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Inter- and Intra-Continental Genetic Variation in the Generalist Conifer Wood Saprobic Fungus *Phlebiopsis gigantea*

Francesco Dovana [1,2], Paolo Gonthier [3] and Matteo Garbelotto [2,*]

[1] Department of Life Science and Systems Biology, University of Turin, Viale P.A. Mattioli 25, I-10125 Torino, Italy; francescodovana@gmail.com
[2] Department of Environmental Science, Policy and Management; 54 Mulford Hall; University of California, Berkeley, CA 94720, USA
[3] Department of Agricultural, Forest and Food Sciences (DISAFA), University of Torino, I-10095 Grugliasco (TO), Italy; paolo.gonthier@unito.it
* Correspondence: matteog@berkeley.edu; Tel.: +1-510-4107058

Abstract: *Phlebiopsis gigantea* (Fr.) Jülich is a well-known generalist conifer wood saprobe and a biocontrol fungus used in several world countries to prevent stump infection by tree pathogenic *Heterobasidion* fungal species. Previous studies have reported the presence of regional and continental genetic differentiation in host-specific fungi, but the presence of such differentiation for generalist wood saprobes such as *P. gigantea* has not been often studied or demonstrated. Additionally, little information exists on the distribution of this fungus in western North America. The main purposes of this study were: (I) to assess the presence of *P. gigantea* in California, (II) to explore the genetic variability of *P. gigantea* at the intra and inter-continental levels and (III) to analyze the phylogeographic relationships between American and European populations. Seven loci (nrITS, ML5–ML6, *ATP6*, *RPB1*, *RPB2*, *GPD* and *TEF1-α*) from 26 isolates of *P. gigantea* from coniferous forests in diverse geographic distribution and from different hosts were analyzed in this study together with 45 GenBank sequences. One hundred seventy-four new sequences were generated using either universal or specific primers designed in this study. The mitochondrial ML5–ML6 DNA and *ATP6* regions were highly conserved and did not show differences between any of the isolates. Conversely, DNA sequences from the ITS, *RPB1*, *RPB2*, *GPD* and *TEF1-α* loci were variable among samples. Maximum likelihood analysis of *GPD* and *TEF1-α* strongly supported the presences of two different subgroups within the species but without congruence or geographic partition, suggesting the presence of retained ancestral polymorphisms. *RPB1* and *RPB2* sequences separated European isolates from American ones, while the *GPD* locus separated western North American samples from eastern North American ones. This study reports the presence of *P. gigantea* in California for the first time using DNA-based confirmation and identifies two older genetically distinct subspecific groups, as well as three genetically differentiated lineages within the species: one from Europe, one from eastern North America and one from California, with the latter presumably including individuals from the rest of western North America. The genetic differentiation identified here among *P. gigantea* individuals from coniferous forests from different world regions indicates that European isolates of this fungus should not be used in North America (or vice versa), and, likewise, commercially available eastern North American *P. gigantea* isolates should not be used in western North America forests. The reported lack of host specificity of *P. gigantea* was documented by the field survey and further reinforces the need to only use local isolates of this biocontrol fungus, given that genetically distinct exotic genotypes of a broad generalist microbe may easily spread and permanently alter the microbial biodiversity of native forest ecosystems.

Keywords: exons; introns; phylogeography; sequence-based

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1. Introduction

There is a current understanding that a strong biogeographical signal characterizes life on our planet [1]. While this has long been clear for animals and plants, the extent of global geographic structuring of microbial populations and species is a controversy ignited by the well-known theory of Baas-Becking [2] stating that "everything is everywhere but the environment selects", which is still partially debated [3]. The fungi occupy a unique position among microbes, due to their extremely diverse life-styles, ranging from obligate biotrophism and host-specific parasitism to generalist parasitism and saprotrophism [4,5]. Fungi can also be endophytic or in symbiosis with host plants, adding further complexity to their biology and to the mechanisms driving host–parasite interactions and associated evolutionary processes [6,7]. While there is probably no one-size-fits-all answer about the biogeography of fungi in general [8,9], an increasing body of evidence points to a strong genetic structuring of fungi present in natural ecosystems and in forests in particular [10,11]. Distance [12], geographic barriers [13], size of host populations [14] and biogeography of host plants [15] all appear to be driving the natural microevolution, phylogeography and evolution of many forest fungi, particularly host–specific ectomycorrhizal and pathogenic fungi. The presence of phylogeographic signals for generalistic fungi is still in question. While a strong biogeographic signal has been recently reported for forest soil fungi in general, including generalist and putatively ubiquitous species [16], other studies did identify endemisms but also uncovered a lack of strong phylogeographic signal in soil fungi [17]. Additionally, the Anthropocene may have erased some of that biogeographical signal, due to the human-mediated long-distance movement of plants, animals and microbes, including fungi, among different world regions [18]. Hence, in-depth studies are still necessary to determine the actual genetic relatedness among populations of a species and among closely related congeneric species from different world regions.

One of the aims of this study was to identify the presence of regional phylogeographic signal for the generalist wood decay saprobe fungus *Phlebiopsis gigantea* (Fr.) Jülich, an organism reported from conifer forests of the northern Hemisphere. Eastern and western North America represent two undisputedly distinct world bioregions [19], with minimal overlap of native plant and animal taxa between the two. The debate is still ongoing regarding the timing and the migration routes of different organisms to/from eastern and western North America and Europe or Asia (see [20]). An older North Atlantic land bridge connecting North America to Europe is in contrast with a more recent Beringial land bridge connecting North America to Asia [21–23]. Different and differently aged migration pathways may explain not only differences in evolutionary and speciation patterns among Eurasian and North American plants and animals, but also some of the differences between eastern and western North America biota [24]. The geological history of the North American continent, and in particular patterns of glaciation and of mountain uplifting [25,26], have been broadly invoked to explain the remarkable differences in the taxonomic composition of plant communities, and in particular of forests, observable when comparing eastern and western North America. Transitional areas with some documented extant or historical overlap in plant, animal and fungal community composition have been identified in Alaskan or western boreal Canadian forests [10,27,28], as well as in central Mexican forests [29–31].

An eastern–western North American taxonomic disjunction can often be inferred by the large number of studies that independently connect eastern North American forest biota to either eastern Asian or western European biota, and western North American to eastern Asian forest biota [24]. Surprisingly, direct comparisons between eastern and western North American forests are less abundant [32–35]. General statements have been made about a closer evolutionary relatedness of taxa within the North American continent, compared to the relatedness of North American taxa to taxa from other continents [20]. However, a specific evaluation of the evolutionary relatedness among individual groups of organisms has shown instead that eastern and western North American taxa, while most often clearly distinct, may be more closely related to either European or eastern Asian

organisms than to each other, depending on their phylogeographic history (see [36]). The different deep histories of forests and forest-dependent organisms in North America, and the unique phylogeography of different species, often repopulating the continent from distinct glacial/climatic refugia [37], have both driven the current-day composition of North American forest biota and may explain such phylogeographic difference. The lack of continuous forest cover and of conifers, in particular in the central part of the continent, where grasslands dominate in the rain shadow of the Rocky Mountains [38], has reinforced the genetic isolation of woody plant populations and of forest-dwelling organisms in general, living on the opposite sea borders of the continent [39].

At least some fungi seem to be part of this East–West continental disjunction. Due to their symbiotic relationships with woody plants, the phylogeography of native ectomycorrhizal fungi has been expected to match the phylogeography of their hosts [40,41]. Hence, it should be no surprise that examples of phylogenetic continuity have been identified between native ectomycorrhizal fungi in central/southern Mexico, eastern US and eastern Asia [42,43], with patterns closely resembling those of their plant hosts [20]. Conversely, congeneric native eastern and western North American ectomycorrhizal fungal species appear to be more distant from one another (see [42,44,45]). Many plant and tree pathogens also coevolved in relationship with their hosts [46]; hence, once again, the phylogeography of many native plant pathogens should match that of their hosts. One of the most intensively studied forest pathogens in North America is *Heterobasidion irregulare* Garbel. & Otrosina [47–49]. *H. irregulare* is an important term of comparison for *P. gigantea*, the fungus here studied [50], for various reasons. Both fungi have a relatively broad host range with a preference for conifers and pines in particular, and both of them are saprobic wood colonizers able to infect freshly cut stumps. The major difference between the two is that *P. gigantea* is not capable of infecting living neighboring trees like *Heterobasidion* does, hence it is often used as a biocontrol agent against *Heterobasidion* spp. [48,51]. In North America, *H. irregulare* is present in eastern, Mexican and western conifer forests and is closely related to the western Eurasian *H. annosum*, suggesting an older North Atlantic migration pathway [30]. Eastern North American and western North American populations of *H. irregulare* are genetically distinct, and ancestral retained polymorphisms of both eastern and western populations are present in Mexico [30].

As mentioned above, humans have greatly modified the world distribution of all living organisms by transporting and introducing them to novel geographic ranges: these introductions not only erase the true phylogeographic signal of the introduced species but also may have significant impacts on the integrity of the ecosystems that receive them. Examples of exotic animals and plants abound across the globe, and an increasing number of studies have proven the same to be true for fungi and fungus-like organisms, with many examples of symbiotic and pathogenic fungi being transported from one region of the world to another [18,52–56]. The number of known cases of long-range movement of ectomycorrhizal fungi is on the rise, thanks to the democratization of next generation sequencing techniques; however, we cite here the specific example of *Amanita phalloides*, a European ectomycorrhizal mushroom introduced in forests on both coasts of the North American continent [57]. One interesting and unexpected outcome of the invasion of forests by *A. phalloides* has been its unusual high productivity of fruitbodies, the deadly "death caps" [58]. Besides its potential ecological consequences, this phenotype's undesirable attributes include its high and lethal toxicity of the mycotoxin present in the mushrooms. A similar enhanced production of fruiting bodies by an exotic fungus has been reported for the North American tree pathogen *Heterobasidion irregulare* [59], introduced by the U.S. military in Italy during World War II [60]. Increased production of fruitbodies leads to increased tree infection; hence, this is also an undesirable ecological trait. Recent evidence has additionally shown that native Italian *H. annosum* genotypes are acquiring *H. irregulare* alleles involved in fruiting through hybridization-mediated genic introgression, further expanding the negative consequences associated with the introduction in Italy of the exotic pathogen [61]. A third example of a fungal introduction is that of *Cronartium ribicola*

J.C. Fisch., the fungus responsible for the lethal disease of five-needle pines known as White Pine Blister Rust, introduced from Eurasia to both North American coasts in the first two decades of the 1900s [62]. The fungal genotypes that started the eastern and western outbreaks came from different Eurasian locations and belonged to genetically different populations. Founder effects were strong enough that the two outbreaks started as genetically differentiated lineages, and that genetic differentiation was further reinforced by over 100 years of isolation. Even if the fungus has colonized the vast majority of eastern *Pinus strobus* L. populations and a large number of western five-needle pines belonging to multiple species, the two outbreaks have yet to merge, due to the lack of forests in the middle of the North American continent [63]. Because of the obvious, although imperfectly understood, connection between genotype and phenotype, the mixing of the genetically different eastern and western *C. ribicola* populations could have dire consequences on the virulence and further adaptation of the pathogen. As such, one of the strongest current recommendations is to prevent any admixing between eastern and western populations of *C. ribicola* in North America. There is currently a ban on plantations of Ribes, the alternate host of *C. ribicola*, in some parts of North America, where the two lineages have come dangerously close to one another and where outbreaks on pines are still on the rise [64].

