Mating Type Distribution of *Phytophthora infestans* (Mont.) de Bary in the Czech Republic in 2007 and 2008

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Abstract

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A total of 195 isolates of *Phytophthora infestans* were collected from late blight-diseased potatoes grown in several localities in the Czech Republic during the years 2007–2008. The isolates were analysed for mating type using the pairing test, CAPS marker assays and PCR assays. Of the 195 tested isolates, 28% were of the A1 mating type and 75% corresponded to the A2 mating type. Furthermore, oospores of *P. infestans* were microscopically detected in leaf samples from one locality.

Keywords: late blight; pathogen; pairing test; CAPS; PCR; oospore detection

The oomycete *Phytophthora infestans* (Mont.) de Bary (1876), the causal agent of the late blight, is the pathogen currently devastating potato and tomato plants and thus causing economically important yield losses in fields and in stores worldwide. In the Czech Republic under favourable cool and moist conditions and without any suitable control steps against the pathogen, *P. infestans* can cause almost 100% yield reduction; therefore, potato growing cannot be managed without frequent fungicide applications for the effective control of this disease. The pathogen is a heterothallic organism with two mating types designated as A1 and A2. When the hyphae of compatible strains of opposite mating type coexist and interact, sexual oospores are formed (Gallegly & Galindo 1958).

The first report of a "new" potato disease came from the North-Eastern USA in 1843 (Stevens 1933). The late blight is especially known due to the starvation and migration of millions of Irish people; it caused the absolute destruction of potato production in Ireland in 1845–1846 (Bourke 1964). According to the theory based on the genetic analysis of *P. infestans* populations from all over the world, the pathogen was introduced from the probable centre of coevolution of the *P. infestans*–*Solanum* spp. pathosystem in Central Mexico by the first migration via international trade in seed potatoes (Fry *et al.* 1993; Fry & Goodwin 1995). The second migration probably occurred in the 1970s, also out of Mexico (Fry *et al.* 1993), where the common occurrence of A1 and A2 mating

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types and oospores in infected plant tissue were first discovered (Gallegly & Galindo 1958). Through importation of infected potatoes shipped from Mexico in 1976 (Niederhauser 1991), the old single clonal lineage known as US-1 genotype (A1 mating type) of *P. infestans* was replaced by new populations with higher genotypic variability and the co-occurrence of the A1 and the A2 mating types (Drenth *et al*. 1994a). Outside Central Mexico, the A2 mating type was first detected in Switzerland (Hohl & Iselin 1984), then in England and in Wales (Gunn 1990) and subsequently e.g. in the Netherlands (Frinking *et al*. 1987), Poland (Sujkowski *et al*. 1994), Finland (Kankila *et al*. 1995), Norway (Hermansen & Amundsen 1995), Hungary (Bakonyi & Érsek 1997), France (Lebreton & Andrivon 1998), and also in the continents of America and Asia (Fry *et al*. 1993; Fry & Goodwin 1995). In the Czech Republic the A2 mating type was first found in 2003 (Mazáková *et al*. 2006).

The spread of the A2 mating type enables sexual reproduction of *P. infestans* and increases risks associated with oospore formation in infected plants (Drenth *et al*. 1995). In connection with the presence of introduced diverse populations that contain both mating types, new aggressive strains able to overcome the resistance of potato varieties and the effects of phenylamide fungicides began to appear. Evidence of oospores in dried herbarium materials existed before the 1980s, showing that the pathogen could also reproduce sexually at that time (Ristaino 1998). However, it is clear that changes due to the introduction of new genotypes have resulted in increased adaptability of the pathogen to chemical control, cultivar resistance and other factors.

The influence of sexual reproduction and changes in *P. infestans* populations have been studied intensively through the use of a number of phenotypic and genotypic markers. The phenotypic traits used for analysing the *P. infestans* populations are mating type, virulence, aggressiveness, and fungicide resistance. The commonly used genotypic markers are isozymes (allozymes), restriction fragment length polymorphism (RFLP) with probe RG57, haplotypes of mitochondrial DNA, amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) (Cooke & Lee 2004). In this study, Czech isolates of *P. infestans* were analysed for mating type with a view to surveying the country-wide distribution of the mating types of *P. infestans*. Furthermore, plant samples collected for the pathogen isolation were used for microscopic detection of oospores in plant tissue.

