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Genetic Variation Among *Phlebiopsis gigantea* Strains Determined by Random Amplified Microsatellite Markers

ZBIGNIEW SIEROTA[1] , JUSTYNA A. NOWAKOWSKA*[2], KATARZYNA SIKORA[1], MARTA WRZOSEK[3], ANNA ŻÓŁCIAK[1] AND MONIKA MAŁECKA[1]

[1] *Department of Forest Protection, Forest Research Institute, Sękocin Stary, Braci Leśnej 3, 05-090 Raszyn, Poland, e- mail: Z.Sierota@ibles.waw.pl; K.Sikora@ibles.waw.pl; A.Zolciak@ibles.waw.pl; M.Malecka @ibles.waw.pl*

[2] *Laboratory of Molecular Biology, Forest Research Institute, Braci Leśnej 3, 05-090 Raszyn, Poland, e-mail: J.Nowakowska@ibles.waw.pl*

[3] *Faculty of Biology, University of Warsaw, Krakowskie Przedmieście 26/28, 00-927 Warszawa, Poland, e-mail: martawrzosek@gmail.com*

**Corresponding author; e-mail: J.Nowakowska@ibles.waw.pl; phone:+48 22 7150467*

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Abstract

The aim of the present study was to examine the genetic variation among European-registered and native strains of *Phlebiopsis gigantea* used as a practical biological agent against root rot in conifers. The high degree of genetic similarity between the *P. gigantea* strain used in product formulations and the *P. gigantea* native strain is crucial for product efficiency against the root rot pathogen *H. annosum* in the forest. Random amplified microsatellite (RAMS) markers were used to detect variation among registered (British and Finnish) and indigenous (Polish) *P. gigantea* isolates. Data were evaluated with using two tests: Band Sharing Index (BSI) and genetic similarity. Higher precision in genetic similarity assessments between isolates resulted from the genetic distance based on UPGMA data, proving strong similarity between six indigenous isolates (PL1, PL2, PL3, PL6, PL7, and PL12), four British (FC14, FC15, FC16, and FC17) and two Finnish and Swedish (VF8 and VF10) isolates. One Polish PL12 isolate was genetically analogous to the registered FC16 from Great Britain, proving their high reciprocal RAMS similarity. Results indicated that there is little reason to fear that foreign isolates pose an invasive threat to non-native forest environments.

Key words: *Phlebiopsis gigantea* isolates, RAMS, biological control, genetic similarity.

Introduction

As reported originally by Rishbeth (1963), *Phlebiopsis gigantea* has been commonly used since the 1960s to control root rot *Heterobasidion annosum* sensu lato in European coniferous stands (Pratt et al. 2000, Garbelotto and Gonthier 2013, Sierota 2013). Initial trials in Poland on the application of *P. gigantea* (British preparation Pg-Suspension Ecological Labs) were initiated at a semi-economic scale in 1971 in the northern areas of Poland; Gdansk Forest Directorate (Sierota 1975). Presently, and in accordance with Council Directive 91/414/EEC and Commission Regulation (EC) No 2229/2004, only registered *P. gigantea* isolates are permitted as biocontrol agents in forests within the European Union (EU). Consequently, no more than 14 *P. gigantea* isolates have been introduced as commercial products (Pg-Suspension and Rotstop) in EU member states, including Poland. Disparate results obtained on the efficacy of *P. gigantea* strains in Europe under both laboratory and field conditions (Małecka et al. 2012, Vainio et al. 2001, Żółciak et al. 2008, 2012) as well as overseas in Canada (Dumas 2013; Dumas and Laflamme 2013) and China (Xingchun et al. 2014) have lead investigators to hypothesise that certain isolates of *P. gigantea* may be equally effective at different forest sites across the northern hemisphere. This raises the question of whether foreign strains selected for registration could supersede more effective native strains.

Little is known about the interactions between *P. gigantea* inocula and endogenous populations of fun-

gi that inhabit forest ecosystems (Vainio et al. 2001). Łakomy and Zarakowski (2000) did not find a significant difference between selected European strains of *P. gigantea* with respect to their activity in decomposition of Scots pine wood. The authors suggested this may be attributed to the low levels of genetic variation in this fungus. On the other hand, Vainio (1998) reported that the *P. gigantea* population could be considered to be widely distributed throughout the boreal forest and genetically highly polymorphic in European *P. gigantea* populations. At the same time, Vainio and Hantula (2000) identified substantial genetic differences between European and North American strains of the fungus, thus they excluded the possibility of fungal transfer between the continents. However, the possibility of hybridization and horizontal genetic transfer cannot be excluded (Vainio et al. 2001, Sun et al. 2009).

