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Wojciech Szewczyk, Hanna Kwaśna, Jolanta Behnke-Borowczyk

Populations of *Armillaria* species in pine plantations in west-central Poland

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Abstract: Two diploid isolates of *Armillaria gallica* and 143 diploid isolates of *A. solidipes* were obtained from 145 samples of rhizomorphs, fruit bodies and infected wood of sessile oak and Scots pines from 5–10-year-old Scots pine plantations in three Forest Districts located 100–350 km apart in west-central Poland. Based on pairings among the 145 isolates, 18 somatic compatibility groups (genets) of *A. solidipes* were distinguished in the three plantations. Sequencing of ITS1/2 rDNA of 18 isolates representing the 18 genets delineated four nuclear haplotypes. This suggests that many of the *A. solidipes* genets are closely related and were possibly established by sib-related basidiospores. With a few exceptions there was general geographical specialization of genets but not haplotypes. Geographical specialization of genets and the dominance of single haplotypes suggest that the *A. solidipes* population results from clonal rather than sexual reproduction. Sequencing of the ITS1/2 and IGS-1 rDNA showed small nucleotide diversity in ITS1/2 rDNA and much more diversity in IGS-1 rDNA of the isolates of *A. solidipes* studied. However, none of these regions has sufficient resolution for the clear differentiation of *A. solidipes* from *A. borealis*. The sequences of the EF 1-alpha gene showed high interspecific variability in *Armillaria* species and very low intraspecific variability in *A. solidipes*. This gene is the most appropriate for reliable identification of biological species and subgroups of *Armillaria*. Its application is the most useful in ecological and epidemiological studies of *Armillaria*.

Additional key words: *Armillaria solidipes*, EF-1 alpha gene, IGS, ITS, somatic incompatibility, population structure

Addresses: W. Szewczyk, H. Kwaśna, J. Behnke-Borowczyk, Department of Forest Pathology, Faculty of Forestry, Poznań University of Life Sciences, Wojska Polskiego 71c, 60-625 Poznań, Poland, e-mail: kwasna@up.poznan.pl

Introduction

Armillaria root rot represents one of the most dangerous diseases of the root systems of pine and other trees in the Northern and Southern Hemispheres. In forestry it causes reduction of tree growth, tree mortality and predisposition to windthrow, which decreases commercial timber production. In Poland in 2012 the pathogen was found to occur with high incidence on 99 215 ha (Małecka et al. 2014).

At present over 40 species of *Armillaria* have been described (Volk and Burdsall 1995; Pegler 2000). Seven species, including *A. borealis* Marxm. et Korhonen, *A. cepistipes* Velen, *A. ectypa* (Fr.) Lamoure, *A. gallica* Marxm., *A. mellea* (Vahl: Fr.) Kumm., *A. solidipes* (Peck) and *A. tabescens* (Scop.: Fr.) Emel., occur in Eu-

rope (Korhonen 1995). Only *A. ectypa* has not been reported from Poland (Szewczyk and Mańka 2002; Szewczyk and Łakomy 2011; Żółciak 1999b, 2005).

Armillaria solidipes and *A. gallica* are the most dangerous in forests. They dominate in fresh mixed coniferous and broadleaved forest, particularly on younger trees (Korhonen 1978; Rishbeth 1985; Hood et al. 1991; Morrison et al. 1991; Żółciak 1999b; Szewczyk and Mańka 2002). The relative importance of *A. solidipes* increases when conifers replace hardwoods (Guillaumin et al. 1993; Legrand and Guillaumin 1993). The presence of *Armillaria* depends on the decomposition of woody substrata and absorptive activities of its mycelium. Mycelium in wood fuels the foraging activities of rhizomorphs which grow in the soil and epiphytically along roots.

Previously identification of *Armillaria* based on characters of its fruit bodies *in situ* and mycelium and rhizomorphs *in vitro* proved not to be efficient because of limited morphological variation. Furthermore fruit bodies are short-lived and could be examined only seasonally. Somatic incompatibility tests performed by pairing haploid tester strains with the isolates in question (Korhonen 1978) were time-consuming, and sometimes ambiguous and difficult to interpret. The reaction tends to be indistinct in diploid-haploid pairings (Guillaumin et al. 1991), and field isolates established from mycelial mats, rhizomorphs and decayed wood are usually diploid (Hintikka 1973).

Other methods for *Armillaria* identification have used immunology (Lung-Escarmant et al. 1985) or analysis of isoenzyme polymorphism (Morrison et al.1985; Mwenje and Ride 1997). All of them were quickly replaced by faster and more reliable molecular techniques, e.g. AFLP, RAPD, RFLP and sequencing (Smith and Anderson 1989; Coetzee et al. 2000; Kim et al. 2000; Dettman and Kamp 2001; Pildain et al. 2009; Wingfield et al. 2009).

Sequencing conventionally targets conserved regions of the ribosomal RNA gene (rDNA), i.e. the internal transcribed spacer (ITS) and intergenic spacer (IGS) (Anderson and Stasovski 1992; Harrington and Wingfield 1995; Banik et al. 1996; Chillali et al. 1998 a, b; Terashima et al. 1998a,b; Pérez-Sierra et al. 1999; Fukuda et al. 2003; Lochman et al. 2004a; Matsushita and Suzuki 2005; Schnabel et al. 2005; Keča et al. 2006; Sekizaki et al. 2008). More recently the translation elongation factor-1 alpha (EF-1 alpha) gene, which encodes an essential part of the protein translation machinery, was shown to have high phylogenetic utility and has been used in studies on fungi. Therefore this gene has been used in studies to establish phylogenetic relationships among *Armillaria* species from the Northern and Southern hemispheres (Maphosa et al. 2006), to elucidate the relationship between two closely related species, *A. gallica* and *A. cepistipes* (Antonin et al. 2009), and for identification of species of *Armillaria* in Japan (Hasegawa et al. 2010).

The objectives of the present research were: (i) to investigate the population structure of *Armillaria* in Scots pine plantations in Poland and (ii) to compare the effectiveness of somatic incompatibility test, and RFLP and sequencing of the ITS1/2, IGS-1 rDNA and EF-1 alpha gene for reliable identification of different species of *Armillaria*. We hypothesized that Scots pine plantations in west-central Poland are colonized by a few species of *Armillaria* and different haplotypes of *A. solidipes* and that there would be geographical specialization in the distribution of *Armillaria* species and haplotypes.

Materials and Methods

Isolates

Isolates of *Armillaria* were obtained from severely diseased, 5–10-year-old Scots pine (*Pinus sylvestris* L.) plantations in three Forest Districts located 100–350 km apart in west-central Poland, in 2005–2008 (Fig. 1, Table 1). The Forest Districts were Siemianice (51.32083°N 16.91000°E), Zielonka (52.5533°N 17.1133°E) and Złotów (53.36346°N 17.04082°E). Two infection centres were chosen randomly in each plantation. The infection centres were surrounded by areas with no symptoms of disease. Four plots were located randomly in each infection centre. Each plot was 0.5 ha, established by marking a 40-m-long radius from the plot's centre. The area sampled in each location, summed from eight plots, was 4 ha.

