Molecular Data Reveal Unrecognized Diversity in the European Ganoderma resinaceum

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Abstract: Ganoderma resinaceum Boud. is commonly found in Mediterranean region, but rarely in Western, Central or Eastern Europe. It is a parasitic basidiomycetous fungus causing stem decay—especially in urban trees. A collection of nine fungal specimens from Slovakia (Central Europe), morphologically identified as G. resinaceum, was recently studied on the basis of sequence data from the internal transcribed spacer (ITS) regions. Analyses showed that the collections clustered into two separate groups. In this study—for the first time—the sequences of other molecular markers, namely partial translation elongation factor (tef1-α) region and partial 25S large subunit ribosomal RNA gene (25S LSU rRNA), as well as matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF MS) were obtained and used to evaluate the genetic variability of G. resinaceum. All these analyses confirm the existence of two previously unrecognized genotypes within the morphospecies.

Keywords: molecular diversity; polypores; Ganoderma resinaceum; intraspecific variability

1. Introduction

Ganoderma resinaceum Boud. (Polyporales, Basidiomycota) is a parasitic fungus, later a saprotrophic, preferring to live on broadleaved woody plants in synanthropic habitats in warmer parts of Europe [1]. Moreover, it is usually used as ethnomedicine for immunoregulation, hyperglycemia and liver disease in the Asian traditional pharmacopoeia [2] and in some parts of West Africa [3]. It was described in 1889 by Boudier [4], who gave it what remains its widely accepted current name. The species has more or less cosmopolitan distribution from the tropics to the southern part of the temperate zone. The European specimens are easily recognized in the field by thick, soft and pale context. However, some G. resinaceum basidiocarps may appear very similar to G. lucidum s. str., but they differ in terms of the ornamentation of the basidiospores [5,6].

Phylogenetic studies of G. resinaceum are relatively scarce. In 2000, internal transcribed spacer (ITS) phylogeny for 248 taxa of Ganodermataceae showed separate populations from Europe and Africa, North America and South America in G. resinaceum lineage [7]. The author [7] assumed, that these were genetically isolated populations. In the study of Hong and Jung [8] based on analysis of nearly complete mitochondrial small-subunit ribosomal DNA sequences it was shown that G. resinaceum forms...
well supported monophyletic group together with *G. lucidum* living on hardwoods from the United States and Taiwan (but not from other territories), *G. pfeifferi* and *G. subumboinense* var. *laevisporum*

Recent molecular phylogenetic studies also showed that the European specimens of *G. resinaceum* grouped in a lineage well separated from the *G. lucidum* agg [9,10]. The genetic diversity of nine fungal collections from the Slovakian urban and suburban areas (Central Europe) morphologically identified as *G. resinaceum* was recently investigated by phylogenetic analysis of internal transcribed spacer DNA sequences. Both analyses showed that the collections clustered into nonhomogeneous group. Multiple sequence alignment indicated the presence of two sequence types with significant statistic support. The two types of sequences differ by six nucleotides in ITS region [6]. In order to analyze the diversity in *G. resinaceum* species in more detail, partial translation elongation factor 1-α (*tef1-α*) and partial 25S large subunit ribosomal RNA (25S LSU rRNA) sequences were obtained and analyzed, as well as protein profiles of *G. resinaceum* strains were compared using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF MS). All abbreviations are explained in Abbreviations.

2. Materials and Methods

2.1. Specimens and Strains Examined

*Ganoderma resinaceum* basidiocarps were collected from a stump (specimen No. 14b, MS947) and living tree trunks (rest of specimens) within the Central Slovakia. The specimens were morphologically identified by routine methods and determined according to standard, widely used identification keys [11–15].

