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Variability of internal transcribed spacer ribosomal DNA sequences of *Fuscoporia gilva* and *Fuscoporia* sp. in Thailand

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The objectives of this study were to examine genetic variation based on the ITS region of the ribosomal RNA gene sequences of *Fuscoporia* sp. and *F. gilva* from Thailand. Considerable levels of genetic variation were found in the Thai samples. The mean intraspecific genetic divergence was 5.74 % for *Fuscoporia* sp. and 5.33 % for *F. gilva*, indicating that they might be composed of several genetically distinct species. Phylogenetic analyses revealed that *Fuscoporia* sp. from Thailand was genetically related to *F. callimorpha* and *F. senex*. However, comparisons of the morphological characters and the level of genetic divergence indicated that they were different taxa. Two divergence lineages among Thai *Fuscoporia* sp. associated with host species preference were found. This finding could indicate the role of host association in genetic divergence and probably speciation. *Fuscoporia gilva* from Thailand was clustered with conspecific species from other geographic regions. Nonetheless, levels of genetic differentiation are highly consistent with their divergent phylogenetic clades. This indicates that specimens from Thailand might represent genetically distinct species. Further study using other genes and an in-depth morphological analysis would be needed to clarify the species status of these important mushrooms.

Key words: *Fuscoporia*, Internal Transcribed Spacer, medicinal mushroom, *Hymenochaetales*.

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Cílem této studie bylo prozkoumání genetické variability založené na DNA sekvencích ITS oblasti ribozomálního RNA genu u druhů *Fuscoporia* sp. a *F. gilva* z Thajska. Mezi thajskými sběry byla zjištěna vysoká míra genetické variability. Průměrná vnitrodruhová genetická divergence byla 5,74 % u *Fuscoporia* sp. a 5.33 % u *F. gilva*, což poukazuje na možnost, že se jedná o skupiny zahrnující více druhů. Fylogenetické analýzy odhalily, že druh *Fuscoporia* sp. z Thajska je geneticky příbuzný druhům *F. callimorpha* a *F. senex*; nicméně míra genetické divergence a studium morfologických znaků dokazují, že se jedná o odlišné taxony. V rámci *Fuscoporia* sp. byly odhaleny dvě genetické linie lišící se vazbou na hostitelské dřeviny. Toto zjištění poukazuje na roli hostitelské vazby při genetické divergenci a pravděpodobně i speciaci. *Fuscoporia gilva* z Thajska tvoří jednu skupinu se sekvencemi stejného druhu z jiných zeměpisných oblastí, přičemž vysoký stupeň genetické diferenciace odpovídá pozicím těchto sekvencí v odlišných fylogenetických kladech. Tyto výsledky naznačují, že sběry z Thajska mohou patřit k odlišným druhům. Podrobnější výzkum založený na fylogenetické analýze většího počtu genů a důkladných morfologických studiích by byl potřebný k ujasnění statusu těchto významných hub.

INTRODUCTION

Many species of the *Hymenochaetaceae* are used as medicinal fungi (Dai et al. 2010). Among these, species belonging to *Phellinus* s.l. have received special attention. Several effects from the extracts of *Phellinus* mushrooms have been reported, including inhibition of pulmonary inflammation (Jang et al. 2004), promotion of dermal wound healing (Bae et al. 2005), immunostimulation (Kim et al. 1996), anti-tumour activity (Han et al. 1999), anti-oxidant and anti-hepatotoxicity activity (Ajith & Janardhanan 2002), and anti-microbial activity (Dulger et al. 2005). Other applications, such as decolorisation of textile indigo dye (Balan & Monteiro 2001), have also been reported. *Phellinus* species are important plant pathogens as well (Chang 1995, Dai et al. 2007).

Phellinus s.l. is the largest genus of the *Hymenochaetaceae* including about 220 species (Dai 2010). Some species previously placed in the genus *Phellinus*, such as *Phellinus gilvus* and *P. torulosus*, were transferred to *Fuscoporia* (Wagner & Fischer 2001, 2002). This has been supported by more recent molecular data, since phylogenetic analysis revealed close relations between *P. torulosus* and *P. gilvus*, and the members of *Fuscoporia* (Jeong et al. 2005). Recently 13 species of *Fuscoporia* have been reported from East Asia (Dai 2010, Ghobad-Nejhad & Dai 2007). However, due to the paucity of information on species distributions in tropical Asia, data on the geographic distribution of each species is not yet available for that area.

