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ORIGINAL RESEARCH PAPER

Notes on some *Phytopythium* and *Pythium* species occurring in oak forests in southern Poland

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Abstract
Phytopythium and *Pythium* species are known to be soil-born oomycete pathogens of forest trees in Europe. Little is known, however, about the presence of these micro-organisms in Polish oak forests. During the period 2007–2009 a comprehensive study of *Phytophthora* species in soils of oak forests in southern Poland was conducted using baiting technique. In this study, baits were also colonized by oomycete resembling *Pythium* species. Based on morphological characteristics and the ITS sequences comparisons, 10 species of *Phytopythium* and *Pythium* were isolated from the soil-root samples, including three putative new species belonging to the genus of *Phytopythium*. The most commonly encountered *Pythium* species was *Pythium anandrum*. The present study demonstrates for the first time that *Phytopythium citrinum* and *Pythium diclinum* can also act as soil-borne organisms in oak forests. In addition, these species were reported for the first time in Poland.

Keywords
Quercus robur; oomycetes; soil; *Phytopythium*; *Pythium*

This issue of Acta Mycologica is dedicated to Professor Maria Lisiewska and Professor Anna Bujakiewicz on the occasion of their 80th and 75th birthday, respectively.

Introduction

The genus *Pythium* Pringsh. including "fungus-like organisms" or "pseudo-fungi" is placed in the kingdom Chromista [1] or kingdom Straminipila [2]. The species of *Pythium* are cosmopolitan, widely distributed throughout the world, and occupy several diverse ecological niches. Some species of *Pythium* are known as pathogens of various plants, including forest and fruit trees; they lead to rot of fruit, rot of roots, and stems, and pre- or postemergence damping-off. For example *Pythium undulatum* H.E. Petersen, causes root rot of *Abies procera* Rehder and *Pseudotsuga menziesii* (Mirb.) Franco in northern Germany [3].

The genus *Pythium* is characterized by filamentous or globose sporangia with zoospores develop in a vesicle, oospores formed in smooth or ornamented oogonia with paragynous or hypogynous antheridia [4]. Many reports have shown that *Pythium* is composed of few morphological groups, whereas recent molecular analyses have shown that the genus *Pythium* is a polyphyletic group that includes several monophyletic groups [5,6]. In a recent study, Uzuhashi et al. [7] restricted the genus *Pythium* to those species with filamentous sporangia and created four new genera to accommodate species with non filamentous sporangia: *Ovatisporangium*, *Elongisporangium*, *Globisporangium* and *Pilasporangium*. In the same year Bala [8] proposed a new genus

Phytopythium for those species with globose to ovoid, often papillate and internally proliferating sporangia. Recently, de Cock et al. [9] provided molecular-based evidence that members of *Pythium* clade K as described by Lévesque and de Cock [5] belong to the *Phytopythium* genus. While recognizing the genus status of remaining species of *Pythium* clades (A–J) is still unclear we prefer to use to the definition of *Pythium* sensu Lévesque and de Cook [5].

Little is known about the occurrence of *Phytopythium* and *Pythium* species in Europe, particularly in forest soils. There were only four reports about isolation of *Pythium* spp. from soils under oak forests [10–13]. These studies reported occurrence of *P. undulatum*, *P. anandrum* Drechsler, *P. aphanidermatum* (Edson) Fitzp., *P. irregulare* Buisman, *P. middletonii* Sparrow, *P. rostratum* Butler and *P. intermedium* de Bary in oak stands in Austria, Germany, Turkey and Sweden. Of all *Pythium* species recorded in these studies, *P. anandrum* and *P. undulatum* are the most commonly reported and are known as fine root pathogens which may affect the health of oak trees in Europe [9–13]. Recently, *Pythium sterilum*, *P. spiculum* B. Paul Belbahri & Lefort, and two *Phytopythium* species, *Phytopythium citrinum*-like (B. Paul) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque and *Phytopythium mercuriale* (Belbahri, B. Paul & Lefort) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque have been associated with soil in declining oak stands in Poland [14]. However, information about the oak-associated *Pythium* and *Phytopythium* species is still very limited.

During the period 2007–2009 a comprehensive study of *Phytophthora* species in soil samples collected from oak forests in southern Poland was conducted using baiting (oak leaves) and selective agar medium technique [15]. In this study, baits were also colonized by other oomycetes, particularly by *Pythium* species. For this reason, our objective was to identify the *Pythium* isolates obtained during *Phytophthora* detecting in oak forests in southern Poland.