Diseased and dead trees were found in each plot. Symptoms of infection included chlorotic needles, dieback of twigs, a layer of resin, debris and fungal

Fig. 1. Location of Scots pine plantations surveyed in Poland

Table 1. Representative *Armillaria* isolates used in this study

Species	Genet based on somatic compatibility test	RFLP of IGS-1 rDNA pattern with enzyme			Haplotype based on sequencing of ITS1/2 rDNA	Origin	Substrate	Location
		*Alu*I	BsmI	*Taq*I				
A. gallica	7023[a]	400 240 180[1]		345 190 160[1]		*Quercus petraea*	Fruit body	Siemianice
A. solidipes	6001[b]	300 200 150[2]	590 290[1]	345 230 150[2]	I	*Pinus sylvestris*	Infected wood	Zielonka
	2603[c]	300 200 150	590 290	345 230 150	I	Soil surface	Rhizomorph	Zielonka
	6036	300 200 150	590 290	345 230 150	I	Soil surface	Rhizomorph	Złotów
	6051[d]	300 200 150	590 290	345 230 150	I	*P. sylvestris*	Infected wood	Złotów
	6095[e]	300 200 150	590 290	345 230 150	I	*P. sylvestris*	Infected wood	Siemianice
	7012[f]	300 200 150	590 290	345 230 150	I	*P. sylvestris*	Fruit body	Siemianice
	7025	300 200 150	590 290	345 230 150	I	Soil surface	Fruit body	Siemianice
	6029[g]	300 200 150	590 290	345 230 150	II	*P. sylvestris*	Infected wood	Zielonka
	6034[h]	300 200 150	590 290	345 230 150	II	*P. sylvestris*	Infected wood	Złotów
	6111[i]	300 200 150	590 290	345 230 150	II	*P. sylvestris*	Infected wood	Siemianice
	7015	300 200 150	590 290	345 230 150	II	*P. sylvestris*	Fruit body	Siemianice
	7018[j]	300 200 150	590 290	345 230 150	II	*P. sylvestris*	Fruit body	Złotów
	6050[k]	400 300 200 150[3]	590 290	345 230 150	III	*P. sylvestris*	Infected wood	Złotów
	8023[l]	400 300 200 150	590 290	345 230 150	III	*P. sylvestris*	Infected wood	Siemianice
	7007	300 200 150	590 290	345 230 150	III	*P. sylvestris*	Fruit body	Zielonka
	7017	300 200 150	590 290	345 230 150	III	*P. sylvestris*	Fruit body	Złotów
	7040	300 200 150	590 290	345 230 150	IV	*P. sylvestris*	Fruit body	Złotów
	8037	300 200 150	590 290	345 230 150	IV	*P. sylvestris*	Infected wood	Złotów

Explanation
Identical to: [a] – 7028, [b] – 6002, 6004, 6005, 6007, 6008, 6012, 6014–6017, 6019, 6021–6023, 6105, 6106, 6108, [c] – 6024, 6107, [d] – 6052–6055, 6057, 6061– 6063, 6065, 6066, 6068– 6079, 6081– 6092, 6129–6131, **7024**, 7027, 7043, 8034–8036, 8038, 8043, [e] – **6097–6103**, **6110**, **6112, 6113**, **6115**, 7029, 7038, **8002**, **8004**, **8009**, **8011**, **8014**, **8020, 8024–8027**, [f] – **7013, 7014**, **7020**, 7036, [g] – 6006, 6018, 6020, 6026–6028, 6030, 6031, 6033, 8001, [h] – 6035, 6037, 6039, 6040, 6043, 6045–6047, 7046, 8032, 8033, 8039–8042, [i] – **8010**, **8021, 8022**, [j] – 7022, [k] – 6116, 6118, 6119, 7049, [l] – **6109**
[1] – pattern typical for *A. gallica*
[2] – pattern typical for *A. solidipes* according to Harrington and Wingfield (1995)
[3] – pattern new for *A. solidipes*
Location of isolates: Number in arial – Siemianice, number in arial **bold** – Zielonka, number in arial underlined – Złotów

tissue forming at the trunk base or around infected roots, white mycelial mats and rhizomorphs under the bark, clusters of fruit bodies at the base of an infected tree. Thirty-six, 37 and 72 samples of rhizomorphs, fruit bodies and infected wood of Scots pines and sessile oaks (*Quercus petraea* Mattuschka) were collected at Siemianice, Zielonka and Złotów, respectively. The number of isolates collected was determined by disease severity, which was moderate at Siemianice (10–20% diseased trees), low at Zielonka (10%), and high at Złotów (more than 20%). At each location 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 (*Q. petraea*).

At Siemianice and Zielonka the mean annual temperature was 7–9°C and at Złotów 6–7°C, and annual precipitation was 700–800, 500 and 500–600 mm, respectively at the three sites.

Within 12 h of collection each sample was surface-sterilized in sodium hypochlorite (7% active chlorine) for 15–30 s and rinsed three times in sterile, demineralized water for 10 min. 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 sodic benzylpenicillin100 mg l^{-1}) (modified from Legrand and Guillaumin 1993). Cultures were incubated at 25°C in darkness and sub-cultured as necessary.

Identification of *Armillaria* with somatic incompatibility tests

The *Armillaria* isolates were initially 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 145 isolates was paired with a haploid tester (monospore isolate) of each of five European *Armillaria* species, i.e. *A. borealis*, *A. cepistipes*, *A. gallica*, *A. mellea* and *A. solidipes*

Table 2. *Armillaria* testers used for comparison

No.	Species	Host	Location	Donor
AB99025	*A. borealis*	*Betula* sp.	Finland, Hartola	Korhonen K.[a]
AC	*A. cepistipes*	*Alnus* sp.	Finland, Tampere	Korhonen K.[a]
AG99929	*A. gallica*	*Juglans regia* L.	Poland, Święciechowa	Łakomy P.[b]
AG99060	*A. gallica*	*Quercus* sp.	Poland, Łopuchówko	Łakomy P.[b]
AG3	*A. gallica*	*Corylus avellana* L.	France, Puy de Dome	Guillaumin J. J.[c]
AM99033	*A. mellea*	Fruit body on the ground	France, Paris	Guillaumin J. J.[c]
AM7	*A. mellea*	Fruit body on the ground	France, Paris	Guillaumin J. J.[c]
AM20051/1	*A. mellea*	*Betula pendula* Roth	Poland, Zielonka	Łakomy P.[b]
AO203013	*A. solidipes*	*Pinus sylvestris* L.	Poland, Międzychód	Łakomy P.[b]

Explanation:
[a] – Finnish Forest Research Institute, Vantaa, Finland; [b] – Department of Forest Pathology, University of Life Science, Poznań, Poland; [c] – Centre INRA de Clermont Ferrand/ Theix/Lyon, UMR Amélioration et Santé des Plantes, Clermont-Ferrand cedex, France.