Because of their medicinal and ecological importance, correct species identification is crucial for further study. DNA sequences are increasingly helpful in species identification (Hebert et al. 2003). The barcoding region of fungi has not yet been formally accepted, but the ITS region is among the most commonly used (Seifert 2009). ITS sequences have been successfully used to delineate species of several fungal species (e.g. Smith & Sivasithamparam 2000, Jageat et al. 2010). However, there is great variation in the intraspecific genetic divergence within the kingdom; thus there is still a problem with using ITS for species identification in the fungi (Nilsson et al. 2008). Despite this limitation, molecular data is useful for revealing cryptic diversity. Recent molecular studies found that one morphological species could be composed of several genetically distinct species (Smith & Sivasithamparam 2000, Jargeat et al. 2010), which indicated the importance of molecular markers for fungal taxonomy (Seifert 2009).

The objectives of this study were to examine ITS sequence variation in two taxa, *Fuscoporia gilva* (Schwein) T. Wagner & M. Fisch. and *Fuscoporia* sp. from Thailand and to assess levels of genetic differentiation with related fungi from other geographic regions. Our results will be useful to reveal the species status of these fungi.

MATERIALS AND METHODS

Sample collection and species identification. Samples of *Fuscoporia* sp. and *F. gilva* were collected in natural habitats in Thailand (Tab. 1). The key to *Fuscoporia* species by Dai (2010) was used for species identification. All specimens used in this study were deposited in the herbarium of the Natural Medicinal Mushroom Museum (MSUT), Faculty of Science, Mahasarakham University, Thailand.

Culture, DNA isolation and PCR amplification. Mycelium was cultured following a method described by Dr. Jae-Mo Sung (Kangwon National University, Korea, pers. comm.). Small pieces of basidiocarps were cut aseptically using a razor blade and sterilised by placing them in 10% Clorox for 10 minutes, then rinsed in sterilised distilled water three times and dried on sterile filter paper. A piece of basidiocarp was placed into a Petri dish and the spores were allowed to discharge into Malt Extract Agar (2 % malt extract, 2 % agar, 0.05 % yeast extract in distilled water) overnight. The plates were incubated at 25° C for 7–15 days.

Genomic DNA was extracted from the mycelium using the Plant Genomic DNA Extraction Kit (RBC Bioscience Corp., Taiwan). Mycelium was ground into a powder in liquid nitrogen and DNA was extracted following the manufacturer's protocol. DNA samples were stored at –20 °C. A fragment of the ITS region was amplified using the primers ITS1 and ITS4 (White et al. 1990). The PCR reaction was performed in a total volume of 50 µl of liquid containing 1x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 2.5 µM of each primer, 0.4 units of *Taq* DNA polymerase, and 2 µl of DNA sample (diluted 1:20 in ddH_2O). The temperature profile was 94 °C for 2 min. followed by 36 cycles of denaturing at 94 °C for 45 s, annealing at 50 °C for 45 s, extension at 72 °C for 1.30 min. and a final extension at 72 °C for 5 min. PCR products were checked on a 1% agarose gel with 0.125 mg/l ethidium bromide. PCR products were cleaned using the PCR purification kit (RBC Bioscience, Taiwan) and were sequenced using the same primers as in the PCR by the DNA Sequencing Service of Macrogen (Seoul, Korea).

Data analysis. Sequences were aligned using the Clustal W algorithm in BioEdit (Hall 1999) with final manual editing. Genetic variation within and between species was calculated based on the Kimura 2-parameter using MEGA 4.1 (Tamura et al. 2007). Maximum parsimony (MP) analysis was conducted in PAUP 4.10b (Swofford 2002) using the default setting. Bootstrap supports were calculated based on 1,000 pseudoreplications. Neighbour-joining (NJ) trees were analysed using the Kimura 2-parameter model by PAUP with the heuristic general search option. Bootstrap support was calculated with permutations of 1,000 pseudoreplications. Phylogeny based on the Bayesian method was analysed with MrBayes version 3.1.2 (Huelsenbeck & Ronquist 2001). The general time-reversible model of substitution with gamma distribution was selected.

Tab. 1. List of fungi analysed in this study.
ATCC – American Type Culture Collection, CBS – Centraalbureau voor Schimmelcultures, KCTC – Korean Collection for Type Cultures, MPUN – Microbiology Laboratory of Pusan National University.

