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GENETIC VARIABILITY OF THE POPULATIONS OF *ZWACKHIA VIRIDIS* (ACH.) POETSCH & SCHIED (LECANOGRAPHACEAE, LICHENIZED ASCOMYCETES) IN THE EASTERN POLAND: GEOGRAPHIC VERSUS HABITAT DISTANCE

ABSTRACT: *Zwackhia viridis* is a crustose lichen being found in forests throughout Poland. It belongs to rare species being labelled primeval forest lichens which are associated with large forest complexes. The eastern populations of *Z. viridis* were examined using RAPD technique in order to check whether geographic distance affects genetic diversity of the populations under study. The analysis of seven populations of that species showed large intraspecific diversity. The greatest genetic similarity occurred between populations from the north-eastern area of the country, about 300 km away from each other. In dendrograms, only these populations have simple matching distance greater than 50%.

KEY WORDS: lichens; random amplified polymorphic DNA (RAPD), genetic variation

1. INTRODUCTION

Zwackhia viridis is a rare lichen in Poland. It can be found both in lowlands and mountains. It mainly grows on the bark of beech, hornbeam and ash trees in natural dense forest stands but is less frequent on the phorophytes growing individually or in small density (*Betula* sp., *Acer* sp., *Quercus* sp., *Populus* sp., etc.), occasionally being reported on the bark of conifers (*Abies alba* and exceptionally

Pinus silvestris) (Wieczorek 2006). *Zwackhia viridis* grows over trees with the bark's pH ranging from moderately acidic (4.9–5.6) to neutral (5.7–7.0). It is a photophobic species which prefers wet locations with poor access to sunlight (Wirth 1995). This species is characterised by a thin thallus, being usually brighter than the bedding, frequently with lines of darker prothallus with numerous, quite regularly distributed fruiting bodies.

Until recently, the available references have described mycobiont molecular analyses at the population level, especially in the aspect of the geographical distribution of lichens. The studies conducted on single populations of different lichen species have been already carried out in the 90s of the last century (Lohtander *et al.* 1998, Heibel *et al.* 1999, Lumbsch and Schmitt 1999, Printzen *et al.* 1999). The use of RAPD technique to study the variability of two lichen species *Graphis scripta* (L.) Ach. and *Phaeographis dendritica* (Ach.) Müll. Arg. has been first reported by Murtagh *et al.* (1999). The authors presented in their paper not only a detailed protocol of the analysis but also emphasised the usefulness of this method to make genetic fingerprints for particular isolates. Since then, this method has been used,

among others, in studies on the variability of *Biatora helvola* (Körb.) Hellb. by Prinzen *et al.* (1999) or *Xanthoria parietina* (L.) Th. Fr. by Honegger *et al.* (2004) and Itten and Honegger (2010).

First molecular population-related study based on the analysis of LSU nrDNA, LSU mtrDNA and ITS1 nrDNA sequences has been carried out by Zoller *et al.* (1999). The authors examined 6 different Swiss populations of *Lobaria pulmonaria* (L.) Hoffm. and identified 6 genotypes. No correlation was found in the study between the occurrence of respective genotypes and their geographical distribution, whereas greater genetic variability

was observed in the specimens reproducing sexually. Further molecular studies have been conducted on *Cavernularia hultenii* Degel. (Prinzen and Ekman 2002, Prinzen *et al.* 2003). Its populations were analysed again, but this time focusing on those from the north-western parts of North America, Newfoundland and North-Western Europe. Although the analyses were performed on a larger number of specimens (300), no dependence of the occurrence of respective genotypes on geographical distribution has been shown. Since then, an interest in this research topic has increased (Zhang and Hewitt 2003, Walser *et al.* 2005, Buschbom 2007,

Table 1. Location of seven natural populations of *Zwackhia viridis* under analysis.