(Table 2). After incubation at 25°C for 4 weeks in darkness 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 somatic compatibility 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. Paired cultures were examined every 7 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. solidipes* (Shaw and Roth 1976). Mycelial plugs of isolates were placed 4 mm apart on 2% MYEA and plates were incubated at 25°C for 4 weeks in darkness. Isolates from the same plot were paired in all possible combinations, including self-pairings as controls. Two isolates were identified as belonging to the same somatic compatibility group if they merged to form a single colony (Rizzo and Harrington 1993). A line of demarcation between the cultures indicated somatic incompatibility. Once somatic compatibility groups were identified within plots, pairings were done between isolates from different plots.

DNA Extraction, PCR Amplification, RFLP Analysis and Sequencing

Representatives of 18 *Armillaria* groups and *Armillaria* testers were grown in 2% malt extract broth (MEB: Difco malt extract 20 g l^{-1}) at 24°C for 3 weeks in darkness. The mycelium was harvested with a strainer, lyophilized and ground to a fine powder in liquid nitrogen. DNA was extracted with the Bead-Beat Micro Gravity (A&A Biotechnology, Gdynia, Poland) from 10 mg of powdered mycelium, according to the protocol provided. The ITS rDNA (between the 3' end of the SSU 18S rDNA and the 5' end of the LSU 28S) and IGS-1 rDNA (between the 3' end of the LSU 28S rDNA and the 5' end of the 5S gene) were amplified respectively with PCR primer pairs: ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') and CNL12 (5' CTGAACGCCTCTAAGTCAG 3') and 5SA (5' CAGAGTCCTATGGCCGTGGAT 3') (Matsushita and Suzuki 2005; Keča et al. 2006). The translation elongation factor-1 alpha gene (EF-1 alpha gene) was amplified with primers EF595F (5' CGTGACTTCATC AAGAACATG 3') and EF1160R (5' CCGATCTTG-TAGACGTCCTG 3') (Maphosa et al. 2006).

Each 25 μl PCR mixture included 0.2 μM of each primer, 2x PCR MixPlus (Taq DNA polymerase 0.1 U μl^{-1}, 4 mM $MgCl_2$, 2 mM deoxyribonucleoside triphosphates (dNTPs)) and 100 ng of DNA (A & A Biotechnology, Poland). For ITS and IGS, PCR conditions included an initial denaturation step at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 42°C for 1 min, and 72°C for 2 min. For EF-1 alpha gene, PCR conditions included an initial denaturation step at 94°C for 1 min, then 30 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min. These were followed by a final extension of 72°C for 10 min and then cooling to 4°C. The amplified regions included 600–650 bp (ITS, EF-1 alpha gene) and 800–880 bp (IGS).

The amplified products of the IGS-1 region were subjected to RFLP analysis using restriction enzymes *Alu*I, *Bsm*I and *Taq*I (MBI Fermentas, St. Leon-Rot, Germany) (Harrington and Wingfield 1995). The PCR reaction mix (18 μl) was subjected to digestion by 10 U of enzyme for 12 h at 37°C (*Alu*I), 3 h at 37°C (*Bsm*I) and 3 h at 65°C (*Taq*I). The products were electrophoresed in gels (2% NuSieve agarose, Cambrex BioScience, Wokingham, UK + 1% standard agarose) in 1 x TBE stained with 0.5 μg ml^{-1} ethidium bromide. Restriction digestion patterns were compared for each enzyme. DNA band sizes were determined by comparison with bands of a $\phi\chi$174 DNA *Hae*III digest and the Low DNA Mass Ladder (Invitrogen Ltd.,

Table 3. *Armillaria* sequences deposited in NCBI GenBank

Species	Origin	NCBI GenBank accession number		
		ITS 1/2 rDNA	IGS-1 rDNA	EF-1 alpha gene
A. borealis	tester	KF523270	KM878699	KM878688
A. cepistipes	tester	KF523271	KM878700	KM878687
A. gallica	tester	KF523268	KM878702	KM878686
A. gallica	tester	KF523269	KM878703	–
A. gallica	tester	KF523272	KM878701	–
A. gallica	7023	KF523265	KM878696	KM878689
A. mellea	tester	KF523273	KM878704	KM878685
A. mellea	tester	KF523274	KM878705	KM878684
A. mellea	tester	KF523275	KM878706	KM878683
A. solidipes	tester	KF523276	KM878707	KM878682
A. solidipes	6036 – haplotype I	KF523263	KM878694	KM878690
A. solidipes	7015 – haplotype II	KF523264	KM878695	KM878691
A. solidipes	8023 – haplotype III	KF523266	KM878698	KM878693
A. solidipes	7040 – haplotype IV	KF523267	KM878697	KM878692

Paisley, UK) using GeneTools gel analysis software (Syngene). Fragments smaller than 100 bp were not scored because they could not be distinguished from primer dimers. Two fragments with difference <10 bp were considered identical.

The amplified fragments of rDNA were purified using Clean-Up Kit (A & A Biotechnology, Gdańsk, Poland). The ITS1/2 rDNA was sequenced with primers ITS1 and M 13 fwd (5' GCCAGGGTTTTCCCAGTCACGA 3'), IGS-1 rDNA with primers CNL12 and 5SA, and EF-1 alpha gene with primers EF595F and EF1160R, at DNA Research Centre (Poznań, Poland), using the BigDye® Terminator v 3.1 Cycle Sequencing Kit (AB Applied Biosystems, Foster City, CA 94404, USA) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA). Sequences were queried against GenBank database using BLAST. Nucleotide sequences of ITS1/2, IGS-1 rDNA, and EF-1 alpha gene of *Armillaria* testers and representative isolates were deposited in NCBI GenBank (Table 3).

DNA Sequence Analysis

ITS1/2, IGS-1 rDNA and EF-1 alpha gene sequences of representative *Armillaria* isolates and testers were compared. They were automatically aligned and manually adjusted using the program Clustal X version 1.8 (Thompson et al. 1997). The total number of characters included in the alignment for ITS1/2, IGS-1 rDNA and EF-1 alpha gene were respectively 418, 584 and 436. Missing and ambiguous data were excluded from the analysis.

Phylogenetic analysis was performed by the neighbor-joining (NJ), maximum likelihood (ML) and maximum parsimony (MS) methods. Neighbor-joining analysis took as input a distance matrix specifying the distance between each pair of isolates. Maximum likelihood analysis was performed with the Tamura-Nei model using heuristic search procedure (Nearest-Neighbor-Interchange, NNI) with an initial tree generated automatically by applying NJ and BJONJ algorithms. Maximum parsimony analysis was performed using the heuristic search procedure (Subtree-Pruning-Regrafting; SPR) with the widest search level (level 3) with 10 replications in the Random Additions method for the initial tree. The percentages of replicate trees in which associated taxa clustered as determined by bootstrap analysis (1000 replicates) were indicated at the nodes of the phylogram. Bootstrap support (BS) values >60% were considered significant in this study. Evolutionary distances were computed using the maximum composite likelihood method and were expressed as number of base substitutions per site. Phylogenetic analysis was performed using MEGA 5.0 (Tamura et al. 2011). Because NJ, ML and MS trees showed similar topologies (data not shown) only the NJ trees are shown in Figs 2–4. The *Schizophyllum commune* Fr. sequence was used as an outgroup sequence after Hasegawa et al. (2010).