Thus, intermixing of genetically distinct fungal populations is seen as something that should be prevented and not facilitated, given the possible detrimental outcomes of such intermixing. Here, we set out to study the presence of both intercontinental and intracontinental genetic differentiation among genotypes of the wood saprobic generalist fungus *Phlebiopsis gigantea*, a fungus used in Europe as a biocontrol agent of forest pathogens belonging to the genus *Heterobasidion* [65,66]. The rationale for the study was threefold. The first was to provide evidence for the presence of geography-driven genetic differentiation in a generalist wood saprobic fungus normally inhabiting mixed coniferous forests. This result would support the presence of a habitat-driven phylogeographic signal for a microbe, even in the absence of strict host specificity. The second was to determine whether this fungus is present in California using both morphology and DNA-based identification, and if so, where and on which hosts. This information could be used to support the introduction of local *P. gigantea* isolates as a biocontrol agent in habitats where it is already present, and to use caution where it is not present. The third rationale was to provide further evidence in favor of or against the use in western North American forests of Rotstop®C Biofungicide WP, a product registered in the US for the control of *Heterobasidion* spp. and based on an eastern North American isolate of *P. gigantea* as a biocontrol agent. Lack of intracontinental genetic differentiation could be used in support of the use of the commercially available biocontrol isolate, while the presence of intracontinental genetic differentiation would speak against it.

2. Materials and Methods

2.1. Survey and Isolation of Phlebiopsis gigantea from Western North American Forests

In 2018, we set out to obtain western North American isolates of *Phlebiopsis gigantea*. Three different approaches were employed. First, a request was sent to forest pathologists and mycologists from the western US to share cultures or herbarium specimens of *Phlebiopsis gigantea*. Second, a survey of California mixed coniferous forests was conducted in person in October and November 2018, when Fall conditions are favorable for the production of fruiting bodies and for sporulation. A transect was laid out from the Pacific Coast all the way to the edges of the Great Basin desert in Nevada, with intensive surveys and field collections conducted in mixed coniferous forest stands located in four distinct California regions where tree felling had occurred within the last two years (Figure 1 and Table 1): (a) coastal low elevation mixed conifer forests around Mendocino (Mendocino County); (b) montane mixed conifer forests on Cobb Mountain (Lake County), in the California Coast Range; (c) montane mixed conifer forests in the mid-elevation of the Eldorado National Forest on the western slopes of the Northern Sierra Nevada, in the interior of California (Eldorado County); and (d) alpine mixed conifer forests in high-elevation stands

of the Tahoe National Forest on the eastern slopes of the Northern Sierra Nevada, in the interior of California at the border with Nevada (Sierra and Placer Counties). Third, at each of 41 sampling points located across the same four regions listed above (Table 1), four freshly cut *Pinus radiata* D. Don wood discs were placed in Petri dishes and left out to trap airborne spores for a period of 24 h as described in [67].

Figure 1. Map of the areas in California that were intensively surveyed for the presence of *Phlebiopsis gigantea*.

Table 1. Sampling points, location, substrate, climate and elevation of intensively surveyed mixed conifer forests in California.

ID	P.g.	Location	County and Ecoregion	Latitude	Longitude	Substrate	Average Yearly Rainfall (mm)	Average Temperature Range	Elevation
P15	No	Van Damme State Park	Mendocino, Coastal	39.277142	−123.782546	Douglas-fir log	1041	5 to 22	65
P25	No	Van Damme State Park	Mendocino, Coastal	39.276701	−123.780552	Conifer log	1041	5 to 22	78
P11	No	Pygmy Forest	Mendocino, Coastal	39.265512	−123.736040	Douglas-fir log	1041	5 to 22	187
P19	No	Pygmy Forest	Mendocino, Coastal	39.266242	−123.734775	Shore pine log	1041	5 to 22	189
P20	No	Pygmy Forest	Mendocino, Coastal	39.266450	−123.766441	Shore pine log	1041	5 to 22	166
P16	No	Airport Rd.	Mendocino, Coastal	39.269930	−123.779402	Bishop pine log	1041	5 to 22	90
P17	No	Airport Rd.	Mendocino, Coastal	39.271307	−123.774745	Bishop pine stump	1041	5 to 22	127
P18	No	Airport Rd.	Mendocino, Coastal	39.271176	−123.771672	Bishop pine log	1041	5 to 22	149
P12	No	Russian Gulch State Park	Mendocino, Coastal	39.329418	−123.808355	Douglas-fir log	1041	5 to 22	20
P23	No	Russian Gulch State Park	Mendocino, Coastal	39.329460	−123.809579	Douglas-fir log	1041	5 to 22	20
P7	No	Cobb Mtn.	Lake, Coast Range	38.811006	−122.712369	Ponderosa pine log	965	−2 to 29	816
P8/U-P8	Yes	Cobb Mtn.	Lake, Coast Range	38.811006	−122.712369	Ponderosa pine log	965	−2 to 29	818
P9/U-P9	Yes	Cobb Mtn	Lake, Coast Range	38.811006	−122.712369	Ponderosa pine log	965	−2 to 29	818
P10	No	Cobb Mtn.	Lake, Coast Range	38.81118	−122.713345	Ponderosa pine log	965	−2 to 29	826
P21	Yes	Cobb Mtn.	Lake, Coast Range	38.809389	−122.711941	Ponderosa pine log	965	−2 to 29	820
P22/U- P22	Yes	Cobb Mtn.	Lake, Coast Range	38.819652	−122.712103	Ponderosa pine log	965	−2 to 29	821
P24/U-P24	Yes	Cobb Mtn.	Lake, Coast Range,	38.819652	−122.712109	Ponderosa pine log	965	−2 to 29	820
P26/U-P26	Yes	Cobb Mtn.	Lake, Coast Range	38.819652	−122.712109	Ponderosa pine log	965	−2 to 29	820
P2	No	Loch Lomond	Lake, Coast Range	38.895662	−122.742704	Ponderosa pine log	965	−2 to 29	785
P6	No	Loch Lomond	Lake, Coast Range	38.887198	−122.729372	Ponderosa pine log	965	−2 to 29	797
P1	No	Tahoe City	Placer, High Sierra Nevada	39.155065	−120.152929	White fir log	787	−8 to 26	1935
P3	No	Tahoe city	Placer, High Sierra Nevada	39.173049	−120.148085	White fir log	787	−8 to 26	1951
P4	No	Tahoe Vista	Placer, High Sierra Nevada	39.250457	−120.108564	White fir log	787	−8 to 26	2180
P5	No	Tahoe Vista	Placer, High Sierra Nevada	39.250457	−120.108564	White fir log	787	−8 to 26	2180
P13	No	Tahoe City	Placer, High Sierra Nevada	39.161265	−120.153599	Ponderosa pine log	787	−8 to 26	1913
P14	No	Tahoe City	Placer, High Sierra Nevada	39.161064	−120.154032	White fir log	787	−8 to 26	1917

Table 1. *Cont.*

ID	P.g.	Location	County and Ecoregion	Latitude	Longitude	Substrate	Average Yearly Rainfall (mm)	Average Temperature Range	Elevation
P27	No	Tahoe City	Placer, High Sierra Nevada	39.161265	−120.153599	Ponderosa pine log	787	−8 to 26	1915
P29	No	Ward Valley	Placer, High Sierra Nevada	39.144342	−120.206932	Lodgepole pine log	787	−8 to 26	2022
P32	No	Ward Valley	Placer, High Sierra Nevada	39.143975	−120.210386	Conifer wood	787	−8 to 26	2031
P28	No	Sierraville	Sierra, High Sierra Nevada	39.491148	−120.306089	Conifer wood	787	−8 to 26	1975
P34	No	Sierraville	Sierra, High Sierra Nevada	39.490843	−120.28722	Ponderosa pine log	787	−8 to 26	1962
P35	No	Sierraville	Sierra, High Sierra Nevada	39.490845	−120.28722	Ponderosa pine log	787	−8 to 26	1962
P36	No	Sierraville	Sierra, High Sierra Nevada	39.490847	−120.28724	Ponderosa pine log	787	−8 to 26	1960
P37	No	Sierraville	Sierra, High Sierra Nevada	39.490847	−120.28724	Ponderosa pine log	787	−8 to 26	1960
P31	No	Sierraville	Sierra, High Sierra Nevada	39.489625	−120.29373	Ponderosa pine log	787	−8 to 26	1974
P30	No	Blodgett Forest	Eldorado, Sierra Nevada	38.880831	−120.648867	Ponderosa pine log	1651	0 to 27	1288
P33	No	Blodgett Forest	Eldorado, Sierra Nevada	38.875021	−120.651343	White fir log	1651	0 to 27	1330
P39	Yes	Blodgett Forest	Eldorado, Sierra Nevada	39.912835	−120.665881	Douglas-fir log	1651	0 to 27	1337
P41	Yes	Blodgett Forest	Eldorado, Sierra Nevada	39.912858	−120.666114	Black oak log	1651	0 to 27	1337
P38	No	Eldorado National Forest	Eldorado, Sierra Nevada	38.830903	−120.383638	Douglas-fir log	1651	0 to 27	1643
P40	No	Eldorado National Forest	Eldorado, Sierra Nevada	38.830903	−120.383638	Douglas-fir log	1651	0 to 27	1643

Isolations were made by plating on standard 2% Malt Extract Agar (MEA) amended with 0.3 g/L (300 ppm) Streptomycin Sulfate diluted in 5 mL 100% ethanol, chips of the interior context of each basidiocarp right at the edges between the fungal fruit body and the wood substrate, making sure the exterior layer of the fruit body had been first excised to avoid contamination. Isolates were then subcultured by transferring one hyphal tip on unamended 2% MEA.

2.2. Molecular Analyses

2.2.1. DNA Extraction

Fungal mycelia were scraped from pure cultures grown on 2% MEA medium for 2 weeks at 20 °C and ground to a fine powder with liquid nitrogen using a mortar and pestle. DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Inc., Valencia, CA, USA) following the manufacturer's instructions.

2.2.2. PCR and DNA Sequencing

DNA sequence data were obtained for seven loci: the internal transcribed spacer (nrITS) of the nuclear ribosomal DNA, the ML5–ML6 DNA region of the mitochondrial large ribosomal RNA (mt LrRNA), a portion of the ATPase subunit 6 (atp6), the RNA polymerase II subunit (*RPB1* and *RPB2*), the glyceraldehyde-3-phosphate dehydrogenase (*GPD*) and the translation elongation factor 1-alpha (*TEF1-α*). The primers used in the PCR reactions and sequencing are shown in Table 2.

Table 2. Primers used for PCR fingerprinting, DNA sequence amplification and sequencing.

