Biol. Bull. NTNU. 1996. 31 (1): 47-53

Analysis of Genetic Diversity of Two Intersterility Groups of *Ganoderma australe* by DNA Sequencing

Chung-Yuh Chang, Zeng-Yung Yeh, and Guey-Jen Lee-Chen*
Department of Biology, National Taiwan Normal University
Taipei, Taiwan 117, Republic of China

ABATRACT

Genetic diversity of two intersterility groups of *Ganoderma australe* (Fr.) Pat. (subgenus *Elfvingia*) was investigated by polymerase chain reaction (PCR) and DNA sequencing. *G. neo-japonicum* Imaz. (subgenus *Ganoderma*) was selected for comparative studies. The DNA fragments containing internal transcribed spacer (ITS) regions were amplified by using oligonucleotide primers complementary to rRNA genes of *Saccharomyces cerevisiae*. The PCR-amplified products were subcloned into pGEM-T vector and sequenced. Phylogenic structures constructed from analyses of ITS regions suggest that two intersterility groups of *G. australe* are genetically differentiated.

Key words: *Ganoderma australe*, intersterility group, internal transcribed spacer, DNA sequencing

Introduction

The genus *Ganoderma*, a member of Aphyllophorales, was established by Karsten in 1881 and composed of over 250 species (Corner, 1983) with worldwide geographical distribution and broad host range including hardwoods, conifers, bamboos and palms. Based on the structure of pilear crust, genus *Ganoderma* is divided into subgenus *Elfvingia* (non-laccate species) and subgenus *Ganoderma* (laccate-containing species) (Corner, 1983). The macroscopic (such as pileus, stipe, context, tube) and microscopic (such as hyphal system, basidiospore) characters have been used to distinguish species within the genus *Ganoderma*. However, characters such as basidiocarp shape, basidiospore size and context color are influenced by environmental factors (Steyaert, 1975; Chen, 1993), the species identification and circumscription are often difficult and controversory (Steyaert, 1980; Corner, 1983; Zhao, 1989). Additional taxonomic characters such as cultural studies, isozyme electrophoretic phenotype and intercompatibility studies have been employed to verify the systematics of the genus *Ganoderma* (Adaskaveg and Gilbertson, 1986; Hseu, 1990; Peng, 1990; Yeh and Chen, 1990; Wang and Hua, 1991).

Recently, sequence characterizations of ribosomal RNA has brought great burgeoning of molecular phylogeny (Hibbett, 1992; Olsen and Woese, 1993). The rDNA repeat of fungi contains coding (functional) regions for 5 8S, 18S and 25S rRNAs along with internal transcribed spacer (ITS) regions (Restrepo and Barbour, 1989). A high level of conservation in the restriction endonuclease recognition sites has been observed in the coding region of rDNA for different species of *Saccharomyces* (Verbeet *et al.*, 1983). The intergenic regions, ITSs, were more variable than the coding regions for all fungi (Bruns *et al.*, 1991). The ITS regions have been applied for fungal systematics, including *Leptosphaeria* (Xue *et al.*, 1992), *Phytophthora* (Lee and Taylor, 1992), Sclerotiniaceae (Carbone and Kohn, 1993), rusts (Zambino and Szabo, 1993), *Talaromyces* and *Pencillium* (LoBuglio *et al.*, 1993), and *Ganoderma* (Moncalvo *et al.*, 1995).

Isolates of *G. australe* (Fr.) Pat., collected from hardwoods of different localities, were examined based on morphology and taxonomy of basidiocarps (Yeh, 1990; Yeh, unpublished data). The mating system of *G. australe* was determined as heterothallic and tetrapolar (Yeh and Chen, 1990). By compatibility and cultural studies, *G. australe* complex is separated into two intersterility groups (Yeh and Chen, 1990). Accordingly, the genetic variation between the two intersterility groups was investigated by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis of rRNA gene (Yeh *et al.*, 1995). Meanwhile, we further examined rDNA sequence

* Corresponding author: Guey-Jen Lee-Chen, FAX: 886-2-9312904

Table 1. List of *Ganoderma* isolates used in this study.