No. population	Name	Latitude	Longitude	No. of sampled thalli	Host tree
I	Olsztyn Lakeland (I)	54°01'18"N	20°24'47"E	6	<i>Fraxinus sp.</i>
II	Olsztyn Lakeland (II)	53°46'14"N	20°28'23"E	6	<i>Acer sp.</i>
III	Knyszyn Forest	53°16'11"N	23°22'25"E	6	<i>Fraxinus sp.</i>
IV	Białowieża Forest	52°40'46"N	23°49'15"E	10	<i>Fraxinus sp.</i>
V	Drohiczyn Plateau	52°21'34"N	23°07'51"E	6	<i>Fraxinus sp.</i>
VI	Świętokrzyskie Mts.	50°56'09"N	20°55'53"E	6	<i>Carpinus betulus</i>
VII	Western Bieszczady Mts.	49°12'21"N	22°24'58"E	7	<i>Fagus silvaticus</i>

Fig. 1. Map presenting seven natural populations of *Zwackhia viridis* used in this study.

Cassie and Piercey-Normore 2008). At present, a number of molecular methods is used in population studies which have been reported by Werth (2010). Unfortunately, many aspects of the genetic diversity of different lichen species populations have not been explained yet.

Bearing in mind many factors that shape the genetic diversity of fungi, including lichens, quite scanty literature in this respect and lack of studies on the genetic variability of the thalli of *Z. viridis* populations, we wanted to find answers to two questions. Firstly, we wished to explore how similar (or distant) are the populations being geographically distant from each other. Secondly, whether the type of bedding, on which these populations occurred, affects the genetic distance of individuals belonging to this species.

2. MATERIAL AND METHODS

2.1. Plant material

Forty seven populations of *Zwackhia viridis* were selected for the study, coming from 7 natural locations situated in the Eastern Poland (Fig. 1, Table 1). In total, 6 to 10 thalli were collected from the bark of three trees growing close to each other from each of these 7 locations. Each of the thalli had numerous well-developed apothecia with fully-developed ascospores. For DNA isolation, over a dozen of fruiting bodies were collected from each thallus. Freshly collected specimens of lichens, being free from any visible symptoms of fungal infection, were used for DNA isolation. The genetic variability of *Z. viridis* was established by the material collected by authors in 2010–2011.

2.2. Extraction of DNA

DNA isolation was performed according to the modified method of Grube *et al.* (1995).

Over a dozen fructifications coming from one analysed specimen were placed in an Eppendorf tube (1.5 mL) each. The material was incubated on Petri dishes in 10 volumes of acetone over 20 minutes. Thereafter, the material was transferred to cooled mortars and ground with liquid nitrogen with a glass rod.

Guanidine buffer (Sigma), in the amount of 100–200 μL , was added to the ground fragments and the entire content was transferred to a clean tube (1.5 mL). The collected material was incubated in a thermoblock at 65°C for 10 minutes. At the further stage of work, tube was centrifuged at 13000 rpm at room temperature for 2 minutes and the supernatant was transferred to a new tube. Guanidine buffer, in the amount of 100 μL , was added again to the precipitate and all of the content was incubated at 65°C for 10 minutes, followed by its centrifugation at 13000 rpm for 2 minutes. This supernatant was combined with the previously obtained one. One volume of phenol-chloroform-isoamyl alcohol mixture (Sigma) was added to the resultant supernatant. The entire content was centrifuged at room temperature for 5–10 minutes and then moved to a new 1.5 mL tube, with one volume of chloroform (Sigma)-isoamyl alcohol (POCH) mixture (24:1) being added to it thereafter. All of the content was centrifuged for 5–10 minutes and the upper phase was then moved to a new tube, with 3 volumes of 6M NaJ (Sigma) and 6 μL glassmilk being subsequently added to it. The entire content was gently stirred and left at 4°C for 24–48 hours. After this time, all of the content was centrifuged for 5 seconds, the supernatant was poured out, whereas the left precipitate was washed three times with 80% ethanol. Next, 20 μL water was added to the washed precipitate and the entire content was centrifuged at 13000 rpm at 20°C for 1 minute. After centrifugation, the supernatant where DNA was isolated had been drawn into a new 1.5 mL Eppendorf tube.

The quantity and quality of DNA were measured using a SmartSpecTMPlus spectrophotometer (BioRad). DNA was diluted with sterile water to a concentration of 50 ng L⁻¹.