Name	Nucleotide Sequence (5′–3′)	Reference	Region
GDP-14f	GTATCGTCCTCCGTAATGCTCTCCT	This study	glyceraldehyde-3-phosphate dehydrogenase (GPD)
GDP-693r	GTCCTTGTTTGAGGGACCATCGAC	This study	glyceraldehyde-3-phosphate dehydrogenase (GPD)
GDP-633f	TAC AAG GTC ATC TCG AAC GCG	This study	glyceraldehyde-3-phosphate dehydrogenase (GPD)
GDP-1134r	GAC ACG ACC TTC TCA TCG GTG	This study	glyceraldehyde-3-phosphate dehydrogenase (GPD)
GPD1	AGCCTCTGCCCAYTTGAARG	[30]	glyceraldehyde-3-phosphate dehydrogenase (GPD)
GPDR	RTANCCCCAYTCRTTRTCRTACCA	[30]	glyceraldehyde-3-phosphate dehydrogenase (GPD)
ML5	CTCGGCAAATTATCCTCATAAG	[68]	introns in the ML5–ML6 DNA region of the mitochondrial large ribosomal RNA (mt LrRNA)
ML6	CAGTAGAAGCTGCATAGGGTC	[68]	introns in the ML5–ML6 DNA region of the mitochondrial large ribosomal RNA (mt LrRNA)
ATP6-34f	GGGTTAAATGCTCCCATTTTTGGT	This study	ATPase subunit 6 (atp6)
ATP6-693r	TGAGAAAACGTAGGCTTGTATAAATGA	This study	ATPase subunit 6 (atp6)
EF625f	GGACCGCTTCAACGAAATCG	This study	translation elongation factor 1-alpha (tef-1a)
EF1437r	CTCGCCTCGATCACCTTACC	This study	translation elongation factor 1-alpha (tef-1a)
EF983F	GCYCCYGGHCAYCGTGAYTTAT	[69]	translation elongation factor 1-alpha (tef-1a)
EF-2218R	ATGACACCRACRGCRACRGTYTG	[69]	translation elongation factor 1-alpha (tef-1a)
RPB1-29f	TGGACTGATGGATCCTCGGT	This study	RNA polymerase II subunit (rpb1)
RPB1-1292r	TCGCCCAGTTTGTACGTCAA	This study	RNA polymerase II subunit (rpb1)
PRB2-5f	TACCTCACAAACTTCCTCGTACG	This study	RNA polymerase II subunit (rpb2)
RPB2-957r	ATGTGCTTCAGACGCTGATAGTA	This study	RNA polymerase II subunit (rpb2)
ITS1F	CTTGGTCATTTAGAGGAAGTAA	[70]	internal transcribed spacer (nrITS) of nuclear ribosomal DNA
ITS4	TCCTCCGCTTATTGATATGC	[68]	internal transcribed spacer (nrITS) of nuclear ribosomal DNA

PCR amplification conditions for the amplification of the nrITS and of the ML5–ML6 DNA region were described in Gardes and Bruns [70] and White et al. [68]. The ATP6, *RPB1* and *RPB2* regions were amplified using the ATP6-PGF/ATP6-PGR primers, the RPB1-PGF/RPB1-PGR primers and RPB2-PGF/RPB2-PGR, respectively. The GPD region including the IV and V introns was amplified using the GPD-PGF1 and the GPD-PGR1 PG-specific primers; the GPD region including the VI intron was amplified using the GPD1 and GPDR or PG-specific primers GPD-PGF2 and GPD-PGR2. Degenerate primers EF983F and EF-2218R [69] or PG-selective primers EF-PGF and EF-PGR were used to amplify the *TEF1-α* region. The new *Phlebiopsis gigantea* selective primers are named with "-PGF" and "-PGR" suffix for forward and reverse, respectively. The PCR conditions of new primers used in this study are reported in Table 3. All *P. gigantea*-specific primers were designed using the software Primer 3 2.3.7 [71] in Geneious v. R 11.1.5 (http://www.geneious.com, [72]) accessed on 30 September 2019 at https://primer3.ut.ee/ (accessed on 20 June 2004) using the draft of the entire *P. gigantea* genome as a template [73].

2.2.3. Alignments and Phylogenetic Analyses

For each single region, sequences were aligned with two or three close referenced sequences available in GenBank using MAFFT v 7.017 [74] in Geneious v. R 11.1.5, setting auto algorithm. Only for separate *GPD* intron/exon analyses was no outgroup chosen. Two concatenated datasets were generated and partitioned. The first one included *TEF1-α*, nrITS, *RPB1*, *RPB2* and *GPD* (exon and IV, V, VI introns), while the second one included *TEF1-α*, nrITS, *RPB1*, *RPB2* and *GPD* (only IV and V introns). Sequences of *Phlebia* sp. FBCC296 retrieved from GenBank were used as an outgroup for these concatenated analyses. A ML maximum likelihood analysis was performed with RAxML v. 8.2.11. [75] in Geneious v. R 11.1.5 implementing the GTR + G model to each partition and a total of 1000 bootstrap replicates.

Table 3. PCR protocols for the use of primers designed in this study.

Regions	Primers	PCR Protocol
ATP6	ATP6-PGF ATP6-PGR	95 °C 2 min; 34 cycles: 95 °C 30 s, 59.5 °C 30 s, 72 °C 2 min; 72 °C 5 min
GPD (IV-V introns)	GPD-PGF1 GPD-PGR1	95 °C 2 min; 34 cycles: 95 °C 30 s, 60 °C 30 s, 72 °C 2 min; 72 °C 5 min
GPD (VI Iintron)	GPD-PGF2 GPD-PGR2	95 °C 2 min; 34 cycles: 95 °C 30 s, 59.5 °C 30 s, 72 °C 2 min; 72 °C 5 min
RPB1	RPB1-PGF RPB1-PGR	95 °C 2 min; 34 cycles: 95 °C 40 s, 57 °C 40 s, 72 °C 2 min; 72 °C 5 min
RPB2	PRB2-PGF RPB2-PGR	95 °C 2 min; 34 cycles: 95 °C 30 s, 56 °C 30 s, 72 °C 2 min; 72 °C 5 min
tef-1a	EF-PGF EF-PGR	95 °C 2 min; 34 cycles: 95 °C 30 s, 57 °C 30 s, 72 °C 2 min; 72 °C 5 min

Overall, the analyses included 13 isolates from California, 9 isolates from the eastern US, four isolates from Europe and 45 GenBank accessions (Table 4 and Table S1).

Table 4. GenBank accession numbers of newly generated *Phlebiopsis gigantea* sequences.

	GenBank Code						
ID Code	**ATP6**	**ITS**	**ML5ML6**	**EFA**	**RPB1**	**rpb2**	**GDP**
MVW11027 US-E AL [1]	MW052838	MW055455	MW067609	MW074132	MW168678	MW239099	MW272459
MVW11111 US-E AL [1]	MW052837	MW055456	MW067610	MW074136	MW168677	MW239078	MW272460
MVW23048A US-E GA [1]	MW052841	MW055457	MW067611	MW074133	MW168682	MW239079	MW272461
MVW24089A US-E GA [1]	MW052840	MW055458	MW067612	MW074134	MW168681	MW239080	MW272462
MVW31044B US-E GA [1]	MW052839	MW055454	MW067613	MW074135	MW168680	MW239081	MW272463
NCII US-E NC [1]	MW052836	MW055459	MW067614	MW074137	MW168675	MW239082	MW272464
P21 US-W CA [2]	MW052834	MW055461	MW067616	MW074139	MW168670	MW239084	MW272466
P22 US-W CA [2]	MW052833	MW055462	MW067617	MW074140	MW168669	no	MW272467
P24 US-W CA [2]	MW052832	MW055463	MW067618	MW074141	MW168668	MW239085	MW272468
P26 US-W CA [2]	MW052831	MW055464	MW067619	MW074142	No	no	MW272469
P29 US-W CA [2]	MW052830	No	MW067620	no	No	MW239077	MW272470
P39 US-W CA [2]	MW052829	MW055465	MW067621	MW074143	MW168664	MW239086	MW272471
P41 US-W CA [2]	MW052828	MW055466	MW067622	MW074144	MW168665	MW239098	MW272472
P9 US-W CA [2]	MW052835	MW055460	MW067615	MW074138	MW168671	MW239083	MW272465
PG0045 EU IT [3]	MW052826	MW055468	MW067624	MW074146	MW168683	MW239088	MW272474
PG16g EU IT [3]	MW052828	MW055467	MW067623	MW074145	MW168684	MW239087	MW272473
PG1862 EU CZ [3]	MW052825	MW055469	MW067625	MW074147	MW168686	MW239089	MW272475
PG1889 EU CZ [3]	MW052824	MW055470	MW067626	MW074148	MW168685	MW239090	MW272476
SC US-E SC [1]	MW052823	MW055471	MW067627	MW074149	MW168674	MW239100	MW272477
SCNC US-E GA [1]	MW052822	MW055472	MW067628	MW074150	MW168676	MW239091	MW272478
U-P22 US-W CA [2]	MW052819	MW055476	MW067632	MW074152	MW168679	MW239094	MW272480
U-P24 US-W CA [2]	MW052818	MW055477	MW067629	MW074153	MW168673	MW239095	MW272481
U-P26 US-W CA [2]	MW052817	MW055474	MW067633	MW074155	MW168672	MW239096	MW272482
U-P8 US-W CA [2]	MW052821	MW055475	MW067630	MW074151	MW168667	MW239092	MW272479
U-P9 US-W CA [2]	MW052820	MW055473	MW067631	MW074154	No	MW239093	no
VA_APP US-E VA [1]	MW052816	MW055478	MW067634	MW074156	MW168666	MW239097	MW272483

[1] *P. gigantea* isolates from eastern North America, came from Dr. Sarah Covert's Lab collection (State: AL = Alabama; GA = Georgia; NC = North Carolina; SC = South Carolina). [2] *P. gigantea* isolates from western North America (State: CA = California), legit. M. Garbelotto and P. Gonthier. [3] *P. gigantea* isolates from Europe (State: CZ = Czech Republic; IT = Italy, legit. P. Gonthier (Italian isolates), L. Jankovsky and P. Sedlák (Czechs isolates).

3. Results

None of the 10 resupinate fruit bodies collected in western US forests by collaborators and sent to U.C. Berkeley were identified as *Phlebiopsis gigantea*. Likewise, all of the 164 woody spore traps employed during the survey in California failed to yield any *P. gigantea* culture, while the vast majority of traps were overgrown by fungal contaminants. A total of 13 *Phlebiopsis gigantea* cultures were obtained from an equal number of resupinate fruit bodies collected in 8 out of 41 California locations. *P. gigantea* was found exclusively in montane mixed conifer forests of the Coast and Sierra Nevada mountain ranges, while it was not found in strictly coastal and in high-elevation inland sites. We produced 174 new

sequences from 26 *Phlebiopsis gigantea* isolates from the West and the East coast of the US and from Europe. The isolate provenance, collectors and GenBank accessions numbers of these sequences are reported in Table 4.