MATERIALS AND METHODS

Sample collection. Throughout epidemics during 2007–2008, samples of *P. infestans*-infected potato leaf and stem tissues were collected from several growers' fields, research station fields, variety testing institute fields and gardens in four regions and nine districts of the Czech Republic (Table 1). The collection of samples was performed systematically according to the ranges of different potato varieties. In gardens, samples were taken from individual plants.

Isolation of pure cultures of** P. infestans.** Leaves or stems with lesions were placed the abaxial side up in a humid chamber, i.e. a Petri dish with moistened filter paper or an inverted Petri dish with 1.5% water agar to support pathogen sporulation. Isolation was performed by trapping sporangia on a small piece of agar with an inoculating needle under a stereomicroscope and transferring the agar piece to a Petri dish with rye A medium (Caten & Jinks 1968). After incubation at 15–18°C in darkness for growth, small mycelial plugs were transferred to a number of Petri dishes with rye A medium to obtain a sufficient amount of mycelia for subsequent analysis.

Mating type determination

One conventional and two molecular biological techniques were used for determination of mating types with the view of obtaining the most accurate results.

Mating type pairing test. The mating type of the isolates was determined by pairing them with reference isolates (A1 02 BASF 05, A2 02 BASF 10) kindly provided by D. E. L. Cooke (SCRI, Dundee, Scotland). The reference isolate was placed on one side of a Petri dish with rye B agar (Caten & Jinks 1968), and a tested isolate was placed on the opposite side. After incubation at 15–18°C in darkness, the presence or absence of oospores at the hyphal interfaces was observed under a light microscope. If a dish yielded oospores, the tested isolate was designated as the opposite mating type of the reference isolate.

Table 1. Localities and numbers of A1 and A2 mating types in Czech *P. infestans* isolates in 2007–2008

Year/locality	Number of tested isolates		
	total	A1	A2
2007 (*n* = 76 isolates)			
Humpolec[g]	3	–	3
Lípa	1	–	1
Horažďovice	40	13	27
Valečov	24	5	19
Černý Dub[g]	8	–	8
2008 (*n* = 119 isolates)			
Semice	20	2	18
Olešná	1	–	1
Valečov	33	–	33
Horažďovice	21	21	–
Lípa	42	5	37
České Budějovice[a]	1	1	–
Holubov[g]	1	1	–

n – number of collected isolates; [a]isolates collected in gardens

DNA extraction. The mycelium of each isolate was harvested from a 6-cm Petri dish, frozen in liquid nitrogen and transferred to a grinding mortar. DNA was extracted by pulverising under liquid nitrogen and using the CTAB (1% CTAB, 50mM Tris-HCl (pH 8.0), 0.7mM NaCl, 10mM EDTA, and 20mM mercaptoethanol) extraction method. The homogenised mycelia were placed in a sterile 1.5-ml microcentrifuge tube and incubated at 65°C for 60 minutes. After incubation, an equivalent volume of chloroform:isoamyl alcohol (24:1) was added to each tube, and the tubes were vortexed for 10 min and centrifuged for 10 min at 7000 × g. The aqueous phase was removed to a new tube. DNA was precipitated in an equivalent volume of isopropanol overnight at –20°C or in liquid nitrogen for 5 min to 10 minutes. After centrifugation (10 min at 7000 × g), the supernatant was discarded, the pellet was washed with 70% ethanol and centrifuged again. The pellet was dried and resuspended in sterile double-distilled water.