2.3. DNA amplification

In the study on the genetic variability in *Z. viridis* lichens, 19 RAPD primers (OPERON Technology) were used. The amplification process was repeated three times for each of seven natural populations of that species.

Amplification reactions were carried out in 25 μL final volume of reaction mixture containing 150 ng DNA, 2.5 LI PCR buf-

fer, 2.5 µL dNTP (1.25 mM), 3.5 µL MgCl₂ (25 mM), 6.25 µL primer (50 ng), and 1U Taq polymerase.

The temperature profile of amplification reaction was established experimentally and included the following stages of PCR: a) initial DNA denaturation: 95°C for 5 min.; b) 40 cycles: (DNA denaturation at 93°C for 30 s, primer annealing at 37°C for 40 s, DNA chain elongation at 72°C for 1–2 min.; c) final elongation at 72°C for 5 min. Amplification was carried out in a DNA thermal cycler (Bio-Rad). Amplification products were separated on 1.5% agarose gel in TBE buffer and then visualised under UV light, using ethidium bromide. The size of amplification products was estimated by comparison with standard molecular weight marker (GeneRuler).

Based on electrophoretic separation, the image of amplified DNA fragments was converted into numerical data, using computer software such as Quantity One® v.22 (BioRad), Bio-Profil Bio-Gene Windows Application v.99.05 (Vilber Lourmat) and Gel Pro-Analyzer™ v.2.0 (Agilent®).

2.4. Data analysis

Based on the band patterns being obtained by means of PC-RAPD technique, the genetic similarity of seven natural populations of *Zwackhia viridis* from the area of Eastern Poland was determined. The occurrence of bands was coded in the form of binary matrix (0 – no band, 1 – band present). The matrix was used to calculate genetic distance (simple

matching method) (Rohlf 2002). The value of genetic distance was calculated based on the bootstrapped matrix being obtained on the ground of 499 random permutations using PhylTools computer package (Buntjer 1997), whereas the value of percent support for the obtained groups using the PHYLIP software package (Felsenstein 2012). To calculate the value of correlation between genetic distance (simple matching) and geographic distance, together with statistical significance, the Mantel test was applied. The matrix of distance between locations was obtained based on the geographic co-ordinates using Geodist computer programme from AFLPdat software package (Ehrich 2006). The Mantel test was conducted using NT-SYSpC software (Rohlf 2002).

The data referring to the occurrence of thalli on respective tree species (beech, hornbeam, ash and maple trees) were recorded in the form of latent dummy variables. To determine relationships between the variability of *Zwackhia* in relation to tree species, canonical correspondence analysis (CCA) was used (Ter Braak 1986). Based on 499 random permutations, a Monte Carlo test of statistical significance for Axis I and all Axes CCA was carried out. In calculations, CANOCO for Windows software package (Ter Braak and Smilauer 2002) was applied.

3. RESULTS

From among 19 RAPD primers being used in the study, only 7 primers gave poly-

Table 2. General characteristics of RAPD marker polymorphism.

Primer	No. of fragments		% polymorphic fragments	Product size range (bp)
	Total	Polymorphic		
A11	11	10	91	158–1484
A18	19	19	100	227–1435
AJ04	20	20	100	198–1351
B01	8	8	100	243–784
D03	18	17	94	158–934
E04	23	23	100	119–1008
E07	19	19	100	207–1054
Total	118	116	98	158–1484
Mean	16.90	16.60	98	–
Min – Max	8–23	8–23	91–100	158–1484

morphic loci in all analysed natural populations of *Zwackhia viridis*: A11, A18, AJ04, B01, D03, E04 and E07 (Table 2). The analysis covered only well-marked and recurrent bands. The total number of bands in the analysis was 118 and included polymorphic loci with size ranging from 119 to 1484 bp. The largest number, 23 products, was obtained for primer E04, whereas the least one, *i.e.* 8 bands, for primer B01 (Table 3), with a mean of 16.6 polymorphic bands per primer. The monomorphic loci were observed only after amplification with primers A11 and D03. These data demonstrate that the genetic variability of the lichens under analysis is very high.

