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Genetic variability in *Fomes fomentarius* reconfirmed by translation elongation factor 1-α DNA sequences and 25S LSU rRNA sequences

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Abstract: The existence of two cryptic species within strains of the wood-decaying fungus *Fomes fomentarius* was revealed recently based on the internal transcribed spacer (ITS) sequence variability. In this study for the first time the sequences of another molecular markers, partial translation elongation factor 1-α (*efa*) region and partial 25S large subunit ribosomal RNA gene were obtained and used to evaluate genetic variability of *F. fomentarius*. Congruent phylogeny was observed for all three markers used confirming the presence of two cryptic species within *F. fomentarius*. Surprisingly, ITS sequence variability within *F. fomentarius* was significantly lower compared to the variability of *efa* sequences (0.023 versus 0.036 nucleotide substitutions per site) questioning the discriminatory power of ITS sequences for fungal species identification.

Key words: Polyporaceae; cryptic species; *Fomes fomentarius*; molecular phylogeny; variability.

Abbreviations: *efa*, translation elongation factor 1-α; ITS, internal transcribed spacer; LSU, 25S large subunit ribosomal RNA.

Introduction

The wood-decaying fungus *Fomes fomentarius* (L.) J. Kickx f. is widely distributed throughout the range of its hosts in the northern hemisphere. It infects a very large range of deciduous and (rarely) coniferous trees. Moreover, in healthy beech (*Fagus sylvatica*) trees it can also operate as an endophyte (Baum et al. 2003). *F. fomentarius* is commonly known as the Tinder Polypore, Hoof Fungus, Ice Man Fungus or Tinder Conk. Its basidioma is also known as the "Mudi" in Chinese, which has been used as a traditional Chinese medicine for many centuries in China for the treatment of various diseases (Chen et al. 2008).

Until recently, no significant genetic variability was observed in *F. fomentarius*. In our previous work (Judova et al. 2012) we observed the existence of two genotypes in this species based on comparison of internal transcribed spacer (ITS) sequences. Two genotypes differ from each other by 3% at the nucleotide level and the presence of 7 bp indel in the ITS2 region is the most remarkable difference. A rapid method was developed for discrimination of these genotypes and analyses suggest that one of genotypes is prevalent on beech (*F. sylvatica*), while other prefers other broadleaves trees (Judova et al. 2012).

The aim of the present study was to re-evaluate genetic variability in *F. fomentarius* seen at the ITS level using other molecular methods based on sequence comparison of both the partial translation elongation factor 1-α (*efa*) region and partial 25S large subunit ribosomal RNA (LSU) gene as well as to compare the discriminatory power of three molecular markers used.

Material and methods

DNA isolation and analysis
All DNA techniques used were in principle as already described (Judova et al. 2012). Shortly, total genomic DNA of *F. fomentarius* strains was prepared using microwave treatment according to Goodwin & Lee (1993) with small modifications. Released DNA was purified by repeated extractions using chloroform-isoamylalcohol (24:1) mixture and DNA was precipitated using isopropylalcohol. Purified DNA was visualized after electrophoresis on 0.8% agarose gels.

PCR amplifications and primers
For PCR amplification, about 50 ng of total DNA was amplified using EF595F (5'-CGTGACTTCATCAAGAACATG-3') and EF1160R (5'-CCGATCTTGTAGACGTCCTG-3') primer pair for the partial translation elongation factor 1-α (*efa*) region (Kauserud & Schumacher 2001) or LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR7 (5'-TACTACCACCAAGATCT-3') primer pair for the partial

Table 1. *Fomes fomentarius* strains used in this study.

