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, 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 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 presence 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
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 [65]. 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® 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 | −123.805957 | Douglas-fir log      | 1041                         | 5 to 22                  | 20        |
| P7  | No             | Cobb Mt. Lake, Coast Range     |                      | 38.811006 | −122.712369 | Ponderosa pine log   | 965                          | −2 to 29                 | 816       |
| P8/U-P8 | Yes         | Cobb Mt. Lake, Coast Range     |                      | 38.811006 | −122.712369 | Ponderosa pine log   | 965                          | −2 to 29                 | 818       |
| P9/U-P9 | Yes         | Cobb Mt. Lake, Coast Range     |                      | 38.811006 | −122.712369 | Ponderosa pine log   | 965                          | −2 to 29                 | 818       |
| P10 | No             | Cobb Mt. Lake, Coast Range     |                      | 38.811118 | −122.713345 | Ponderosa pine log   | 965                          | −2 to 29                 | 826       |
| P21 | Yes            | Cobb Mt. Lake, Coast Range     |                      | 38.809389 | −122.711941 | Ponderosa pine log   | 965                          | −2 to 29                 | 820       |
| P22/U-P22 | Yes        | Cobb Mt. Lake, Coast Range     |                      | 38.819652 | −122.712103 | Ponderosa pine log   | 965                          | −2 to 29                 | 821       |
| P24/U-P24 | Yes        | Cobb Mt. Lake, Coast Range     |                      | 38.819652 | −122.712109 | Ponderosa pine log   | 965                          | −2 to 29                 | 820       |
| P26/U-P26 | Yes        | Cobb Mt. 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      |
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.

| 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      | 38.912835 | −120.665881 | Douglas-fir log    | 1651                        | 0 to 27                 | 1337      |
| P41 | Yes  | Blodgett Forest | Eldorado, Sierra Nevada      | 38.912838 | −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      |
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.