In the present study, we compared foreign and native Polish *P. gigantea* strains using the simple random amplified microsatellite technique (RAMS) (Ziętkiewicz et al. 1994) with indigenous isolates from Poland previously applied as the "PgIBL" preparation throughout Poland's forests (Pratt et al. 2000). The RAMS method has been successfully used for identification of *Ganoderma boninense* strains (Zakaria et al. 2005, Nudin and Siddiquee 2012) as well as for the determination of molecular differences between *P. gigantea* strains (Vainio 1998, Vainio et Hantula 2000, Hantula et al. 1996) and other fungi (Guo et al. 2004, Guarro et al. 2005). It was expected that the RAMS analysis, based on the methodology described by Vainio and Hantula (2000), would accurately indicate genetic similarities between the registered and indigenous (Polish) strains of *P. gigantea* and also show that non-native fungi isolates do not pose an invasive threat. Therefore, registered strains could be equally effective as biocontrol agents against root rot as native strains. The genetic similarity for the RAMS markers was evaluated in order to identify similarities between the studied strains.

Materials and Methods

Phlebiopsis gigantea isolates

The 12 single-spore isolates of *P. gigantea* strains tested in this study originated in Finland, Great Britain, and Poland. Finnish strains were isolated from *P. gigantea* fruit bodies growing on Norway spruce *Picea abies* logs or stumps while those from Great Britain were isolated from infected lodgepole pine *Pinus contorta* and Scots pine *Pinus sylvestris* trees or stumps (Table 1). The pure cultures were obtained in 2012 and re-isolated on malt extract agar (MEA). Native Polish strains were previously isolated, mainly from *P. sylvestris* stumps that were naturally infected

Table 1. Descriptions of tested *P. gigantea* isolates

Strain code	Strain origin	Strain name and number in culture collection	Strain origination
		Registered strains	
VF8	Verdera, Finland	VRA 1835, ATCC 90304, Used in Rotstop (Rotstop-F)	Fruit body on Norway spruce log, Loppi (Finland)
VF10	Verdera, Finland	VRA 1984, DSM 16 201	Fruit body on *P. abies* stump, Råberg near Uppsala (Sweden)
FC14	Forestry Commission, GB	IMI 390101	*P. contorta* stump, Mull (GB), 1998
FC15	Forestry Commission, GB	FOC PG SP log 5, IMI 390098	Information not available; NRS Roslin strain
FC16	Forestry Commission, GB	FOC PG B20/5, IMI 390096, Used in PG Suspension	*P. sylvestris* tree, NRS Roslin strain, 1996
FC17	Forestry Commission, GB	FOC PG BU 3, IMI 390099	*P. sylvestris* tree, Buchan (GB), 1999
		Native strains (not registered)	
PL1	Department of Forest Phytopathology, Forest Research Institute, Poland (ZFL IBL)	ZFL IBL/Ph.g.1	*P. sylvestris* stump No 1, Chojnów Forest District (FD), 2003
PL2	Department of Forest Phytopathology, Forest Research Institute, Poland (ZFL IBL)	ZFL IBL/Ph.g.2	*P. sylvestris* stump No 2, Chojnów FD, 2002
PL3	Department of Forest Phytopathology, Forest Research Institute, Poland (ZFL IBL)	ZFL IBL/Ph.g.3	*P. sylvestris* stump No 3, Chojnów FD, 2002
PL6	Department of Forest Phytopathology, Forest Research Institute, Poland (ZFL IBL)	ZFL IBL/Ph.g.6	*P. sylvestris* stump No 5, Chojnów FD, 2003
PL7	Department of Forest Phytopathology, Forest Research Institute, Poland (ZFL IBL)	ZFL IBL/Ph.g.7	*P. sylvestris* stump No 6, Chojnów FD, 2003
PL12	Department of Forest Phytopathology, Forest Research Institute, Poland (ZFL IBL)	ZFL IBL/Ph.g.12	Fruit body on *P. sylvestris* stump, Nidzica FD, 2004

after thinning in early spring 2002-2003. The description of the isolates used in the study is shown in Table 1. All of the native isolates were cultivated on 2% MEA media at 20°C and transferred from agar to agar using beech sawdust every year.