Analysis of the individual populations showed that intraspecific genetic diversity was considerably lower and ranged between 20% in population III and 40% in population II. For an individual population, the highest number of alleles was recorded for locus E04. For that locus, 15 alleles (Table 4) were found in population II. The average number of alleles per locus in a population was 5.5 which was three times lower than the general average number of alleles per locus (16.6). As far as the number of alleles in a population is concerned, populations II (39%) and IV (38%) were the most polymorphic, whereas the lowest polymorphism was observed in

Table 3. List of RAPD primer sequences and their annealing temperature (Ta).

Primer	5'→3' sequence	Ta
A11	CAATCGCCGT	32°C
A18	AGGTGACCGT	32°C
AJ04	GAATGCGACC	32°C
B01	GTTTCGCTCC	32°C
E04	GTGACATGCC	32°C
E07	AGATGCAGCC	32°C
D03	GTCGCCGTCA	34°C

Table 4. Percentage and number of polymorphic fragments in seven natural populations of *Z. viridis* based of RAPD markers.

Primer	Populations													
	I – Olsztyn Lakeland I		VII – Western Bieszczady Mts.		III – Knyszyn Forest		V – Drohiczyń Plateau		IV – Białowieża Forest		II – Olsztyn Lakeland II		VI – Świętokrzyskie Mts.	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
A11	3	27	2	18	1	9	3	27	3	27	6	55	2	18
A18	6	32	12	63	1	5	6	32	7	37	5	26	8	42
AJ04	7	35	3	15	3	15	4	20	11	55	3	15	0	0
B01	1	12	6	75	1	12	6	75	5	62	2	25	5	62
D03	5	28	9	50	7	39	2	11	8	44	7	39	3	17
E04	8	35	2	9	11	13	11	48	6	26	15	65	11	48
E07	7	37	5	26	0	0	9	47	5	26	8	42	8	4
Total	37	31	39	33	24	20	41	35	45	38	46	39	37	31
Mean	5	29	6	37	3	13	6	37	6	40	7	38	5	33
Min– Max	1–8	12–37	2–12	9–63	0–11	5–39	3–11	11–48	3–11	27–62	2–15	15–65	0–11	0–47

Fig. 2. Phenogram clustered by the UPGMA method, using simple matching distance. Support was given to a value of > 50%.

Table 5. Genetic similarity matrix (UPGMA) of seven natural population of *Zwackhia viridis* under study based on the PCR–RAPD analysis

	II – Olsztyn Lake- land (II)	VII – Western Bieszczady Mts.	III – Knyszyn Forest	V – Drohiczyn Plateau	IV – Białowieża Forest	I – Olsztyn Lake- land (I)	VI – Świętokrzyskie Mts.
II – Olsztyn Lakeland (II)	1						
VII – Western Bieszczady Mts.	0.48	1					
III – Knyszyn Forest	0.34	0.41	1				
V – Drohiczyn Plateau	0.41	0.45	0.37	1			
IV – Białowieża Forest	0.42	0.52	0.41	0.31	1		
I – Olsztyn Lakeland (I)	0.37	0.36	0.40	0.40	0.53	1	
VI – Świętokrzyskie Mts.	0.41	0.54	0.42	0.46	0.44	0.39	1

Table 6. Two-way Mantel (1967) test.

Tests for association:	
Matrix correlation:	$r = 0.14870$
Raw Mantel statistic	$Z = 2875.1459$
Approximate Mantel t-test:	
Prob. random $Z < \text{obs. } Z$:	$p = 0.7652$

Table 7. Summary of the Monte Carlo test based on 499 random permutations.

	F-ratio	P-value
Test of significance of the first canonical axis: eigenvalue = 0.316	0.871	0.9900
Test of significance of all canonical axes: Trace = 0.837	0.800	0.9700

the Knyszyn Forest population – only 20% (Table 4).