Strain	Host	ITS genotype	GenBank accession number		
			ITS	*Efa*	LSU
1 FF001AP	*Acer platanoides*	B	FJ865438	JX481268	JX470534
7 FF007NA	*Negundo aceroides*	A	FJ865440	JX481269	JX470537
9 FF009AH	*Aesculus hippocastanum*	B	FJ865441	JX481267	JX470538
ITS12 FF012FS	*Fagus sylvatica*	A	GQ184603	JX481270	JX470535
13 FF013Tsp	*Tilia* sp.	B	FJ865443	JX481266	JX470536

large subunit ribosomal RNA (LSU) region (Vilgalys & Hester 1990).

PCR was performed in a MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories, Richmond, USA). The reaction mixtures (50 μL) contained 200 μM of each deoxyribonucleotide triphosphate, 1 μM of each primer, 1.25 U *Taq* DNA polymerase (Invitrogen, Paisley, UK), 5 μL 10× PCR buffer (Invitrogen, Paisley, UK), 2 mM $MgCl_2$ and 50 ng of template DNA. The PCR cycling conditions involved an initial cycle of 94 °C for 5 min, followed by 36 cycles of 30 s at 94 °C, 35 s at 55 °C, 40 s at 72 °C, and a final 10 min extension step at 72 °C for *efa* sequences. For LSU sequences a program was used consisting of initial cycle of 94 °C for 5 min, followed by 36 cycles of 1 min at 95 °C, 45 s at 47 °C, and 1.5 min at 72 °C.

Amplification products were visualized by electrophoresis through 0.8% agarose gels, purified using Wizard SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA), and selected amplicons were sequenced in both directions using the same primer pair as for PCR at Macrogen sequencing facility (Macrogen, Seoul, Korea). The sequences obtained were submitted to the GenBank database (Benson et al. 2013).

Phylogenetic analyses

The *efa* and LSU sequences of *F. fomentarius* strains obtained during this study were assembled using DNA Baser version 2.2 software (Heracle BioSoft S.R.L., Romania) and compared against GenBank database using BLASTn and BLASTx algorithms (Altschul et al. 1990).

All phylogenetic analyses were conducted using MEGA software version 5 (Tamura et al. 2011). DNA sequences were aligned using Muscle algorithm (Edgar 2004) and phylogenetic relationships were constructed by all phylogeny reconstruction methods available in the software. Phylogenetic robustness of trees obtained was tested by bootstrap analysis after 1,000 replications.

Results

Strains of *Fomes fomentarius* selected from our previous study for its variability on hosts from urban and suburban areas in Slovakia (Central Europe) were typed to the ITS1 or ITS2 genotype on the basis of ITS-RFLP analysis and nucleotide sequence comparison of ITS region and used for further analyses (Table 1).

Variability of partial translation elongation factor 1-α (efa) region sequences

Of about 500 bp long partial nucleotide sequence of translation elongation factor 1-α (*efa*) region of all studied strains were obtained. The sequences show highest similarity at nucleotide level to other polypores, e.g., members of *Trametes* (84%), *Coriolopsis* (83%), and *Ganoderma* (81%) using BLASTn algorhitm. Conserved introns were detected in all *efa* sequences. At the amino acid level, the sequences were practically identical – the single difference observed was the substitution of isoleucine in genotype A by a valine in genotype B. Both genotype A and B derived amino acid sequences exhibited high degree of sequence similarity (over 95%) to translation elongation factor sequences of many polypores but agarics and bolets as well (e.g., *Trametes villosa*, *Armillaria borealis*, *Boletus carminipes*; data not shown).

For phylogenetic analyses the *efa* nucleotide sequences of studied strains were compared to *efa* sequences of other polypores. Unfortunately, there have been no additional *F. fomentarius efa* sequences in the GenBank database. Sequence analysis clearly confirmed the existence of two *F. fomentarius* genotypes. The sequences of ITS genotype A grouped to a branch separated from ITS genotype B with strong statistical support using all phylogeny reconstruction methods available in MEGA-5 software (bootstrap values 100 for all methods used, Fig. 1). The mean interpopulation diversity was 0.036 base substitutions per site, while mean intrapopulations diversity was 0.009 only. The same level of variability as seen for *F. fomentarius* sequences was observed, e.g., for *Trametes versicolor* and *T. pubescens* species pair (Fig. 1).