Cleaved amplified polymorphic sequence (CAPS) assay. Each PCR was conducted in a 25-µl reaction mixture consisting of 2.5 µl 1× buffer for *Taq* polymerase (Fermentas), 1.5 µl $MgCl_2$ (1.5mM), 0.25 µl dNTP (0.4µM of each nucleotide), 0.4 µl primer mix (0.4µM of each primer), 0.5 µl *Taq* polymerase (2.5 U) (Fermentas), 18.85 µl ddH_2O and 1 µl template DNA. Reactions were carried out in a thermal cycler PTC 200 (MJ Research, Watertown, USA) under thermal cycling parameters as follows: initial denaturation for 5 min at 94°C, 29 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 53°C, and primer extension for 1 min at 72°C, and final extension for 4 min at 72°C. For PCR amplification of DNA fragments of both mating types, the primer set W16-1 (5'-AACACGCACAAGGCATATAAATGTA-3') and W16-2 (5'-GCGTAATGTAGCGTAACAGCTCTC-3') was used (Judelson *et al*. 1995). PCR products were cleaved with the restriction enzyme *Hae*III (*Bsu*RI) (Fermentas) with recognition site GGCC to distinguish the two mating types (Judelson *et al*. 1995). Restriction products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide (1 mg/ml) and visualised on a UV transilluminator. Sizes of amplified DNA fragments and cleaved products were specified by cloning of the amplified fragments into the pTZ57R/T vector (Fermentas) and transformation of competent *E. coli* DH5α cells. Plasmid DNA was extracted using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, USA) and sequenced (Genomac International, s.r.o.

Prague, Czech Republic). Bioinformatics analyses of obtained DNA sequence data were performed by BLAST, ClustalW, NEBcutter V2.0.

Polymerase chain reaction (PCR) assay. The primer pair S1-a (5'-AGGATTTCAACAA-3') and S1-b (5'-TGCTTCCTAAGG-3') was used for detection of the specific DNA fragment of the A1 mating type (Judelson 1996). The PCR was conducted as described above, except that the following cycling parameters were used: initial denaturation for 5 min at 94°C, 35 cycles of denaturation for 1 min at 94°C, optimised primer annealing for 1 min at 46.8°C, and primer extension for 1 min at 72°C, and then final extension for 4 min at 72°C. Electrophoresis of amplified products was conducted on 1% agarose gels containing ethidium bromide.

Detection of oospores in leaf tissues. Some of the leaves with two or more lesions used for the pathogen isolation were also used in the oospore survey in both years. Leaf tissues were cleared by boiling in 96% ethanol for 10 min, mounted in glycerol (Cohen *et al*. 1997) or slightly homogenised, and examined microscopically for the presence of oospores.

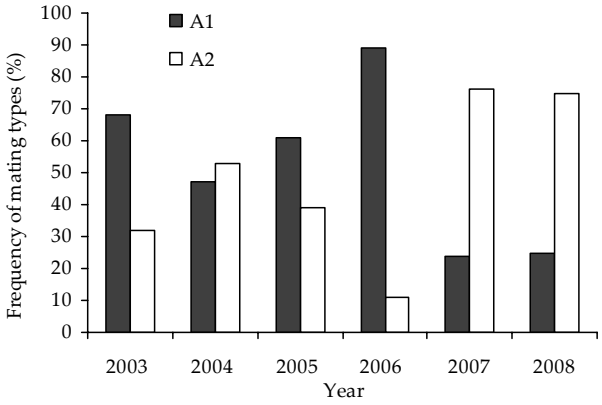
RESULTS

Collection and isolation of *P. infestans*

During the two-year sampling period, 481 samples of blighted plant tissue were collected from potato crops. Out of these, 195 isolates were maintained in pure cultures, of which 76 and 119 isolates were from 2007 and 2008, respectively. The majority of isolates were from potato leaves; three of them were from stems (2007).

Mating type pairing test

Isolates belonging to both mating types were found among the tested Czech *P. infestans* isolates. Of the 195 isolates tested by the pairing test, 25% were the A1 and 75% were the A2 mating type. No self-fertile isolate was found. From the 2007 collection, 18 and 58 isolates were designated as mating type A1 and mating type A2, respectively. From 2008, 30 isolates represented the A1 mating type and 89 isolates corresponded to the A2 mating type (Table 1). The majority of isolates from both years were the A2 mating type (76% from 2007 and 75% from 2008) (Figure 1). When more than one isolate were collected from a field, the proportion of the A1 mating type was in the range 0–33% in 2007 and 0–100% in 2008. The frequency of A2 varied from 67% to 100% in the first year of the survey and in the second year, the frequency of A2 varied from 0% to 100%.



