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Diversity of *Armillaria ostoyae* in Scots pine plantations in Poland

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Abstract: Incidence of *Armillaria* root disease and the population structure of associated *Armillaria* spp. were studied in 5–17-year-old Scots pine plantations in west-central Poland. Two infection centres (1.14–9.30 ha) in each of three forest districts (Siemianice, Zielonka and Złotów) were intensively sampled. Root collars were examined for mycelial fans, decayed wood, and rhizomorphs. Twenty two isolates of *Armillaria ostoyae* collected from epiphytic rhizomorphs from 20 living and two dead trees in the six infection centres were identified with somatic incompatibility group. Only one somatic incompatibility group for *A. ostoyae* was found. Twenty one isolates produced rhizomorphs on oak-wood discs submerged in a sand-forest soil substrate. Isolates from Siemianice formed the smallest rhizomorph networks and those from Złotów the most abundant. There were 16 different genets among 22 isolates of *A. ostoyae* distinguished by RAPD analysis. Genetic similarity among genets was 25.6–97.5%. The large diversity in *A. ostoyae* suggests that sexual reproduction may occur in nature more often than expected.

Additional key words: *Armillaria ostoyae*, diversity, genets, RAPD-PCR, rhizomorphs

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Introduction

Armillaria root rot can severely damage the root system of forest trees, causing reduced growth, predisposition to windthrow and tree mortality, with a consequent negative effect on commercial timber production. *Armillaria ostoyae* (Romagn.) Herink infects conifers of the Pinaceae Lindley. It is a common pathogen in Poland. In 2012 *Armillaria* root rot occurred on 99 215 ha (Anonymous 2012). The rela-

tive importance of *A. ostoyae* from a commercial point of view increases when conifers replace hardwoods (Guillaumin et al. 1993; Legrand and Guillaumin 1993).

Armillaria (Fr.) Staude is an obligate wood inhabitant, obtaining all its nutrients from the degradation of woody substrates. The fungus forms a highly differentiated root-like aggregation of hyphae. These rhizomorphs ramify through the soil, being fuelled by the mycelium growing in wood. Rhizomorphs

also grow epiphytically along roots (Baumgartner and Rizzo 2001).

The ability to colonize a wood substrate and then to produce rhizomorphs that spread out into the surrounding soil enables *Armillaria* to form infection centres that can develop from a single tree to cover many hectares (Shaw and Roth 1976; Van der Kamp 1993; Peet et al. 1996; Ferguson et al. 2003).

Rhizomorphs are 1–5 mm in diam., have a melanized outer protective cortex layer and central gas-filled cavity and pores which allow them to translocate water and nutrients and participate in aeration and gas exchange (Granlund et al. 1984; Cairney et al. 1988; Pareek et al. 2001, 2006). Considering that they grow towards rather than away from the soil surface (Morrison 1976), they may help mycelium to respire in the low oxygen and high carbon dioxide environment beneath the root bark (Baumgartner et al. 2011). Rhizomorphs may affect the activity of other soil organisms, including mycorrhizal fungi (Leake et al. 2004).

Rhizomorphs are produced during various stages of wood decay, but the extent of growth is host species-dependent and influenced by habitat and environmental conditions, including composition and nutritional status of the soil and the presence of primary and secondary colonizers (Weinhold 1963; Pearce and Malajczuk 1990; Prospero et al. 2006). Rhizomorphs are formed mostly in the upper layer of the soil profile (A–B horizon up to 30 cm) owing to their dependence on an adequate oxygen supply (Redfern 1973; Morrison 1976; Rishbeth 1978; Singh 1981).

The extent, development and functions of *Armillaria* rhizomorphs in undisturbed ecosystems have been described by Thompson and Rayner (1983). The behaviour of rhizomorphs in pure culture and their spatial distribution in soil have been studied in terms of nutrient translocation and growth strategies by Bolton and Boddy (1993), Mihail and Bruhn (2005), Watkinson et al. (2005) and Lamour et al. (2007).

Much effort has been made to understand the biology and the epidemiology of *Armillaria*. Morrison (1989, 2004) first observed that rhizomorph growth habit affects saprotrophic and pathogenic growth of *Armillaria* and its virulence.

The ecological significance of *Armillaria* rhizomorphs is still not fully recognized. One of the areas where further investigation is needed is the genetic basis of pathogenicity and virulence (Gregory et al. 1991), which is linked to the ability to form rhizomorphs. In further studies, Prospero et al. (2004) showed that the virulence of Swiss isolates of *A. ostoyae* on 2-year-old Norway spruce (*Picea abies* (L.) H.Karst) was positively correlated with its ability to produce rhizomorphs. Susceptibility of Norway

spruce to *A. ostoyae* infection varied and depended on the provenance of the host. Infection by *A. ostoyae* was associated with significantly more lesions on roots than infection by *A. cepistipes* Velen. The virulence of *A. ostoyae* and *A. cepistipes* was not correlated with their wood-degrading capability.

Many studies on *Armillaria* have included population structure analyses. These often result in the identification of genets – genetically distinct secondary mycelia of the same species coexisting within a population. They are considered as units within a population. Knowledge of the size, distribution and behaviour of genets provides important information on the ecology, establishment, variation, activity and pathogenicity of *Armillaria*.

Studies on the population structure of pathogenic *Armillaria* species have been made in Canada, USA and Europe. In Canada, the most common species in nine infection centres in the central interior of British Columbia were *A. ostoyae* and *Armillaria sinapina* Bérubé & Dessureault (Dettman and Kamp 2001). The population structure of *A. ostoyae* was consistent with a clonal reproductive strategy, and infection centres were occupied by single *A. ostoyae* genets or ramets. The population structure of *A. sinapina* resulted from more frequent basidiospore infections, and infection centres were occupied by multiple (18) genets. In the USA, the most common *Armillaria* species on six tree species in approximately 16100 ha of dry, mixed-conifer forest of the Blue Mountains of north-east Oregon was *A. ostoyae* (96.4%) (Ferguson et al. 2003). Only 3.6% of isolates belonged to North American Biological Species X (NABS X). The most common species in more than 500 km² of mixed oak forests of central Massachusetts was *A. gallica* Marxm. & Romagn (Brazee et al. 2012). Here, there were 38 genets within 153 isolates. The genets ranged in distribution from 5 to 33 per hectare, with a mean of eight and the average genet occupying 0.13 ha. In the Swiss Alps, the incidence of *A. ostoyae* and *A. cepistipes* varied greatly (Prospero et al. 2004). Six genets were identified in each species. Genets of the same species overlapped rarely and only on the borders. There was strong intraspecific competition in both *Armillaria* species.

