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POPULATION STRUCTURE OF *PHYTOPHTHORA CACTORUM* IN EUROPE

DOCTORAL THESIS

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1. Abreviations

AFLP – Amplified Fragment Length Polymorphism

AMOVA – Analysis of Molecular Variance

ANOVA – Analysis of Variance

Cox I – cytochrom oxidase gene locus

D_{Nei} – Nei´s gene diversity index

DW – Rarity index

GenBank – open access sequence database produced and maintained by National Center for biotechnology Information (NCBI)

ITS – Internal Transcribed Spacer, the region nuclear DNA coding ribosomal RNA

L:W ratio – Length to width ratio of sporangial dimensions

MCMC – Markov Chain Monte Carlo, algorhitm for sampling from distribution of probability

ML hybrid index – Maximum likelihood hybrid index

Paa – *P. alni* subsp. *alni*

Pam – *P. alni* subsp. *multiformis*

Pau – *P. alni* subsp. *uniformis*

PCA – Principal Component Analysis

Pheca I - phenolic acid decarboxylase gene locus

RAMS – Random Amplified MicroSatellites

RAPD – Random Amplified Polymorphic DNA

SAR – Monophyletic group of eucaryotic organisms including Stramenopiles, Alveolata and Rhizaria

Tukey´s HSD – Tukey´s honest significant difference test

Φ_{ST} – Fixation index; probability, that any of two randomly sampled alleles in a populations are identical by descent

2. Introduction

Genus *Phytophthora* (Chromista, Peronosporomycota, Peronosporales) comprises primary plant pathogens causing damages of a number of species. The species *Phytophthora cactorum* was already described at the beginning of 19th century (under the name of *Peronospora cactorum*, Lebert & Cohn 1870) as plant pathogen causing root rot on *Cactae*. This species presents serious problems not only on forestr trees, but also on ornamental and fruit trees, and in agriculture, especially in strawberry farms. Among important Central European forest trees, especially *Fagus sylvatica*, *Acer* spp., *Tilia cordata*, *Fraxinus excelsior*, and other trees are attacked. The pathogen is able to attack healthy plants regardless the age, and causes damages mainly on fine roots of broad-leaved trees and significantly limits water and nutrients supply of above-ground parts of the plant. Infection often leads to quick death of the host, or to secondary infections. Infection of seedlings is known as damping-off. Nowadays, *P. cactorum* is worldwide distributed throughout the temperate climate zone, but it was probably spread out from a small area, which remains unknown. *P. cactorum* was firstly recorded in the current Czech Republic in 1960s as pathogen of horticultural hosts *Dahlia* spp. (Cejp 1961b). Hybridization events among *P. cactorum* and other species have been recently discovered using molecular biology techniques. Fertile hybrids of *P. cactorum* with *P. hedraiandra*, and *P. nicotianae* have been detected. Hybridization events play a major role in the evolution of the genus *Phytophthora* by providing more genes for mutations and selection to work with and by combining different parental sets of crucial effectors in pathogenicity. This work aims to describe the population structure of *P. cactorum* including hybrids, based on morphological and molecular data, and to evaluate changes in resistancy to chemicals associated with hybridization events.

2.1 Genus *Phytophthora*

The genus *Phytophthora* comprises a group of obligatory parasitic oomycetes, many of which are primary plant pathogens causing important economic loss of numerous herbaceous and woody plant species (Jung et al. 2016). Most of these pathogens have very limited ability of saprotrophic growth and their vegetative stage survive on live tissues of host plants (Erwin & Ribeiro 1996). Today, the genus is placed within the supergroups Chromalveolata or SAR (Burki et al. 2007), where heterotrophic species resembling fungi are considered more primitive. The genus is placed to subkingdom