3.1. Results of the Analysis for Each Locus

3.1.1. Mitochondrial Gene *atp6* Encoding the Sixth Subunit of ATP Synthase

The twenty-six newly generated *atp*6 sequences (609 bp each) did not show any differences among them or when compared to a *P. gigantea* sequence available in GenBank (KF147751). The dataset used included 30 sequences, twenty-seven of *P. gigantea* and three of outgroup taxa (*Caudicicola gracilis, Phanerochaete sordida* and *Physisporinus vitreus*). The aligment included 609 positions, and all sequences of *P. gigantea* in the ML analysis formed a well-supported clade (MLB = 100) without any discernable subclades (Figure 2).

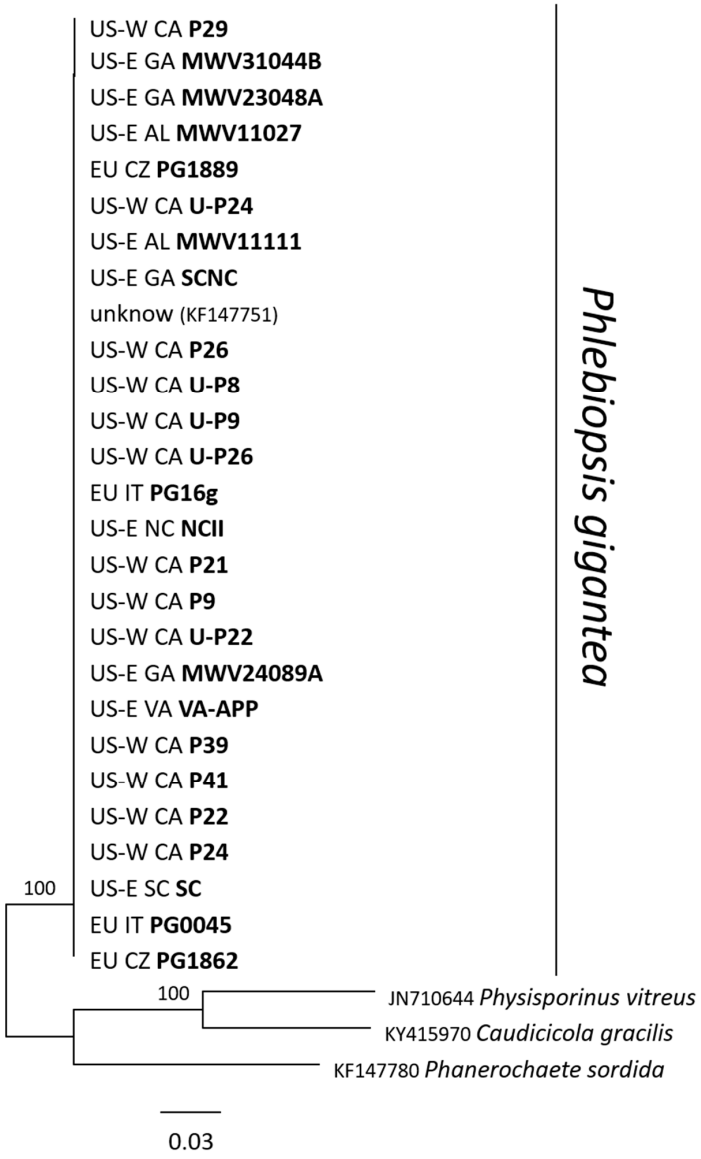

Figure 2. Maximum likelihood phylogram obtained from the *atp*6 sequence alignment of *Phlebiopsis gigantea*. *Caudicicola gracilis, Phanerochaete sordida* and *Physisporinus vitreus* were used as outgroup taxa. ML bootstrap percentages ≥70% are given above clade branches. Labels indicate geographic area, state, isolate code (in bold for sequences newly generated) and GenBank code brackets for sequences retrieved from NCBI (National Center for Biotechnology Information).

3.1.2. Partial Mitochondrial Large Subunit rRNA, ML5-ML6

The newly generated 26 sequences were 355 bp in length and did not show any differences among them or when compared to GenBank *P. gigantea* sequence AF518718. Conversely, the GenBank *P. gigantea* sequence MN473235 from Colorado was characterized by two single nucleotide deletions. All 28 *P. gigantea* ML5-ML6 sequences were devoid of any insertion, and an ML analysis clustered all of them together in a strongly supported clade (MLB = 100) without any subclades (Figure 3).

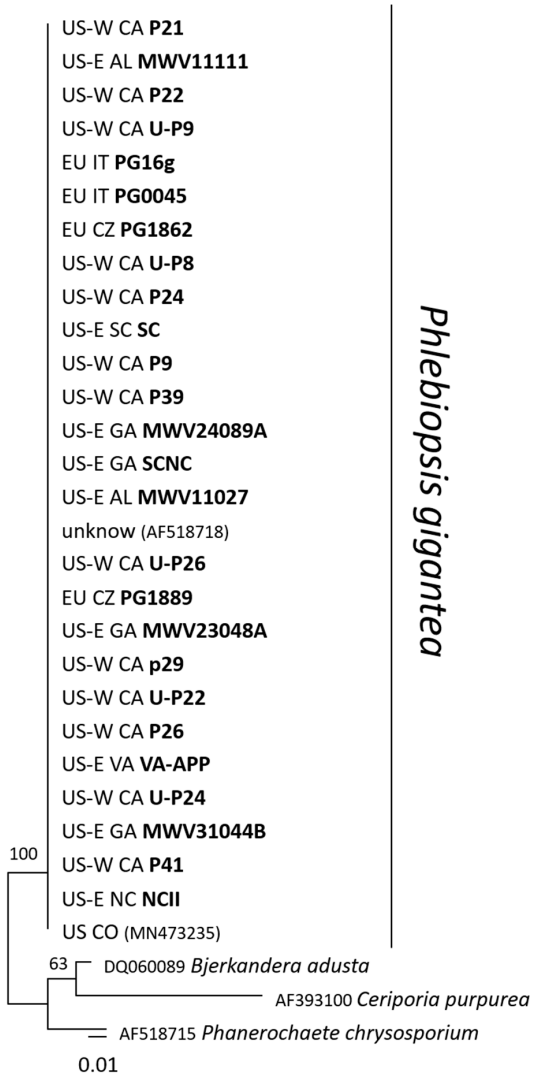

Figure 3. Maximum likelihood phylogram obtained from the partial large mitochondrial rRNA subunit (region between **ML5 and ML6**) sequence alignment of *Phlebiopsis gigantea* genotypes. *Bjerkandera adusta, Ceriporia purpurea* and *Phanerochaete chrysosporium* were used as outgroup taxa. ML bootstrap percentages ≥70% are given above clade branches. Labels indicate: geographic area, state, isolate code (in bold for sequences newly generated) and GenBank code brackets for sequences retrieved from NCBI.

3.1.3. Internal Transcribed Spacer (nrITS)

Twenty-five new nrITS sequences were generated in this study, and pairwise distances between them ranged from 0.00% to 1.74% (average distance = 0.19%). In the ITS1 region, two sequences (isolates PG16g from Italy and SC from South Carolina) had one five bps (ATTTA) insertion. The nrITS data matrix included 58 sequences of *P. gigantea*, 25 from this study and 28 retrieved from GenBank, and comprised 596 characters. In the ML analysis, all sequences of *P. gigantea* formed a well-supported (MLB = 85) monophyletic

clade characterized by the presence of two distal subclades, defined here as Clade A and B. Clade A included four isolates from the US West Coast (P-9, U-P9, U-P22 and P41) (MLB = 76), while Clade B included three western US isolates (P21, P24 and PU-24) (MLB = 80) (Figure 4). All other California isolates fell within the main basal *P. gigantea* clade. The sequences belonging to Clade A and Clade B differed from each other only for one SNP. In our dataset, ten sequences from Europe and the East US Coast showed an ATTTA insertion in the ITS1 region, while one sequence from Sweden presented one insertion of two nucleotides (AA) in the same position. The ML analysis was not able to segregate the samples on the basis of geographic origin.

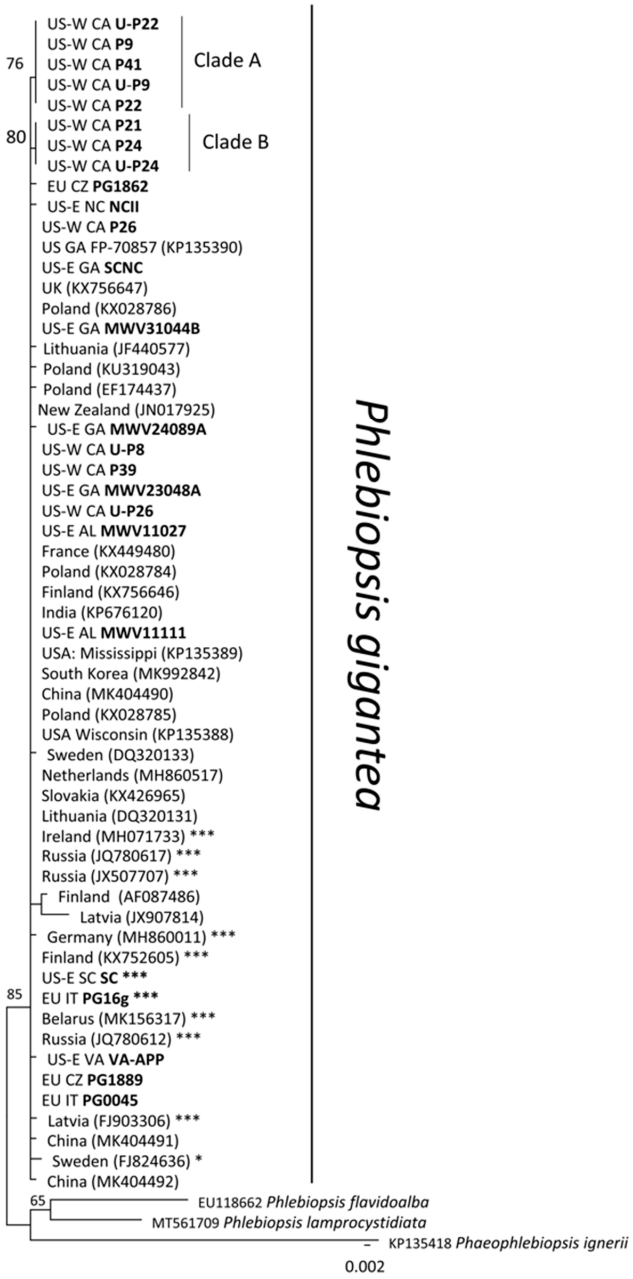

Figure 4. Maximum-likelihood phylogram obtained from the ITS sequence alignment of *Phlebiopsis gigantea* sequences. *Phlebiopsis flavidoalba*, *Phlebiopsis lamprocystidiata* and *Phaeophlebiopsis ignerii* were used as outgroup taxon. ML bootstrap percentages ≥70% are given above clade branches. Labels indicate: geographic area, state, isolate code (in bold for sequences newly generated) and GenBank code brackets for sequences retrieved from NCBI. * insertion of -AA- in ITS1 region. ***: insertion of -ATTTA- in ITS1 region.

