Intrastrain internal transcribed spacer heterogeneity in *Ganoderma* species

D.-M. Wang and Y.-J. Yao

Abstract: Intrastrain internal transcribed spacer (ITS) heterogeneity is first reported from *Ganoderma*, a fungal genus within Basidiomycetes. ITS amplification products from 4 strains, representing 4 *Ganoderma* species, were cloned and sequenced. Two to five different ITS types were found within a single strain. The clone sequences were analyzed along with other sequences from *Ganoderma* retrieved from GenBank. The results show that sequence variation within strains varies considerably with species and the heterogeneity may occur in the 3 parts (ITS1, ITS2, and 5.8S) of the ITS region.

Key words: intragenomic ITS heterogeneity, polymorphism, *Ganoderma*.

Introduction

*Ganoderma* P. Karst. (Basidiomycetes, Ganodermataceae), a cosmopolitan fungal genus with a high species diversity in the tropics (Gilbertson and Ryvarden 1986; Kirk et al. 2001; Núñez and Ryvarden 2000; Ryvarden and Gilbertson 1993; Ryvarden and Johansen 1980; Zhao and Zhang 2000), is well known for its economical, especially in Chinese traditional medicine, and ecological importance. *Ganoderma* is a fungus of champignons appartenant aux basidiomycètes. Les produits d’amplification de l’ITS de 4 souches, représentant 4 espèces de *Ganoderma*, ont été clonés et séquencés. Les séquences des clones ont été analysées en conjonction avec celle de *Ganoderma* issus de GenBank. Les résultats démontrent que les variations de séquence à l’intérieur des souches varient considérablement selon l’espèce et que l’hétérogénéité peut être retrouvée dans 3 parties (ITS1, ITS2 et 5,8S) de la région ITS.


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Moncalvo et al. 1995a; Smith and Sivasithamparam 2000) suggest that the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) is a suitable molecular marker for phylogenetic analysis of species within the genus. ITS sequences, ITS–RFLP and ITS–SSCP profiles have also been employed to discriminate species in the genus (Gottlieb et al. 2000; Moncalvo et al. 1995b, 1995c; Smith and Sivasithamparam 2000).

Within species, ITS regions are homogenized by the process of concerted evolution, e.g., gene conversion (Hillis et al. 1991) and unequal crossing over (Coen and Dover 1983). A growing number of ITS polymorphisms within a single individual, however, have also been widely reported in fungi (e.g., Fatehi and Bridge 1998; Ko and Jung 2002; O’Donnell and Cigelnik 1997; Okabe et al. 2001; Waalwijk et al. 1996; Yao et al. 1992). For the ITS polymorphisms, some occur only in the ITS1 region (e.g., Ko and Jung 2002), whereas others may be found in the ITS2 region (e.g., O’Donnell and Cigelnik 1997). The nonorthologous sequences might form different clusters in phylogenetic trees (e.g., Waalwijk et al. 1996), and the analyses based on these sequence data would obscure the true phylogenetic relationships, e.g., *Fusarium* species (O’Donnell and Cigelnik 1997; O’Donnell et al. 1998) and *Trichaptum abietinum* (Ko and Jung 2002).

In *Ganoderma* species cultivated in China, the ITS region was also adopted to infer phylogenetic relationships and ITS heterogeneity was detected within some strains from different species by direct sequencing of the PCR products. In the present study, ITS heterogeneity within 4 strains representing 4 species was characterized by sequencing clones and phylogenetic analyses. The results of this work are reported here.
Materials and methods

Fungal cultures
Twenty-eight *Ganoderma* strains were processed for DNA sequencing in an investigation on species phylogeny; among them, 4 from the China General Microbiological Culture Collection Centre (CGMCC) were found to be with intrastrain ITS heterogeneity and used in this study (Table 1). Their identification was carried out by the CGMCC (China General Microbiological Culture Collection Centre 1997). The strains were subcultured at room temperature in the dark in 100-mL flasks containing 40 mL of liquid potato dextrose medium for 2 weeks or more and gently shaken by hand twice a day at 12-h intervals. The culture fluid was drained off. The mycelium was dried in oven at 65 °C before DNA extraction.