Variability of partial 25S large subunit ribosomal RNA gene sequences

Of about 850 bp long partial 25S large subunit ribosomal RNA gene sequences were obtained for all studied strains. The sequences showed more than 99% similarity at the nucleotide level to other *F. fomentarius* LSU sequences. The sequences were aligned against other 25S LSU sequences available in the GenBank database and the phylogenetic relatedness was assessed by all phylogeny reconstruction methods available in MEGA-5 software. All these methods confirmed genetic variability in *F. fomentarius*, however, the observed diversity level and bootstrap support values are lower than those obtained for the *efa* sequences (the mean interpopulation diversity 0.006 base substitutions per site). Strains classified as belonging to the ITS genotype A grouped separately from those belonging to the ITS genotype B. As documented in Figure 2, the genetic distance between *F. fomentarius* LSU sequences is similar that between

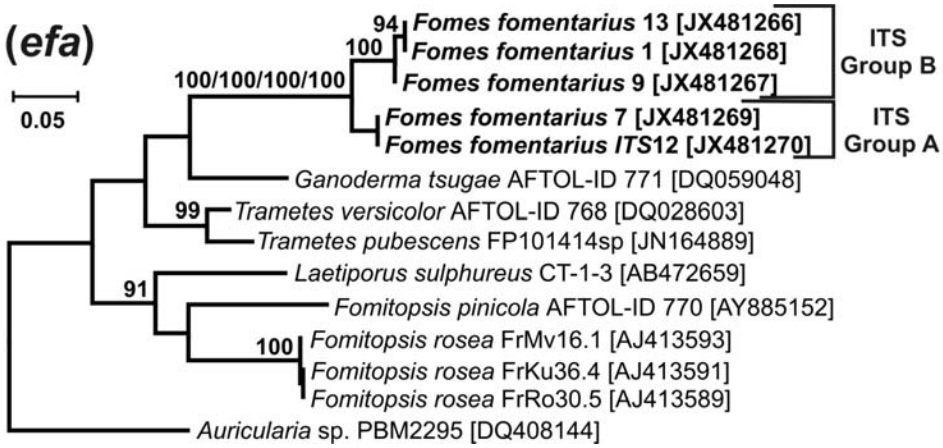

Fig. 1. Phylogenetic relationship of *Fomes fomentarius* and related species inferred from partial nucleotide sequences of the translation elongation factor 1-α (*efa*) region. The tree was constructed using the maximum likelihood algorithm. The numbers at nodes are bootstrap values after 1,000 repetitions. The *efa* sequence of *Auricularia* sp. PBM2295 was used as an outgroup. For *F. fomentarius* branch, bootstrap values for maximum likelihood/neighbour joining/minimum evolution/maximum parsimony phylogeny reconstruction methods are shown. The sequences obtained in this study are shown in bold.