Material and methods

Study sites

The survey was conducted during May–June and September–October 2007–2009 in 29 pedunculate oak (*Quercus robur* L.) stands in the southern part of Poland. Sampling sites were selected from areas characterized by different tree health status and site conditions. The characteristics of the study sites are given in Tab. 1.

Sampling and isolation methods

In each stand 6 mature trees (tree age >50 years), which were considered to be representative for the health status of the stand, were chosen. The crown status of these trees was assessed according to Balcì and Halmschlager [11]. Three soil samples were taken with fine roots (soil-root monoliths without the organic part ca. 25 × 25 × 25 cm), 100–150 cm from the trunk and spaced in three directions around the stem base of each tree. Soil from three monoliths was mixed and a sub-sample of this mixture was put in a plastic bag and transported to the laboratory.

Isolation was performed using the oak leaf baiting method described by Jung et al. [10,16]. Each sample was mixed thoroughly, then two 200 mL subsamples were flooded with 400 mL of distilled water in a plastic boxes (18 × 11 × 7 cm), and baited by floating 2- to 6-day-old *Q. robur* leaves (10–15 leaves per each box) at room temperature. After 3–6 days, discolored leaves were taken to *Phytophthora* spp. isolation. For this purpose, leaves were washed under tap water, dried on filter paper, cut into small pieces (2 × 2 mm) and placed on PARPNH medium (V8 juice agar amended with 10 mg/L pimaricin, 200 mg/L ampicillin, 10 mg/L rifampicin, 25 mg/L PCNB, 50 mg/L nystatin and 50 mg/L hymexazol). Emerging cultures were purified by transferring small pieces of mycelium from individual colonies to fresh V8 juice agar (100 mL/L Vega's juice (Tymbark®, Poland), 900 mL/L distilled water, 15 g/L agar, 3 g/L $CaCO_3$).

Tab. 1 Characteristics of the study sites (in bold the sites where *Phytopythium*/*Pythium* spp. were found).

		Geographic coordinates							
Sites No.	Site	Longitude	Latitude	Forest Type[1]	Tree age	Soil texture[2]	Soil moisture[3]	Soil pH[4] $CaCl_2$	Crown status[5]
1	Ispina 1	20.362721°	50.096364°	Q	130	silty clay loam	M	3.69	SD
2	**Ispina 2**	**20.359564°**	**50.081254°**	**D**	**165**	**silty clay loam**	**M**	**3.75**	**SD**
3	Wola Batorska 1	20.269651°	50.032460°	P	120	sand	M	2.83	SD
4	Wola Batorska 2	20.249682°	50.027453°	Q	195	silty clay loam	M	3.57	D
5	Stanisławice 1	20.335946°	49.991910°	P	65	sand	MM	3.55	SD
6	Stanisławice 2	20.324853°	49.932567°	P	125	sand	M	2.92	SD
7	Krzyszkowice	20.016746°	50.002342°	D	150	silt loam	MM	3.75	D
8	Ostrowy Tuszowskie	21.643837°	50.326383°	Q	135	sand	MM	3.61	SD
9	**Przyborów 1**	**15°45′06″**	**51°47′27″**	**Q**	**150**	**sandy loam**	**PFWT**	**4.82**	**D**
10	**Przyborów 2**	**15°45′07″**	**51°46′58″**	**Q**	**140**	**sandy loam**	**PFWT**	**5.39**	**D**
11	Babice	18°16′03″	50°08′01″	Q	120	sandy loam	MM	3.77	SD
12	Maleniska	22°19′59″	50°16′00″	D	70	sand	MM	3.32	H
13	Przedbórz	19°54′17″	51°05′41″	P	60	sand	MD	3.46	SD
14	Rączna	19°44′52″	50°00′26″	D	70	sand	MD	3.51	H
15	**Radymno**	**23°04′00″**	**49°56′60″**	**P**	**115**	**loamy sand**	**MM**	**4.22**	**D**
16	Pomorsko	15°28′47″	52°02′44″	Q	150	sand	PFWT	5.20	H
17	Piskorowice	22°34′40″	50°14′04″	P	80	loamy sand	MM	3.75	SD
18	Pociękarb	18°04′44″	50°18′58″	Q	150	sandy loam	MM	3.14	SD
19	Bierdzany	18°09′10″	50°48′19″	D	80	loamy sand	MM	3.33	H
20	Rzędzów	18°08′22″	50°44′18″	D	50	sand	MM	3.52	SD
21	Chełmiec	16°04′58″	51°02′13″	Q	100	silt loam	MM	3.6	H
22	Moszna	17°46′25″	50°25′31″	D	90	loamy sand	MM	3.32	SD
23	Leśniki	15°50′22″	52°07′39″	P	105	sand	MD	5.94	SD
24	**Laski**	**15°48′32″**	**52°10′27″**	**D**	**135**	**sandy loam**	**MM**	**3.29**	**D**
25	**Kręcko**	**15°04′01″**	**52°10′27″**	**D**	**125**	**sand**	**MM**	**5.18**	**SD**
26	**Zwierzyniec**	**18°45′31″**	**50°55′30″**	**Q**	**140**	**sandy loam**	**MM**	**3.99**	**H**
27	**Głubczyce**	**17°38′39″**	**50°09′50″**	**Q**	**135**	**silt loam**	**M**	**3.68**	**H**
28	Mikolin	17°40′48″	50°47′41″	Q	120	silt loam	M	3.89	SD
29	Czyżowice	18°22′39″	49°59′33″	Q	90	silty clay loam	MM	3.58	SD