The objectives of this study were to investigate: (i) the incidence and population structure of *Armillaria* spp. in Scots pine plantations in west-central Poland; (ii) the ability of the Polish *A. ostoyae* isolates to produce rhizomorphs *in vitro*; (iii) effects of location on abundance of rhizomorphs produced; (iv) intraspecific genetic variability among *A. ostoyae* isolates; (v) reproductive strategy of *A. ostoyae* evaluated on the basis of its genetic variability.

Material and methods

Isolates

The 22 isolates of *Armillaria* used in this study were obtained from epiphytic rhizomorphs attached to roots and were collected between 2005 and 2013 from six infection centres in more or less severely diseased 5–17-year-old Scots pine (*Pinus sylvestris* L.) plantations in Siemianice Forest District (51.32083°N 16.91000°E), Zielonka Forest District (52.5533°N 17.1133°E) and Złotów Forest District (53.36346°N 17.04082°E). Isolates R3, R12 and R26 were from Siemianice I (2.95 ha), and R6, R27, R28 and R29 from Siemianice II (2.28 ha). Isolates R1 and R8 were from Zielonka I (9.30 ha), and R11, R13 and R30 from Zielonka II (3.52 ha). Isolates R4, R7, R14 and R15 were from Złotów I (3.60 ha), and R5, R9, R10, R16, R20 and R25 from Złotów II (1.14 ha). The infection centres in different Forest Districts were 300–400 km apart (Fig. 1, Table 1). The two infection centres in Siemianice were 50 km apart, in Zielonka 5 km apart and in Złotów 30 km apart. Characteristics of soils (top 30 cm of mineral soil) are in Table 1. In all three locations the previous stand was composed of Scots pine (95–135 years old) with admixture of larch (*Larix decidua* Mill.), spruce (*Picea abies* (L.) H. Karst.) and oak (*Quercus petraea* (Mattuschka) Liebl.). In Siemianice and Zielonka the mean annual temperature was 7–9°C and in Złotów 6–7°C, and annual precipitation was 700–800, 500 and 500–600 mm, respectively.

Table 1. Characteristics of topsoil in the sample areas

Soil characteristics	Siemianice	Zielonka	Złotów
Texture	Sandy loam	Sandy loam	Sandy loam
Sand:clay:silt	54:10:36	56:12:32	55:12:33
pH in KCl ¹	4.030	3.940	3.955
pH in H ₂ O	4.720	4.695	4.510
Organic carbon (%) ²	0.357	0.553	0.419
Total nitrogen (%) ³	0.040	0.056	0.046
C:N	8.92	9.87	8.91
Extractable phosphorus P ₂ O ₅ (mg/kg) ⁴	171.00	197.00	195.00
Extractable potassium (mg/kg) ⁵	10.95	12.10	10.00
Extractable magnesium (mg/kg) ⁵	1.05	1.35	1.00
Extractable calcium (mg/kg) ⁵	4.30	4.30	5.10
Extractable sodium (mg/kg) ⁵	0.75	1.00	0.90

¹ – analysed with potentiometer according to norm PN-ISO 103390:1997

² – analysed chemically according to norm PN-ISO 10694:2002

³ – analysed chemically according to norm PN-13878:2002

⁴ – analysed chemically with method of Egner-Riehm

⁵ – analysed chemically according to procedure PB-05 ed.2



Fig. 1. Map of Poland with infection centres in Siemianice, Zielonka and Złotów Forest Districts

At each infection centre, four plots were randomly established. From each plot centre, a variable radius (20–50 m) was established to locate isolates of *Armillaria*, primarily from living trees exhibiting symptoms and signs of infection (chlorotic needles, die-back of twigs, layer of resin, debris, and fungal tissue forming at the trunk base or around infected roots, white mycelial mats and rhizomorphs under bark, or clusters of fruit bodies at the base of the infected tree) or recently killed trees. Mean plot size was 0.48 ha with a range of 0.12–0.85 ha. The total area of all 24 plots sampled was 11.52 ha. The infection centres were surrounded by areas with no symptoms of disease. The number of isolates collected depended on the severity of *Armillaria* infection, which was low at Zielonka (10% diseased trees per plot), moderate at Siemianice (10–20%) and high at Złotów ($\geq 20\%$).

Within 12 h after collection, each rhizomorph was surface sterilized in sodium hypochlorite (7% active chlorine) for 15–30 s and rinsed twice in sterile, demineralized water for >15 s. The pieces were dried between paper towels and placed on 2% malt extract agar (MEA: Difco malt extract 20 g l⁻¹, Bacto Agar 15 g l⁻¹, thiabendazole 230 mg l⁻¹ (added in 1 ml concentrated lactic acid, 85–90%), streptomycin 100 mg l⁻¹, polymyxin sulphate 50 mg l⁻¹ and benzylpenicillin sodium 100 mg l⁻¹; modified from Legrand and Guillaumin 1993). Cultures were incubated in the dark at 25°C and subcultured as necessary.