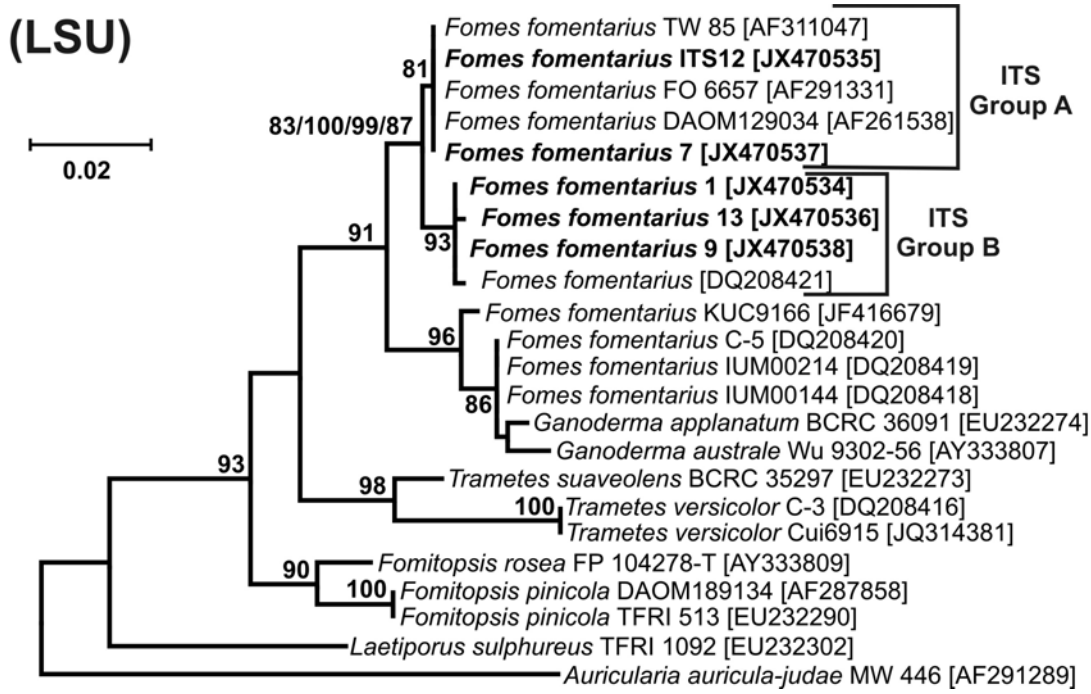

Fig. 2. Phylogenetic relationship of *Fomes fomentarius* and related species inferred from partial nucleotide sequences of the 25S large subunit ribosomal RNA (LSU) gene. The tree was constructed using maximum likelihood algorithm. The numbers at nodes are bootstrap values after 1,000 repetitions. The LSU sequence of *Auricularia auricula-judae* MW 446 was used as an outgroup. For *F. fomentarius* branch, bootstrap values for maximum likelihood/neighbour joining/minimum evolution/maximum parsimony phylogeny reconstruction methods are shown. The sequences obtained in this study are shown in bold.

Ganoderma species *G. applanatum* and *G. australe*. Surprisingly four *F. fomentarius* strains from South-Eastern Asia (GenBank accession numbers DQ208418-DQ208420 and JF416679 – all originating from South Korea) formed well supported separate branch outside the *F. fomentarius* sequences of European origin (Fig. 2). However, phylogenetic analyses placed these sequences to the branch shared with *Ganoderma* spp. LSU sequences indicating that these sequences either came from a new unrecognized species morphologically similar to European ones or the specimens were misidentified.

Discussion

The application of DNA based methods has dramatically improved our ability to reveal speciation and cryptic diversity in Basidiomycota. Recent studies have shown that many morphospecies are complex or aggregates of taxa with distinct geographic, ecological or pathological traits, comprising several biological and/or phylogenetic species (e.g., Le Gac et al. 2007; Geml et al. 2008; Stubbe et al. 2010; O'Donnell et al. 2011).

The nuclear ribosomal RNA genes, which include the small-subunit (SSU) and large-subunit (LSU)

rRNA genes and the internal transcribed spacer (ITS) region that separates the two rRNA genes, have been used for fungal phylogenetics for more than two decades. In our previous work (Judova et al. 2012) we observed genetic variability in *F. fomentarius* based on comparison of ITS sequences indicating that *F. fomentarius* population in Central Europe is probably composed of two sympatric cryptic species differing by 3% at the ITS sequence level. However, a relatively high level of intragenomic variation of ITS sequences was reported for some species, e.g. up to 3.6% in *Mycosphaerella punctiformis* (Simon & Weiss 2008) or basidiomycete *Laetiporus* (Lindner & Banik 2011).

