Identification of *Phytophthora alni* Subspecies in Riparian Stands in the Czech Republic

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Abstract

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In the Czech Republic, *Phytophthora alni* was first confirmed in 2001 and the pathogen has been quickly spreading and occupying almost the whole area of the country. The pathogen attacks *Alnus glutinosa* or *A. incana* to a lesser extent and causes considerable losses of alder trees along hundreds of kilometres of riverbanks. The aim of our work was to perform the identification of *P. alni* isolates at the subspecific level using PCR and to determine the frequencies and distribution of particular subspecies. The allele-specific PCR primers focused on allele diversity of orthologs of *ASF*-like, *TRP*1, *RAS-Ypt*, and *GPA*1 genes were selected for identification. Eighty-eight per cent of the 59 analysed isolates belonged to *P. alni* ssp. *alni* while 12% were *P. alni* ssp. *uniformis. P. alni* ssp. *multiformis* has not been recorded in the country till now. The two subspecies differed in distribution. *P. alni* ssp. *alni* dominated in riparian stands along broader rivers in lowlands and the results confirmed the more effective spreading of *P. alni* ssp. *alni* based on its higher aggressiveness and ecological advantage. *P. alni* ssp. *uniformis* was acquired rather from riparian stands of small watercourses at higher altitudes. The insular distribution of *P. alni* ssp. *uniformis* may represent the remains of its former occurrence. Therefore, *P. alni* ssp. *uniformis* may be an indigenous subspecies suppressed by the more aggressive related taxon.

Keywords: *Phytophthora alni; Alnus*; alder disease; PCR; riparian stand

Phytophthora alni is a destructive oomycetous pathogen of alder trees (*Alnus* spp.). The respective disease and damage of alder stands has recently become a crucial problem in many European countries. Since 1993, the *Phytophthora* alder disease has occurred mainly along riverbanks and locally in orchards, shelterbelts and woodland plantations (Brasier *et al.* 1995; Gibbs 1995) and later it was also found in forest plantations and nurseries (Jung & Blaschke 2004). The disease was found mainly in *Alnus glutinosa* (L.) Gaertn., *A. incana* (L.) Moench, and *A. cordata* Desf. in the majority of the Western to Central European countries (Santini *et al.* 2001; Brasier *et al.* 2004; Jung & Blaschke 2004; Érsek & Nagy 2008). The disease caused significant losses of alder trees in several countries (Gibbs *et al.* 1999; Streito *et al.* 2002; Jung & Blaschke 2004). The

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causal agent, *Phytophthora alni* Brasier et S.A. Kirk, was of a hybrid origin and comprised a range of phenotypically diverse heteroploid populations (Brasier *et al.* 2004). Therefore three subspecies: *alni*, *multiformis*, and *uniformis* were recognised within *P. alni*. Ioos *et al.* (2006) revealed that *P. alni* subsp. *alni* (*Paa*) was a descendant of hybridisation between *P. alni multiformis* (*Pam*) and *P. alni uniformis* (*Pau*). This hypothesis was later confirmed by Bakonyi *et al.* (2007). In addition to these subspecies, diverse isolates have been recovered that represent the backcross offspring with *P. cambivora*, or previously undefined variant types of *P. alni* (Brasier *et al.* 2004; Jung & Blaschke 2004; Érsek & Nagy 2008). Bakonyi *et al.* (2007) evidenced variability within *Paa* mitochondrial DNA which may indicate the multiple origin of this subspecies. Reliable PCR methods for subspecies identification were developed in previous years. Two methods were based on anonymous RAPD markers (Ioos *et al.* 2005; Bakonyi *et al.* 2006), another approach (Ioos *et al.* 2006) was targeted on introns in four nuclear orthologous genes and distribution of single allele within the genome of *P. alni* individuals. The development of a reliable identification method enabled to study the distribution of subspecies and their ecological preferences. Small-scale population study seemed to be necessary, because the distribution, frequency and pathogenicity of particular subspecies were found to be highly diverse (Brasier & Kirk 2001; Brasier *et al.* 2004; De Merlier *et al.* 2005), which can significantly affect the process of recent invasion.