Statistical analysis

The coefficients of genetic similarity (S) of the isolates were calculated using the formula of Nei and Li (1979). The data were scored and entered in a computer as binary matrices where 0 coded for absence and 1 for presence of a band formed by RFLP of IGS rDNA. Estimates of similarity were based on the total number of shared fragments. The principal component and the average linkage cluster analyses were performed using the statistical package GenStat v. 7.1.

Measures of haplotype diversity (H_d) were based on the estimated haplotype frequencies in the sample (Nei 1987). Differences among *A. solidipes* haplotypes were analysed with the total number of polymorphic sites (S), nucleotide diversity per site

between two sequences (Π, Nei 1987) and the average number of pairwise nucleotide differences between two sequences (*k*, Tajima 1983). Haplotypes were reconstructed using PHASE v. 2.1 (Stephens and Donnelly 2003), as implemented in DNASP v. 5.10 (Librado and Rozas 2009). Sites with gaps were excluded from analyses.

Results

Isolates

Thirty-six, 37 and 72 diploid isolates of *Armillaria* were obtained from rhizomorphs, fruit bodies and infected wood of Scots pines and sessile oaks from two infection centres in, respectively, Siemianice, Zielonka and Złotów Forest Districts (Table 1).

Identification of *Armillaria* with somatic incompatibility tests

In somatic incompatibility tests performed with haploid testers, two isolates were identified as *A. gallica* and 143 as *A. solidipes*. In somatic compatibility tests performed for all within-centre and between-centre pairings, the 143 isolates of *A. solidipes* formed colonies with no dark lines or barrier zones. However, there were different levels of somatic compatibility: completely compatible interaction, intermediate reaction, and partly compatible reaction showing a sparse zone and pigment formation. There were also differences in colony morphology (velvety, crustose, flat, rhizomorphogenic, light or dark brown with age), formation of rhizomorphs, rhizomorphs morphology (compact or open due to intensity of branching, submerged and aerial, cylindrical or flat) and growth rate. On the basis of these differences 18 somatic compatibility groups were distinguished (a–l + single isolates). Each group was represented by 1–45 isolates. Four groups were from Zielonka (including b, c, g), eight from Złotów (including d, h, j, k) and six from Siemianice (including e, f, i, l) (Table 1).

PCR Amplification, RFLP and Sequence Analysis

For 143 isolates, PCR amplification of the ITS1/2 and IGS-1 rDNA yielded single strong bands of 600–650 bp. RFLP patterns of IGS-1 rDNA with *Alu*I, *Bsm*I or *Taq*I yielded 2-4 fragments of rDNA (Tables 1, 4).

Testers and two *A. gallica* isolates from *Q. petraea* (including 7023) gave *Alu*I pattern = 400 240 180 (typical for *A. gallica*) and *Taq*I pattern = 345 200 160. Testers and 136 *A. solidipes* isolates from *P. sylvestris* gave *Alu*I pattern = 300 200 150, *Bsm*I pattern = 590 290 and *Taq*I pattern = 345 230 150 (all typical for *A. solidipes*). Two *A. solidipes* isolates (6050, 8023), although having typical *A. solidipes* patterns for *Bsm*I (590 290) and *Taq*I (345 230 150), gave a new *Alu*I pattern (400 300 200 150) which was not recorded by Harrington and Wingfield (1995). The similarity coefficient values for the 143 *A. solidipes* pairwise genotype combinations (0.5555–1.0) showed genetic similarity between 55.5% and 100%. Seven isolates had 55.5% similarity to each other. The other 136 isolates were identical.

Table 4. RFLP patterns of the IGS-1 rDNA from *Armillaria* generated with digestion enzymes

Species	*Alu*I	*Bsm*I	*Taq*I
A. borealis	300 200 130 300 200 150		345 240 150
A. cepistipes	400 200 180 300 200 150		345 190 160
A. gallica	400 240 180 400 250 240 180 380 230 180		
A. mellea	320 180 150 300 150		
A. solidipes	300 200 150	590 290	345 230 150 500 190 150

Sequencing of ITS1/2 rDNA confirmed the identification of *A. gallica* (7023). Sequencing of ITS1/2 rDNA from the 18 *A. solidipes* isolates from 18 somatic compatibility groups delineated four nuclear *A. solidipes* haplotypes (Table 1). The largest haplotype I (represented by isolate 6036) was identical to *A. solidipes* tester KF523276 and included 99 isolates. Haplotype II (represented by isolate 7015) included 33 isolates. Haplotype III (represented by isolate 8023) included nine isolates, and haplotype IV (represented by isolate 7040) included two isolates. Haplotype III included isolates with two *Alu*I patterns. Isolates within haplotypes were isolated from fruit bodies, rhizomorphs and infected wood from the three locations surveyed, which were located 100–350 km apart, with no geographical or substrate specialization. In the ITS1/2 rDNA phylogenetic tree, three *A. solidipes* isolates, 6036, 8023 and 7040, representing haplotypes I, III and IV, joined, non-significantly, *A. solidipes* tester KF523276 (BS 53%), while isolate 7015, representing haplotype II, joined, non-significantly, *A. borealis* (BS 44%) (Fig. 2). This was because of the small amount of nucleotide diversity (Π = 0.00012); the number of nucleotide substitutions in the 418 nucleotide base-pair region was 0–2 (Table 5).

Sequencing of IGS-1 rDNA of representative isolates (6036, 7015, 7040, 8023) from four haplotypes did not confirm clearly the identification by sequencing of ITS1/2 rDNA. In the IGS-1 rDNA phylogenetic trees only isolate, 7015, joined significantly *A. solidipes* (BS 76%). The other isolates (6036, 8023, 7040) were significantly included in the *A. borealis* +

Table 5. Nucleotide polymorphism in ITS 1/2 rDNA sequences of *Armillaria*

Species	Nucleotide position									
	7	78	270	273	296	331	373	408	410	414
A. borealis tester KF523270	C	C	C	C	C	A	C	G	A	G
A. cepistipes KF523271	C	C	C	C	T	G	T	–	G	A
A. gallica tester KF523268	C	T	C	C	C	G	T	–	A	A
A. gallica tester KF523269	C	C	C	C	C	G	T	–	A	A
A. gallica tester KF523272	T	C	C	C	C	G	T	–	A	A
A. gallica 7023	T	C	C	C	C	G	T	–	A	A
A. solidipes tester KF523276	C	C	T	C	C	G	T	G	A	G
A. solidipes 6036 – haplotype I	C	C	T	C	C	G	T	G	A	G
A. solidipes 7015 – haplotype II	C	C	C	C	C	A	T	G	A	G
A. solidipes 8023 – haplotype III	C	C	C	C	C	G	T	G	A	G
A. solidipes 7040 – haplotype IV	C	C	C	A	C	G	T	G	A	G

A. solidipes clade (BS 96%, Fig. 3). This was because of much greater nucleotide diversity (Π = 0.00077); the number of nucleotide substitutions in the 584 nucleotide base-pair region was 4–18 (Table 6).

