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Genetic variation between and within two populations of *Rhabdocline pseudotsugae* Sydow in Germany

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Abstract: Knowledge of the ecology and biology of *Rhabdocline pseudotsugae* Sydow is still at a very early stage. Recent results indicate the existence of an endophytic stage of the fungus in the form of symptomless infections in various Douglas fir tissue types. This study represents the first description of genetic variation between and within two populations of *R. pseudotsugae*. Needles featuring fruiting bodies of *R. pseudotsugae* were collected from sample areas in Saxony and North Rhine-Westphalia (in Germany), with different fungal genotypes distinguished using the start codon targeted (SCoT) polymorphism and branch point signal sequences (BPS) techniques. Five of the 20 primers tested could be selected for further investigation. A total of 349 fragments were amplified at an average of 69.8 fragments per primer. Results showed the two sample areas to exhibit a high degree of both genetic variability and genetic diversity. A clear differentiation between the sample areas was not observed. There was nevertheless a clear connection between the genetic distance and spatial distribution of the fruiting bodies of *R. pseudotsugae* within the sample areas themselves.

Keywords: Rhabdocline needle cast; start codon targeted polymorphisms; branch point signal sequences; genetic structure; fungal distribution
1. Introduction

Rhabdocline needle cast is one of the most significant fungal diseases known to affect the Douglas fir (Pseudotsuga menziesii [Mirb.] Franco) and is caused by five fungi within the genus Rhabdocline. These obligate biotrophic parasites exclusively infect North American species within the genus Pseudotsuga. To date Rhabdocline pseudotsugae is the only of the five species to have been confirmed to exist in Europe (Stephan 1973, Catal et al. 2010). From a European perspective, R. pseudotsugae was first observed in Scotland in 1914 but was almost non-existent in England until 1922 (Wilson & Wilson 1926). A relatively short time later the fungus had spread from Great Britain to large swaths of the European mainland. The presence of R. pseudotsugae was confirmed almost simultaneously in Germany, Denmark and the Netherlands (v. Geyr 1930, Van Vloten 1932, Liese 1939). By 1995 it had been reported in a further 14 European countries including Poland, Sweden, Norway, France, Switzerland, Greece and Italy (Boyce 1948, Bonifacio et al. 1970, Anonymous 1995). As a basic principle, R. pseudotsugae can now be found at any location where susceptible Douglas firs are cultivated, with susceptibility varying to a particularly considerable extent between different Douglas fir varieties. To give an example, Douglas firs of the viridis variety are generally less susceptible than Douglas firs of the glauca variety. The fungus can nevertheless lead to major damage throughout the viridis variety, whereas not all trees of the glauca variety are affected to the same extent (Stephan 1980, Chastagner 2001). The typical symptoms of R. pseudotsugae are orange-yellow to rusty red fruiting bodies and often massive premature needle loss which can lead to substantial loss of increment and tree death if trees are subjected to repeated fruiting cycles (Stephan 1981). The fungus is spread by ascospores which infect newly sprouted Douglas fir needles in spring. The fungus’s one-year development cycle is therefore optimally adapted to the needles’ seasonal growth cycle (Van Vloten 1932, Brandt 1960, Stephan 1981). This notwithstanding, current studies also demonstrate the presence of R. pseudotsugae in the seeds of Douglas firs, thus potentially identifying a new source of infection (Morgenstern et al. 2014).

Detailed, substantiated knowledge of the ecology and biology of harmful organisms is a prerequisite
for the targeted, efficient control thereof as well as the development of suitable plant protection measures. Genetic structure is of central importance in this regard. In addition reflecting a pathogen’s evolational history and potential for adaptation, population genetic structures also provide insights into the way in which a pathogen spreads. In recent years molecular genetic methods have therefore been applied to identify and trace the spread of fungal pathogens. Work in this area has included analysis of the geographic distribution of *Sphaeropsis sapinea* (Fr.) Dyko & Sutton (Stanisz et al. 1999). The distribution mechanisms and invasive history of *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya were determined by studying the population genetic structure of the fungus at a European level (Kraj et al. 2010, Gross et al. 2014, Burokiene et al. 2015) and comparing it with populations in Japan (Gross et al. 2014). Winton et al. (2007) studied genetic variation in *Phaeocryptopus gäumannii* (Rohde) Petrak as well as its potential for recombination between different lineages. Comparable work has also examined *Melampsora larici-populina* Klebahn (Barrès et al. 2008, Elefsen et al. 2014), *Cronartium ribicola* J.C. Fischer (Et-touli et al. 1999, Hamelin et al. 2000) und *R. parkeri* Sherwood, J.K. Stone & G.C. Carroll (McCutcheon et al. 1993). At present, studies of the genetic structure of *R. pseudotsugae* are not known to exist.

The aim of the work presented in this paper was to investigate genetic variation in *R. pseudotsugae* and in turn describe genetic variation within and between populations of the fungus. Results were to provide a foundation for conclusions on the distribution strategies pursued by the fungus for the development of suitable monitoring and management strategies in the future.