DNA extraction, ITS amplification, cloning, and sequencing
DNA extraction mainly followed Yao et al. (1999), but β-mercaptoethanol was omitted in this protocol. Samples of around 20 mg of dried culture were ground to a fine powder and then transferred to 1.5-mL Eppendorf tubes. Each sample in 600 µL of preheated cetyltrimethylammonium bromide lysis buffer was incubated in a water bath at 65 °C for 30 min to 1 h. An equal volume of chloroform–isoamylol (24:1) was added and mixed by inverting the tubes for 10 min. After centrifugation at 10 000 g for 10 min, the supernatant was transferred to another 1.5-mL Eppendorf tube followed by extraction of the chloroform–isoamylol (24:1) step once more. To precipitate the total DNA, 2 volumes of cold absolute alcohol and 1/10 volumes of sodium acetate (pH 5.2) were used and the mixture was incubated overnight at −20 °C. The pellet was collected by centrifugation at 10 000 g for 20 min and washed in cold 70% ethanol. After drying at room temperature, the DNA preparation was resuspended in 30–100 µL of Tris–EDTA buffer (pH 8.0), depending on the yield. The crude extracts containing unquantified amounts of DNA were used as templates for PCR amplification.

The entire ITS of the nrDNA region, including the interving 5.8S gene, was amplified from the total DNA using the primer pair ITS5/4 (White et al. 1990), and negative controls with no template DNA were included. Each reaction mixture (50 µL) contained 3 mmol/L MgCl₂, 0.1 µmol/L each primer, 0.2 mmol/L each dNTP, 2 units of Promega *Taq* polymerase, 1 µL of template DNA, and buffer as provided by Promega. Template DNA was taken directly from the undiluted product of extraction for ITS amplification. Reaction mixtures were placed in a GeneAmp® 9700 thermal cycler and exposed to the following PCR profile: predenaturing at 95 °C for 1 min, denaturing at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 45 s, 30 cycles at 72 °C for 7 min, and then a 4 °C soak. To test the results of PCR, 2 µL of each of the PCR products was checked on 1% agarose gels. These reactions were purified using the QIAquick PCR purification kit from Qiagen and the products eluted in 30 µL of elution buffer (supplied by the manufacturer).

Purified PCR products were sequenced using the ABI PRISM Big Dye Terminator Cycle sequencing ready reac-

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fied taxa or unpublished work were not included in the final analysis.

Additional ITS sequences from 2 poroid fungi, *Rigidoporus ulmarius* (Sowerby: Fr.) Imaz. and *Polyporus tuberaster* Jacq.: Fr., the type species of *Polyporus* Fr. s. str., were selected as outgroups because they are within the same order, Polyporales (Kirk et al. 2001), although taxonomically distinct from *Ganoderma*. Inclusion of the outgroups created more gaps in the data set but did not change the alignment of the ingroup. Some portions of the sequences were not aligned very well by the computer programme and these were further adjusted manually before phylogenetic analysis.

Forty sequences were included for the phylogenetic analysis and a data matrix containing 775 base pairs of nucleotides was established. The principles of base exclusion (303 sites excluded) and analysis parameter preferences were the same as those in the initial parsimony analysis and 5000 replicates with 2 trees were saved for each replicate. Bootstrap analysis (Hillis and Bull 1993) was performed by 5000 replications with simple addition sequences to obtain estimates of reliability for nodes.

## Results

### Number of ITS types within the 4 *Ganoderma* strains

The 4 strains of *Ganoderma* species with intrastrain ITS heterogeneity were found among 28 strains of 7 species examined and subjected to further cloning for screening. Thirty-six sequences were obtained from 40 positive recombinant colonies after cloning. Ten clones were successfully

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<td><em>Rigidoporus ulmarius</em> 333  England AY593868</td>
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*Ganoderma* cultures with intragenomic ITS heterogeneity.
Sequences in Smith and Sivasithamparam (2000) that have not been submitted to GenBank.
sequenced for each strain, except for the strain of *G. fornicatum*, which had only 6 clones sequenced. Up to 5 diverse ITS types were detected from the strains named as *G. applanatum var. gibbosum* and *G. fornicatum* but only 2 from each strain of *G. japonicum* and *G. neojaponicum*. Among the 5 different types of ITS sequences obtained from *G. applanatum var. gibbosum*, Type 1 was shared by 3 clones sequenced, Types 2, 4, and 5 by 2, and Type 3 by 1. In the 6 clones sequenced for the *G. fornicatum* strain, only 2 shared 1 identical sequence (Type 3), whereas the other 4 clones had unique sequences. The 2 types of sequences from each strain of *G. japonicum* and *G. neojaponicum* were shared by 8 (Type 1) and 2 (Type 2) clones, respectively. These sequences from cloning have been submitted to GenBank as listed in Table 1.