The topology of the phylogenetic trees was similar for ITS1/2 and IGS-1 rDNA. Both contained three major clades, i.e. *A. borealis* + *A. solidipes*, *A. gallica* and *A. mellea*. The *Armillaria cepistipes* lineage joined the *A. gallica* clade.

Sequencing of the EF-1 alpha gene of representative isolates (6036, 7015, 7040, 8023) from four haplotypes confirmed clearly the identification by somatic

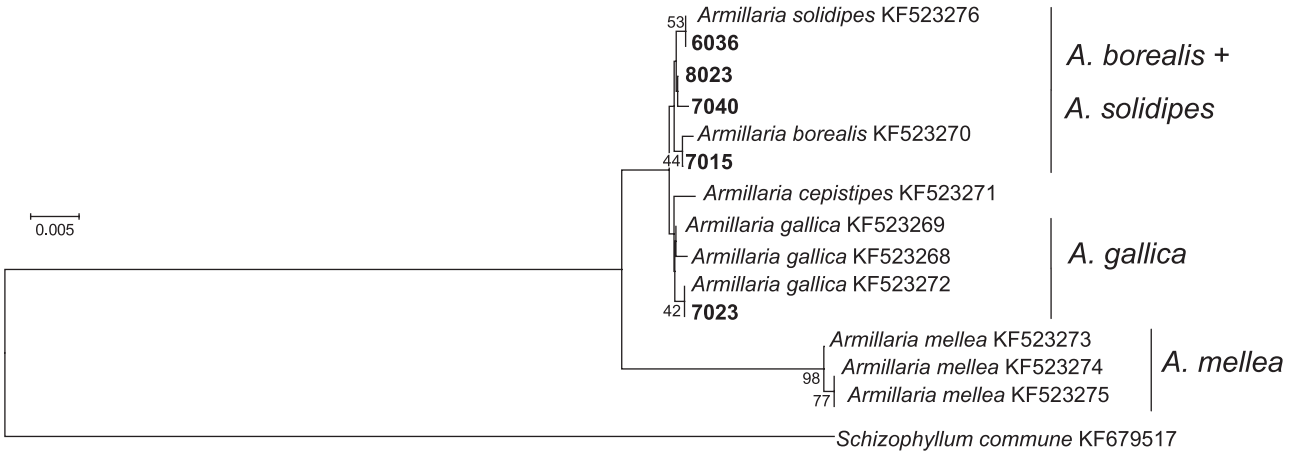

Fig. 2. Phylogenetic tree obtained by neighbor-joining analysis of ITS1/2 rDNA sequences of *Armillaria*. Gaps and missing data were excluded from the analysis. Bootstrap values (1000 replicates) are listed above the branches. Codes in bold indicate the Polish specimens of *Armillaria*. Scale bar indicates the percentage of difference between sequences

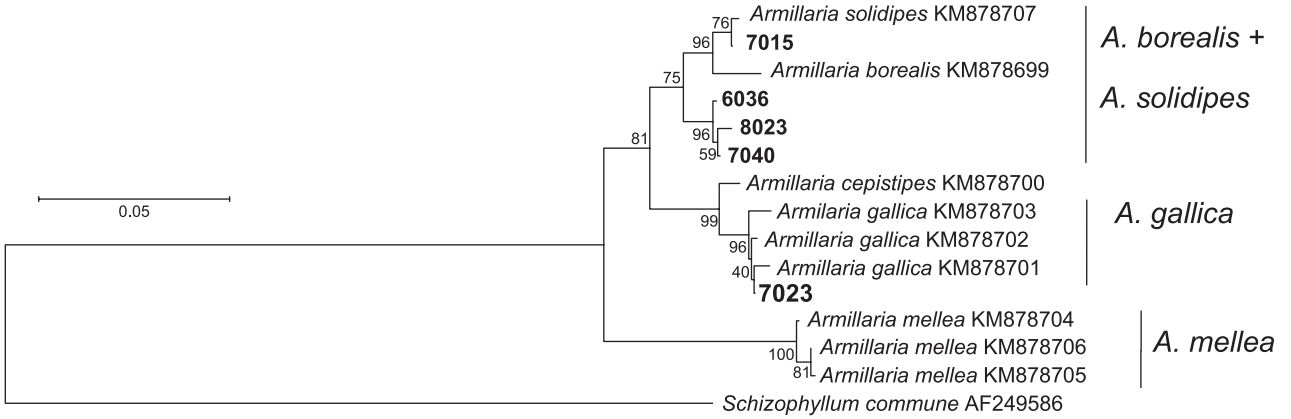

Fig. 3. Phylogenetic tree obtained by neighbor-joining analysis of IGS-1 rDNA sequences of *Armillaria*. Gaps and missing data were excluded from the analysis. Bootstrap values (1000 replicates) greater than 50% are listed above the branches. Codes in bold indicate the Polish specimens of *Armillaria*. Scale bar indicates the percentage of difference between sequences

Table 6. Nucleotide polymorphism in IGS-1 rDNA sequences of *Armillaria*

Species	Nucleotide position																																				
	23	24	37	48	61	62	63	123	124	157	243	301	302	303	310	311	316	328	329	330	331	332	336	344	356	357	359	366	367	378	391	393	396	398	401	405	406	407
A. borealis KM878699	C	T	T	A	C	–	–	G	G	G	A	G	C	G	C	G	A	–	–	–	G	C	G	T	C	G	T	A	T	C	G	A	G	G	A	C	G	G
A. cepistipes KM878700	C	T	C	G	A	C	G	G	G	G	G	A	G	A	A	G	A	A	A	C	G	C	A	–	T	C	T	A	C	T	A	T	T	A	C	T	A	G
A. gallica KM878701	A	A	C	G	A	C	–	–	–	–	G	A	G	A	A	G	G	A	A	C	G	C	A	–	C	T	T	A	T	T	G	T	T	A	C	T	A	T
A. gallica KM878702	C	T	C	G	C	A	–	–	–	G	G	A	G	A	A	G	G	A	A	C	G	C	A	–	C	T	T	A	C	T	G	T	T	A	C	T	A	T
A. gallica KM878703	C	T	C	G	A	C	–	G	G	G	G	A	G	A	T	G	G	A	A	C	G	C	A	–	C	T	T	A	C	T	G	T	T	A	C	T	A	T
A. gallica 7023	C	T	C	G	A	C	G	G	G	G	G	A	G	A	A	G	G	A	A	C	G	C	A	–	C	T	T	A	C	T	G	T	T	A	C	T	A	T
A. solidipes tester – KM878707	C	T	C	A	C	–	–	G	G	G	A	–	C	G	C	G	A	–	–	–	G	C	G	T	C	G	C	A	T	T	G	T	G	A	A	T	G	G
A. solidipes 6036 – haplotype I	C	T	C	A	A	C	G	G	G	G	A	G	C	G	C	A	A	–	–	–	A	A	T	–	T	G	T	A	T	T	G	T	G	A	A	T	G	G
A. solidipes 7015 – haplotype II	C	T	C	A	A	C	G	G	G	G	A	–	C	G	C	G	A	–	–	–	G	C	G	T	C	G	C	A	T	T	G	T	G	A	A	T	G	G
A. solidipes 8023 – haplotype III	C	T	C	A	A	C	G	G	G	G	A	G	C	G	C	A	A	–	–	–	A	A	T	–	C	G	T	C	T	T	G	T	G	A	A	T	G	G
A. solidipes 7040 – haplotype IV	C	T	C	A	A	C	G	G	G	G	A	G	C	G	C	A	A	–	–	–	A	A	T	–	T	G	T	A	T	T	G	T	G	A	A	T	G	G