The following nine *G. resinaceum* specimens were used in this study. (No. 14b, GenBank Acc. No. MN017824): in the town of Zvolen, Študentská street, *Acer negundo* L., 48.573703, 19.117391, 19 October 2015; (No. MS8, Acc. No. MN017824): in the capital city of Bratislava, Slávičie údolie cemetery, *Gleditsia triacanthos* L., 48.157439, 17.067742, 24 August 2017; (No. SADS1, Acc. No. MK415360): the roadside near the village of Dolná Strehoľava in the Veľký Krtíš District, *Salix alba* L., 48.246074, 19.486921, 5 September 2018; (No. MS131, Acc. No. MN017820): in the town of Lučenec, cemetery, *Fraxinus excelsior* L., 48.322267, 19.662514, 3 July 2018; (No. MS133, Acc. No. MN017821): ibid., *Fraxinus excelsior* L., 48.322267, 19.662514, 3 July 2018; (No. GSP, Acc. No. MN017822): Počúvadlo lake in the Banská Štiavnica District, *Quercus* sp., 48.409732, 18.854544, 22 July 2017; (No. MS947, Acc. No MT397405): in the town of Piešťany, Winterova street, unknown host, 48.5884939, 17.8385169, 8 August 2019; (No. MS1211, Acc. No. MT397406): in the town of Topoľčianky, Tovarnícke park, *Quercus rubra* L., 48.567439, 18.146153, 19 September 2019; (No. MS1212, Acc. No. MT397407): in the town of Topoľčianky, Tovarnícke park, *Quercus rubra* L., 48.567744, 18.145275, 19 September 2019.

In total, 10 morphologic features were observed for all *G. resinaceum* specimens: presence/absence of stipe; annual/perennial growth; substrate attachment; presence/absence of resinous layer; shape of pileus; weight of basidiocarp; color of pileus, stipe, context and tubes; pileus margin; and basidiospore size and shape.

The following *Ganoderma* species were used in the study of *Ganoderma* spp. variability by MALDI–TOF MS: *Ganoderma pfeifferi* (No. G1ZH): in the town of Žiar nad Hronom, Štefan Moyes park, *Acer platanoides* L., 48.585158, 18.864481, 9 January 2015; (No. M2): in the Topoľčianky park in the Zlaté Moravce District, *Tilia platyphyllos* Scop., 48.422639, 18.414302, 25 October 2015. *Ganoderma lucidum* (No. GLP): in the Biela hora forest near the town of Michalovce, unknown host, 48.769188, 21.931371, 7 October 2017. *Ganoderma applanatum* (No. CA009ND): in the private garden in Korňa in the Čadca District, unknown host, 49.411580, 18.56588, 18 October 2015; (No. JTGA): on the Kučelach hill, Muránska planina Mts, *Fagus sylvatica* L., 48.754554, 19.882483, 25 March 2017; (No. BBURP1): in the town of Banská Bystrica, the calvary on the Urpin hill, unknown host, 48.729336, 19.149725, 9 April 2017; (No. K60): in the Polánsky forest in the Ostrava District, Czech Republic, unknown host, 49.791864, 18.210538, 7 May 2018.
Isolations were performed within 24 h after collection of the samples in the field. All strains were obtained on 2% malt extract agar in a Petri dish from trama of basidiocarps. The strains were incubated at 24 ± 1 °C in darkness. The remaining part of each specimen was dried and stored as voucher specimen. Herbarium specimens are deposited in the Herbarium of the Department of Biology and Ecology, Faculty of Natural Sciences, Matej Bel University in Banská Bystrica, Slovakia. Strains are being preserved at the Mycological laboratory of the Matej Bel University in Banská Bystrica, Slovakia.

In nomenclature of fungi, The Index Fungorum [16] database was followed. In nomenclature of woody plants, The International Plant Names Index database [17] was followed.

2.2. DNA Isolation and Analysis

All DNA techniques used were in principle as already described by Beck et al. [6]. Shortly, for the isolation of G. resinaceum total genomic DNA fresh basidiocarps were grinded by oscillating mill (MM200, Retsch GmbH, Haan, Germany). A small amount of basidiocarp 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 in microwave oven 10 times (600 W for 4 s). Then, 300 µL of fresh lysis solution was again added and the mixture was incubated at 100 °C for 2 min. 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, the DNA was precipitated with a 0.7-volume of isopropyl alcohol and 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).