The use of Start Codon Targeted Polymorphism (SCoT) and Branch Point Signal Sequences (BPS) marker techniques represented a relatively new approach. Both marker techniques are based on conserved branch point signal sequences or short conserved regions that flank the ATG translation start codon (Collard & Mackill, 2009, Xiong et al. 2011a) and facilitate comparisons between genetic structures without the need for detailed sequencing information on the target organism. The markers have already been applied in studies of *Arachis hypogaea* L. (Xiong et al. 2011a, b), *Mangifera indica* L., *Musa* spp. and *Dimocarpus longan* Loureiro (Xiong et al. 2011a) as well as *Dendrobium nobile*...
Lindley (Bhattacharyya et al. 2013), *Jatropha curcas* L. (Mulpuri et al. 2013), various species of *Cicer* (Amirmoradi et al. 2012) and *Boehmeria nivea* (L.) Gaudichaud-Beaupré (Satya et al. 2015). Both marker techniques promise a higher degree of polymorphism and better reproducibility of results when compared with the randomly amplified polymorphic DNA (RAPD) technique. The work presented in this paper therefore also includes the assessment of their suitability for the study of population genetics in obligate biotrophic fungi.

2. Material and Methods

2.1 Sampling

Two German sample areas created by Staatsbetrieb Sachsenforst in 1993 in order to test the suitability of various hybrid Douglas firs for cultivation were made available for the purposes of the planned studies. Information on the hybrids used was provided by Staatsbetrieb Sachsenforst and is presented in Figure 1. The first sample area located close to the Saxon town of Cunnersdorf (50° 51' N, 14° 7' E) covered a total area of 1.3 ha and consisted of 110 plots planted with a total of 42 different hybrids. Each plot was originally planted with 5 x 5 Douglas firs arranged in a 2 m x 2 m formation. In addition, the stand was broken up in a north-south direction by a row of common beech (*Fagus sylvatica* L.) every two rows of plots. The second sample area located close to the town of Bad Berleburg in North Rhine-Westphalia (50° 51' N, 14° 7' E) covered a total area of 1.1 ha and consisted of 108 plots planted with a total of 36 different hybrids. Each plot was again originally planted with 25 trees arranged in a 2 m x 2 m formation per plot. Both sample areas exhibited massive damage caused by *R. pseudotsugae* and *P. gäumannii* over a number of years. In the autumn of 2004 the number of trunks at the Cunnersdorf location was schematically reduced by up to 50 % in order to reduce the intensity of infestation. No such treatments have been carried out at the sample area in Bad Berleburg to date.

Douglas fir branches featuring fruiting bodies of *R. pseudotsugae* were collected from both sample areas in late May 2013. Samples were taken from one tree per plot provided there were Douglas firs
exhibiting infestation with *R. pseudotsugae* on the plot. This led to the collection of samples from a total of 101 plots at the Cunnersdorf site and 83 plots at the Bad Berleburg site. The intensity of infestation with *R. pseudotsugae* on each plot was also rated. The rating system used is shown in Figure 1.

In addition to the fruiting bodies of *R. pseudotsugae* plant material was also taken from 3 Douglas firs of the *glauca* variety and 5 Douglas firs of the *viridis* variety. The Douglas firs selected did not exhibit any sign of fungal infection at the time when the samples were taken.

### 2.2 Sample preparation and amplification

The fruiting bodies were prepared in a laboratory immediately after the collection of samples from the two sample areas. Working with the aid of a binocular microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) set to 32-fold magnification, sterile dissecting needles were used to extract sample material directly from individual fruiting bodies of *R. pseudotsugae*. In order to minimise the risk of mixing the various fungi, the Douglas fir needles selected exhibited little or no infestation with *P. gäumannii* and clearly separated fruiting bodies of *R. pseudotsugae*. The material extracted was transferred to 20 µl of dilution buffer from a Phire Plant Direct PCR Kit (Finnzymes [Part of Thermo Fisher Scientific], Espoo, Finland) and stored at -20°C.

Genetic variation was analysed by preparing a total of 8 SCoT polymorphism primers (Collard & Mackill 2009) and 12 BPS primers (Xiong et al. 2011a) and tested them on 7 independent *R. pseudotsugae* samples. Amplification was carried out with the aid of a Phire Plant Direct PCR Kit (Finnzymes [Part of Thermo Fisher Scientific]). Table 1 provides a detailed description of the reaction mixture and cycler programme.

All *R. pseudotsugae* samples taken from Cunnersdorf and Bad Berleburg were analysed using 5 primers (see Table 2) selected on the basis of the results of preliminary testing and direct PCR. In the case of all samples primers BPS 1, SCoT 35 and SCoT 36 exhibited far lower amplification than in preliminary testing. As a result all samples were nested in a second PCR (TopTaq Mastermix, Qiagen GmbH, Hilden, Deutschland) using the same primer. Table 3 presents a description of the nested PCR
preparation and cycling protocol used. The annealing temperatures of the selected primers are listed in
Table 2.