Species	Nucleotide position																																				
	412	418	422	425	430	440	448	452	469	470	471	472	488	490	491	494	495	496	497	499	508	520	523	526	527	530	531	533	540	550	558	562	563	565	578	581	582	584
A. borealis KM878699	C	C	A	C	T	C	A	T	–	–	–	–	T	C	A	T	G	G	T	T	G	G	G	C	T	T	G	G	G	C	T	A	G	C	T	T	G	G
A. cepistipes KM878700	C	C	C	C	T	C	A	–	G	A	C	T	C	T	–	G	C	C	A	G	G	G	A	T	C	C	G	C	T	T	T	G	G	T	T	C	A	G
A. gallica KM878701	C	C	C	T	T	C	A	T	–	–	–	–	C	T	A	T	G	G	T	T	–	G	G	T	C	C	G	C	T	T	C	T	G	C	T	C	A	G
A. gallica KM878702	C	C	C	T	T	C	A	T	–	–	–	–	C	T	A	T	G	G	T	T	G	G	G	T	C	C	G	C	T	T	C	G	G	C	T	C	A	G
A. gallica KM878703	C	C	C	T	T	C	A	T	–	–	–	–	C	T	A	T	G	G	T	T	G	G	G	T	C	C	S	C	T	T	C	G	G	C	T	C	A	C
A. gallica 7023	C	C	C	T	T	C	A	T	–	–	–	–	C	T	A	T	G	G	T	T	G	G	G	T	C	C	G	C	T	T	C	G	G	C	T	C	A	G
A. solidipes tester – KM878707	C	C	A	C	C	T	A	T	–	–	–	–	T	C	A	T	G	G	T	T	G	G	G	T	T	T	G	G	G	C	T	A	G	C	G	T	A	G
A. solidipes 6036 – haplotype I	C	C	A	C	T	C	G	T	–	–	–	–	C	C	A	T	G	G	T	T	G	A	G	T	T	T	G	G	T	C	T	A	T	C	T	C	A	G
A. solidipes 7015 – haplotype II	C	–	A	C	C	T	A	T	–	–	–	–	T	C	A	T	G	G	T	T	G	G	G	T	T	T	G	G	G	C	T	A	G	C	T	T	A	G
A. solidipes 8023 – haplotype III	T	C	A	C	T	C	G	T	–	–	–	–	C	C	A	T	G	G	T	T	G	A	G	T	T	T	G	G	T	C	T	A	T	C	T	C	A	G
A. solidipes 7040 – haplotype IV	T	C	A	C	T	C	G	T	–	–	–	–	C	C	A	T	C	C	T	T	G	A	G	T	T	T	G	G	T	C	T	A	T	C	T	C	A	G

Table 7. Nucleotide polymorphism in EF-1 alpha gene of *Armillaria*

Species	Nucleotide position																																			
	8	20	26	56	59	68	74	101	109	116	117	118	120	124	126	131	132	133	138	140	148	151	186	198	252	291	304	310	313	319	324	328	330	333	372	426	430
A. borealis tester – KM878688	T	C	C	C	T	G	T	T	G	G	C	C	C	G	T	–	C	T	T	A	C	G	C	C	C	C	C	C	G	C	A	C	T	-	C	C	A
A. cepistipes KM878687	C	T	C	C	T	A	T	C	G	G	C	T	C	A	C	–	G	T	C	T	T	T	T	C	A	T	T	C	C	T	G	T	T	T	C	C	A
A. galllica tester – KM878686	C	T	C	C	T	A	C	C	G	A	C	T	C	G	C	–	G	T	C	A	C	T	C	C	C	T	T	T	C	T	G	T	C	T	T	T	A
A. galllica 7023	C	T	C	C	G	A	C	C	G	A	C	T	C	G	C	–	G	T	C	A	C	T	C	C	A	T	T	C	C	T	G	T	C	T	T	T	A
A. solidipes tester – KM878682	T	C	C	T	T	G	C	C	R	A	T	C	T	A	C	A	C	C	G	A	C	T	C	T	C	C	C	T	G	C	G	C	T	T	C	T	A
A. solidipes 6036 – haplotype I	T	C	C	T	T	G	C	C	G	A	T	C	T	A	C	A	C	C	G	A	C	T	C	T	C	C	C	T	G	C	G	C	T	T	C	C	A
A. solidipes 7015 – haplotype II	T	C	T	T	T	G	C	C	G	A	T	C	T	A	C	A	C	C	G	A	C	T	C	T	C	C	C	T	G	C	G	C	T	T	C	C	A
A. solidipes 8023 – haplotype III	T	C	T	T	T	G	C	C	G	A	T	C	T	A	C	A	C	C	G	A	C	T	C	T	C	C	C	T	G	C	G	C	T	T	C	C	A
A. solidipes 7040 – haplotype IV	T	C	T	T	T	G	C	C	G	A	T	C	T	A	C	A	C	C	G	A	C	T	C	T	C	C	C	T	G	C	G	C	T	T	C	C	T

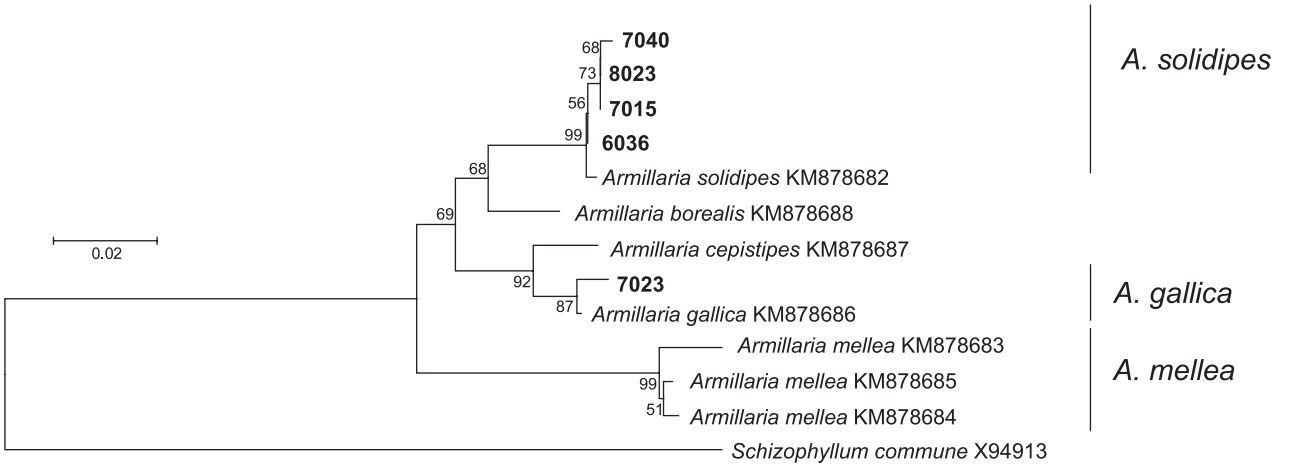

Fig. 4. Phylogenetic tree obtained by neighbor-joining analysis of EF-1α gene of *Armillaria*. Gaps and missing data were excluded from the analysis. Bootstrap values (1000 replicates) are listed above the branches. Codes in bold indicate the Polish specimens of *Armillaria*. Scale bar indicates the percentage of difference between sequences