Identification of *Armillaria* with somatic incompatibility tests

The *Armillaria* isolates were identified using somatic incompatibility tests following the methods described by Korhonen (1978). Mating tests were performed on 2% malt yeast extract agar (MYEA: Difco malt extract 20 g l⁻¹, yeast extract 2 g l⁻¹, agar 20 g l⁻¹) in Petri dishes. Each of the unknown 22 isolates was paired with a haploid tester (monospore isolate) of each of *A. borealis* Marxm. & Korhonen, *A. cepistipes*, *A. gallica*, *A. mellea* (Vahl) P. Kumm., *A. ostoyae* and *A. tabescens* (Scop.) Emel. Plates were incubated at 25°C in the dark for 4 weeks. The mating reaction was scored on the basis of the appearance of the mycelium. If the two cultures were confluent, this indicated that the isolates were the same species (the same so-

matic incompatibility group). A gap between the two cultures, with a reaction zone consisting of sparse to absent mycelia (i.e. mutual inhibition) indicated an antagonistic reaction between two genetically distinct isolates (Korhonen 1978; Dettman 1999). Paired cultures were examined every seven days until a clear and obvious compatibility or incompatibility reaction was observed. Any pairing that gave ambiguous or intermediate results was repeated.

Somatic incompatibility tests with diploid isolates of *Armillaria* collected throughout the plots were done to distinguish genetically distinct individuals (genets) within *A. ostoyae* (Shaw and Roth 1976). Mycelial plugs of isolates were placed 4 mm apart on 2% MYEA and plates were incubated at 25°C in the dark for 4 weeks. Isolates from the same plot were paired in all possible combinations, including self-pairings

Table 2. Effect of location on *A. ostoyae* rhizomorph production

Infection centre	Previous forest type	Isolate	Number of rhizomorphs		Number of rhizomorph apices		Rhizomorph length (mm)		Rhizomorph dry weight (mg)	
			Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Siemianice I	Fresh broadleaf forest (FBF)	R3	3.67c	4.72	6.33b	5.43	72.80c	62.60	2.83b	2.14
		R12	12.83bc	15.57	18.67b	24.78	192.30c	238.40	27.33b	55.72
		R26	10.43bc	13.51	15.42b	20.43	168.20c	198.60	17.48b	35.92
Mean		8.97	11.26	13.47	16.88	144.43	166.53	15.88	31.26	
Siemianice II	Fresh mixed broadleaf forest (FMBF)	R6	6.17bc	10.57	8.17b	13.59	72.70c	126.70	2.17b	3.92
		R27	10.23bc	13.27	16.42b	14.88	70.17c	122.52	2.12b	3.90
		R28	8.98bc	13.81	15.56b	16.53	57.25c	124.12	1.98b	3.32
		R29	8.26bc	10.60	8.27b	16.23	71.71c	116.43	1.87b	2.92
Mean		8.41	12.06	12.10	15.30	67.95	122.44	2.03	3.51	
Zielonka I	Fresh broadleaf forest (FBF)	R1	37.33bc	27.65	55.00b	47.34	1 296.80b	893.10	48.33b	33.40
		R8	10.67bc	13.38	10.83b	13.76	34.50c	45.20	0.75b	0.69
Mean		24.0	20.51	32.91	30.52	665.65	469.15	24.54	17.04	
Zielonka II	Fresh mixed broadleaf forest (FMBF)	R11	19.67bc	20.82	20.33b	21.39	137.70c	146.10	4.17b	4.57
		R13	9.00bc	19.26	10.00b	20.00	76.30c	152.40	4.00b	8.00
		R30	14.25bc	10.58	15.45b	20.67	100.53c	151.15	3.98b	7.88
Mean		14.30	16.88	15.26	20.68	104.84	149.88	4.05	6.81	
Złotów I	Fresh mixed broadleaf forest (FMBF)	R4	50.00b	42.79	54.17b	47.60	764.20bc	706.10	24.17b	24.85
		R7	1.50c	1.76	1.5b	1.76	4.00c	4.60	0.25b	0.27
		R14	13.17bc	10.96	14.17b	11.18	84.20c	59.60	2.83b	2.56
		R15	0.00c	0.00	0.00b	0.00	0.00c	0.00	0.00b	0.00
Mean		21.55	18.50	23.28	20.18	284.13	256.76	9.08	9.22	
Złotów II	Fresh broadleaf forest (FBF)	R5	116.00a	26.08	147.00a	33.56	2 327.80a	863.70	269.67a	224.61
		R9	22.50bc	40.80	28.17b	49.77	172.50c	90.60	9.50b	7.04
		R10	96.00a	36.54	121.83a	51.94	2 290.00a	1 074.70	114.67b	65.56
		R16	12.17bc	14.59	13.33b	15.54	82.70c	95.10	2.42b	3.83
		R20	32.33bc	23.09	40.33b	27.81	665.20bc	470.60	28.50b	20.08
		R25	8.33bc	10.86	10.33b	15.13	108.70c	175.60	12.50b	28.18
Mean		47.88	25.32	60.16	32.29	941.15	461.71	72.87	58.21	
		LSD _{0.001}	44.32		56.59		904.90		117.85	
Mean squares from analysis of variance										
Isolate			6 498.2**		10 480.1**		3 512.6**		27 223**	
Residual			507.2		826.8		211.3		3 586	

Different letters (a,b,c) indicate statistically significant differences between isolates (ANOVA, $P < 0.001$ or $P < 0.05$).

**Significant at $P < 0.001$.

as controls. Two isolates were identified as belonging to the same somatic incompatibility group (SIG) (Rizzo and Harrington 1993) if they merged to form a single colony. A line of demarcation between the cultures indicated somatic incompatibility. Once SIGs were identified within plots, pairings were done between isolates from different plots.

Production of rhizomorphs in oak segments

Discs (2 cm diameter, 1 cm long) from live, freshly cut branches of 20-year-old *Quercus robur* L. were used as a woody substrate to produce rhizomorphs *in vitro*. The discs were washed with 70% ethanol and autoclaved twice at 121°C for 60 min. Next, the discs were placed onto 20-day-old *A. ostoyae* cultures growing on 2% MEA in Petri dishes and incubated in the dark at 22°C for 30 days. Four discs were inoculated with each of the 22 *Armillaria* isolates. When the discs became covered with white-cream to light-brown *A. ostoyae* mycelium they were placed in non-sterile moist river sand in sealed jars. After 14 weeks of incubation in the dark at 22°C (Rishbeth 1982, 1984), when *A. ostoyae* was well established and initial, single, short rhizomorphs had formed on the surface of the discs, the discs were carefully transferred from the jars to plastic bags containing a substrate consisting of forest soil, sand and humus (1:1:1). The composition of this substrate resembled the composition of the forest soil and was intended to induce the formation of fully melanized rhizomorphs. After 10 weeks in the dark at 23°C, the number of rhizomorphs, number of rhizomorph apices, rhizomorph length (mm), and dry weight of rhizomorphs (mg) were determined (Łakomy 1998). The results are presented as the mean of four replicates (four wood pieces inoculated with one isolate) from one jar.

