The Journal of Microbiology, June 2000, p.62-65 Vol. 38, No. 2

Sequence Comparison of Mitochondrial Small Subunit Ribosomal DNA in *Penicillium*

Soon Gyu Hong, Yoon-Dong Park, Wonjin Jeong, and Kyung Sook Bae*

Korean Collection for Type Cultures, Genetic Resources Center,
Korea Research Institute of Bioscience and Biotechnology,
P.O. Box 115 Yusong, Taejon 305-600, Republic of Korea

(Received March 23, 2000 / Accepted May 3, 2000)

Partial sequence comparisons of mitochondrial small subunit rDNA (mt SSU rDNA) were used to examine taxonomic and evolutionary relationships among seven *Penicillium* species: two monoverticillate species, two biverticillate species, and three terverticillate species. Amplified fragments of mt SSU rDNA highly varied among seven species in size, suggesting the existence of multiple insertions or deletions in the region. A phylogenetic tree was constructed by exhaustive search of parsimony analysis. The phylogenetic tree distinguished two statistically supported monophyletic groups, one for two monoverticillate species and the other for three terverticillate species and one biverticillate species, *P. vulpinum*. The phylogenetic relationship of *P. waksmanii*, the biverticillate species, was not clear.

Key words: *Penicillium*, phylogeny, mt SSU rDNA

Penicillium is a genus of Hyphomycetes that produces conidia in chains from the verticils of phialides (18). Since teleomorphic information on the *Penicillium* species is limited, *Penicillium* taxonomy has depended primarily on anamorphic features such as size and color of colony and color and head structures of conidia and conidiophore (17, 21). Some physiological characteristics and secondary metabolite profiles were also applied to a further subdivision of these species (4, 8). However, these characteristics show significant variations within an isolate, and so may lead to confusion in delineating species (1). Such confusion consequently resulted in discrepancies among taxonomists in the classification of *Penicillium*. For example, Pitt (16) placed *Penicillium vulpinum* (Cooke et Massee) Seifert et Samson under biverticillate species but Stolk *et al.* (23) listed it as terverticillate species. In order to clarify these different views regarding the classification of *Penicillium* species, new and reliable investigative approaches are required.

Mitochondrial DNA (mt DNA) has several advantages when studying fungal taxonomy as well as evolution. Fungal mt DNA is smaller than nuclear DNA in size, ranging from 19 kb in the ascomycetous yeast, *Schizosaccharomyces pombe*, to 170 kb in the basidiomycete *Agaricus bitorquis* (5). Because of small size, it is easy to manipulate and study finger-printing patterns using restriction endonucleases. It has also been reported that the fungal mitochondrial genome shows high polymorphisms suitable for studying taxonomic relationships between or among species (14). Particularly, the sequence of mitochondrial small subunit ribosomal DNA (mt SSU rDNA) has been successfully used in recent years for fungal systematics and evolution (11, 22).

In this study, we present partial sequence comparisons of mt SSU rDNA to examine taxonomic and evolutionary relationships among mono-, bi-, and terverticillate *Penicillium* species. Besides the practical considerations regarding the taxonomic and evolutionary relationships among *Penicillium* species, the present study was designed to determine whether mt SSU rDNA could be used to resolve discrepancies among taxonomists for taxonomic positions of other problematic *Penicillium* species.

Materials and Methods

Strains

Seven *Penicillium* species examined in this study were obtained from KCTC (Korean Collection for Type Cultures): two monoverticillate, two biverticillate, and three terverticillate species (Table 1).