Phytophthora alni has spread quickly in the Czech Republic. The pathogen was first isolated from damaged alder trees in 2001. Six years later, *P. alni* was identified in about 60 alder stands, mostly in the western part of the country. So far, the pathogen has caused considerable losses of alder trees along hundreds of kilometres of riverbanks and has been spreading beyond control (Černý *et al.* 2008; Černý & Strnadová 2010). Thus the area seemed to be very suitable to perform research focused on the identification of strains of *P. alni* at the subspecific level and on the clarification of distribution of particular subspecies.

MATERIAL AND METHODS

Extensive search of damaged riparian stands was done across the whole country during 2005 to 2010. *P. alni* isolates were obtained from infected tissues according to Černý *et al.* (2011) from symptomatic bark of native alders (*Alnus glutinosa* and *A. incana*) and were deposited in the culture collection of Silva Tarouca Research Institute for Landscape and Ornamental Gardening, Průhonice, Czech Republic. The identity of *P. alni* isolates was verified by morphological traits of sexual and asexual structures and growth characteristics of colonies according to Brasier *et al.* (2004). The allele-specific PCR primers focused on allele diversity of orthologs of *ASF*-like, *TRP*1, *RAS-Ypt*, and *GPA*1 genes (Ioos *et al.* 2006) were selected for the more exact PCR identification of isolates at the subspecific level. The DNA was extracted from freshly grown cultures using a DNeasy Plant mini kit (Qiagen, Carlsbad, USA) according to the manufacturer's protocol. The PCR was performed under the following temperature regime: 94°C/3 min, 58–62°C/30 s, 72°C/1 min (1×), 94°C/30 s, 58–62°C/30 s, 72°C /1 min (33×) and 94°C/30 s, 58–62°C/30 s, 72°C/7 min (1×). Annealing temperatures were 58°C (*RAS-Ypt*) or 62°C (*ASF*-like, *TRP*1, and *GPA*1 genes). The PCR products were checked by agarose electrophoresis.

The coordinates and altitude of locations were determined with the use of Garmin GPSMAP 60CSx and controlled with the use of MapSource 6.15.4 (Garmin Ltd., Olathe, USA) with TOPO Czech 3.1. (Garmin Czech, Prague, Czech Republic) as a map base. The width of watercourses was estimated visually. In the case of broader rivers the estimation was corrected using the Google Earth 6.2 application (Google Inc., Mountain View, USA). The data on pathogen distribution were processed by the Mann Whitney U-test in nonparametric statistics in Statistica 7.1 package (StatSoft Inc., Tulsa, USA).

The habitat types were classified according to Chytrý *et al.* (2010).

RESULTS

In total, 59 isolates of *P. alni* were analysed. They were acquired from the major part of the country (with the exception of the northeastern part where no disease was found). The results revealed the prevalence of *Paa* – 52 isolates (88.14%) were determined as *Paa* (Table 1 and Figure 1). Only 7 isolates were determined as *Pau* – 11.86% of all isolates.

Paa was very frequent in the whole area of the alder. Distribution of *Pau* had an apparently insular

