

Genetic variability of *Chalara fraxinea*, dieback cause of European ash (*Fraxinus excelsior* L.)

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Abstract A total of 159 colonies of *Chalara fraxinea* were isolated between 2005 and 2006 from dying trees of European ash (*Fraxinus excelsior* L.) aged between 3 and 10 years. They derived from five regions of Poland differing by geographic location and climatic conditions. On the basis of 90 RAMS markers, pathogen intra- and inter-population variability, as well as its dependency on geographic distance and climatic conditions in the regions of strain origin, was analysed. The applied measures of intrapopulation genetic variability (genetic distance, Nei's unbiased diversity, Shannon's Information Index and percentage of polymorphic loci) allowed for differentiation of two strain groups: the first deriving from lowlands and the second from uplands and mountainous areas. Strains in lowlands were characterised by smaller number of markers, smaller number of polymorphic loci and smaller intrapopulation genetic variability. Positive and statistically significant correlation was shown between variability of isolates and elevation of regions above sea level. Pair-wise genetic distances between groups of isolates (Nei's unbiased genetic distance) from particular regions were not significantly correlated with the corresponding geographic distances. On the basis of AMOVA, it was shown that 85% of variability was within-region differences and 2% between-region differences, whereas differences between lowlands and uplands were 13%. Principal Components Analysis (PCA) for the investigated regions confirmed the results from Nei's genetic distance matrix.

Keywords *Chalara fraxinea* · Genetic variability · Tree diseases · RAMS markers · Population genetics

Introduction

At the beginning of the 1990s, the first symptoms of increased dieback of ash (*Fraxinus excelsior* L.) were observed in north-eastern Poland. In the subsequent years, the disease symptoms were observed in all regions of the country, both in lowlands and in the mountains (Kowalski 2001; Kowalski and Łukomska 2005; Przybył 2002). Ash trees also started to display dieback symptoms in many countries of northern, western and, recently, also southern Europe (Bakys et al. 2009b; Engesser et al. 2009; Halmschlager and Kirisits 2008; Ioos et al. 2009; Kowalski and Holdenrieder 2008; Lygis et al. 2005; Ogris et al. 2010; Szabo 2008; Talgo et al. 2009). Tree stands of all age classes are suffering from the disease, both of natural and artificial afforestation, regardless of the occupied habitat, in forests and in urban areas (Bakys et al. 2009b; Halmschlager and Kirisits 2008; Kowalski and Holdenrieder 2008; Schumacher et al. 2007a; Talgo et al. 2009). Sick trees display a wide range of symptoms: leaf necrosis, wilting, premature leaf shedding, cankers on shoots, branches and stems, wood discoloration, dying of whole branches or their apices, top dying, and dying of whole trees and stands (Bakys et al. 2009a; Engesser et al. 2009; Kirisits and Halmschlager 2008; Kowalski 2006; Kowalski and Holdenrieder 2008; Schumacher et al. 2007b; Skovsgaard et al. 2010; Thomsen et al. 2007).

Numerous potentially pathogenic fungi from the genera *Cytospora*, *Diplodia*, *Fusarium*, *Nectria* and *Phomopsis* were identified in tissues of sick and decaying ash trees (Kowalski and Łukomska 2005; Przybył 2002; Schumacher et al.

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2007b; Vasiliauskas et al. 2006). In Poland, a new species of anamorphic fungus, *Chalara fraxinea* T. Kowalski, was discovered on sick ash trees (Kowalski 2006). In the entire country, it is the most frequent fungus found on ash trees with dieback symptoms (Kowalski 2007, 2009). In recent years, its presence has been recorded in a majority of European countries where ash trees have started to die: in Lithuania, Austria, Germany, Sweden, Denmark, Finland,

