Common Ash Stand Affected by Ash Dieback in the Wolica Nature Reserve in Poland

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Abstract

The ash stand in Wolica reserve (Poland), affected with ash dieback, was studied. Isolations performed from collected ash tissues and rhizosphere soil samples revealed 28 isolates of *Hymenoscyphus fraxineus* and 27 isolates of *Phytophthora* spp., respectively. The vitality and defoliation of 198 and 176 trees were studied, respectively in 2012 and 2013. In 2013 only one tree was completely vital, while 83 trees were within the degeneration phase. These results suggested that trees being classified in this class are the most vital and the natural genetic resistance should be sought among this vitality class in the future. In total, 112 trees were classified to the stage 2 of vitality, for which large deformation of shoots is typical. Further, monitoring of defoliation in 2013 revealed that the largest number of trees in the stand (126) were moderately damaged trees (defoliation 26-60%), while 47 trees had over 60% of defoliation. The synthetic damage index was 1.58 in 2012 and 1.66 in 2013 indicating that advanced disease processes are occurring in this stand. In addition, sampling, isolation, morphological and molecular identifications of *Phytophthora* species were performed. After the isolation tests, *P. megasperma*, *P.* sp. hungarica, and *P. plurivora* were obtained. These results were confirmed after the PCR and ITS sequencing. This is the first report of *P.* sp. hungarica and *P. megasperma* in the stands of common ash in Poland. The natural genetic variation of the *Fraxinus excelsior* genome was studied to improve understanding of its role in the adaptation and tolerance processes facing ash dieback phenomenon. Six nuclear microsatellite markers and four chloroplast microsatellite markers have been used in order to assess the genetic diversity of *Fraxinus excelsior* stand in Poland, categorized into three different Roloff' classes of vitality 0+1, 2 and 3. We demonstrated lack of correlation between three different vitality classes of ash trees and their nuclear or chloroplast genetic differentiation. Nevertheless, the observed heterozygosity (H_O) value was significantly different between vitality classes 2 and 3 assessed with nuclear SSR markers (p = 0.000183 in HSD Tukey test, $p < 0.05$). Also private (*A*p) alleles distribution of chloroplast SSR markers significantly differ (p = 0.000 in HSD Tukey test, $p < 0.05$) between the vitality classes 0+1 and 3 of ash trees. Those data suggest that DNA differentiation of *F. excelsior* at local spatial scale may be driven by gene based tolerance.

Keywords: Ash dieback, *Fraxinus excelsior*, defoliation, vitality, *Phytophthora* spp., nuclear and chloroplast SSR markers

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Introduction

The phenomenon of dieback of European ash (*Fraxinus excelsior* L.) has been observed in the last decade in most European countries (Halmschlager and Kirisits 2008, Szabó 2009, Chandelier et al. 2010, Rytkönen et al. 2011, Husson et al. 2011). This new disease causing mass mortality of trees forced foresters to refrain temporary from cultivating ash as forest tree species, any more. For the first time the symptoms were observed in the early 1990s in North-Eastern Poland and Lithuania (Kowalski 2006, Timmermann et al. 2011). Since that time the disease spread across Europe causing severe mortality of ash trees. The research on ash dieback in Poland was conducted by many researchers but the findings of Professor Tadeusz Kowalski from the University of Agriculture in Cracow led to the discovery and identification of a new fungus species *Chalara fraxinea* Kowalski (Kowalski 2006) an asexual stage of *Hymenoscyphus pseudoalbidus* Queloz. It is accepted to be the cause of mass dieback of ash trees in Europe (Kowalski and Holdenrieder 2009 a, b). The currently recognized scientific name for the fungus causing ash dieback in Europe is *Hymenoscyphus fraxineus* (Baral et al. 2014). According to available data, the last country where *H. fraxineus* was recorded on common ash is Bosnia and Herzegovina (Stanivuković et al. 2014). Next to common ash, narrow-leafed ash was also reported to be susceptible on infections with *H. fraxineus* (Kirisits et al. 2010).

Parallel to studies of *Chalara* in ash dieback phenomenon, the appearance of pathogens from the *Phytophthora* genus in the rhizosphere soil of ash trees was connected with rot of root collars and decay and loss of fine roots, leading consequently to the dieback of many trees (Orlikowski et al. 2011, Akilli et al. 2013). In the last decade, many studies reported the occurrence of soil borne *Phytophthora* species in relation to the severe damage of significant forest tree species. The fungal- or -algae like pathogens of the genus *Phytophthora* are distributed worldwide (Erwin and Ribeiro 1996). Till now, over 140 *Phytophthora* species have been described from a broad range of plant hosts in agriculture and forestry (Abad 2014). Nevertheless, the knowledge about *Phytophthora* species associated with ash trees is still very limited, and there are few reports about *Phytophthora* isolations from ash trees (Orlikowski et al. 2004, Orlikowski et al. 2011). Also, the studies carried out by Orlikowski et al. (2007 a) have demonstrated that *Phytophthora* species were present in the rhizosphere of ash affected by the fungus *H. fraxineus*.

The Wolica nature reserve was established by virtue of the order of the Ministry of Forestry and Wood Industry, of the Government of Poland on August 4, 1984. The area is situated in central part of Poland in Forest District Chojnów (RDLP in Warsaw). Among the large number of different species in this reserve, including ecologically very valuable noble hardwoods, department 374c contains European ash dominated stand. The first symptoms of ash dieback in this stand were recorded during the year 1998. Since then, the number of trees with characteristic ash dieback symptoms has grown every year. This phenomenon continues until present. Also, this nature reserve of lowland forest ecosystem was known as an area to be free from alien plant pathogens of the *Phytophthora* genus.

Genetic structure can play an important role in determining fitness of forest tree populations at equilibrium in changing environmental conditions. Gene-based tolerance is the key in modern forest management actions, often examined at the DNA differentiation level of the individual tree in forest stand (Hamrick et al. 1992, Nguyen et al. 2015). Forest tree species have complex genomes, the biggest in size and still poorly understood in structure and function. Nevertheless, several types of molecular markers have been applied in population genetics studies including reproductive mechanisms, gene flow, hybridization processes or adaptive ecology. The nuclear microsatellite DNA loci are nowadays considered to be the most precise tool to determine the genotype of living organisms (Li et al. 2002, Esudero et al. 2003, Semagn et al. 2006). The microsatellite fragments (simple sequence repeats - SSRs) are built from short nucleotide repetitions, usually comprising from 2 to 9 base-pairs, uniformly distributed over the genome. They are characterized by high polymorphism and high mutation rates in comparison to coding sequences (Epperson 2005). Detailed research based on nuclear microsatellite markers for European ash populations was performed by Heuertz et al. (2003, 2004) excluding, however, the populations of ash from Poland.

Another type of markers, the chloroplast SSR loci were also applied to elucidate the genetic variation of many oak, beech or ash populations (Petit et al. 2002, Vornam et al. 2004, Heuertz et al. 2006). Interestingly, the transition zone between chloroplast microsatellite DNA haplotypes has been previously reported in Poland, proving the genotypic richness of the studied ash populations (Heuertz et al. 2003).

The positive correlation between the level of heterozygosity and the adaptation of population during the evolution of many organisms has been observed. The opposite (negative) trend may also occur, when the elimination of harmful alleles and inbreeding process leads to the impoverishment of a gene pool (Reed and Frankham 2003). The selection agents may favour homozygous alleles in the genome, but sometimes they could favour heterozygous alleles by choosing the genetic information suitable for the advantageous adaptive features of a population (Whitlock 2002).

Nevertheless, little is known about the health status of stands affected with ash dieback and about the ongoing

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processes inside the declining stands, especially in the frame of searching for natural genetic resistance against the ash dieback (McKinney et al. 2014). It was recorded in many cases in Poland that stands after appearance of first symptoms and rapid spreading of the disease turn into a stagnation phase according to Roloff vitality classes (Pacia et al. 2011). The application of molecular tools to study the relationship between European ash dieback and natural genetic tolerance/susceptibility has been fairly limited. It seems to be crucial that dead ash trees have to be replaced with more tolerant genotypes before this species completely disappear from forest ecosystem. In this scope, the predictive methodology based on molecular markers may help in successful management of European ash disease mainly caused by pathogenic fungus *H. fraxineus*. Our studies were based on general hypothesis that greater variety of gene pool assessed with SSR markers is positively correlated with vitality class of Roloff among sixty investigated ash trees in Wolica Natural Ash Reserve in central Poland.

Due to the lack of silviculture measurements, which have never been applied in Wolica reserve (Chojnów FD), a study was performed with aims to: i) determine the health status of this ash stand (expressed by defoliation and vitality degrees of trees), ii) confirm the presence of known fungus *H. fraxineus* responsible for ash dieback iii) monitor the occurrence of soil-borne pathogens from the *Phytophthora* genus in the rhizosphere soil of damaged trees, iv) to perform genetic studies on both nuclear and chloroplast SSR markers with the interaction to Roloff vitality classes.