2.3. PCR Amplification and Sanger Sequencing

The ITS region was amplified with the primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC TAT TTA TAA TCC AGG CTA C) according to White et al. [18], tef1-α was amplified with the primers EF595F (5′-CGTGACTTACATCAAGAAAGTG-3′) and EF1160R (5′-CCGATCTTGTAGACGTCCTG-3′) and 25S LSU rRNA gene was amplified with the primers LR0R (5′-ACCCGCTGAACTTAAGC-3′) and LR7 (5′-TACTACCACCAAGATCT-3′) according to Pristaš et al. [19]. Polymerase chain reaction (PCR) was performed according to Pristaš et al. [19] in a T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., Singapore). The PCR reaction mixture (50 µL) contained 10 µL of 5x HOT FIREpol® Blend Master Mix with 10-mM MgCl₂ (Solis BioDyne, Tartu, Estonia), 37 µL of PCR Grade Water (Solis BioDyne, Tartu, Estonia), 1 µL of each primer (10-pmol/ µL) and 1 µL of diluted genomic DNA (50 ng). The conditions for PCR cycling for ITS amplification were: 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. Conditions of PCR cycling for tef1-α amplification: an initial cycle for 5 min at 94 °C, followed by 36 cycles at 94 °C for 30 s, 35 s at 55 °C, 40 s at 72 °C and a final extension at 72 °C for 10 min. The conditions for PCR cycling for LSU amplification were: an initial cycle for 5 min at 94 °C, followed by 36 cycles at 95 °C for 1 min, 45 s at 47 °C and 1.5 min at 72 °C.

The amplification products were visualized after electrophoresis on 1.5% agarose gel and purified using ExoSAP-IT (Affymetrix, Inc., Cleveland, OH, USA). The PCR products were sequenced in both directions using the same primers as for PCR at SEQme s.r.o. (Dobřiš, Prague, Czech Republic).

The sequences were deposited into the GenBank database under accession numbers MK995651, MK995652, MK995653, MK995654, MK995655 and MK995656 for MS131, MS133, GSP, MS8, 14b and SADS1 specimens partial LSU rRNA gene sequence, under accession numbers MN017820, MN017821, MN017822, MN017823, MN017825, MN017824, MT415668, MT415669 and MT415670 for MS131, MS133, GSP, MS8, 14b, SADS1, MS947, MS1211 and MS1212 specimens partial tef1-α sequences and under accession numbers MT397405, MT397406 and MT397407 for MS947, MS1211 and MS1212 specimens ITS sequences, respectively.
2.4. Phylogenetic Analyses

The sequences from both primers were assembled using DNA Baser software version 2.0 (Heracle BioSoft S.R.L., Pitești, Romania) and compared against GenBank database using BLASTn algorithm [20]. Assembled sequences were aligned using ClustalW algorithm [21] and phylogenetic trees were constructed using the Maximum parsimony method. As outgroups Trametes spp. sequences were used—GenBank entry JF437649 for ITS based tree and KX880919 for tef1-α based tree. Trametes originated sequences are used in similar phylogenetic studies focused on genetic variability of Ganoderma [22]. The robustness of the trees was evaluated using bootstrap analysis with 1000 repetitions. All phylogenetic analyses were performed using MEGA software version 6.0 [23].

2.5. MALDI–TOF MS Analysis

Cells extract preparation was carried out according to Pristaš et al. [24]. The samples of mycelium (about 50 µg) of G. resinaceum strains SADS1 and GSP grown on malt agar were resuspended in 600 µL of distilled water (LC–MS Chromasolv, Sigma-Aldrich, USA) and the samples were incubated at 95 °C for 5 min. Then, 900 µL of absolute ethanol was added, and the mixture was centrifuged twice at maximum speed for 2 min, and the supernatant was discarded. To the pellet, 100 µL of 70% formic acid and 100 µL of 100% acetonitrile were added and vortexed vigorously. The sample was centrifuged again at maximum speed for 2 min. One microliter of the supernatant was placed on the steel MALDI target plate and left to dry at room temperature. Subsequently, the dried sample was covered with 1 µL of the matrix (IVD matrix HCCA-portioned, Bruker Daltonics GmbH, Leipzig, Germany; a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and left to dry.