Table 1 Origin of *Chalara fraxinea* isolates examined

No.	Strain no.	Forest District	Coordinates
Region I			
1	816, 816b, 818, 818b, 819, 820, 820b, 823, 825, 827	Lebork	54°32'N, 17°44'E
2	830, 831/2, 833, 833a, 833b, 833/C, 835, 836, 839, 840	Starogard	53°58'N, 18°31'E
Region II			
3	1001, 1002, 1003, 1004, 1006, 1008, 1010, 1012, 1018, 1030	Kanczuga	49°58'N, 22°24'E
4	717, 721, 725, 726, 732, 737, 738, 1256, 1257, 1259, 1265, 1266	Mircze	50°39'N, 23°54'E
Region III			
5	331, 336, 338, 4020, 4029, 4031a, 4031b, 4032, 4044, 4046, 4048, 4059, 4060, 4070	Jedrzejew	50°38'N, 20°17'E
6	441, 442, 450, 456, 457, 458, 842, 1218/a, 1230, 1235, 1298	Pinczow	50°31'N, 20°31'E
7	1282, 1284, 1286, 1287, 1289, 1292, 1297, 1300, 1303/2, 1304, 1311	Pinczow-Michalow	50°29'N, 20°27'E
Region IV			
8	498, 499, 625, 628, 631, 1043, 1045, 1050, 625b	Miechow	50°21'N, 20°01'E
9	304, 305, 310, 315/1, 315/2, 319/1, 319/2, 326, 329, 331, 333, 324	Niepolomice	50°02'N, 20°12'E
10	270, 419, 420, 473, 474, 485, 511, 514, 526, 534, 541, 542, 544, 546, 550, 551, 561, 562, 595, 506/1, 506/4, 506/5, 508/1, 508/2, 509/3, 509/4, 509/6, 510/2, 528/1	Ojcow	50°12'N, 19°49'E
Region V			
11	363, 364, 365/1, 365/2, 1118, 1119, 1120, 1122, 1124, 1135	Andrychow	49°51'N, 19°20'E
12	353, 354, 355, 357	Limanowa	49°42'N, 20°25'E
13	424, 425/1, 425/2, 425/3, 426, 428	Losie	49°34'N, 21°05'E
14	382, 383, 384, 385, 405, 1174, 1174b, 1176, 1182, 1183, 1184	Nowy Targ	49°29'N, 20°01'E

Norway, Czech Republic, Slovakia, Slovenia, Switzerland, France, Croatia, Hungary and Italy (Bakys et al. 2009a; Barić and Diminić 2009; Barklund 2006; Engesser et al. 2009; Halmschlager and Kirisits 2008; Ioos et al. 2009; Jankovsky and Holdenrieder 2009; Kowalski and Holdenrieder 2008; Ogris et al. 2010; Schumacher et al. 2007b; Szabo 2008; Thomsen et al. 2007). Pathogenicity of *C. fraxinea* with respect to *Fraxinus excelsior* has recently been demonstrated a number of times (Bakys et al. 2009b; Kirisits et al. 2009; Kowalski and Holdenrieder 2009a; Ogris et al. 2010; Talgo et al. 2009). Taking into account the significant economic and ecological threat, *C. fraxinea* was included in the EPPO Alert List and in the NAPPO Phytosanitary Alert System.

Recently, its teleomorph has been detected and assigned to *Hymenoscyphus albidus* (Roberge ex Desm.) W. Phillips (Kowalski and Holdenrieder 2009b), which had been known from Europe since 1851 as a saprotroph, not causing ash tree disease (Desmazières 1851; Ellis and Ellis 1997). It produces abundant apothecia on previous year ash leaf rachises in the litter, while ascospores are distributed in a period from July to September. Further research provided molecular evidence for the existence of, apart from *H. albidus* s. str., another morphologically similar fungus which was described as a new cryptic species *Hymenoscyphus pseudoalbidus*. According to the data collected so far, *H. pseudoalbidus* occurs on *Fraxinus excelsior* with disease symptoms, whereas *H. albidus* is present in ash tree stands without dieback (Queloz et al. 2010).