Genomic DNA was extracted from the Douglas fir needles with the aid of a DNeasy Plant Mini Kit (Qiagen) in accordance with the instructions provided by the manufacturer. The Douglas fir samples were amplified at a DNA concentration of approximately 30 ng/µl in a 25 µl PCR preparation (TopTaq MasterMix, Qiagen) as described in the case of the aforementioned nested PCR preparation (see Table 3). PCR was repeated three times in order to verify the reproducibility of the results delivered.

The PCR products were checked and quantified by means of gel electrophoresis in a 1.5 % agarose gel. Products were stained with ethidium bromide (Serva Electrophoresis GmbH, Heidelberg, Germany) and visualized under UV light using the Bio-Vision system (Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany). Fragment length was measured by comparing the fragments against the GeneRuler 100 bp Plus DNA Ladder (Life Technologies GmbH, Darmstadt, Germany) with the aid of the Bio-1D software solution (Vilber Lourmat Deutschland GmbH).

2.3 Data analysis

Hierarchical cluster analysis was carried out in order to facilitate the grouping of the various genotypes according to their genetic distance with the aid of SCoT and BPS markers. This involved the transfer of the marker bands measured to a 0/1 matrix (0 = no band, 1 = band exists) and the determination of the pairwise genetic distance between two samples as described by Nei & Li (1979). The distance between clusters was calculated with the aid of the UPGMA (unweighted pair-group method using arithmetic averages) algorithm. The clusters identified were visualized using a dendrogram and validated by means of a bootstrap test (resampling: 10,000). Cluster analysis was carried out using the "parPvclust()" function included in the "pvclust" package (Version 2.0.0) with the aid of the "parallel" package (Version 2.3.2) included in the statistics program R.

Genetic diversity within the collectives was characterised according to expected heterozygosity \( H_e = 1 - \sum p_i^2 \) (\( p_i = \) marker band frequency) and averaged across all of the loci analysed (Berg & Hamrick 1997). A Kruskal–Wallis one-way analysis of variance was carried out in order to investigate the intensity of
infestation exhibited by the hybrid varieties. Therefore the null hypothesis was: “There is no difference between the hybrid varieties”. The level of significance was set at \( p = 0.05 \). In the case of significant differences between the hybrid varieties a post-hoc test was carried out in order to determine which groups differed from one another. This also involved the execution of a pairwise test for multiple comparisons of mean rank sums (Nemenyi test) comparing all groups. Chi-squared was used as the distance metric (Nemenyi, 1962). The Kruskal–Wallis one-way analysis of variance was carried out using the "kruskal.test()" function included in the "stats" package (Version 3.2.3) of the statistics program R. The post-hoc test was executed using the "posthoc.kruskal.nemenyi.test()" function included in the "PMCMR" package (Version 4.1).

### 3. Results

#### 3.1 Intensity of infestation with Rhabdocline pseudotsugae

Figure 1 shows the intensity of infestation with \( R. \) pseudotsugae recorded at the sample areas in Cunnersdorf and Bad Berleburg. Regarding the intensity of infestation there are no spatial structures in evidence either between or within the sample areas. At 11.9 \%, Cunnersdorf exhibits almost two and a half times as many plots affected by major infestation (category 3) as Bad Berleburg (4.8 \%). If categories 2 (moderate infestation) and 3 (major infestation) are grouped together the proportion of plots exhibiting significant damage as a result of infestation is nevertheless relatively high (47.5 \% in Cunnersdorf, 47.0 \% in Bad Berleburg).

The significance (p value) calculated with the Kruskal–Wallis one-way analysis of variance shows a value of 1.506e-06 (chi-squared = 41.754; df = 8). As the p value was below the level of significance the null hypothesis was rejected. Significant differences between the hybrid varieties in terms of intensity of infestation were therefore observed. The results of the post-hoc test are presented in Table 4. From an overall perspective there was no difference between the varieties in terms of intensity of infestation. The following hybrid varieties were the only to exhibit significant differences (p value \(< 0.05\): \( caesia \times caesia \) and \( glauca \times viridis \); \( caesia \times caesia \) and \( viridis \times glauca \); \( viridis \times caesia \) and...
3.2 Polymorphism detection and marker band frequency

A total of 5 of the 20 SCoT and BPS primers tested exhibited a high proportion of polymorphic bands and were therefore selected for the analysis of genetic variation in *R. pseudotsugae*.

The selected primers amplified a total of 349 fragments with between 3167 and 218 base pairs (bp) in the *R. pseudotsugae* samples analysed. The Cunnersdorf and Bad Berleburg sample areas yielded 272 and 253 fragments respectively. A total of 176 fragments were identified in samples from both sample areas. 96 fragments were detected in samples from Cunnersdorf only, 77 in samples from Bad Berleburg only. When grouped according to the primers used, the incidence of new fragments in either of the two sample areas was between 50 % (SCoT 3) and 56.4 % (SCoT 35) in the case of primers BPS 1, SCoT 3, SCoT 33 and SCoT 35. Only primer SCoT 36 was found to possess a lower proportion of different fragments (32.8 %).