DNA extraction

Isolates were grown in 2% malt extract broth (MEB: Difco malt extract 20 g l⁻¹) at 24°C in the dark for 3 weeks. The mycelium was harvested with a strainer, lyophilized and ground to a fine powder in liquid nitrogen. Extraction buffer (1 ml; 100 mM Tris-Cl pH 8.4, 1.4 M NaCl, 25 mM EDTA pH 8, 2% CTAB, hexadecyltrimethylammonium bromide) was added to ~0.5 g powdered mycelium and incubated at 60°C for 2 hours. The mycelium powder-buffer suspension was divided into two parts and centrifuged (17 900 g, 20 min) to remove cell debris. Isoamyl alcohol:chloroform (1:24 v/v) extractions were performed on the aqueous phase until a clean interphase was obtained. Nucleic acids were precipitated using 96% ice-cold ethanol. The precipitate was col-

lected by centrifugation (17 900 g, 30 min), washed with cold 70% ethanol, dried, and eluted in sterile distilled water. Contaminating RNA was removed by adding RNase A (0.01 mg ml⁻¹) (Roche Diagnostics, Mannheim, Germany).

RAPD-PCR assay

Random amplified polymorphic DNA (RAPD) analysis was applied to detect polymorphisms in *A. ostoyae* isolates and to distinguish *A. ostoyae* genets. Seven decamer oligonucleotides (5'-3') were used: OPA-1 (CAGGCCCTTC), OPA-2 (TGCCGAGCTG), OPA-3 (AGTCAGCCAC), OPA-4 (AATCGCGCTG), R-25 (CCCGGCCTTCC), R-28 (ATGGATCCGC) and UBC-31 (CCCGGCCTTCC), obtained from oligo.pl (Institute of Biochemistry and Biophysics, Polish Academy of Science). These oligonucleotides were selected on the basis of high-intensity bands, hypervariability, and good definition of amplified DNA fragments, and have been used for molecular characterization and detection of polymorphisms in *Armillaria* spp. by Smith et al. (1992), Guillaumin et al. (1996), Schulze et al. (1997) and Dettman and van der Kamp (2001). RAPD analysis generally followed the method of Williams et al. (1990). The final RAPD-PCR reaction concentrations were: 2.0 mM MgCl₂, 100 μM deoxynucleotide triphosphates, 0.2 μM primer, 2.0 units *Taq* polymerase, 10 × PCR buffer, 10 ng DNA template, with a final volume of 25 μl per reaction tube. Cycling conditions were: initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 40°C for 30 s, 72°C for 30 s, and then cooling at 4°C. Amplification was carried out in a T3 thermocycler (Biometra, GmbH, Göttingen Germany). The amplification products were separated on 1.5% agarose gels at 4.75 V/cm, stained with ethidium bromide, and photographed under ultraviolet illumination. Only bands that were clearly resolved and consistent across all amplifications were recorded. The molecular weight of each band was estimated by comparing gel migration distances with those of the PCR marker, 50–2 000 bp (SIGMA, Cat. No. P9577, Saint Louis, MO 63103, USA). Each RAPD pattern describes a single genet.

Statistical analysis

To determine whether isolates collected from different locations differed in their ability to produce rhizomorphs *in vitro*, the number of rhizomorphs, number of rhizomorph apices, length of rhizomorph and dry weight of rhizomorphs were analysed with one-way analysis of variance (ANOVA, $P < 0.001$ or $P < 0.05$). The least significant differences (LSDs) for each variable were calculated and homogeneous groups for number of rhizomorphs, number of rhizo-

Table 3. Similarity among 22 isolates of *A. ostoyae* expressed by Mahalanobis distance

Location	Isolate	Siemianice						Zielonka						Złotów									
		R3	R12	R26	R6	R27	R28	R29	R1	R8	R11	R13	R30	R4	R7	R14	R15	R5	R9	R10	R16	R20	R25
Siemianice	R3	0.000						3.156															
	R12	0.611	0.000		0.607			3.282	0.783	1.059				2.695	0.791								
	R26	0.511	0.332	0.000	0.527			3.122	0.743	1.009	0.178			2.455	0.752	0.428	1.476	5.252	0.750	5.406			
	R6	0.204		0.000	0.000			3.179						2.536				5.022	0.678	5.326	0.369	1.555	0.172
	R27	0.320	0.688	1.324	1.253	0.000		2.871	0.838	0.616	1.343			2.345	4.720	1.238	0.387	5.276	0.666	5.335	0.469	1.476	1.456
	R28	0.199	0.567	1.224	0.393	1.555	0.000	3.259	5.401	0.286	0.262			2.423	5.519	0.234	0.639	5.316	5.320	5.452	0.769	1.562	1.456
	R29	0.200	0.757	1.425	0.488	0.256	1.224	0.000	2.979	0.124	0.376	0.282		2.789	0.612	0.438	0.597	5.234	0.706	5.291	0.876	1.276	1.256
	R1							0.000															
Zielonka	R8	0.663			0.464			3.387	0.000					2.323	0.546			5.511					
	R11	1.062			0.861			3.319	0.465	0.000			1.924	0.972				5.247	0.762	5.119			
	R13	0.457	0.687		0.256			3.227	0.238	0.626	0.000		2.363	0.362				5.502	0.866	5.332			
	R30	0.201	1.628	1.474	5.293	1.123	1.378	3.056	4.401	0.318	0.162	0.000	2.538	4.420	0.731	0.457	4.123	4.325	5.190	1.069	1.345	1.156	
	R4	2.688						2.704					0.000										
	R7	0.312			0.263			3.276						2.638	0.000			5.742					
Złotów	R14	0.690	0.767		0.488			3.302	0.124	0.386	0.262		2.226	0.612	0.000		5.416	0.706	5.291				
	R15	0.310	0.828		0.318			3.261	0.638	1.060	0.448		2.696	0.092	0.702	0.000	5.785	1.238	5.541				
	R5	5.665						4.832					4.427				0.000						
	R9	1.064			0.940			3.460	0.774				2.381	1.162			5.196	0.000					
	R10	5.450			5.393			2.881	5.401				3.514	5.520			3.923	5.320	0.000				
	R16	0.618	0.728		0.417			3.277	0.126	0.453	0.198		2.265	0.550	0.072	0.638	5.442	0.723	5.301	0.000			
	R20	1.594	1.628		1.510			2.032	1.525	1.333	1.443		1.359	1.651	1.418	1.686	4.606	1.593	3.896	0.419	0.000		
	R25	0.344	0.532		0.204			3.197	0.411	0.787	0.212		2.476	0.330	0.430	0.397	5.448	0.901	5.349	0.369	0.476	0.000	