3.1.4. RNA Polymerase II Subunit RPB1

Twenty-three new *RPB1* sequences were generated in this study and the pairwise distances among them ranged from 0.00% to 0.46% (average distance = 0.15%). The Rpb1 alignment consisted of 1158 bps and included twenty-six *P. gigantea* sequences. *Phlebiopsis* sp., *Phlebiopsis crassa and Rhizochaete radicata* were used as outgroup taxa. All *P. gigantea* sequences clustered in a monophyletic clade comprising a major basal clade and two distal subclades, defined as Clade A and Clade B. Six sequences from the US East coast were grouped in Clade A (MLB = 100) and all four sequences from Europe were grouped in Clade B (MLB = 100) (Figure 5).

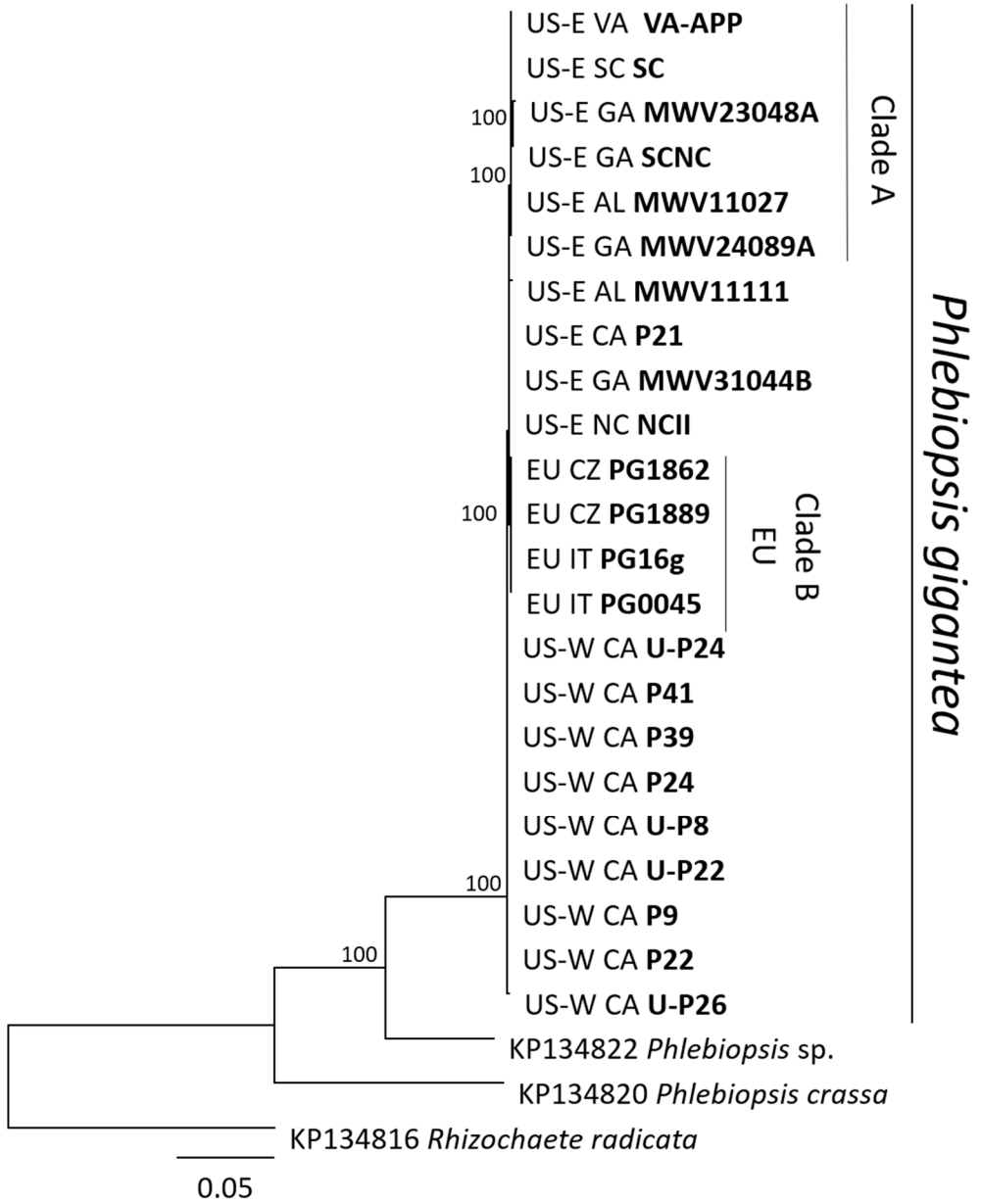

Figure 5. Maximum likelihood phylogram obtained from the *RPB1* sequence alignment of *Phlebiopsis gigantea*. *Phlebiopsis* sp., *Phlebiopsis crassa* and *Rhizochaete radicata* were used as outgroup taxa. ML bootstrap percentages ≥70% are given above each clade. Labels indicate: geographic area, state, isolate code (in bold for sequences newly generated) and GenBank code brackets for sequences retrieved from NCBI.

3.1.5. RNA Polymerase II Subunit RPB2

Twenty-four new *RPB2* sequences were generated in this study and the pairwise distances among them ranged from 0.00% to 0.91% (average distance = 0.19%). The *RPB2* sequences alignment consisted of 884 sites and included thirty sequences. *Phlebia* sp., *Scopuloides hydnoides* (Cooke & Massee) Hjortstam & Ryvarden and *Trametes elegans* (Spreng.) Fr. were used as outgroup taxa. Six sequences from the US East coast were grouped together in Clade A (MLB = 70) while all seven sequences from Europe, of which four newly generated in this study and three retrieved from GenBank, grouped together in Clade B (MLB = 70) (Figure 6).

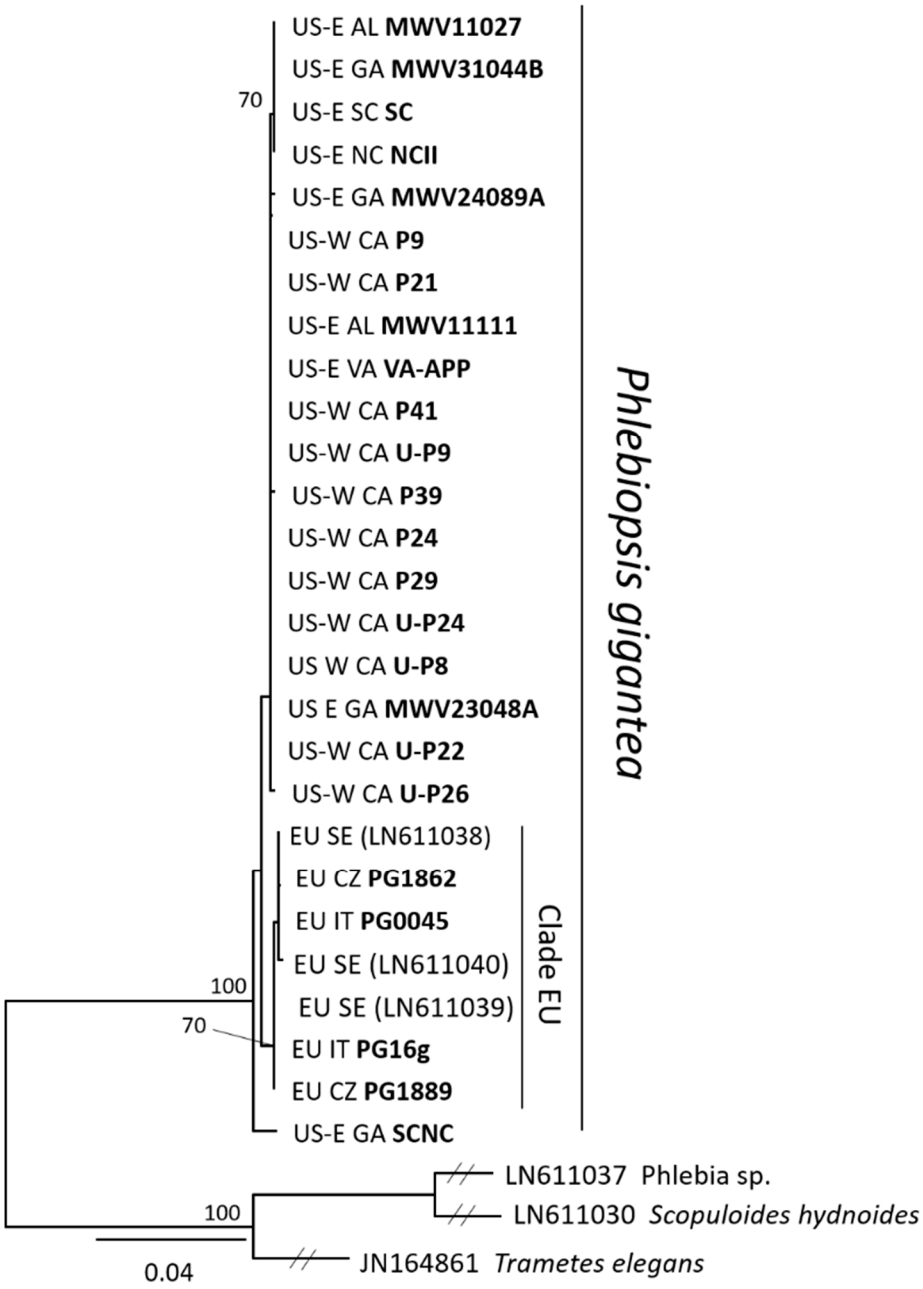

Figure 6. Maximum likelihood phylogram obtained from the *RPB2* sequence alignment of *Phlebiopsis gigantea*. *Phlebia* sp., *Scopuloides hydnoides* and *Trametes elegans* were used as outgroup taxa. ML bootstrap percentages ≥70% are given above each clade. Labels indicate: geographic area, state, isolate code (in bold for sequences newly generated) and GenBank code brackets for sequences retrieved from NCBI.

3.1.6. Translation Elongation Factor 1-alpha (*TEF1-α*)

Twenty-five new *TEF1-α* sequences were generated in this study, and the pairwise distances among them ranged from 0.00% to 2.83% (average distance = 0.82%). The *TEF1-α* alignment consisted of 797 sites and included 29 *P. gigantea* sequences. Sequences of *Phanerochaete chrysosporium* Burds. (AY885155) and *Phanerina mellea* (Berk. & Broome) Miettinen (LC387382) were used as outgroup taxa. Twenty-two sequences from the US and a single UK sequence were grouped in Clade A (MLB = 99%), and five sequences from Europe were grouped in Clade B (MLB = 84%) (Figure 7). In Clade A, a subclade A1 included two sequences from the US East coast (SCNC and MWV24089A) and one from the UK (GenBank: KU886025). We note, though, that sequence KU886024 from Poland fell in the Clade B, and the same was observed for all other sequences from the UK, that, although unavailable in GenBank, are reported in Wit et al. [76], questioning the actual phylogenetic positioning or validity of the UK sequence KU886025. The phylogenetic analysis of the *TEF1-α* region identified two main genetic intraspecific A and B lineages, with average within-group distances of 0.02% and 0.11%, respectively. The average distance between the two clade instead was 2.42%. *TEF1-α* Clade A included all American genotypes and the single questionable sequence from one UK isolate. *TEF1-α* Clade B instead was limited only to Europe. The topology of the *TEF1-α* tree was identical when analyzing intronic and exonic portions of the locus separately.