[1] Q – pure *Quercus robur* stand; P – mixture with *Pinus sylvestris*; D – mixture with other deciduous species. [2] Soils texture according to USDA (United States Department of Agriculture). [3] M – moist; MM – moderately moist; D – dry; MD – moderately dry; PFWT – periodically fluctuating water table. [4] The pH was measured with a glass electrode in a 0.01 m $CaCl_2$ suspension and in deionized H_2O. [5] H – healthy (class 1 according to Balcì and Hamschlager [10]); SD – slightly damaged (class 2); D – declining (class 3 and 4).

Identification of *Pythium* and phylogenetic analysis

All *Pythium* cultures were grouped according to morphological characters using a Nikon Eclipse 50i microscope (Nicon® Corporation, Tokyo, Japan) and an Invenio 5S digital camera (DeltaPix®, Maalov, Denmark) with Coolview 1.6.0 software (Precoptic®, Warsaw, Poland). *Pythium* structures and colony characteristics were compared with the descriptions of species given in the literature [17]. The morphology of colonies was described from 7-day-old colonies growing on carrot (CA), cornmeal agar (CMA), potato-dextrose agar (PDA), malt agar (MEA) and V8 agar medium. Sporangia were obtained by flooding agar discs taken from growing margins of 7-day-old colonies with unsterile soil extract (ratio 1:10). From each morphological group, isolates were selected for DNA sequencing and were deposited in the Fungal Culture Collection of the Department of Forest Pathology, Mycology and Tree Physiology, Hugo Kołłątaj University of Agriculture, Cracow, Poland.

Genomic DNA from 18 strains (Tab. 2) was isolated using ArchivePure DNA Yeast/Gram-positive Bacteria Kit (5 PRIME, Inc. Gaithersburg, MD) with modified time of incubation with lytic enzyme solution (2 h, 37°C) and cell lysis solution (4 h, 64°C). Nuclear ITS rDNA region internal transcribed spacers (ITS1 and ITS2) and 5.8S subunits were amplified with primer set ITS5/ITS4 [18]. The reaction mixtures and conventional PCR protocols were the same as in Hubka and Kolarik [19]. Custom purification of PCR amplicons and sequencing was conducted at Macrogen Inc. (Seoul, South Korea) using the same primers.

Tab. 2 Cultures used in this study and GenBank accession numbers for sequences.