Data on genetic variability provide valuable insight in dispersal mechanisms and local adaptation of pathogens. We studied the genetic variability of *Ch. fraxinea* isolates from necrotic lesions on ash trees in different regions of Poland.



Fig. 1 Schematic map of Poland with designation of *Chalara fraxinea* strains provenience regions

Table 2 Climatic characteristics of regions where *Chalara fraxinea* strains were isolated

Region	Elevation (m)	Average annual temperature (°C)	Annual sum of precipitation (mm×m ⁻²)	Number of days with snow cover
Region I	60	7.2	770	50
Region II	192	7.5	675	80
Lowland	126	7.4	723	65
Region III	233	7.6	594	87
Region IV	308	7.7	656	74
Region V	476	6.5	792	99
Upland and mountain	339	7.3	681	87

Materials and methods

Isolates

The research encompassed 159 isolates of *Chalara fraxinea* obtained between 2005 and 2006 from local necroses on live shoots or dead shoots of *Fraxinus excelsior* (Table 1). They derived from five regions of Poland, where disease symptoms occurred on young, 3- to 10-year-old ash tree stands. In every region, isolates derived from 2–4 Forest Districts (Table 1, Fig. 1). One region represented northern Poland (Region I), one eastern Poland (Region II), whereas three regions represented southern Poland (Region III, IV and V). Regions differed by geographic location and elevation above sea level, as well as climatic conditions, such as average annual temperatures, annual sum of precipitation and number of days with snow cover (Table 2).

Fungi isolation was performed during 24 (as an exception 48) h from collection of shoots in the field. From each shoot, a section whose length ranged between 8 and 10 cm with symptoms of local necrosis was cut out or, in the case of decayed shoot tops, from a section bordering on a live shoot. After superficial disinfection (1 min ethanol 96%, 5 min NaOCl 4%, 30 s ethanol 96%), parts of shoots whose dimensions were 5×2×2 mm were cut out and

placed on the surface of 2% of malt extract agar (MEA; 20 g l⁻¹ malt extract (Difco; Sparks, MD, USA), 15 g l⁻¹ agar Difco supplemented with 100 mg l⁻¹ streptomycin sulphate) in Petri dishes. From every shoot, 6–12 fragments were isolated. Incubation was conducted in the dark at room temperature. Among many colonies of various fungi, between 1 and 12 colonies of *Ch. fraxinea* appeared. Among them, 1 colony was selected at random. The growing mycelium was transferred to new MEA plates and incubated at 20°C in the dark.

Molecular analyses

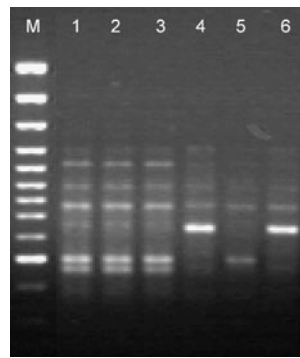
Extraction of genomic DNA was conducted in line with the procedure of Carlson et al. (1991). Ribosomal DNA fragments containing ITS1, 5.8 S and ITS2 were amplified using ITS1 and ITS4 primers (White et al. 1990). PCR reaction was conducted in a volume of 50 µl containing: PCR buffer, MgCl₂ 1.5 mM, dNTP 200 mM (Fermentas, Burlington, Canada), primer 1 µM, Taq DNA polymerase 1 U (Qiagen, Valencia, CA, USA), template DNA 10 ng. PCR was conducted in Biometra T3 thermocycler (Biometra, Goettingen, Germany) programmed as follows: initial denaturation at 95°C for 5 min, subsequently 36 cycles consisting of denaturation at 95°C for 1 min, annealing for 45 s at 54°C, elongation at 72°C for 2.5 min. Elongation of the last cycle was extended to 8 min. Purification and sequencing of amplification products using ITS1 primer were carried out at MacroGen Europe, Netherlands. BLAST searches were conducted using Geneious 5.1 software (Biomatters, New Zealand) (Drummond et al. 2010).