DNA extraction

Prior to DNA extraction, both the aerial and outer wood mycelia were removed from the wood substrate and homogenized in the presence of liquid nitrogen using a mortar and pestle. Total DNA of each isolate was extracted from 100 mg of homogenized mycelium using GenElute Plant Genomic DNA Miniprep Kit (Sigma Aldrich) according to the manufacturer's protocol.

PCR amplification

PCR amplifications were performed with the primer proposed by Hantula et al. (1996): 5' DHB$(CGA)_5$3' consistent with the RAMS method and primer structure, where D = (G/A/T), H = (A/T/C), B = (G/T/C) (Vainio and Hantula 2000, Hantula et al. 1996). The amplification was carried out in a 50 µl reaction mix containing 50-100 ng of genomic DNA, 2 µM of primer, 200 µM each of dNTP, 1 mM $MgCl_2$, 1U Taq polymerase, 1×Q solution, and 1×PCR buffer (Qiagen Ltd., Valencia, CA, USA). The reaction was carried out in a PTC-200^{TM} Programmable Thermal Controller (MJ Research Inc.) for 35 denaturation cycles at 95 °C for 30 s, annealing at 61 °C for 45 s, and extension at 72 °C for 2 min with an initial denaturation at 95 °C for 3 min before cycling and final extension at 72 °C for 10 min after cycling. A minimum of three independent reactions were run for each DNA sample.

Electrophoresis and gel analysis

Following DNA amplification, the PCR products were purified from the residual reagents using a GeneClean kit (MP Biomedicals, CA, USA) and separated by electrophoresis in a Basica LE agarose (Prona, Spain) with SynerGel additive (Diversified Biotech, MA, USA) at concentrations of 0.85% and 0.9%. Electrophoresis proceeded in a TBE-buffer (45 mM Tris-borate, pH = 8.0; 1 mM EDTA) at medium voltage (80-100 V) and the products were visualised using GelRed staining under UV light. Optimal resolution for the RAMS markers of different lengths was obtained. For evaluation of band length, a GBP 3000 bp ladder (Genoplast, Poland) was used. Amplification products were analyzed via qualitative assessments (presence = 1, absence = 0), which were then followed by the creation of microsatellite occurrence matrices. All fragment bands were counted according to the BIO-PROFIL Bio-Gene V99.05 software. Similarities between isolates were assessed according to Lynch (1990) and based on a Band Sharing Index (BSI) calculated according to the formula: BSI = 2Sab/(Sa + Sb), where Sa and Sb are total numbers of bands in strains a and b, respectively, and Sab is the number of bands shared between the strains. A strong similarity between strains was indicated, when BSI > 0.75. A matrix of bands for genetic analysis was used (PAST software, Hammer et al. 2001, http://folk.uio.no/ohammer/past/).

A dendrogram of the genetic similarities between isolates, built on a UPMGA algorithm of genetic distance (*d*), was generated using MEGA 6 software (Tamura et al. 2013) after 999 permutations of binary RAMS data for haploid *P. gigantea* isolates in GenALEx v.6.501 software (Peakall and Smouse 2012).

Results

The results indicated both genetic similarity and differentiation of the *P. gigantea* strains we examined. In total, 22 different RAMS markers (bands) were scored from the amplification profiles (Figure 1). The PL1 isolate showed the greatest number of bands (16), while PL12 showed the fewest (7). Two bands were observed in all of the isolates tested (+/- 270 bp and +/- 260 bp) and two markers were unique. One of the unique bands was only characteristic of PL1 (+/- 230 bp) and the other for FC14 (+/- 290 bp).

The high degree of polymorphism observed in the RAMS markers was characterised by the presence of a unique banding pattern for each isolate. However, based on BSI values calculated for each isolate (Table 2), some genetic similarity between isolates originating from different countries was noted and BSI indices greater than 0.75 were detected in comparisons made between 15 pairs.

