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Genetic (non)-homogeneity of the bracket fungi of the genus *Ganoderma* (Basidiomycota) in Central Europe

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Abstract
During systematic surveys of urban, rural and forest areas in Slovakia and Moravia (Czech Republic) and occasional botanical excursions in Northern Hungary, seventy-five collections of *Ganoderma* spp. were gathered during 2015 – 2018. Taxa identification was accomplished through observation of morphological characteristics of their mature, sporulating and undeformed basidiocarps. The genetic diversity of the collections was investigated by using internal transcribed spacer (ITS1/4) of ribosomal DNA sequences comparisons. Both analyses showed that the 75 collections clustered into six clades, namely, *G. applanatum*, *G. adspersum*, *G. resinaceum*, *G. pfeifferi*, *G. lucidum* and *G. carnosum* in accordance with the morphospecies concept.
The sequence comparison demonstrated genetic homogeneity of all *G. resinaceum* clade. Multiple sequence alignment indicated the presence of two *G. resinaceum* sequence types with significant statistic support and, probably, the existence of two cryptic taxa. The twenty-eight collections clustered with the *G. applanatum* group and the twenty other collections were grouped with morphologically very similar *G. adspersum* group. However, ITS sequences show no close similarity between these two species.

Key words – basidiospores – cryptic taxa – interspecific and intraspecific variations – ITS – phylogeny – polypores – wood-decay

Introduction

Ganoderma (Ganodermataceae, Agaricomycetes, Basidiomycota) is a well-known white-rot wood-decay genus of bracket macrofungi (polypores) because of its medicinal properties (Bishop et al. 2015, Hapuarachchi et al. 2016) and phytopathological importance (Schwarze & Ferner 2003, Ryvarden & Melo 2014). The genus differs from other polypores by the crust-like upper surfaces of their basidiocarps, double-walled basidiospores with an enlarged or truncated apex and exosporium with ornamentations (Karsten 1881, Moncalvo 2000, Niemelä & Miettinen 2008, Hennicke et al.

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2016, Costa-Rezende et al. 2017). The hyphal system is arboreal: the generative hyphae are hyaline, thin-walled, clamped; the arboriform hyphae are yellowish brown, thick-walled; basidiospores are (ellipsoid-, cylindric-) ovoid; cystidia absent (Breitenbach & Kranzlin 1986, Gilbertson & Ryvarden 1987, Hansen & Knudsen 1997, Bernicchia 2005).

Ganoderma species were classified primarily on the basis of morphological features, such as an appearance of pileus surface (dull or laccate, resinous deposits), colour of the context, presence of the stipe, and shape, size and ornamentation of the basidiospores (Breitenbach & Kranzlin 1986, Gilbertson & Ryvarden 1987, Hansen & Knudsen 1997, Bernicchia 2005, Torres-Torres & Dávalos 2012, Ryvarden & Melo 2014, Costa-Rezende et al. 2017). However, over the past three decades, there has been substantial progress in our understanding of genetic variability within these traditionally recognized wood-decay macrofungi. Molecular systematics has been shown to be a valuable tool in their current taxonomy (Papp 2019). The first fundamental works on phylogenetic studies of *Ganoderma* using the internal transcribed spacer (ITS) and large subunit (LSU) rDNA sequences are those of Moncalvo et al. (1994, 1995a, 1995b), Bae et al. (1995), Hseu et al. (1996), and Sokoł et al. (1999) in the 1990s. The second major advance work was in the late 2000's, when Guglielmo and co-workers carried out PCR assays with taxon-specific primers for the early diagnostics of the most important phytopathological species (Guglielmo et al. 2007, 2008, 2010). In molecular studies of *Ganoderma*, the most common sequenced marker is the ITS of nuclear DNA (e.g. Moncalvo 2000, Smith & Sivasithamparam 2000, Moncalvo & Buchanan 2008, Douanla-Meli & Langer 2009, Cao et al. 2012, Park et al. 2012, Wang et al. 2012, Zhou et al. 2015, Hapuarachchi et al. 2019, Hennicke et al. 2016, Xing et al. 2016, 2018), which is one of the most preferred regions for genetic identification of fungi (Raja et al. 2017). ITS was accepted as the universal barcode marker for fungi by a consortium of mycologists (Schoch et al. 2012, Raja et al. 2017). In addition, many other markers have been used for phylogenetic studies within *Ganoderma* genus, such as nuclear large subunit ribosomal DNA (nrLSU, Costa-Rezende et al. 2017), mitochondrial small subunit ribosomal DNA (mtSSU) (Hong & Jung 2004), protein coding genes: translation elongation factor 1-α (tef1-α), RNA polymerase II largest subunit (RBP1), RNA polymerase II second largest subunit (RPB2), β-tubulin (β-tub) and intergenic spacer region (IGS) (e.g. Cao et al. 2012, Park et al. 2012, Wang et al. 2012, Zhou et al. 2015, Costa-Rezende et al. 2017). Due to the development of molecular methods, many new species of *Ganoderma* are being described, e.g. *Ganoderma ellipsoideum* Hapuar., T.C. Wen & K.D. Hyde, sp. nov. (Hapuarachchi et al. 2018) and *Ganoderma casuarinicola* J.H. Xing, B.K. Cui & Y.C. Dai, sp. nov. (Xing et al. 2018).