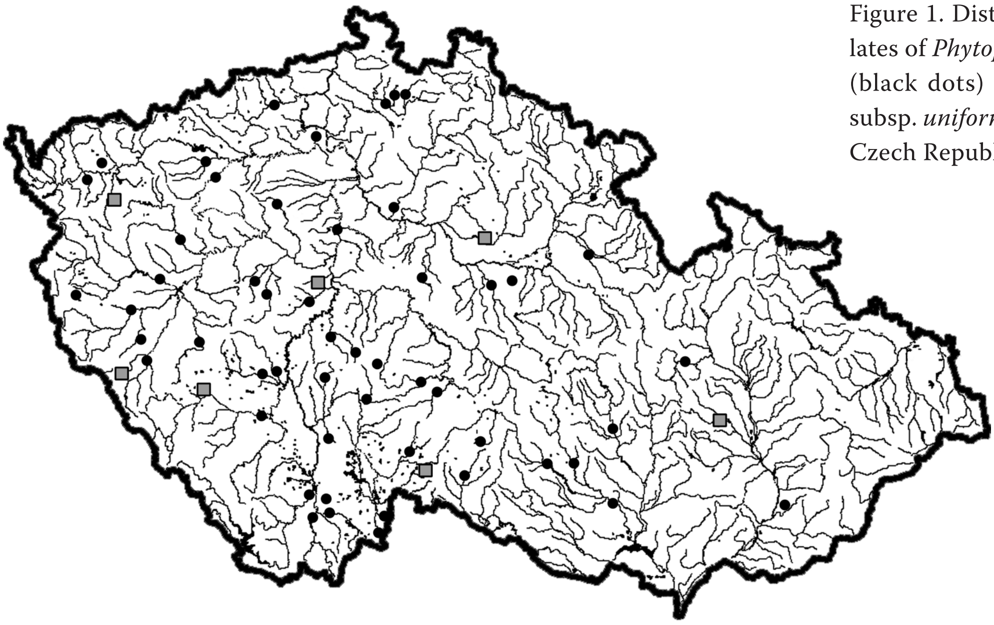

Figure 1. Distribution of studied isolates of *Phytophthora alni* subsp. *alni* (black dots) and *Phytophthora alni* subsp. *uniformis* (grey squares) in the Czech Republic

pattern although this subspecies seems to be also evenly distributed across the country (Figure 1).

The data on the altitude of particular locations and width of watercourses were collected and evaluated. The data on the altitudes of locations showed a normal distribution, but according to the low P value ($P < 0.10$, Lilliefors test) the normal distribution did not have any strong statistical support and their transformation did not lead to better results. The data describing the width of watercourses did not have a normal distribution even after log transformation ($P < 0.05$, Lilliefors test). Moreover, the sizes of both sets were different. On the other hand, the homogeneity requirements were fulfilled in both cases ($P > 0.10$, Levene's test) and the data could be analysed by the non-parametric Mann Whitney U-test.

The distribution of both subspecies significantly differed according to the width of respective watercourses ($P = 0.01$). *Paa* was distributed along watercourses of different types, but it was frequently found on banks of broad lowland rivers. The width of watercourses surrounded by alder stands infected by *Paa* varied from ca. 1 to nearly 190 m (Table 1). The median of estimates was nearly 10 m, which meant that about one half of *Paa* isolates originated from riparian stands of rivers ca. 10–30 m in width (Figure 2), with low banks and slow flow in broad valleys at medium and lower altitudes. *Paa* was also found on pond