D_{critical} = 4.73Table 4. Similarity coefficient values for pairwise combinations of 22 *A. ostoyae* genets based on 177 RAPD-PCR products

Location	Isolate	Siemianice						Zielonka						Złotów									
		R3	R12	R26	R6	R27	R28	R29	R1	R8	R11	R13	R30	R4	R7	R14	R15	R5	R9	R10	R16	R20	R25
Siemianice	R3	1						0.4750															
	R12	0.3548	1		0.2941			0.3704	0.2857	0.2985			0.3188	0.3250			0.2899	0.2857	0.2564				
	R26	0.3688	1	1	0.2531			0.8725	0.2957	0.8585	0.4505		0.5088	0.5480	0.5396	0.5500	0.2999	0.2557	0.2544	0.4265	0.2488	0.6348	
	R6	0.4894			1			0.4186					0.4752				0.5941						
	R27	0.4324	0.4952	0.5894	0.5186	1		0.4186	0.4862	0.7694	0.5941		0.5752	0.4832	0.3186	0.4654	0.6741	0.5941	0.5186	0.4852	0.5941	0.4286	
	R28	0.5224	0.5152	0.5199	1	0.6358	1	0.5486	0.5262	0.6584	0.5242		0.5872	0.4712	0.6286	0.5554	0.6651	0.5122	0.4886	0.5152	0.4841	0.8416	
	R29	0.4564	0.5258	0.5249	0.5123	1	0.7488	1	0.5231	0.5262	0.7654	0.5167	0.5456	0.5672	0.3890	0.5134	0.6780	0.5122	0.4237	0.4789	0.4823	0.4386	
	R1							1															
	R8	0.3659			0.3182			0.3514	1				0.6517	0.5800			0.4494						
	Zielonka	R11	0.4086			0.5859		0.3765	0.4138	1			0.5000	0.5586			0.5200	0.4870	0.4954				
R13		0.4444	0.3562		0.5524		0.4176	0.3871	1	1		0.5094	0.5641			0.5283	0.4628	0.5043					
R30		0.4234	0.7898	0.6899	0.5024	0.4568	0.7211	0.9281	0.5478	0.4562	0.2644	0.9687	1	0.4986	0.4612	0.7881	0.5244	0.5280	0.5021	0.3637	0.8789	0.7813	0.5376
R4		0.4421					0.5517						1										
R7		0.4906			0.5357		0.5102		0.6364	0.3529	0.3797	0.3765	0.9027	1			0.7611						
R14		0.4054	0.3750		0.4000		0.4000	0.6269	0.4348	0.3500	0.3488	0.4691	0.4691	0.4691	0.3333	0.4667							
Złotów	R15	0.4000	0.3265		0.3704		0.5287	0.6269	0.4348	0.3500	0.3488	0.5366	0.4731	0.8852	1	0.4390	0.3505	0.4396					
	R5	0.5474					0.5287	0.6269	0.4348	0.3500	0.3488	0.6863											
	R9	0.5636			0.5172		0.5172	0.3137	0.5577			0.6128	0.5781				0.6325	1					
	R10	0.6154			0.5091		0.5091	0.5000	0.4490			0.6667	0.7213				0.8649	0.6984	1				
	R16	0.5000	0.3448		0.4000		0.9474	0.3333	0.3596	0.4000		0.5275	0.5098	0.6286	0.5915	0.5275	0.3585	0.5200	1				
	R20	0.4474	0.2800		0.4634		0.7647	0.3429	0.3704	0.3678		0.4096	0.4255	0.5484	0.5079	0.4337	0.4082	0.4348	1	1			
	R25	0.4762	0.3103		0.4000		0.9211	0.3077	0.3371	0.3789		0.5055	0.5098	0.6000	0.5634	0.5275	0.3585	0.5234	1	1	1		

morph apices, length of rhizomorph and dry weight of rhizomorphs were determined. Similarity between each pair of evaluated isolates with respect to the four parameters analysed was expressed by Mahalanobis distance. This method provides a relative measure of a data point's distance (residual) from a common point. It is used to identify and gauge similarity of an unknown sample set to a known one. It takes into account the correlations of the data set and is scale-invariant. It has a multivariate effect size, more effective than principal component analysis (Mahalanobis 1936; Seidler-Łożykowska and Bocianowski 2012). The Mahalanobis distance was suggested as a measure of 'polytrait' object similarity, whose significance was verified by means of critical value D_{critical} called "the least significant distance". Graphical distribution of isolates on a plane, considering all the parameters, was possible through the application of canonical variates analysis (Camussi et al. 1985). The relationship among the parameters was analysed using correlation coefficients (Kozak et al. 2010). The coefficients of genetic similarity (S) of the isolates were calculated using the formula of Nei and Li (1979). The association between molecular markers (genetic loci indicated by bands) and the number of rhizomorphs, number of rhizomorph apices, length of rhizomorphs and weight of rhizomorphs for the 22 isolates was estimated using regression analysis (Hastie and Tibshirani 1990). The molecular markers were treated as independent variables and considered in individual models. Data analysis was performed using the statistical package GenStat v. 7.1

Results

Isolates

Two infection centres for *Armillaria* root rot were identified in the 5–17 year-old plantations of Scots pine in each of the three forest districts (Siemianice, Zielonka and Złotów) in west-central Poland. Twenty two isolation attempts from epiphytic rhizomorphs taken from these infection centres yielded 22 viable cultures of *Armillaria* (Table 2).