Within *Ganoderma*, over 250 (up to 400) species have been described worldwide, most of them from the tropics (Moncalvo et al. 1995a, Richter et al. 2015). Only seven species naturally occur in Central Europe (Kotlaba 1984, Sokół 2000, Bernicchia 2005, Papp & Szabó 2013, Ryvarden & Melo 2014). Although many molecular studies have been conducted within the genus, especially within *G. lucidum* complex (e.g. Cao et al. 2012, Wang et al. 2012, Zhou et al. 2015), available data from Central Europe are rare. The aim of the present study, therefore, was to analyze both interspecific and intraspecific genetic variability in this genus using molecular methods based on our ITS sequences in accordance with the morphospecies concept from Central Europe.

Materials & Methods

Fungal Collections

A total of seventy-five basidiocarps of *Ganoderma* spp. were collected in Central Europe. Most of the collections were acquired during systematic surveys of urban, rural and forest areas in Slovakia and Moravia (Czech Republic) and occasional botanical excursions in northern Hungary during 2015 – 2018. A schematic map illustrating their spatial distribution is given in Fig. 1.

The collections of the recorded species are also documented and voucher specimens are deposited in the Herbarium of the Department of Biology and Ecology, Faculty of Natural Sciences, Matej Bel University in Banská Bystrica, Slovakia. The specimens were morphologically identified by routine methods and determined according to standard, widely used keys (Breitenbach

& Kranzlin 1986, Gilbertson & Ryvarden 1987, Hansen & Knudsen 1997, Bernicchia 2005, Ryvarden & Melo 2014). In order to observe basidiospores (without the outer wall and expanded vesicular apex), slide preparations mounted in 5% KOH with cotton blue were drawn from dried tissue for each specimen and measured with maximum magnification (with immerse objective 100x) of a MOTIC light microscope (Motic Company, Germany). Thirty basidiospores from five basidiocarps of each species were measured for width and length of the inner wall. The nomenclature of fungi follows Index Fungorum (Cooper & Kirk 2019).

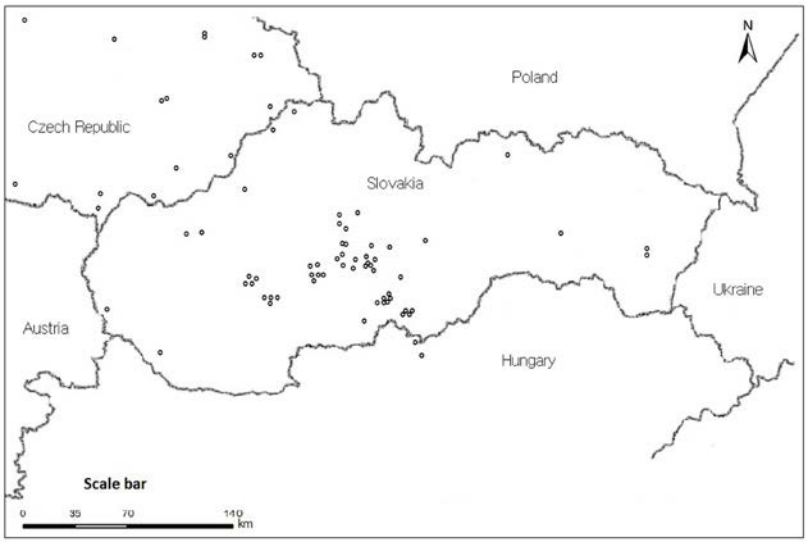

Figure 1 – Schematic map illustrating the spatial distribution of *Ganoderma* collections in Central Europe

DNA Isolation

Tissues from 75 fresh *Ganoderma* basidiocarps were ground using oscillating mill (MM200, Retsch GmbH, Haan, Germany). Total genomic DNA was extracted using the modified method described by Gašparcová et al. (2017). A small amount of fungal tissue (about 100 mg) was suspended in 300 µl of lysis solution (2% w/v CTAB, 100 mM Tris–HCl, 20 mM EDTA, 1.4 M NaCl, pH 8.0) and heated 10 times in a microwave oven (600 W for 4 s). Then 300 µl of fresh lysis solution was added again and the mixture was incubated at 100 °C for 2 min. After that the mixture was extracted using 500 µl chloroform and centrifuged for 3 min. The purified upper aqueous phase was transferred to a new microcentrifuge tube. Extraction using chloroform was repeated once more. Then DNA was precipitated with 0.7 vol. of isopropyl alcohol. Then samples were centrifuged for 10 min at maximum speed (12 000 g). The obtained DNA pellet was washed in 1 ml of 70% ethanol, centrifuged for 5 min at 12 000 g, and dissolved in 50 µl of PCR Grade Water (Solis BioDyne, Tartu, Estonia).