Material and methods

Monitoring of healthiness of ash trees

The study area and inventory of tree health

The stand of ash trees, where the research was conducted is located in the partial nature reserve (coordinates 51.1°N, 10.5°E) in Wolica in central Poland. The total area of the reserve is 50.39 ha, including the separated stand of ash (374c) which makes the object of the research area of 1.77 ha.

Sampling, isolation and identification of H. fraxineus from symptomatic ash tissues

Sampling, isolation and identification were done in 2012, according to the methodology of Kowalski (2006). Samples with symptomatic tissues were collected over the whole stand area from 30 chosen 22-year-old ash trees (2-3 samples per tree). Tissue samples were collected from infected stems and shoots up to 3 m high (of 20 m high trees), by cutting and placing them in separated plastic bags, immediately subjected to the laboratory analysis. Isolation was done in the way that 3-4 mm big pieces were taken between necrotic and healthy areas, using the scalpel sterilized in 70% ethanol and burned on the open flame. Pieces were surface sterilized for 3 minutes in 1% sodium hypochlorite with 4% active chlorine, three times washed in sterile distilled water and plated placed onto Malt Extract Agar media-MEA (18 g/l of Malt Extract (MERCK, Germany), and 18 g/l of agar (BTL, Poland). Petri dishes were incubated at 18-20°C in the dark, and observed daily for the presence of hypha. After appearance of first hypha, they were transferred with sterile mycological needle onto fresh MEA media and incubated at ~20°C in the dark.

Four-weeks-old cultures, incubated at ~20°C in the dark, were observed under the light microscope ZEISS Axioskop 2, equipped with Nikon Ds-fi1 camera, and NIS Elements AhR4® software. In parallel, pure cultures were incubated for four weeks on the MEA media for the purposes of colony shape patterns.

Identification of isolates was done based on the colony shape of pure cultures, incubated for four weeks in the dark at 20°C, the shape and size of phyalides observed in the pure cultures and based on previously registered symptoms. Not to rely solely on morphological characters the molecular based methods were employed for the confirmation of the presence of *H. fraxineus.*

Vitality test based on the extent of trees damage

The vitality test was performed using the method developed by Roloff (1989), in which the vitality is shown as tree growth potential and its ability to regenerate the damaged crown. The basis for the assessment of the vitality is the architecture of shoots produced in the upper part of the crown. This assessment takes into account not only the current condition but also the changes visible in the form of shoot deformation as an effect of ongoing processes over past several years. The vitality assessment of ash trees in department 374c was conducted in May 2012 and 2013. The analyses of vitality and defoliation were conducted by the same observer to minimize subjectivity and biases, and that is why the calibration was not necessary. All the trees were classified into one of four groups distinguished on the basis of differences in vitality (Table 1).

Table 1. Vitality degrees (Roloff 1989)

Vitality degree	Damage level
0	Exploration phase, untouched trees, vital ones
1	Degeneration phase, weakened trees
2	Stagnation phase, damaged trees
3	Resignation phase, badly damaged trees, decaying

Assessment of defoliation

The assessment of crown defoliation of ash trees was carried out in May 2012 and 2013. It was performed according to the criteria ICP Forests protocol shown in Table 2. In our experiment the defoliation was estimated as

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the lack of leaves in the canopy at the beginning of the vegetation season in order to avoid damage caused by insects or to be attributed to premature shedding as a result of *H. fraxineus* activity and in this way affecting on our evaluation. Example of ash tree growing in Wolica reserve, described as defoliation 40% (ICP Forests 26-60%) and according to Roloff 2 vitality class (stagnation phase) is shown in Figure 1.

Table 2. Defoliation degrees according to the damage scale

Defoliation (%)	Damage level
0 - 10	0 (no symptoms of damage)
11 - 25	1 (slight damage)
26 - 60	2 (moderate damage)
above 60	3 (strong damage)

Figure 1. Example of ash tree growing in Wolica reserve, described as defoliation 40% (ICP Forests 26-60%) and according to Roloff 2 vitality class (stagnation phase)

The synthetic damage index of trees

Based on the obtained data concerning defoliation and vitality the synthetic damage index of trees was calculated (Dmyterko et al. 2003). Compared to the assessment of vitality and assessment of defoliation applied separately, the synthetic damage index allows for a more objective assessment of the health of trees and stands (Dmyterko 1998). The synthetic damage index was calculated according to the method of Dmyterko et al. (2003), using the following formula:

[1] Syn = 1/2 (0.03 * Def + Wit)

where *Def* – defoliation is expressed in percentage, *Wit* - tree vitality is expressed by the degree of damage. The damage index for the whole separation was achieved following the methodology of Dmyterko (1998):

[2] Syn_wydz = ½ [(0.03 * total sum Def + Wit) / N],

where the *Def* sum - the sum of the percentage of defoliation, *Wit* - the sum of degrees of vitality, *N* - number.

In addition, for assessment of vitality and defoliation, the photographic documentation of the trees was collected from the points which allowed clear separation of crowns from the background of neighbouring trees.

Sampling, isolation and morphological identification of Phytophthora species

Sampling and isolation of pathogens from the *Phytophthora* genus were performed according to the methodology of Jung (2009) and Jung et al. (1996, 2000). Soil and fine roots were collected in the form of soil monoliths ~25×25×25 cm, and two monoliths per tree were taken at the distance of 0.8-1 m from the tree base, and mixed before the isolation. In total, nine symptomatic trees were randomly chosen and sampled from different parts of the stand. Isolation tests were performed using the method of bait (Jung et al. 1996, 2000, Jung 2009), and young pedunculate oak and beech leaves were used as baits. After appearance of the first necrotic spots on the leaves the small pieces of damaged plant tissues were cut with a sterile scalpel and placed onto selective agar media (V8A PARPNH) (Jung at al. 1996, 2000, Jung 2009). The first hypha was taken and transferred on the fresh V8 agar media.

The obtained isolates were morphologically characterized by flooding the pieces of young colonies, grown on CA media, in the non-sterile soil extract, and with observation of four-weeks-old cultures, incubated at 20-22°C in the dark (Erwin and Ribeiro 1996). Sexual and asexual structures were observed at ×400 magnifications using the ZEISS Axioskop 2 microscope, and 50 structures per observed isolate were measured using the Nikon Ds-fi1 camera and NIS Elements AR 4® software. Observed features were compared with identification keys (Stamps et al. 1990, Erwin and Ribeiro 1996), as well as with papers and reports with recently described species (e.g. Jung et al. 1999, 2002, 2003, Brasier et al. 2003, Jung and Nechwatal 2008, Jung and Burgess 2009, Bakonyi et al. 2012).

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Molecular identification of obtained Phytophthora spp. isolates

After the detailed morphological classification, small pieces from the edges of young colonies were transferred on liquid V8 media (900 ml/l of distilled water, 100 ml/l of V8 juice (Tymbark, Poland), 3 g/l $CaCO_3$), and incubated at 22-25ºC in the dark. After 3-5 days of incubation, the mycelium was collected and smashed in liquid nitrogen, after washing in sterile distilled water. The DNA was extracted by using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich® GmbH, Germany), according to the company recommendations. ITS amplifications of *Phytophthora* isolates were performed using the ITS4 and ITS6 primers (White et al. 1990, Cooke et al. 2000). The amplification reaction mixture contained 1 x PCR buffer [75mm Tris-HCl (pH 9.0), 50mM KCl, 20 mM $(NH_4)_2$ SO4]; 1×Q solution, 0.2 mM dNTPs, 0.25 mM of each primer; 1 mM $MgCl_2$; 1U of *Taq* Polymerase (Qiagen Ltd., Valencia, CA, USA); and 1 µL of mycelial DNA in a total volume of 25 µL. Reactions were performed in PTC-200™ Programmable Thermal Controller (MJ Research, Inc.) machine, and PCR protocol was as follows: 3 min of initial DNA denaturation at 94°C and 35 cycles of amplification (30 sec of annealing at 55°C, 60 sec of elongation at 72°C), and 5 min of final elongation at 72°C.

Amplified products were analyzed by 1.5% TBE-agarose gel electrophoresis, stained with 6×Orange DNA Loading Dye, and visualized under a UV transilluminator. The presence of a single band (ca. 800 bp) was considered as a positive reaction. The PCR products were cleaned using the A&A Biotechnology (Gdynia, Poland) Clean-up kit, following the manufacturer's protocol. Sequencing was conducted on CEQ™8000 9.0.25 automated sequencer (Beckman Coulter®, Fullerton, USA), using ITS4 and ITS6 primers. The consensus sequences were aligned from two-directional sequencing (Zhang et al. 2000), and compared with sequences in GenBank (http://blast.ncbi.nlm.nih.gov /Blast.cgi) using the ClustalW program (Thompson et al. 1994) and MEGA6 software (Tamura et al. 2013).