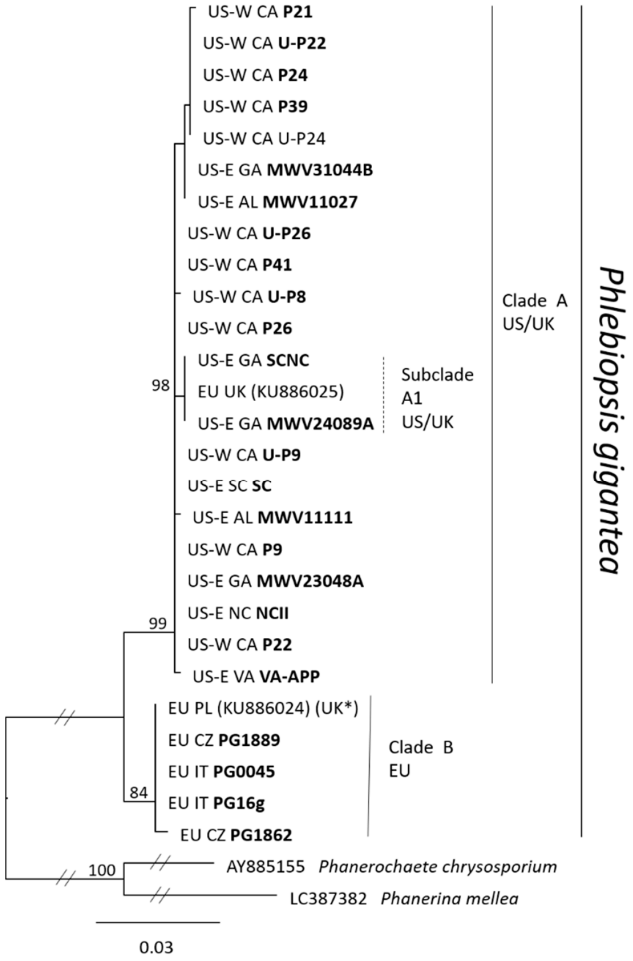

Figure 7. Maximum likelihood phylogram obtained from the *TEF1-α* sequence alignment of *Phlebiopsis gigantea*. *Phanerochaete chrysosporium* and *Phanerina mellea* were used as outgroup taxa. ML bootstrap percentages ≥70% are given above each clade. Labels indicate: geographic area, state, isolate code (in bold for sequences newly generated) and GenBank code brackets for sequences retrieved from NCBI.

3.1.7. Glyceraldehyde-3-Phosphate Dehydrogenase (*GPD*)

Twenty-five new partial *GPD* sequences (all sequences included the IV, V and VI introns) were generated in this study; the pairwise distances among them ranged from 0.00% to 5.93% (average distance = 2.47%). The *GPD* alignment consisted of 962 sites and included 32 sequences in total. Twenty-nine were Pg sequences, 25 from this study and four retrieved from GenBank (without IV and V introns), while *Phlebia* sp. (LN611076), *Phanerochaete chrysosporium* (AB272086) and *Cryptococcus amylolentus* (Van der Walt, D.B. Scott & Klift) Golubev (XM019141641) were used as outgroup taxa. In the ML analysis, 19 sequences from the US and Europe were grouped in Clade A (MLB = 100%), and within it, 12 sequences from California (all from the West Coast) formed a well-supported clade A1 (MLB = 86%) (Figure 8). Ten sequences from Europe and East Coast formed a well-supported Clade B (MLB = 89%), and within it, four sequences from Europe formed an independent sub-clade (MLB = 87%). In Clade B, five sequences from the US East Coast had a deletion of seven nucleotides (-TATGCCT-) in the V intron. The average distance between *GPD* clades A and B was 4.96%.

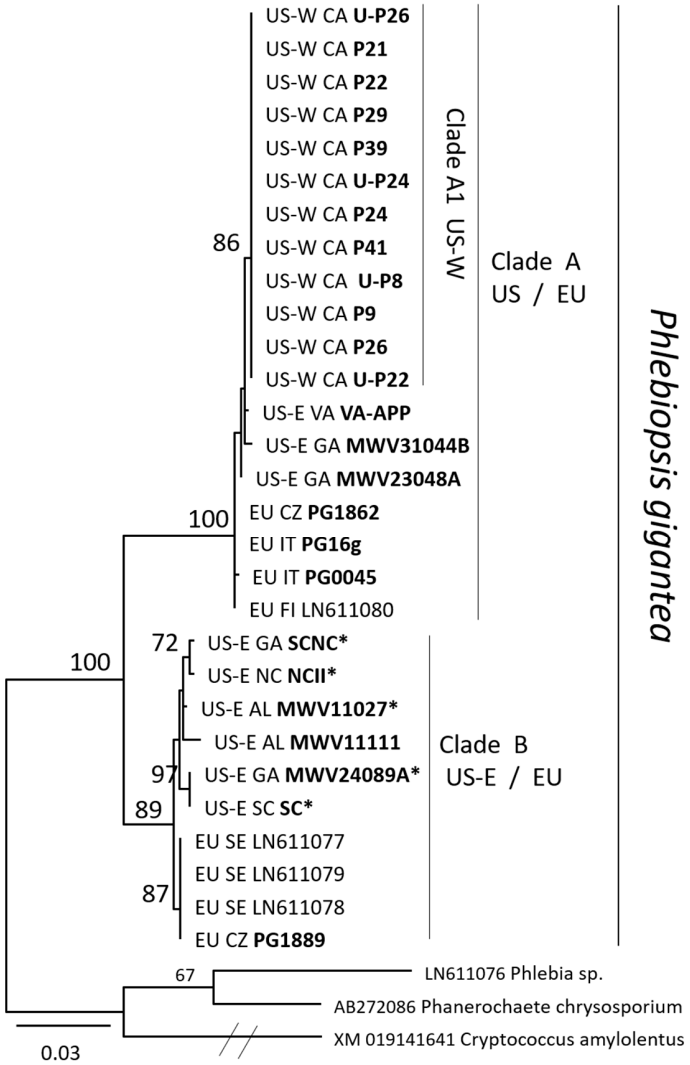

Figure 8. Maximum likelihood phylogram obtained from the *GPD* sequence alignment of *Phlebiopsis gigantea*. *Phlebia* sp. (LN611076), *Phanerochaete chrysosporium* (AB272086) and *Cryptococcus amylolentus* (XM019141641) were used as outgroup taxa. ML bootstrap percentages ≥70% are given above each clade. Labels indicate: geographic area, state, isolate code (in bold for sequences newly generated) and GenBank code brackets for sequences retrieved from NCBI. * presence of deletion (-TATGCCT-) in the V intron.

Independent analyses of IV, V and VI introns and of the exon of *GPD* identified significant incongruencies in the results, mostly regarding the relationship among isolates from the three major geographic regions studied here. In the two separate analyses of the IV and V introns (Figure 9A,B), eastern US and EU isolates fell in the same monophyletic clade and were more closely related to each other than to western US isolates. In the VI intron and exon analyses (Figure 9C,D); instead, one clade included eastern US, western US and EU isolates, while another included only eastern US and EU isolates. It is interesting that, although lacking statistical support, all western US isolates fell in a separate subclade in the analysis of the exonic sequence. It is also noteworthy that, in spite of the incongruencies, all western US isolates always fell in the same monophyletic clade. We also note that all clades were supported by an MLB = 100%.

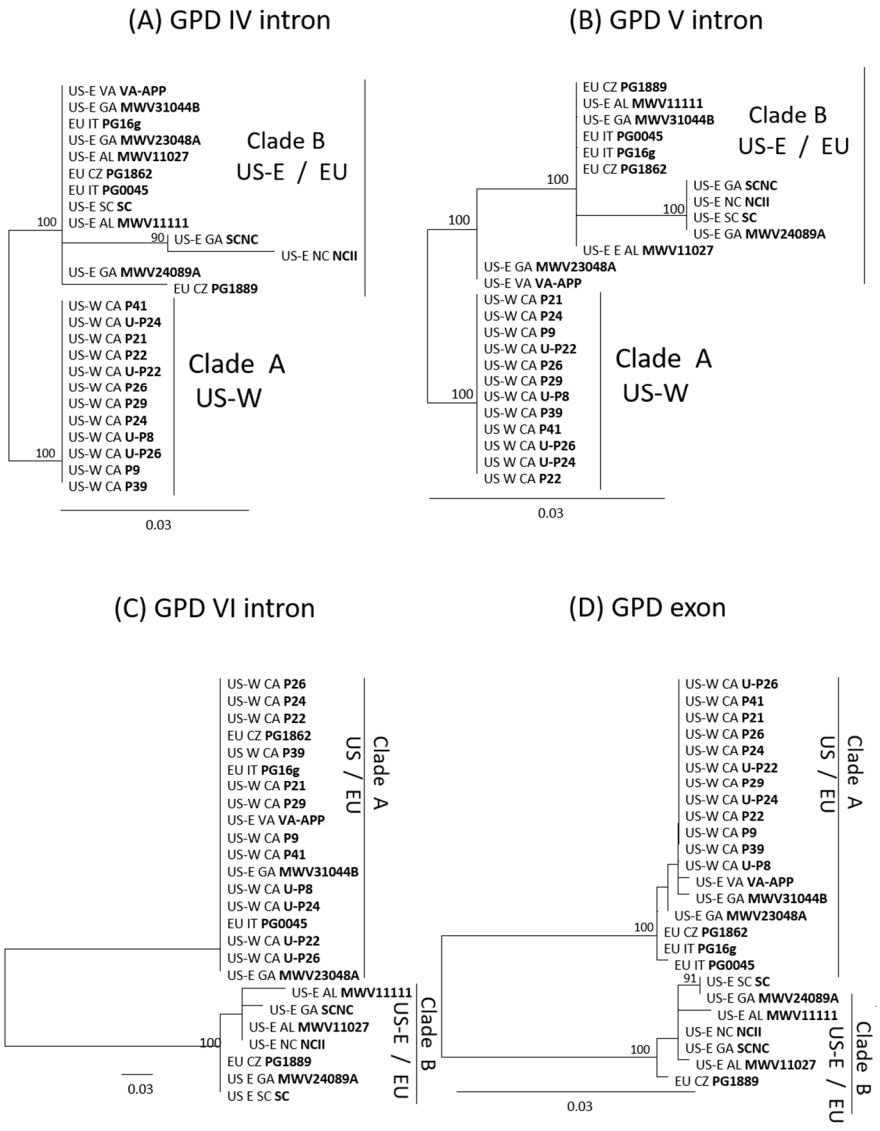

Figure 9. Maximum likelihood phylogram obtained from (**A**) IV intron of *GPD*; (**B**) V intron of *GPD*; (**C**) VI intron of *GPD*; (**D**) partial exon of GPD. ML bootstrap percentages ≥70% are given above each clade. Labels indicate: geographic area, state, isolate code.