Chromobiota, phylum Oomycota, order Peronosporales (Kalina & Váňa 2010). The origin of this phylum is supposed in photosynthetic algae, whose photosynthesis ability was secondarily lost. This theory is confirmed by the existence of motile flagellated zoospores in water (Duncan & Cooke 2002). Organisms of the phylum Oomycota are characterised by a filamentous thallus forming aseptate hyphae. True septa are less common or emerge in older hyphae and separate reproductive organs. Cell membranes constitute cellulose matrix filled with amorphous mixture of β-1,3, β-1,4- and β-1,6-glucans, the energy-storage component is mycolaminaran (Kalina & Váňa 2010). Order Peronosporales, is classified in subclass Peronosporomycetidae within class Peronosporomycetes. The order comprises soil and water saprotrophic and parasitic organisms with several genera (e.g. *Peronospora* and *Phytophthora*) having important economic impact (Kalina & Váňa 2010). *Pythium,* a genus similar and related to *Phytophthora,* comprises both saprotrophic and parasitic species, while the genus *Phytophthora* is mostly composed of obligatory parasites with very limited saprotrophic capabilities. Both genera are diploidic in vegetative stage of life-cycle, meiosis occuring only during sexual process. These two genera differ in the formation of zoospores in sporangium. Zoospores of *Phytophthora* mature inside zoosporangium, while zoospores of *Pythium* mature in the thin-walled vesicle that is produced from the sporangium. *Phytophthora* create terminal sporangia of roughly ovoid or oval shape, while *Pythium* form sporangia intercalary. These two genera also differ in the morphology of their gametangia: *Phytophthora* create amphigynous (surrounding oogonial stalk) and paragynous (attached to oogonium close to stalk) antheridia. *Pythium* create only paragynous antheridia attached to oogonium at different places (Erwin & Ribeiro, 1996). Since many obligatory parasitic *Phytophthora* species form deciduous sporangia propagated by air, and their sporangia germinate directly from, a general similarity to genera *Bremia* and *Peronospora* has been pointed out (Brasier & Hansen 1992; Thines 2014).

In host plant tissues, pathogenic *Phytophthora* spp. produce hyaline intracellular or intercellular mycelia, often with haustoria. Hyphae are ca. 3–8 μm wide, smooth or distinctively twisted or intercalary or terminaly thickened. Septa are formed only when separates gametangia, sporangia, and chlamydospores, rarely also empty parts of older hyphae. Chlamydospores are oval or globose, colourless, ageing could get faint brown tint. Sporangia develop terminally at sporangiophores undifferentiated from other

hyphae, colourless, of species-specific shape: globose to ovoid, with papilla in some species. Mature sporangium in water environment loosens motile zoospores that always develop inside sporangium, or entire sporangium germinates directly by hypha. Zoospores are roughly ovoid cells with one diploid nucleus and two flagella without cell membrane (Erwin & Ribeiro, 1966; Waterhouse, 1963b).

In *Phytophthora*, two basic strategies of sexual reproduction occur: homothallism and heterothallism, but some species include both homothallic and heterothallic strains. Homothallic species are able to form gametangia – oogonia and antheridia – within single isolate, while in heterothallic species, two mating types could be distinguished – A^1 a A^2 – and a partner from the second mating type is needed for the gametangial induction (Ko, 1978). During mating, mating hormone 1α (MH-1α) stimulates gametangia production (Ko, 2007; Harutyunyan et al. 2008). Oogonia are usually terminal and separated by thick septum from maternal hypha. Oogonia are colourless, but could darken with age, and ornamentation could appear on the surface. Antheridium can be amphigynous – surrounding oogonium stalk, or paragynous - attached to oogonium close to the stalk. Oogonium contains only one oospore and is completely filled (plerotic oospore), or has space between oospore wall and oogonium wall – aplerotic oospore (Waterhouse, 1963; Erwin & Ribeiro, 1996).

Genus *Phytophthora* used to be divided into six groups according to the morphological features (Waterhouse, 1963; Stamps et al., 1990). This classification was based on 25 morphological macroscopic features (colony shape and other mycelial characteristics on agar media), microscopic characters and physiological features (cardinal temperatures). The most important morphological characters for identification are shape and size of sporangia, and presence of papilla, position of antheridium (amphigynous/paragynous), and type of sexual reproduction (homothallic/heterothallic). Morphological characteristics used to be (Rosenbaum, 1917) and still are in use for identification of *Phytophthora* species, even though various works reveiled a considerable variability. Stamps (1953) reports high variability of morphological features (colony pattern and morphology and size of reproductive organs) even within one monosporic isolate of *P. cactorum*. Phylogenetic study (Cooke et al., 2000) neglected division of the genus *Phytophthora* into six groups based on morphology, because the groups do not reflect phylogenetic relationships among species. Therefore, the authors divided *Phytophthora* into 8 clades, based on phylogenic analysis of DNA sequences of the ITS region of