Identification of *Armillaria* with somatic incompatibility tests

In somatic incompatibility tests performed with six testers representing six European *Armillaria* spp., the tested isolates produced confluent mycelium only with the *A. ostoyae* tester indicating that all 22 isolates were *A. ostoyae*. In somatic incompatibility tests performed for all within-centre and between-centre pairings, the tested isolates formed homogenous col-

onies with no dark lines or other barrier zones, indicating that they belong to one and the same somatic incompatibility group of *A. ostoyae*.

Production of rhizomorphs in oak segments

Twenty one out of 22 *A. ostoyae* isolates initiated rhizomorphs in soil *in vitro*. Isolate R15 (from Złotów I) did not produce rhizomorphs despite mycelial growth on/within the oak discs. The branching pattern of the rhizomorphs was dichotomous. Each rhizomorph network grew from one or several initials formed on the oak disc. The mean number of rhizomorphs produced was 8.41–47.88, the number of rhizomorph apices was 12.10–60.16, rhizomorph length was 67.95–941.15 mm and rhizomorph dry weight was 2.03–72.87 mg (Table 2). Isolates from Siemianice and Złotów formed, respectively, the smallest and the largest rhizomorph networks. The individual rhizomorph parameters were often similar in isolates from Siemianice and Zielonka, and often significantly different (ANOVA, $P < 0.001$) from isolates from Złotów. Among isolates from Złotów there were four homogeneous groups for number of rhizomorphs, three for rhizomorph length, and two homogeneous groups each for number of rhizomorph apices and rhizomorph weight. The two isolates with most similarity expressed by the smallest Mahalanobis distance (0.072) were from Złotów I (R14 and R16, Table 3). The two isolates with the least similarity (Mahalanobis distance = 5.785) were from Złotów II (R5) and Złotów I (R15). Similarities and differences among isolates with respect to the four parameters analysed are illustrated in Fig. 2, which presents the dispersion of isolates on a plane in the system of the first two canonical variates. The first and second canonical variates elucidated 74.76% and 18.38% of the multivariate variability of the isolates, revealing that isolates R1 (Zielonka I), R4 (Złotów I) and R5, R10, R20 (Złotów II) are different from each other and are different from the other 17 isolates, which grouped together. The relationship between all four of the rhizomorph parameters analysed was shown by the positive and significant correlation coefficients 0.88–0.99 (at $P < 0.001$, data not presented).

The seven random amplified polymorphic DNA (RAPD) primers used to characterize the 22 *A. ostoyae* isolates yielded a total of 177 bands (10–27 per primer), of which 153 were polymorphic (Fig. 3). Their products ranged in size from 260 to 2000 bp. Each primer produced 8–15 different bands. Eleven isolates produced five RAPD patterns: R6 and R28, R12 and R26, R27 and R29 from Siemianice; R11 and R13 from Zielonka; R16, R20 and R25 from Złotów. Isolates with the same RAPD patterns were located

within areas of 1.14–3.52 ha. The other 11 isolates produced different RAPD patterns. Isolates with the same RAPD pattern were considered as identical. Based on the RAPD patterns, 16 distinct multilocus genets were detected among the 22 *A. ostoyae* isolates. Each genet consisted of 1–3 isolates. The similarity coefficient values for the 22 *A. ostoyae* pairwise genotype combinations (0.2488–0.975) showed genetic similarity between genets ranging from 24.88 to 97.50% (Table 4). The similarity coefficient was ≥ 0.500 for only 47% of pairwise genet combinations. The degrees of genetic similarity between isolate R1 (Zielonka I) and isolates R16 and R25 (Złotów II) were among the highest, even though they originated from two infection centres located 200 km apart.

Significant associations between nine markers (genetic loci) and the number of rhizomorphs and number of rhizomorph apices were demonstrated by regression analyses (Table 5). The percentage variation accounted for in regressions on number of rhizomorphs ranged from 23.0% for OPA2–40 and OPA2–41 to 52.4% for OPA4–101. The percentage variation accounted for in regressions on the number of rhizomorph apices ranged from 20.4% for OPA3–58 to 53.4% for OPA4–101. Significant associations were found between eight markers and rhizomorph length and between three markers and rhizomorph weight (Tables 6, 7). The percentage variation accounted for in regressions on rhizomorph length ranged from 21.5% for OPA3–59 to 43.1% for OPA4–101. In regressions on rhizomorph weight, the percentage variation accounted for ranged from 18.6% for OPA3–59 to 45.6% for OPA4–101.

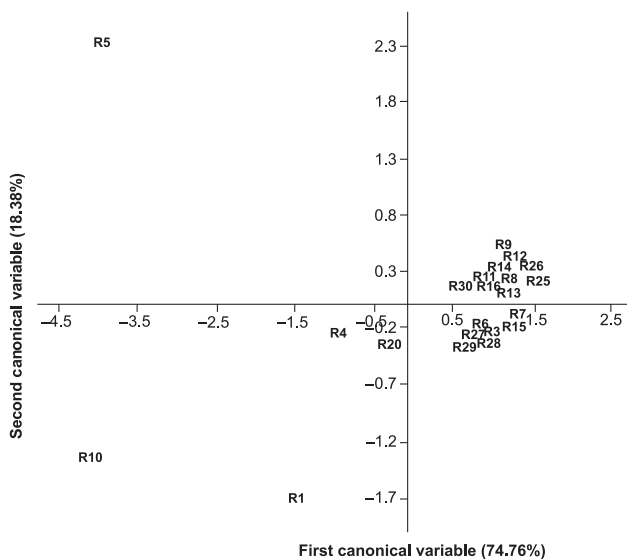


Fig. 2. Distribution of isolates in the plane of the first two canonical variables calculated for the four traits together: number of rhizomorphs, number of rhizomorph apices, length of rhizomorph and dry weight of rhizomorphs