Genetic polymorphism of isolates was determined by RAMS method (Random Amplified Microsatellites) (Hantula et al. 1996; Zietkiewicz et al. 1994). Eight primers were tested, among which most relevant ones were selected on the basis of electrophoretic profiles and repeatability of results (Table 3). DNA amplification of fungus was conducted in 10 µl of reactive mixture consisting of: PCR buffer, MgCl₂ 1 mM, dNTP 200 mM, 1 µM primer, Taq DNA polymerase 0.2 U, DNA 10 ng. Cycling parameters

Table 3 Primers used, number of amplified products and range of their lengths

Primer name	Sequence (5'→3')	Temperature of annealing (°C)	Number of amplified products	Length of obtained products (bp)
CGA	DHBCGACGACGACGACGA	61	29	144-1776
ATG	DHBATGATGATGATGATG	46	16	218-1919
AGT	DHBAGTAGTAGTAGTAGT	30	13	383-1219
AGC	DHBAGCAGCAGCAGCAGC	60	21	234-1738
ACA	BDBACAACAACAACAACA	49	11	186-1550

Fig. 2 Banding pattern obtained from PCR using AGC primer. *M* marker, *lines 1–3* strains from region I, *lines 4–6* strains from region V



were: initial denaturation at 95°C for 5 min, subsequently 36 cycles consisting of denaturation at 95°C for 1 min, annealing for 45 s at temperatures depending on the type of primer (Table 3), elongation at 72°C for 2.5 min. Elongation of the last cycle was extended to 8 min. Amplification products were resolved in 1.5% agarose gel (Prona, Madrid, Spain) containing 0.2 µg/ml of ethidium bromide in the 1 × TBE buffer. GeneRuler 100 bp Ladder Plus (Fermentas) was used as a marker for the length of DNA fragments. The lengths of amplified products were determined using BIO 1 D++ software (Vilber Lurmat, Torcy, France). Every PCR reaction was performed twice for the purpose of confirming repeatability of results.

Data analysis

The RAMS bands that could be scored unequivocally were included in the analysis. They were scored as present (1) or absent (0) to create a binary matrix. Homogeneity in distribution frequency of allele for isolates deriving from individual regions was ascertained on the basis of the conducted chi-squared test. Binary data were used for the calculation of Dice's similarity coefficient $S_D = [2a / (2a + b + c)]$, where *a* is the number of bands common for isolate *x* and *y*, *b* is the number of bands present only in isolate *x*, and *c* is the number of bands present only in isolate *y*.

Similarity coefficient matrix was calculated with the use of SPSS Statistics 17.0 (SPSS, Chicago, IL, USA).

The intrapopulation genetic variation analysis between isolates obtained from particular regions was quantified on the basis of several measures: (1) genetic distance calculated as $D = 1 - S_D$; (2) mean of Nei's unbiased diversity (Hedrick 2000; Nei 1972); (3) Shannon's Information Index (Lewontin 1972; Shannon and Weaver 1949); and (4) percentage of polymorphic loci. Calculations of these diversity estimates were performed with Popgene version 1.32 (Yeh et al 1997) and GenAlex 6.3 (Peakall and Smouse 2006) software.

Analysis of molecular variance (AMOVA) was carried out in GeneAlex 6.3 to study the occurrence of population structure. In this analysis, the total variation in the marker frequencies was divided into within- and between-region components. Variability components were expressed as a percentage of the entire variability, whereas statistical significance of components was tested with the use of 1,000 permutations. Genetic distance between groups of isolates from the analysed regions was expressed by means of Nei's unbiased genetic distance (Hedrick 2000; Nei 1972).

The Principal Components Analysis (PCA), which graphically presents between-region variability, was performed based on genetic distance between region pairs, calculated according to Huff et al. (1993) method. The above-mentioned statistical analyses were performed with the GeneAlex 6.3 (Peakall and Smouse 2006) software.