MALDI–TOF MS analysis was performed on Microflex LT instrument (Bruker Daltonics GmbH) using Flex Control software (version 3.0). All samples were analyzed at least in duplicates taken at different time of cultivation. For comparisons, MALDI spectra of several other Ganoderma species from the collection of strains at the Mycological laboratory of the Matej Bel University in Banská Bystrica were added to the analysis and MSP (Main Spectrum Profiles) dendrograms were created using UPGMA algorithm implemented in the MALDI Biotyper 3.0 software (Bruker Daltonik GmbH).

3. Results

The same strains of Ganoderma resinaceum as in the study of Beck et al. [6] and three new strains were used in the following analyses. ITS sequences of G. resinaceum were found to form two separate genotype groups. Genotype A (specimens from Fraxinus excelsior, Quercus rubra and Quercus sp.) differed from genotype B (specimens from Gleditsia triacanthos, Acer negundo, Salix alba) by two transitions in ITS1 region and three transitions and one indel in ITS2 region (Figure 1A). While all G. resinaceum ITS sequences within genotype B were practically identical, limited variability was observed among genotype A sequences.

BLASTn analysis of G. resinaceum type A and B ITS sequences against GenBank database yielded a multiple hits within Ganoderma ITS sequences. The phylogenetic analysis of G. resinaceum ITS sequences indicated that variability seen in Slovak strains is not accidental and the existence of two genotypes was confirmed with strong statistical support (bootstrap value 85, Figure 2). The genotype A strains are reported worldwide mainly from Quercus spp. and genotype B strains are reported from South Europe mainly and from wide spectrum of hosts different from Quercus spp. (Figure 2). Based on these data G. resinaceum must be considered a complex of at least two sibling species. ITS sequence analysis clearly separated G. resinaceum from closely related G. weberianum and G. sessile species.

To confirm variability observed at ITS level, other molecular markers were used to identify diversity among G. resinaceum specimens and to elucidate relations between G. resinaceum and G. weberianum/G. sessile species complexes.
3.1. Variability of Partial Translation Elongation Factor Tef1-α Region Sequences

The partial gene tef1-α was amplified and completely sequenced from all studied specimens. Alignment of genotype A and B tef1-α gene sequences revealed multiple (14) variable nucleotide positions between two genotypes. Among them 8 transitions and 5 transversions were detected. The rest of variable positions were ambiguous nucleotides detected in genotype A only (Figure 2).

A sequence comparison of tef1-α gene sequences of *G. resinaceum* specimens from Central Europe against GenBank database showed perfect homology to the *G. resinaceum* strain CBS 194.76 tef1-α gene sequence (GenBank accession number KJ143934) for tef1-α of genotype B sequences. Genotype A tef1-α gene sequences showed slightly lower similarity (96–98%) to the *G. resinaceum* tef1-α gene sequences. Phylogenetic analysis strongly supported the existence of two lineages of *G. resinaceum* (Figure 3) with bootstrap value of 89. Most *G. resinaceum* tef1-α gene sequences available in GenBank database, mainly of Greek origin, belong to the genotype B. To the genotype A, in addition to sequences reported in this study, belong two specimens from Belgium. Surprisingly, sequence comparisons of tef1-α gene indicated that genotype A could be more closely related to *G. resinaceum*/*sessile* group of isolates from China and USA than to the genotype B. However, statistical support for this branching is relatively low (below 80) and further studies are necessary to assess true relatedness in *G. resinaceum*/*sessile* group.

3.2. Variability of Partial 25S Large Subunit Ribosomal RNA Gene Sequences

The gene 25S LSU rRNA was amplified and completely sequenced from all specimens (except MS947, MS1211 and MS1212 specimens). Sequence comparisons against GenBank database showed similarity over 99% to the LSU sequences of several *Ganoderma* spp., e.g., *G. philippii* (Bres. & Henn. ex Sacc.) Bres., *G. lucidum* (Curtis) P. Karst., *G. applanatum* (Pers.) Pat. At the time of submission there were no LSU sequences of *G. resinaceum* available in the GenBank database. Again, sequence comparisons confirmed the existence of two *G. resinaceum* genotypes. However, the observed diversity between LSU sequences of genotypes A and B was very low represented by a single transition (C→T) in sequenced LSU fragment (data not shown) only. Similar to the tef1-α gene sequences comparison *G. resinaceum* genotype A is practically identical at LSU sequence level from *G. sessile* (data not shown).