As shown in Table 5, an average of 69.8 fragments per primer was observed with results ranging between 58 fragments (SCoT 36) and 81 fragments (SCoT 33). Each fragment was found in an average of 8.8 % of the samples analysed. This notwithstanding, 48.7 % of fragments were observed in a maximum of 10 samples, 47.0 % of fragments in 11 to 50 samples and 4.3 % of fragments in more than 50 samples. The most frequently encountered fragment (SCoT 35 – 565 bp) was identified in 43.5 % of samples. No single fragment was detected in all of the samples analysed. The proportion of polymorphic fragments therefore was 100 %.

The relative frequencies with which the various marker bands were observed are summarised in Table 5. Values of between 0.02 % and 1.42 % were determined for the overall collective across all of the gene loci investigated. In terms of the primers used, frequencies ranged between 0.08 % (SCoT 38, SCoT 33) and 7.51 % (SCoT 3).

Expected heterozygosity *H*<sub>e</sub> was determined to be close to the maximum value of 1 in the case of every gene locus both within the individual sample areas and across the collective as a whole (Table 5). The selected primers amplified a total of 54 fragments with between 2393 bp and 323 bp in the
Douglas firs under investigation. All three sets of PCR results displayed an identical fragment pattern. An average of 10.8 fragments per primer was observed. The proportion of polymorphic bands was at 77.8%. Eighteen fragments were only found in Douglas fir samples; 36 fragments were confirmed to be present in *R. pseudotsugae* samples. In the case of primers BPS 1, SCoT 3, SCoT 33 and SCoT 35 the incidence of new fragments in Douglas fir samples ranged between 53.9 % (BPS 1) and 25.0 % (SCoT 3). SCoT 36 yielded the lowest proportion of new fragments (9.1 %).

### 3.3 Cluster analysis

The fragments measured were used to calculate the genetic distance (Nei & Li 1979) between the 184 *R. pseudotsugae* samples and 8 Douglas fir samples. Genetic distance varied between 0.043 and 0.221 (mean = 0.141) within the Cunnersdorf sample area and between 0.054 and 0.223 (mean = 0.144) within the Bad Berleburg sample area. The figures for the overall collective were between 0.043 and 0.249 (mean = 0.148). The genetic distance within the Douglas fir samples was determined to range between 0.003 and 0.027 (average = 0.016).

Cluster analysis of the entire collective resulted in the division of the 184 *R. pseudotsugae* samples and 8 Douglas fir samples with p values of between 66 % and 100 % into 8 main clusters (see Figure 2). Clusters I to III contain between 2 (Cluster I) and 29 (Cluster II) *R. pseudotsugae* samples found in Cunnersdorf only. Clusters IV to VI contain *R. pseudotsugae* samples from Bad Berleburg only and a total of between 17 samples (Cluster V) and 31 samples (Cluster VI). Cluster VII comprises the 8 Douglas fir samples. Cluster VIII groups together 6 samples from Bad Berleburg and 64 samples from Cunnersdorf. From an overall perspective the dendrogram illustrates clear differentiation between *R. pseudotsugae* samples and Douglas fir samples. In the case of the *R. pseudotsugae* samples there is nevertheless no clearly discernible differentiation between the two sample areas Cunnersdorf and Bad Berleburg (see Figure 2).

The Bad Berleburg sample area exhibits 4 main clusters containing between 2 samples (Cluster III) and 35 samples (Cluster I) at p values of between 66 % and 96 % (Figure 3). By transferring these main clusters to a schematic map of the sample area it is possible to demonstrate a clear spatial...
distribution. Cluster I dominates the area to the north of the forest track and also includes outliers in
the southern section of the sample area, with plots 83, 89 and 96 forming a cluster with plots 8, 13 and
32 at \( p = 94 \% \). The 32 plots included in Cluster II dominate the central section of the sample area.
Cluster II comprises 2 plots which nevertheless combine with Cluster IV to form a group at \( p = 95 \% \).
Both clusters are restricted to the southern section of the sample area (see Figure 3).
The Cunnersdorf sample area exhibits 4 main clusters containing between 6 samples (Cluster I) and 40
samples (Cluster IV) at \( p \) values of between 92 \% and 100 \% (Figure 4). By transferring these main
clusters to a schematic map of the sample area it is again possible to demonstrate a clear spatial
distribution. Samples in Cluster I are restricted to a narrow strip in the western section of the sample
area. The 29 plots included in Cluster II dominate the central section of the sample area, whereas
samples from Cluster III only occur in the western section. Samples from Cluster IV dominate in the
eastern section. Plots 8, 9, 12, 19, 25, 30 and 44 nevertheless represent outliers found far further west
than the other 33 samples in Cluster IV (see Figure 4).

