this study, I tried to analyze the DNA sequences of some kickxellaleans to construct more reliable phyletic trees of the order. For this purpose, nuclear encoded 18S and 28S rRNA genes were selected; because

large comparable data are accessible at the present and the preceding studies (O'Donnell et al. 1998, Tanabe et al. 2000) have revealed that the sequences are supportive enough to determine clades within the Kickxellales.

*Myconymphaea yatsukahoi*, *Mycoemilia scoparia* (tentative name), and *Ramicandelaber brevisporus* (tentative name) were selected as the fungal materials because they were probable keystone species newly added in the present study and represented the morphological groups of the chapter 4.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Preparation of fungal materials

The isolates of *M. yatsukahoi*, *M. scoparia* (tentative name), and *R. brevisporus* (tentative name) were incubated in submerged cultures with the quarter-strength and agar free ME-YE medium at room temperature. Mycelia were harvested from these cultures and frozen at -30 °C, then physically broken up with a hammer. The total DNA was extracted from the broken mycelia with a DNeasy Plant Mini Kit (QIAGEN).

### 6.2.2 Polymerase chain reaction and sequencing of rDNA

From the extracted DNA, 18S and 28S rDNA were amplified with primer sets PNS1, NS2, NS3, NS4, NS5 and NS8Z (White et al. 1990, O' Donnell et al. 1998; Fig. 6-1) and NS1 and NL4 (O' Donnell 1993; Fig. 6-2), respectively. Polymerase chain reactions (PCR) were carried out in 50  $\mu$ l reaction volumes containing 0.5  $\mu$ l of 25  $\mu$ M each primer, 20.5  $\mu$ l of distilled water, 25  $\mu$ l of HotStarTaq™ (QIAGEN), 2.5  $\mu$ l of 10 $\times$ PCR buffer, and 1  $\mu$ l of the extracted DNA as the template. The PCR was executed in a GeneAmp PCR System 2400 (Perkin-Elmer) under the following conditions: 95 °C for 15 min; 45 cycles of 94 °C for 20 sec and 60 °C for 1.5 min; 72 °C for 10 min. Then, the amplified DNA was purified with a QIAquick PCR Purification Kit (QIAGEN).

Sequencing reactions were done manually using BigDye Terminator Cycle Sequencing FS Ready Reaction (Perkin-Elmer Applied Biosystems) in a GeneAmp PCR System 2400 (Perkin-Elmer), and the products were purified with DyeEX Spin Columns (QIAGEN). The sequence data were collected using an ABI PRISM™ 377-18 automated DNA sequencer.

### 6.2.3 Phylogenetic analyses

The 18S and 28S rDNA sequences determined in this study were aligned with

the sequences obtained from the rRNA WWW Server (University of Antwerp, <http://rrna.uia.ac.be/>) (18S rDNA) or the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) (28S rDNA) using Clustal W 1.81. Fifty-four (18S rDNA) and thirty-eight (28S rDNA) strains employed for the analyses are shown in Tables 6-1, 6-2. Positions with gaps were ignored for the analyses.

Phylogenetic trees were constructed using the neighbor-joining method (NJ method) and the maximum parsimony method (MP method) that had been programmed into PAUP\* 4.0b8 (Swofford 2001). NJ trees were constructed based on the HKY85 distance estimation model (Hasegawa et al. 1985). Due to the extensive base substitution in the 18S rRNA gene of the Dimargaritales, only the NJ tree was adopted as the results of 18S rDNA analyses. This is because the MP method is less faithful than the NJ method when the evolutionary rates of lineages are uneven (Saitou & Imanishi 1989, Hasegawa & Fujiwara 1993). For rooting these trees, 6 species of the Choanoflagellida (18S rDNA) and 8 species of the Ascomycota and the Basidiomycota (28S rDNA) were selected as outgroups. The reliability of each branches was evaluated by bootstrapping (Felsenstein 1985) with 1,000 resamplings in PAUP\* 4.0b8 (Swofford 2001).

### 6.3 RESULTS

Almost full sequences of 18S rDNA and the D1-D2 region of 28S rDNA of the species analyzed were determined. About 18S rDNA, the 5' ends of the sequences were analyzed only from 3' with the NS2 primer since the PNS1 primer (O' Donnell et al. 1998) did not suite well.

In the 18S rDNA analyses, the MP tree was largely concordant with the NJ tree except that *S. spiralis* directly connected to the root of the cluster that included the *Coemansia* clade, the Harpellales clade, and the cluster consisted of *S. minutus* and *M. scoparia* (tentative name). In this dissertation, only the NJ tree was presented in Fig. 6-3 and employed for discussions about the phylogeny. In the 28S rDNA analyses, the NJ and MP trees were coincident in general.