| Name   | Nucleotide Sequence (5’–3’) | Reference | Region                                      |
|--------|----------------------------|-----------|---------------------------------------------|
| GDP-14f| GTATCGCTCTCGTGAATGCTCTCCT  | This study | glyceraldehyde-3-phosphate dehydrogenase (GPD) |
| GDP-693r| GTCCTTCGTTTGGGAGACATCGAC  | This study | glyceraldehyde-3-phosphate dehydrogenase (GPD) |
| GDP-633f| TAC AAG GTC ATC TGC 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) |
| GDP1 | AGCCTCCTCCCAATTGAAARG | [30] | introns in the ML5–ML6 DNA region of the mitochondrial large ribosomal RNA (mt LrRNA) |
| GPDR | RTANC CCCTRTCRTRCTACCA | [30] | introns in the ML5–ML6 DNA region of the mitochondrial large ribosomal RNA (mt LrRNA) |
| ML5  | CTGGCAAAATATTCCTCAAG     | [68] |                                       |
| ML6  | CAGTATAAACGTGCTATGGTC    | [68] |                                       |
| ATP6-34f| GGGTTAAAATGCTGCCCAATTGGT  | This study | ATPase subunit 6 (atp6)                   |
| ATP6-693r| TGGAGAAAACTGCTGGTATTAGTA | This study | ATPase subunit 6 (atp6)                   |
| EF625f| GGAGCCCTCTGAACAGAAATCG  | This study | translation elongation factor 1-alpha (tef-1α) |
| EF1427r| CTGGCCCTGATACAACTTAC    | This study | translation elongation factor 1-alpha (tef-1α) |
| EF983f| GCYCCYGGHCAYCRGTYTG     | [69] | RNA polymerase II subunit (rpB1)          |
| EF-2218R| AGTACACCRACRGRACRGTYYTG | [69] | RNA polymerase II subunit (rpB1)          |
| RPB1-29f| TGACTGTATGCTCTCCGT    | This study | RNA polymerase II subunit (rpB1)          |
| RPB1-1292r| TCAGCCCATTTGTACGTCAA   | This study | RNA polymerase II subunit (rpB2)          |
| PRB2-5f| TACCCTCAAAACTTCTCTTGACG | This study | RNA polymerase II subunit (rpB2)          |
| PRB2-957r| ATGTTCTCCAGACGCTTATAGTA| This study | RNA polymerase II subunit (rpB2)          |
| ITS1F| CTCTGCCATATTAGAGGATGAA | [70] | internal transcribed spacer (nrITS) of nuclear ribosomal DNA |
| ITS4 | TCCTCCGCTTATGTATGC      | [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 GPD2 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. FBCB296 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.
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 | ATP Primers | ITSCPrimers | MLSPrimers | EFPrimers | RPB1 | rpb2 | GDP |
|--------------|-------------|-------------|------------|-----------|------|------|-----|
| MW052838     | MW05455     | MW067609    | MW074132   | MW168678  | MW230909 | MW272459 |
| MW052837     | MW05456     | MW067610    | MW074136   | MW168677  | MW230907 | MW272460 |
| MW052841     | MW05457     | MW067611    | MW074133   | MW168682  | MW230909 | MW272461 |
| MW052840     | MW05458     | MW067612    | MW074134   | MW168681  | MW230908 | MW272462 |
| MW052839     | MW05454     | MW067613    | MW074135   | MW168680  | MW230910 | MW272463 |
| MW052836     | MW05459     | MW067614    | MW074137   | MW168675  | MW230902 | MW272464 |
| MW052833     | MW05462     | MW067617    | MW074140   | MW168669  | no      | MW272466 |
| MW052832     | MW05463     | MW067618    | MW074141   | MW168668  | MW230905 | MW272468 |
| MW052831     | MW05464     | MW067619    | MW074142   | No        | no      | MW272469 |
| MW052830     | No          | MW067620    | no         | No        | MW230907 | MW272470 |
| MW052829     | MW05465     | MW067621    | MW074143   | MW168664  | MW230906 | MW272471 |
| MW052828     | MW05466     | MW067622    | MW074144   | MW168665  | MW230908 | MW272472 |
| MW052835     | MW05460     | MW067615    | MW074138   | MW168671  | MW230903 | MW272465 |
| MW052826     | MW05468     | MW067624    | MW074146   | MW168683  | MW230908 | MW272474 |
| MW052825     | MW05467     | MW067623    | MW074145   | MW168684  | MW230907 | MW272473 |
| MW052824     | MW05469     | MW067625    | MW074147   | MW168686  | MW230905 | MW272475 |
| MW052823     | MW05470     | MW067626    | MW074148   | MW168685  | MW230900 | MW272476 |
| MW052822     | MW05471     | MW067627    | MW074149   | MW168674  | MW230910 | MW272477 |
| MW052821     | MW05472     | MW067628    | MW074150   | MW168676  | MW230901 | MW272478 |
| MW052819     | MW05476     | MW067632    | MW074152   | MW168679  | MW230904 | MW272480 |
| MW052818     | MW05477     | MW067629    | MW074153   | MW168673  | MW230905 | MW272481 |
| MW052817     | MW05474     | MW067633    | MW074155   | MW168672  | MW230906 | MW272482 |
| MW052820     | MW05473     | MW067630    | MW074151   | MW168667  | MW230902 | MW272479 |
| MW052816     | MW05478     | MW067631    | MW074156   | MW168666  | MW230907 | 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 atp6 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 alignment 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 atp6 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](image-url)  
**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 \textit{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 \textit{Phlebiopsis gigantea} sequences. \textit{Phlebiopsis flavidoalba}, \textit{Phlebiopsis lamprocystidiata} and \textit{Phaeophlebiopsis igneri} were used as outgroup taxon. ML bootstrap percentages $\geq 70\%$ 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).