Species	Host	Locations/Source	Voucher number	GenBank accession number	Reference
Fuscoporia sp.	*Peltophorum dasyrachis*	Si Saket Province, Thailand	MSUT451	JF461321	This study
	P. dasyrachis	Si Saket Province, Thailand	MSUT380	JF461322	This study
	Shorea obtusa	Amnatchareon Province, Thailand	MSUT618	JF461320	This study
	S. obtusa	Amnatchareon Province, Thailand	MSUT558	JF461323	This study
Fuscoporia sp.	Unknown	CBS 182.34, USA	–	AY558649	Jeong et al. (2005)
Fuscoporia gilva (Schwein.) T. Wagner & M. Fisch.	*Shorea obtusa*	Udon Thani Province, Thailand	MSUT30	JF461328	This study
	S. obtusa	Si Saket Province, Thailand	MSUT395	JF461324	This study
	S. obtusa	Sakon Nakon Province, Thailand	MSUT549	JF461329	This study
	S. obtusa	Sakon Nakon Province, Thailand	MSUT650	JF461326	This study
	S. obtusa	Maha Sarakham Province, Thailand	MSUT653	JF461327	This study
	S. obtusa	Mukdahan Province, Thailand	MSUT739	JF461325	This study
	Unknown	China	–	FJ481039	Jiang et al. Unpublished data
	Unknown	ATCC 26729	–	AF250932	Park et al. (2001)
	Unknown	KCTC 6653		AY558620	Jeong et al. (2005)
Fuscoporia torulosa (Pers.) T. Wagner & M. Fisch.	Unknown	Czech Republic	–	AM269803	Guglielmo et al. (2007)
	Quercus cerris	Czech Republic	–	EF068234	Tomšovský & Jankovský (2007)

Tab. 1. – continuation
ATCC – American Type Culture Collection, CBS – Centraalbureau voor Schimmelcultures, KCTC – Korean Collection for Type Cultures, MPUN – Microbiology Laboratory of Pusan National University.

Species	Host	Locations/Source	Voucher number	GenBank accession number	Reference
Fuscoporia callimorpha (Lév.) Groposo, Log.-Leite & Góes-Neto	Hardwood	Florida, USA	JV090487	JF692190	Vlasák et al. (2011)
	Hardwood	Florida, USA	JV090487	JF692191	Vlasák et al. (2011)
	Hardwood	Florida, USA	JV031220.5J	JF692192	Vlasák et al. (2011)
	Hardwood	US Virgin Islands, USA	JV040914J	JF692193	Vlasák et al. (2011)
Fuscoporia ferrea (Pers.) G. Cunn.	Unknown	CBS 444.48, Canada	–	AY558617	Jeong et al. (2005)
	Thuja plicata	Canada	–	DQ516525	Lim et al. (2007)
Fuscoporia ferruginosa (Schrad.) Murrill	Unknown	KCTC 6652, India	–	AY558616	Jeong et al. (2005)
	Unknown	KCTC 6652	–	AY189700	Nam et al. Unpublished data
Fuscoporia senex (Nees & Mont.) Ghob.-Nejh.	Unknown	CBS 442.76, India	–	AY558647	Jeong et al. (2005)
Phellinus tremulae (Bondartsev) Bondartsev & P.N. Borisov	*Populus tremula*	Germany	–	AY340066	Fischer & Binder (2004)
Phellinus rimosus (Berk.) Pilát	Unknown	China	–	DQ103885	Jiang et al. Unpublished data
Phellinus linteus (Berk. & M.A. Curtis) Teng	Unknown	MPNU 7016	–	AF153009	Kim et al. 2005
	Unknown	–	–	AF080457	Kim et al. 2003

Markov chains were run for 2,000,000 generations with a sampling frequency of every 100th generation. The point where log likelihood is stationary was analysed with Tracer version 1.5 (Rambaut & Drummond 2004). Trees sampled before this point were discarded as burn-in. The remaining trees of each run were included in posterior probability calculations. ITS sequences of *Ganoderma lucidum* (Curtis) P. Karst. from the GenBank database (AF079584) were used as an outgroup.