Isolate	Locality	Altitude (m)	intersterility group
G. australe (Fr.)Pat. TAI-01	Taipei, Taiwan	< 500	1
G. australe FL-01	Florida, USA	< 500	1
G. australe TAI-08	Taoyuan, Taiwan	1650	2
G. australe TAI-17	Taoyuan, Taiwan	1650	2
G. neo-japonicum Imaz. CCRC 36049	Miaoli, Taiwan	< 500	

Table 2. List of oligonucleotide primer sequences and positions on *S. cerevisiae* rDNA repeat.

Name	Nucleotide sequence 5' → 3'	Position on *S. cerevisiae* rRNA
BMB	GTACACACCGCCCGTCG	1624-1640 in 17S rRNA
5.8S	CGCTGCGTTCTTCATCG	50-34 in 5.8S rRNA
5.8S-R	TCGATGAAGAACGCAGC	34-50 in 5.8S rRNA
LR1	GGTTGGTTTCTTTTCCT	72-56 in 25S rRNA

variation on the above two groups by DNA sequencing. Type strain of *G. neo-japonicum* Imaz. CCRC 36049 (subgenus *Ganoderma*) was selected for comparative studies. Phylogenic structures based on analyses of ITS regions suggest that two intersterility groups of *G. australe* are genetically differentiated.

Materials and Methods

Culture and DNA Preparation

Isolates used are listed in Table 1. Identification of *G. australe* collected in Taiwan was based on Yeh (1990). *G. australe* FL-01, which was compatible with monokaryotic culture of *G. australe* TAI-01 (Yeh, unpublished data) was collected in Florida, USA. Type culture of *G. neo-japonicum* CCRC 36049 was obtained from Culture Collection and Research Center, Hsinchu, Taiwan. The culturing, harvesting, and DNA preparation were done as previously described (Yeh *et al.*, 1995).

PCR Amplification

Precautions were taken to avoid cross-contamination from handling of many identical PCR reactions (Kwok and Higuchi, 1989). The oligonucleotide primers used and their locations on the rDNA are shown in Table 2. Primers BMB and 5.8S were used to amplify fragment containing ITS 1. Primers 5.8S-R and LR1 were used to amplify fragment containing

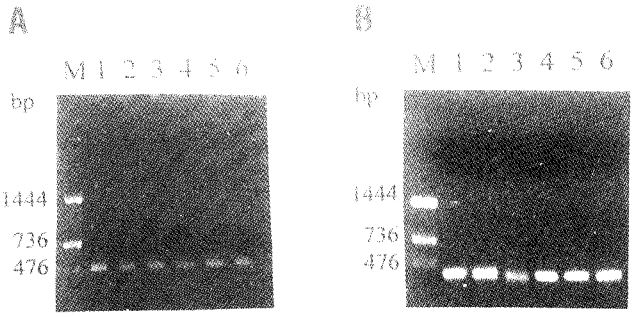

Figure 1. Photographs of 1.4% agarose gel electrophoresis of PCR-amplified DNA fragment containing ITS 1 (**A**) or ITS 2 (**B**). Lane M (*Taq*I digest of pUC19 DNA) is used as size markers. Lanes 1-2, *G. neo-japonicum* CCRC 36049. Lanes 3-6, *G. australe* isolates TAI-01, FL-01, TAI-08, and TAI-17.

ITS 2. The PCR reactions were conducted with 1 U DynaZyme DNA polymerase (Finnzymes Oy) in a total volume of 25 µl, using 200 µM dNTP, 0.08 µM primers, 100 ng DNA and buffer conditions recommended by the manufacturer. Thirty-five PCR cycles were performed on an automated thermocycler (OminiGene, HYBAID), using the following parameters: 95°C denaturation for 30 s, 57°C annealing for 45 s, and 72°C extension for 1 min. A final 7 min at 72°C was added after the final cycle to ensure complete polymerization of any remaining PCR products. PCR products were checked by running 2 µl of each reaction mixture on agarose minigels.