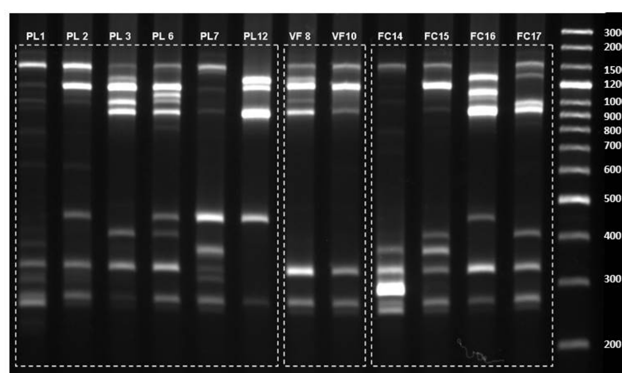

Figure 1. Electrophoretic band pattern obtained for the *P. gigantea* strains we examined.

A description of the isolates is given in Table 1. Right column is GBP 3000bp ladder [Genoplast]

VF8 (used in Rotstop, Finland) was genetically similar to PL3, VF10, FC15 and FC17 strains, while FC16 (used in Pg-Suspension, Great Britain) was similar to the native PL6 strain. The greatest genetic similarity value was found between PL3 and VF8, which shared 9 of 10 markers (BSI index for both strains = 0.9). Interestingly, we also observed two of the strains, PL3 and FC15, seemed to be related to other tested isolates. PL12 was genetically distinct when compared to other isolates (BSI values ranged from 0.3 to 0.7). Two EU-registered isolates, i.e. VF8 and VF10, were genetically similar to each other (BSI values = 0.77) despite the fact that VF8 originated from Finland while VF10 was from Sweden. The average BSI value for Polish strains was 0.629 and comparable levels of similarity were observed for the British and Finnish strains (0.641 and 0.633, respectively). No unreproducible bands were observed.

Interestingly, one Polish isolate (PL12 from the Nidzica Forest District) was separated by the greatest genetic distance d = 29.419 from other Polish isolates (PL1, PL2, PL3, PL6, and PL7 from the Chojnów Forest District) while being more similar to the FC16 British isolate (d = 19.007). The Finnish and British *P. gigantea* isolates were grouped into one cluster with the exception of one British isolate (FC14), which was separated from the others by the greatest distance d = 23.264 (Figure 2). The bootstrap values revealed a small probability for clustering (less than 0.95), which provides strong evidence for the high degree of genetic similarity between the strains.

Discussion

The performance of *P. gigantea* strains originating from specific forest environments and then introduced to non-native sites may not be as effective or may even threaten the natural diversity of local fungi populations in tree stumps (Pratt et al., 2000). The most effective *P. gigantea* strain for the control the root rot pathogen *H. annosum* in Europe (Great Britain, Finland, Sweden, France, or Poland), North America (Canada), and Asia (China), needs to be a vigorous isolate possessing genetically similarity to native strains and combined with an efficacious product formulation.

Table 2. Band sharing indices (BSI) for the tested strains

	PL2	PL3	PL6	PL7	PL12	VF8	VF10	FC14	FC15	FC16	FC17
PL1	**0.814**	0.615	0.666	0.740	0.434	0.615	0.583	0.689	0.692	0.538	0.666
PL2	-	0.571	0.636	**0.818**	0.444	0.666	0.413	0.583	0.571	0.571	0.636
PL3	-	-	**0.761**	0.571	0.588	**0.900**	**0.777**	0.695	**0.800**	0.700	**0.857**
PL6	-	-	-	0.636	0.555	0.666	0.413	0.666	0.666	**0.761**	0.636
PL7	-	-	-	-	0.588	0.666	0.631	0.583	**0.761**	0.571	0.545
PL12	-	-	-	-	-	0.588	0.533	0.300	0.400	**0.705**	0.444
VF8	-	-	-	-	-	-	**0.777**	0.608	**0.800**	0.700	**0.857**
VF10	-	-	-	-	-	-	-	0.571	**0.777**	0.555	0.736
FC14	-	-	-	-	-	-	-	-	**0.761**	0.434	0.583
FC15	-	-	-	-	-	-	-	-	-	0.500	**0.761**
FC16	-	-	-	-	-	-	-	-	-	-	0.666

Bold numbers indicate BSI > 0.75

Based on unweighted pair group method with arithmetic mean (UPGMA) clustering, the Polish *P. gigantea* isolates PL1, PL2, and PL7 as well as PL3 and PL6 were grouped into a single cluster according to the dendrogram of genetic similarity (Figure 2).