Correlation between the DNA differentiation of ash trees and vitality classes in Wolica Reserve

Population differentiation was determined at a local spatial scale in Wolica Ash Reserve, where three different health classes among sixty ash trees were examined, i.e. class 0 and 1 – 0-25 % of defoliation (represented by 23 trees), class 26 – 60 % of defoliation (21 trees) and class above 60 % of defoliation (16 trees, respectively).

From all investigated trees total DNA was extracted using the NucleoSpin® Plant II (Macherey-Nagel), following the manufacturer's protocol except some modifications in volume: 600 µl of PL2, 150 µl of PL3 and 900 µl of PC buffer. Six nuclear microsatellite and four chloroplast loci in two multiplexes (A and B) were amplified. The multiplex A consisted in five loci: Femsatl-4, Femsatl-8, Femsatl-19, ccmp3, ccmp6; and multiplex B in five loci: Femsatl-11, Femsatl-16, M2-30, ccmp7, ccmp10 (Sutherland et al. 2010). The Multiplex PCR Kit (Qiagen®) was used for both multiplexes preparation and the PCR reactions were carried out in 10 µl reaction volumes with 5–50 ng DNA, 10 pmol of each primer labelled with fluorescent WellRED dyes (Beckman Coulter, Inc.), 5 µl Multiplex PCR Master Mix, followed by the PCR thermal amplification profile described by Sutherland et al. (2010). All samples were genotyped and the allele lengths were scored on a CEQ 8000 sequencer (Beckmann-Coulter, Inc.).

The observed (n_a) and expected (n_e) number of alleles, observed (H_E) and expected heterozygosity (H_O), mean Shannon index (I), general heterozygosity (h), inbreeding coefficient of an individual relative to the subpopulation (F_{IS}), inbreeding coefficient of an individual relative to the total (F_{IT}), and fixation index (F_{ST}) were calculated in GenAlEx 6.501 software (Peakall and Smouse 2012). The frequency of private alleles (Ap) was determined for the unique alleles present only once in analysed vitality classes of trees.

For nuclear SSR markers, F statistics was conducted with ANOVA, and the model-based clustering method with STRUCTURE 2.1 software (Pritchard et al. 2000). STRUCTURE assigned individual multilocus genotypes probabilistically to a defined number K from 2 to 3 clusters or gene pools, and was run three times for 100 000 iterations after a burn-in period of 50 000 on the total dataset (n = 60).

The test of departure from Hardy–Weinberg equilibrium was chosen for $P = 0.05$. Gene flow among groups of trees was estimated from F_{ST} using the formula $N_m = 0.25(1/ F_{ST} - 1)$ (where N is effective population size and m is a fraction of migrants per generation).

For both types of markers, the Pearson coefficient (r) of correlation between genetic parameters, i.e. n_a, n_e, I, H_O, H_E and Ap and their distribution in three groups of trees (representing classes: 0 and 1, 2 and 3) was analyzed in STATISTICA ver. 10.

Results

Monitoring of healthiness of ash trees

Registered symptoms, isolation and identification of Hymenoscyphus fraxineus

Different symptoms were recorded on ash trees in this stand, including increased crown transparency, wilting and premature shedding of leaves, dying of shoots and branches, dieback of the top of the crowns, decline of trees, and necrosis and canker on the stems, branches and shoots. The asexual stage of *H. fraxineus* (*Chalara fraxinea*) was isolated from 27 out of 40 plated, randomly chosen sam-

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ples, taken from the edges of necrotic zones and surface sterilized as described above. In total, 28 isolates were obtained.

Most of the isolates were of white to light orange colony color, after four weeks incubation at 20°C in the dark, and with hyaline, slightly immersed. Also, few isolates had an intensive orange to brown color of the colonies. Color from the bottom side varied from light orange, light brown to, less often dark brown. Growth of the colonies was slow, and after three weeks at 20°C in the dark on MEA media, total colony diameter averaged 24.4±2.22 mm, with range of 21-28 mm (N=5).

In the four-weeks-old cultures, incubated in the dark at 20°C, numerous phialides were observed on both terminal hypha and on branched or simple phialophores. Phialides were subcylindrical, obclavate and less often spindle shaped, with average total length of 19.7±3.42 µm (range 14.3-25.6 µm). Width of ventral part of phialides averaged 4.4±0.41 µm, with range 3.7-5.3 µm. Phialoconidia were observed in chains, or they were dispersed in droplets. Phialoconidia were hyaline, unicellular, with smooth walls and cylindrical shape, and with one or two oil droplets.

Vitality assessment

The assessment of vitality was performed for 198 ash trees in 2012 and for 176 in 2013. In 2012, only one tree was classified as class 0 – intact - vital class of trees. The trees weakened in the degeneration stage (class 1) included 83 trees (42%). The largest number of trees (100 trees, 51%) was classified into the second class of vitality - trees damaged in a phase of stagnation. The third class (resignation phase, trees badly damaged and dying) included 14 ash trees (7%) (Table 4). In 2013 the vitality dropped in class 1 and 2 reaching values 63 and 92 trees, respectively. In class 3 the number of trees increases from 14 to 20 trees. The obtained results indicate going on long-term stress impact, lasting perhaps even more than a decade.

Assessment of defoliation

The assessment of defoliation was carried out for 198 ash trees in 2012. There were no trees being intact (the degree of defoliation of 0-10 %). Most trees (126 trees) belonged to the range of 26-60 % of defoliation. A large number of trees belonged to defoliation level above 60% (47 trees). Trees slightly damaged (range 11 - 25%) included 25 ash trees. The number and percentage of trees in each class is shown in Table 3.

In 2013, only further negative changes in the health of the examined trees were noticed. The number of healthy trees has been further reduced (Table 3). Of the 198 trees 22 died and 176 left showing different level of crown damage. The year 2013 proved further developing of the disease process.

Table 3. Number of trees representing defoliation classes expressed in percentage in the year 2012 and 2013

Defoliation (%)	2012		2013	
	Number of trees	(%)	Number of trees	(%)
0-10	0	0	0	0
11-25	25	13	16	9
26-60	126	63	121	69
above 60	47	24	39	22
Total	198	100	176	100

Table 4. Number of trees representing vitality classes expressed as percentage of trees in the years 2012 and 2013

Degree of vitality	2012		2013	
	Number of trees	(%)	Number of trees	(%)
0	1	0	1	1
1	83	42	63	36
2	100	51	92	52
3	14	7	20	11
Total	198	100	176	100

Synthetic damage index

The calculated values of the synthetic damage index for each tree are included in the range from 0.8 (the lowest value) to 2.7 (maximum value), with the theoretical, possible range from 0 to 3. The modification of the model for the determination of synthetic damage index for a single tree allowed for the calculation of the synthetic damage index for the entire stand. The value of this index amounted to 1.58 in 2012 and 1.66 in 2013. The obtained result indicates advancing disease processes occurring in the examined stand.

Isolation and identification of Phytophthora species

After the isolation tests, seven out of nine, randomly taken samples, were positive for the presence of *Phytophthora* species, and 27 isolates were obtained. All the isolates were obtained from rhizosphere soil using the baiting techniques. The highest isolation frequency was from samples number 5 and 9, respectively. Positive and negative samples and number of obtained isolates are shown in Table 5.

After the morphological identification, three different species were identified: *P. plurivora*, *P. megasperma* Drechsler and *P.* sp. hungarica (Table 5). *P. plurivora* and *P. megasperma* are well described species in the literature, but little information was available about *P.* sp. hungarica. The species is homothallic with mycelium slightly sparse aerial in the middle and pressed in the edges. Colony was with shape of rosette and with regular edges on V8, CA, and irregular on MEA and PDA.

