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Phylogenetic Relationships between *Phlebiopsis gigantea* and Selected *Basidiomycota* Species Inferred from Partial DNA Sequence of Elongation Factor 1-Alpha Gene

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Abstract: *Phlebiopsis gigantea* (Fr.) Jülich has been successfully used as a biological control fungus for *Heterobasidion annosum* (Fr.) Bref., an important pathogen of pine and spruce trees. The *P. gigantea* species has been known for many years, but our understanding of the relationship between various isolates of this fungus has been substantially improved through the application of DNA sequence comparisons. In this study, relationships between *P. gigantea* and selected *Basidiomycota* species was determined, based on elongation factor 1-alpha (*EF1α*) partial DNA sequence and *in silico* data. A total of 12 isolates, representing the most representatives of *P. gigantea*, with diverse geographic distributions and hosts, were included in this study. Phylogenetic trees generated for sequences obtained in this research, grouped the European taxa of *P. gigantea* and partial sequence of the genome deposed in NCBI database, in a strongly supported clade, basal to the rest of the strains included in the study. *P. gigantea* isolates originating from Poland, Finland, Sweden, Great Britain and partial sequence of genome formed a monophyletic group. Within this group, isolates of *P. gigantea* constituted two subclades, showing their partial difference like the two SNPs (single nucleotide polymorphisms) between one and the rest of isolates. The intron and exon relationships among *P. gigantea* isolates were moreover resolved. The results obtained using the *EF1α* region should be useful in the selection of more efficient *P. gigantea* isolates for limiting forest tree root pathogens.

Keywords: *Phlebiopsis gigantea*; *EF1α*; introns; exons; phylogenesis

1. Introduction

Phlebiopsis gigantea (Fr.) Jülich has widely been used as a biological control of the fungus *Heterobasidion annosum* (Fr.) Bref., the causative factor of conifer root rot infections [1–12]. In the literature this species is described with multiple synonymous names, e.g., *Corticium giganteum* (Fr.) Fr., *Peniophora gigantea* (Fr.) Massee, *Peniophora gigantea f. pruinosa* Pilát, *Phanerochaete gigantea* (Fr.) S.S. Rattan, *Phlebia gigantea* (Fr.) Donk. The fungus, after the taxonomic revision made by Jülich (1978) In: Parmasto and Hallenberg [13], was finally placed in the new genus *Phlebiopsis*, which is now commonly accepted. The fungus is a typical saprotroph, regularly colonizing dead parts of coniferous woods, occurring mostly in the Northern Hemisphere, but it has also has a worldwide distribution; for example, few strains have been retrieved from South Africa, New Zealand and Canada [9,14].

According to the Index Fungorum database (www.indexfungorum.org) the taxonomic status of *P. gigantea* is well recognized and there are at least 23 species included in the genus *Phlebiopsis*, based on morphological characteristics. A number of techniques have been employed to identify *P. gigantea* and traditionally, the morphological characters of the spores were used for this purpose. Although the classical methods are reasonably easy and fast to apply [15], molecular techniques confirm identification of this fungus and are very useful for identification of species [16,17].

Various molecular markers used in barcoding, like ITS 1/2, β-tubulin, histone H3 and elongation factor α (syn. *EF1α*), are the most commonly applied in fungal taxonomy. Their application allows determination of fungal genotypes at a species level [18–20]. Comparisons of DNA sequence data are increasingly being used in order to gain knowledge concerning the phylogenetic relationships among *P. gigantea* isolates [7,10,11,21,22]. Many studies have utilised DNA sequence data of the *EF1α* gene for phylogenetic analyses including a wide range species of fungi [23,24].