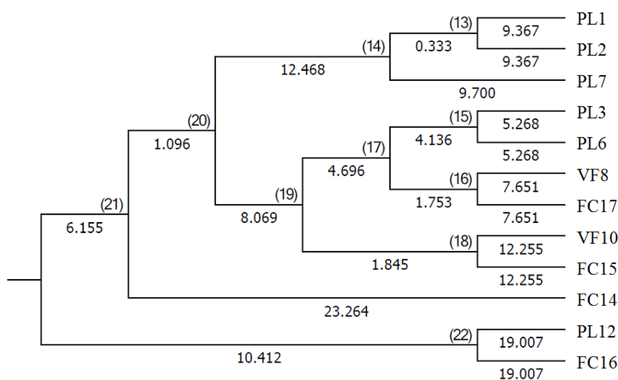

Figure 2. Dendrogram of genetic similarity based on UPGMA analysis. Branch length illustrates the genetic distance between isolates and numbers in brackets represent the value of bootstraps (as %).

In the current study, two complementary methods for evaluating genetic similarity between 12 isolates originating from the Forestry Commission – Great Britain (four isolates), Verdera – Finland (two) and Poland (six non EU-registered isolates) were used to examine the degree of genetic similarity between strains using the RAMS technique.

The comparison of RAMS markers based on Band Sharing Index (BSI) calculations presented in this study are broadly similar to those obtained by Vainio and Hantula (2000), who used the same primers for PCR amplification. Consistent with the findings of Vainio and Hantula (2000), we observed the same

number of bands for Rotstop and for VF8. Surprisingly, however, the length of some bands differed. The RAMS banding pattern is sensitive to the conditions of PCR amplification. In this study, Taq DNA polymerase was used for the amplification of RAMS markers whereas Vainio et al. (1998, 2000, and 2001) used a DynaZyme DNA polymerase, which may have affected the size and number of the resulting bands. Several investigators have highlighted important disadvantages of using unspecified markers. Saikia and Kadoo (2010) recommended using DNA fragments identified using RAMS or RAPD and converted to simple and reliable molecular markers such as Sequence Characterized Amplified Region (SCAR) or Sequence Tagged Site (STS). Additionally, the microsatellite markers described by Liu et al. (2009) are useful for examining the population structure and biogeography of some European and North American *P. gigantea* strains.

The tests of genetic similarity used in our study (BSI calculation and genetic distance data, see Vainio and Hantula (2000)) are valuable tools that can be used studying future similar work. The close comparison of the genetic similarity between isolates based on UPGMA clustering revealed the greatest RAMS-based relationship between Polish *P. gigantea* PL1, PL2, PL3, PL6, and PL7 isolates, whereas one Polish PL12 isolate was genetically similar to the British FC16 isolate. Two EU-registered isolates, e.g. VF8 and VF10 had greater similarity to the British isolates FC17 and FC15.

The nuclear microsatellite DNA loci are considered to be the most precise tools for determining the genotypes of living organisms. These markers are characterized by high degree of polymorphism and reproducibility. Taking the microsatellite loci-based genetic structure into account, the RAMS markers revealed that all the investigated *P. gigantea* isolates differ from several registered (derived from different countries in Europe) and native *P. gigantea* strains. Differences in strain were also found at a global level, which suggests low gene flow among *P. gigantea* populations (Dumas and Laflamme 2013, Vainio and Hantula 2000, Kauserud and Schumacher 2003).

It is possible that the PL12 Polish strain and British FC16 one (Pg-Suspension) may have a common origin since their BSI indexes both reached approximately 0.705 and they were separated by a low genetic distance (d = 19.007) based on the dendrogram of genetic similarity. The PL12 strain was isolated in the northern region of Poland (Nidzica Forest District), spatially close to where the first biological trials were conducted in 1970, when an unknown British isolate was applied as Pg-Suspension Ecological Laboratories (Sierota 1975).

Conclusions

Genetic variation of the *P. gigantea* strains tested here, based on RAMS markers, indicates considerable genetic similarity among *P. gigantea* strains that were derived across Europe. The sample size of the analyzed isolates was small and so the results should be interpreted with caution.

Nevertheless, the results of the current study confirm that the selection of *P. gigantea* strains used in commercial products is appropriate and that their current application in forestry practices throughout the continent can be considered as being safe.

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