In this study, genetic variability within *F. fomentarius* was confirmed by analysis of other rRNA marker – LSU sequences. Despite rRNA markers protein-coding genes are now widely used for species identification in mycology; several different markers, e.g. gene for translation elongation factor 1-α were used for study of population structure of *Phellinus nigrolimitatus* (Kauserud & Schumacher 2002) and could provide a deeper view of variability of fungal species.

Both partial translation elongation factor 1-α (*efa*) region and partial 25S large subunit ribosomal RNA (LSU) gene sequences comparison used in this study confirmed the existence at least two different cryptic species within *F. fomentarius* with strong statistical support. Congruent phylogeny was observed between ITS based either on *efa* or LSU phylogeny. ITS group A strains grouped to the separate branch out of the ITS group B isolates in all phylogeny analyses used. Stronger support and higher level of divergence was obtained for *efa* than for LSU sequences. Our data indicate that similar situation is found between *Fomitopsis pinicola* and *F. rosea* species. Genetic divergence between *F. fomentarius* species complex is comparable to the divergence seen between some other polypore species included in the current comparisons (Fig. 1).

However, analysis of LSU sequences strongly supported the existence of third *F. fomentarius* genotype. The most probable explanation of this observation is misidentification of the specimens. The sequences of South Korean origin (GenBank: JF416679 and DQ208418-DQ208420) were grouped outside the *F. fomentarius* sequences of European origin (Fig. 2). Another *F. fomentarius* LSU sequence from South Korea (GenBank: DQ208421) clustered with European ITS genotype B sequences. Based on the differences in Korean *F. fomentarius* LSU sequences, variability in *F. fomentarius* has already been postulated (Lee et al. 2006). However, phylogenetic analyses placed these sequences to the branch shared with *Ganoderma* spp. LSU sequences indicating that these sequences either came from a new unrecognized species morphologically similar to European *F. fomentarius* or the specimens were misidentified. Other experiments will be necessary to evaluate differences between South-Eastern Asia and European specimens.

Our data clearly demonstrate that *F. fomentarius* is composed of (at least) two sympatric cryptic species. For the first time the sequences of another two molecular markers, partial translation elongation factor 1-α region and partial 25S large subunit ribosomal RNA gene were obtained from the studied set of isolates and used to evaluate genetic variability of *F. fomentarius*. All three genetic markers (ITS, *efa*, and LSU) confirmed genetic variability within *F. fomentarius* species complex. Surprisingly, non-coding ITS sequence diversity within *F. fomentarius* taxa was lower compared to the variability of *efa* sequences (0.023 versus 0.036 nucleotide substitutions per site) coding for functional protein. For several other species (e.g. *Penicillium* spp.; Skouboe et al. 1999) it was documented that the variability of the ITS region is sometimes not high enough to separate it at the level of species.

Due to an insufficient evolutionary pressure, rapidly evolving ITS sequences have been widely used for species discrimination in mycology. The data presented here indicate that for some fungal species, partial translation elongation factor 1-α region sequences show higher degree of variability and discrimination power compared to currently used ITS sequences. There is a relative lack of fungal *efa* sequences in the GenBank (Benson et al. 2013); however, the same situation as seen for *F. fomentarius* taxa (0.023 versus 0.036 nucleotide substitutions per site for ITS versus *efa* variability) was observed for several pairs of closely related species. For example, for *Cortinarius aurilicis*/*C. iodes* (0.102 versus 0.151), *Grifola sordulenta*/*G. frondosa* (0.114 versus 0.182) or *Monilinia laxa*/*M. fructicola* (0.009 versus 0.020). For all these species LSU marker shows the lowest discriminatory power.

Until recently, despite these results, no clear differences were observed between basidiomata and/or strains, which would be suitable for reliable separation of *F. fomentarius* taxa. DNA analysis of three DNA markers provides a clear evidence for genetic non-homogeneity of *F. fomentarius* in Central Europe. Another study, aimed mainly on strains from Asia, could bring new data on true variability of *F. fomentarius*.

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