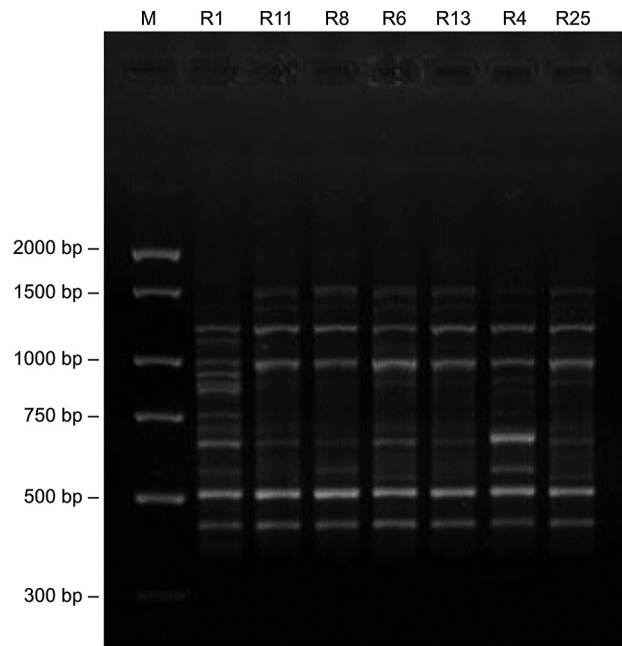


Fig. 3. *Armillaria ostoyae* RAPD-PCR products amplified by primer OPA-4. Lane M shows PCR marker

Discussion

Armillaria ostoyae was the only *Armillaria* species found in the six 5–17-year-old Scots pine plantations in the three forest districts in west-central Poland. This agrees with Żółciak (2007), who found only one species of *Armillaria* (*A. ostoyae*) in Scots pine in Poland. She analysed 356 samples from 105 infection centres located all over Poland. Members of *Pinaceae* are the main hosts for *A. ostoyae* and in Scots pine plantations the fungus is usually the primary and dominant pathogen. Generally, once *Armillaria* is established in a root system, secondary infection may occur via direct root–root contact and grafts with non-infected trees, via rhizomorphs growing through the soil or airborne basidiospores in autumn (Redfern and Filip 1991). Absence of other *Armillaria* species seems to result from strong competitive properties of *A. ostoyae*. It seems to inhibit the spread of other *Armillaria* species, restricting them to relatively small sizes (Dettman and van der Kamp 2001). We expected to find *A. cepistipes*, which may occur sympatrically with *A. ostoyae* in the same stand (Legrand et al. 1996; Marxmüller and Holdenrieder 2000; Prospero et al. 2003). This species was not recorded, however. It is considered rare in Poland and, according to Żółciak (2007), is restricted to Norway spruce (*Picea abies* (L.) H. Karst) and fir (*Abies alba* Mill.).

We showed that majority of our *A. ostoyae* isolates (21 out of 22) formed rhizomorphs on oak wood submerged in a substrate based on sand and forest soil. One isolate failed to form rhizomorphs. This is not unusual. Failure of certain isolates of *A. ostoyae* to produce rhizomorphs was also observed by Prospero

Table 5. Molecular markers associated with number of rhizomorphs and number of rhizomorph apices of *A. ostoyae* isolates

Molecular marker	Number of rhizomorphs				Number of rhizomorph apices			
	Estimate of regression coefficient	P	% variation accounted	S.E. of observations	Estimate of regression coefficient	P	% variation accounted	S.E. of observation
OPA2-23	73.8	0.024	24.9	28.5	94.4	0.023	25.4	36.1
OPA2-25	42.4	0.018	27.2	28.1	54.5	0.017	28.0	35.5
OPA2-32	73.8	0.024	24.9	28.5	94.4	0.023	25.4	36.1
OPA2-34	73.8	0.024	24.9	28.5	94.4	0.023	25.4	36.1
OPA2-40	37.0	0.030	23.0	28.9	46.1	0.033	21.9	36.9
OPA2-41	37.0	0.030	23.0	28.9	46.1	0.033	21.9	36.9
OPA3-58	35.4	0.029	23.3	28.8	42.7	0.039	20.4	37.3
OPA3-59	40.1	0.016	28.2	27.9	48.3	0.024	24.8	36.2
OPA4-101	62.5	<0.001	52.4	23.3	80.2	<0.001	53.4	29.3

et al. (2004). In their studies, 83.4% of the *A. ostoyae* isolates formed rhizomorphs on hazel (*Corylus avellana* L.) segments placed in soil substrate composed of peat and wood fibre (80% + 20%) amended with slow-release fertilizer and horn powder. The oak-wood discs used in our study proved to be a good substrate for rhizomorph formation. When inoculated with *A. ostoyae* and placed in moist river sand the first initial rhizomorphs developed after 14 weeks, in the initial stage of wood decay. Prospero et al. (2004) observed single rhizomorphs after 30 months when using colonized hazel segments for inoculation of Scots pine seedlings in pots.

Generally, the nutritional and environmental status of the original location can affect future *Armillaria* behaviour. Good nutrition and favourable soil conditions, preferably with higher acidity and lower moisture, may have positive effects on future activity, including the formation of rhizomorphs (Morrison 1974, 1976; Redfern 1978). Isolates from Siemianice and Złotów produced, respectively, the smallest and the most abundant rhizomorph networks. These differences probably did not result from differences in the nutritional status of soil or previous forest type since these were similar. Less annual precipitation was recorded for Złotów, however. Adaptation of the local *A. ostoyae* to dryer conditions may result in the development of a more abundant rhizomorph net-

work in the substrate, which dried slowly but continuously over 24 weeks in jars and plastic bags.