Results of the frequency of mating types in 2003–2006 samples (Mazáková *et al*. 2006; Hausvater *et al*. 2007)

Figure 1. Frequencies of A1 and A2 mating types in Czech *P. infestans* samples obtained in 2003–2008

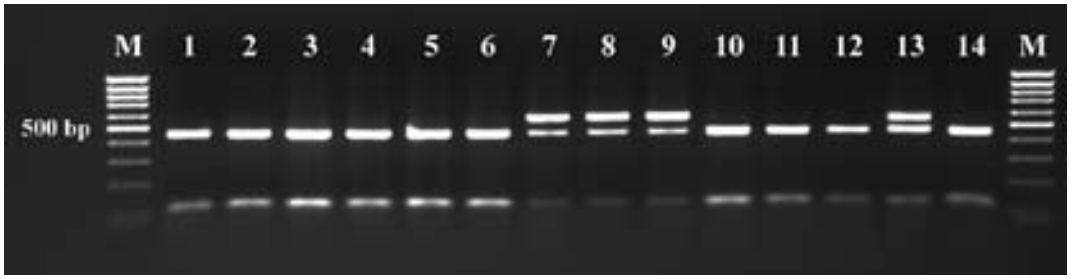
CAPS assay

Each of the 195 isolates was tested using the primer set W16-1 and W16-2 followed by cleavage of the PCR product with the restriction enzyme *Hae*III (*Bsu*RI). This primer pair is specific for both mating types and produces a unique PCR amplicon of approximately 557 bp. Three fragments (557 bp, 457 bp, 100 bp) or one uncleaved product (557 bp) (data not shown) are obtained after digestion of amplified DNA of A1 mating type isolates by the restriction enzyme, while

Table 2. Localities and numbers of leaflets with *P. infestans* oospores

Locality	Year/leaflets	
	2007/ l^o (l^n)	2008/ l^o (l^n)
Lípa	0 (0)	5 (20)
Valečov	0 (20)	0 (30)
Horažďovice	0 (20)	0 (20)

l^o – number of leaflets with oospores; l^n – number of analysed leaflets



The A1 mating type is shown in lanes 7, 8, 9, 13; lanes 7–9: cleaved PCR products of isolates collected in Horažďovice (2008); lane 13: reference isolate of the A1 mating type. The A2 mating type is shown in lanes 1–6, 10–12, 14; lanes 1–3 contain cleaved PCR products of isolates collected in Semice (2008); lanes 4–6: of isolates collected in Valečov (2008); lanes 10–12, of isolates collected in Lípa (2008); lane 14 contains products of the reference isolate of the A2 mating type; lane M: MassRuler^{TM} DNA Ladder Low Range (Fermentas)

Figure 2. Restriction enzyme digestion using *Bsu*RI (*Hae*III) of the PCR products amplified from *P. infestans* DNA with primer pairs W16-1 and W16-2

two fragments of 457 bp and 100 bp are obtained from cleaved amplified DNA from A2 mating type isolates (Figure 2).