incompatibility tests. All *A. solidipes* isolates joined, mostly significantly, *A. solidipes* tester KM878682 (BS 56–99%) (Fig. 4). The separation of *A. borealis* from *A. solidipes* was significantly supported (BS 68%). This was because of very little nucleotide diversity (Π = 0.00001); the number of nucleotide substitutions in the 436 nucleotide base-pair region was 1–2 (Table 7).

Haplotype diversity within *A. solidipes* was moderate (*Hd* = 0. 48239). The average nucleotide diversity and the average number of pairwise differences per sequence for four *A. solidipes* haplotypes was higher in IGS-1 rDNA than in ITS1/2 rDNA and the EF-1 alpha gene (Table 8).

Table 8. Nucleotide diversity for *A. solidipes* haplotypes

A. solidipes	*h*	H_d	*S*	Π	*k*
ITS 1/2	4	0.48239	3	0.00012	0.75
IGS -1	4	0.48239	21	0.00077	9.75
EF-1 alpha gene	4	0.48239	2	0.00001	0.50

h – total number of haplotypes
H_d – haplotype diversity
S – total number of polymorphic sites
Π – nucleotide diversity per site between two sequences
k – average number of pairwise differences per sequence

Discussion

The virulence of *Armillaria* species to trees varies from weakly to strongly pathogenic (Kile et al. 1991). Recognition of the population structure and distribution of *Armillaria* species is important for evaluation of disease impact and application of management practices aimed at preventing infection. Correct recognition of the most common species in northern European forests, i.e. *A. solidipes* and *A. gallica*, is of particular importance because of the differences in their pathogenicity. The first species is the most common and the most pathogenic on conifers in central Europe. The second is an opportunistic pathogen in weakened tree hosts, and is a common and ecologically important saprophytic wood-decay fungus (Żółciak 1991, 1999a,b, 2005, 2007; Mańka 2005).

On the basis of somatic incompatibility tests, only one *A. gallica* and 18 *A. solidipes* genets were detected in 5–10 year-old Scots pine plantations located 100–350 km apart in west-central Poland. Three or four groups of genets and between one and four single-isolate *A. solidipes* genets were detected in an area of 4 ha on each site. This diversity is less than the three and six *A. solidipes* genets per 1 ha found in North America (Worrall 1994), France (Legrand et al. 1996) or Norway (Prospero 2003). The number of genets detected probably depends on the size of the sampling area but also on the kind of substrate and method of sampling. Worrall (1994) and Prospero (2003) analysed soil and stumps. Legrand et al. (1996) analysed rhizomorphs, fruit bodies and mycelia from stumps. We analysed rhizomorphs, fruit bodies and infected wood. The sampling in our study provided representatives of large-, medium-, and small–sized *A. solidipes* genets. There was, generally, geographical specialization of genets. There were, however, some exceptions; in case of four isolates, i.e. 3%, (7024, 7029, 7038, 7036) there was no compatibility between morphology and location. No other *Armillaria* species were recorded although we expected to find *A. cepistipes*, which is reported as one of the most common *Armillaria* species in Europe (Legrand et al. 1996; Marxmüller and Holdenrieder 2000). It tends, however, to colonize Norway spruce and fir rather than Scots pine (Prospero 2003; Żółciak 2007).

The ITS1/2 and IGS-1 rDNA regions and the EF-1 alpha gene were assessed for their value in identification of Polish forest *Armillaria* species. The first procedure was RFLP analysis of IGS-1 rDNA with

three enzymes. Since Harrington and Wingfield (1995) first identified band patterns with *Alu*I and *Bsm*I for *Armillaria* species, this approach has been successfully used for studies of North American, European, South African and Japanese species of *Armillaria* (Chillalli et al. 1998; Coetzee et al. 2000; Mwenje et al. 2003; Hasegawa et al. 2010). Only two isolates gave the enzyme pattern typical for *A. gallica*. The majority of our isolates gave a pattern typical for *A. solidipes*. However, a few *A. solidipes* isolates gave a new pattern with *Alu*I. This, in diploid isolates of *A. solidipes*, seems to result from heterozygosity and the existence of variable multicopies in the rDNA array (Pérez-Sierra et al. 1999; Kim et al. 2000; Dunne et al. 2002; Smith-White et al. 2002; Lochman et al. 2004a,b; Schnabel et al. 2005; Keča et al. 2006). Hybrid RFLP patterns of the IGS-1 rDNA region have also been discovered in other *Armillaria* species (Kim et al. 2006; Antonin et al. 2009).

Sequencing of the ITS1/2 and IGS-1 rDNA showed small nucleotide diversity ($\Pi = 0.00012$) in ITS1/2 rDNA and much more ($\Pi = 0.00077$) in IGS-1 rDNA of the isolates of *A. solidipes* (Table 8). However, none of these regions has sufficient resolution for the clear differentiation of *A. solidipes* from *A. borealis*. Sequencing of ITS1/2 rDNA allowed differentiation of four haplotypes within 18 *A. solidipes* genets. This suggests that many of the *A. solidipes* genets are closely related and were possibly established by sib-related basidiospores. However, only representatives of three haplotypes with nucleotide substitution of 0–2 bp joined the *A. solidipes* tester (Fig. 2). The representative of the fourth haplotype, despite similar low nucleotide substitution of 2 bp, 0.3% nucleotide polymorphism and 99.7% nucleotide similarity, joined *A. borealis*. The phylogenetic tree obtained by neighbor-joining analysis of IGS-1 rDNA presents a completely different effect: the representative of haplotype II (7015), with nucleotide substitution of 2 bp compared with the *A. solidipes* tester, joined *A. solidipes*, while representatives of three other haplotypes, with nucleotide substitution of 16–19 bp compared with the *A. solidipes* tester, formed a subclade in the *A. borealis* and A. *solidipes* clade.