DNA Sequencing

PCR-amplified DNA fragments containing ITS regions were gel purified, subcloned into pGEM-T (Promega), and sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) with T7 DNA polymerase (Pharmacia LKB Biotechnology AB). The sequencing products were then separated on a 6% polyacrylamide gel, and the separation was monitored on line with an automated laser fluorescent DNA sequencer (A.L.F.; Pharmacia LKB Biotechnology AB).

Data Analysis

DNA sequences were aligned with the HIBIO DNASIS program (HITACHI) and later adjusted visually. The sequences of *G. australe* TW (GenBank SAO705IT and GAS2, 1994) were included for analyses. The cladistic analyses of sequencing data from separate ITS 1 and ITS 2 and from combined values were performed by maximum parsimony with Phylogenetic Analysis

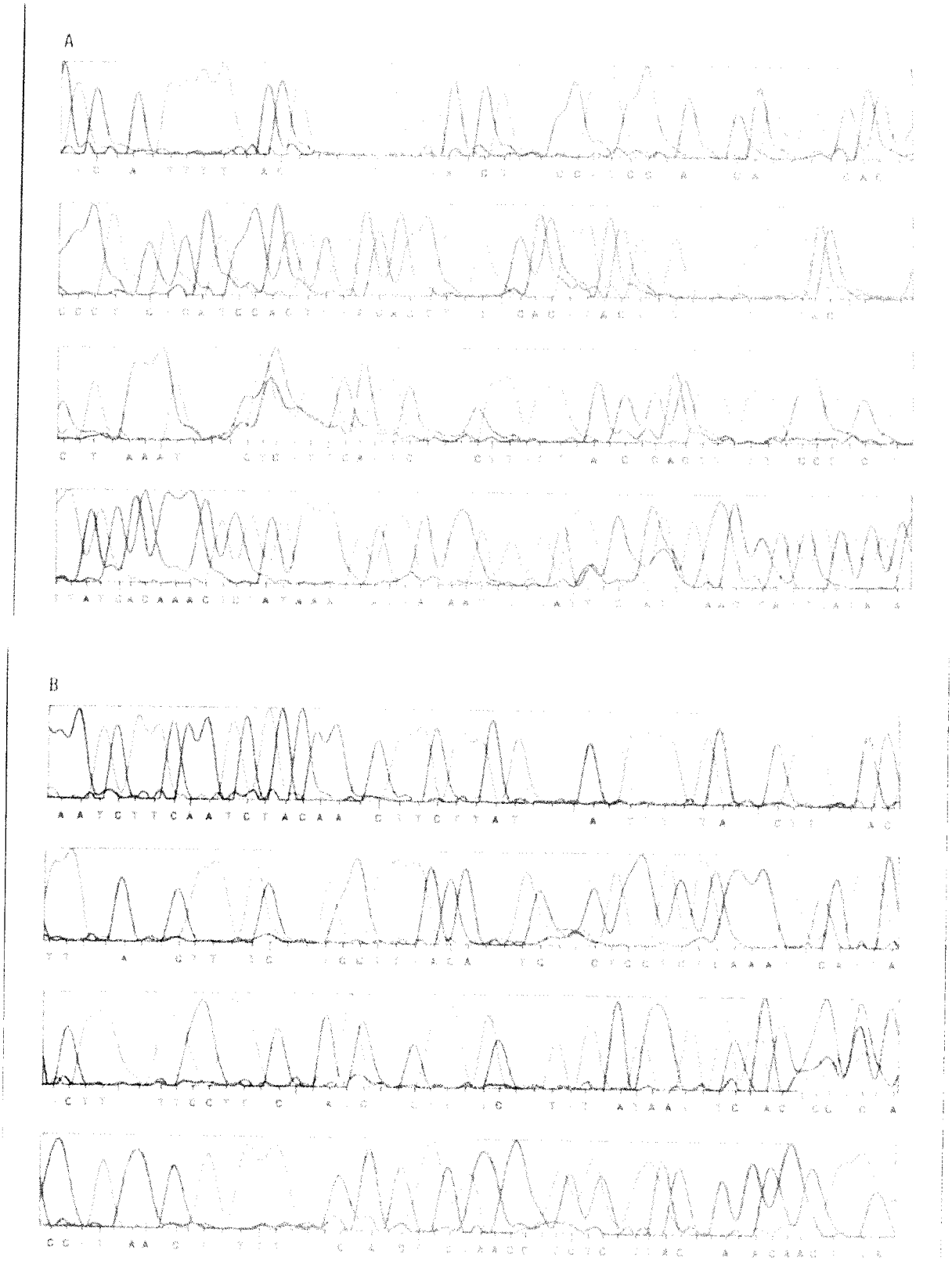

Figure 2. The sequencechromatograms of ITS1-F (**A**) and ITS2 (**B**) from *G. australe* FH-01 isolate.