**Characteristics of the ITS types**

Amplification of the ITS region of *Ganoderma* species using the primer pair described above gave PCR products of 666–680 bp. The lengths of the ITS1, ITS2, and the 5.8S genes in the 4 strains were 195–203, 189–201, and 158 bp, respectively. The detectable mutations occurred at sites 104, 430, 447, 468, 473, and 474 in the ITS types of the *G. applanatum var. gibbosum* strain, except for the long deletions in Type 5, which lost the whole ITS1 and most of 5.8S, but the existing sequence, however, is identical with that of Type 4 from the same strain; at sites 251, 265, 330, 345, 429, 443, and 566 in *G. fornicatum*, at sites 101 and 155 in *G. japonicum*, and at sites 64, 65, 184, 189, 198, 202, 265, 357, 403, and 558 in *G. neojaponicum* (Fig. 1). The number of base changes in the entire ITS region, including transitions and (or) indels, ranged from 1 to 10 between ITS types within strains. In the *G. applanatum var. gibbosum* strain, the base changes range from 1 to 5, except for Type 5. One to 6 changes were found in the *G. fornicatum* strain, 2 in *G. japonicum*, and 10 in *G. neojaponicum*. There are 2 substitutions found in the 5.8S gene in the *G. fornicatum* and *G. neojaponicum* strains, respectively. Locations of ITS sequence variations within strains were diverse in the 4 *Ganoderma* strains. The heterogeneity occurred in the entire ITS region of *G. fornicatum* and *G. neojaponicum*, in ITS1 and ITS2 of *G. applanatum var. gibbosum*, and only in ITS1 of *G. japonicum*. The magnitude of variation in individual ITS1 and ITS2 varied with strain. In the *G. fornicatum* strain, base changes did not show the distinct preferences of the 2 internal transcribed spacers, whereas most variations occurred in ITS1 of the *G. neojaponicum* strain and in ITS2 of the *G. applanatum var. gibbosum* strain. However, all base changes were located in ITS1 of the *G. japonicum* strain.

**Phylogenetic analysis**

A total of 472 bp of the ITS1 and ITS2 regions were used in the analysis after exclusion of the sequences of 5.8S, 18S, and 28S. Of the characters, 126 were parsimony informative. With this alignment, 12 most parsimonious trees were retained. One of them is shown in Fig. 2. The other 11 trees differed from Fig. 2 in the arrangement of sequences within Group IV. In the phylogenetic tree, sequence types from the same strain nested together and the corresponding 4 clades were distant from each other. The clades comprising these types were depicted as I, II, III, and IV successively (Fig. 2).

In Group I, 5 ITS types of the *G. fornicatum* strain and 1 *G. fornicatum* strain from GenBank submitted by Moncalvo et al. (1995c) (strain No. RSH 0814 collected from Taiwan) formed a clade that received 86% bootstrap support and 2 *G. tropicum* strains from GenBank served as sister taxa to this clade. Within the clade, 3 ITS types of *G. fornicatum* clustered with 63% bootstrap support, and the other 2 ITS types and the additional sequence from GenBank are only 2 steps different from each another. From the strain information provided by the CGMCC, AS 5.539 from Taiwan, 0814 = ATCC 76536 (see Table 1), and the cultural characters of the strains by Hsu (1990) and by the present study suggested that the 2 strains are derived from the same original culture.

Group II comprised sequences from the *G. formosanum*, *G. sinense*, and *G. japonicum* strains in GenBank and was considered as the single species *G. sinense* by Moncalvo et al. (1995c). The 2 ITS types of the *G. japonicum* strain investigated in this study clustered with the sequences from GenBank, having 100% bootstrap support. In fact, there were very few step changes among these sequences in Group II as indicated by the phylogenetic analysis. In addition, the 2 *G. japonicum* strains in Group II are apparently duplicates from the same strain, AS 5.69, housed in the CGMCC, although ACCC 5.69 was used for this strain by Moncalvo et al. (1995c). There is no such number in the ACCC. Similar mistakes were made for other strain numbers prefixed with ACCC in Moncalvo et al. (1995a, 1995c), e.g., ACCC 5.151 for *G. gibbosum*, as seen in Table 1.

Two ITS types of the *G. neojaponicum* strain formed Group III. This group was very strongly supported by bootstrap analysis (100%) and well separated from the other clades. Between the 2 ITS types of *G. neojaponicum*, there were 7 base substitutions and 3 indels in the entire ITS region (Fig. 1) and 5 step changes in the phylogenetic tree (Fig. 2).