![Figure 1](image1.png)

Figure 1. Sequence diversity between *Ganoderma resinaceum* genotypes. (A) Comparison of aligned ITS sequences of *G. resinaceum* genotypes A and B; (B) comparison of aligned partial translation elongation factor 1-α (tef1-α) sequences of *G. resinaceum* genotypes A and B.
Figure 2. Genetic diversity between *Ganoderma resinaceum* genotypes and related *Ganoderma* species documented by internal transcribed spacer (ITS) sequence analysis. Maximum parsimony phylogenetic tree documenting phylogenetic relatedness of *Ganoderma resinaceum* specimens from Slovakia based on internal transcribed spacer sequences comparison. The sequences obtained through this study are underlined. Numbers at nodes are bootstrap values after 1000 repetitions (only values over 80 are shown). ITS sequence of *Trametes versicolor* (GenBank accession number JF437649) was used as an outgroup. The geographical origin and host plant of specimens are shown (if available). The bar indicates the number of nucleotide changes over the whole sequence.
positions between two genotypes. Among them 8 transitions and 5 transversions were detected. The rest of variable positions were ambiguous nucleotides detected in genotype A only (Figure 2).

A sequence comparison of tef1-α gene sequences of G. resinaceum specimens from Central Europe against GenBank database showed perfect homology to the G. resinaceum strain CBS 194.76 tef1-α gene sequence (GenBank accession number KJ143934) for tef1-α of genotype B sequences. Genotype A tef1-α gene sequences showed slightly lower similarity (96–98%) to the G. resinaceum tef1-α gene sequences. Phylogenetic analysis strongly supported the existence of two lineages of G. resinaceum (Figure 3) with bootstrap value of 89. Most G. resinaceum tef1-α gene sequences available in GenBank database, mainly of Greek origin, belong to the genotype B. To the genotype A, in addition to sequences reported in this study, belong two specimens from Belgium. Surprisingly, sequence comparisons of tef1-α gene indicated that genotype A could be more closely related to G. resinaceum/sessile group of isolates from China and USA than to the genotype B. However, statistical support for this branching is relatively low (below 80) and further studies are necessary to assess true relatedness in G. resinaceum/sessile group.

Figure 3. Genetic diversity between Ganoderma resinaceum genotypes documented by tef1-α sequence analysis. Maximum parsimony phylogenetic tree documenting phylogenetic relatedness of Ganoderma resinaceum specimens from Slovakia based on tef1-α region sequences comparison. The sequences obtained through this study are underlined. Numbers at nodes are bootstrap values after 1000 repetitions (only values over 80 are shown). Tef1-α sequence of Trametes versicolor (GenBank accession number KX880919) was used as an outgroup. The bar indicates the number of nucleotide changes over the whole sequence.

3.3. MALDI–TOF MS Analysis

The two strains studied were derived from SADS1 (genotype B) and GSP (genotype A) specimens and their protein profiles were compared using MALDI–TOF MS analysis. Well defined spectra were obtained from both strains showing multiple peaks in m/z ratios from 3000 to 12,000. While both, the genotype A and genotype B strains showed the same dominant peak with m/z ratio about 6224,
they differed by other major peaks. For genotype A strain GSP peaks with \( m/z \) ratios 4087, 3497, 3108 and 7001 with relative intensity 84, 68, 62 and 58%, respectively were detected, for genotype B SADS1 strain we observed peaks with \( m/z \) ratio 3497, 7001, 5650 and 4701 with relative intensity 77%, 72%, 63% and 63%, respectively, (Figure 4).

