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Molecular identification of *Fomes fomentarius* in hosts from urban and suburban areas in Slovakia

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Abstract

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Intraspecific, genetic diversity was studied in the wood-decaying fungus *Fomes fomentarius* using the internal transcribed spacer region (ITS) sequence analysis. Fourteen different isolates originating from six host plant genera from the Slovakian urban and suburban areas (Central Europe) were compared. Based on ITS sequences comparison, two different genotypes were found. Genotype A consists of the two isolates from *Fagus sylvatica* and *Negundo aceroides*, whereas genotype B consists of isolates from all host plants (excluding *Negundo aceroides*). There only exists one host tree species (*Fagus sylvatica*) that hosts both fungal genotypes. Moreover, this host clearly represents two different genotypes from the same kind of urban and suburban habitat. Our study appears to be the first report of the heterogeneity within the ITS region of *F. fomentarius* from urban trees. Our data indicate that the occurrence of *F. fomentarius* genotypes is affected by characters such as host plant species and kinds of urban habitats, but not by geographical location.

Keywords

Fomes fomentarius, hosts, ITS region, urban habitats

Introduction

Fomes fomentarius (L.) J. Kickx f. is one of the most important fungi in the temperate and boreal forests of the northern hemisphere causing white rot in forest trees. On healthy *Fagus sylvatica* trees it can also operate as an endophyte (Baum et al., 2003). Although there are some studies on *F. fomentarius* in the forest ecosystems (Baum et al., 2003; Schwarze et al., 2004; Schmidt, 2006), there is little information available about properties of this polypore collected from urban and suburban areas. Between 1999 and 2001 a pilot study was carried out within COST Action E-12 on the main pests and diseases in urban forests and trees in 18 European countries. From 8 of them it has been reported among fungi causing decay mentioned as important (Konijnendijk et al., 2005). The occurrence of *F. fomentarius* basidiomes in urban Slovakia during the years 1982–2010 has been described previously and account of its localities have been presented (Gáperová and Gáper, 2011; Gáper et al., 2011). Some trees were common hosts wherever they occurred (e.g. *Aesculus hippocastanum*, *Acer* spp., *Populus* spp. and *Tilia* spp.). Other trees, although widespread and common (e.g. *Negundo aceroides*), hosted this polypore only occasionally. The aim of the present study was to analyze

intraspecific variability in *F. fomentarius* isolates using molecular methods based on available ITS sequences from the Slovakian urban and suburban areas. We questioned also whether the host ranges, geographical factors, and kinds of urban habitat of *F. fomentarius* reflect its genetic diversity.

Material and methods

Isolates

Fomes fomentarius basidiomes were collected from a living or dead tree trunks and stumps within the capital city Bratislava (Western Slovak Region, specimens no. 2, 4) and the Central Slovak Region (the others specimens). Localities within Central Slovak Region were distant from each other by 0.5–79.2 km. Urban and suburban habitats are divided into five categories according to the placement and function they have (Jeffrey, 2002; Konijnendijk et al., 2005; Kunca, 2009; Modranský, 2012): (1) Urban "Public" open city spaces (public parks, pocket parks, both paved and non-paved squares and pockets of greenery in the cities and towns: planted streets, promenades, tree-lined allees, courtyards and patios), (2) Urban "Public" open village spaces (public parks, pocket parks, both paved and non-paved pockets of greenery in the villages: planted streets, tree-lined allees), (3) Quasi-natural habitats associated with enginered features (transport corridor verges: roadsides), (4) Suburban Gardens and residential landscaping: private garden areas, (5) Nearly natural habitats: foothills of settlement Mountains.

Isolations were performed within 24 hours of the collection of samples in the field. All pure cultures were obtained on 2% malt extract agar in a Petri dish from trama of basidiomes (Júdová et al., 2012). The cultures were incubated at 24 ± 1°C in darkness. The remaining part of each specimen was dried and stored as voucher specimens. Herbarium specimens are deposited in the herbarium of the Department of Biology and General Ecology, Faculty of Ecology and Environmental Sciences, Technical University in Zvolen (KBVE). Pure cultures were isolated from 14 specimens growing on *Acer platanoides* L., *Aesculus hippocastanum* L., *Fagus sylvatica* L., *Negundo aceroides* Moench, *Populus alba* L., *Populus tremula* L., *Populus* sp. and *Tilia* sp. Cultures are preserved at the Mycological laboratory of the University of Matej Bel in Banská Bystrica, Slovakia under numbers 1 FF001AP–ITS14 FF014PA. Obtained ITS sequences were deposited in GenBank database (Accession Nos. FJ865438–FJ865443 and GQ184597–GQ184604).