4. Discussion

4.1. Sampling and molecular genetic methods

The aim of the study presented in this paper was to describe the genetic structure of \( R. pseudotsugae \) in
two stands of Douglas firs in Germany. The achievement of that aim represented a special challenge,
not least because of the limited amount of knowledge available on the life of the fungus. The
successful cultivation of the obligate biotrophic needle parasite has yet to be reliably reported. The
report on the cultivation of the fungus from marine sponges (Porifera) published by Sweet et al. (2015)
is to be viewed with a critical eye given both the limited amount of knowledge currently available on
the biology of \( R. pseudotsugae \) and the author’s own unsuccessful attempts at cultivation. With this in
mind, the study at hand therefore required the preparation of infected needles featuring fruiting bodies
of \( R. pseudotsugae \). Another critical issue which accompanied the investigation of the genetic structure
of \( R. pseudotsugae \) was the selection of an appropriate marker technique. In recent years the
application of molecular genetic methods has been restricted to the development of fungus-specific
primers designed to facilitate early infection detection (Catal 2002, Catal et al. 2010) and
investigations into the spread of the fungus (Morgenstern et al. 2013, 2014). BPS and SCoT marker
techniques (Collard & Mackill, 2009, Xiong et al. 2011a) were selected for the study at hand due to the
lack of species-specific markers suitable for the analysis of the population genetics of *R. pseudotsugae*
(e.g. SSR).

The adoption of the aforementioned techniques for the investigation of *R. pseudotsugae* was inevitably
accompanied by issues related to sample contamination and the reproducibility of results. Both of
these issues are of relevance to the interpretation of genetic data and therefore require brief discussion
here. It is important to underline that contamination with foreign DNA cannot be ruled out completely.
As far as was possible the risk of contamination was minimized by selecting ripe, self-enclosed
fruiting bodies of *R. pseudotsugae*, with direct PCR used in order to minimize the amount of material
required. Contamination with material from closely related species within the genus *Rhabdocline* can
essentially be ruled out due to the fact that to date only *R. pseudotsugae* has been verified to exist in
Europe (Stephan 1973, Catal et al. 2010). The degree to which a sample may be contaminated with
Douglas fir DNA is more difficult to assess. As a result eight Douglas firs were included in the study.
The results of the cluster analysis showed the Douglas fir samples to form an independent cluster and
therefore clearly differentiate themselves from the *R. pseudotsugae* samples. Douglas fir DNA can
therefore be assumed to have exerted only a limited influence on the analysis of the *R. pseudotsugae*
samples.

Reproducibility of results is a fundamental weakness of anonymous marker techniques. The BPS and
SCoT marker techniques selected use much longer oligonucleotides as PCR primers and therefore
promise a high level of both polymorphism and reproducibility of results (Collard & Mackill, 2009,
Xiong et al. 2011a). Reproducibility was successfully verified with the aid of DNA samples taken
from the Douglas firs. Another aspect which requires particularly careful consideration when using
anonymous marker techniques is DNA concentration. As a general rule it is recommended that the
amount of DNA used is defined as precisely as possible and optimized with the aid of testing. Tests carried out on the genus *Fusarium* using RAPD-PCR have nevertheless found DNA concentrations of between 2.5 ng and 250 ng to exert a negligible influence on amplification (Möller et al. 1994). In the study at hand the direct PCR technique selected did not permit the determination of DNA concentration. As each sample was nevertheless to be assumed to include a minimal amount of DNA attempts were made to optimize amplification by varying the amount of template used. Nested PCR was also carried out with the aim of enhancing amplification. Although the technique increases the risk of band artefacts a comparison of the number of fragments amplified did not identify any significant differences between nested (BPS 1, SCoT 35, SCoT 36) and unnested primers (SCoT 3, SCoT 33). It is not possible to rule out the occurrence of false positive and false negative fragments. The distance coefficient defined by Nei & Li (1979) was therefore used to factor this issue into the quantification of genetic differences. Band artefacts exert less of an influence on the Nei & Li distance coefficient than on other methods, thus making it a preferential means of describing genetic relationships (Lamboy 1994).

4.2. Genetic structure of the Rhabdocline *pseudotsugae* collectives

From an overall perspective the *R. pseudotsugae* collectives studied were characterized by a high degree of both genetic variability and genetic diversity. They also exhibited clear spatial differentiation within the two sample areas. By contrast, and despite the considerable geographic distance between them, it was not possible to determine any clear differentiation between the sample areas. The fact that genetic structure can be influenced by myriad factors means that the interpretation of results of this type is not always simple.

The genetic variability of the *R. pseudotsugae* collectives far exceeded the number of fragments observed in previous work using BPS and SCoT markers (Xiong et al. 2011a, b, Amirmoradi et al. 2012, Bhattacharyya et al. 2013, Mulpuri et al. 2013, Satya et al. 2015). Comparisons are nevertheless hindered to a particularly significant extent by the differing size of the sample groups and the various types of species included in the study. In the case of *R. pseudotsugae* a high degree of genetic diversity...
was nevertheless to be expected due to the high level of genetic variability generally observed in organisms with sexual reproductive systems (Winton et al. 2007, Kraj & Kowalski 2008, Kraj et al. 2010, Burokiene et al. 2015).