The objectives of this study were to obtain DNA partial sequences for *EF1α* for *P. gigantea* strains, and to compare them with other some *Basidiomycota* species from the NCBI database. This gene is a highly conserved ubiquitous protein involved in translation that has been suggested to have desirable properties for phylogenetic inference [25]. It has been successfully used in phylogenetic studies as a phylogenetic marker for *Eukaryotes*, *Acomycetes* and *Basidiomycota* [23–25] Additionally, the partial sequences (intron and exon partial regions) obtained in the study of *EF1α* gene isolates of *P. gigantea* from Poland, Finland, Sweden and Great Britain, and partial genome sequence (gi:752829739) deposited in GenBank by Hori et al. [26] were investigated. To date, in studies on the differentiation of chosen *P. gigantea* isolates on the known activity of linear growth and wood decay [3,4,11,27] the *EF1α* gene has not been studied.

Our study provides an additional gene region useful for testing taxonomic groupings and phylogenetic relationships, previously identified based on the other gene regions like ITS [2,3]. Several factors can affect biological activity of the fungus. Grossbard [28] reported, that the presence of some fungi in soil can modify biological traits of co-occurring taxons. Schardl and Craven [29] described that the variation in enzyme and decay activity of fungal isolates in time may suggested risks in lost or change the molecular and biochemical characteristics. The cause is showed in possible hybridisation from the mating of clearly homozygous individuals. Żółciak et al. [3] and Sierota et al. [27] suggested the changes in the activity of different *P. gigantea* isolates with time, its origin, and wood density. For the effective use of competitive fungi used in biopreparations against pathogens in biological control (e.g., *P. gigantea*), there is a need for periodic exchange of strains for more effective ones [4]. Checking the utility of the *EF1α* region can be a valuable clue and can help in making decision regarding the selection of the most effective *P. gigantea* isolates as a competitor of *Heterobasidion* spp.

2. Materials and Methods

2.1. Cultivation of Isolates

Twelve previously identified and tested isolates of *P. gigantea* [11] were used in the experiment: six from Poland (not registered as biocontrol agent) and one from Finland, one from Sweden and four from Great Britain (registered as biocontrol agent) (Table 1). The number of Polish isolates was limited to six due to difficulties in obtaining homogeneous single-spore cultures, while the Finnish and British isolates were accepted as previously tested and approved. Isolates were grown on potato dextrose agar (PDA) medium (Difco™, Sparks, MD, USA) in Petri dishes for ten days at 20 °C according to Kwaśna et al. [30].

Table 1. List of *P. gigantea* isolates used in the study.

Polish Isolate Codes	Location Coordinates/Forest District/Country	Host	Substrate	Collector	Collection (Institution Name, Isolate Code, Country)
PL Pg_1	x:52.09859 y: 20.85479 Chojnów FD, Poland	*Pinus sylvestris* L.	Stump wood	A. Żółciak	Forest Research Institute, 01.06.08.1.5; 02.10.23.1.2; 03.11.04.1.1; 03.11.13.1.3; 99.09.10.1.1; 04.11.19.1.2 Poland
PL Pg_2					
PL Pg_6					
PL Pg_9					
PL Pg_{11} (Accession number: KU886024)	x:51.22000 y: 20.33157 Barycz FD, Poland		Fruitbody on stump	Z. Sierota	
PL Pg_{12}	x:53.42508 y: 20.59593 Nidzica FD, Poland		Stump wood	A. Żółciak	
SE Pg_8	Råberg near Uppsala, Sweden	*Picea abies*(L.) Karst.		K. Korhonen	Lallemand Plant Care - Verdera Oy, VRA 1984 *, Finland
FI Pg_{10}	Loppi, Finland		Log wood		Lallemand Plant Care - Verdera Oy, VRA 1835 *, Finland
GB Pg_{14}	Mull, Great Britain	*Pinus contorta* Dougl. ex Loud.	No data	No data	Forestry Commission, FOC PG 410.3 *, Great Britain
GB Pg_{15}	Roslin, GB	No data			Forestry Commission, FOC PG SP log 5 *, Great Britain
GB Pg_{16} (Accession number: KU886025)	NRS, GB	*Pinus sylvestris* L.			Forestry Commission, FOC PG B 20/5 *, Great Britain
GB Pg_{17}	Buchan, GB				Forestry Commission, FOC PG BU 3 *, Great Britain

* isolates registration in European Union.