PCR amplification and Sanger Sequencing

The nuclear ribosomal ITS region was amplified with the primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) according to White et al. (1990). The polymerase chain reaction (PCR) was performed in a T100TM Thermal Cycler (Bio-rad Laboratories, Inc., Singapore). PCR reaction mixture (50 µl) contained 10 µl of 5x HOT FIREpol® Blend Master Mix with 10 mM $MgCl_2$ (Solis BioDyne, Tartu, Estonia), 1 µl of each primer (10 pmol/µl), 37 µl of PCR Grade Water (Solis BioDyne, Tartu, Estonia) and 1 µl of diluted genomic DNA (50 ng). Conditions of PCR cycling: an initial denaturation for 5 min at 94 °C, followed by 35 cycles at 94 °C for 30 s, 45 s at 52 °C and 45 s at 72 °C, a final extension at 72 °C for 10 min. Then PCR products were visualized after electrophoresis on 1.5% agarose gel, purified using ExoSAP-IT (Affymetrix, Inc., Cleveland, Ohio, USA) according to the manufacturer's

instructions, then sequenced in both directions using the same primers as for PCR at SEQme s.r.o. sequencing service (Dobříš, Czech Republic).

Phylogenetic analyses

About 600 bp ITS region was sequenced from all studied basidiocarps. In total, 75 ITS sequences were generated during this study and deposited in the GenBank database. The sequences from both primers were assembled using DNA Baser software (Heracle Biosoft, Romania) and resulted sequences were submitted to the GenBank database (For the list of GenBank accession numbers see Table 1). For phylogenetic analyses, sequences obtained were aligned using the clustalw algorithm and the phylogenetic tree was constructed using the Neighbor-Joining method. The reliability of the estimated phylogenetic tree was evaluated using bootstrapping with 1000 repetitions. For all phylogenetic analyses, MEGA software version 7 was used (Kumar et al. 2016). As many *Ganoderma* entries in the GenBank are misidentified or lack some important details (e.g. host species or locality), the sequences obtained during this study were used for the molecular analyses. The data obtained (alignment and tree file) were deposited to the Treebase database (Submission ID: 24333). For sequence comparisons, blastn analysis (Altschul et al. 1990) was performed against the GenBank database (Benson et al. 2013).

Results

The species/specimens recognized in the study, their geographical locations in Central Europe and their hosts are given in Table 1.

Table 1 GenBank accessions numbers of the 75 specimens of *Ganoderma* spp. used in this study.

Species/specimens No.	Geographical origin	Host	GenBank accession No.
Ganoderma applanatum			
JTGA	Slovakia	*Fagus sylvatica*	MK415277
FS8G	Slovakia	*Fagus sylvatica*	MK415253
MS115	Slovakia	*Tilia* sp.	MK415297
3GL	Czech Republic	unknown	MK415242
MS116	Slovakia	*Tilia* sp.	MK415298
MS117	Slovakia	*Tilia* sp.	MK415299
CA009ND	Slovakia	unknown	MK415248
BBURP5	Slovakia	unknown	MK415247
G171	Slovakia	*Quercus robur*	MK415260
K63	Czech Republic	unknown	MK415284
K60	Czech Republic	unknown	MK415283
K15	Czech Republic	unknown	MK415278
BB010ND	Slovakia	unknown	MK415245
BBURP1	Slovakia	unknown	MK415246
DT211	Slovakia	*Fraxinus excelsior*	MK415251
K28	Czech Republic	*Populus* sp.	MK415279
K33	Czech Republic	*Alnus* sp.	MK415281
DT212	Slovakia	*Fraxinus excelsior*	MK415252
K36	Czech Republic	unknown	MK415282
B47	Czech Republic	*Fagus sylvatica*	MK415243
G2	Slovakia	*Populus tremula*	MK415264
D3	Slovakia	*Fagus sylvatica*	MK415249
G012	Slovakia	*Quercus* sp.	MK415254
G013	Slovakia	unknown	MK415255
GVF	Slovakia	*Fagus sylvatica*	MK415275
GNRT	Slovakia	*Tilia* sp.	MK415273
VaG	Czech Republic	*Acer pseudoplatanus*	MK415313
MS15	Slovakia	*Fagus sylvatica*	MK415308
Ganoderma resinaceum			
Type A			
MS131	Slovakia	*Fraxinus excelsior*	MK415304
MS133	Slovakia	*Fraxinus excelsior*	MK415305

Table 1 Continued.