The formation of spatial structures within and between the *R. pseudotsugae* collectives studied requires more differentiated analysis. As a general rule the genetic distances identified within and between the sample areas were relatively low. Although the use of bulk samples (i.e. samples containing multiple ascospores) can lead to the underestimation of genetic distance, it is nevertheless clear that the genetic structures of the *R. pseudotsugae* collectives studied exhibit a relatively high degree of correlation. This is reflected particularly distinctly in the low level of genetic differentiation between the two sample areas (Cunnersdorf and Bad Berleburg). Gene flow and migration counteract differentiation between populations. The wind-assisted distribution of fungal spores is not uncommon amongst fungi, and facilitates exchange between populations even if separated by considerable distances. Intensive gene flow has been observed in examples such as *M. larici-populina* (Barrès et al. 2008) and *H. fraxineus* (Kraj et al. 2010, Burokiene et al. 2015) in Europe as well as *C. ribicola* in North America (Et-touli et al. 1999, Hamelin et al. 2000). Although *R. pseudotsugae* is also distributed by wind-carried ascospores, distribution over large distances is nevertheless somewhat unlikely due to their gelatinous sheath (Van Vloten 1932). The *R. pseudotsugae* collectives studied are therefore assumed to have originated from the same population. One decisive reason for this assumption is the way in which the stands were created. *Rhabdocline pseudotsugae* has already been confirmed to exist in Douglas fir buds, cambial tissue and embryos (Morgenstern et al. 2013, 2014). As the sampled Douglas firs were bred by crossing specific seed and pollen parents it is not possible to rule out the transmission of the fungi in the flowers or pollen of an infected parent. When the sample areas were created in 1993 it would therefore only have been possible for a limited number of genotypes to spread, with new genetic variants only able to emerge further to the sexual reproduction of *R. pseudotsugae*. It is unfortunately unknown when the symptoms of Rhabdocline needle cast were first observed in the sample areas. The low level of genetic differentiation nevertheless shows that there was insufficient time between
planting and sampling for significant differentiation to occur. The spatial distribution of the clusters within the individual sample areas essentially supports the hypothesis that it is ascospores that spread the fungus within a restricted area. Intensive gene flow within the sample areas would lead to a far more homogeneous picture. Distribution by means of ascospores – and in turn exchange between genetic variants – nevertheless occurs. This notwithstanding, reproduction is likely to tend to take place within an individual tree or between trees which stand close together. This is reflected in the considerable similarities between neighbouring plots observed in subordinate clusters in Bad Berleburg in particular. Although the data yielded by samples taken from Cunnersdorf broadly confirms this reproductive hypothesis it also describes the somewhat more differentiated distribution of genetic variants within the main clusters. To give an example, the reduction in the size of the Cunnersdorf stand may have influenced the genetic structure within the sample area. The removal of potential hybrid parents also increased the distance between host trees, thus hindering the spread of *R. pseudotsugae* and in turn gene flow. The potential role of seed and pollen parents as sources of infection certainly played a role in the formation of the clusters described in this paper. Data on the genetic structure of *R. pseudotsugae* within parent trees was not available. A Douglas fir parent is nevertheless generally used as a flower or pollen donor by multiple descendants, which may promote the distribution of particular genotypes of the fungus. This could explain the outliers observed in Bad Berleburg (Cluster I) and Cunnersdorf (Cluster IV).

The variety of the Douglas firs sampled does not appear to exert a significant impact on the development of spatial structures, with *R. pseudotsugae* found to be evenly distributed throughout both sample areas. With the exception of three hybrids no significant differences were identified between hybrid varieties in terms of their intensity of infestation. Potential partners were therefore theoretically available for *R. pseudotsugae* throughout both sample areas. It is also entirely conceivable that genetic variability has been influenced by local conditions. Similar situations have already been discussed in work on fungi including *H. fraxineus* (Kraj et al. 2010), *G. abietina* (Kraj & Kowalski 2008) and *C. ribicola* (Hamelin et al. 2000). In the present study the regular planting of beech in Cunnersdorf and
the mixing of Douglas firs with spruces (as observed at both sample areas) may well have impeded
gene flow between populations of *R. pseudotsugae*. Definitive clarification of this issue would require
the consideration of not only understorey vegetation but also the level of canopy cover, the vitality of
host trees and weather conditions during spore dispersal.

From an overall perspective the results of the study presented in this paper demonstrate that the
ascospore-based distribution of *R. pseudotsugae* seems to be restricted to short distances and that
infected plant material plays a far greater role in the spread of the fungus than previously assumed.

Other issues of interest naturally include the extent to which genetic structure changes over the long
term, the factors which have the greatest influence on the changes involved (e.g. selection, mutation,
genetic drift, gene flow and migration) and how the resultant genetic structure differs from that of
other populations. Regardless of new insights in these areas, it is important that the findings on the
distribution of *R. pseudotsugae* presented in this paper are given greater consideration in the nursery
sector and that existing management strategies are expanded to include suitable control systems.