3.1.8. Inference of Combined *TEF1-α*, nrITS, *RPB1*, *RPB2* and *GPD* (Partial Exon and IV, V and VI Introns Included)

In the *TEF1-α*, nrITS, *RPB1*, *RPB2* and *GPD* combined analysis, all western US sequences, three from the eastern US and three from Europe, grouped in Clade A. Within

Clade A, all 12 sequences from the western US formed a distinct clade (MLB = 69%, Clade A1 US-W in Figure 10), while three from Europe formed a well-supported clade (MLB = 96%, Clade A2 EU in Figure 10). Six sequences from the eastern US grouped in Clade "B1 US-east" and PG1889 from Europe is the basal terminal taxon of *P. gigantea*.

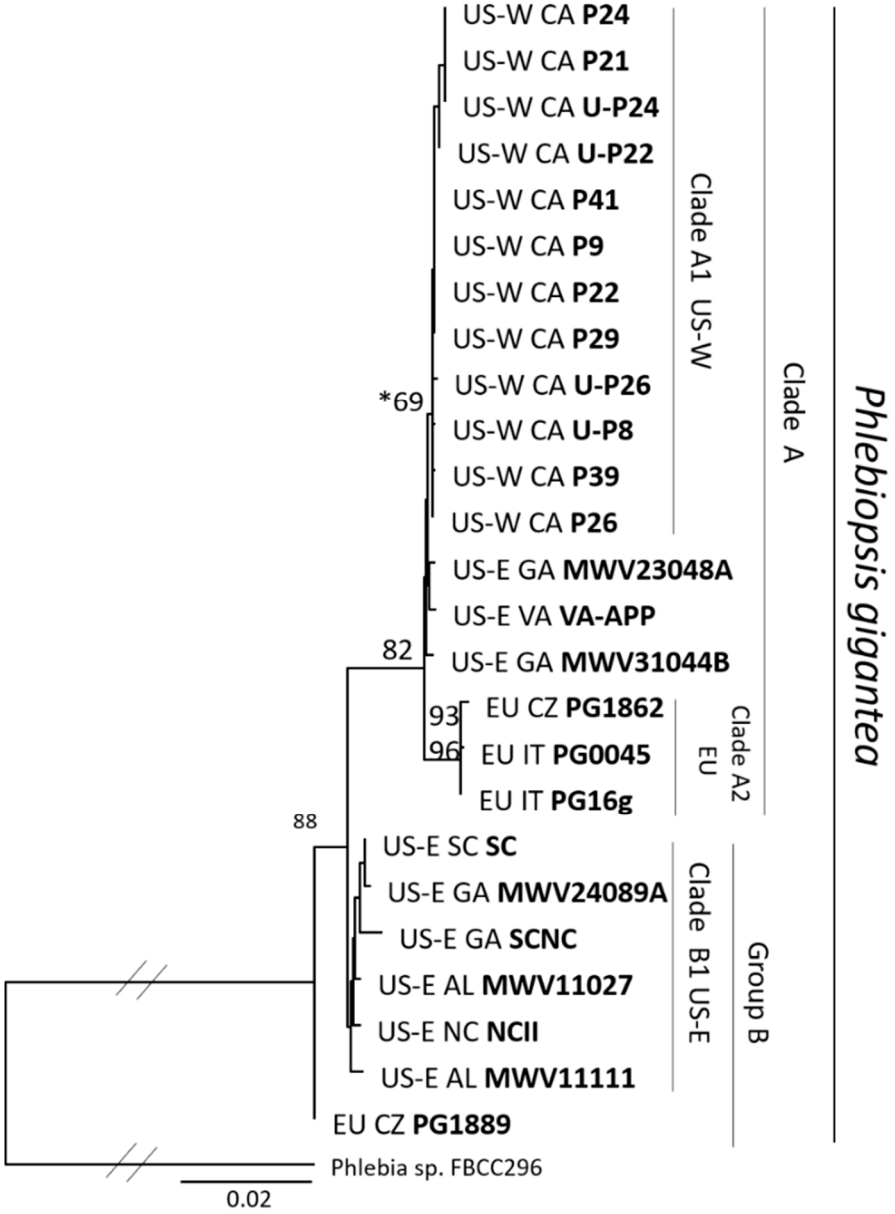

Figure 10. Maximum Likelihood phylogram obtained from the *TEF1-α*, nrITS, *RPB1*, *RPB2* and *GPD* combined sequence alignment. *Phlebia* sp. *(FBCC296)* was used as outgroup taxon. Only MLB values ≥69% are given above clade branches.

3.1.9. Phylogenetic Inference of Combined *TEF1-α*, nrITS, *RPB1*, *RPB2* and *GPD* (Only IV and V Introns Included)

In *TEF1-α*, nrITS, *RPB1*, *RPB2* and *GPD* (exon and VI intron excluded) combined analysis, all isolates from US West coast form a supported clade (MLB = 77%, Figure 11), and all isolates from Europe grouped in well-supported Clade B.

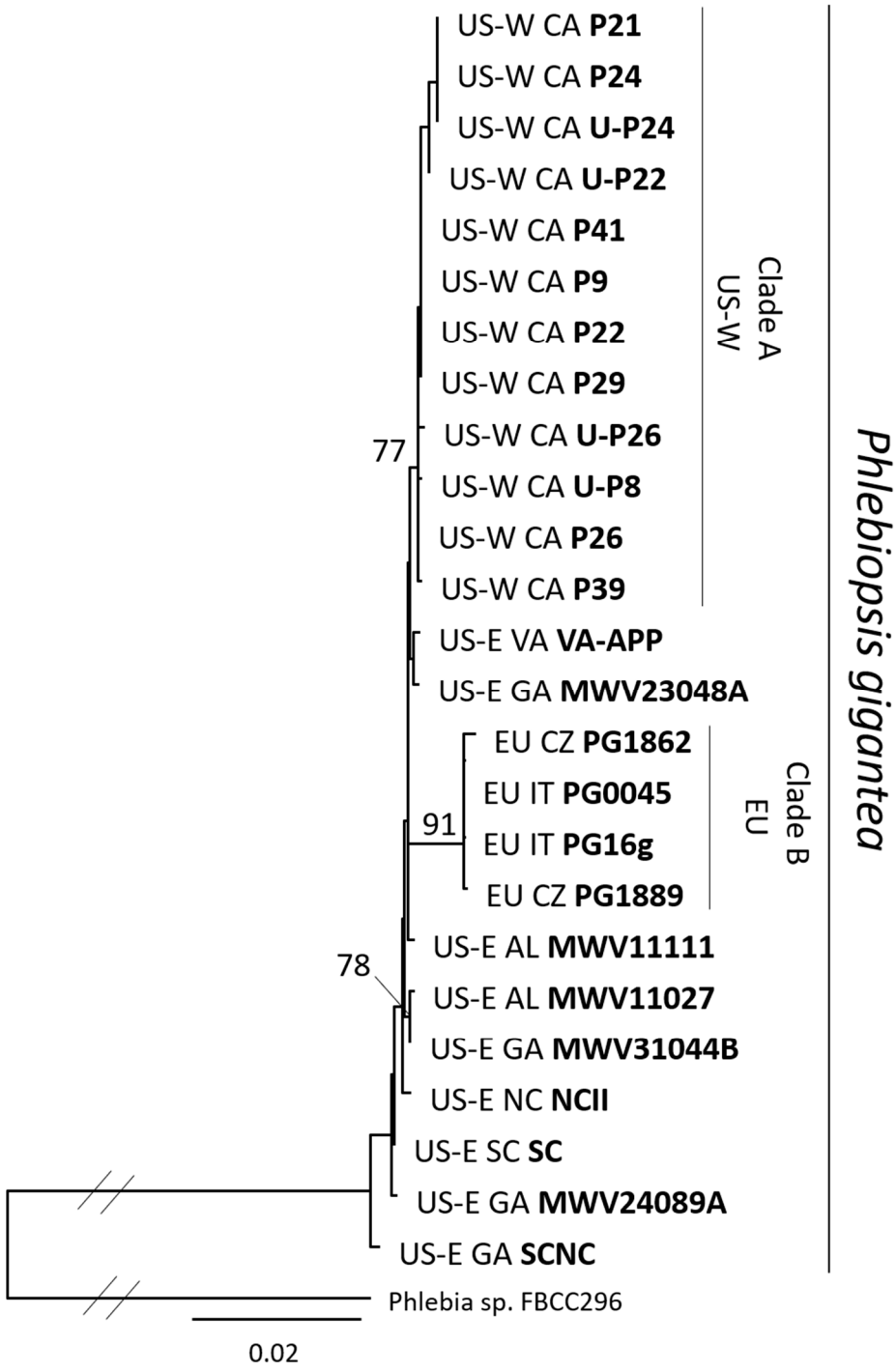

Figure 11. Maximum Likelihood phylogram obtained from the *TEF1-α*, nrITS, *RPB1*, *RPB2* and *GPD* (IV and V introns only) combined sequence alignment. *Phlebia* sp. (FBCC296) was used as outgroup taxon. Only MLB values ≥70% are given above clade branches.

4. Discussion

The main aim of this study was to investigate whether deep genetic structuring could be identified in the generalist saprobic wood-colonizing fungal species *Phlebiopsis gigantea* when comparing isolates from conifer forests in different world regions, specifically from western Europe, eastern North America and western North America. While regional genetic structure has been identified in many fungal species displaying some degree of host-specificity [77,78], much fewer cases have been presented analyzing generalist fungi. The presence of a phylogeographic signal and of genetically distinct groups of this fungus

in different world regions would provide a significant contribution to the understanding of the processes that have led to regional differences in biodiversity and microbial community composition. However, and furthermore, *P. gigantea* has also been long used as a biocontrol of Heterobasidion root disease in northern Europe [48,51], and a product based on an eastern US isolate of the fungus has been recently made commercially available in the US for the control of tree stump infection by the forest pathogen *Heterobasidion irregulare*. Very little information was available on the presence of *P. gigantea* in western US conifer forests with species identification based on both morpohology and DNA sequence data. Assessing its presence, investigating some of its host and environmental requirements, and determining whether western North American genotypes may be undistinguishable from eastern North American genotypes are all questions that should be answered before utilizing the commercially available product in western North American forests. Twenty-six isolates of *P. gigantea* collected from conifers in eight states spanning from western North America to the Czech Republic in Europe were sequenced and analyzed using single- and multi-locus phylogenies. The 13 specimens collected specifically for this study by the authors represent the first records of *P. gigantea* from California or the western US to be identified with absolute confidence thanks to DNA sequence data and were isolated from logs of *Pinus ponderosa* Lawson & C. Lawson (11), *Pseudotsuga menziesii* (Mirb.) Franco (1) and *Quercus kelloggii* Newberry (1). This result underlines the ability of *P. gigantea* to colonize different tree species that belong to different families, both conifers and angiosperms. A search of the US National Fungus Collections Fungus-Host Database dated April 04, 2021 [79] showed that while most *P. gigantea* records are from conifers, at least two previous records from angiosperms exist. In California, the main substrate for *P. gigantea,* not surprisingly, was pine, and in particular Ponderosa pine, one of the most widespread pine species across the western US. *P. gigantea* basidiocarps were not found in the mild coastal mixed conifer forests surveyed in this study. Based on our field observations, we believe that the competition among wood decay fungi may be very strong in this region characterized by very wet and year-long mild climate. The vast majority of fruiting bodies observed during the survey were in fact produced by fungi that notoriously can colonize standing trees as endophytes. By the time these trees are felled or fail on their own, the wood appeared to be already significantly decayed; thus, niches of healthy wood available to an early saprobic wood colonizer as *P. gigantea* are rather limited. The survey in alpine high Sierra Nevada mixed conifer stands was also unsuccessful. The ecology and floristic composition of these sites are extremely different from those in coastal forests and are driven by extreme temperatures, relatively low precipitation in the form of rain and high levels of snow precipitation, resulting in distinctively drier ecosystems. Floristically, different varieties of *Pinus contorta* Douglas ex Loudon are found on the coast and in the high Sierra Nevada, but the main substrate on which *P. gigantea* was found (see below), i.e., *Pinus ponderosa,* is only present in the Sierra Nevada sites and not in the low-elevation truly coastal sites. It is interesting, though, that in spite of the presence of what we know now is a common host for this fungus, no *P. gigantea* basidiocarps were found on Ponderosa pine logs in high-elevation mixed conifer stands. We suggest this may be due to the dryer type of forest typical of the High Sierra Nevada. The two regions where *P. gigantea* fruiting bodies were found (Figure 1), i.e., Cobb Mountain (Coast Range) and the mid elevation Eldorado National Forest (Sierra Nevada), are geographically distant and ecologically disjunct, being separated by the hot and arid foothills of the coastal and Sierra Nevada mountain ranges and by the agricultural Sacramento valley. Nonetheless, they have significant ecological and floristic similarities. Both comprise montane mixed conifer forests, with a significant co-dominance of Ponderosa pine and abundant precipitation. Douglas-fir, tanoaks and black oaks are also present in both regions. Average temperatures are similar between the two and range between values close to zero and the upper twenties centigrade. We can confirm that all logs on which *P. gigantea* was fruiting had been cut in the previous 1–2 years and were only showing signs of incipient decay, without any significant physical advanced deterioration. Although our survey effort was too small