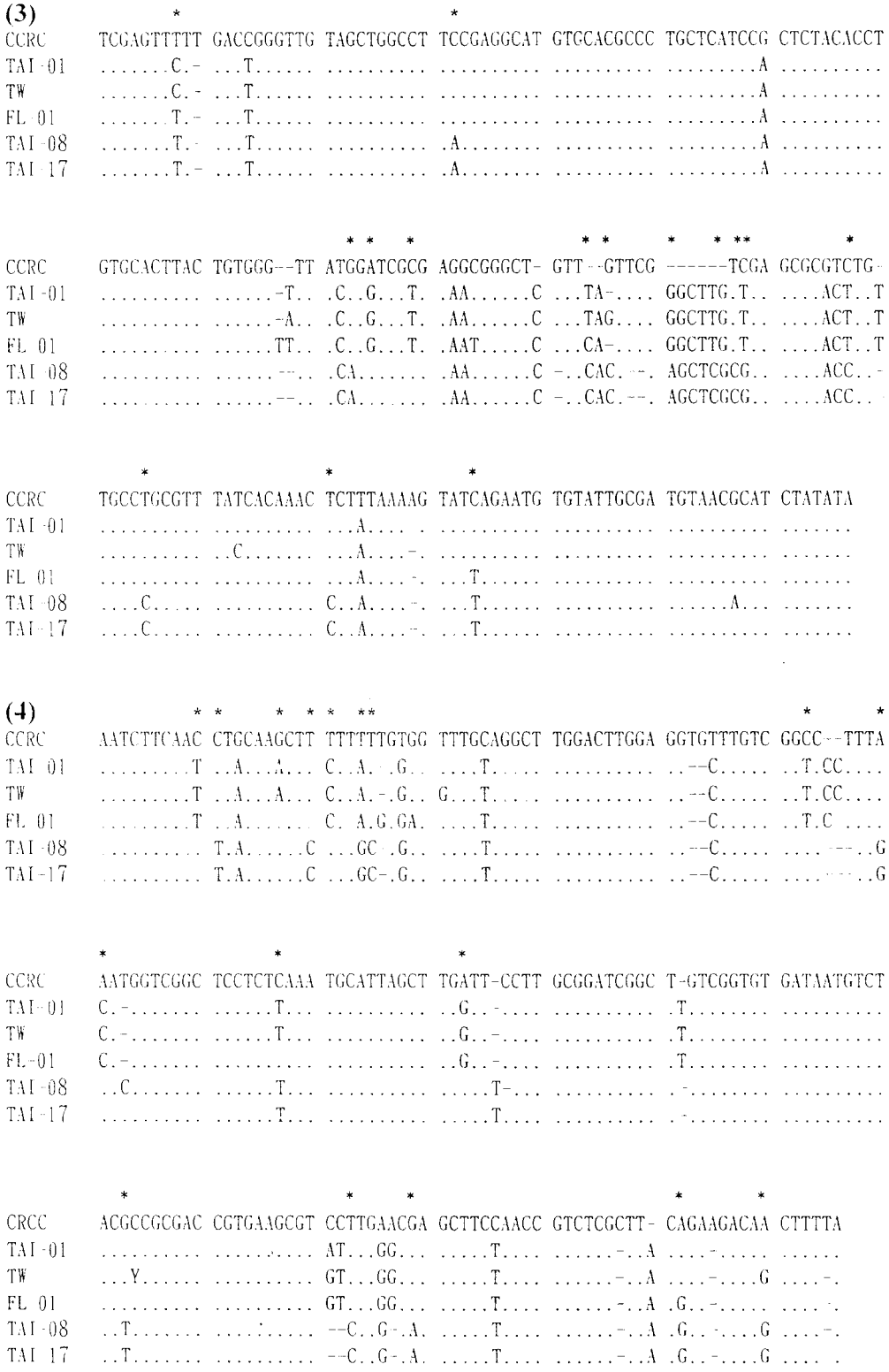

Figure 3-4. Aligned sequence data of ITS 1 **(3)** and ITS 2 **(4)** from isolates listed in Table 1. DNA sequences were aligned with the HIBIO DNASIS program (HITACHI). A dash (-) indicates a gap inserted to maintain alignment. Dots (.) in the sequences indicate conserved bases. Stars (*) indicate phylogenetically informative sites as defined in the PAUP manual. The sequences of *G. australe* TW were from GenBank SAO705IT (ITS 1) and GAS2 (ITS 2), 1994.

Table 3. The numbers of indels of different lengths in amplified ITS 1 and ITS 2 regions.

	ITS 1 (bp)						ITS 2 (bp)						Total
	1	2	3	4	5	6	1	2	3	4	5	6	
TAI-01	4	0	0	0	0	0	5	1	0	0	0	0	10
TW	3	0	0	0	0	0	6	1	0	0	0	0	10
FL-01	3	0	0	0	0	0	6	1	0	0	0	0	10
TAI-08	4	2	0	0	0	0	7	2	1	0	0	0	16
TAI-17	4	2	0	0	0	0	6	2	1	0	0	0	15
CCRC	2	2	0	0	0	1	3	1	0	0	0	0	9
Total	20	6	0	0	0	1	33	8	2	0	0	0	70

Table 4. Percentage[a] sequence divergence between taxa, based on nucleotide sequence of ITS 1[b] and ITS 2[b].

	1	2	3	4	5	6
1. TAI-01	-	1.5	2.5	8.5	8.0	7.3
2. TW	1.5	-	3.0	8.5	8.0	7.3
3. FL-01	2.5	3.0	-	7.0	6.5	7.3
4. TAI-08	8.4	8.4	8.4	-	0.5	8.9
5. TAI-17	8.4	8.4	8.4	0.5	-	8.4
6. CCRC	8.7	9.8	9.2	9.5	8.9	-

[a]Pairwise comparisons of percentage sequence divergence as determined in PAUP.

[b]Values above and below the diagonal are the ITS 1 and ITS 2 regions, respectively.