Species/specimens No.	Geographical origin	Host	GenBank accession No.
GSP	Slovakia	*Quercus* sp.	MK415274
Type B			
MS8	Slovakia	*Gleditschia triacanthos*	MK415309
14b	Slovakia	*Negundo aceroides*	MK415241
SADS1	Slovakia	*Salix alba*	MK415311
Ganoderma lucidum			
GL4	Slovakia	unknown	MK415270
K87	Czech Republic	unknown	MK415286
K84	Czech Republic	*Quercus* sp.	MK415285
GL5	Slovakia	*Quercus* sp.	MK415271
GLP	Slovakia	unknown	MK415272
GL	Slovakia	*Quercus* sp.	MK415269
Ganoderma carnosum			
GCAND	Czech Republic	unknown	MK415267
G177	Slovakia	unknown	MK415261
BB001ND	Slovakia	unknown	MK415244
GVZV	Slovakia	*Larix decidua*	MK415276
K91	Czech Republic	*Picea abies*	MK415287
ZV012PN	Slovakia	*Pinus nigra*	MK415314
GC011ND	Slovakia	unknown	MK415266
SG4	Slovakia	*Picea* sp.	MK415312
Ganoderma pfeifferi			
M2	Slovakia	*Tilia platyphyllos*	MK415295
MS10	Hungary	*Quercus robur*	MK415296
G16T	Slovakia	*Fagus sylvatica*	MK415259
G15T	Slovakia	*Fagus sylvatica*	MK415258
G1ZH	Slovakia	*Acer platanoides*	MK415263
PMS13	Slovakia	*Fagus sylvatica*	MK415310
LeG1	Czech Republic	*Fagus sylvatica*	MK415290
Ganoderma adspersum			
LV1	Slovakia	*Aesculus hippocastanum*	MK415291
G1	Slovakia	*Quercus* sp.	MK415256
G13	Slovakia	*Tilia cordata*	MK415257
GDS	Slovakia	*Fraxinus excelsior*	MK415268
ZV17	Slovakia	unknown	MK415315
K29	Czech Republic	unknown	MK415280
LC7	Slovakia	*Tilia cordata*	MK415289
LC013TC	Slovakia	*Tilia cordata*	MK415288
LVC4	Slovakia	unknown	MK415293
G2ZH	Slovakia	*Tilia platyphyllos*	MK415265
MS137	Slovakia	*Quercus petraea*	MK415306
DT210	Slovakia	unknown	MK415250
MS125	Slovakia	*Fraxinus excelsior*	MK415302
LVC5	Slovakia	*Tilia platyphyllos*	MK415294
LV2	Slovakia	*Aesculus hippocastanum*	MK415292
MS124	Slovakia	*Fraxinus excelsior*	MK415301
MS126	Slovakia	*Fraxinus excelsior*	MK415303
MS143	Slovakia	*Acer* sp.	MK415307
G1TT	Slovakia	*Tilia cordata*	MK415262
MS120	Slovakia	unknown	MK415300

Species determination through observation of morphological features of their mature, sporulating and undeformed basidiocarps showed that the 75 *Ganoderma* collections in Central Europe divided into six morphospecies: *G. applanatum* (Pers.) Pat., *G. adspersum* (Schulzer) Donk, *G. resinaceum* Boud., *G. pfeifferi* Bres., *G. lucidum* (Curtis) P. Karst., and *G. carnosum* Pat. *G. valesiacum* Boud., the seventh known, extremely rare European species, was not recorded and, therefore, it is not included in this study. The ITS sequences comparisons confirmed the existence

of 6 statistically well supported clades in concordance with the morphological identification (Fig. 2), however different levels of intra- and inter-clades variabilities were observed (Tables 2, 3).

The largest group of basidiocarps was identified in *G. applanatum*. The clade *G. applanatum* included 28 isolates from Slovakia and Czech Republic, most commonly occurring on stumps of unknown trees or *Fagus sylvatica* L. No intra-species genetic variability was observed in this clade (Table 3) and sequence comparisons indicated that sequences obtained in our study are identical to the *G. applanatum* sequences retrieved from GenBank, e.g. strain 407 from Poland (MH320562), strain CBS 187.31 from Germany (MH855178), strain 7411 from China (MG279158).