In Group IV, 5 ITS types of the *G. applanatum var. gibbosum* strain clustered together with the sequence of *G. gibbosum* from GenBank. However, the clade was not well supported by bootstrap analysis. Sequences of the *G. incrassatum* and *G. australe* strains from GenBank served as sister taxa to this clade. The 2 *G. incrassatum* strains of different geographical origin (one from southeastern Asia and the other from Australia) formed a subclade with 97% bootstrap support.

**Discussion**

The intrastrain ITS heterogeneity is first reported for *Ganoderma* in this study, although the ITS region has been subjected to extensive sequencing for phylogenetic inference of species within the genus with 291 entries of ITS sequences in GenBank at the moment (October 2004). However, this phenomenon may not be exceptional among *Ganoderma* strains. Four out of twenty-eight strains tested in this study were found to be heterogeneous in ITS sequence. The number of ITS types may vary among strains, as 5 were found in the *G. applanatum var. gibbosum* and *G. fornicatum* strains and 2 in *G. japonicum* and
Fig. 1. Alignment of the 14 type sequences from the 4 *Ganoderma* strains representing 4 *Ganoderma* species. Dots and dashes indicate identity and deletion, respectively.
It is evident that all of the possible ITS types have not been exhausted in the former 2 strains, but much more work is required to find more new types in the latter 2. There are only 2 ITS types found in the 10 clones sequenced in the 2 strains. It may not be easy to find any new ITS types from them, but there are certainly more ITS types in the *G. applanatum* var. *gibbosum* and *G. fornicatum* strains, especially in the latter where 5 ITS types were found.
in 6 successfully sequenced clones. Further, the number of different ITS types are not equally distributed in strains. The ratio for the 2 ITS types in the *G. japonicum* and *G. neojaponicum* strains was 4:1 and was 3:2:2:2:1 in the *G. applanatum* var. *gibbosum* strain, although any meaningful ratio cannot be drawn from the *G. fornicatum* strain because 5 ITS types were found in 6 clones.

Currently, there are several methods that have been employed to detect intragenomic ITS heterogeneity, e.g., cloning and sequencing (e.g., Paskewitz et al. 1993; Vogler and DeSalle 1994), ITS-RFLP (Gernandt et al. 2001), SSCP (Gasser et al. 2001), and temperature-gradient gel electrophoresis (Schlötterer 1995). It is possible to screen a large number of clones, especially by SSCP, to detect the types of variations and to do representative sampling for sequencing.

As shown in Fig. 1, the site variations may occur in any parts of the ITS sequences but may be different among species and strains as in ITS1 and ITS2 or the entire ITS region or ITS1 only. However, it cannot be concluded whether this is the nature of species or of strain because only 1 strain of each of the 4 species was detected to have ITS heterogeneity. If more ITS types are obtained, the site variations may be greater. It is likely that site variations in ITS sequence of the *G. fornicatum* strain are more than those that have been detected when more ITS types are found. It is interesting to see the 4 substitutions found in the 5.8S gene in the *G. fornicatum* and *G. neojaponicum* strains. These variations will be discussed further below.

As indicated in the Results section, the *G. fornicatum* RSH 0814 and *G. japonicum* ACCC 5.69 strains, accessed as Z37067 and Z37087 and as Z37065 and Z37102, respectively, for both ITS1 and ITS2 in GenBank, have the same origin as the AS 5.539 and AS 5.69 strains under the same names investigated in this study. However, no ITS heterogeneity was reported for those sequences (Moncalvo et al. 1995c). Sequence comparison shows that those from GenBank represent additional ITS types to the types detected from the 2 strains in the present study. The combination of Z37067 and Z37087 from *G. fornicatum* RSH 0814 has 1 substitution, 2 deletions, and 2 unreadable sites compared with the other ITS types obtained in this investigation. There are more variable sites in the combination of Z37065 and Z37102, with 1 substitution, 1 additional deletion, 4 unreadable sites, and 4 double-peaked sites. Further comparison of the data of electrophoregrams may provide more information.

Ancient hybridization or gene duplication or low concerted evolution might explain the ITS heterogeneity present in the 4 *Ganoderma* strains. Different ITS types shared by strains/species are often cited for the detection of hybridization (Agatsuma et al. 2000; Okabe and Matsumoto 2003; Tang et al. 1996; Widmer and Baltisberger 1999) and the postulation of species phylogeny (Ko and Jung 2002). ITS types from the 4 *Ganoderma* strains are clustered together with the types from the same strain in the phylogenetic analysis but sometimes with sequences from other strains, e.g., Groups I, II, and IV in Fig. 2. However, there is no ITS type shared by different strains observed in the present study. The strains clustered in Group II are possibly of the same species as suggested by Moncalvo et al. (1995c), although they are under different names. Apparently, the available evidence at the moment cannot directly support the hypothesis that the ITS heterogeneity in the 4 *Ganoderma* strains results from hybridization.