Using Parsimony (PAUP, version 3.0, Swofford, 1990). Parsimony analyses were conducted using heuristic searches with TBR branch swapping, pairwise addition of 10 random replicates, accelerated transformation (ACCTRAN), and retention of multiple most parsimonious trees (MULPARS). The fit of character data on phylogenetic hypotheses was evaluated and calculated by the consistency index, CI and the retention index, RI. The confidence of the internal branches of the tree was tested by bootstrapping with 200 replicates of heuristic searches on the 50% majority rule trees. The nodes with bootstrap values greater than 0.70 are significantly supported with >95% probability.

Results and Discussion

Amplification and sequencing of rDNA

The locations of primer sites for PCR amplification of portions of the rDNA repeat are shown in Table 2. These oligonucleotide primers represent highly conserved consensus regions between published rRNA sequences for several fungi and eukaryotes (White *et al.*, 1990). Fragments containing ITS 1 and ITS 2 were successfully amplified from all isolates tested (Figure 1). The length of amplified fragments is about 400 bp for both ITS regions. No gross length heterogeneity was observed. The PCR-amplified products were subcloned into pGEM-T vector and sequenced by automated DNA sequencer. The sequencing results from ITS regions of *G. australe* FL-01 isolate are shown in Figure 2. The ITS regions from *G. australe* FL-01 are 204 bp for ITS 1 and 198 bp for ITS 2 in length.