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Table 5. Number of obtained isolates from different samples taken

Isolated species and number of obtained isolates	Sample Number									Total
	No 1	No 2	No 3	No 4	No 5	No 6	No 7	No 8	No 9	
P. plurivora	1	-	1	-	8	3	3	3	3	22
P. megasperma	-	-	-	-	-	-	-	-	3	3
P. sp. hungarica	-	-	-	-	-	-	-	-	2	2
Total	1	-	1	-	8	3	3	3	8	27

Table 6. BLAST analyses of sequenced ITS region for selected isolates

No of Sample	Isolate	Species	GenBank Access Number	The closest sequence in the GenBank	Identities (%)	Query cover (%)	E-value
1	IBL277	*P. plurivora*	JX274427	GU259247	100	100	0.0
2	IBL280	*P. plurivora*	JX274426	KM052583	100	100	0.0
3	IBL282	*P. plurivora*	JX274421	KM052583	100	100	0.0
4	IBL287	*P. plurivora*	JX274420	KF234680	100	100	0.0
5	IBL290	*P. plurivora*	JX274424	EU240194	100	100	0.0
6	IBL293	*P. plurivora*	JX274425	KM052583	100	100	0.0
7	IBL294	*P. plurivora*	JX274422	KM052583	100	100	0.0
8	IBL297	*P. megasperma*	JX274423	HM004230	100	100	0.0
9	IBL301	*P.* sp. hungarica	JX274428	EF522144	100	100	0.0
10	IBL302	*P.* sp. hungarica	JX274429	EF522144	100	100	0.0

Species produced big oogonia with average dimensions of 34.38±3.64 × 34.31±3.63 µm, and range of 27.10-45.30 × 26.58-43.80 µm (N=53). Size of antheridia averaged 15.0±3.40 × 10.5±2.20 µm and range 8.0-22.5 × 5.82-17.3 µm (N=32), and they were mostly paragynous with only few amphigynous recorded. Oospores were aplerotic, and few were plerotic with average dimensions 26.7±3.21 × 27±3.04 µm, and range 20.31-33.82 × 19.88-33.5 µm (N=51). Oospore wall thickness ranged from 0.8-3.3 µm, with mean of 1.84±0.89 µm. Sporangia produced in non-sterile extract were nonpapillate, persistent and with regular, ovoid to obpyriform shape. Dimensions of sporangia were 48.2±6.17 × 31.2±2.95 µm, with range 32.23-62.31 × 24.73-37.35 µm (N=55). Length to breadth ratio averaged 1.55±0.19, and range 1.11-1.94. Growth rate at 20°C on CA media was 3.52±0.24 mm/day. The species belongs to ITS clade 6, *sensu* Cooke *et al*. (2000), and it was identified as *Phytophthora* sp. hungarica. Presence of this species, as well as the presence of *P. megasperma* and *P. plurivora* was confirmed during the molecular identification and ITS sequencing (Table 6).

Used ITS4 and ITS6 primers successfully amplified ITS region of selected isolates, and ~700-1100bp products were obtained, respectively. In total ten isolates were sequenced, seven from *P. plurivora*, two from *P.* sp. hungarica and one isolate from *P. megasperma*. After BLAST analyses of obtained sequences, identity of all the obtained sequences with the closest sequence in the GenBank was 100%, with 0% of gaps and Expect value, respectively (Table 6). Sequences were submitted to the GenBank and assigned accession numbers are shown in Table 6.

This is the first report of *P. megasperma* and *P.* sp. hungarica on *Fraxinus excelsior* in Poland.

Correlation between the DNA differentiation of ash trees and vitality classes in Wolica Reserve

Allele frequency

Among highly polymorphic nuclear microsatellite markers selected for this study, the most variable was locus M2 (27 alleles), while the least polymorphic was locus F16 (5 alleles). Among four chloroplast SSR loci examined, the highest variation was observed in ccmp6 and ccmp10 loci (with 5 different haplotypes), and the lowest in ccmp3 locus (2 haplotypes).

Private alleles were present both in nuclear and chloroplast DNA loci, with prevalence in number of 55 alleles in 5 loci in highly polymorphic nuclear SSR markers in comparison to 5 alleles in 3 chloroplast loci examined.

Genetic variation

A summary of genetic variation measures for six nuclear and four chloroplast SSR loci was given in Tables 7 and 8, respectively. Concerning the nuclear SSR marker variation, the highest numbers of observed (n_a) and expected (n_e) alleles per locus, as well as the highest diversity coefficient of Shannon (I) were found in ash trees belonging to the vitality class 0+1. Conversely, the parameters n_a, n_e and Shannon index (I) had the lowest values in the most damaged class 3 of ash trees (Table 7).

The most heterogeneous trees examined with nuclear SSR markers belonged to the Roloff class 2 (H_E = 0.731)

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Table 7. Genetic differentiation parameters based on nuclear SSR markers in studied ash trees

Vitality class	Genetic parameters of nSSR variability							
	n_a	n_e	I	H_O	H_E	F_{IS}	F_{IT}	F_{ST}
0+1	6.611	4.915	1.571	0.694	0.711	0.083	0.128	-
2	6.000	4.544	1.557	0.643	0.731	0.163	0.228	-
3	5.111	3.837	1.373	0.787	0.685	-0.127	-0.006	-
Total:	11.611	6.188	1.954	0.694	0.775	0.142	0.160	0.020**

n_a and n_e, observed and expected number of alleles; *I,* mean Shannon index; H_O and H_E, observed and expected heterozygosity; F_{IS}, inbreeding coefficient relative to the subpopulation; F_{IT}, inbreeding coefficient relative to the total; F_{ST}, fixation index. **Significant deviation from 0: $p < 0.05$

and class 3 (H_O = 0.787). Total mean genetic value of observed heterozygosity was lower than the expected one, which is in accordance with general observations made for different forest tree populations across Europe, e.g. *Fraxinus excelsior* (Heuertz et al. 2004), *Pinus sylvestris* (Kosińska et al. 2007), *Picea abies* (Nowakowska 2009) and *Quercus petraea* (Kremer et al. 2002).

All groups of trees had similar level of genetic differentiation, according to F_{ST} value indicating only 2% differences between vitality classes. The inbreed coefficient F_{IS} and F_{IT} suggest 14.2% and 16.0% of heterozygosity losses respectively between all classes of vitality (Table 7).

Concerning the chloroplast SSR marker variation, the highest observed numbers of alleles per locus (n_a) was found in ash trees belonging to the vitality class 2. Conversely, the highest parameters of: expected number of alleles (n_e), Shannon index (*I*), Nei's (1973) heterozygosity (*h*) and private allele content (*A*p) characterized the most damaged class 3 of ash trees. Because the trees from the class 2 of Roloff had no private allele, their genetic variability was lower than in class 3 and 0+1 (Table 8).

Based on analysis of molecular variance (ANOVA) among trees assessed with nuclear SSR markers, no pairwise Pearson correlation (r = -0.11 – 0.49) was observed among genetic parameters and tree classes of trees (Table 9). The observed heterozygosity (H_O) value was only significantly different between classes 2 and 3 (p = 0.0332 in HSD Tukey test, for p value < 0.05). The other genetic parameters (n_a, n_e, H_E, I and F) showed no statistically significant differences between different classes of trees (p > 0.05).

Similarly, no pairwise Pearson correlation (r = -0.12 – 0.51) was observed among genetic parameters and tree classes of trees assessed with chloroplast SSR markers (Table 9). The only significant statistical differences were observed between private allele presence (*A*p) in the different class of vitality (p = 0.0001 in HSD Tukey test, for p value < 0.05).

Table 8. Genetic parameters differentiation based on chloroplast SSR markers in studied ash trees

Vitality class	Genetic parameters of cpSSR variability				
	n_a	n_e	I	h	*A*p
0+1	2.750	2.151	0.822	0.503	0.178
2	3.000	1.870	0.776	0.459	0.000
3	2.750	2.257	0.854	0.534	0.429
Total:	2. 833	2.093	0.817	0.499	0.607**

n_a and n_e, observed and expected number of alleles; *I,* mean Shannon index; *h*, Nei's (1973) heterozygosity; *A*p, private allele content. *Significant deviation from 0: $p < 0.05$

Table 9. Statistic correlations between vitality classes of trees and genetic parameter values based on Pearson *r* coefficient, N=60, *p* value < 0.05

	nuclear SSR markers					
	n_a	n_e	I	H_O	H_E	F
Vitality Class	0.337455	0.489680	0.271914	-0.114483	0.034685	0.088720
	chloroplast SSR markers					
	n_a	n_e	I	h	*A*p	-
Vitality Class	-0.026841	-0.055929	-0.124202	-0.124202	0.505501	-

n_a and n_e, observed and expected number of alleles; *I,* mean Shannon index; H_O and H_E, observed and expected heterozygosity; *F*, ANOVA correlation coefficient.

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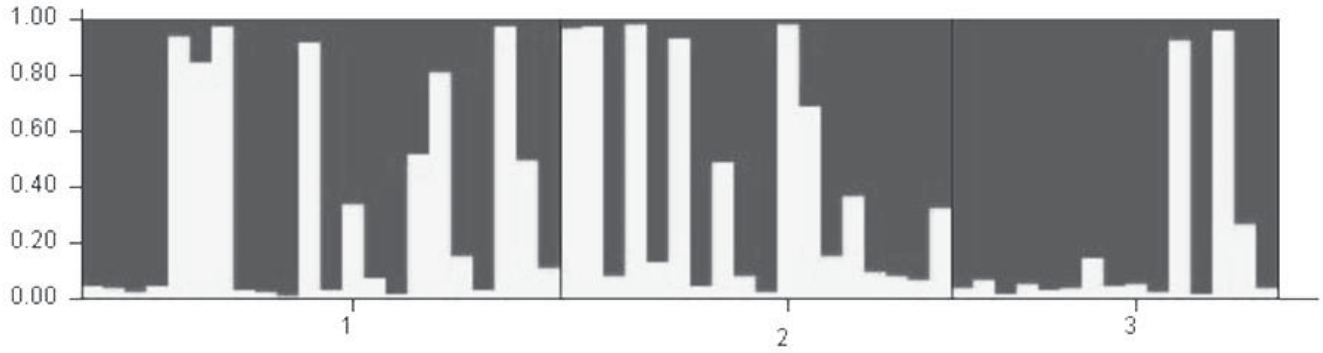

Figure 2. Clustering analysis between three vitality classes of ash trees and their genotypes based on nuclear microsatellite marker data in K-structure algorithm

Surprisingly, the most healthy class of trees (0+1) was not statistically different in any genetic parameter (based on nuclear or chloroplast type marker data) from the other classes.