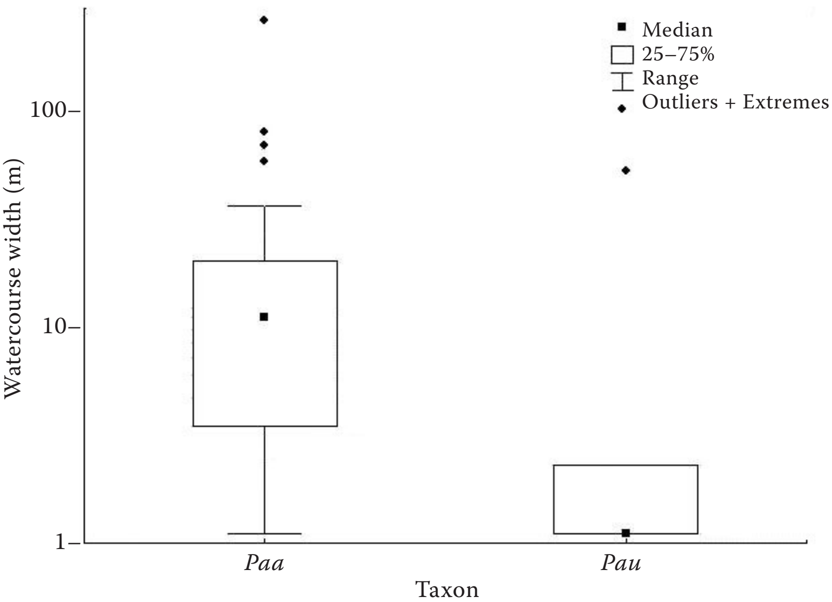

Figure 2. Widths of watercourses associated with the occurrence of *Phytophthora alni* subsp. *alni* (*Paa*) and *Phytophthora alni* subsp. *uniformis* (*Pau*)

Table 1. List of isolates included in the study

Sub-species	Region	Locality (District)	Coordinates	Isolate number	Host	Stand	Habitat	Width of water-course (m)
Paa	Karlovy Vary	Jenišov (Karlovy Vary)	50°14'16.68"N; 12°47'7.97"E	P 146.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	402
		Sokolov (Sokolov)	50°10'11.46"N; 12°40'8.94"E	P 186.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	430
	Plzeň	Bdeněves (Plzeň-sever)	49°46'9.94"N; 13°13'50.51"E	P 169.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	326
		Borek (Tachov)	49°38'12.73"N; 12°46'42.32"E	P 171.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	464
		Horšovský Týn (Domažlice)	49°31'56.74"N; 12°57'19.07"E	P 136.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	374
		Malechov (Klatovy)	49°27'28.16"N; 13°15'50.15"E	P 004.06	*A. glutinosa*	riparian stand	ash-alder alluvial forest	377
		Mladotice (Plzeň-sever)	49°58'36.4"N; 13°20'24.67"E	P 206.08	*A. glutinosa*	riparian stand	ash-alder alluvial forest	366
		Radonice (Domažlice)	49°27'12.28"N; 12°59'43.92"E	P 133.07	*A. glutinosa*	riparian stand	alder carr	401
		Srby (Klatovy)	49°31'16.82"N; 13°36'5.76"E	P 131.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	413
	Ústí nad Labem	Holedeček (Louny)	50°18'7.8"N; 13°35'2.85"E	P 227.08	*A. glutinosa*	riparian stand	ash-alder alluvial forest	217
		Hrdly (Litoměřice)	50°29'31.7"N; 14°9'34.8"E	P 231.08	*A. glutinosa*	riparian stand	alder carr	153
		Ohníč, Dolánky (Teplice)	50°35'26.76"N; 13°51'15.37"E	P 230.08	*A. glutinosa*	riparian stand	alder carr	189
		Sedčice (Louny)	50°18'36.57"N; 13°26'32.48"E	P 229.08	*A. glutinosa*	riparian stand	alder carr	231
	Central Bohemian	Církvice (Kolín)	49°55'3.22"N; 15°0'50.16"E	P 105.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	360
		Dolní Bučice (Kutná Hora)	49°55'41.92"N; 15°28'45.97"E	P 044.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	223
		Ješetice (Benešov)	49°34'53.25"N; 14°36'30.73"E	P 084.07	*A. glutinosa*	pond bank	alder carr	507
		Jince (Příbram)	49°47'57.47"N; 13°58'35.28"E	P 042.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	370
		Kačice (Kladno)	50°9'28.24"N; 13°59'8.08"E	P 051.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	382
		Nový Knín (Příbram)	49°47'7.16"N; 14°17'8.64"E	P 141.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	301
		Osek (Beroun)	49°49'21.65"N; 13°52'3.64"E	P 060.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	371
		Sedlčany (Příbram)	49°39'26.27"N; 14°25'47.81"E	P 137.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	340
		Sojovice (Mladá Boleslav)	50°12'5.25"N; 14°45'10.39"E	P 199.07	*A. glutinosa*	riparian stand	willow-poplar forest	173
	Praha	Praha (Praha)	50°7'19.8"N; 14°23'41.11"E	P 377.10	*A. glutinosa*	riparian stand	willow-poplar forest	179
	Liberec	Heřmaničky (Česká Lípa)	49°36'3.45"N; 14°35'22.36"E	P 028.06	*A. glutinosa*	riparian stand	alder carr	255
		Velenice (Česká Lípa)	50°42'54.83"N; 14°39'36.35"E	P 052.07	*A. glutinosa*	riparian stand	alder carr	283
		Velký Grunov (Česká Lípa)	50°42'27.38"N; 14°42'39.06"E	P 039.07	*A. glutinosa*	riparian stand	alder carr	289
	South Bohemian	Hamr (České Budějovice)	48°52'26.36"N; 14°29'53.54"E	P 223.08	*A. glutinosa*	riparian stand	ash-alder alluvial forest	421
		Jarošov n. Než. (Jindřichův Hradec)	49°11'22.06"N; 15°4'8.51"E	P 130.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	474