In order to determine dependency of climatic conditions (Table 2) of studied regions on intrapopulation measures of genetic variability, correlation ratios were calculated using Statistica 8.0 (Stat Soft, Tulsa, OK, USA).

Results

On the basis of morphological researches and analysis of ribosomal DNA sequences containing ITS1, 5.8 S and

Table 4 Characteristics of intrapopulation variability of *Chalara fraxinea* isolates from particular regions

Region	Number of bands	Percent of polymorphic loci	Genetic distance (D)	Mean of unbiased diversity	Shannon Information Index
Region I	52	53.33	0.204±0.008	0.151±0.019	0.225±0.027
Region II	55	61.11	0.349±0.017	0.201±0.021	0.293±0.028
Lowland	53.5	57.22	0.283±0.01	0.179±0.02	0.271±0.027
Region III	85	92.22	0.392±0.005	0.288±0.019	0.428±0.024
Region IV	87	95.56	0.432±0.003	0.291±0.018	0.438±0.022
Region V	85	93.33	0.486±0.006	0.331±0.016	0.482±0.021
Upland and mountain	85.7	94.70	0.432±0.003	0.305±0.016	0.462±0.021

Table 5 Matrix of Pearson rank correlation coefficients (*r*) for intrapopulation measures and climatic conditions (*P* values shown in parentheses)

	Genetic distance (D)	Shannon Information Index	Mean of unbiased diversity	Percent of polymorphic loci
Shannon Information Index	0.947 (0.014)			
Mean of unbiased diversity	0.955 (0.011)	0.999 (<0.001)		
Percent of polymorphic loci	0.881 (0.048)	0.977 (0.004)	0.964 (0.008)	
Elevation [m]	0.947 (0.015)	0.901 (0.037)	0.918 (0.028)	0.797 (0.106)
Average annual temperature (°C)	-0.548 (0.339)	-0.486 (0.407)	-0.527 (0.361)	-0.304 (0.619)
Annual sum of precipitation (mm×m ⁻²)	-0.155 (0.804)	-0.196 (0.752)	-0.166 (0.789)	-0.324 (0.594)
Number of days with snow cover	0.898 (0.039)	0.821 (0.089)	0.846 (0.071)	-0.704 (0.184)

ITS2 we identified 159 isolates of *Chalara fraxinea*. Analysed sequences were closest to *Chalara fraxinea* sequence No. FJ429385 (percent of identical sites: 99.6–100%).

On the basis of repeatability of results and the degree of polymorphism of electrophoretic profiles, among eight tested RAMS primers, the five most appropriate ones were selected (Table 3). The applied primers allowed for obtaining 90 amplification products in total. The example gel image, showing the banding pattern obtained using the

AGC primer for regions I and V, is presented in Fig. 2. The number of obtained amplicons and the range of their length depended on the applied primer and the region of isolates origin (Tables 3 and 4). None of the obtained amplification product (RAMS marker) was monomorphic for the group of isolates deriving from individual regions. Similarly, no pair of isolates with the same haplotype was ascertained for the applied primers. Chi-squared test showed that the obtained data were homogenous (the homogeneity test was insignificant at $P < 0.01$), which allowed for the application of variance analysis (ANOVA) for evaluation of differences between the mean genetic distance (D) values for the regions.

On the basis of applied measures of intrapopulation genetic variability, the analysed regions of occurrence of *Chalara fraxinea* were divided into two groups. The first one encompassed lowland regions (Region I and Region II), whereas the second one highland regions (Region III and Region IV) and a mountainous region (Region V). Isolates derived from lowland regions were characterised by a lower number of obtained amplification products and a smaller number of polymorphic loci, as well as smaller values of measures of intrapopulation genetic variability. All the above-listed indices were lower by over 60% in the case of lowland regions in comparison to regions located higher up (Table 4). Additional cluster analyses for each individual region and for the combined low and high elevation regions revealed the presence of several genetically distinct groups, which, however, were not related to the geographic origin of the specimens (data not shown).