ribosomal RNA gene, and supported by the phylogenic analysis of the mitochondrial cytochrome oxidase gene (Martin & Tooley, 2003). More recently, the genus *Phytophthora* has been divided into ten clades based on more gene regions (28S subunit of ribosomal RNA gene, 60S ribosomal protein L10, beta tubulin, elongation factor 1α, heat shock protein, TigA fusion protein) (Blair et al., 2008). These clades only overlap partially with six groups defined morphologically (Waterhouse, 1963). Therefore, morphological similarities of phylogenetically distant species do not reflect phylogenetic relationships, but can be caused by convergent evolution (Förster et al., 2000), and morphological and genetic variability are not always in correlation (Dudzinsky et al., 1993). Division of the genus into ten clades (1-10), based on molecular data is today widely accepted (Cooke et al., 2000; Kroon et al., 2004; Kroon et al., 2012; Martin et al. 2012), although it seems probable, that the number of clades is not yet final (Yang et al, 2014). Nevertheless, morphological characteristics are still important in routine identification of *Phytophthora* species and opinions on classification and taxonomic status of some species are still in progress.

2.2 Ecological importance of genus *Phytophthora*

The genus *Phytophthora* comprises several of the most important plant pathogens causing problems in agriculture, in intensive forest management, and also in natural ecosystems. Research of the genus *Phytophthora* started in the first half of 19th century, when during Ireland famine in 1845-46 potato blight, caused by *Phytophthora infestans,* destroyed the whole potato harvest and the population of Ireland decreased two millions. Potato blight gradually spread over the European continent, causing the mortality of the population dependent on potato production. The causal agent of the disease was identified in 1845 and named *Botrytis infestans* Mont. The change into *Phytophthora −P. infestans* (Mont.) de Bary was published in 1876 (de Bary, 1876). Also other currently recognised *Phytophthora* spp. were described till the end of 19th century: *P. cactorum* Leb. and Cohn, *P. nicotianae* Breda le Haan, *P. phaseoli* Thaxt., *P. colocasiae* Racib. About 50 species were described in 1900-1950 (Waterhouse, 1963b). Thanks to the development of molecular-genetic methods, the understanding of *Phytopthora* diversity has increased considerable, and today, more than 150 species are recognised in the genus (Jung et al., 2016).

Probably the best-known *Phytophthora* species is *P. infestans*, parasite of potatoes and other *Solanum* spp. *P. infestans* is a heterothallic species with origin in a small area in Mexico, which over its dispersal through North America and Europe underwent two bottleneck events, reducing its genetic diversity to just one strain with clonal reproduction (Goodwin, 1997). Subsequent migration events from 1970s introduced more strains of the 2nd mating type from America to Europe. Sexual recombination resulted in a significant increase of the virulence variability and infection pressure of this pathogen (Goodwin, 1997). Today, large sums of money are spent on fungicides fighting against this pathogen, without which potato production would be impossible. In USA, 1.2 billions USD are invested each year, which represents 10 % of the total costs for chemical protection in agriculture (Fernandez-Cornejo et al., 2014). The history of *P. infestans* invasion illustrates the role of sexual recombination for spreading as well as agressivity of *Phytophthora* pathogens. Simultaneously also documents importance of quarantine procedures, which are able to restrict the invasion of uninfected areas by another mating type, and subsequent increase of genetic variability of pathogen.

Another major member of the genus *Phytophthora* is *Phytophthora ramorum*. This species is an important pathogen with very broad host spectrum (Grünwald et al., 2008), causing extensive death of entire forest ecosystems particularly in the west of USA, and also in Great Britain and other Western European countries. This species was first isolated in 1993 as Rhododendron pathogen and formally described in 2001 (Werres et al., 2001). *P. ramorum,* also a heterothallic species, spreading probably only through few clonally reproducing strains NA1, NA2, EU1, recently another EU2 lineage was described (de la Mata Saez et al. 2015). In the USA most of the isolates are comprised in a single genotype, while European isolates are clustered into unique, closely related genetic lineages. Both populations were found on separate introductions from a third, unknown location (Ivors et al. 2004). Although there are evidences that indicate that genetical lineages of *P. ramorum* splited up before the introduction to Europe and North America (Goss et al. 2009a) there are also other evidences of recombination between lineages in history. The population of *P. ramorum* has undergone a complex development (Goss et al., 2009b).