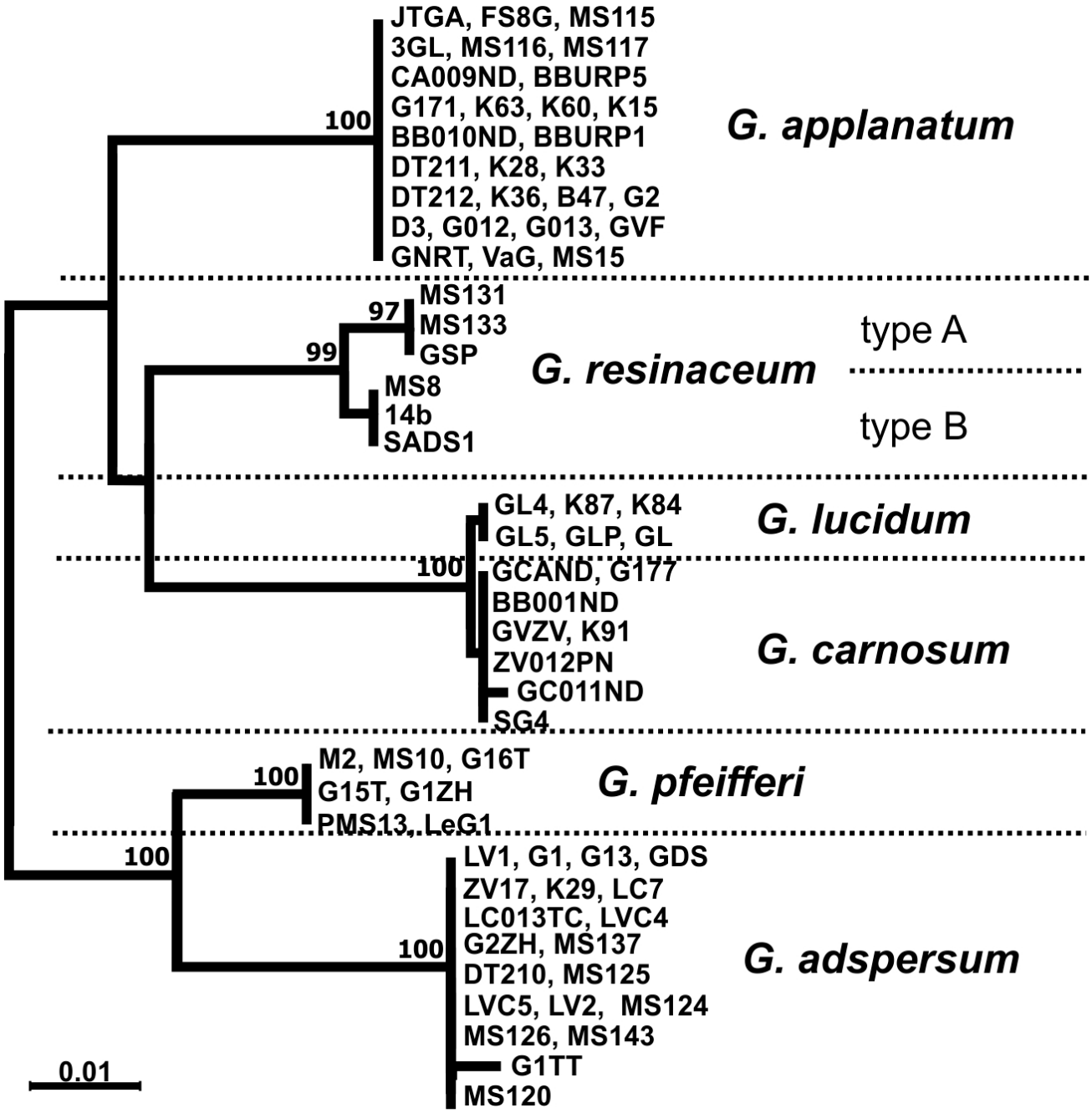

Figure 2 – Unrooted phylogenetic tree documenting the relatedness among Central European collections of *Ganoderma* spp. The tree was inferred using the Neighbor-Joining method, the evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Numbers at nodes shown the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test after 1000 repetitions.

Table 2 The evolutionary divergences between groups of *Ganoderma* spp. from Central Europe based on ITS sequence comparisons. The number of base differences per sequence from averaging over all sequence pairs between groups are shown.

	***G. resinaceum* type B**	***G. applanatum***	***G. carnosum***	***G. adspersum***	***G. pfeifferi***	***G. lucidum***	***G. resinaceum* type A**
***G. resinaceum* type B**	0.0						
G. applanatum	21.0	0.0					
G. carnosum	23.1	25.1	0.0				
G. adspersum	31.1	33.1	36.2	0.0			
G. pfeifferi	28.0	27.0	34.1	17.1	0.0		
G. lucidum	23.8	25.8	0.9	35.4	33.3	0.0	
***G. resinaceum* type A**	4.0	23.0	23.1	32.1	28.0	23.8	0.0

Table 3 The average evolutionary divergence within groups of *Ganoderma* spp. from Central Europe based on ITS sequence comparisons. The number of base differences per sequence from averaging over all sequence pairs within each group are shown.

	***G. resinaceum* type B**	***G. applanatum***	***G. carnosum***	***G. adspersum***	***G. pfeifferi***	***G. lucidum***	***G. resinaceum* type A**
The Number of Base Differences within the Group	0	0	0.25	0.2	0	0.429	0

The second largest clade consists of 19 isolates from Slovakia and 1 from Czech Republic and was classified as *G. adspersum* species. The isolates showed very low intra-species genetic variability (Table 3) and sequences obtained were practically identical to the ITS sequences of *G. adspersum* isolates Dai 13191 isolate from China, 4137 isolate from Belgium, or JV 1106/7 isolate from USA (MG279153, MG066632, and KF605651 respectively). The *G. adspersum* group is morphologically very similar to more common *G. applanatum* group. Specimens in both groups had sessile basidiocarps and were attached directly to the stump or tree´s trunk with no stipe. However, ITS sequences showed that the two groups are phylogenetically well separated and, therefore, these molecular methods have been useful for species delimitation. The basidiospores of the two groups had also different sizes (µm): 8.4 – 11.0 × 6.0 – 8.4 for basidiocarps of *G. adspersum* and 6.0 – 10.0 × 4.5 – 6.5 for *G. applanatum* (Table 4).