The applied measures of intrapopulation genetic variability of isolates within regions were significantly correlated (Table 5). Genetic distance among isolates in individual regions increased along with growth of elevation of a given region above sea level (Tables 2 and 4). A positive, statistically significant correlation between measures of intrapopulation genetic variability of isolates and elevation of regions above sea level was ascertained (Table 5). A scatter plot of pair-wise genetic distance (D) and average elevation of regions illustrated a gradual increase in genetic distance between regions at increasing elevations (Fig. 3). The R^2 value (0.897) suggested that almost 90% of total variation in genetic distance was explained by changes in elevations between regions. It was also ascertained that genetic distance (D) was significantly correlated with the number of days with snow cover in the examined regions (Table 5).

Out of 90 RAMS markers obtained, 7 occurred with a frequency decreasing along with increase in the region's elevation above sea level. They were obtained with the use of primers: ATG (ATG_1259 bp), AGT (AGT_650), AGC (AGC_1738, AGC_1216, AGC_1080, AGC_490) and

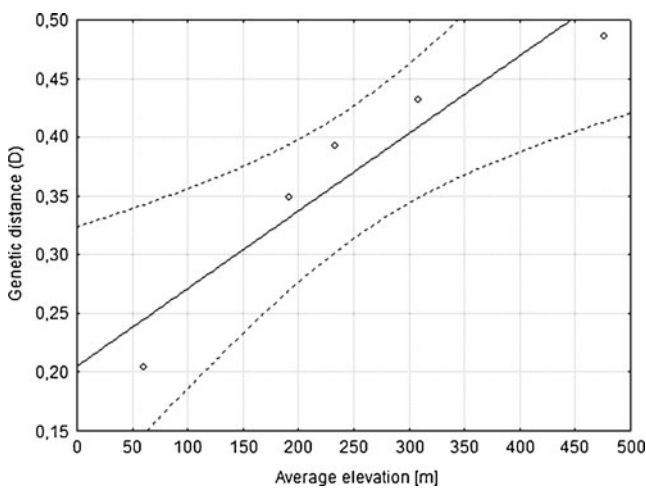


Fig. 3 Scatter plots of intrapopulation variance (expressed as genetic distance coefficient (D)) as a function of the average elevation of the regions investigated. The solid line is a predicted value from the regression equation ($P=0.015$, $R^2=0.897$), dashed lines=95% confidence interval

Table 6 Frequency of random amplified microsatellite bands according to region elevation and correlation coefficients (r)

Marker	Region (elevation [m])					r	P
	I (60)	II (192)	III (233)	IV (308)	V (476)		
ATG_1259	0.950	0.889	0.880	0.864	0.645	-0.907	0.034
AGT_650	0.950	0.955	0.750	0.677	0.640	-0.801	0.103
AGC_1738	0.950	0.591	0.500	0.400	0.355	-0.898	0.038
AGC_1216	0.750	0.591	0.472	0.340	0.290	-0.946	0.015
AGC_1080	0.650	0.500	0.472	0.400	0.323	-0.976	0.004
AGC_490	0.800	0.636	0.583	0.580	0.548	-0.864	0.059
ACA_643	0.700	0.682	0.417	0.260	0.226	-0.883	0.047
Average	0.821	0.688	0.583	0.500	0.438		

ACA (ACA_643). Five of them showed statistically significant, negative correlation with the elevation of a region above sea level (Table 6).