2.2. DNA Extraction

Total fungal DNA of *P. gigantea* was extracted from mycelium grown on PDA by using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the protocol. Quality of the DNA was checked with an Infinite 200 PRO multimode plate reader (Tecan, Group Ltd., Männedorf, Switzerland).

2.3. Primers and PCR Conditions

The PCR reactions were done in 25 µL volumes using a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Each reaction contained 1 unit (0.25 µL) of Taq DNA polymerase (recombinant) (Thermo Scientific, Life Technologies Inc., Carlsbad, CA, USA), 2.5 µL of 10 × Taq buffer, 2 mM of each dNTP (0.5 µL), 1.5 mM of $MgCl_2$ (1.5 µL), 12.5 pmol of forward/reverse primers (0.125 µL), and 20 ng (1 µL) of DNA.

The primers and the touchdown PCR reaction conditions were used according to modified procedure of Rehner [18]. Amplicons of the partial region of the *EF1&* gene were generated using two overlapping primer combinations, 526F (5′ GTC GTY GTY ATY GGH CAY GT 3′) × 1567R (5′ AC HGT RCC RAT ACC ACC RAT CTT 3′) and EFdF (5′ AAG GAY GGN CAR ACY CGN GAR CAY GC 3′) × 2218R (5′ AT GAC ACC RAC RGC RAC RGT YTG 3′). For 526F × 1567R primers the touchdown PCR was as follows: 5 min at 94 °C (an initial denaturation), 30 s at 94 °C (denaturation) and 45 s at 60 °C (annealing) in the first cycle, successively reducing the Tm by 1 °C per cycle over the next 9 cycles to a

final Tm 50 °C, which was used in the remaining 36 cycles. An extension step per cycle was 1 min 30 s at 72 °C. The final extension step was 1 cycle of 7 min at 72 °C.

PCR parameters for EFdF × 2218R primers were as follows: 5 min at 94 °C, 45 cycles of (30 s at 94 °C, 30 s at 63 °C, 1 min 30 s at 72 °C) and 7 min at 72 °C. Amplicons were run in 1.5% (w/v) ethidium bromide-stained agarose gels (Sigma-Aldrich, St. Louis, MO, USA) and the bands were visualized under UV illumination. PCR products were sequenced using Sanger's sequencing method at the Polish Academy of Sciences Institute of Biochemistry and Biophysics (Warsaw, Poland).

2.4. DNA Sequence Analysis

Sequencing results were analyzed using the BLAST algorithm on The National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/) database and MEGA version 5 [31]. The DNA sequences were aligned using Clustal W version 2.0. [32]. Genetic variability was estimated for the sequences of *P. gigantea* and realigned separately for intron and exon regions using FGENESH 2.6 [33,34].

The obtained sequences were analyzed using Tajima's Neutrality Test. The number of sequences (m), number of segregating sites (S), mean number of segregating sites (Ps), nucleotide diversity (π) and estimates of Theta (Θ) per site as well as the Tajima test statistic (D) were estimated using MEGA5 program [35,36].

EF1α partial gene sequences of *P. gigantea* and selected *Basidiomycota* species from the NCBI database were analyzed using the maximum likelihood approach for phylogeny reconstruction and tested by bootstrapping with 1,000 replicates. Missing and ambiguous characters were excluded from the analysis. Phylogenetic trees were generated based on maximum likelihood method [37]. Two sequences of *P. gigantea* were deposited in GenBank NCBI (Accession numbers: KU886024 and KU886025, www.ncbi.nlm.nih.gov.genbank/).

3. Results

EF1α Partial Gene of P. gigantea Analysis

The region of the *EF1α* gene was successfully amplified for all *P. gigantea* isolates described in this study. All amplifications yielded a single band approximately 1,500 bp long. The final analyzed length partial sequence of the *EF1α* gene was 1,411 bp, the mRNA length was 1,403 bp. The structure of *EF1α* partial gene from the *P. gigantea* (Accession numbers: KU886024 and KU886025) is shown in Figure 1.