![Phlebiopsis gigantea phylogram](https://example.com/phylogram.png)

**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).

![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.](image)

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.](image-url)
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%.

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.
3.1.9. Phylogenetic Inference of Combined 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.

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 morphology 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 suggests 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 inter-regional 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.

Author Contributions: Conceptualization, M.G.; methodology, M.G., P.G. and F.D.; field work M.G. and P.G.; formal analysis, F.D.; data curation, F.D.; writing—original draft preparation, M.G. and F.D.; writing—review and editing, P.G.; funding acquisition, M.G. and P.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the USDA Forest Service, State and Private Forestry, Pacific Northwest and Alaska Regions, Agreement 18-CA-11062765-742 awarded to MG through the Forest Service Pesticide Impact Assessment Program and by the European Union’s Horizon 2020 research and innovation program under grant agreement No 634179 (EMPHASIS).

Data Availability Statement: All sequence data submitted to GenBank.

Acknowledgments: The collections in the Sierra Nevada were possible thanks to the help by Victor Marquez. The authors gratefully acknowledge Libor Jankovsky and Petr Sedlák at the Mendel University in Brno for providing *P. gigantea* isolates from the Czech Republic. Our gratitude also goes to Irene “Blakey” Lockman for supporting and managing the project. The authors are grateful to Doug Schmidt and Tina Popenuck for their invaluable technical support.

Conflicts of Interest: The authors declare no conflict of interest.
References

1. Hubbell, S.P. *The Unified Neutral Theory of Biodiversity and Biogeography*; Princeton University Press: Princeton, NJ, USA, 2001; pp. 1–448.

2. Baas-Becking, L.G.M. *Geobiologie van Inleiding Tot de Milieukunde*; W.P. Van Stockum & Zoon: The Hague, The Netherlands, 1934; pp. 1895–1963.

3. Green, J.; Bohannan, B.J.M. Spatial scaling of microbial biodiversity. *Trends Ecol. Evol.* 2006, 21, 501–507. [CrossRef]

4. Garnica, S.; Riess, K.; Schön, M.E.; Oberwinkler, F.; Setaro, S.D. Divergence Times and Phylogenetic Patterns of Sebacinales, a Highly Diverse and Widespread Fungal Lineage. *PLoS ONE* 2016, 11, e0149531. [CrossRef] [PubMed]

5. Sato, H.; Tsujino, R.; Kurita, K.; Yokoyama, K.; Agata, K. Modelling the global distribution of fungal species: New insights into microbial cosmopolitanism. *Mol. Ecol.* 2012, 21, 5599–5612. [CrossRef]

6. Smith, S.E.; Read, D.J. *Mycorrhizal Symbiosis*; Academic Press: Cambridge, UK, 2008; pp. 1–787.

7. Tedersoo, L.; May, T.W.; Smith, M.E. Ectomycorrhizal lifestyle in fungi: Global diversity, distribution, and evolution of phylogenetic lineages. *Mycorrhiza* 2010, 20, 217–263. [CrossRef] [PubMed]

8. Halling, R.E.; Osmundson, T.W.; Neves, M.-A. Pacific boletes: Implications for biogeographic relationships. *Mycol. Res.* 2008, 112, 437–447. [CrossRef] [PubMed]

9. Geml, J.; Tulloss, R.E.; Laursen, G.A.; Sazanova, N.A.; Taylor, D.L. Evidence for strong inter-and intracontinental phylogeographic structure in Amanita muscaria, a wind-dispersed ectomycorrhizal basidiomycete. *Mol. Phylogenet. Evol.* 2008, 48, 694–701. [CrossRef]

10. O’Donnell, K.; Rooney, A.P.; Mills, G.L.; Kuo, M.; Weber, N.S.; Rehner, S.A. Phylogeny and historical biogeography of true morels (Morchella) reveals an early Cretaceous origin and high continental endemic and provincialism in the Holarctic. *Fungal Genet. Biol.* 2011, 48, 252–265. [CrossRef]