To the *G. resinaceum* clade six isolates were classified. However phylogenetic analysis identified significant genetic diversity within this clade. Two types of sequences were identified, marked as type A and type B in Fig. 2. Type A includes isolates MS131, MS133 and GSP, and type B includes isolates MS8, 14b and SADS1 (Fig. 2). Isolates of type A were recorded on *Fraxinus*

excelsior L. and *Quercus* sp., isolates of type B on *Gleditschia triacanthos* L., *Negundo aceroides* Moench and *Salix alba* L. While no inter-type variability was observed (Table 3), the two types of sequences differ by 4 nucleotides in ITS2 region (Table 2) indicating possible genetic non-homogeneity of the species and the existence at least two cryptic species within *G. resinaceum*. The non-homogeneity of *G. resinaceum* is probably widely occurred as the blastn analysis confirmed that both types of sequences already have been reported. E.g. sequences identical to the *G. resinaceum* type A sequences were reported for isolate 7 from Poland (KY196415) or CBS 747.84 isolate from Korea (JQ520198). The sequences identical to the *G. resinaceum* type B sequences were reported for DP2 isolate from *Celtis australis* L. in Italy (AM906060) or for F-1 isolate from France (JN588588). The differences between type A and type B isolates were accompanied by the differences in morphology and spore size. The basidiocarps of type A were sessile with no stipes, and basidiospore size of 9.6 – 14.4 × 6.0 – 8.4 µm. Type B specimens had sessile basidiocarps with or without stipes and basidiospore size of 9.6 – 12.0 × 7.2 – 9.6 µm. In addition, the length to width ratio of type A basidiospores was 1.7 and type B 1.34 (Table 4). The observed genetic differences between two types of *G. resinaceum* sequences were found to be 4 times higher than those for generally accepted and validly described *G. lucidum* and *G. carnosum* species (Table 2).

The *G. lucidum* clade consists of six isolates from *Quercus* sp. or unidentified trunks of probably broadleaf trees. The isolates showed limited intra-species variability (Table 3) and sequence comparison showed complete identity to the other *G. lucidum* sequences in the GenBank e.g. to MS183CA isolate from California, USA (MG911000) or to ZBS1 isolate from Russia (MF419230). The *G. lucidum* specimens had basidiospores of size 8.0 – 10.5 × 5.5 – 7.0 µm (Table 4).

To the *G. carnosum* clade eight isolates growing on coniferous trees *Larix decidua* Mill., *Picea abies* (L.) H. Karst., *Picea* sp. and *Pinus nigra* Arn. were classified. The isolates showed very limited genetic diversity and sequence comparison showed complete identity to the other *G. carnosum* sequences in the GenBank database e.g. to JV 1208/10KN isolate from USA (KF605626). The *G. carnosum* specimens had basidiospores of size 9.0 – 12.0 × 6.0 – 8.5 µm (Table 4).

Surprisingly very low genetic diversity was observed between *G. lucidum* and *G. carnosum* clades (Table 2). Despite the strong statistic support, (bootstrap value 100, see Fig. 2), the clades differ in general by a single nucleotide polymorphism questioning the validity of these taxa. Specimens in both clades were morphologically very similar: basidiocarps with stipe and very similar basidiospores, but basidiospores of *G. carnosum* were longer and wider (Table 4). They also differed in the colour of the basidiocarp surface (*G. lucidum* has lighter) and host preferences.

The *G. pfeifferi* clade consists of seven isolates from Slovakia, Czech Republic and Hungary. Basidiocarps grew on four species of hardwoods, mainly on *F. sylvatica*. ITS sequences of these isolates did not show intra-species genetic variability (Table 3) and were identical to the ITS sequences of e.g. Dai 12153 isolate from China, JV 0511/11 isolate from USA, or GPF2 isolate from Poland (MG279164, KF605660, and JN00887 respectively). The *G. pfeifferi* had basidiospores of size 9.5 – 14.4 × 7.0 – 10.0 µm. The length to width ratio of basidiospores was similar to *G. resinaceum* type B, 1.33. These two groups of *Ganoderma* basidiocarps had the widest spores among all studied species (Table 4).

Table 4 Basidiospores of Central European isolates of *Ganoderma* analysed in this study.

Species (Type) Code	Length (Min – Max) × Width (Min – Max) Mm	Length to Width Ratio (Average Value)
Ganoderma adspersum	**8.4 – 11.0 × 6.0 – 8.4**	**1.50**
DT210	9.0 – 11.0 × 6.0 – 7.5	1.53
LV2	9.0 – 11.0 × 6.0 – 7.5	1.47
ZV17	9.6 – 10.8 × 6.0 – 8.4	1.56
LC013TC	9.6 – 10.8 × 6.0 – 8.4	1.56
G13	8.4 – 10.8 × 6.0 – 8.4	1.39

Table 4 Continued.