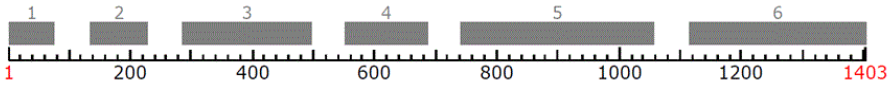

Figure 1. The structure of *EF1α* partial gene from the *Phlebiopsis gigantea*. The positions for exons are presented respectively: exon 1 (1–75), exon 2 (134–228), exon 3 (284–496), exon 4 (550–686), exon 5 (739–1056), exon 6 (1113–1403). (mRNA) 1–6 exon (s): 1–1403. The numbers indicate the positions of the bases identified using FGENESH software.

Genetic diversity was calculated for all obtained sequences including exons and introns of 12 partial sequences of the *EF1α* gene and partial genome sequence (gi:752829739) of *P. gigantea* (Figure 1) and separately for exons and introns (Table 2) The number of segregating sites (*S*) and mean number of segregating sites (*Ps*) were 32 and 0.022679 respectively. The value of the Θ, expressing the total variability was 0.007308 for analyzed 13 partial sequences mentioned above. The nucleotide diversity (π) was 0.005761. The value of Tajima's *D* was below zero (−0.931820).

In the I_1 and I_2 intron region the number of segregating sites was one. The remaining introns I_3, I_4 and I_5 showed three and six segregating sites, respectively. The value of the Θ, expressing the total variability ranged from 0.005556 to 0.034526 for all intron part sequences. The nucleotide diversity (π)

ranged from 0.004863 to 0.025641. The analysis of exons of the coding regions showed no segregating sites for partial E_1, however the number of segregating sites ranged from one for E_2 to seven for E_6. The value of the Θ was zero for partial E_1 and the highest 0.007544 for E_6. Among exons the nucleotide diversity (π) ranged from zero to 0.006176. The value of Tajima's *D* was below zero for all intron and E_2–E_6 exon regions (Table 2).

Table 2. Genetic polymorphism and neutrality tests of *EF1α* partial gene sequences of 12 Polish isolates and partial genome sequence (gi:752829739) of *P. gigantea*, including introns and exons.

Region	Sequence	*bp*	*m*	*s*	P_s	Θ	π	*D*
Intron	I_1	58	13	1	0.017241	0.005556	0.004863	−0.274290
	I_2	55	13	1	0.018182	0.005859	0.005128	−0.274290
	I_3	53	13	3	0.056604	0.018240	0.013546	−0.813698
	I_4	52	13	3	0.057692	0.018591	0.013807	−0.813698
	I_5	56	13	6	0.107143	0.034526	0.025641	−0.950320
Exon	Partial E_1	75	13	0	0.000000	0.00000E+000	0.000000	*n/c*
	E_2	95	13	1	0.010526	0.003392	0.002969	−0.274290
	E_3	213	13	4	0.018779	0.006052	0.004093	−1.099317
	E_4	137	13	3	0.021898	0.007057	0.006176	−0.394391
	E_5	318	13	3	0.009434	0.003040	0.002661	−0.394391
	E_6	299	13	7	0.023411	0.007544	0.006174	−0.688629

Abbreviations: *bp*, base pairs; *m*, number of sequences; *s*, number of segregating sites; $P_s = s/m$, mean number of segregating sites; $\Theta = P_s/a_1$, estimates of Theta per site; π, nucleotide diversity; *D*, Tajima's *D* Tajima test statistic; *n/c*, no changes.

Bootstrap values supported the separation of species into distinct clades (Figure 2). Among partial 12 sequences of *P. gigantea* the closest one to the partial genome sequence of *P. gigantea* (gi: 752829739) was GB Pg 16 (KU886025) from Great Britain. The couple of single mutations differentiated GB Pg 16 sequence from the rest of identical isolates of this fungus.