In all rDNA sequence-based trees (Figs 6-3, 6-4, 6-5), the Kickxellales *sensu* Benjamin (1979) was divided into three clades named *Coemansia* clade, *Spiromyces* clade, and *Ramicandelaber* clade, and the three species belonged to each clade. That is, *Mycoemilia* (tentative name) grouped with the *Spiromyces* species and composed the *Spiromyces* clade, *R. brevisporus* (tentative name) constructed *Ramicandelaber* clade independently, and *Myconymphaea* coupled with all the other

genera and composed the *Coemansia* clade with them.

In the 28S rDNA sequence-based trees, the *Spiromyces* clade and the remaining Kickxellales made a sister group supported by low bootstrap values (60% and 84%). In the 18S rDNA sequence-based tree, the *Ramicandelaber* clade and the Dimargaritales united with a Chytridiomycetes sp. (55%), while in the 28S trees, the clade consisted a larger clade with the *Conidiobolus* Bref. 1884 (Entomophthorales, Zygomycetes) and *Basidiobolus* Eidam 1886 (Basidiobolales, Zygomycetes) with low bootstrap values (34% and 46%). The larger clade that includes the *Coemansia* clade and the *Spiromyces* clade principally formed a sister clade with the Harpellales.

The lineages that were strongly supported included the *Coemansia* clade (bootstrap = 100%) in the 18S tree, and the *Spiromyces* clade (100%) and *Ramicandelaber* clade (100%) in the 28S trees. Moderate bootstrap values were obtained for the *M. scoparia* (tentative name)-*S. minutus* clade (93%) and the *Coemansia* and *Spiromyces*-Harpellales clade (87%) in the 18S tree, and the clade that coupled with *Myconymphaea* within the *Coemansia* clade (84% and 99%). In all the trees, large clades that contained *Coemansia* clade, *Spiromyces* clade, and *Ramicandelaber* clade were poorly supported.

#### 6.4 DISCUSSION

The rDNA sequence-based trees supported the *Coemansia* clade with high bootstrap value, and this clade united with *Spiromyces* clade in relatively poor bootstrap support. The larger cluster consisted of these two clade and the Harpellales clade made a sister group. The *Ramicandelaber* coupled with the Dimargaritales in the low bootstrap confidence. Thus, the exclusion of the species of the *Ramicandelaber* and *Spiromyces* clades from the Kickxellales appears to be appreciated, while the accurate relationships between the three clades of the Kickxellales and the Harpellales and the Dimargaritales remain unclear. Consequently, the morphological grouping of the chapter 4 was supported by the rDNA sequences analyses; each morphological group was identified to be phylogenetically independent.

*Myconymphaea* constructed a clade with *Linderina* and other genera of the *Coemansia* group. This result corroborates the taxonomical placement of *Myconymphaea* into the Kickxellales with high bootstrap value (100%), in spite of its septal plug nature intermediate between the Kickxellales and the Dimargaritales.

In showing the polyphyly in the Kickxellales, the present analyses concurred with O'Donnell et al. (1998) and Tanabe et al. (2000). The present study, however, demonstrated that the order was much more polyphyletic in comparison with these previous studies; A new clade, the *Ramicandelaber* clade was established, and a new genus, *Mycoemilia* (tentative name) was added to the *Spiromyces* clade that had been monogeneric.

Although the 18S and 28S rDNA sequences could identify the clades within the kickxellids, the sister group relationships between them are still undecided. Taking an example, the NJ tree of 18S rDNA sequences in the present analyses opposed Tanabe et al. (2000) concerning with the sister relationships between the *Coemansia* clade and the Dimargaritales. Such ambiguity is probably due to the insufficient sampling of taxa, in addition to the extensive base substitution in the gene of the Dimargaritales (Tanabe et al. 2000). The fact demands that we find more isolates of unknown taxa for building trees that are more consistent.

The rRNA genes would be the best choice at the present, but they are not the perfect ones. That is, analyses of the sequences of the genes could not show clear relationships at the species level. For example, in the 18S rDNA tree, *S. minutus* was in closer relationship with *M. scoparia* (tentative name) than *S. spiralis*. To take another example, by both the 18S and 28S rDNA analyses, the phyletic relationships between the species of the *Coemansia* clade were unidentified due to the robust similarities in the sequences of the genes of them. Probably, sequencing of another genes is needed to solve the relationships at the species level. The RNA polymerase II largest subunit gene, that had been regarded as the most probable candidate for it, was proved to be not so much useful phylogenetically (Tanabe et al. 2001). Thus, sequencing of other genes is required for further phylogenetic study.