Specimens and isolates examined

No. 1: Starohorské vrchy Mts.: the Laskomerská dolina valley, the Laskomer private garden area, ca. 2.5 km NE of the town of Banská Bystrica, 20 Oct., 2008, (KBVE 1293, 1 FF001AP); no. 2: the city of Bratislava, the municipal part of Petržalka, Janko Kráľ Park, 11 Nov., 2007, (KBVE 1294, ITS2 FF002AP); no. 3: the town of Banská Bystrica, Štadlerovo nábrežie promenade, 2 June, 2008, (KBVE 1295, ITS3 FF003Tsp); no. 4: the city of Bratislava, the municipal part of Petržalka, Janko Kráľ Park, 10 Nov., 2007, (KBVE 1296, ITS4 FF004Tsp); no. 5: in the village of Horný Tisovník in the Veľký Krtíš District, 25 March, 2008, (KBVE 1297, 5 FF005Psp); no. 6: the roadside near the village of Senné in the Veľký Krtíš District, 15 April, 2008, (KBVE 1298, ITS6 FF006PT); no. 7: the roadside near the town of Zvolen, 25 June, 2007, (KBVE 1299, 7 FF-007NA); no. 8: in the village of Dolná Strehová in the Veľký Krtíš District, I. Madácha Park, 31 Aug., 2005, (KBVE 1300, ITS8 FF008AH); no. 9: ibid., 31 May, 2008, (KBVE 1301, 9 FF009AH); no. 10: Starohorské vrchy Mts.: the Laskomerská dolina valley, foothills of Starohorské vrchy Mts., ca. 4.5 km NE of the town of Banská Bystrica, 31 May, 2008, (KBVE 1302, 10 FF010FS); no. 11: ibid., ca. 4 km NE of the town of Banská Bystrica, 22 Oct., 2008, (KBVE 1994, ITS11 FF011FS); no. 12: ibid., ca. 4.5 km NE of the town of Banská Bystrica, 31 May, 2008, (KBVE 1304, ITS12 FF012FS); no. 13: in the village of Kováčová in the Zvolen District, Spa Park, 19 Sept., 2008, (KBVE 1305, 13 FF013Tsp); no. 14: the roadside near the village of Mýtna in the Lučenec District, 19 Sept., 2008, (KBVE 1306, ITS14 FF014PA).

To the aim of our research, we compared our data with three other ITS sequences available in Genbank.

DNA isolation, amplification and analysis

Total genomic DNA of *F. fomentarius* isolates was prepared using microwave treatment according to Goodwin and Lee (1993) with small modifications. 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 deoxynucleotide triphosphate, 1 µM of each primer, 1.25 U Taq DNA polymerase (Invitrogen, Paisley, UK), 5 µl 10x PCR buffer (Invitrogen, Paisley, UK), 2 mM $MgCl_2$, and 50 ng template DNA. For amplification of ITS1-5.8S-ITS4 nuclear DNA region ITS1 and ITS4 primers were used (White et al., 1990). The PCR cycling conditions involved an initial cycle of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 45 s, and 72 °C for 45 s, with a final cycle step at 72 °C for 10 min.

Amplification products were visualized by electrophoresis through 0.8% agarose gels. A 1 kb DNA or 100 bp DNA ladders (invitrogen) were used as a molecular mass standard. ITS amplicons were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and sequenced in both directions using the same

primer pair as for PCR at Macrogen sequencing facility (Macrogen, Seoul, South Korea). Sequences obtained were compared against GenBank database using blastN algorithm (Altschul et al., 1990). The sequences were typed into A and B genotype based on the absence or presence of 7 bp signature sequence TCGTTTG (Júdová et al., 2012).

Results

To evaluate phylogenetic relatedness among *Fomes fomentarius* isolates ITS region from all 14 urban strains was amplified by PCR and sequenced. Continuous ITS sequences of at least 730 bp were obtained from every isolate analysed and deposited in GenBank database under accession numbers FJ865438–FJ865443 and GQ184597–GQ184604 (Tables 1–2). Comparison of sequences with ITS sequences available in GenBank indicated that all isolates belong to the *F. fomentarius* species with sequence similarity values to other isolates of this species higher than 97% (data not shown). Three additional sequences originated, however, from natural habitat, available in GenBank, were used in the analysis from Slovakia for comparison purpose (Tables 1–2): KYJ3: Vihorlat Mts.: Kyjovský prales primeval forest reserve, ca. 60 km NE of the town of Košice; 980706.7: Kremnické vrchy Mts.: Badínsky prales primeval forest reserve, ca. 10 km SW of the town of Banská Bystrica; KYJ7: Vihorlat Mts.: Kyjovský prales primeval forest reserve, ca. 60 km NE of the town of Košice.