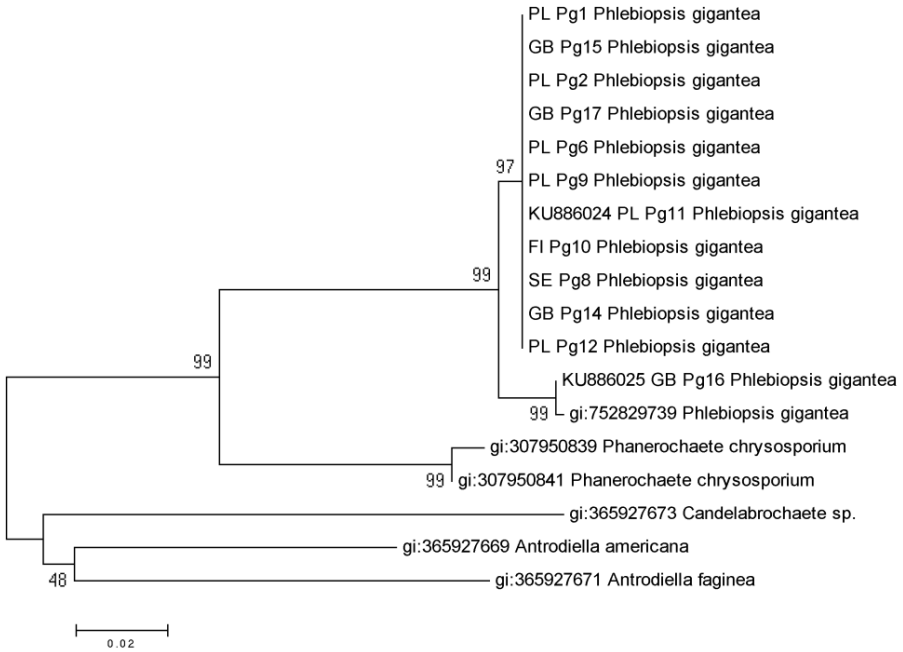

Figure 2. Phylogenetic tree for *Phlebiopsis gigantea* isolates and other species from the *Phanerochaetaceae*, based on *EF1α* gene partial sequences. Branch length values were shown, the tree was reconstructed using the maximum likelihood approach and tested by bootstrapping (1,000 replicates).

For *Phanerochaetaceae* family, two major and well supported groups were identified. One of the group represented all *P. gigantea* and *Phanerochaete chrysosporium* Burds. partial sequences (97–99% bootstrap support). The second group included: *Candelabrochaete* sp., *Antrodiella Americana* Ryvarden & Gilb. and *Antrodiella faginea* Vampola & Pouzar (48% bootstrap support). Phylogenetic analysis of partial sequences of *EF1α* gene for *P. gigantea* and selected *Basidiomycota* species, showed separate clades for following orders: *Polyporales*, *Agaricales*, *Boletales*, *Atheliales*, *Russulales* and *Sebacinales* (Figure 3).