Taxon	Isolate number[1]	Site	Accession No.	Closest match in BLAST	Accession of match	Identity (%)
Phytopythium citrinum	121HR09	Zwierzyniec	KC602480	*Phytopythium citrinum*	HQ 643379	100.0
	143HR09	Zwierzyniec	KC602481	*Phytopythium citrinum*	HQ 643379	100.0
	35HR09	Zwierzyniec	KC602482	*Phytopythium citrinum*	HQ 643379	100.0
	48HR09	Zwierzyniec	KC602483	*Phytopythium citrinum*	HQ 643379	100.0
	99HR09	Głubczyce	KC602484	*Phytopythium citrinum*	HQ 643379	100.0
Phytopythium cf. *citrinum* A	444HR08	Przyborów 2	KC602485	*Pythium* sp. GD33b	EF152505	99.7
	447HR08	Przyborów 2	KC602486	*Pythium* sp. GD33b	EF152505	99.7
Phytopythium cf. *citrinum* B	428HR08	Przyborów 2	KC602487	*Pythium* sp. GD33b	EF152505	100.0
	520HR08	Przyborów 1	KC602488	*Pythium* sp. GD33b	EF152505	100.0
Phytopythium sp. 1	68HR09	Zwierzyniec	KC602492	*Pythium* sp. B57	JN863966	95.9
Phytopythium sp. 2	451HR08	Przyborów 2	KC602493	*Pythium* sp. PV So7	EU669081.1	94.0
Phytopythium sp. 3	474HR08	Przyborów 1	KC602494	*Phytopythium* sp. MAB-2011e	AB690623.1	97.5
Pythium anandrum	304HR07	Ispina 2	KC602489	*Pythium anandrum*	HQ643435	100.0
	305HR07	Ispina 2	KC602490	*Pythium anandrum*	HQ643435	100.0
Pythium diclinum	27HR09	Laski	KC602491	*Pythium diclinum*	JQ898459	100.0
Pythium intermedium	2HR09	Kręcko	KC602495	*Pythium intermedium*	AY083936	100.0
Pythium undulatum	719HR08	Radymno	KC602496	*Pythium undulatum*	EU240049	100.0
	725HR08	Radymno	KC602497	*Pythium undulatum*	EU240049	100.0

[1] KFL: Fungal Culture Collection, Department of Forest Pathology, Mycology and Tree Physiology, Hugo Kołłątaj University of Agriculture, Cracow, Poland.

Sequences were compared with the data from GenBank using a BLAST search. All sequences were aligned online with MAFFT v6 [20], using the E-INS-i option with a gap-opening penalty of 1.53 and an offset value of 0.00.

Datasets were analyzed using maximum likelihood (ML) and Bayesian inference (BI). For the ML and Bayesian analyses, the best-fit substitution models for each data set were established using the corrected Akaike information criterion (AICc) in jModelTest 0.1.1 [21]. The selected model for the ITS was GTR+I+G.

ML searches were conducted in PhyML 3.0 [22], via the Montpelier online server (http://www.atgc-montpellier.fr/phyml/) with 1000 bootstrap replicates. BI analyses based on a Markov chain Monte Carlo (MCMC) were performed with MrBayes v3.1.2 [23]. The MCMC chains were run for 10 million generations using the best fitting model. Trees were sampled every 100 generations, resulting in 100 000 trees from both runs. The burn-in value for each dataset was determined in Tracer v1.4.1 [24].

All sequences generated in this study were deposited in the NCBI GenBank (Tab. 2) and are presented in the phylogenetic tree (Fig. 1, Fig. 2). *Pythium* clades were designated according to Lévesque and de Cock [5].

Results

Morphological investigation showed that ten groups producing sporangia and sexual structures in culture were collected. The ITS data confirmed that these groups represented taxa belonging to the phylogenetically related genera, *Phytopythium* and *Pythium*. Groups with affinity to the genus *Phytopythium* represented one known species (*Phytopythium citrinum*) and five unknown species named here as *Phytopythium* cf. *citrinum* A, *Phytopythium* cf. *citrinum* B, *Phytopythium* sp. 1, *Phytopythium* sp. 2 and *Phytopythium* sp. 3. In turn, the genus *Pythium* was represented by four species including *Pythium anandrum*, *P. diclinum* Tokun., *P. intermedium* and *P. undulatum* (Fig. 1, Fig. 2). Polygenetic analyses of the ITS sequences of *Pythium* isolates placed them in three clades (B, F, H) within *Pythium* (in Lévesque, de Cock [5]). *Phytopythium* cf. *citrinum* A and *Phytopythium* cf. *citrinum* B in phylogenetic analyses were most closely related to isolate *Pythium* sp. GD33b, while the three unknown species were most closely related to different species in the genus *Phytopythium* (Fig. 2, Tab. 2). Their identity and morphological features are still under investigation and descriptions of these taxa will be provided in a later publication.