Species (Type) Code	Length (Min – Max) × Width (Min – Max) Mm	Length to Width Ratio (Average Value)
Ganoderma applanatum	**6.0 – 10.0 × 4.5 – 6.5**	**1.50**
FS8G	8.0 – 10.0 × 5.0 – 6.5	1.57
BURP1	8.0 – 9.5 × 5.0 – 6.0	1.51
GNRT	7.0 – 10.0 × 4.5 – 6.5	1.52
G013	7.0 – 8.0 × 5.0 – 5.5	1.51
GVF	6.0 – 9.6 × 4.8 – 6.0	1.45
Ganoderma carnosum	**9.0 – 12.0 × 6.0 – 8.5**	**1.42**
G177	9.6 – 12.0 × 7.2 – 8.4	1.36
GC011ND	9.0 – 11.0 × 6.0 – 7.5	1.43
GCAND	10.8 – 12.0 × 7.2 – 8.4	1.43
GVZV	9.0 – 11.0 × 6.5 – 8.5	1.40
K91	9.0 – 11.0 × 6.0 – 8.0	1.50
Ganoderma lucidum	**8.0 – 10.5 × 5.5 – 7.0**	**1.50**
GL	8.5 – 10.5 × 5.5 – 7.0	1.48
GLP	8.5 – 10.0 × 6.0 – 7.0	1.48
K84	8.0 – 10.0 × 6.0 – 7.0	1.46
K87	9.0 – 10.0 × 6.0 – 6.5	1.53
GL4	8.0 – 10.0 × 5.5 – 6.0	1.57
Ganoderma pfeifferi	**9.5 – 14.4 × 7.0 – 10.0**	**1.33**
MŠ10	12.0 – 14.4 × 8.4 – 9.6	1.43
PMS13	10.0 – 11.0 × 7.0 – 8.0	1.41
LeG1	10.0 – 14.0 × 8.0 – 10.0	1.23
M2	10.0 – 11.0 × 7.0 – 9.0	1.29
G15T	9.5 – 11.0 × 7.0 – 8.5	1.27
Ganoderma resinaceum	**9.6 – 14.4 × 6.0 – 8.4**	**1.70**
Type A		
MS131	9.6 – 14.4 × 6.0 – 8.4	1.75
MS133	10.0 – 12.5 × 6.5 – 7.0	1.66
GSP	10.0 – 12.5 × 6.5 – 7.5	1.70
Ganoderma resinaceum	**9.6 – 12.0 × 7.2 – 9.6**	**1.34**
Type B		
MS8	9.6 – 12.0 × 7.2 – 9.6	1.31
SADS1	10.0 – 12.0 × 8.0 – 9.0	1.36

Discussion

A combination of morphological and molecular methods was used to analyze the diversity of *Ganoderma* spp. in Central Europe region. From this territory, seven species were reported previously (Kotlaba 1984, Sokół 2000, Gáperová 2001, Papp & Szabó 2013, Ryvarden & Melo 2014). In this study, among the collection of 75 basidiocarps collected during 2015 – 2018 years, six *Ganoderma* species were recorded with the dominance of *G. applanatum* and *G. adspersum*. The *G. valesiacum* species was not recorded. This species belongs to the *G. lucidum* complex, however, *G. lucidum* and *G. valesiacum* taxa could be conspecific (Ryvarden & Gilbertson 1993, Ryvarden & Melo 2014, Hapuarachchi et al. 2015). According to literature, *G. valesiacum* differs only by the length of the stipe (shorter than *G. lucidum* or rudimentary), the cracked crust of pileus, white context and host preference of *Larix* (Sokół 2000, Ryvarden & Melo 2014). From Central Europe territory, there is a very limited number of *G. valesiacum* specimens and it is commonly found in natural coniferous forests at higher altitudes, mainly from the Alps (Plank & Wolkinger 1981, Kotlaba 1984, Sokół 2000, Bernicchia 2005).

Ganoderma adspersum is very similar to the widespread *G. applanatum*. Both species can be distinguished by the size of their basidiospores; in *G. adspersum*: they are somewhat longer and larger. In addition, older tube layers are not whitening in its basidiocarps (Breitenbach & Kränzlin 1986, Ryvarden & Gilbertson 1993, Bernicchia 2005). In many cases, the combination of

morphological and anatomical criteria of the basidiocarps and isolates, alone, however, may still not be sufficient, and the two species can often be confused (Peterson 1987, Leonard 1998, Moncalvo 2000). Our ITS sequences show, no close similarity for these species and, therefore, these molecular methods have been useful for their delimitation. This has been suggested by Moncalvo (2000) and Vlasák (2015) and subsequently confirmed by Guglielmo et al. (2008) and De Simone & Annesi (2012) based mainly on the analysis of the Italian *G. adspersum* isolates and the *G. applanatum* isolates originating from other parts of the Europe. Similarly, Badalyan et al. (2012) confirmed this similarity between sequences generated outside the Europe based on the analysis of the Armenian *G. adspersum* isolates. While no intra-species genetic differences were observed in *G. applanatum*, very low diversity was detected in *G. adspersum*. *G. pfeifferi* can be easily distinguished in the field by its very dark context and thick, cracked and wrinkled resinous-waxy layer covering the pileus surface, especially from older basidiocarps of both *G. lucidum* and *G. resinaceum*. Similar to *G. applanatum* no intra-species genetic differences were observed in this species.