Between the most important species of the genus *Phytophthora* belongs *Phytophthora cinnamomi,* which destroys the whole ecosystem in Australia or New Zealand and threatens several plant species with extinction. In Southern Europe, especially in Spain *P. cinnamomi* causes dieback on *Quercus suber* (Sun & Walsh, 1998; Corcobado et al.,

2013; Jung et al., 2016). *Phytophthora ×alni*, causes extensive alder decline mainly in Europe, and North America (Ioos et al., 2006). Other species, such as *P. plurivora*, *P. ×cambivora*, *P. cactorum*, and *P. cinnamomi,* are also important pathogens in European forestry (Jung et al., 2016).

2.3 Interspecies hybridization, forming of new species of *Phytophthora* spp.

Interspecies hybridization under natural conditions is prevented by genetic, geographical, and ecological barriers. The most developed reproductive barriers are expected in species having common or overlapping area of distribution and ecological niches. On the other hand, geographically separated, i.e. allopatric populations that evolved in isolation or in different geographic regions, could lack these reproductive barriers and hybridize in case of physical contact (Olson & Stenlid, 2002). Different *Phytophthora* spp. could encounter as a result of anthropogenic activities, mainly through worldwide trade of plant material. Places where various *Phytophthora* species could co-occur are primarily nurseries (Brasier, 2000; Brasier, 2008).

Three types of hybridization are described in *Phytophthora:* first two – sexual process and somatic hybridization by zoospore fusion are experimentally documented (Kroon et al., 2012), while the third one – somatic fusion is hypothesised as merging of mycelia of two distinct individuals (English et al., 1999). In most of the published studies, nuclear genes are inherited biparentally whereas mitochondrial genes are inherited maternally (Förster & Coffey, 1990; Whittaker & Assinder, 1994), which corresponds with sexual process (Burgess, 2015).

One of the first studies about hybridization was carried out by Boccas et al. (1981), in which heterothallic species *P. capsici*, and *P. palmivora* were paired in the laboratory. Only one hybrid isolate was confirmed using the protein pattern (Boccas, 1981). Another hybrid progeny was mentioned by Goodwin & Fry (1994) on *P. infestans*, and *P. mirabilis*. They documented maternal heritability of mitochondrial DNA on these new hybrids. Change of host spectrum had been discovered in these strains – hybrid strains did not efficiently infest typical hosts of parental species.

Phytophthora ×alni is currently the best studied hybrid species, where heterothallic *P. ×cambivora*, and homothallic *P. fragariae* were originally regardered as parental species (Brasier et al., 1999). This hybrid species was considered a group of heteroploid

crossbreeds comprising three subspecies – *P. alni* subsp. *alni* (*Paa*), *P. alni* subsp. *uniformis* (*Pau*), and *P. alni* subsp. *multiformis* (*Pam*) (Brasier et al., 1999). However, there is an intense discussion about the origin of these three subspecies. Brasier et al. (1999) assumed that *Pau*, and *Pam* evolved from *Paa*, which emerged from somatic fusion. On the other hand, Ioos et al. (2006) assumed emergence of *Paa* from cross-breeding of the other two subspecies derived by autopolyploidization or backcrossing with *P. ×cambivora*. Husson et al. (2015) define *P. ×alni* as hybrid of *Pau* and *Pam*, describing both as separate species. *P. uniformis* is species native in North America, while *P. ×multiformis* originates in Europe by hybridization (Aguayo et al., 2012). According to this, *P. ×alni* is triploid cross of diploid *P. uniformis* and tetraploid *P. ×multiformis*. Husson et al. (2015) consider origin of *P. ×multiformis* in interspecies hybridization of two unknown species, and suppose that *P. ×alni* has emerged relatively recently and whole species has been undergoing rapid evolution associated with other genetic changes. Moreover, the increase of virulence in *P. ×alni* compared to parental species occurred. Today, *P. ×alni* comprises 90 % of records in all three species originally classified as subspecies of *P. alni*.