Ten different *Phytopythium* and *Pythium* species were isolated from rhizosphere soil in 8 of the 29 oak stands. Among them, *Pythium anandrum* was most frequently recorded (4.0%) and showed the widest geographical distribution (4 stands). *Phytopythium citrinum*, *Phytopythium* cf. *citrinum* B, *Phytopythium* sp. 1 and *Phytopythium* sp. 3 were found in two stands. On average, the isolation frequency of *Phytopythium citrinum*, *Phytopythium* cf. *citrinum* B, *Phytopythium* sp. 1 and *Phytopythium* sp. 3, in the oaks forests was 2.3%, 1.7%, 2.3% and 2.9%, respectively. Other species were sporadically isolated from rhizosphere soil (Tab. 3).

Among the six identified soil textures, *Phytopythium* spp. and *Pythium* spp. were most frequently isolated from soil developed from sandy loam. These organisms occurred on sites with a mean soil pH range ($CaCl_2$) ranging from 3.29 to 5.39, and were most often isolated from soil samples taken from declining trees (14 out of 36) and from pure *Q. robur* stands (4 out of 29; Tab. 1).

Discussion

This is the first extensive report demonstrating the presence of *Phytopythium* and *Pythium* species in oak forests in Poland. Based on morphological characteristics and ITS rDNA sequence analysis, 10 species of *Phytopythium* spp. and *Pythium* spp. were isolated from the soil-root samples, including three putative new species. The most commonly encountered *Pythium* species was *Pythium anandrum*. The present study demonstrates for the first time that *Phytopythium citrinum* and *Pythium diclinum* can