Paa	South Bohemian	Klikov (Jindřichův Hradec)	48°54'18.34"N; 14°54'24.3"E	P 195.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	421
		Litvínovice (České Budějovice)	48°57'42.66"N; 14°27'23.69"E	P 226.08	*A. glutinosa*	pond bank	alder carr	388
		Malý Pěčín (Jindřichův Hradec)	49°6'26.09"N; 15°26'45.65"E	P 012.06	*A. glutinosa*	riparian stand	alder carr	469
		Mirotice (Písek)	49°25'53.04"N; 14°2'8.94"E	P 132.07	*A. glutinosa*	riparian stand	alder carr	410
		Nová Ves (České Budějovice)	48°55'15.16"N; 14°32'12.91"E	P 222.08	*A. glutinosa*	riparian stand	ash-alder alluvial forest	445
		Nová Ves n. Luž. (Jindřichův Hradec)	48°48'34.86"N; 14°55'58.87"E	P 193.07	*A. glutinosa*	riparian stand	alder carr	467
		Sezimovo Ústí (Tábor)	49°23'23.04"N; 14°40'36.93"E	P 197.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	392
		Trhové Sviny, Jedovary (České Budějovice)	48°52'28.91"N; 14°34'57.57"E	P 221.08	*A. glutinosa*	riparian stand	ash-alder alluvial forest	439
		Varvažov (Písek)	49°26'19.32"N; 14°7'37.18"E	P 135.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	388
		Vlksice, Prachárna (Písek)	49°28'46.31"N; 14°26'58.83"E	P 016.06	*A. glutinosa*	riparian stand	ash-alder alluvial forest	468
		Zhoř u Mladé Vožice (Tábor)	49°32'2.46"N; 14°46'30.88"E	P 210.08	*A. glutinosa*	riparian stand	ash-alder alluvial forest	448
		Žimutice (České Budějovice)	49°12'8.51"N; 14°30'43.23"E	P 024.06	*A. glutinosa*	riparian stand	alder carr	442
		Zátaví (Písek)	49°17'18.88"N; 14°7'9.81"E	P 050.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	365
	Hradec Králové	Týniště nad Orlicí (Rychnov n. Kněžnou)	50°7'47"N; 16°4'14.44"E	P 217.08	*A. glutinosa*	riparian stand	willow-poplar forest	249
	Pardubice	Holotín (Pardubice)	49°57'0.14"N; 15°35'22.62"E	P 140.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	328
	Vysočina	Čakovice (Pelhřimov)	49°27'36.42"N; 15°10'24.65"E	P 061.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	517
		Naloučany (Třebíč)	49°12'58.18"N; 16°9'19.79"E	P 339.09	*A. glutinosa*	riparian stand	ash-alder alluvial forest	362
		Řídelov (Jihlava)	49°14'18.086"N, 15°24'6.003"E	P 145.07	*A. glutinosa*	pond bank	alder carr	621
		Samšín (Pelhřimov)	49°29'37.77"N; 15°4'49.1"E	P 047.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	461
		Vladislav (Třebíč)	49°12'33.62"N; 15°59'25.68"E	P 006.06	*A. glutinosa*	riparian stand	ash-alder alluvial forest	387
	Olomouc	Kozov (Olomouc)	49°42'33.75"N; 16°52'26.14"E	P 063.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	293
	South Moravian	Ivančice (Brno-venkov)	49°5'51.26"N; 16°23'59.58"E	P 240.08	*A. glutinosa*	riparian stand	willow-poplar forest	215
		Štěpánovice (Brno-venkov)	49°22'0.38"N; 16°23'39.5"E	P 298.09	*A. glutinosa*	riparian stand	ash-alder alluvial forest	259
	Zlín	Březolupy (Uherské Hradiště)	49°8'6.19"N; 17°35'24.46"E	P 079.07	*A. glutinosa*	deposit of harvested timber	ash-alder alluvial forest	225
Pau	Karlovy Vary	Krásné Údolí (Karlovy Vary)	50°4'30.48"N; 12°52'50.5"E	P 270.09	*A. incana*	riparian stand	montane grey alder gallery	671
	Plzeň	Horažďovice (Klatovy)	49°19'28.23"N; 13°40'47.55"E	P 220.08	*A. glutinosa*	riparian stand	ash-alder alluvial forest	427
		Pocinovice (Klatovy)	49°19'33.23"N; 13°8'27.01"E	P 239.08	*A. glutinosa*	riparian stand	alder carr	433
	Central Bohemian	Čisovice (Praha-západ)	49°51'49.5"N; 14°18'4.02"E	P 213.08	*A. glutinosa*	riparian stand	ash-alder alluvial forest	357
		Žiželice (Kolín)	50°08''7.5"N; 15°23'52.88"E	P 198.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	209
	South Bohemian	Kunžak (Jindřichův Hradec)	49°7'28.38"N; 15°11'33.98"E	P 144.07	*A. glutinosa*	riparian stand	ash-alder alluvial forest	557
	Olomouc	Prostějov (Prostějov)	49°2758.2"N; 17°4'20.24"E	P 299.09	*A. glutinosa*	riparian stand	ash-alder alluvial forest	241