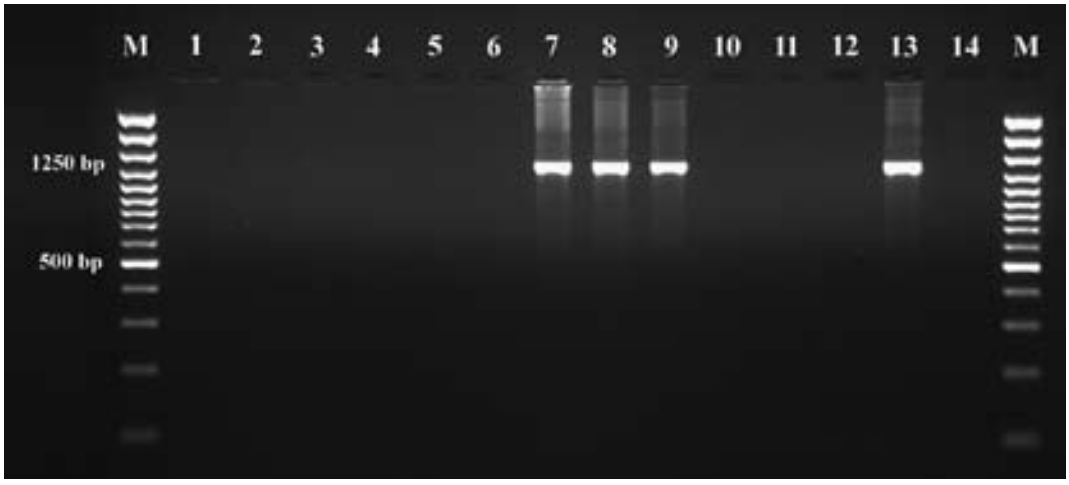
PCR assay

The primer set S1-a and S1-b was also used for testing DNA of all isolates. These primers are specific to the A1 mating type and, after PCR reaction and electrophoresis, allow the observation of a targeted amplified fragment of approximately 1250 bp (Figure 3).

On the whole, results of the molecular assay firmly corresponded to those of the pairing tests.

Detection of oospores in leaf tissues

Leaflets with two or more lesions that were used for the pathogen isolation were also utilised for the oospore detection. The leaf tissue for this survey was selected with respect to the field size and number of collected samples (Valečov, Horaždovice, Lípa), thus increasing the possibility of observing oospores. No oospores of *P. infestans*



The A1 mating type is shown in lanes 7, 8, 9, 13; lanes 7–9, isolates collected in Horažďovice (2008); lane 13: reference isolate of the A1 mating type. The A2 mating type is shown in lanes 1–6, 10–12, 14: lanes 1–3: isolates collected in Semice (2008); lanes 4–6: isolates collected in Valečov (2008); lanes 10–12: isolates collected in Lípa (2008); lane 14: reference isolate of the A2 mating type; lane M: O'GENE RULER 100 bp DNA Ladder plus (Fermentas)

Figure 3. Agarose gel electrophoresis of the products of *P. infestans* DNA amplified using primers S1-a and S1-b

were detected in samples collected at chosen sites in the Czech Republic in the first year of the survey. The first observed occurrence of oospores was in 2008 in five leaves that originated from a field located in Lípa (Table 2).

DISCUSSION

Phytophthora infestans belongs to a group of organisms that are able to reproduce both by asexual and sexual means. Although asexual reproduction largely accounts for the pathogenic development and distribution of this species, during the last thirty years the possibility of its sexual reproduction has been widely discussed. For heterothallic pathogens like *P. infestans*, the common presence of both mating types is necessary for the formation of sexual oospores, which were first detected in this species in plant tissue originating from Mexico (Gallegly & Galindo 1958). As Hohl & Iselin (1984) reported that isolates of the A2 mating type were present outside Mexico, i.e. in Switzerland since 1981, reports of the occurrence of the A2 mating type as well as of new populations of *P. infestans* consisting of both mating types have come from many countries all over the world.