ITS sequences variation

Alignment of nucleotide sequences in the ITS regions is shown in Figures 3 and 4. The size of ITS 1 ranged from 195 to 204 bp and sequences were aligned in 207 positions, of which 29 (14.0 %) were variable and 15 (7.2 %) were phylogenetically informative. ITS 2 ranged from 192 to 201 bp and sequences were aligned in 206 positions, of which 31 (15.0 %) were variable and 17 (8.3 %) were phylogenetically informative. The frequency of nucleotide substitutions was similar in the two ITS regions. Variations were mostly located in the central region in ITS 1 and close to the termini in ITS 2 as reported (Moncalvo *et al.*, 1995).

A number of 1-6 bp insertions/deletions (indels) contribute to the length variation were observed. Indels of different size are catalogued in Table 3. In 70 indel events, 53 (75.7 %) are single base indels, 14 (20.0 %) are two-base indels, 3 (4.3 %) are three- to six-base indels. In 19 *Ganoderma* species studied, the lengths of ITS 1 and ITS 2 ranged from 197 to 204 bp and 185 to 196 bp, respectively (Moncalvo *et al.*, 1995).

Genetic Distance and Phylogeny Reconstruction

Pairwise comparison of ITS sequence divergence between isolates is shown in Table 4. Nucleotide divergence among isolates ranged from 0.5 to 9.8 %. Three *G. australe* group 1 isolates (TAI-01, TW and FL-01) diverged in 1.5-3.0 % in both ITS regions. *G. australe* FL-01 was shown to be closely related to group 1 isolates of *G. australe* in Taiwan by cultural and intercompatibility studies (Yeh, unpublished data). Two *G. australe* group 2 isolates (TAI-08, TAI-17) diverged in 0.5 % in both ITS regions. Sequence divergence between two groups of *G. australe* were 6.5-8.5 % in ITS 1 and 8.4 % in ITS 2, which is close to those between *G. neo-japonicum* and *G. australe* (7.3-8.9 % in ITS 1, 8.7-9.8 % in ITS 2).

Fifteen phylogenetically informative sites were unambiguously aligned in the ITS 1 region and seventeen in the ITS 2 region. Phylograms depicted were produced from ITS 1, ITS 2 and combined values of ITS

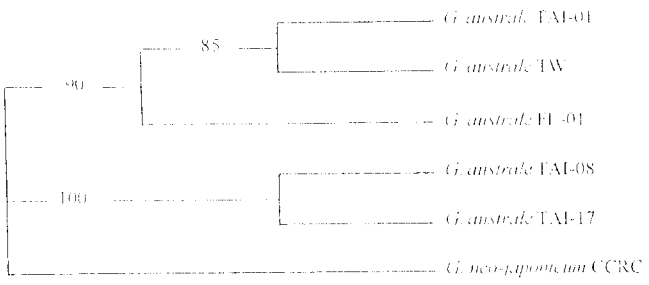

Figure 5. Phylogenetic relationships of isolates inferred from sequences of ITS regions. Only cladistically informative sites as defined in the PAUP were used in the phylogenetic analysis. Phylogram was generated from 200 bootstrap replications. The confidence levels of bootstrap replications (numbers in percentage) above the branches are averaged from ITS 1, ITS 2 and combined values of ITS regions.