Acknowledgments

We would like to express our thanks to the state forest authorities for the supply of plant material. We
would also like to thank all anonymous referees for their helpful comments and for contributing to the
improvement of the manuscript. The study was financially supported by the Federal Ministry of

Literature cited

Amirmoradi, B., Talebi, R. & Karami, E. 2012. Comparison of genetic variation and differentiation
among annual *Cicer* species using start codon targeted (SCoT) polymorphism, DAMD-PCR, and ISSR

Institute, CAB International, Wallingford, UK.

poplar rust fungus *Melampsora larici-populina*: Evidence for isolation by distance in Europe and


### Table 1. Direct PCR: pipetting instructions and cycling protocol

<table>
<thead>
<tr>
<th>Phire Plant Direct PCR Kit</th>
<th>per sample</th>
<th>Cycle step</th>
<th>Temp.</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase free water</td>
<td>8.1 µl</td>
<td>Initial denaturation</td>
<td>98°C</td>
<td>5'</td>
<td>1</td>
</tr>
<tr>
<td>2x Phire Plant PCR Buffer</td>
<td>10.0 µl</td>
<td>Denaturation</td>
<td>98°C</td>
<td>5''</td>
<td></td>
</tr>
<tr>
<td>Primer (10 µM)</td>
<td>1.0 µl</td>
<td>Annealing</td>
<td>T&lt;sub&gt;A&lt;/sub&gt;°C</td>
<td>5''</td>
<td>40</td>
</tr>
<tr>
<td>Phire Hot Start II DNA Polymerase</td>
<td>0.4 µl</td>
<td>Extension</td>
<td>72°C</td>
<td>30''</td>
<td></td>
</tr>
<tr>
<td>Dilution template</td>
<td>0.5 µl</td>
<td>Final extension</td>
<td>72°C</td>
<td>1'</td>
<td>1</td>
</tr>
<tr>
<td>∑</td>
<td>20.0 µl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. List of primers used (Eurofins Genomics GmbH, Ebersberg, Germany) including primer name, primer sequence, annealing temperature (T<sub>A</sub>) and bibliographical references

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5'-3'</th>
<th>T&lt;sub&gt;A&lt;/sub&gt;°C</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPS 1</td>
<td>GCGACGGGTGTACTGAC</td>
<td>54</td>
<td>Xiong et al. 2011a</td>
</tr>
<tr>
<td>SCoT 3</td>
<td>CAACAATGGCTACCACC</td>
<td>56</td>
<td>Collard &amp; Mackill, 2009</td>
</tr>
<tr>
<td>SCoT 33</td>
<td>CCATGGCTACCACC</td>
<td>61</td>
<td>Collard &amp; Mackill, 2009</td>
</tr>
<tr>
<td>SCoT 35</td>
<td>CATGGCTACCACCGC</td>
<td>63</td>
<td>Collard &amp; Mackill, 2009</td>
</tr>
<tr>
<td>SCoT 36</td>
<td>GCAACAATGGCTACCAC</td>
<td>56</td>
<td>Collard &amp; Mackill, 2009</td>
</tr>
</tbody>
</table>

### Table 3. Nested PCR: pipetting instructions and cycling protocol

<table>
<thead>
<tr>
<th>TopTaq Master Mix</th>
<th>per sample</th>
<th>Cycle step</th>
<th>Temp.</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase free water</td>
<td>9.5 µl</td>
<td>Initial denaturation</td>
<td>94°C</td>
<td>3'</td>
<td>1</td>
</tr>
<tr>
<td>2x Phire Plant PCR Buffer</td>
<td>12.5 µl</td>
<td>Denaturation</td>
<td>94°C</td>
<td>30''</td>
<td></td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1.0 µl</td>
<td>Annealing</td>
<td>T&lt;sub&gt;A&lt;/sub&gt;°C</td>
<td>30''</td>
<td>35</td>
</tr>
<tr>
<td>Primer (10 µM)</td>
<td>1.0 µl</td>
<td>Extension</td>
<td>72°C</td>
<td>1'</td>
<td></td>
</tr>
<tr>
<td>PCR template</td>
<td>1.0 µl</td>
<td>Final extension</td>
<td>72°C</td>
<td>10'</td>
<td>1</td>
</tr>
<tr>
<td>∑</td>
<td>25.0 µl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Post-hoc test: p values for pairwise comparisons between Douglas fir varieties using the Nemenyi test with chi-squared approximation for independent samples (1<sup>st</sup> position = seed parent; 2<sup>nd</sup> position = pollen parent; v = viridis; g = glauca; c = caesia), significant differences are marked in bold

<table>
<thead>
<tr>
<th>Varietät</th>
<th>cc</th>
<th>cg</th>
<th>cv</th>
<th>gc</th>
<th>gg</th>
<th>gv</th>
<th>vc</th>
<th>vg</th>
</tr>
</thead>
<tbody>
<tr>
<td>eg</td>
<td>0.978</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cv</td>
<td>0.999</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>gc</td>
<td>0.214</td>
<td>1.000</td>
<td>0.915</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gg</td>
<td>0.981</td>
<td>1.000</td>
<td>1.000</td>
<td>0.992</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gv</td>
<td><strong>0.041</strong></td>
<td>0.991</td>
<td>0.633</td>
<td>0.999</td>
<td>0.884</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vc</td>
<td>1.000</td>
<td>0.968</td>
<td>0.997</td>
<td>0.488</td>
<td>0.975</td>
<td>0.216</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5. *R. pseudotsugae* samples: Total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (PPB), absolute frequency of bands (aFB), relative frequency of bands (rFB) and expected heterozygosity ($H_e$) per locus and across all loci analysed (pool), split according to the sample area in Cunnersdorf (Cu, 101 samples), the sample area in Bad Berleburg (83 samples) and the overall collective (184 samples)