Paa – *Phytophthora alni* subsp. *alni*; *Pau* – *Phytophthora alni* subsp. *uniformis* banks and in the surroundings of landings of harvested alder timber, which supported its high invasive potential.

On the contrary, *Pau* was more or less limited to small watercourses at different altitudes. The pathogen was found predominantly in riparian stands of narrow watercourses of 1–2 m in width (median of estimates was only 1 m) and only one isolate was acquired from the riparian stand of a lowland river 40 m in width.

The two subspecies, *Paa* and *Pau*, differed in vertical distribution but the difference did not have statistical support (Mann-Whitney U-test, $P > 0.05$). *Paa* was more frequent at lower altitudes in comparison with *Pau*, the average elevation of subspecies distribution is as follows: *Paa* – 360 m and *Pau* – 413 m. A real difference in the vertical distribution of both subspecies could be more distinct because the uppermost isolates from identical watercourses were usually analysed to minimise the possibility of repeating the analysis of identical and downstream spreading genotypes.

The habitat distribution of both subspecies seems to be generally similar according to the habitat type, but some quite minor differences were also identified. *Paa* was predominantly isolated from ash-alder alluvial forests (32 records) and alder carrs (14 records). Moreover, 4 records were made in willow-poplar forests of lowland rivers. *Pau* was isolated from 5 affected ash-alder alluvial forests, one alder carr and one montane grey alder gallery. The most characteristic and common habitats of both pathogens are ash-alder alluvial forests and alder carrs. However, *Paa* unlike *Pau* was found also in willow-poplar forests of lowland rivers whereas *Pau* was found in one montane grey alder gallery.