RESULTS AND DISCUSSION

The ITS sequences of *Fuscoporia* sp. and *F. gilva* were deposited in GenBank under accession numbers JF461320–JF461329 (Tab. 1). *Fuscoporia* sp. and *F. gilva* from Thailand show considerable levels of genetic variation. Intraspecific genetic divergence based on the Kimura 2-parameter (K2P) for *Fuscoporia* sp. ranges from 0.30 to 6.87 % with a mean of 5.74 %. For *F. gilva* it ranges from 0.75 to 5.65 % with a mean of 5.33 %. Sequence variations found in *Fuscoporia* sp. and *F. gilva* from Thailand were much higher than in other fungal species. For example, European *F. torulosa* samples have sequence variations of less than 1 % (Tomšovský & Jankovský 2007), and intraspecific genetic variation of Australian *Ganoderma* species ranges from from 0.75 to 1.5 % (Smith & Sivasithamparam 2000). Sequence variations found in *Fuscoporia* sp. and *F. gilva* from Thailand were almost double the value of the mean ITS variation in Basidiomycetes (3.33 %) (Nilsson et al. 2008). The high intraspecific genetic variation in *Fuscoporia* sp. and *F. gilva* is mainly due to the existence of deep divergent clades as revealed by the phylogenetic analysis.

All three phylogenetic analyses revealed a similar tree topology; therefore only the maximum parsimony tree is shown (Fig. 1). There are three major clades (I, II, and III) among the 29 ingroup sequences included in the analyses. *Fuscoporia* sp. from Thailand forms Clade I with *F. senex*, *F. callimorpha* and *Fuscoporia* sp. CBS 182.34 from the USA. Clade I was further divided into four subclades (I-1, I-2, I-3, and I-4) with strong bootstrap support. *Fuscoporia* sp. from Thailand comprised Clade I-1 with *Fuscoporia* sp. CBS 182.34 from the USA, *F. callimorpha* from the USA, and *F. senex* from India. European *F. torulosa* formed Subclade I-2, *F. ferrea* from Canada Subclade I-3 and *F. ferruginosa* Subclade I-4.

The close relationship between *Fuscoporia* sp. from Thailand and *F. senex* and *F. callimorpha* was supported by their morphology. These three fungal species shared the following characters: pileate basidiocarps and circular pores with 7–9 pores per mm. The basidiospores are ellipsoid, hyaline, and thin-walled. However, *Fuscoporia* sp. from Thailand can be distinguished from these species by the combinations of the following characters. The basidiospores are larger in