Culture conditions and isolation of genomic DNA

After growth of mycelia on Potato Dextrose agar plate at 25°C for 7-10 days, suspensions of conidia were inoculated into flasks containing 24 g potato dextrose broth (Difco)/L of tap water, followed by incubating at 25°C for

* To whom correspondence should be addressed.
(Tel) 82-42-860-4610; (Fax) 82-42-860-4677
(E-mail) ksbae@mail.kribb.re.kr

Table 1. Seven *Penicillium* species based on Pitts taxonomic scheme and their characteristic PCR product size amplified from mt SSU rDNA

Species name	Strain	Type	PCR procuct size	GenBank Acc. No.
Penicillium lividum	KCTC 6261 (NRRL 754)	Monoverticillate	508 bp	AF241470
Penicillium thomi	KCTC 6271 (NRRL 2077)	Monoverticillate	468 bp	AF241471
Penicillium vulpinum	KCTC 6267 (NRRL 2031)	Biverticillate	478 bp	AF241467
Penicillium waksmanii	KCTC 6263 (NRRL 777)	Biverticillate	599 bp	AF241472
Penicillium aurantiogriseum var. *neoechinulatum*	KCTC 6257 (NRRL 13486)	Terverticillate	478 bp	AF241468
Penicillium chrysogenum	KCTC 6052 (ATCC 10106)	Terverticillate	470 bp	AF241466
Penicillium verrucosum	KCTC 6265 (NRRL 965)	Terverticillate	474 bp	AF241469

30 h with shaking at 180 rpm. Mycelia were harvested by vacuum filtration and rinsed in distilled water. Mycelia were freeze-dried overnight, followed by grinding with sea sand to a fine powder. Isolation of genomic DNA followed the method of Hwang *et al.* (12).

Primer synthesis and amplification of mt SSU rDNA

Primers used for the polymerase chain reaction (PCR) were designed on the basis of the mt SSU rDNA sequence of *P. chrysogenum* KCTC 6052 (22) and synthesized by the phosphoramidite method on a DNA synthesizer (Applied Biosystem Model 392). The primers were 5'-GATGGCTCTAACTGAACAC-3' (15-36 nt) and 5'-GCACGTAGTTTGGTCAAG-3' (483-466 nt). Amplificaiton reaction mixtures were 100 μl in volume, and contained 10 mM Tris-HCl (pH 8.8), 500 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin, 0.5% Tween-20, 0.5% NP-40, 20 μM primers, 250 μM each of dATP, dCTP, dGTP and dTTP (Perkin-Elmer-Cetus), 100 ng of template DNA, and 5 units of *Taq* DNA polymerase (Perkin-Elmer-Cetus). The reaction mixtures were overlaid with mineral oil (Sigma) and placed in an MJ Research DNA thermal cycler (model PTC-100-60). Amplification reactions were performed after 5 min of pre-incubation at 95°C to enhance denaturation of template DNA. The amplification protocol consisted of 40 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min.

Cloning and sequencing

The PCR products were purified by GenClean kit (Bio101), followed by treating with Klenow DNA polymerase to generate blunt-ended termini. These fragments were ligated into an *Eco*RV site of pBluescript. Clones carrying partial sequences of mt SSU rDNA were constructed in *E. coli* XL1-Blue, following the standard protocol described by Sambrook *et al.* (19). Cloned sequences were determined by the dideoxy termination method using Sequenase (US Biochemical, version 2.0).

Phylogenetic analysis

The mt SSU rDNAs partially sequenced from seven *Penicillium* species were aligned on the basis of the secondary structure model using the PHYDIT program (3). Ambiguously aligned V1 and V2 domains were excluded from further phylogenetic analyses.

Phylogenetic relationships were estimated using the parsimony criterion and exhaustive search option of PAUP* 4.0 beta version (24). To evaluate the strength of support for branches in parsimony trees, we used bootstrap program from PAUP*. In addition to using parsimony methods, distance-based trees based on Kimura's two-parameter model and neighbor-joining methods were generated with NEIGHBOR from the PHYLIP 3.5 package (7).

Results and Discussion

Sequence comparisons

As shown in Fig. 1 and Table 1, the sizes of the amplified fragments varied among seven species, suggesting the existence of length mutations in mt SSU rDNA. In particular, the fragments amplified from *P. lividum* and *P. waksmanii* were much larger than those from other species (Table 1). From the sequence alignment (Fig. 1), we could determine that *P. lividum* and *P. waksmanii* had multiple insertions in stem 6 (V1 domain) and in stems 9, 10 and 11 (V2 domain) of the prokaryotic SSU rRNA secondary structure model, respectively (15). The results indicate that the regions amplified are highly susceptible to mutations such as insertions and deletions. This finding may be consistent with Dujon's report (6) that optional intron, insertion or deletion occurs in high degrees, causing size differences among fungal mitochondrial DNAs. High degrees of insertions and deletions were also found in other fungal mitochondrial DNAs (2, 9, 10, 13).