Eleven isolates produced five RAPD patterns and belonged to the same five genets (2–3 isolates per genet). The other 11 isolates belonged to separate genets. This suggests high variation in these populations of *A. ostoyae*. Genets of pathogenic *A. ostoyae* are usually significantly larger than those of saprotrophic *Armillaria* species (Bendel 2006), and detection of so much variation was unexpected. A similarity coefficient was used to assess genetic variability among genets. When the presence or absence of RAPD markers (bands) produced by the seven primers was analysed, the similarity coefficient values produced for pairwise genet combinations of the 22 *A. ostoyae* were 0.2488–0.975 and were similar to the range (0.333–0.950) reported by Dettman and van der Kamp (2001) for pairwise genet combinations of 21 *A. ostoyae* isolates. Therefore similar variation was obtained with a similar number of isolates by us and Dettman and van der Kamp (2001). It was also noted that greater genetic similarity was often associated with geographic spatial proximity. This also agrees with Dettman and van der Kamp (2001) who reported an association between genetic similarity and spatial proximity of *A. ostoyae* genets in British Columbia (Canada).

The frequent occurrence of separate genets of *A. ostoyae* in three locations in Poland is not fully consistent with the general reproductive strategy of *Armillaria*, which is 'clonal' (vegetative, mitotic from an original initial mycelium). The dominance of 'clonal' reproduction of *Armillaria* in western United

Table 6. Molecular markers associated with length of rhizomorphs of *A. ostoyae* isolates

Molecular marker	Estimate of regression coefficient	P	% variation accounted	S.E. of observation
OPA2-23	1909	0.010	32.4	629
OPA2-25	923	0.030	23.0	671
OPA2-32	1909	0.010	32.4	629
OPA2-34	1909	0.010	32.4	629
OPA3-59	837	0.035	21.5	678
UBC31-74	815	0.044	22.0	707
UBC31-76	815	0.044	22.0	707
OPA4-101	1335	0.003	43.1	593

Table 7. Molecular markers associated with dry weight of rhizomorphs of *A. ostoyae* isolates

Molecular markers	Estimate of regression coefficient	P	% variation accounted	S.E. of observation
OPA2-25	86.2	0.019	26.7	57.7
OPA3-59	69.9	0.047	18.6	60.8
OPA4-101	121.1	0.002	45.6	51.3

States, eastern England and Australia was reported by Shaw and Roth (1976), Rishbeth (1978) and Kile (1983). In Australia the majority (up to 71%) of infection centres studied were occupied by single genets (Kile 1983).

'Clonal' reproduction via rhizomorphs or airborne asexual propagules occurs in *A. ostoyae* in west-central Poland. This is supported by the occurrence of identical genets. The population structure of *A. ostoyae* here was, however, more consistent with a sexual reproductive strategy, i.e. successive infections by basidiospores transferred from outside sources. Several earlier studies have produced indirect evidence of dispersal and infection via basidiospores (Rishbeth 1978, 1985, 1988; Kile 1983; Smith et al. 1994; Worrall 1994). Up to now, however, this has been considered rare in nature. The results of Stenlid and Gustafsson (2001) and Power et al. (2008) confirm, however, that dispersal and infection via basidiospores is possible in indigenous and non-indigenous forests. Many *Armillaria* basidiospores are released, thus providing potential inoculum for spread to other plantations and establishment of new infection centres. Given the enormous number of basidiospores produced by *Armillaria* fruiting bodies, it is probable that some will travel over large distances (Stenlid and Gustafsson 2001). The small proportion of spores dispersed over longer distances is crucial for the establishment of the pathogen in distant localities and for achieving genetic exchange between populations. According to Hood et al. (2002, 2008), *A. novae-zelandiae* is capable of colonizing freshly cut wood of *P. radiata* by means of basidiospores, and establishment of the pathogen in pine debris produced during site preparation may result in new infection centres in succeeding rotations. Knowledge of pathogen spread may help to predict the presence and extent of establishment of *A. ostoyae* in new stands.

The high genetic similarity (similarity coefficient values >0.9) found between isolate R1 from Zielonka I and R16 and R25 from Złotów II located more than 200 km away could be the result of successive, long-distance transfers of asexual propagules by a vector. Spread via rhizomorphs can be discounted because of the distance. The typical size of an infection centre occupied by a single genet of *A. ostoyae* ranges from 6.01– (0.01–0.44) –15.83 ha (Shaw and Roth 1976; Van der Kamp 1993; Worrall 1994; Legrand et al. 1996; Peet et al. 1996; Dettman and van der Kamp 2001; Ferguson et al. 2003).

The possibility of the development of new genets from inbreeding, genetic recombination or somatic mutation can not be discounted. The likelihood of somatic mutation is greater in genets advanced in size and age. A large-scale mutation such as a chromosomal rearrangement or segment deletion may produce additional RAPD markers. The high degree

of polymorphism in *A. ostoyae* DNA may have resulted partly from a long-lasting and permanent host effect, resulting from a genetically diverse population of spruce and other admixture species in the previous forest. Spruce (*Picea*), its major host group, survived in Europe in several separate refuges, resulting from events mediated by the last Ice Ages (Silverton 1987). Local isolation led to its genetic diversity, which could be transferred to its pathogens. The type of forest grown previously in the sample areas was fresh broadleaved forest or fresh mixed broadleaved forest. Apart from spruce, both had admixture of oak (*Quercus*), larch (*Larix*), hornbeam (*Carpinus*) and wild black cherry (*Prunus*). Previous exposure to alternative hosts, which generally causes relaxation or shifts in selection pressure, could contribute to greater genetic diversity (Burdon 1993; Burdon and Silk 1997).

The present study suggests that sexual reproduction in *A. ostoyae* (via basidiospores) may occur in Poland more often than was so far suspected. Such reproduction results in populations of larger numbers of relatively small fungal individuals (Dettman and van der Kamp 2001).

We found the genetic association between gene, phenotype and function of *A. ostoyae*. The molecular genetic basis of adaptive variation is of fundamental importance for evolutionary dynamics, but is still poorly known. Only in very few cases has the relationship between genetic variation at the molecular level, phenotype and function been established in natural populations (Kivimäki et al. 2007).

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