Genetic differentiation and gene flow

It is generally considered that a value of $N_m > 1$ characterises large gene flow within a given population (Slatkin and Barton 1989). In our investigation, the value of $N_m = 24.72$ obtained indicates a high gene flow between investigated classes of trees.

No particular grouping of investigated ash tree genotypes was revealed by STRUCTURE clustering analysis proving that there is no relationship between the vitality class and genetic structure of a single tree (Figure 2).

Discussion

The examined stand of ash trees is in an advanced disease stage. Different symptoms, indicative for infections with the aggressive and invasive fungus *H. fraxineus* were recorded. After isolation tests, the presence of this pathogen was confirmed and 68% of plated tissue samples were positive for the presence of *H. fraxineus*, what corresponded to the previously published data (Kowalski 2006).

In the Forest District of Chojnów the separated areas with a dominant ash tree amount to 16.85 ha, which accounts for only 0.2% of the total forest area of this Forest District. In the last 10 years within the sanitary cuts more than 500 m^3 of ash timber was obtained, mainly dying trees (according to SILP - State Forests Information System). Since 2000, *Fraxinus excelsior* has not been introduced to forest plantations and its production in nurseries has not been continued. If the situation does not change, probably within the next few years ash species is likely to lose its dominant position on still such a small area. In the Wolica reserve the mortality of trees is high, when we started to count and permanently mark all ash trees in autumn 2011 there were 230 specimens. In 2012 in May when evaluating defoliation and vitality only 198 trees remained and the next year we counted 176 trees, which stayed alive.

Currently, ash dieback is one of the most important phytosanitary problems in Europe (Queloz et al. 2011). However, *H. fraxineus* was present in some areas (e.g. Switzerland) for a long time before the outbreak of the disease (Engesser et al. 2009). After the contamination it might take some years for building up of inoculums and the detection of first symptoms. This probably explains why the ongoing devastating epidemic, particularly on *Fraxinus excelsior*, is still developing.

Concerning the role of particular organisms in ash dieback phenomenon, next to *H. fraxineus* some authors suggested the role of *Phytophthora* species in complex of factors of ash dieback phenomenon (Orlikowski et al. 2011, Akilli et al. 2013). During the research carried out in the Forest District Chojnów in the reserve Wolica the incidence of three species from the *Phytophthora* genus was confirmed, namely, *P. plurivora, P. megasperma* and *Phytophthora* sp. hungarica. Their role in the process of dieback is under investigation. Especially, *P. plurivora* is a species highly damaging fine roots of broad-leaved trees, as well as the tissues of hosts belonging to several different genera, including ashes (Jung and Burgess 2009, Orlikowski et al. 2011).

The other two species are new in Poland and found for the first time in ash stands in general as well as little studied. Namely, *Phytophthora* sp. hungarica was previously isolated from soils under the alder trees (Bakonyi et al. 2012), and the role of this species in different syndromes of forest trees decline is little studied. On the other hand, *P. megasperma* was reported as a pathogen of *Malus pumilla* Mill. and *Brassica* spp. (Erwin and Ribeiro 1996). Also, this species was recently reported as being involved in Walnut trees (*Juglans regia* L.) decline in Italy (Belisario et al. 2012). However, the role of these two species in ash decline phenomenon need further research, particularly having in mind that they were isolated from only one out of nine collected rhizosphere soil samples in this stand. The

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role of these pathogenic organisms in the decline of observed ash stand in Wolica reserve should be tested through the pathogenicity tests. Because of the fact that their direct impact in ash decline phenomenon was not confirmed in several other studies (Bakys et al. 2009, Schumacher et al. 2010, Husson et al. 2012) the future field surveys in this respect in other declining ash stands in Poland are required. There is also a room for an alternative hypothesis e.g. that these *Phytophthora* species have been detected because we have looked for them, but we could also probably have detected them in ash stands before the arrival of the dieback. However, species from the *Phytophthora* genus were recognized as frequent pathogens in forest and ornamental nurseries from where they could be introduced to newly established plantations and planted forests (Brasier and Jung 2006, Pérez-Sierra and Jung 2013). This is the possible scenario of origin of detected *Phytophthora* species in this ash stand, established in the Wolica reserve c.a. 20 years ago. Water is also a very important source of inoculum of these pathogenic organisms, including both the water from irrigation reservoirs and natural streams (Orlikowski et al. 2007 b, Hulvey et al. 2010, Reeser et al. 2011).

The shoots of examined ashes were infested with *Chalara fraxinea* (anamorph of *Hymenoscyphus fraxineus*) that is why in a further stage of the research it is planned to determine the relationship between the incidence of the fungus *C. fraxinea* attacking stems of trees, and fine root pathogens of the genus *Phytophthora*. Efforts will be also taken aiming at awakening the resistance of trees to these pathogens through the use of preparations containing phosphites. Similar tests were completed successfully in Australia, where they are applied to a large scale against phytophthoras (Jackson et al. 2000, Wilkinson et al. 2001, McCarren et al. 2009), but not yet against *H. fraxineus* in the affected areas.

In addition to the fact that some other studies (e.g. by Schumacher et al. 2010) failed to detect *Phytophthora* in dying ash stands, Husson et al. (2012) suggested *H. fraxineus* as a cause of collar rots on ash trees. Moreover, Enderle et al. (2013) reported the frequent presence and isolation of *Armillaria gallica* Marxm. and Romagn from cankers at the stem base of declining ash trees, and previously Skovsgaard et al. (2010) reported association between the disease and the symptoms of *A. gallica*. It is, however, common that secondary pathogens affect weakened trees, and Enderle et al. (2013) suggested that recorded collar rots on ash trees could be possibly caused by both *Armillaria* species and *H. fraxineus*. However, presence of *Armillaria* species in the necrotic, as well as in asymptomatic tissues close to observed cankers was confirmed in this stand using molecular tools (Oszako, unpublished data), but in the isolation trials we failed to obtain *Armillaria* spp., and future field surveys and isolation trials are required.

One of the most important challenges in silviculture and management of the stands affected with ash dieback is infection spreading and post infection stand status, in the perspective of planning of future measurements. The obtained results showed that the most of the trees (112) were judged to belong to the stagnation phase (stage 2 of the vitality), for which large deformations of shoots is typical. This phase is characterized by slow growth and mainly shoots in clusters that grow in the crown. This fact is confirmed by the results of defoliation, which is a commonly used criterion for assessing the degree of damage to trees and stands. It was found that the largest share in the stand constituted moderately damaged trees (defoliation 26-60%).

Obtained results are very important in the perspective of searching for natural resistance against invasive *H. fraxineus* (McKinney et al. 2014). However, the natural resistance to the ash dieback disease was recorded in several studies in Denmark and Sweden (McKinney et al. 2011, 2012, Kjær et al. 2012, Stener 2013), and the presence of genetic variations of dieback resistance was proved through the progeny inoculation studies (Lobo et al. 2015). Based upon this, presence of highly resistant, not affected trees is very important. Also, and their resistance should be checked via large scale pathogenicity-provenance tests.

Many diversity conservation programs and the forest tree management require the detailed knowledge of the genetic distribution of endangered species, like *F. excelsior* suffering from ash-dieback phenomenon. The richness of the gene pool of each population is basically determined by multilocus allele occurrence in the genome (Escudero et al. 2003).

Our results showed that genetic variation of declining ash trees in the Wolica Reserve was not significantly correlated with the defoliation rate of ashes. The microsatellite DNA-based research on genetic variation in dieback resistance in *Fraxinus excelsior* in Denmark also proved no correlation between inheritability of the Femsatl loci and susceptibility of trees measured by necrosis development due to *Chalara fraxinea* artificial infection, which is consistent with the neutral nature of the SSR markers (Lobo et al. 2015). But the results obtained from the analysis of 60 ash trees from Wolica Reserve naturally subjected to *H. fraxinea* infection indicate that there is a little 2% variation between three Rollof's classes examined, with one significant difference in observed heterozygosity value between the classes 2 and 3. The highest observed heterozygosity in class 3, suggests the highest differences in investigated microsatellite loci among the most damaged trees comparing to other classes. Nevertheless, more studies with bigger number of investigated ashes displaying signs of dieback is needed to confirm this observation.