The insufficient effectiveness of ITS1/2 rDNA analysis for identification of *Armillaria* species from forest trees was reported earlier (Chillali et al. 1998b; Potyralska et al. 2002; Keča et al. 2006). Potyralska et al. (2002) found considerable similarity in the ITS1/2 rDNA sequences of Polish isolates of *A. borealis*, *A. cepistipes*, *A. gallica* and *A. solidipes*. Studying European isolates with RFLP, Chillali et al. (1998b) found more than 80% similarity in the ITS1/2 rDNA of *A. borealis* and *A. solidipes*. However, since ITS1/2 rDNA has been used successfully for differentiation of Northern Hemisphere *Armillaria* species from Australian and Japanese isolates (Dunne et al. 2009; Hasegawa et al. 2010), we decided to check its effectiveness again in our studies. A further reason was that the ITS region is the formal fungal barcode and is the primary choice for molecular identification of fungi from a number of sources (Schoch et al. 2012).

The occurrence of four haplotypes of *A. solidipes*, despite low nucleotide diversity in the ITS1/2 rDNA, seems to result from mutation. This is often found in diploid organisms (Buckler 1997). The 5.8S gene sequence is highly conserved but the ITS1 and ITS2 sequences are more variable and may be highly polymorphic (Nilsson et al. 2008). Further, many intergenic spacers may exist as a mosaic of functional elements and inactivated pseudogenes at different stages in sample decay (Degnan et al. 2011).

The phylogenetic trees obtained by neighbor-joining analysis of ITS1/2 and IGS-1 rDNA sequences of *Armillaria* show a high level of similarity between *A. borealis* and *A. solidipes* (Figs 2, 3). This phenomenon has been found earlier among isolates from Europe (Czech Republic, Montenegro, Poland, Serbia and Slovakia), North America and Japan (Anderson and Stasovski 1992; Keča et al. 2006; Hasegawa et al. 2010). We showed, however, satisfactory effectiveness of IGS-1 rDNA in identification of *A. cepistipes, A. gallica* and *A. mellea*, which agrees with Keča et al. (2006). The average nucleotide substitution between *A. cepistipes* and *A. gallica* of 14.5 bp was, however, more than that (8.7) found by Keča et al. (2006).

The analyses of ITS1/2 and IGS-1 rDNA showed much less diversity in the isolates of *A. solidipes* than was found previously using RAPD analysis, in which 16 distinct multilocus haplotypes were found among 22 *A. solidipes* isolates from Scots pine plantations in west-central Poland (Szewczyk et al. 2014).

The general geographical specialization of genets detected with somatic incompatibility tests was not supported by sequencing of the ITS1/2 rDNA. The three most common haplotypes were detected in each of three locations with no geographical specialization.

The elongation factor-1 alpha gene has so far been used rarely for identification of European *Armillaria* species. Only Antonin et al. (2009) used it for differentiation of *A. gallica* and *A. cepistipes*. Other studies using the EF-1 alpha gene included *Armillaria* from the northern and southern hemispheres (Maphosa et al. 2006), Japan (Hasegawa et al. 2010) and North America (Brazee et al. 2011, 2012; Ross-Davis et al. 2012). In our studies the EF-1 alpha gene analysis allowed recognition of *A. solidipes* (BS 99%) and clear separation of *A. borealis* from *A. solidipes* and *A. cepistipes* from *A. gallica* (BS 68% and 92%, respectively). There was very low nucleotide diversity in the *A. solidipes* EF-1 alpha gene ($\Pi = 0.00001$). Since the EF-1 alpha gene sequence was also found to be the most suitable for identifying different species of Japanese *Armillaria* (Hasegawa et al. 2010) it seems to

be the most useful for recognition of forest *Armillaria* species in the Northern Hemisphere. The EF-1 alpha gene is a highly conserved ubiquitous protein with high resolution necessary for phylogenetic studies of species (Roger et al. 1999) and its usefulness as a phylogenetic marker is confirmed by our studies. One of the obvious benefits of its application is its relatively easy amplification because of its large copy numbers and the possibility of amplification directly from decayed wood, mycelial fans, rhizomorph tissues or fungal cultures without DNA extraction.

The phylogenetic trees constructed with sequence data of the ITS1/2, IGS-1 rDNA and the EF-1 alpha gene were roughly similar in topology. Isolates of *A. borealis* + *A. solidipes, A. gallica* and *A. mellea* formed independent clades, and the single isolate of *A. cepistipes* had a more or less independent lineage which joined *A. gallica* clades. Although the overall similarity values differed, trends such as: (i) the similarity of *A. borealis* and *A. solidipes,* and of *A. cepistipes* and *A. gallica,* and (ii) the separation of *A. mellea*, were common to all three trees. Such differentiation is consistent with findings of Korhonen (1995) who, 20 years ago, on the basis of morphological and biological features only, divided northern hemisphere *Armillaria* species into three groups: *A. ostoyae* group, which included *A. borealis* and *A. ostoyae* (=*A. solidipes*); *A. gallica* group, which included *A. cepistipes* and *A. gallica*; and *A. mellea* designated as a single-species group.

Genetic variability is strongly associated with climatic factors such as temperature and precipitation, which affect the frequency and abundance of fructification and sporulation (Straatsma et al. 2001). The scarcity of fruit bodies in dry conditions may inhibit the formation of new genets, favour the existence of old ones and eliminate competition (Worrall 1994; Ferguson et al. 2003; Bendel et al. 2006). Total annual precipitation in west-central Poland over the past 10 years averaged 500–800 mm, with 25–30% of that total falling in June–October when mild temperatures persist and fungi are most likely to fruit, and so should have stimulated *Armillaria* sporulation and hence increased the genetic variability. This may have been counterbalanced, however, by (i) the size of the *A. solidipes* genet which can spread by rhizomorphs on 20–965 ha (Ferguson et al. 2003) and (ii) a high degree of genetic stability of *Armillaria* established over more than 1500 years (Smith et al. 1992). Geographical specialization detected by somatic incompatibility tests suggest that the population structure of *A. solidipes* in west-central Poland results rather from clonal reproduction (i.e. vegetative, mitotic, from an original initial mycelium, mostly via rhizomorphs or airborne asexual propagules). The dominance of clonal reproduction of *Armillaria* has also been reported from western United States, eastern England and Australia (Shaw and Roth 1976; Rishbeth 1978; Kile 1983). The molecular analyses show, however, that sexual reproduction, with dispersal and infection via basidiospores, may be more frequent than expected. Domination of single haplotypes (i.e. haplotypes I and II) and the rare occurrence of others in pine plantations in west-central Poland suggest, however, that site-directed conservative gene flow (including intraspecific crossings) dominates over random genetic drift, and supports the theory of genetic structure of the pathogen's population, i.e. that it is typically composed of many different physiological races but with a few dominants (Burdon 1993).

Conclusions

Armillaria solidipes is the most common *Armillaria* in *P. sylvestris* plantations in west-central Poland. Geographical specialization of genets and the dominance of single haplotypes suggest that its population results from clonal rather than sexual reproduction. The sequences of the EF 1-alpha gene showed high interspecific variability in *Armillaria* species and very low intraspecific variability in *A. solidipes* in Poland. This gene is the most appropriate for reliable identification of biological species and subgroups of *Armillaria*. Its application is the most useful in ecological and epidemiological studies of *Armillaria.*

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