![Figure 4. Comparison of normalized matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF MS) spectra of G. resinaceum genotype A. (right) GSP strain and genotype B; (left) SADS1 strain.](image)

Despite the presence of some common peaks in MALDI–TOF spectra, MSP dendrogram analysis clearly placed GSP and SADS1 strains to the separate branches with distance value over 500 (Figure 5) when compared against MALDI–TOF spectra of several Ganoderma spp. MALDI–TOF analysis confirmed that diversity between two genotypes seen at DNA level is accompanied by a diversity at the protein profile level.

![Figure 5. Similarity dendrogram of Ganoderma spp. strains based on MALDI–TOF spectra comparisons. The vertical line at distance value 500 indicates cutoff score for species delineation. Numbers after strains designation indicate biologic replicates.](image)

All molecular data obtained through this study indicate the existence of up to now unrecognized diversity in the European G. resinaceum species.
3.4. Morphologic Features

*G. resinaceum* basidiocarps of genotype A were annual, sessile, nonstipitate, ungulate with thick margins, whereas genotype B were annual or perennial with tube layers non-separated by context tissue, sessile or stipitate, applanate with thin margins or ungulate with thick margins. Specimens of both genotypes share the same pileal surface (glossy with resinous layer) and almost identical coloration of pileal surface, context, margins and color of tube layers. Only the context color was lighter—brown beige to sand yellow in genotype A and darker—brown beige to ochre brown in genotype B.

Our previous study showed that basidiospore sizes range between 9.6–14.4 × 6.0–8.4 µm in genotype A and 9.6–12.0 × 7.2–9.6 µm in genotype B [6]. In this study, we introduce a measure of new *G. resinaceum* specimens: sizes of genotype A basidiospores were 11.0–13.0 × 7.0–8.0 µm (MS1211 specimen) and 10.5–12.5 × 7.0–8.0 µm (MS1212 specimen), respectively. Sizes of genotype B basidiospores were 9.0–11 × 6.0–8.0 µm (14b specimen). These measurements correspond with our previous results: basidiospores of genotype A are longer and shorter than those of genotype B [6]. This also confirms length and width ratio, which is 1.66 in genotype A (MS1211; MS1212) and 1.35 in genotype B (MS947 specimen).

4. Discussion

The application of molecular, mainly DNA based methods, to systematics have revolutionized the discovery and subsequent description of species diversity also in wood-decaying polypores [25,26]. In particular, DNA sequence data reveal the diversity within previously recognized morphotaxa with distinct geographic or pathologic traits, e.g., *Fomes fomentarius* (L.) Fr. [27], *Heterobasidion* spp. [28,29], *Letiporus* spp. [30–32] and *Phellinus* spp. [33,34].

*Ganoderma* genus has long been regarded as one of the most important genera of medicinal fungi worldwide with more than 300 species described. The research interest focused mainly on *G. lucidum* complex [10] and variability of other species has not been studied in detail. That is why there is very limited data on variability of *G. resinaceum* species. In the only published study by Ayissi and Mossebo [35], the *G. resinaceum* specimens collected in tropical Africa showed some striking variations in the macro- and microscopic characteristics compared to descriptions of specimens from temperate regions. The variability was however studied in specimens from Africa only and morphologic diversity observed was not correlated with any molecular data.

Beck et al. [6] observed intraspecies variability in *G. resinaceum* based on comparison of ITS sequences indicating that *G. resinaceum* population in Central Europe is composed of two genotypes differing by six nucleotide positions in ITS region. The nuclear ribosomal RNA genes, which include the small-subunit (SSU) and LSU rRNA genes as well as the ITS region that separates the two rRNA genes, have been used for fungal studies for two or three decades. In this study, genetic variability within *G. resinaceum* observed at ITS level was confirmed by analyses of other DNA markers. Both, the partial teF1-α region and the partial 25S LSU rRNA gene sequence comparisons confirmed the existence of two different genotypes within *G. resinaceum*. Significant differences in discriminatory power were detected among DNA markers used. While diversity between genotypes at ITS level was 0.007 base differences per site, those for teF1-α and LSU were 0.022 and 0.0009 base differences per site, respectively. First signs of genetic diversity within *G. resinaceum* were observed by Moncalvo [7]. Study of phylogenetic inferences based on multilocus sequences by Hernández et al. [36] also showed that *G. resinaceum* represents a species complex which confirms larger diversity in Europe than expected.