Total genetic differentiation level of ash trees from Wolica Reserve based on nuclear SSR markers was medium (H_O = 0.694, H_E = 0.775), and comparable to another

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studies performed for this species (Yazdani et al. 2003). Previous studies based on nuclear and chloroplast DNA markers showed similar level of genetic variation (G_{ST} = 0.198) in Polish ash populations to the level of G_{ST} = 0.110 found in Angiosperms (Nowakowska et al. 2004, Hamrick 1992). The observed in present study significantly different heterozygosity (H_O) value only between classes 2 and 3 may suggest some genetically based differences between the DNA variation and the degree of damages. Moreover, high gene flow observed among all classes of trees for nuclear SSR markers (N_m = 24.72) may indicate favourable condition for combination of alleles, guaranteeing the survival and adaptation to changing environmental conditions (Reed and Frankham 2003).

In Poland and Lithuania, the preliminary assessment of genetic variability of the population of 13 European ash using RAPD analysis was carried out, but these studies have not assumed correlation analysis of molecular data with the data on the degree of dieback stands (Nowakowska et al. 2004). Detailed studies on the identification of SCARs markers associated with resistance of Polish ash provenances to infection caused by *Hymenoscyphus fraxineus* showed some genetic differences between individuals resistant and susceptible to infection, but the incidence of those molecular markers failed to allow unequivocal identification of the trees resistant throughout the studied populations (Kowalski 2012).

On the other side, the chloroplast SSR markers have been used to determine the postglacial migration routes in Europe thanks to their maternal inheritance in deciduous tree species and low mutation rate. In our study, the significant statistical differences observed between presence of private chloroplast DNA allele (*A*p) distribution in the different class of vitality may suggest some adaptive phenomenon of single trees in the particular Wolica Reserve of ash.

The Polish ash stands studied with cpDNA markers revealed some moderate differentiation due to the presence of only two haplotypes of ccmp genes (Heuertz et al. 2004). Additionally, the same genes were revealed in *F. excelsior* and *F. angustifolia*, suggesting the possible hybridization among ash species (Heuertz et al. 2006). Such phenomenon is commonly observed in *Quercus* (Belahbib et al. 2001), *Betula* (Palme et al. 2004) and *Populus* (Lexer et al. 2005) species. Evolutionary driven forces like hybridization play an important role in tolerance and adaptation processes of ash species in Europe.

Plant genomes contain a large number of resistance genes against various pathogens (Rajesh et al. 2015). During evolution the plants developed several mechanisms against the pathogenic invaders, based on gene-for-gene interaction concept (Manion 1981). Many genes and secondary-metabolite compounds are up- and down-regulated during tolerance / resistance pathway in plants subjected to external physical stress factors (like heavy metals, heat-shock, UV, Nowakowska 1998) or biotic factors (pathogens and bacteria, Jones and Dangl 2006). The resistance genes are constitutively expressed and induced as soon as a pathogen is perceived, which subsequently triggers the plant defence responses (Rajesh et al. 2015). Recently, the associative transcriptomics research performed in Denmark helped to discover specific DNA sequences and gene-expression variants across ash trees scored for disease symptoms and identified markers strongly associated with canopy damage (Harper et al. 2015). The latest study revealed some SNPs differences between susceptible and tolerant Danish *F. excelsior* trees, and even made possible a single-nucleotide based distinction between moderately tolerant ash species (*F. mariensii*) and highly tolerant ash species (*F. mandhsurica*, *F. americana* and *F. ornus*). Such a new method using rapidly identifying molecular markers associated with tolerant trait variation across a set of trees, and taking into account both gene sequence variation and gene expression variation, can be very effective in in stands protection measures.

The further studies based on DNA polymorphism assessment performed on larger group of trees may help to understand the genetic basis of the pathogen tolerance developed by some European ash trees.

Conclusions

New strategies of ash population restitution in Poland may be designed thanks to predictive molecular markers identifying tolerant and susceptible trees, with emphasis on propagation of seeds from the most tolerant-ones. Molecular markers, both nuclear and chloroplast microsatellite loci, constitute good tools to support conservation and management of forest genetic resources, via assessment of the gene-pool diversity of ash populations, and to predict potential tolerance or susceptibility of this species against harmful pathogen like *Hymenoscyphus fraxineus*. More advanced studies, based on resistance-gene identification should be done to elucidate those mechanisms on the molecular level in order to promote marker-assisted silviculture in European ash restitution in Poland.

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References

Abad, G.Z. 2014. The Taxonomy of *Phytophthora*: What is done and what is needed for the correct identification and diagnostics of species in the Genus. *Phytophthora* in Forests and Natural Ecosystems. Proc. 7th IUFRO Working Party 7-02-09 Meeting, 2014: 28.

Akilli, S., Ulubaş Serçe, Ç., Katircioğlu, Y.Z. and Maden, S. 2013. *Phytophthora* dieback on narrow leaved ash in the Black Sea region of Turkey. *Forest Pathology* 43: 252-256.

Bakonyi, J., Nagy, Z.A., Burgess, T., Szigethy, A., Nechwatal, J., Koltay, A., Woodward, S., Belbahri, L. and Jung, T. 2012. Characterization of the two informally designated ITS Clade 6 taxa *Phytophthora* taxon Forestsoil and *P.* sp. hungarica. *Phytophthora* in Forests and Natural Ecosystems. Proc. 6th IUFRO Working Party 7-02-09 Meeting, 2012: 83.

Bakys, R., Vasaitis, R., Barklund, P., Ihrmark, K. and Stenlid, J. 2009. Investigations concerning the role of *Chalara fraxinea* in declining *Fraxinus excelsior*. *Plant Pathology* 58: 284-292.

Baral, H.O., Queloz, V. and Hosoya, T. 2014. *Hymenoscyphus fraxineus*, the correct scientific namefor the fungus causing ash dieback in Europe. *IMA Fungus* 5: 79-80.

Belahbib, N., Pemonge, M-H., Ouassou, A., Sbay, H., Kremer, A. and Petit, R.J. 2001. Frequent cytoplasmic exchanges between oak species that are not closely related: *Quercus suber* and *Q. ilex* in Morocco. *Molecular Ecology* 10: 2003-2012.

Belisario, A., Luongo, L., Galli, M. and Vitale, S. 2012. First Report of *Phytophthora megasperma* Associated with Decline and Death of Common Walnut Trees in Italy. *Plant Disease* 96(11): 1695.

Brasier, C.M. and Jung, T. 2006. Recent developments in *Phytophthora* diseases of trees and natural ecosystems in Europe. Progress in Research on *Phytophthora* Diseases of Forest Trees. Proc. 3rd IUFRO Working Party 7.02.09 Meeting, 2004: 5-16.

Brasier, C.M., Cooke, D.E.L., Duncan, J.M. and Hansen, E.M. 2003. Multiple new phenotypic taxa from trees and riparian ecosystems in *Phytophthora gonapodyides – P. megasperma* ITS Clade 6, which tend to be high-temperature tolerant and either inbreeding or sterile. *Mycological Research* 107: 277-290.

Chandelier, A., Delhaye, N. and Helson, M. 2010. First report of the Ash Dieback pathogen *Hymenoscyphus pseudoalbidus* (anamorph Chalara fraxinea) on *Fraxinus excelsior* in Belgium. *Plant Disease* 95: 220.

Cooke, D.E.L., Drenth, A., Duncan, J.M., Wagels, G. and Brasier, C.M. 2000. A Molecular Phylogeny of *Phytophthora* and Related Oomycetes. *Fungal Genetics and Biology* 30: 17-32.

Dmyterko, E. 1998. Metody określania uszkodzenia drzewostanów dębowych [Methods for specifying damage to oak stands]. *Sylwan* 142(10): 29-38 (in Polish).

Dmyterko, E., Wojtan, R. and Bruchwald, A. 2003. Stan zdrowotny drzewostanów jesionowych (*Fraxinus excelsior* L.) Nadleśnictwa Mircze [Health condition of ash stands (*Fraxinus excelsior* L.) in Mircze Forest District]. *Sylwan* 147(12): 9-18 (in Polish).

Enderle, R., Peters, F., Nakou, A. and Metzler, B. 2013. Temporal development of ash dieback symptoms and spatial distribution of collar rots in a provenance trial of *Fraxinus excelsior*. *European Journal of Forest Research* 132: 865-876.

Engesser, R., Queloz, V., Meier, F., Kowalski, T. and Holdenrieder, O. 2009. Das Triebsterben der Eshe in der Schweiz [Ash dieback in Switzerland]. *Wald und Holz* 6: 24-27 (in German).

Epperson, B.K. 2005. Mutation at high rates reduces spatial structure within populations. *Molecular Ecology* 14: 703-710.

Erwin, D.C. and Ribeiro, O.K. 1996. *Phytophthora* diseases worldwide. APS Press, St. Paul, Minnesota, USA, 562 pp.