11. Peay, K.G.; Garbelotto, M.; Bruns, T.D. Evidence of dispersal limitation in soil microorganisms: Isolation reduces species richness on mycorrhizal tree islands. *Ecology* 2010, 91, 3631–3640. [CrossRef]

12. Amend, A.; Garbelotto, M.; Fang, Z.; Keeley, S. Isolation by landscape in populations of a prized edible mushroom Tricholoma matsutake. *Conserv. Genet.* 2010, 11, 795–802. [CrossRef]

13. Bonito, G.M.; Gryganskyi, A.P.; Trappe, J.M.; Vilgalys, R. A global meta-analysis of Tuber ITS rDNA sequences: Species diversity, host associations and long-distance dispersal. *Mol. Ecol.* 2010, 19, 4994–5008. [CrossRef]

14. Tallbot, J.M.; Bruns, T.D.; Taylor, J.W.; Smith, D.P.; Branco, S.; Glassman, S.I.; Erlandson, S.; Vilgalys, R.; Liao, H.L.; Smith, M.E.; et al. Endemism and functional convergence across the North American soil mycobiome. *Proc. Natl. Acad. Sci. USA* 2014, 111, 6341–6346. [CrossRef]

15. Tedersoo, L.; Bahram, M.; Põõme, S.; Kõljalg, U.; Yorou, N.S.; Wijesundera, R.; Ruiz, L.V.; Vasco-Palacios, A.M.; Thu, P.Q.; Suija, A.; et al. Global diversity and geography of soil fungi. *Science* 2014, 346, 1256688. [CrossRef]

16. Hoeksema, J.D.; Averill, C.; Bhatnagar, J.M.; Brzostek, E.; Buscardo, E.; Chen, K.H.; Liao, H.L.; Nagy, L.; Policelli, N.; Ridgeway, J.; et al. Ectomycorrhizal Plant-Fungal Co-invasions as Natural Experiments for Connecting Plant and Fungal Traits to Their Ecosystem Consequences. *Front. For. Glob. Chang.* 2020, 3, 84. [CrossRef]

17. Donoghue, M.J.; Moore, B.R. Toward an integrative historical biogeography. *Integr. Comp. Biol.* 2003, 43, 261–270. [CrossRef] [PubMed]

18. Xiang, Q.Y.; Soltis, D.E.; Soltis, P.S. The eastern Asian and eastern and western North American floristic disjunction: Congruent phylogenetic patterns in seven diverse genera. *Mol. Phylogenetics Evol.* 2008, 43, 3631–3640. [CrossRef]

19. Wen, J.; Nie, Z.L.; Ickert-Bond, S.M. Intercontinental disjunctions between eastern Asia and western North America in vascular plants highlight the biogeographic importance of the Bering land bridge from late Cretaceous to Neogene. *J. Syst. Evol.* 2016, 54, 469–490. [CrossRef]

20. Donoghue, M.J.; Bell, C.D.; Li, J. Phylogenetic patterns in Northern Hemisphere plant geography. *Int. J. Plant Sci.* 2001, 162, S41–S52. [CrossRef]

21. Ruddiman, W.F.; Kutzbach, J.E. Forcing of late Cenozoic northern hemisphere climate by plateau uplift in southern Asia and the American West. *J. Geophys. Res. Atmos.* 1989, 94, 18409–18427. [CrossRef]

22. Zinck, J.W.; Rajora, O.P. Post-glacial phytogeography and evolution of a wide-ranging highly-exploited keystone forest tree, eastern white pine (Pinus strobus) in North America: Single refugium, multiple routes. *BMC Evol. Biol.* 2016, 16, 1–17. [CrossRef] [PubMed]