Analysis on ITS sequence data clearly indicated the presence of two sequence types (genotypes) in studied *F. fomentarius* isolates. All isolates typed to the B genotype showed the presence of 7 bp signature sequence TCGTTTG in ITS1 region while isolates typed to the A genotype lack this sequence (data not shown). The absence or presence of this sequence was used for discrimination of *F. fomentarius* genotypes from natural forest reserves in Vihorlat Mountains (Júdová et al., 2012).

The majority of our urban and suburban isolates formed large group together with the strain KYJ7 isolated from basidiome collected in the Kyjovský prales primeval forest reserve on adult *Fagus sylvatica* (genotype B, Table 1). The rest our urban and suburban

Table 1. List of the 13 isolates of *Fomes fomentarius* (genotype B), of which 12 are used in the study (shown in bold) and 1 is additional sequence available from Slovakia in GenBank

Strain No.	Slovak Region	Host	Habitat*	Accession No.
1 FF001AP	**Central**	***Acer platanoides***	**(4)**	**FJ865438**
ITS2 FF002AP	**Western**	***Acer platanoides***	**(1)**	**GQ184597**
ITS3 FF003Tsp	**Central**	***Tilia* sp.**	**(1)**	**GQ184598**
ITS4 FF004Tsp	**Western**	***Tilia* sp.**	**(1)**	**GQ184599**
5 FF005Psp	**Central-southern**	***Populus* sp.**	**(2)**	**FJ865439**
ITS6 FF006PT	**Central-southern**	***Populus tremula***	**(3)**	**GQ184600**
ITS8 FF008AH	**Central-southern**	***Aesculus hippocastanum***	**(2)**	**GQ184601**
9 FF009AH	**Central-southern**	***Aesculus hippocastanum***	**(2)**	**FJ865441**
10 FF010FS	**Central**	***Fagus sylvatica***	**(5)**	**FJ865442**
ITS11 FF011FS	**Central**	***Fagus sylvatica***	**(5)**	**GQ184602**
13 FF013Tsp	**Central**	***Tilia* sp.**	**(2)**	**FJ865443**
ITS14 FF014PA	**Central-southern**	***Populus alba***	**(3)**	**GQ184604**
KYJ7	Eastern	*Cerasus avium*	(6)	HQ189535

*Habitat: (1) – Urban "Public" open city space; (2) – Urban "Public" open village space; (3) – Quasi-natural habitat associated with engineered features; (4) – Suburban Garden and residential landscaping; (5) – Nearly natural habitat; (6) – Natural habitat.

Table 2. List of the 4 isolates of *Fomes fomentarius* (genotype A), of which 2 are used in the study (shown in bold) and 2 are additional sequences from Slovakia available in GenBank

Strain No.	Slovak Region	Host	Habitat*	Accession No.
7 FF007NA	**Central**	***Negundo aceroides***	**(3)**	**FJ865440**
ITS12 FF012FS	**Central**	***Fagus sylvatica***	**(5)**	**GQ184603**
KYJ3	Eastern	*Fagus sylvatica*	(6)	HQ189534
980706.7	Central	*Fagus sylvatica*	(6)	EU162056

*Habitat: (3) – Quasi-natural habitat associated with engineered features; (5) – Nearly natural habitat; (6) – Natural habitat.

strains (7 FF007NA and ITS12 FF012FS) fell to the two sequences (HQ189534 and EU162056) originating also from *Fagus sylvatica* host collected in the Kyjovský prales primeval forest reserve and Badínsky prales primeval forest reserve (genotype A, Table 2).

This study demonstrates that both fungal genotypes are widespread on different hosts over large areas. The genotype B (Table 1) consists of thirteen fungal strains from six host genera, among which are three from *Tilia* spp., two from *Acer platanoides*, *Aesculus hippocastanum* and *Fagus sylvatica*, and are each from *Cerasus avium* (from natural forest only), *Populus alba*, *Populus tremula* and *Populus* sp. The genotype A (Table 2) consists of four strains from two host genera, among which are three from *Fagus sylvatica* (the one of its from natural forest) along with one from *Negundo aceroides*. Yet only one woody host plant (*Fagus sylvatica*) has both fungal genotypes. Moreover, there is the observation, that different European beech trees growing within a few meters of each other have different fungal genotypes (Accession Nos. FJ865442, GQ 184602, GQ 184603), so it is likely that the geographical features do not reflect the genetic diversity of *F. fomentarius*.