DISCUSSION

The rate of *Paa* in the Czech population of the subspecies complex corresponded with overall European data – *Paa* took up ca. 89% of the European population of the *P. alni* complex (Brasier *et al.* 2004). In contrast to *Paa*, *Pau* was rather rare in the Czech Republic and *Pam* was not recorded at all.

The differences in abundance and distribution of *Paa* and *Pau* and absence of *Pam* in the Czech Republic can be elucidated at least partially in the light of knowledge of their distribution in Europe, pathogenicity and ecology.

Paa has been found in Ireland, UK, Sweden, Germany, Netherlands, Belgium, France, Spain, Poland, Czech Republic, Austria, and Hungary up to the present time (Szabó *et al.* 2000; Nagy *et al.* 2003; Brasier *et al.* 2004; De Merlier *et al.* 2005; Ioos *et al.* 2005; Cerny *et al.* 2008; Pintos Varela *et al.* 2010; Solla *et al.* 2010; Trzewik & Orlikowska 2010). The distribution area of *Paa* overlaps the area of *Pam* in the West (UK and northwestern part of continental Europe) and the area of *Pau* in almost the whole continental Europe. According to the fact that *Paa* is a hybrid of *Pam* and *Pau* (Ioos *et al.* 2005, 2006; Bakonyi *et al.* 2007; Érsek & Nagy 2008), *Paa* may have its origin somewhere in northwestern continental Europe – in the area of coincidence of both, *Pau* and *Pam*. Although *Pau* has recently been much less frequent than *Paa* (Brasier *et al*. 2004), it has the most extensive area – it is known from Sweden, Lithuania, Germany, Netherlands, Belgium, France, Spain, Czech Republic, Austria, Italy, Hungary, and Slovenia (Szabó *et al.* 2000; Santini *et al.* 2001; Nagy *et al.* 2003; Brasier *et al.* 2004; De Merlier *et al*. 2005; Ioos *et al.* 2005; Munda *et al.* 2006; Pintos Varela *et al.* 2012). The subspecies was also found in North America (Adams *et al.* 2010). Therefore, *Pau* can be an indigenous subspecies there, currently suppressed by the more aggressive *Paa* (Ioos *et al.* 2006). The distribution of *Pam* is the most restricted and local – it was found in the UK, Netherlands, Belgium, France (Britanny) and Germany (Brasier *et al.* 2004; De Merlier *et al*. 2005; Ioos *et al.* 2005).

The absence of *Pam* in the Czech Republic may be explained by the position of the Czech river system in the continental watershed. The particular isolation from river systems in adjacent areas – especially from Germany in the west – as possible sources of *Pam* inoculum may play an important role. Another reason for *Pam* absence in the Czech Republic can be the limited import of alder planting stock from the countries of Western Europe. Moreover, the potential occurrence of *Pam* in the area can be overlooked and suppressed by invasion of *Paa*. Nevertheless, the exact distribution of *Pam* and *Pau* in Europe seems to be known insufficiently.

Likely, the success of *Paa* in riparian stands of broader watercourses at lower altitudes in comparison with *Pau* is connected with its higher aggressiveness (Brasier & Kirk 2001; De Merlier *et al.* 2005), and also with the more effective sporangial production of *Paa* in warmer water

(Chandelier *et al.* 2006) and with higher survival success of its zoospores in water with higher electrical conductivity (Kong *et al.* 2012). Higher values of both factors are characteristic of slow and polluted lower reaches of rivers (e.g. Vega *et al.* 1998). Greater damage to alders in slow lower reaches with higher summer temperature of water (Thoirain *et al.* 2007) and water pollution (Gibbs *et al.* 1999) was confirmed. However, the epidemiology of the disease is poorly understood and many other factors can play a potential role in the pathogen spread and disease development.

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