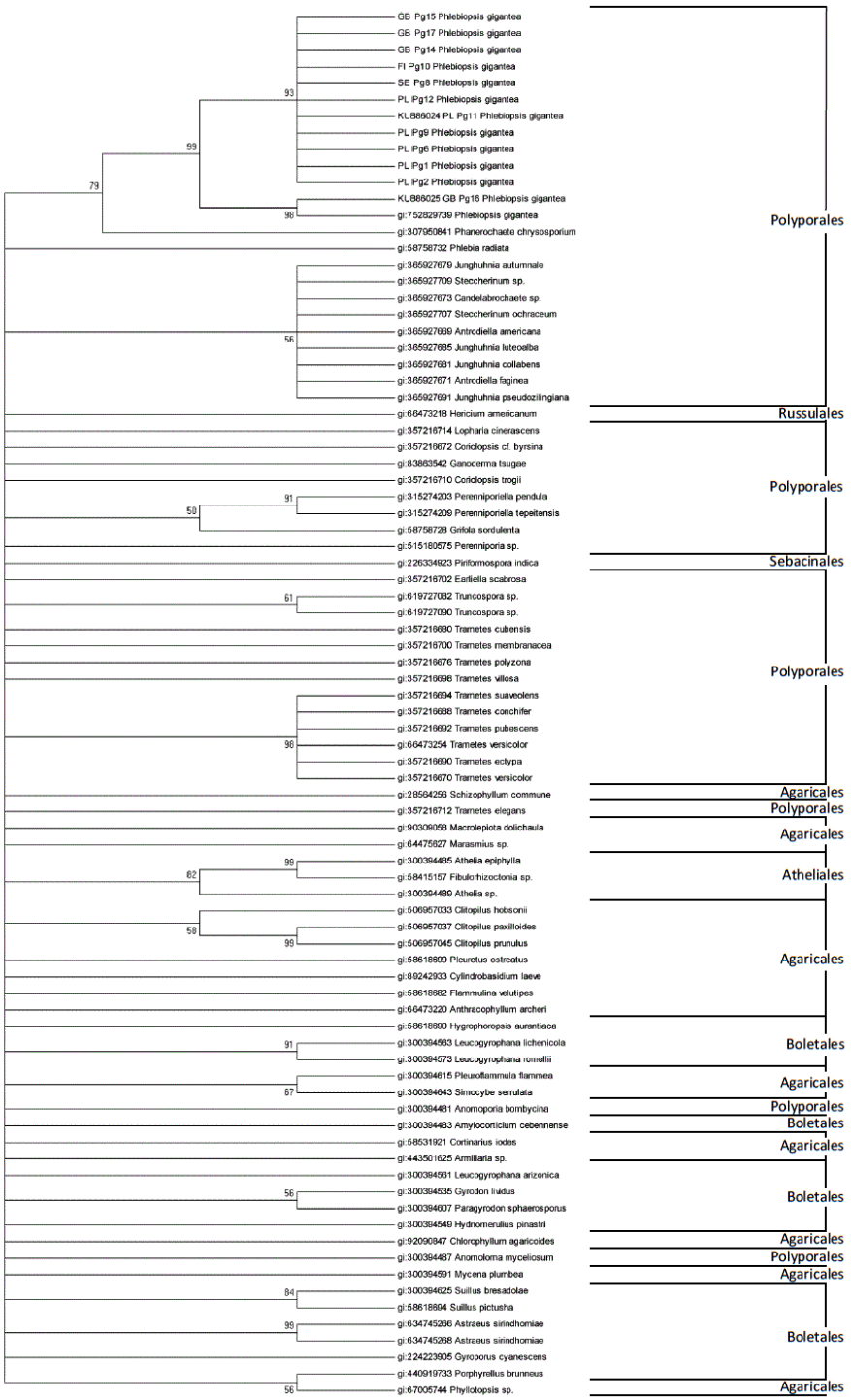

Figure 3. Phylogenetic tree for *Phlebiopsis gigantea* isolates and selected *Basidiomycota*, based on the *EF1α* partial gene sequences. The tree was reconstructed using the maximum likelihood approach and tested by bootstrapping (1000 replicates).

4. Discussion

In this study, DNA partial sequence for the *EF1α* gene of *Phlebiopsis gigantea* was successfully identified and analyzed. All *P. gigantea* isolates yielded PCR products of similar size, indicating that the amplified partial gene region does not include large indels, and therefore is a suitable choice for phylogenetic studies. The aligned sequences showed considerable homology among *P. gigantea* but various species-specific nucleotide substitutions and indels were observed among all *Basidiomycota* species. No sequence variation was observed among all *P. gigantea* except of one isolate (GB Pg 16) from Great Britain and partial sequence of *P. gigantea* genome (gi: 752829739).

The remarkable variation was observed between different strains. This is consistent with previous studies employing ITS region and genetic fingerprinting using random amplified microsatellite (RAMS) markers in taxonomic studies of *P. gigantea* [21].

For the analyzed *P. gigantea EF1α* partial gene the occurrence of exons and introns was identified. Introns represented the so-called 3rd introns group, spliced during the maturation of RNA with the participation of spliceosome [38]. The size of introns ranged from 52 to 58 bp, at the average length of the gene from 0.75 to 1,000 bp of gene.