In view of the urban habitats, the majority of our isolates originating from the Western Slovak Region and the Central Slovak Region and from all urban and suburban habitats fell to the genotype B (Table 1). Two other isolates (genotype A) come from two different urban habitats ("Quasi natural habitat associated with engineered features" and "Nearly natural habitat" respectively) within the Central Slovak Region (Table 2). The host *Fagus sylvatica* clearly represents two different genotypes (Accession Nos. FJ 865442 and GQ184603) from the same kind of urban habitat ("Nearly natural habitat").

In summary, our results indicate that at least in the two above mentioned kinds of urban and suburban habitat both the genotypes are found sympatrically, so it is likely that the *Fomes fomentarius* includes two cryptic species. The data suggest that genotype A seems to be linked preferably with natural like habitats. No sequence originated from *Fomes fomentarius* basidiomes grown within the "Urban public open spaces" was observed. In contrary, from 12 our sequences of genotype B only 5 sequences originated from *Fomes fomentarius* strains grown within natural like habitats.

Discussion

The nuclear ribosomal, particularly highly variable ITS regions are highly variable sequences widely used in distinguishing fungal species. The ITS regions are highly conserved within most species – with intraspecific similarities usually higher than 99% – but are variable between species, making it suitable for use in taxonomy (Gomes et al., 2002). In natural forest reserves in Vihorlat Mountains (Eastern Slovakia), it consists of two sequence types (genotypes) showing different host preferences. The genotype A is a pathogen of *Fagus sylvatica* and *Betula pendula*, and the genotype B occurs on *Fagus sylvatica*, *Quercus robur* and *Cerasus avium*. Both the genotypes were found sympatrically, so it is likely that the *Fomes fomentarius*, a single described morphological species, should include two sympatric cryptic species (Júdová et al., 2012). Molecular analysis of *F. fomentarius* strains from urban and suburban areas in Slovakia also clearly identified two genotypes of strains with overall ITS sequence similarity values 97% only (data not shown), indicating complex genetic structure of *F. fomentarius* population.

Among polypores it is common to find species complexes within the traditional morphological species (Holdenrieder and Greig, 1998; Kraj and Kowalski, 2010; Tomšovský et al., 2010; Vasaitis et al., 2009 and others). For example, the ribosomal DNA sequences, including sequences from the internal transcribed spacer (ITS) and also large subunit (LSU) regions, have been used to define species and infer phylogenetic relationships in genus *Laetiporus* and to confirm the existence of cryptic species described with mating compatibility, ITS-RFLP, morphology and host preference data

(Vasaitis et al., 2009; Lindner and Banik, 2011 and others). According to the data made available here, it is the first report of the heterogeneity within the ITS region among *F. fomentarius* isolates from urban areas within different kinds of urban habitats in Slovakia. Our findings are of high importance for polypore ecology and proper procedures aimed at the control and restriction of the epidemic spread of fungi over large urban areas. Similarly, our previous analysis of *F. fomentarius* basidiomes from Slovakia´s natural forests (Júdová et al., 2012) clearly identified two genotypes based on ITS sequence comparisons. ITS sequence variability observed in these experiments must be further analysed. The general line of investigation will continue to obtain valuable data regarding the association between *F. fomentarius* genotypes and various woody plant species within different kinds of habitat, including forest ecosystems, in Europe. There are two main questions. First, why is there only one tree species that has two *F. fomentarius* genotypes? Second, why are there only three kinds of habitat associated with both fungal genotypes occurring?

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Molekulárna identifikácia *Fomes fomentarius* na drevinách v urbánnom a suburbánnom prostredí na Slovensku

Súhrn

Drevokazný trúdnik *Fomes fomentarius* je bežne sa vyskytujúcou hubou na celom území Slovenska. Najčastejší je v bukových porastoch, často kolonizuje aj dreviny v mestskom prostredí, môže rásť aj endofyticky. Skúmaním 14 izolátov metódou založenou na PCR amplifikácii ITS sekvencií a ich následnom štiepení sme v urbánnom a suburbánnom prostredí Slovenska detekovali jeho dva genotypy. Údaje o genetickej typizácii sme spracovali tabelárne. Genotyp A kolonizuje *Fagus sylvatica* a *Negundo aceroides*, genotyp B má širší okruh hostiteľských drevín. *Fagus sylvatica* je jediným druhom, ktorý kolonizujú obidva genotypy. Genotyp B rastie vo všetkých kategóriách urbánnej vegetácie, pravdepodobne na celom území Slovenska.

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