Taxonomic position of P. vulpinum and evolutionary relationships of seven species.

Parsimony analysis produced two most parsimonious trees. One of them was presented in Fig. 2. It correlated with the neighbor-joining tree based on Kimura's two-parameter model. The other tree was different in the relationships among three terverticillate species and *P. vulpinum*. Three terverticilate species made the monophyletic group and *P. vulpinum* was related to them as a sister group. However, monophyly of four species, including

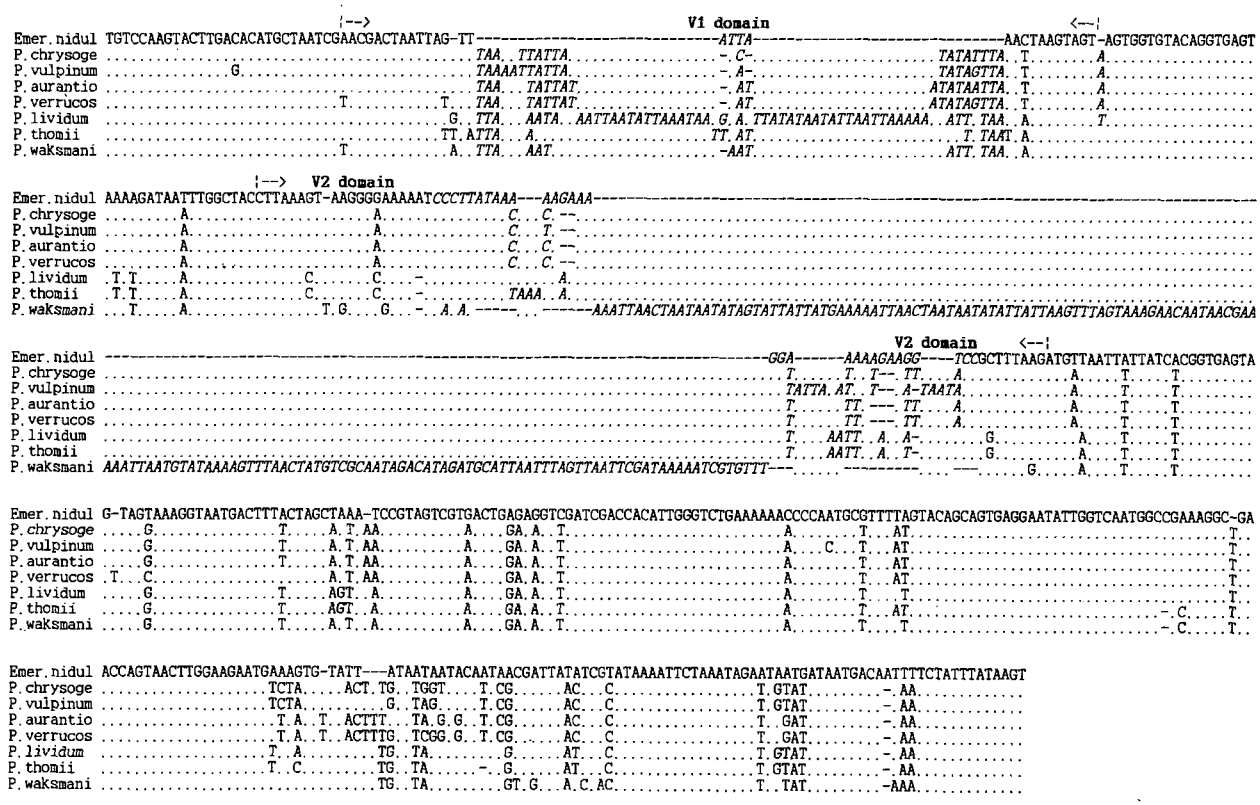

Fig. 1. Aligned sequences of mt SSU rDNA from seven *Penicillium* species. Nucleotides presented in italics represent nucleotides excluded from the phylogenetic analysis, due to ambiguities in alignment.