Escudero, A., Iriondo, J.M. and Torres, M.E. 2003. Spatial analysis of genetic diversity as a tool for plant conservation. *Biological Conservation* 113: 351-365.

Halmschlager, E. and Kirisits, T. 2008. First report of the ash dieback pathogen *Chalara fraxinea* on *Fraxinus excelsior* in Austria. *Plant Pathology* 57(6): 1177.

Hamrick, J.L., Godt, M.J.W. and Sherman-Broyles, S.L. 1992. Factors influencing levels of genetic diversity in woody plant species. *New Forests* 6: 95-124.

Harper, A.L., McKinney, L.V., Nielsen, L.R., Havlickova, L., Li, Y., Trick, M., Fraser, F., Wang, L., Fellgett, A., Sollars, E.S.A., Janacek, S.H., Downie, J.A., Buggs, R.J.A., Kjær, E.D. and Bancroft, I. 2015. Molecular markers for tolerance of European ash (*Fraxinus excelsior*) to dieback disease identified using Associative Transcriptomics. *Scientific Reports* 6: 19335. DOI: 10.1038/srep19335.

Heuertz, M., Carnevale, S., Fineschi, S., Sebastiani, F., Hausman, J.F., Paule, L. and Vendramin, G.G. 2006. Chloroplast DNA phylogeography of European ashes, *Fraxinus* sp. (*Oleaceae*): roles of hybridization and life history traits. *Molecular Ecology* 15: 2131-2140.

Heuertz, M., Hausman, J.F., Hardy, O.J., Vendramin, G. G., Frascaria-Lacoste, N. and Vekemans, X. 2004. Nuclear microsatellites reveal contrasting patterns of genetic structure between western and southeastern European populations of the common ash (*Fraxinus excelsior* L.) *Evolution* 58(5): 976-988.

Heuertz, M., Vekemans, X., Hausman, J.F., Palada, M. and Hardy, O.J. 2003. Estimating seed versus pollen dispersal from spatial genetic structure in the common ash. *Molecular Ecology* 12: 2483-2495.

Hulvey, J., Gobena, D., Finley, L. and Lamour, K. 2010. Co-occurrence and genotypic distribution of *Phytophthora* species recovered from watersheds and plant nurseries of eastern Tennessee. *Mycologia* 102: 1127-1133.

Husson, C., Caël, O., Grandjean, J.P., Nageleisen, L.M. and Marçais, B. 2012. Occurrence of *Hymenoscyphus pseudoalbidus* on infected ash logs. *Plant Pathology* 61: 889-895.

Husson, C., Scala, B., Caël, O., Frey, P., Feau, N., Ioos, R. and Marçais, B. 2011. *Chalara fraxinea* is an invasive pathogen in France. *European Journal of Plant Pathology* 130: 311-324.

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Jackson, T.J., Burgess, T., Colquhoun, I. and Hardy, G.E.S.J. 2000. Action of the fungicide phosphite on *Eucalyptus marginata* inoculated with *Phytophthora cinnamomi*. *Plant Pathology* 49: 147-154.

Jones, J.D.G. and Dangl, J.L. 2006. The plant immune system. *Nature* 444: 323-329.

Jung, T. 2009. Beech decline in Central Europe driven by the interaction between *Phytophthora* infections and climatic extremes. *Forest Pathology* 39: 73-94.

Jung, T. and Burgess, T.I. 2009. Re-evaluation of *Phytophthora citricola* isolates from multiple woody hosts in Europe and North America reveals a new species, *Phytophthora plurivora* sp. nov. *Persoonia* 22: 95-110.

Jung, T. and Nechwatal, J. 2008. *Phytophthora gallica* sp. nov., a new species from rhizosphere soil of declining oak and reed stands in France and Germany. *Mycological Research* 112: 1195-1205.

Jung, T., Blaschke, H. and Neumann, P. 1996. Isolation, identification and pathogenicity of *Phytophthora* species from declining oak stands. *European Journal of Forest Pathology* 26: 253-272.

Jung, T., Blaschke, H. and Oßwald, W. 2000. Involvement of soilborne *Phytophthora* species in Central European oak decline and the effect of site factors on the disease. *Plant Pathology* 49: 706-718.

Jung, T., Cooke, D.E.L., Blaschke, H., Duncan, J.M. and Oßwald, W. 1999. *Phytophthora quercina* sp. nov., causing root rot of European oaks. *Mycological Research* 103: 785–798.

Jung, T., Hansen, E. M., Winton, L., Oßwald, W. and Delatour, C. 2002. Three new species of *Phytophthora* from European oak forests. *Mycological Research* 106: 397-411.

Jung, T., Nechwatal, J., Cooke, D.E.L., Hartmann, G., Blaschke, M., Oßwald, W.F., Duncan, J.M. and Delatour, C. 2003. *Phytophthora pseudosyringae* sp. nov., a new species causing root and collar rot of deciduous tree species in Europe. *Mycological Research* 107: 772-789.

Kirisits, T., Matlakova, M., Mottinger-Kroupa, S., Halmschlager, E. and Lakatos, F. 2010. *Chalara fraxinea* associated with dieback of narrow-leafed ash (*Fraxinus angustifolia*). *Plant Pathology* 59: 411.

Kjær, E.D., McKinney, L.V., Nielsen, L.R., Hansen, L.N. and Hansen, J.K. 2012. Adaptive potential of ash (*Fraxinus excelsior*) populations against the novel emerging pathogen *Hymenoscyphus pseudoalbidus*. *Evolutionary Applications* 5: 219-228.

Kosińska, J., Lewandowski A. and Chałupka, W. 2007. Genetic variability of Scots pine maternal populations and their progenies. *Silva Fennica* 41: 5-12.

Kowalski T. 2012. Zamieranie jesionu – aspekty taksonomiczne sprawcy choroby [Ash dieback – taxonomic aspects of the perpetrator of the disease]. *Sylwan* 156(4): 262-269 (in Polish).

Kowalski, T. 2006. *Chalara fraxinea* sp. nov. associated with dieback of ash (*Fraxinus excelsior*) in Poland. *Forest Pathology* 36: 264-270.

Kowalski, T. and Holdenrieder, O. 2009a. Pathogenicity of *Chalara fraxinea*. *Forest Pathology* 39: 1-7.

Kowalski, T. and Holdenrieder, O. 2009b. The teleomorph of *Chalara fraxinea*, the causal agent of ash dieback. *Forest Pathology* 39: 304-308.

Kremer, A., Kleinschmit J., Cottrell J., Cundall, E.P., Deans, J. D., Ducousso, A., König, A. O., Lowe, A.J., Munro, R.C., Petit, R.J. and Stephan, B.R. 2002. Is there a correlation between chloroplastic and nuclear divergence, or what are the roles of history and selection on genetic diversity in European oaks? *Forest, Ecology and Management* 156: 75-85.

Lexer, C., Fay, M.F., Joseph, J.A., Nica, M-S. and Heinze, B. 2005. Barrier to gene flow between two ecologically divergent *Populus* species, *P. alba* (white poplar) and *P. tremula* (European aspen): the role of ecology and life history in gene introgression. *Molecular Ecology* 14: 1045-1057.

Li, Y.-C., Korol, A.B., Fahima, T., Beiles, A. and Nevo, E. 2002. Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. *Molecular Ecology* 11: 2453-2465.

Lobo, A., McKinney, L.V., Hansen, J.K., Kjær E.D. and Nielsen L.R. 2015. Genetic variation in dieback resistance in *Fraxinus excelsior* confirmed by progeny inoculation assay. *Forest Pathology* 45: 379-387.

Manion, P.D. 1981. Tree Disease Concepts. Englewood Cliffs: Prentice-Hall, 399 pp.

McCarren, K.L., McComb, J.A., Shearer, B.L. and Hardy, G.E.S.J. 2009. Phosphite impact on the *in vitro* production and viability of selfed oospores by *Phytophthora cinnamomi*. *Forest Pathology* 39: 124-132.

McKinney, L.V., Nielsen, L.R., Hansen, J.K. and Kjær, E.D. 2011. Presence of natural genetic resistance in *Fraxinus excelsior* (*Oleaceae*) to *Chalara fraxinea* (Ascomycota): an emerging infectious disease. *Heredity* 106: 788-797.

McKinney, L.V., Nielsen. L.R., Collinge, D.B., Thomsen, I.M., Hansen, J.K. and Kjær, E.D. 2014. The ash dieback crisis: genetic variation in resistance can prove a long term solution. *Plant Pathology* 63: 485-499.

McKinney, L.V., Thomsen, I.M., Kjær, E.D. and Nielsen, L.R. 2012. Genetic resistance to *Hymenoscyphus pseudoalbidus* limits fungal growth and symptom occurrence in *Fraxinus excelsior*. *Forest Pathology* 42: 69-74.

Nei, M. 1973. Analysis of gene diversity in subdivided populations. Proc. of the National Academy of Sciences USA 70: 3321-3323.