to draw final conclusions, and further considering that the presence of *P. gigantea* was determined only by the presence of visible fruiting bodies without any direct isolation from wood, we believe that some useful inferences can be made based on the results of this study. These inferences have relevance for the distribution of *P. gigantea* in the West as well as for its disease control efficacy and volunteer dispersal, if employed as a biocontrol agent against pathogens belonging to the *Heterobasidion* species complex [48,51]. First, *P. gigantea* not unlike what is reported for pathogenic *Heterobasidion* species, seems to be unfavored by extremely wet and mild conditions [80], possibly because of the species richness of wood-inhabiting fungal communities in areas characterized by this type of climate. Conversely, its presence in mesic montane forests on Ponderosa pine may suggest its use as a biocontrol may be promising on this host in these environments. These are areas known to have significant *Heterobasidion* root disease, and Ponderosa pine is one of the main hosts affected by the disease.

However, in western North America, the distribution of Heterobasidion root rot [48] and of Ponderosa pine [81] is much broader and includes drier sites like the High Sierra ones surveyed in this study and more inland western conifer stands. The presence of *P. gigantea* may be naturally limited in these drier and/or warmer sites, and its efficacy in these conditions, if any, will need to be evaluated carefully. In fact, it has been reported that warmer temperatures are unfavourable to the establishment of *P. gigantea* in stumps [82]. We should also consider whether it may be appropriate to introduce a microbial control agent in areas where its natural presence may be marginal [83], questioning again its use in drier western pine stands if its rarity in these areas were to be confirmed by further studies. Finally, the fact that in mesic California forest environments, *P. gigantea* was found on logs of three different host species, including an angiosperm, indicates that the fungus has the potential to spread in mesic natural ecosystems way beyond the pine hosts on which it would be mostly employed to prevent infection by *Heterobasidion*. This generalism is a further reason to exercise caution in the use of *P. gigantea* as a biocontrol [84]: the use of exotic isolates, in fact, could easily result in their spread and in the possible displacement of native less fit isolates [85], with unpredictable ecological and evolutionary consequences [61,86].

Multilocus analysis revealed that levels of genetic variation and taxonomic resolutions were different when analyzing each of the seven genetic loci considered in this study. The mitochondrial ML5 and ML6 rDNA and the *ATP6* locus did not show variability within the species. Being strongly conserved, they may be used as a species-specific diagnostic marker to facilitate the identification of this notoriously difficult to identify species, especially in California and other western North American regions where it has been little studied [87,88]. The nrITS region showed some moderate intraspecific variability but without any clear association with the geographic origin of the genotypes. In 2000, a study conducted by Vainio and Hantula [89] pointed out a "considerable" level of intraspecific variation in both nrITS and random amplified microsatellite markers (RAMS), highlighting a clear differentiation between the European and North American populations. Our nrITS maximum likelihood analysis as well as the same analysis by Vainio et al. [90] were not able to separate the samples on a geographic basis but confirmed the presence of genetic polymorphisms. *RPB1*, *RPB2* and *TEF1-α* maximum-likelihood analyses (maximum distances between sequences up to 0.46%, 0.91% and 2.83%, respectively) supported the difference between European and American samples as previously reported [89], but could not differentiate between samples from western North America and those from eastern North America. The placement of a sequence of a single UK isolate with North American isolates in the *TEF1-α* tree may be either an artifact or the result of a recent introduction of a US genotype in the UK. It should be noted that sequences from other UK isolates used in the same study clustered as expected within the European clade.

High intraspecific genetic variability was detected in the *GPD* locus (distances among sequences up to 5.93%); hence it is no surprise that this locus provided the greatest resolution both by itself and when combined with the other loci. GPD and ML analyses of

all loci combined clearly separated western US from eastern US and European genotypes but also identified two subspecific groups. The first included European, eastern US and western US genotypes, while the second included only eastern US and European genotypes. Combined, these analyses suggest: (a) the presence of retained ancestral polymorphisms responsible for the structuring of the species in two subspecific genetic groups; (b) occasional interbreeding resulting in incongruencies in the assignment of genotypes to each group when using different loci and likely to prevent the formation of intersterility groups [50,90]; (c) western and eastern US genotypes are more related to each other than to European genotypes, suggesting a shared more recent ancestry; (d) western and eastern US genotypes are different; (e) both subspecific groups are present in eastern US and in Europe, while only one group is present in California, although more sampling in the West needs to be done to confirm this at the western North American scale; (f) western US genotypes are derived from eastern US genotypes and European genotypes are more closely related to eastern US than to western US genotypes: this pattern suggest an older Atlantic migration pathway of this fungus in between continents, however, whether *P. gigantea* may have originated first in Europe vs. eastern North America cannot be resolved in the current study.

Many of the results match the results reported by Linzer et al. [30] for the ecologically similar *Heterobasidion irregulare*. Other studies using anonymous genetic markers or SSRs have identified the presence of genetic differences between eastern North American and European *P. gigantea* genotypes, and the lack of such differences within Europe [89–91]. Results from these studies are consistent with significant regional-level migration of this organism accompanied by the presence of a large genetic pool minimizing drift-associated evolutionary processes. Our approach using sequence-based multi-locus phylogenies was aimed at identifying evolutionary-level divergence among metapopulations stronger than the presence of population-level genetic structuring detectable using highly polymorphic anonymous or SSR markers [90–92]. While it could be argued that the genetic differentiation between eastern and western North American populations is not strong on an evolutionary scale, such a difference is likely to be much stronger than the structuring identified by other studies based on other genetic markers mentioned above. Likewise, while a stronger genetic divergence has been identified among host-associated ectomycorrhizal fungi, with species in eastern North America being related to but distinct from sister western North American species [42,44,45], a pattern of subspecific genetic structuring comparable to the one here identified for *P. gigantea* has emerged for the wood-inhabiting fungal pathogen *H. irregulare* [30]. As for *P. gigantea*, limited mitochondrial sequence variation in *H. irregulare* is in contrast with moderate variability and continental divergence in exonic nuclear sequences and high coast-to-coast divergence in sequences of DNA insertions or introns [30]. Recent research has identified nuclear-mitochondrial communication as an essential function for wood-inhabiting fungi, in part explaining the high conservation of the mitochondrial code and of nuclear genes involved in nucleus-mitochondrion communication [93,94]. In *P. gigantea*, the presence of two interbreeding but genetically distinct subspecific groups may be the results of continental-level repopulations from different refugia, as suggested for the white truffle *Tuber borchii* [95]. On the other hand, as suggested for the ecologically similar wood-inhabiting fungus *H. irregulare*, a relatively recent post-glacial connectivity between eastern and western North America through Central Mexico may explain the low phylogenetic divergence between populations of *P. gigantea* from the two different sides of the North American continent [30].

We are aware this study only addresses sequence variation without addressing variation in genic expression, which ultimately is responsible for phenotypic variation. Nonetheless, we believe the identification of intraspecific genetic variation in genotypes from different world regions is a first step necessary and sufficient to advise against the interregional movement of genotypes for the following reasons. First, increasing sequence variation in any given world region may favor the evolution of novel alleles, even if the variation imported from a different region is not immediately associated with phenotypic variation. Second, even if sequence variation in genotypes from a world region is synony-

mous (i.e., different alleles code for the same proteins) to sequence variation extant in a different region, that sequence variation may be associated with differences in expression of that same protein due to protein folding constraints [96], with obvious immediate effects on the fitness of individual genotypes. Third and lastly, any sequence variation resulting in the expression of novel gene products may have an immediate effect on genotypic fitness. Because all three scenarios above lead to phenotypic changes, we believe the interregional movement of genetically distinct genotypes should not be facilitated by humans.

5. Conclusions

In this study, we confirm for the first time the presence of *P. gigantea* in western North America using DNA data. These western isolates of *P. gigantea* are distinguishable from eastern US isolates using a phylogenetic approach. In this study, we have further confirmed this conifer wood-colonizing fungus is a generalist with a preference for hosts in the genus *Pinus*. The presence of genetic differentiation between eastern and western North American *P. gigantea* isolates indicates that even wood saprobic generalist fungi are characterized by a phylogeographic signal that, in most likelihood, matches the signal and history of the mixed conifer forests in which they are found. This phenomenon could be defined as a coevolutionary process between a microbe and a type of habitat, e.g., mixed conifer forests, rather than a specific host. Furthermore, it is commonly understood that the introduction of exotic organisms, including fungi, may have undesirable outcomes on the integrity of natural or even artificial ecosystems. Here, we surmise that the introduction of exotic isolates from genetically differentiated subgroups of a species may be equally deleterious. Exotic isolates, in fact, may outcompete and replace native isolates by having greater growth and fruiting rates. Additionally, they may disproportionately use local resources, or they may accelerate the evolution of native populations by exchanging alleles through hybridization-mediated interspecific genic introgression. We as others before us believe that these and other concerns apply to the introduction of exotic fungal biocontrol agents as well [91,97,98]. A further and unique complication of this particular biocontrol agent is its lack of host specificity [84]. In fact, although normally found in conifer-dominated forests, the ability of *P. gigantea* to grow on a broad range of woody substrates, as further confirmed by this study, would make its management difficult once it is applied in a forest setting.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/f12060751/s1, Table S1: GenBank Accession numbers used in this study with species and geographic provenance.

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