23. Adams, J.M.; Faure, H. Preliminary vegetation maps of the world since the last glacial maximum: An aid to archaeological understanding. *J. Archaeol. Sci.* 1997, 24, 623–647. [CrossRef]
84. Clercq, P.D. Dark clouds and their silver linings: Exotic generalist predators in augmentative biological control. *Neotrop. Entomol.* 2002, 31, 169–176. [CrossRef]

85. Hulcr, J.; Gomez, D.F.; Skelton, J.; Johnson, A.J.; Adams, S.; Li, Y.; Justino, M.A.; Smith, M.E. Invasion of an inconspicuous ambrosia beetle and fungus may affect wood decay in Southeastern North America. *Biol. Invasions* 2021, 23, 1339–1347. [CrossRef]

86. Brasier, C.M.; Buck, K.; Paolletti, M.; Crawford, L.; Kirk, S. Molecular analysis of evolutionary changes in populations of *Ophiostoma novo-ulmi*. *Investig. Agrar.-Sist. Recur.* 2004, 13, 93–103.

87. Kretzer, A.M.; Bruns, T.D. Use of atp6 in fungal phylogenetics: An example from the Boletales. *Mol. Phylogenetics Evol.* 1999, 13, 483–492. [CrossRef] [PubMed]

88. Bruns, T.D.; Szaro, T.M.; Gardes, M.; Cullings, K.W.; Pan, J.J.; Taylor, D.L.; Horton, T.R.; Kretzer, A.; Garbelotto, M.; Li, Y. A sequence database for the identification of ectomycorrhizal basidiomycetes by phylogenetic analysis. *Mol. Ecol.* 1998, 7, 257–272. [CrossRef]

89. Vainio, E.J.; Hantula, J. Genetic differentiation between European and North American populations of *Phlebiopsis gigantea*. *Mycologia* 2000, 92, 436–446. [CrossRef]

90. Vainio, E.J.; Korhonen, K.; Hantula, J. Genetic variation in *Phlebiopsis gigantea* as detected with random amplified microsatellite (RAMS) markers. *Mycol. Res.* 1998, 102, 187–192. [CrossRef]

91. Sierota, Z.; Nowakowska, J.A.; Sikora, K.; Wrzosek, M.; Zółciak, A.; Malecka, M. Genetic variation among *Phlebiopsis gigantea* strains determined by Random Amplified Microsatellite Markers. *Balt. For.* 2015, 21, 178–183.

92. Liu, A.Z.; Samils, N.; Higgins, B.; Stenlid, J.; Slippers, B.; Nairn, C.J.; Covert, S.F. Microsatellite markers for the wood decay fungus *Phlebiopsis gigantea*. *Conserv. Genet.* 2009, 10, 1529–1532. [CrossRef]

93. Giordano, L.; Sillo, F.; Garbelotto, M.; Gonthier, P. Mitonuclear interactions may contribute to fitness of fungal hybrids. *Sci. Rep.* 2018, 8, 1–7.

94. Olson, Å.; Stenlid, J. Mitochondrial control of fungal hybrid virulence. *Nature* 2001, 411, 438. [CrossRef]

95. Bonuso, E.; Zambonelli, A.; Bergemann, S.E.; Iotti, M.; Garbelotto, M. Multilocus phylogenetic and coalescent analyses identify two cryptic species in the Italian bianchetto truffle, *Tuber borchii* Vittad. * Conserv. Genet.* 2010, 11, 1453–1466. [CrossRef]

96. Chothia, C.; Finkelstein, A.V. The classification and origins of protein folding patterns. *Annu. Rev. Biochem.* 1990, 59, 1007–1035. [CrossRef]

97. Nicolotti, G.; Gonthier, P.; Varese, C. Effectiveness of some biocontrol and chemical treatments against *Heterobasidion annosum* on Norway spruce stumps. *Eur. J. Plant Pathol.* 1999, 29, 339–346. [CrossRef]

98. Simberloff, D. Risks of biological control for conservation purposes. *Biocontrol* 2012, 57, 263–276. [CrossRef]