Nguyen, D.T., Cleary, M.R., Enderle, R., Berlin, A. and Stenlid, J. 2015. Analyses of the ash dieback pathogen, *Hymenoscyphus fraxineus*, suggest role of tree species diversity on colonization and population structure differentiation. *Forest Pathology*: DOI: 10.1111/efp.12236.

Nowakowska, J. 1998. Two germin isoforms genes expression and oxalate oxidase activity induced by stress. *Acta Physiologiae Plantarum* 20(1): 19-33.

Nowakowska, J.A. 2009. Mitochondrial and nuclear DNA differentiation of *Picea abies* populations in Poland. *Dendrobiology* 61: 119–129.

Nowakowska, J.A., Jabłonowski, S., Mockeliunaite, R. and Bieniek, J. 2004. Genetic variability within and among Polish and Lithuanian populations of common Ash (*Fraxinus excelsior* L.) based on RAPD analysis. *Baltic Forestry* 10(1): 57-64.

Orlikowski, L.B., Oszako, T., Duda, B. and Szkuta, G. 2004. Występowanie *Phytophthora citricola* na jesionie

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wyniosłym w szkółkach leśnych [Occurrence of *Phytophthora citricola* on ash (*Fraxinus excelsior*) in forest nurseries]. *Leśne Prace Badawcze* 4: 129-136. (in Polish)

Orlikowski, L.B., Oszako, T., Trzewik A. and Orlikowska, T. 2007 a. Occurrence of *Phytophthora ramorum* and other *Phytophthora* species in nurseries, trade stands, forests and water. *Journal of plant protection research,* 47: 445-455.

Orlikowski, L.B., Ptaszek, M., Rodziewicz, A., Nechwatal, J., Thinggaard, K. and Jung, T. 2011. *Phytophthora* root and collar rot of mature *Fraxinus excelsior* in forest stands in Poland and Denmark. *Forest Pathology* 41: 510-519.

Orlikowski, L.B., Trzewik, A. and Orlikowska, T. 2007 b. Water as potential source of *Phytophthora citricola*. *Journal of Plant Protection Research* 47: 125-132.

Pacia, A., Nowakowska, J.A., Milenkovic, I., Kraj, W. and Oszako, T. 2011. Polish case study of ash decline in natural reserve Wolica (Chojnów Forest District) and in southern Poland. Book of Abstract of the COST ACTION FP1103 FRAXBACK, 1st MC/WG Meeting, 2011: 32-33

Palme, A.E., Su, Q., Palsson, S. and Lascoux, M. 2004. Extensive sharing of chloroplast haplotypes among European birches indicates hybridization among *Betula pendula*, *B. pubescens* and *B. nana*. *Molecular Ecology* 13: 167–178.

Peakall, R. and Smouse, P.E. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* 28: 2537-2539.

Pérez-Sierra, A. and Jung, T. 2013. *Phytophthora* in woody ornamental nurseries. In: Lamour K. (Editor), *Phytophthora* A Global Perspective. CABI, Plant protection series, 2, p. 166-177.

Petit, R.J., Csaikl, U.M., Bordács, S., Burg, K., Coart, E., Cottrell, J., van Dam, B., Deans, J.D., Dumolin-Lapègue, S., Fineschi, S., Finkeldey, R., Gillies, A., Glaz, I., Goicoechea, P.G., Jensen, J. S., König, A.O., Lowe, A.J., Madsen, S.F., Mátyás, G., Munro, R.C., Olalde, M., Pemonge, M.-H., Popescu, F., Slade, D., Tabbener, H., Taurchini, D., de Vries, S.G.M., Ziegenhagen, B., Kremer, A. 2002. Chloroplast DNA variation in European white oaks phylogeography and patterns of diversity based on data from over 2600 populations. *Forest Ecology Management* 156: 5–26.

Pritchard, J. K., Stephens, M. and Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155: 945-959.

Queloz, V., Grünig, C.R., Berndt, R., Kowalski, T., Sieber, T.N. and Holdenrieder, O. 2011. Cryptic speciation in *Hymenoscyphus albidus*. Forest Pathology 41: 133-142.

Rajesh, M.K., Rachana, K.E., Naganeeswaran, S.A., Shafeeq, R., Thomas, R.J., Shareefa, M., Merin, B. and Anitha, K. 2015. Identification of expressed resistance gene analog sequences in coconut leaf transcriptome and their evolutionary analysis. *Turkish Journal of Agriculture and Forestry* 39: 489-502.

Reed, D.H. and Frankham, R. 2003. Correlation between fitness and genetic diversity. *Conservation Biology* 17(1): 230-237.

Reeser, P.W., Hansen, E.M., Sutton, W., Remigi, P. and Adams, G.C. 2011. *Phytophthora* species in forest streams in Oregon and Alaska. *Mycologia* 103: 22-35.

Roloff, A. 1989. Kronenentwicklung und Vitalitätsbeurteilung ausgewählter Baumarten der gemässigten Breiten [Crown development and vitality evaluation of selected tree species of the temperate latitudes]. Schriften aus der Forstlichen Fakultät der Universität Göttingen und Niedersächsischen Forstlichen Versuchsanstalt, (93) Frankfurt am Main (in German).

Rytkönen, A., Lilja, A., Drenkhan, R., Gaitnieks, T. and Hantula, J. 2011. First record of *Chalara fraxinea* in Finland and genetic variation among isolates sampled from Åland, mainland Finland, Estonia and Latvia. *Forest Pathology* 41(3): 169-174.

Schumacher, J., Kehr, R. and Leonhard, S. 2010. Mycological and histological investigations of *Fraxinus excelsior* nursery saplings naturally infected by *Chalara fraxinea*. *Forest Pathology* 40: 419-429.

Semagn, K., Bjørnstad, Å. and Ndjiondjop, M.N. 2006. An overview of molecular marker methods for plants. *African Journal of Biotechnology* 5(25): 2540-2568.

Skovsgaard, J.P., Thomsen, I.M., Skovgaard, I.M. and Martinussen, T. 2010. Associations among symptoms of dieback in even-aged stands of ash (*Fraxinus excelsior* L.). *Forest Pathology* 40: 7-18.

Slatkin, M. and Barton, N.H. 1989. A comparison of three indirect methods for estimating average levels of gene flow. *Evolution* 43: 1349-1368.

Stamps, D.J., Waterhouse, G.M., Newhook, F.J. and Hall, G.S. 1990. Revised tabular key to the species of *Phytophthora*. Mycol. Papers CAB International Mycological Institute, Kew, Surrey, No. 162.

Stanivuković, Z., Karadžić, D. and Milenković, I. 2014. The first report of the parasitic fungus *Hymenoscyphus fraxineus* (Kowalski) Baral, Queloz, Hosoya on the common ash in Bosnia and Herzegovina. *Šumarstvo* 3-4: 19-34. (in Serbian with English abstract and summary)

Stener, L.G. 2013. Clonal differences in susceptibility to the dieback of *Fraxinus excelsior*. *Scandinavian Journal of Forest Research* 28(3): 1-12.

Sutherland, B.G., Belaj, A., Nier, S., Cottrell, J.E., Vaughan, S., Hubert, J. and Russell, K. 2010. Molecular biodiversity and population structure in common ash (*Fraxinus excelsior L.*) in Britain: implications for conservation. *Molecular Ecology* 19(11): 2196-2211.

Szabó, I. 2009. First report of *Chalara fraxinea* affecting common ash in Hungary. *Plant Pathology*, 58: 797.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* 30: 2715-2729.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acid Research* 22(22): 4673-4680.

Timmermann, V., Børja, I., Hietala, A.M., Kirisits, T. and Solheim, H. 2011. Ash dieback: pathogen spread and diurnal patterns of ascospore dispersal, with special emphasis on Norway. *EPPO Bulletin* 41: 14-20.

Vornam, B., Decarli, N. and Gailing, O. 2004. Spatial distribution of genetic variation in natural beech stands (*Fagus sylvatica* L.) based on microsatellite markers. *Conservation Genetics* 5: 561-570.

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White, T.J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White (Editors.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, USA, p. 315-322.

Whitlock, M.C. 2002. Selection, load and inbreeding depression in a large metapopulation. *Genetics* 160(3): 1191-1202.

Wilkinson, C.J., Holmes, J.M., Dell, B., Tynan, K.M., McComb, J.A., Shearer, B.L., Colquhoun, I.J. and Hardy, G.E.S.J. 2001. Effect of phosphite on in planta zoospore production of *Phytophthora cinnamomi*. *Plant Pathology* 50: 587-593.

Yazdani, R., Scotti, I., Jansson, G., Plomion, C. and Mathur, G. 2003. Inheritance and diversity of simple sequence repeat (SSR) microsatellite markers in various families of *Picea abies*. *Hereditas* 138(3): 219–227.

Zhang, Z., Schwartz, S., Wagner, L. and Miller, W. 2000. A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology* 7(1/2): 203-214.

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