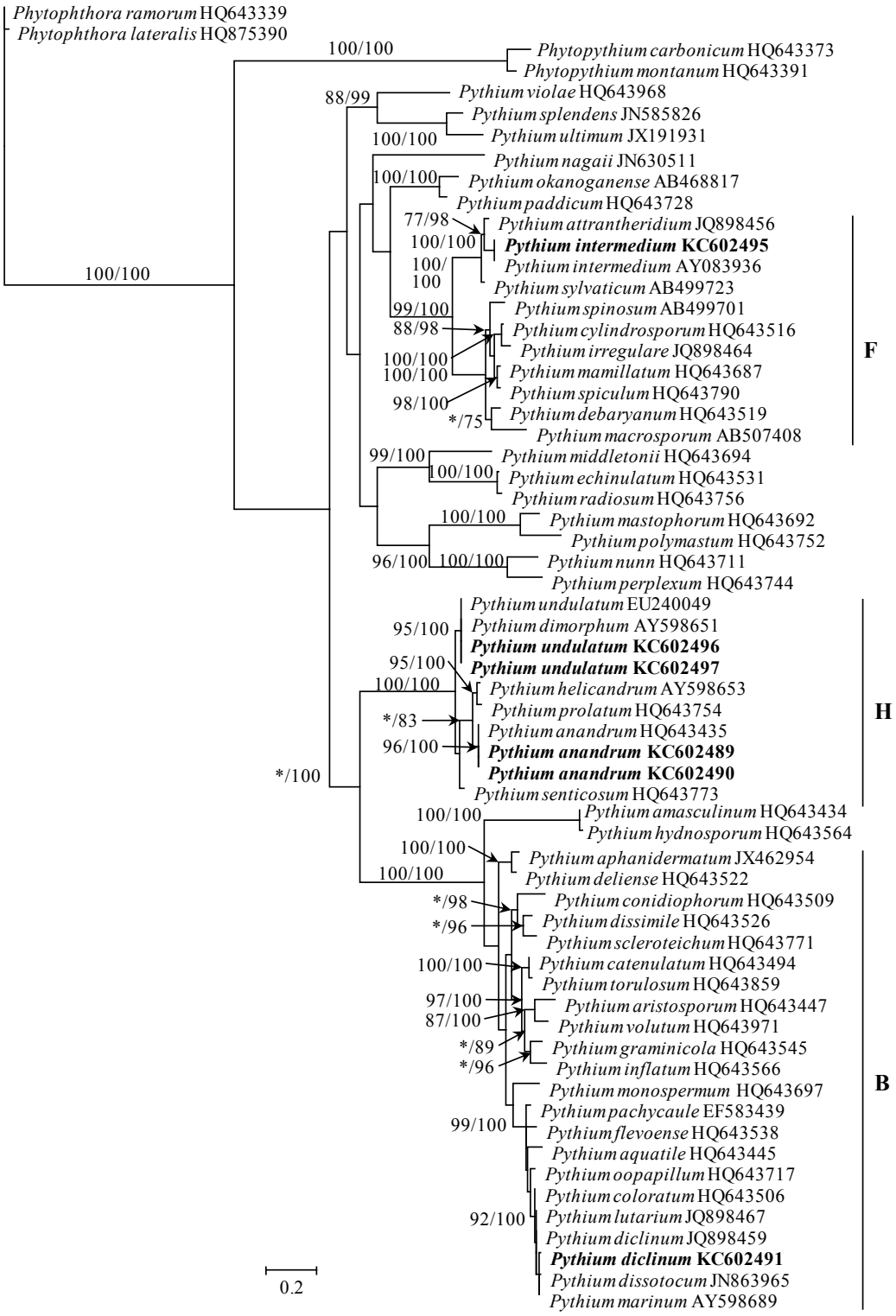

Fig. 1 Phylogram obtained from the analyses of ITS sequence data, revealing the identity of *Pythium* spp. isolated from soil in oak stands in the southern part of Poland. Sequences obtained during this study are presented in bold type. The phylogram was obtained from maximum likelihood (ML) analyses. The bootstrap values (>75%) for ML and posterior probabilities (>75%) that were obtained from Bayesian (BI) analyses are presented at nodes as follows: ML/BI. * Bootstrap values <75%.

also act as soil-borne oomycete species in oak forests. In addition, these species were reported for the first time in Poland.

This study has shown that the assemblage of *Phytopythium*/*Pythium* spp. occurring in the rhizosphere soil of oak forests in Poland was quite diverse. The occurrence of