Genetic distance among strain groups deriving from individual regions was calculated with the use of unbiased Nei's genetic distance (Table 7). The matrix of pair-wise genetic distances between regions was not significantly correlated with the corresponding matrix of geographic distances (Mantel tests; $r=0.418$, $P=0.133$; Fig. 4). Regions located lowest (I and II) were characterised by smallest genetic distance, in spite of being most distanced geographically. Pairs of regions that were geographically close were most distanced genetically—they were located higher above sea level (regions III, IV and V) and characterised by greater intrapopulation variability (Tables 2 and 4). Correlation analysis has shown that Nei's genetic distance was significantly correlated with differences in elevation above sea level (Pearson rank correlation coefficient: $r=0.769$, $P=0.009$) and differences in measures of intrapopulation variability of isolates for regions of their occurrence (Tables 4, 7).

The AMOVA of distance matrix for the five investigated regions permitted partitioning of the overall variation into three levels. The proportion of variation attributable to within-region differences was 85% ($P=0.001$), between-region differences only 2% ($P=0.005$), whereas differences between lowlands (Regions I and II) and uplands (Regions III–V) were 13% ($P=0.001$) (Table 8).

Table 7 Pairwise population matrix of Nei's unbiased genetic distance

Region	Region I	Region II	Region III	Region IV
Region II	0.003			
Region III	0.046	0.025		
Region IV	0.072	0.041	0.003	
Region V	0.101	0.068	0.013	0.007

Principal Components Analysis (PCA) for investigated regions confirmed the results from Nei's genetic distance matrix (Table 7). A close relationship was revealed between groups of isolates from lower placed Regions I and II, and between three regions from southern Poland, which are located higher. It should be stressed that the most distanced group of isolates was obtained from Region V, which is placed on the highest elevation (Fig. 5).

Discussion

The study describes the genetic variability of *Ch. fraxinea*, a serious pathogen of ash trees. The applied molecular markers showed a high genetic variability and an interregional genetic structure of the fungal populations, depending on geographic location and climatic conditions. RAMS markers are frequently used to characterise the genetic variability because they are highly polymorphic and, for the analysis, only a small amount of mycelium is needed (Grünig et al. 2001; Kraj and Kowalski 2008; McDonald 2004; Vainio and Hantula 1999).

The genetic variability of *Ch. fraxinea* was determined at multiple loci across the genome. A potentially more sensitive measure of genetic variability is determination of

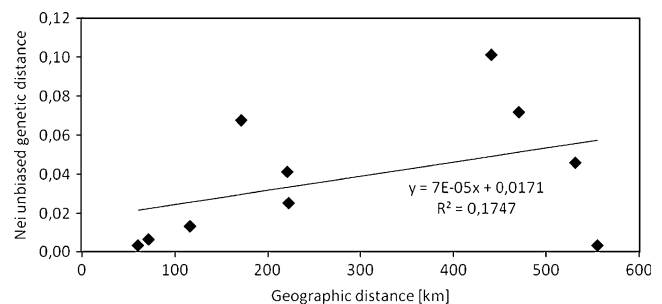
**Fig. 4** The relationship between geographic and Nei unbiased genetic distance among groups of *Chalara fraxinea* isolates from five regions of Poland

Table 8 Analysis of molecular variance (AMOVA) for *Chalara fraxinea* strains from five regions of Poland based on RAMS markers

Source of variation	df	Sum of square	Variance	% of variance	Φ	<i>P</i>
Among lowland–upland	1	134.509	1.882	13	0.132	0.001
Among Regions	3	58.960	0.233	2	0.019	0.005
Within Regions	154	1,862.304	12.093	85	0.149	0.001
Total	158	2,055.77	14.208	100		

the number of allele on a locus; however, this measure cannot be applied in this case.

We did not find individuals with identical haplotypes. This leads to the conclusion that the only method of *Ch. fraxinea* propagation is creation of ascospores. It was observed by Kirisits et al. (2009) who showed that conidia did not germinate on MEA, V8 agar or on agar medium containing an extract from ash leaves that stimulated mycelial growth. Also, Rytönen et al. (2010) observed a large proportion of haplotypes (14 among 32 isolates) and high genetic variability of fungus.