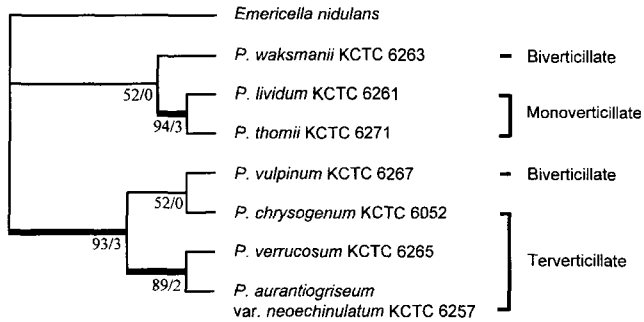

Fig. 2. One of the two most parsimonius trees constructed by the exhaustive search option of PAUP*, which correlated with the distance-based neighbor-joining tree. *Emericella nidulans* was used as an outgroup. Bootstrap values were calculated by 1000 bootstrap-resamplings of the data set using the parsimonius criterion. Branches supported by bootstrap values higher than 70% in both distance and parsimony analyses were indicated by thick lines. Decay indices were calculated by the filtering option of PAUP 4.0. The tree was constructed from 29 parsimony-informative sites among 392 total aligned sites. Consistency and retention indices (CI and RI) were 0.871 and 0.750, respectively.

three terverticillate species and *P. vulpinum*, was maintained in the two most parsimonious trees and one distance-based tree and supported by a high bootstrap value and decay index. Therefore, it is clear that *P. vulpinum* is closely related to the terverticillate species rather than monoverticillate or other biverticillate species in phylogenetic analysis of mt SSU rDNA. Placement of *P. vulpinum* in the subgenus *Penicillium*, the terverticillate taxon, was previously suggested by Stolk *et al.* (23) based on the fact that acuminate phialide necks found in the subgenus *Biverticillium* were not observed in *P. vulpinum*. They also observed clearly different structures of conidiophore and phialide morphology. Furthermore, secondary metabolites produced by *P. vulpinum* were different from those produced by biverticillate species (20). For example, this fungus produces patulin and roquefortine C, which were not produced by the biverticillate species. Thus, the results suggest that *P. vulpinum* may be taxonomically closer to the terverticillate species rather than the biverticillate species.

P. waksmanii, the biverticillate species, was presented to have a close relationship with the monoverticillate species in Fig. 2. However, monophyly of *P. waksmanii* and two monoverticillate species was not strongly supported by a bootstrap value. Moreover, a strict consensus tree of the two most parsimonious trees did not retain the group. Therefore, it is premature to assign relationships among three groups based on its branching type, namely mono-, bi- and terverticillate.

In conclusion, the results indicate that sequence of mt SSU rDNA is a useful means for studying systematics and evolution of the *Penicillium* species. The sequence divergence of mt SSU rDNAs clearly separated those species

into three different groups, corresponding with their branch type. However, relationships among the three groups were not clear. The results suggest that *P. vulpinum* is taxonomically more affiliated with terverticillate species than with the biverticillate species.

Acknowledgments

We are very grateful to Drs. Stephen W. Peterson and Martha Christensen for critical comments on the manuscript. We also appreciate Dr. Stephen W. Peterson for providing the cultures. This study was supported by the Korean Ministry of Science and Technology.