It is not known exactly how long the A2 mating type has been present in the Czech Republic, though regular monitoring of mating types began in 1994 (Hausvater & Rasocha 1999). Isolates of the A2 mating type were first detected in this country in 2003 (Mazáková *et al*. 2006) and have been found every year subsequently (Hausvater *et al*. 2007), likewise in the present study. Regarding other Central European countries, the occurrence of the A2 mating type was reported in Hungary (Bakonyi & Érsek 1997), Poland (Spielman *et al*. 1991; Sujkowski *et al*. 1994), Austria (Rauscher 2003), and Slovakia (Forišeková & Heldák 2004). During the period 2003–2008, the frequency of the A2 mating type oscillated between 11% and 76%, and it was predominant in 2004 (Mazáková *et al*. 2006), 2007 and 2008. Based on Eucablight data, the A1 mating type was a predominant mating type in Europe in 2005 and 2006, while in 2007 and 2008 the frequency of the A2 mating type was higher than that of A1 mating type. The A2 mating type was predominant in Poland during 2005–2007 (Anonymous 2009) in contrast to previous years when the frequency of the A1 mating type was higher (Zarzycka *et al*. 2002; Śliwka *et al*. 2006). During 2006, a higher proportion of the A2 mating type was noted in Wales and England, during 2007 in Germany, England, Scotland and Wales and during 2008 in England, Scotland, and Wales (Anonymous 2009). In other countries, the frequency of the A2 mating type varied, being 36–49% in Finland, Norway, Denmark and Sweden (Lehtinen *et al*. 2008). The A2 mating type was quite widely detected in the 1990's. However, the proportion was much lower than that of the A1 mating type, being 1–3% in England and Wales (Day & Shattock, 1997) and 2–5% in France (Lebreton *et al*. 1998). Increased frequency of the A2 mating type was observed in the 1990's in Ireland (O'Sullivan *et al*. 1995), Poland (Sujkowski *et al*. 1994) and in the Netherlands (Drenth *et al*. 1994b). Goodwin *et al*. (1995) found that 61% of isolates were of the A2 mating type in the USA during 1992. However, as Hermansen *et al*. (2000) pointed out, it may be difficult to compare frequencies in different countries because the provided data are often based on different numbers of isolates and monitoring sites.

Based on Mazáková *et al*. (2006) and Hausvater *et al*. (2007) and on the results presented here, both mating types were clearly present in one field from which more than one isolate was tested, indicating a strong potential for sexual reproduction. Lehtinen *et al*. (2008) mentioned 29–56% of the fields in Nordic countries with the presence of both mating types. The presence of both mating types in one field was also reported in Hungary (Bakonyi *et al*. 2002; Nagy *et al*. 2006), Poland (Śliwka *et al*. 2006) and other European countries (Cooke *et al*. 2003). We found both mating types in potato growing areas as well as in private gardens. It is known that some of the A2 mating type isolates from the Lípa locality originated from infected seed imported from the Netherlands and Germany. However, it is not possible to deduce whether there is a definitive correlation between the presence of the A2 mating type and imports trade, because the origin of potato varieties is not known in any case. The occurrence of both mating types was also detected from one variety, or even a single plant, especially in 2005 (data not published), but also in 2007 when two isolates of the opposite mating type from one plant were also detected. However, it is difficult to deduce any correlation between mating type and potato variety in the

lack of knowledge regarding the source of primary and secondary infection.

For comparative purposes we used molecular techniques to determine the mating type in a faster and less time-consuming way. Little information is available about the region of the *P. infestans* genome related to the mating type (Cooke & Lee 2004), thus we chose the CAPS marker W16 (Judelson *et al*. 1995) and the primer set S1-a, b which is specific to the A1 mating type (Judelson 1996). Using these two markers (W16 and S1), results comparable with those of the pairing test were obtained. However, the sizes of the fragments amplified using the W16 primer pair and the sizes of the cleaved products were different from those reported by Judelson *et al*. (1995). Therefore, we performed a sequence analysis of the amplified products to determine the true size of our amplicons (557 bp). Despite different sizes of the amplified fragments obtained, the CAPS marker used in our analysis appears to be suitable for distinguishing the two mating types of *P. infestans*. In the region of the genome that contains this marker, the A2 mating type is present in the homozygous state, whereas the A1 mating type is present in the heterozygous state; therefore only the DNA fragment of one allele is cleaved. In some cases, the A1 mating type was homozygous and no allele was cleaved (data not shown).

In 2008, oospores were found in five leaves from the Lípa locality in the Vysočina region in the Czech Republic, where both mating types were detected. It appeared to be only a question of time when oospores would be detected throughout the Czech Republic, because they have already been found in fields in other countries, including Sweden (Andersson *et al*. 1998), the Netherlands (Turkensteen *et al*. 2000), Norway (Hermansen *et al*. 2000) and Finland (Lehtinen & Hannukkala 2004). The annual occurrence of the A2 mating type, the presence of both mating types in the same field and the detection of oospores all confirm the probability of sexual reproduction of *P. infestans* in the Czech Republic. However, further investigations are necessary to elucidate the significance of the oospores in the epidemiology of potato blight worldwide including the Czech Republic.

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