<table>
<thead>
<tr>
<th>Locus</th>
<th>TNB</th>
<th>NPB</th>
<th>PPB [%]</th>
<th>aFB</th>
<th>rFB [%]</th>
<th>$H_e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPS 1</td>
<td>Overall</td>
<td>68</td>
<td>68</td>
<td>100</td>
<td>1 – 59</td>
<td>0.21 – 5.66</td>
</tr>
<tr>
<td>Cu</td>
<td>50</td>
<td>50</td>
<td>100</td>
<td>2 – 33</td>
<td>0.41 – 6.80</td>
<td>0.969</td>
</tr>
<tr>
<td>BB</td>
<td>50</td>
<td>50</td>
<td>100</td>
<td>1 – 43</td>
<td>0.18 – 7.71</td>
<td>0.967</td>
</tr>
<tr>
<td>SCoT 3</td>
<td>Overall</td>
<td>64</td>
<td>64</td>
<td>100</td>
<td>1 – 74</td>
<td>0.10 – 7.51</td>
</tr>
<tr>
<td>Cu</td>
<td>44</td>
<td>44</td>
<td>100</td>
<td>1 – 38</td>
<td>0.17 – 6.45</td>
<td>0.966</td>
</tr>
<tr>
<td>BB</td>
<td>52</td>
<td>52</td>
<td>100</td>
<td>1 – 36</td>
<td>0.25 – 9.09</td>
<td>0.963</td>
</tr>
<tr>
<td>SCoT 33</td>
<td>Overall</td>
<td>81</td>
<td>81</td>
<td>100</td>
<td>1 – 63</td>
<td>0.08 – 5.29</td>
</tr>
<tr>
<td>Cu</td>
<td>66</td>
<td>66</td>
<td>100</td>
<td>1 – 52</td>
<td>0.14 – 7.42</td>
<td>0.971</td>
</tr>
<tr>
<td>BB</td>
<td>54</td>
<td>54</td>
<td>100</td>
<td>1 – 37</td>
<td>0.20 – 7.54</td>
<td>0.969</td>
</tr>
<tr>
<td>SCoT 35</td>
<td>Overall</td>
<td>78</td>
<td>78</td>
<td>100</td>
<td>1 – 80</td>
<td>0.09 – 6.94</td>
</tr>
<tr>
<td>Cu</td>
<td>62</td>
<td>62</td>
<td>100</td>
<td>1 – 55</td>
<td>0.15 – 8.46</td>
<td>0.971</td>
</tr>
<tr>
<td>BB</td>
<td>50</td>
<td>50</td>
<td>100</td>
<td>1 – 31</td>
<td>0.20 – 6.18</td>
<td>0.970</td>
</tr>
<tr>
<td>SCoT 36</td>
<td>Overall</td>
<td>58</td>
<td>58</td>
<td>100</td>
<td>1 – 71</td>
<td>0.08 – 5.57</td>
</tr>
<tr>
<td>Cu</td>
<td>50</td>
<td>50</td>
<td>100</td>
<td>1 – 54</td>
<td>0.16 – 8.50</td>
<td>0.962</td>
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<tr>
<td>BB</td>
<td>47</td>
<td>47</td>
<td>100</td>
<td>1 – 40</td>
<td>0.16 – 6.26</td>
<td>0.969</td>
</tr>
<tr>
<td>Pool</td>
<td>Overall</td>
<td>349</td>
<td>349</td>
<td>100</td>
<td>1 – 80</td>
<td>0.02 – 1.42</td>
</tr>
<tr>
<td>Cu</td>
<td>272</td>
<td>272</td>
<td>100</td>
<td>1 – 55</td>
<td>0.03 – 1.80</td>
<td>0.968</td>
</tr>
<tr>
<td>BB</td>
<td>253</td>
<td>253</td>
<td>100</td>
<td>1 – 43</td>
<td>0.04 – 1.66</td>
<td>0.968</td>
</tr>
</tbody>
</table>
Figure 1. Rating system for intensity of infestation with *R. pseudotsugae* including indication of the hybrid varieties found on each plot (v = viridis; g = glauca; c = caesia; na = data not available): (a) Rating system for *R. pseudotsugae* according to Röllich 2011; (b) Sample area in Bad Berleburg; (c) Sample area in Cunnersdorf
**Figure 2.** Dendrogram (UPGMA) showing p-values for the overall collective (*R. pseudotsugae* samples from Bad Berleburg and Cunnersdorf, Douglas fir samples)
**Figure 3.** Bad Berleburg sample area: Dendrogram (UPGMA) including p values and schematic map of the sample area, colour-coded according to clusters.
Figure 4. Cunnersdorf sample area: (a) Dendrogram (UPGMA) including p values; (b) schematic map of the sample area, colour-coded according to clusters.