G. lucidum complex consists of three central European species namely, *G. lucidum*, *G. carnosum*, and *G. resinaceum*. Recent molecular studies have revealed that the commercially cultivated '*G. lucidum*' (reishi or "Lingzhi") = *G. linghzi* in East Asia is a different species from *G. lucidum* s. str. *Ganoderma linghzi* in East Asia now represents several non-European species, such as *G. lingzhi*, *G. sichuanense*, and *G. multipileum*, whereas *G. lucidum* s. str. only occurs in Europe and some parts of China (Moncalvo et al. 1995b, Cao et al. 2012, Hennicke et al. 2016, Papp et al. 2017, Loyd et al. 2018). *Ganoderma carnosum* and *G. lucidum* s. str. are also very similar in their early stages of basidiocarp development, and the two species are not easy to distinguish from each other based on morphological and anatomical criteria of basidiocarps. Our ITS sequences show, however, a close similarity of these species and the only criterion for dividing them seems to be the host preferences. Moreover, there are two kinds of sexual spores with a different size: smaller, thin-walled basidiospores termed "proterospores", which are ready to germinate at the beginning of the sporulation period, and larger, double-walled normal basidiospores, which are hard to germinate (Nuss 1982). In our study, however, no "proterospores" were recorded. In accordance with Ryvarden & Melo (2014), we conclude that the mature *G. carnosum* basidiocarps have dark brown to black upper surface and larger normal basidiospores, while the *G. lucidum* basidiocarps have orange red to bay upper surface and smaller normal basidiospores. In the phylogenetic analysis, based on ITS and partial transcription elongation factor 1-α sequences data *G. lucidum* groups together with *G. carnosum* (Xing et al. 2016).

The *G. resinaceum* group is distinguished in the field by thick, soft and pale context. As we mentioned above, *G. resinaceum* basidiocarps of type B also can form a stipe. There is only one such finding in our study in a very wet area. These *G. resinaceum* basidiocarps may appear very similar to those of *G. lucidum*. But *G. resinaceum* has finely punctulate basidiospores whereas *G. lucidum* has coarse, rough basidiospore ornamentation (Steyaert 1972). We partially agree with the opinion of Kotlaba & Pouzar (2009) that the basidiospore ornamentation is the only reliable feature between these two species in the above mentioned cases. *Ganoderma lucidum* basidiospores are shorter than those of *G. resinaceum* (both types).

As mentioned above, ITS was adopted as the universal barcode marker for fungi. ITS is the fastest evolving region of the nuclear ribosomal RNA cistron, it shows the highest genetic variation making it suitable for species-level identification (Raja et al. 2017). Moreover, it is successfully amplified by PCR and therefore is widely used (Schoch et al. 2012). Some authors (e.g. Cao & Yuan 2013, Hapuarachchi et al. 2018) have described new species of *Ganoderma* spp. based only on ITS sequences. However, if the studied taxa show low interspecific variability for ITS (our *G. lucidum* and *G. carnosum* isolates), secondary markers should be used to accurately clarify their genetic diversity (Schoch et al. 2012). On the other hand, higher variability can be observed among the sequences within one valid species (2 genotypes of *G. resinaceum*). In such cases, sequencing of protein-coding genes is also recommended. These genes sometimes evolve faster than ITS and thus exhibit a higher degree of variability. The translation elongation factor 1-α is one of the most

commonly used protein-coding gene in the mycology (Raja et al. 2017). Also, Pristaš et al. (2013) found that tef-1α sequences of two genotypes of wood-decay polypore *Fomes fomentarius* show a higher degree of variability and discriminatory power compared to ITS (Pristaš et al. 2013). The tef-1α marker has also been used to study the genetic variability of *Ganoderma* spp. (Xing et al. 2016, 2018, Elliott et al. 2018). However, there is limited number of sequences available in the GenBank database compared to the frequently sequenced ITS region.

Conclusions

Seventy-five *Ganoderma* basidiocarps were separated into six clades based on morphology and phylogenetic analysis of ITS sequences: *Ganoderma applanatum*, *G. adspersum*, *G. resinaceum*, *G. pfeifferi*, *G. lucidum*, *G. carnosum*. No intra-species genetic differences observed in *G. applanatum* and *G. pfeifferi* and very low variation observed in *G. adspersum*. ITS sequences showed no close similarity for morphologically similar taxa *G. applanatum* and *G. adspersum*. Significant intra-species genetic diversity was observed in *G. resinaceum* clade. It was four times higher than the inter-species variability between valid species *G. lucidum* and *G. carnosum*. In *G. resinaceum* clade, two types of spores were identified, named as type A and B, based on the size and shape of the spores. Further studies are needed to clarify the genetic variability within the clade *G. resinaceum* and between the clades *G. carnosum* and *G. lucidum*.

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