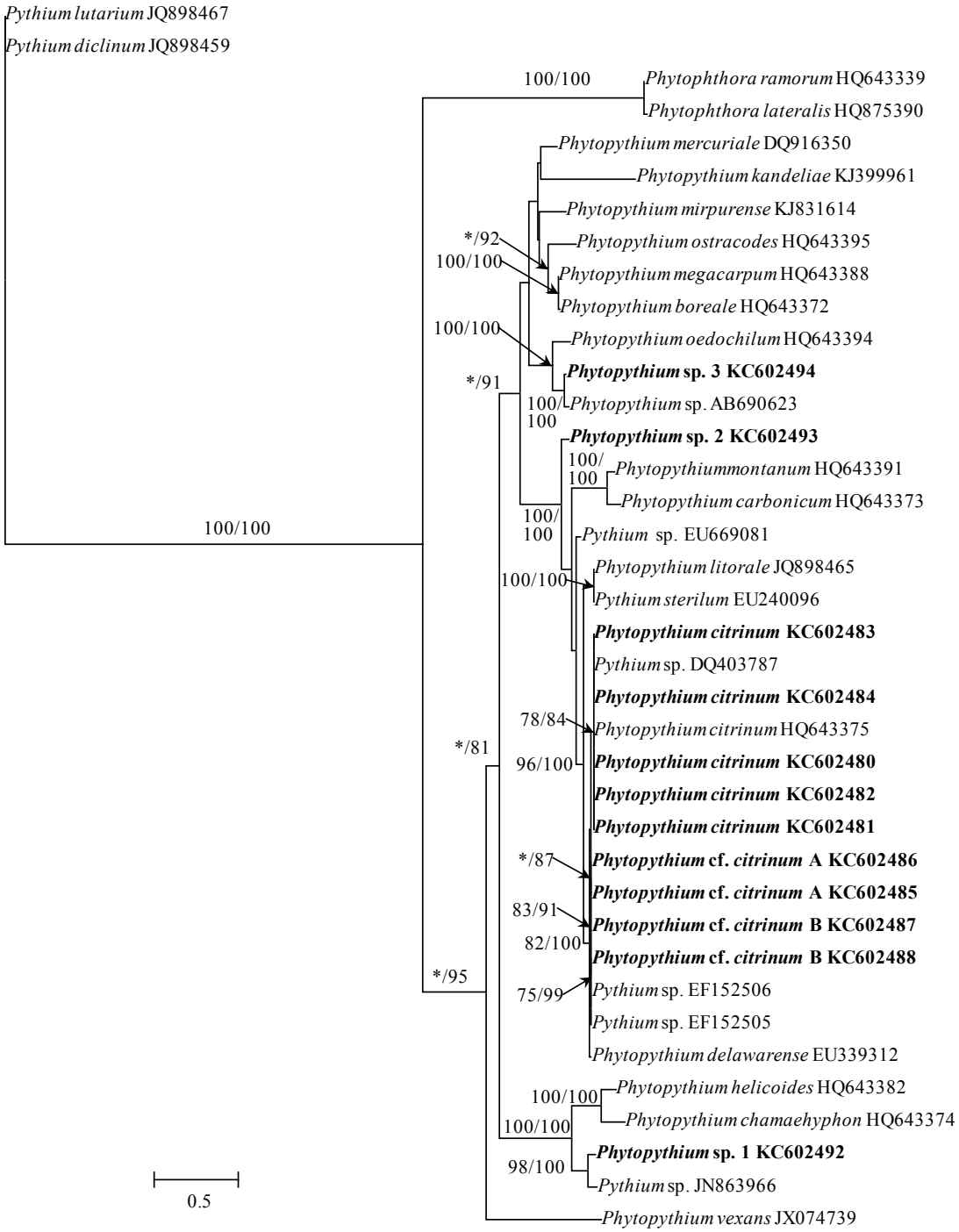

Fig. 2 Phylogram obtained from the analyses of ITS sequence data, revealing the identity of *Phytopythium* spp. isolated from soil in oak stands in the southern part of Poland. Sequences obtained during this study are presented in bold type. The phylogram was obtained from maximum likelihood (ML) analyses. The bootstrap values (>75%) for ML and posterior probabilities (>75%) that were obtained from Bayesian (BI) analyses are presented at nodes as follows: ML/BI. * Bootstrap values <75%.

Pythium species in oak stands is in accordance with results from previous studies [10,11,13,14]. Of all the *Pythium* species recorded in association with the rhizosphere soil of oak stands in Europe, *P. anandrum* and *P. undulatum* are the most commonly reported. Consistent with Jung et al. [10], Balcì and Halmshlager [11] and Jönsson et al. [13], we isolated *P. anandrum* most frequently. However, in contrast to these studies but similar to Cordier et al. [14], we additionally relatively often isolated species resembling *Phytopythium citrinum*. The level of *Pythium* diversity found in our survey was certainly underestimated because the typically selective medium for

Tab. 3 Isolation frequencies of *Phytopythium* spp. and *Pythium* spp. from soil samples in oak stands in Poland and soil pH ranges.

Taxon	pH range ($CaCl_2$)	Number of positive soil samples	Frequency of isolation (%)	Percent of positive stands	Site number
Phytopythium citrinum	3.68–3.99	4	2.3	6.9	26, 27
Phytopythium cf. *citrinum* A	5.39	1	0.6	4.2	10
Phytopythium cf. *citrinum* B	4.82–5.39	3	1.7	6.9	9,10
Pythium anandrum	3.75–4.22	7	4.0	13.8	2, 15, 24, 26
Pythium diclinum	3.29	1	0.6	4.2	24
Pythium intermedium	5.18	1	0.6	4.2	25
Pythium undulatum	4.22	1	0.6	4.2	15
Phytopythium sp. 1	3.99–5.39	4	2.3	6.9	10, 26
Phytopythium sp. 2	4.82	2	1.1	4.2	9
Phytopythium sp. 3	4.82–5.39	5	2.9	6.9	9, 10
Total		23	13.2	27.6	2, 9, 10, 15, 24, 25, 26, 27

Pythium has not been used. Therefore further studies will be needed to fully characterize the oak-associated *Pythium* species.

Among the species of the genus *Pythium*, only *P. undulatum* is known to be pathogenic to oak [16]. Weber et al. [3] showed also the aggressiveness of *P. undulatum* on roots of *A. procera* and *P. menziesii*. In addition, Shafizadeh and Kavanagh [25] have also shown the pathogenicity of *P. undulatum* on *Picea sitchensis* (Bong.) Carr., *Picea abies* (L.) Karst. and *Pinus contorta* Dougl. ex Loud. In the present study, this root pathogen has been recorded only on one site suggesting that rather does not play important role in the destruction of root systems of oaks. However, the majority of *Phytopythium* spp. and *Pythium* spp. isolates have been obtained from declining stands indicating a possible association between the presence of these organisms and health status of trees. Similar relationships have been revealed for soil-borne *Phytophthora* spp. in several European countries [10–12,26,27].

It was shown that *Phytopythium* and *Pythium* species are widespread on a range of different soil textures with a mean soil pH ($CaCl_2$) between 3.29 and 5.39. Our results resembled those of Jung et al. [10] and Balcì and Halmshlager [11] who mentioned that *Pythium* species in Germany and Austria occurred in similar site conditions like *Phytophthora* species in oak forests.

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