Among the most important sources of genetic variability of fungi are: selection of various alleles and genotypes, depending on the local environment, limitations of gene flow and founder effects and epidemic spread to new host plant populations (Hedrick 2000; Wang et al. 1997). All the measures of intrapopulation variability applied in our study showed that genetic variability is positively correlated with elevation above sea level. Comparison of intrapopulation genetic variability among isolates from lowlands and areas at higher elevations revealed clear differences. No specific molecular markers characteristic for distinguished groups of regions (lowlands and uplands) were identified, yet for five markers a statistically significant, negative correlation between their frequency and increasing elevation of the region's location above sea level was shown (Table 6). According to our data, the genetic variability of *Ch. fraxinea* isolates is not connected to the geographic distance of regions of their occurrence, as it has been found for other

fungi. For example, the main factors influencing genetic variability of *Gremmeniella abietina* (Lagerb.) Morelet are climatic conditions and thickness of snow cover (Kraj and Kowalski 2008; Uotila et al. 2006). Higher levels of genetic variability of isolates from sites at higher elevations were related to the more harsh and variable climate. Strains of *Sclerophoma pythiophila* (Corda) v. Hoehn deriving from various regions also displayed high level of intrapopulation variability, dependant on climatic conditions (Kraj 2009). For *Ch. fraxinea* in Poland, the effect of climatic conditions on the level of intrapopulation variability was confirmed by AMOVA analysis (Table 8), which showed a much higher (13%) share of variability among isolates deriving from lowlands and uplands in comparison to only 2% share in variability between geographically distant regions. This means that 87% of the differences can be explained by elevation. We assume that the high genetic variability level of *Ch. fraxinea* is related to the need for adaptation to climate. Development and spreading of fungal pathogens depend strongly on humidity and temperature and coping with these factors is essential for survival (Huber and Gillespie 1992). The high genetic variability of *Ch. fraxinea* populations, which possibly evolved in response to climatic conditions, could also contribute to virulence. Isolates of *Ch. fraxinea*, depending on temperature, showed a significant variety of the growth rate in vitro (Kowalski and Bartnik 2010). Such growth differences occurred not only among isolates from distant origins, but also among isolates deriving from the same forest district. Inoculation experiments showed tolerance of individual ash trees against *Ch. fraxinea* both in the greenhouse (Bakys et al. 2009a) and in the field (Olrik et al. 2007).

High levels of genetic variation are characteristic for sexually reproducing organisms with a wide geographical distribution (Hedrick 2000; James et al. 1999) and the contrary is true for species with a restricted distribution, small populations or in organisms that reproduce asexually (Burdon and Roelfs 1985). The high genetic variability is somewhat surprising for a pathogen, which has probably been introduced in Europe (Queloz et al. 2010). All strains in our study were clearly determined as *Ch. fraxinea* with the teleomorph *H. pseudoalbidus* by ITS sequencing. Nevertheless, we cannot exclude completely that cryptic species might be detected within this taxon by application of markers with higher resolution (e.g. AFLPs).

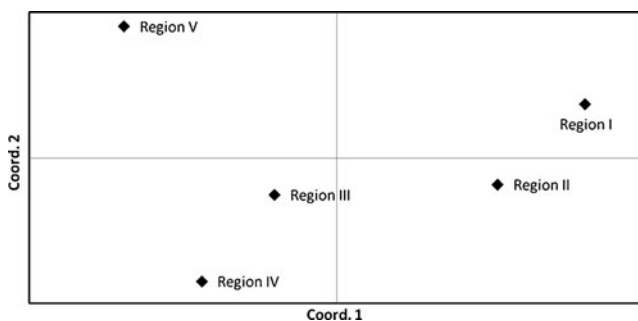


Fig. 5 Principal Components Analysis (PCA) for *Chalara fraxinea* strains from five regions of Poland made via a matrix genetic distance. Projection into 1st and 2nd coordinates. Percentage of variation explained by the first three axes: 95.80%, 2.88% and 1.32%

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