References

1. Bridge, P.D., L. Hudson, Z. Kozakiewicz, A.H. Onions, and R.R.M. Paterson. 1987. Investigation of variation in phenotype and DNA content between single-conidium isolates of single *Penicillium* strains. *J. Gen. Microbiol.* 133, 995-1004.
2. Bruns, T.D., T.J. White, and J.W. Taylor. 1991. Fungal molecular systematics. *Annu. Rev. Ecol. Syst.* 22, 525-564.
3. Chun, J. 1995. Computer-assisted classification and identification of actinomycetes. Ph. D. Thesis. University of Newcastle, UK.
4. Cigler, T.D., D.I. Fennell, G.A. Sansing, R.W. Detroy, and G.A. Bennett. 1973. Mycotoxin-producing strains of *Penicillium viridicatum*: classification into subgroups. *Appl. Microbiol.* 26, 271-278.
5. Dujon, B. 1983. Mitochondrial genes, mutants and maps: a review, p. 1-24. *In* R.J. Schweyen, K. Wolf, and F. Kaudewitz (eds.), Mitochondria. Walter de Gruyter & Co., Berlin.
6. Dujon, B. 1989. Group I introns as mobile genetic elements: fact and mechanistic speculations-a review. *Gene* 82, 91-113.
7. Felsenstein J. 1993. PHYLIP (Phylogeny inference package) version 3.5c. University of Washington.
8. Frisvad, J. 1981. Physiological criteria and mycotoxin production as aids in identification of common asymmetric Penicillia. *Appl. Environ. Microbiol.* 41, 568-579.
9. Garber, R.C. and O.C. Yoder. 1985. Mitochondrial DNA of the filamentous ascomycete *Cochliobolus heterostrophus. Curr. Gent.* 22, 191-195.
10. Gonzalez, P. and J. Labarere. 1998. Sequence and secondary structure of the mitochondrial small-subunit rRNA V4, V6, and V9 domains reveal highly species-specific variations within the genus *Agrocybe. Appl. Environ. Microbiol.* 64, 4149-4160.
11. Govind, N.S., K.L. McNally, and R.K. Trench. 1992. Isolation and sequence analysis of the small subunit ribosomal RNA gene from the euryhaline yeast *Debaryomyces hansenii. Curr. Genet.* 22, 191-195.
12. Hwang, B.-K., A.W.A.M. DeCock, G. Bahnweg, H.H. Prell, and R. Heitefuss. 1991. Restriction fragment length polymorphisms of mitochondrial DNA among *Phytophthora capsici* isolates from pepper (*Capsicum annuum*). *System. Appl. Microbiol.* 14, 111-116.
13. Matin, F.N. 1990. Variation in the ribosomal DNA repeat unit within single-oospore isolates of the genus *Pythium. Genome* 33, 585-591.
14. Moulinier, T., G. Barroso, and J. Labarere. 1992. The mitochondrial genome of the basidiomycete *Agrocybe aegerita*: molecular cloning, physical mapping and gene location. *Curr. Genet.* 21, 499-505.
15. Neefs, J.-M., Y. Van de Peer, P.D. Rijk, S. Chapelle, and R.D. Wachter. 1993. Compilation of small ribosomal subunit RNA structures. *Nucl. Acids Res.* 21, 3025-3049.
16. Pitt, J.I. 1979. The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. Academic Press, London.
17. Pitt, J.I. 1989. Recent developments in the study of *Penicillium* and *Aspergillus* systematics. *J. Appl. Bacteriol. Sym. Suppl.* 18, 37S-45S.
18. Raper, K.B. and C. Thom. 1949. A manual of the Penicillia. Williams and Wilkins, Baltimore, Maryland.
19. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular clonig, a laboratory manual. Cold Spring Harbor Laboratory Press, New York.
20. Samson, R.A., A.C. Stolk, and J.C. Frisvad. 1989. Two new synnematous species of *Penicillium. Stud. Mycol.* 31, 133-143.
21. Samson, R.A., A.C. Stolk, and R. Hadlock. 1976. Revision of the subsection *Fasciculata* of *Penicillium* and some allied species. *Stud. Mycol.* 11, 1-47.
22. Sheen, J., Y.H. Kho, and K.S. Bae. 1993. Genomic sequence of mitochondrial genes coding for ATPase subunit 6 and small subunit ribosomal RNA from *Penicillium chrysogenum*: a key for molecular systematics on fungi. *Nucleic. Acids Res.* 21, 4393.
23. Stolk, A.C., R.A. Samson, J.C. Frisvad, and O. Filtenborg. 1990. The systematics of the terverticillate Penicillia, p. 121-137. *In* A.R. Samson and J.I. Pitt (eds.), Modern concepts in *Penicillium* and *Aspergillus* classification. Plenum Press, New York and London.
24. Swofford, D.L. 1998. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.