Heterokaryotic status was concluded to cause intrasporal ITS heterogeneity in the species of *Glomales* whose spores contain large numbers of nuclei (Hijri et al. 1999). Different nuclei were also reported to produce different ITS types in a *Sclerotium rolfsii* strain (Okabe et al. 2001). Independent mutations in copies within a single nucleus could also contribute multiple forms of the gene cluster within the chromosomes (numerous tandem repeated arrays within individual cells) as demonstrated in lichen fungi (DePriest 1993), insects (Schäfer and Kunz 1985), and nematodes (Zijlstra et al. 1995). The strains used in this study are binucleate because they were tissue isolations of *Ganoderma*. Different ITS regions associated with particular nuclei or polymorphism within a single nucleus in the *Ganoderma* strains requires further study by using monokaryotic cultures.

Inherent mechanisms involving slippage events during DNA duplication may cause minor intragenomic ITS heterogeneity, e.g., microsatellite repeats and binucleotide repeat dispersal (Conole et al. 2001; Gasser et al. 2001). Such mechanisms may also be involved in base indels or transitions of the ITS sequences from *Ganoderma* strains, as few sequence mutations occur in the ITS types.

Polymorphic sequences may be attributed to subrepeat (Gernandt and Liston 1999; Murrell et al. 2001; van Herwerden et al. 1998, 1999) or pseudogenes (Gernandt et al. 2001), but few changes in sequence length and base compositions suggest that the 2 possibilities should be discounted.

Paralogous ITS sequences may result from varying PCR conditions or contamination (Ramey et al. 2000). In the present study, however, the same experimental conditions with negative controls were employed to amplify the ITS regions from 28 cultivated strains and only 4 were found to have ITS heterogeneity. The sequence profiles before and after cloning were compared and the ambiguous peaks began with the mutation sites, especially indels. Nonspecific amplification or contamination was excluded from the experiments. Misinterpretation of ITS type diversity, however, may result from PCR errors. Analysis of the variation sites in all of the ITS types detected from the 4 *Ganoderma* strains was conducted to find any possible errors of misamplification during the PCR experiments. Most of the sites with variable nucleotides were found to be shared by different types or different clones of the ITS region. It is unlikely that the variations at these sites were caused by PCR errors, which occur randomly. There were 2 site variations in the 5.8S region found in the *G. neojaponicum* strain, substitution of adenine by guanine. These 2 variations were supported by 8 clones, although they are unique to the strain in such a conserved region. Type 5 from *G. applanatum* var. *gibbosum*, similar to that of *Onococalamus* reported by Baker et al. (2000), has a major deletion of the whole ITS and part of 5.8S, but it is shared by 2 clones. The difficulty in determining the true site variation occurs in those variations found in the ITS sequences from the *G. fornicatum* strain because 5 ITS types were found in the 6 clones sequenced. There are 2 sites of variation (Sites 265 and 443, Fig. 1) of the *G. fornicatum*
strain shared by more than 1 ITS type and another unique site (Site 429) to Type 3 supported by 2 clones. The other 4, Sites 251, 330, 345, and 566, were found in only 1 of the ITS types (Type 1 or 5) determined from a single clone. They cannot be verified in the present study, but further sequencing of more clones may provide useful information.

Acknowledgements

The authors acknowledge the reading of early drafts by Dr. F.-Y. Bai and the constructive comments on the manuscript provided by 2 anonymous reviewers. This research was supported by a project grant (30270006) and the National Science Fund for Distinguished Young Scholars (30025002) from the National Nature Science Foundation of China and the Key Research Direction of Innovation Programme (KSCX2-SW-101C) and the scheme of Introduction of Overseas Outstanding Talents operated by the Chinese Academy of Sciences.

References


Hsu, R.S. 1990. An identification system for cultures of *Ganoderma* species. Ph.D. dissertation, National Taiwan University, Taipei.


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4. L. B. Connell, R. Redman, R. Rodriguez, A. Barrett, M. Iszard, A. Fonseca. 2010. Dioszegia antarctica sp. nov. and Dioszegia cryoxerica sp. nov., psychrophilic basidiomycetous yeasts from polar desert soils in Antarctica. *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY* **60**:6, 1466-1472. [CrossRef]