ITS marker has been used for many years for identification of fungi as a barcode [37]. However, for several fungal groups the discriminatory power of ITS sequences is not sufficient for reliable species delineation and other DNA markers were proposed as additional barcodes, including teF1-α and LSU sequences [38] used in our study. The data presented indicate that teF1-α is a superior marker over ITS as the diversity of the protein-encoding teF1-α gene sequences is three times higher compared to non-coding ITS sequences. Despite the advantages of ITS marker it was shown that the resolution power of ITS, especially at higher taxonomic levels, is inferior to that of many protein coding genes.
Instead, tef1-α marker is widely used for identification of mainly medically important ascomycetous fungi [38]. Similar situation, the significantly lower ITS sequence variability compared to the tef1-α variability (0.023 versus 0.036 nucleotide substitutions per site), was detected in the study on variability within *Fomes fomentarius* by Pristaš et al. [19].

The discriminatory power of LSU sequences was found to be very low with only one nucleotide position being polymorphic over the entire 25S LSU rRNA sequence range analyzed (1100 bp).

Recently, a new method to determine relatedness of microorganisms, including fungi, emerged–MALDI–TOF mass spectrometry. In comparison to ITS, LSU and tef1-α markers based on analysis of DNA sequences, MALDI–TOF is based on analysis of protein profiles. In our recent study, we found the MALDI–TOF discriminatory power to be comparable with generally used ITS–PCR–RFLP methods.

Representatives of each *G. resinaceum* genotype were analyzed using this approach and distance values over 500 were detected, confirming differences between *G. resinaceum* genotypes seen at DNA level. This distance level (500) has been used for species delineation in different taxonomic groups, mainly for bacteria, e.g., [39–41], pathogenic oomycetes [42] and rarely for Basidiomycota, including wood-decay fungi [24]. However, lower interspecies value distances were observed for *Mucoraceae* [43], Hymenochaetaceae and Polyporaceae [44].

Comparison of *G. resinaceum* protein spectra against spectra of other central European *Ganoderma* spp. placed both genotypes to the separate branch with *G. lucidum* as a sister branch (Figure 5). Similar results were reported by Hong and Jung [8] who showed that *G. resinaceum* from Europe and the North American *G. lucidum* were sister taxa based on mitochondrial small-subunit ribosomal DNA sequences. However, according to more recent studies, the North American *G. lucidum* that Hong and Jung [8] refer to, is probably *G. sessile* [9,45].

The data presented here indicate the existence of substantial genetic diversity in European *G. resinaceum* specimens and the species could be considered a complex of at least two species. The sequence comparisons suggest that sister group to *G. resinaceum* complex is *G. weberianum/G. sessile* complex showing similar degree of genetic variability. Incongruent phylogenetic grouping obtained using different molecular markers makes the phylogenetic analyses in this complex very difficult.

5. Conclusions

All molecular data obtained in the current study reveal up to now unrecognized diversity in the European *G. resinaceum* and they may suggest much higher variability within the whole *G. resinaceum* group [8]. Phylogeny based on tef1-α and LSU gene sequence comparisons indicates that the sequences of genotype A could be more closely related to the *G. sessile* sequences from isolates from USA than to the sequences of genotype B. However, no such grouping between *G. resinaceum* and *G. sessile* sequences was observed for ITS sequences and further studies are necessary to understand the true diversity within *G. resinaceum* group.

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Abbreviations

agg. aggregatum
CTAB cetrimonium bromide
DNA deoxyribonucleic acid
EDTA ethylenediaminetetraacetic acid
ibid. ibidem
ITS internal transcribed spacer
LSU large subunit ribosomal RNA
MALDI–TOF MS matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MgCl₂ magnesium chloride
MSP main spectrum profiles
NaCl sodium chloride
PCR polymerase chain reaction
RFLP restriction fragment length polymorphism
RNA ribonucleic acid
s. str. sensu stricto
SSU small subunit ribosomal RNA
tef1-α partial translation elongation factor 1-α
Tris-HCl tris hydrochloride
UPGMA unweighted pair group method with arithmetic mean

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