regions. *G. neo-japonicum* (subgenus *Ganoderma*), which is shown more distant related while it is compared to *G. australe* (subgenus *Elfvingia*), is selected as an outgroup for analyses. Different analyses produced the same topological tree (Figure 5). *G. australe* TAI-01, TW and FL-01 fall in one group, whereas TAI-08 and TAI-17 fall in another group. The two groups are reproductively separated by compatibility and cultural studies (Yeh and Chen, 1990; Yeh, unpublished data). *G. australe* TAI-08 and TAI-17 (group 2 isolates) were collected from mountain area (altitude above 1600 m) whereas *G. australe* TAI-01 (group 1 isolate) and TW were collected from lower land (altitude below 500 m) in Taiwan (Yeh, 1990; Hseu, 1990). *G. australe* FL-01 was collected from lower plain in Florida, USA (Yeh, unpublished data). *G. australe* is habitated primarily in subtropical and tropical regions. Our results demonstrate that *G. australe* nabitating in two different ecological environments (temperate region of above 1600 m and subtropical region below 500 m) are genetically differentiated.

In conclusion, the phylogenic structures of *G. australe* complex revealed by PCR and RFLP analysis of rRNA gene (Yeh *et al.*, 1995) and DNA sequencing analysis of ITS regions suggests that the two intersterility groups are genetically differentiated.

Acknowledegment

We thank Drs. Z. C. Chen, C. Y. Chien and S. Huang for helpful discussion and comments. Gratitude is extended to Drs. K. Fang and T. Y. Chiang for reviewing the manuscript and expertise on computer software. Portion of this study was supported by grant (NSC84-2311-B-003-002), the National Science Council, Executive Yuan, Taipei, Taiwan, R. O. C.

References

Adaskaveg, J. E. and R. L. Gilbertson. 1986. Cultural studies and genetics of sexuality of *Ganoderma lucidum* and *G. tsugae* in relation to the taxonomy of the *G. lucidum* complex. *Mycologia* 78:694-705.

Bruns, T. D., T. J. White, and J. W. Taylor. 1991. Fungal molecular systematics. *Annual Rev. Ecol. Syst.* 22:525-564.

Carbone, I. and L. M. Kohn. 1993. Ribosomal DNA sequence divergence within internal transcribed spacer 1 of the Sclerotiniaceae. *Mycologia* 85:415-427.

Chen, C. S. 1993. Methods for inducing various morphological fruiting body of *Ganoderma tsugae* Murr. *Trans. Mycol. Soc. R. O. C.* 8:9-16.

Corner, E. J. H. 1983. Ad Polyporaceae I. *Amauroderma* and *Ganoderma*. *Beih. Nova Hedwigia* 75:1-182.

Hibbett, D. S. 1992. Ribosomal RNA and fungal systematics. *Trans. Mycol. Soc. Japan* 33:533-556.

Hseu, R. S. 1990. An identification system for cultures of *Ganoderma* species. Ph.D. Dissertation, National Taiwan University, Taipei, Taiwan, R. O. C. 169 pp. (In Chinese)

Karsten, P. A. 1881. Enumeratio Boletinearum et Polyporearum Fennicarum, Systemate novo dispositarum. *Rev. mycol.* 3:16-19.

Kwok, S. and R. Higuchi. 1989. Avoiding false positives with PCR. *Nature* (London) 339:237-238.

Lee, S. B. and J. W. Taylor. 1992. Phylogeny of five fungus-like protoctistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. *Molec. Biol. Evol.* 9:636-653.

LoBuglio, K. F., J. I. Pitt, and J. W. Taylor. 1993. Phylogenetic analysis of two ribosomal DNA regions indicates multiple independent losses of a sexual *Talaromyces* state among asexual *Penicillium* species in subgenus *Biverticillium*. *Mycologia* 85:592-653.

Moncalvo, J. M., H.-H. Wang, R.-S. Hseu. 1995. Phylogenetic relationships in *Ganoderma* inferred from the internal transcribed spacers and 25S ribosomal DNA sequences. *Mycologia* 87:223-238.

Olsen, G. J. and C. R. Woese. 1993. Ribosomal RNA: a key to phylogeny. *FASEB J.* 7:113-123.