The findings referring to genetic similarity allowed construction of Dice genetic similarity matrix (Table 5). Low bootstrap values show that dendrogram topology is unstable (Fig. 2). Among the natural populations of *Z. viridis* being analysed, those from the Western Bieszczady Mts. and the Świętokrzyskie Mts.

proved to be the most similar (0.54), as well as those from the Olsztyn Lakeland (I) and the Białowieża Forest (0.53). On the other hand, the least similarity was observed between the natural populations of *Z. viridis* from the Drohiczyn Plateau and the Białowieża Forest (0.31) (36.6%) and those from the Olsztyn Lakeland (II) and the Knyszyn Forest (0.34) (Table 5). Although the natural populations

from location III are sisterly to those from location II in dendrogram, yet with no greater statistical support.

The Mantel's test (Table 6) did not show any statistically significant correlation between genetic distance and geographic distance for respective regions.

It also turned out that genetic variability did not affect the type of substrate.

The data obtained in the Monte Carlo test showed that there is no statistically significant effect of substrate on genetic distance (Table 7).

4. DISCUSSION AND CONCLUSIONS

The study showed differences between isolated populations of *Z. viridis* from different regions of the Eastern Poland, even though these populations were morphologically identical and indistinguishable. All the thalli being analysed had well-developed organs of sexual and asexual reproduction in the form of well-developed pycnidia with mature conidiospores. The material used in this study included only organs of sexual reproduction based on earlier studies (Zoller *et al.* 1999), where greater genetic diversity of the thalli populations forming organs of sexual reproduction (apothecia) had been showed, irrespectively of the size of population.

The interpopulation genetic variability of *Zwackhia viridis* thalli is large, as in many other lichen species (Heibel *et al.* 1999, Printzen *et al.* 1999, Zoller *et al.* 1999, Printzen and Ekman 2002, Lindblom and Ekman 2006, Mattsson *et al.* 2009, Yüzbaşıoğlu *et al.* 2011). Within the seven groups being analysed, the largest genetic similarity occurred between the populations in location I and IV (Fig. 2). In dendrogram, only these populations have a statistical support being greater than 50%. The thalli collected from these locations were large, well-developed, had a large number of developed fruiting bodies and grew over the bark of the same phorophyte. The results obtained are probably indicative of a common source of origin of these two populations, but this requires more detailed molecular studies.

The fact of no affiliation between populations from two localities in Olsztyn Lakeland (Olsztyn Lakeland II and Olsztyn Lakeland I)

is confusing. Geographic distance between these two populations is the least from among all other populations. One would expect the highest similarity between them but they are the only populations being most distant genetically (least interrelated), even though the populations from location III are sister ones to those from location II. It is worth noting here that individuals of the population growing within the Olsztyn Lakeland (II) grew over the bark of maple tree, so they grew on a different substrate than the populations of adjacent geographic regions which grew over the bark of ash trees. It is puzzling why the north-eastern populations being nearest to one another are so clearly genetically isolated from each other. The cases of great genetic diversity of individuals, even in local lichen populations, are known from literature (Dyer *et al.* 2001, Printzen and Ekman 2002, Printzen *et al.* 2003). The reason for that may be the habitat isolation which leads to a reduction of gene flow between populations (Heibel *et al.* 1999, Lindblom and Ekman 2006). The effect of substrate type on different conditions for development of lichens of that species may also be taken into account. This is confirmed by the study on *Hypogymnia physodes* (L.) Nyl. where it has been emphasised that intraspecific genetic diversity may be associated with substrate richness in different nutrients (Mattsson *et al.* 2009).

Other groups form variable combinations, out of which the south-eastern populations from the Western Bieszczady Mts. and the Świętokrzyskie Mts. form only a stable group but with no statistical support.

Summing up, it was found that genetic diversity of the populations under study does not result from the isolation model (isolation by distance). According to this, the probability of hybridisation or migration decreases together with increasing geographic distance. The lack of correlation between genetic variability and geographic distance has been also showed, among others, in the study on *Lobaria pulmonaria* populations being carried out in Switzerland (Zoller *et al.* 1999). Most probably, the reason for genetic diversity between the populations of that species may be a limited gene flow associated with different habitat conditions.

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