Among representatives of *Basidiomycota* six introns in gene are observed average [39]. Compared to higher *Eukaryota*, introns occurring in fungi are relatively short [40]. In the case of the analyzed gene, introns size corresponds to the average size of introns identified in representatives of the *Fungi* kingdom (50–200 bp) [41]. The length of introns of *EF1α* gene was slightly smaller than the average for the *Puccinia graminis* (0.65 kb, NCBI accession number: X73529.1) and *Neurospora crassa* gene (0.81 kb) [42].

Despite the randomness and variability of the structure of introns, in the case of I_5 of *EF1α* partial gene, a larger number of segregating sites (six) was identified. In the sequences of other introns this number were from 1 to 3. Furthermore, the characteristic feature of the identified intron sequences was the presence of the sequence GT on the donor side (5′) and the sequence AG at the acceptor side (3′). They are essential in the process of identifying and splicing by spliceosome. Identified GT and AG sequences are considered as the most commonly occurring canonical dinucleotide fragments, respectively starting and ending the introns [43].

Intraspecies analysis of *EF1α* partial gene showed small variability within the coding and non-coding regions. Introns of analyzed *EF1α* partial gene were well conserved among all tested isolates of *P. gigantea.* The evolution of the structure of all introns and E_2–E_6 exon regions of *EF1α* partial gene was neutral. Isolates representing *P. gigantea* from Poland, Great Britain, Finland and Sweden had similar partial sequences of *EF1α* gene and were grouped together. The only British isolate (GB Pg 16 = FOC PG B 20/5) of this fungus showed coupled single mutations like single nucleotide polymorphisms (SNPs) and was very similar to *P. gigantea* (gi: 752829739) with identity 99% and query cover 100%.

Results of the study using DNA-RAMS markers indicated genetic similarity among isolates collected in Finland and Great Britain [10]. Additional studies using the previously mentioned markers showed that Polish isolate (PL 12) of *P. gigantea* was genetically analogous to FC 16 from Great Britain [11]. Vainio and Hantula [21] also showed that European and North American ITS/A alleles of *P. gigantea* were identical, while ITS/C alleles were different. The authors mentioned that the analysis of molecular variation and neighbor joining analysis using 28 RAMS markers revealed a considerable degree of differentiation between Europe and North America [21].

The main advantage of this study is the phylogenetic analysis of *EF1α* partial DNA sequence data for *P. gigantea* in comparing it to species belonging to the same family—*Phanerochaetaceae* and selected *Basidiomycota* species. Phylogenetic trees showed that *P. gigantea* is closely related to *Phanerochaete chrysosporium. EF1α* is also the first protein-coding gene and first single-copy gene used for phylogenetic analysis of *P. gigantea*. The whole genome sequence of *P. gigantea* (gi: 752829739, accession number: AZAG01000080) was published by Hori et al. [26]. The whole genome sequence size is approximately 30 Mbp and number of predicted genes (11,891). Sequence data from the majority of isolates belonging

to the different species showed unique species-specific substitutions, allowing the isolates to be differentiated into clades representing the species.

The results of this study demonstrate that the *EF1α* region is useful for phylogenetic analysis and classification of *Polyporales* species. This is a large and taxonomically difficult order, which include several genera, for example: *Phlebiopsis*, *Phanerochaete*, *Phlebia*, *Junghuhnia*, *Steccherinum*, *Androniella*, *Ganoderma*, *Coriolopsis*, *Perenniporiella* and *Trametes*.

5. Conclusions

Our in silico studies showed a relationship between *P. gigantea* and selected *Basidiomycota* based on elongation factor 1-alpha partial DNA sequence. The partial *EF1α* gene sequence of *P. gigantea* isolates originating from Poland, Finland, Sweden and Great Britain formed a monophyletic group, except for one British isolate GB Pg 16 (FOC PG B 20/5). The results of our study proved that the partial sequence of *EF1α* gene is useful for phylogenetic analysis of the *Phanerochaetaceae* family.

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Conflicts of Interest: The authors declare there were no personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results.

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