Peng, J. T. 1990. Identification and culture conservation of the wild *Ganoderma* species in Taiwan. Publ. Taiwan Agric. Res. Inst. (TARI), Wufeng, Taiwan, R. O. C. 155 pp. (In Chinese)

Restrepo, B. I. and A. G. Barbour. 1989. Cloning of 18S and 25S rDNAs from the pathogenic fungus *Cryptococcus neoformans*. *J. Bacteriol.* 171:5596-5600.

Sanger F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-termination inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.

Steyaert, R. L. 1975. The concept and circumscription of *Ganoderma tornatum*. *Trans. Brit. Mycol. Soc.* 65:451-467.

Steyaert, R. L. 1980. Study of some *Ganoderma* species. *Bull. Jard. Bot. Belg.* 50:135-186.

Swofford, D. L. 1990. PAUP 3.0. Phylogenetic analysis using parsimony. Illinois Natural History Survey, Champaign, Illinois.

Verbeet, M. P., J. Klootwijk, H. Van Heerikhuizen, R. Fontijn, E. Vreugdenhil, and R. J. Planta. 1983. Molecular cloning of the rDNA of *Saccharomyces rosei* and comparison of its transcription initiation region with that of *Saccharomyces carlbergensis*. *Gene* 23:53-63.

Wang, B. C. and J. Hua. 1991. A cultural atlas of some *Ganoderma* cultures. Publ. Food Industr. Res. Developm. Inst. (FIRDI), Hsinchu, Taiwan, R. O. C. 131 pp.

White, T., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. Innis, D. Gelfand, J. Sninsky, and T. White (eds.), *PCR Protocols*, Academic Press, New York, pp. 315-322.

Xue B., P. H. Goodwin, and S. L. Annis. 1992. Pathotype identification of *Leptosphaeria maculans* with PCR and oligonucleotide primers from ribosomal internal transcribed spacer sequences. *Physiol. Mol. Plant Pathology* 41:179-188.

Yeh, Z. Y. 1990. Taxonomic study of *Ganoderma australe* complex in Taiwan. Ph.D. Dissertation, National Taiwan University, Taipei, Taiwan, R. O. C. 110 pp. (In Chinese)

Yeh, Z. Y. and Z. C. Chen, 1990. Preliminary investigation of *Ganoderma australe* (Subgen. *Elfvingia*) in Taiwan. *Taiwania* 35:127-141.

Yeh, Z. Y., C. Y. Chang, and G. J. Lee-Chen. 1995. Genetic Diversity of two intersterility groups of *Ganoderma australe* in Taiwan. *Biol. Bull. Natn. Taiwan Normal Univ.* 30:117-124.

Zambino, P. J. and L. J. Szabo. 1993. Phylogenetic relationships of selected cereal and grass rusts based on rDNA sequence analysis. *Mycologia* 85:401-414.

Zhao. J. D. 1989. The Ganodermataceae in China. *Biblioth. Mycol.* 132:1-176.

(Accepted: 1996. 5. 17)

利用 DNA 定序法探討南方靈芝兩群雜交不孕性的遺傳變異

張君玉　葉增勇　李桂楨

國立臺灣師範大學生物學系

摘　要

本研究在以聚合酶鏈反應 (PCR) 和 DNA 定序法 (DNA sequencing)，探討南方靈芝 *Ganoderma australe* (Fr.) Pat. 種內的兩群雜交不孕性(intersterility group) 的遺傳變異，並以不同亞屬的新日本靈芝 *G. neo-japonicum* Imaz. 作為比較。以互補於酵母菌的 rRNA 基因的引子與 DNA 聚合酶，將 rRNA 基因重複單位的內部轉錄間隔片段 (ITS 1、ITS 2) 選殖出來。置入 pGEM-T 載體後，將篩選出的純系進行 DNA 序列分析。再以 PAUP 軟體，分析各樣品 ITS 1、ITS 2 序列的歧異度，建築演化樹狀圖。我們的結果顯示南方靈芝種內的兩群雜交不孕性已呈現顯著的遺傳分化。

關鍵詞：南方靈芝、雜交不孕性群、內部轉錄間隔片段、DNA 定序法