Man in 't Veld et al. (1998) described hybridization between homothallic *P. cactorum* and heterothallic *P. nicotianae* in hydroponics cultures of ornamental plants using isozyme analysis and RAPD. According to their results, the hybrids are morphologically almost indistinguishable from the parental species (Man in 't Veld et al., 1998). In hybrid strains, low percentage of fertile oospores and high number of aborted oogonia is mentioned (Bonants et al., 2000). Hybridization of *P. cactorum*, and *P. nicotianae* is corroborated also by other works, based on ITS sequences and AFLP DNA-fingerprinting (Bonants et al., 2000; Hurtado-Gonzales et al., 2009). The host spectrum of the hybrid differs from the parental species' spectrum (Bonants et al., 2000). Hybrid isolates have 13 heterozygous positions in the DNA sequence of phenolic acid decarboxylase (Pheca I) gene, containing nucleotides of both maternal species, and contrarily, the mitochondrial cytochrome oxidase (cox I) gene is found only in genotype of *P. nicotianae* (Hurtado-Gonzales et al., 2009). Based on AFLP study, repeated hybridization of these two species has been confirmed. Hybrid of *P. cactorum* and *P. nicotianae* was later described as *Phytophthora ×pelgrandis* (Nirenberg et al., 2009).

In further publications the occurrence of natural hybrid of homothallic species *P. cactorum* and *P. hedraiandra* was mentioned (Man in 't Veld et al., 2007; Man in 't Veld

et al., 2012). This hybrid was isolated in the Netherlands from several herbaceous and woody plants and its morphology is more or less similar to that of *P. cactorum*. Hybrid species was formally described as *Phytophthora ×serendipita* (Man in 't Veld et al., 2012). The sequence analysis of *cox I* gene of *P. ×serendipita* revealed that hybrid individuals include genotypes of both maternal species. Therefore, the hybridization occurs repeatedly and that both homothallic maternal species could supply antheridium and oogonium during the interspecific mating. Typical *P. ×serendipita* includes *cox I* genotype of *P. cactorum*, but ITS region of *P. hedraiandra* (Man in 't Veld et al., 2012). The relatively wide distribution of *P. ×serendipita* confirms the ability of the hybrid to survive and spread (Man in 't Veld et al., 2007; Man in 't Veld et al., 2012).

Another example of interspecific hybridization in *Phytophthora* is *P. andina*. Strains of this hybrid were originally identified as *P. infestans sensu lato* (Ordoñez et al., 2000), but later, the new species *P. andina* was described. *P. andina* is closely related to *P. infestans* (Oliva et al., 2010) and both species are indistinguishable according to ITS sequences. The sequencing of six nuclear genes revealed three genetic lineages of *P. andina* as consequence of the hybridization between *P. infestans* and an undescribed *Phytophthora* sp. (Goss et al., 2011; Wang et al. 2016). The three genetic lineages of *P. andina* have different host spectrum, which also differs from that of *P. infestans* (Adler et al., 2004; Oliva et al., 2010).

Bertier et al., (2013a, 2013b) confirmed the hybrid strains emerging from *P. porri*, and *P. primulae*/*P.* taxon parsley from *Phytophthora* clade 8b. Similarly to previous examples, the change of host spectrum of these hybrid strains was documented. Here, this phenomenon is attributed to polyploidization and transmission of RxLR effectors associated with host specificity within *Phytophthora* (Whisson et al., 2007). Therefore, polyploidization increases the number and new combinations of RxLR effectors, the virulence and aggressiveness of hybrid specimens and causes changes in the host spectrum (Bertier et al., 2013b).

Conversely, Burgess et al. (2015) describes hybrids strains of sympatrically occurring species as common phenomenon. Hybridization was recorded among five related *Phytophthora* species from group VI, naturally occuring in Australian watercourses that seasonally dry up. From thirty possible hybrid combinations, twelve were isolated from the environment, which indicates that no reproductive barriers exist among parental species. Hybrid strains are mostly sterile and thus could be considered temporary hybrid

clones. Sterility in these strains could be explained as an adaptive strategy, when postzygotic reproductive barrier between sympatric species prevents formation of new generations of hybrids (Burgess, 2015).

Interspecies hybridization enhances virulence and pathogenicity changes of *Phytophthora* spp. and host spectrum change of both parental species, extending their distribution area, as hybrids could better fit in a particular niche (Depotter et al. 2016). A remarkable increase of the aggressiveness of *P. ×alni* has been reported, conversely, other hybrids (*P. capsici* × *P. nicotianae*), show significantly lesser virulence in comparison with parental species (Olson & Stenlid, 2002). Hybridization within *Phytophthora* is widespread. Hybrid strains show increased genetic plasticity, and thus also ability to better adapt to the environmental conditions (Kroon et al., 2012).