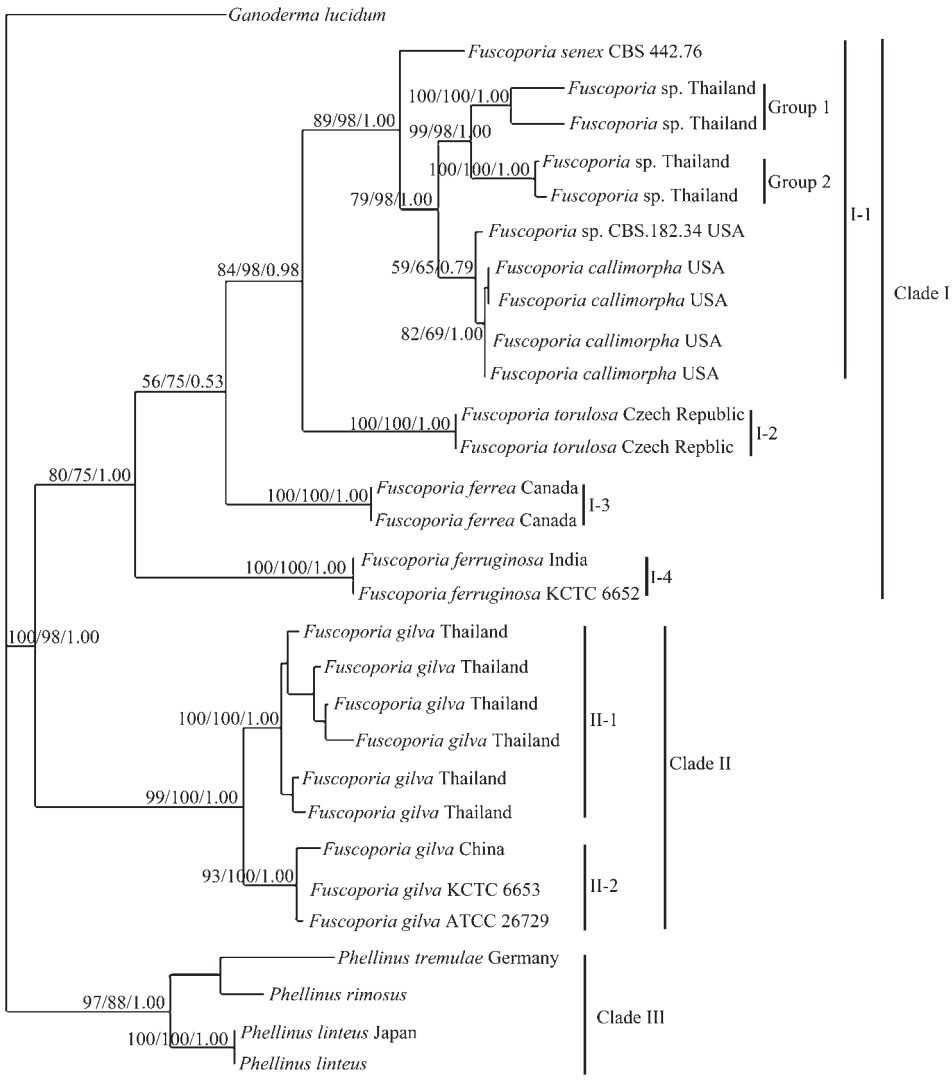

Fig. 1. Maximum parsimony tree of *Fuscoporia* sp. and *Fuscoporia gilva* in Thailand and related taxa based on ITS sequences. The ITS sequence of *Ganoderma lucidum* (AF079584) was used as an outgroup. Bootstrap values for neighbour-joining, maximum parsimony and posterior probability for Bayesian analyses are shown above the nodes. Scale bar represents 10 changes.

Fuscoporia sp. (4.5–5.0 × 3.5–4.0 µm) from Thailand and *F. senex* (4–4.9 × 3.2–4 µm) (Dai 2010) than in *F. callimorpha* (3.5–4.2 × 2.2–2.9 µm) (Vlasák et al. 2011). The *Fuscoporia* sp. from Thailand was different from *F. senex* by the skeleton

hyphae not changing in potassium hydroxide (KOH) in the former species, but swollen in the latter species (Dai 2010). Differences between Thai *Fuscoporia* sp. on the one hand and *F. callimorpha* and *F. senex* on the other were also supported by the levels of genetic divergences. Average K2P distance between Thai *Fuscoporia* sp. and *F. callimorpha* was 6.50 %, and when compared with *F. senex* it was 7.30 %. Based on the morphological characters and the DNA sequences, *Fuscoporia* sp. from Thailand most likely represents a different species.

Fuscoporia sp. from Thailand was further divided into two groups (1 and 2) with strong bootstrap support (Fig. 1). These two groups have a high level of genetic divergence (5.53 % K2P genetic distance). This might indicate the existence of cryptic diversity in this species. Interestingly this separation was consistent with host species differences; individuals belonging to Group 1 were found on *Peltophorum dasyrachis* and those of Group 2 they were found on *Shorea obtusa*. This finding might indicate that host species preference could play a role in genetic divergence and probably speciation. Our results contrast with previous findings. For example, Campanile et al. (2004) found no genetic differentiation between *F. torulosa* on different hosts based on RAPD fingerprints. Similarly, Tomšovský & Jankovský (2007) found no genetic differentiation between *F. torulosa* samples of basidiocarps attached at different positions on host trees.

Fuscoporia gilva comprised Clade II. The *F. gilva* clade was divided into two subclades (II-1 and II-2) with strong bootstrap support (100 %). Samples of this species from Thailand comprised Subclade II-1, whereas samples from other geographic regions formed Subclade II-2. The two subclades have considerable levels of genetic divergence (7.70 % K2P genetic distance). Because the sequence divergence values of the samples from different geographic regions were higher than the 3 % threshold value traditionally accepted for conspecific species (Hughes et al. 2009), the results strongly suggest that they are genetically distinct species. These finding might indicate that samples of *F. gilva* from Thailand represent a genetically distinct species.

Three species of *Phellinus*, *P. tremulae*, *P. rimosus* and *P. linteus*, comprised Clade III with strong bootstrap support. Separation of *Fuscosporia* from *Phellopilus* and *Phellinus* was consistent with the recent transfer of *F. gilva* and *F. torulosa* from *Phellinus* to *Fuscoporia* (Wagner & Fischer 2001, 2002).

In conclusion, the ITS sequences of *Fuscoporia* sp. and *F. gilva* from Thailand indicated that they are genetically distinct from morphologically closely related taxa from other geographic regions. This raises an important issue concerning the systematics of these species and makes further study of these fungi using other gene sequences and in-depth morphological examination (e.g. scanning electron microscope) necessary to clarify the taxonomic status of the Thai samples.

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