2.4 Characterisation of selected species classified in *Phytophthora* 1a clade

Species of *Phytophthora* clade 1a (Cooke et al., 1996; Kroon et al., 1999; 2012; Martin et al., 2012) related and morphologically similar to *P. cactorum* are characterised below. Also the characteristics of two hybrid species, whose parent is *P. cactorum,* are provided.

Phytophthora cactorum

Phytophthora cactorum was described under the name of *Peronospora cactorum* causing root rot of *Cactae* in 1870 (Lebert & Cohn, 1870). In 1876, De Bary proposed the new genus *Phytophthora*, based on species *Phytophthora* (formerly *Peronospora*) *infestans*. In the same year, Hartig described this species again as *Phytophthora fagi* (Hartig, 1876) – the name is a synonym of *P. cactorum*. In 1881, De Bary classified *P. cactorum*, together with *Peronospora sempervivi* as summarizing species *Phytophthora omnivora*. In 1886, Schröter proposed the currently used combination *Phytophthora cactorum* (Lebert & Cohn) J. Schröt (Lebert & Cohn, 1870; Tucker, 1931; Tucker, 1933).

Mycelium of *P. cactorum* is coenocytic, usually aseptate. Hyphal diameter has been pointed to be ca. 6 μm (Waterhouse & Waterston, 1966) and 2–14 μm (Blackwell, 1943). Individual hyphae could be irregularly swollen, but without typical thickening (Erwin & Ribeiro, 1996). Hyphae branch out nearly at right angles, branched hyphae are slightly thinner at the base. The nucleus of the new hypha is near the base, another nuclei are placed along the whole length at ca 10–20 μm intervals. Older hyphae have cell walls

lined with layer of protoplasm only, while the centre of the hypha is occupied by vacuole with oil storage substances. In young hyphae, septa are formed only at the base of reprodutive organs, in older hyphae they can appear more frequently. Hyphae morphology depends on the cultivation medium. The hyphae are mostly thinner, longer and less branched in water than in nutritious medium, where they are more branched, shorter, and thicker, especially in drier media (Blackwell, 1943).

Morphology of the colony is also dependent on growth medium. Mycelium could be formed either by densely growing aerial hyphae or by flat, sessile, thin ones (Blackwell, 1943). Colony growing on cultivating V8 medium (200 ml of V8 juice, 3g of CaCO3, 15 g of agar, topped up with distilled water to 1 l, pH ca. 7–7.5, Miller, 1955), or CA (200 g of carrot homogenised in 500 ml of distilled water and filtrated over filtering paper, topped up to 1 l, with 15 g of agar added; Erwin & Ribeiro, 1996) is stellate to rosaceous and the mycelium is flattened. Colonies are approximately circular, compact, without sharp margin, with sparse, fuzzy, aerial hyphae (Erwin & Riberiro, 1996).

Sporangia enable quick reproduction and dispersal of *Phytophthora* (Darmono & Parke, 1990). *P. cactorum* usually form terminal or intercalar sporangia with oval to globose papilla. Shape of sporangia is widely oval to pyriform, ovoid or globose. Septum separates sporangia from sporangiophores. Mature sporangia detaches with 3–4 μm long pedicel, separated from sporangiophore at the septum. Sporangiophores most often grow separately or in sympodia, and could have smaller diameter than regular hyphae. Sporangia dimensions are as follows: 31.4±4.8×26.4±4.0 μm, length to width ratio (L:W) = 1.2±0.1 (Erwin & Ribeiro, 1996); 36–50×28–35 μm, L:W = 1.3–1.4:1 (Waterhouse & Waterston, 1966); 28×20–45×33 μm, usually 36×28 μm (Blackwell, 1943). According to Blackwell (1943), the size of sporangia also depends on the composition of the growth medium. Caducity of sporangia and the length of their pedicel are considered important discriminative characters, even though proportion of deciduous sporangia depends on the age of the culture, the growth medium and temperature. Contrarily, length of pedicel is independent on cultivation conditions and is significantly uniform (Erwin et al., 1983). Sporangial walls are made from two layers. Inner layer is thinner, mucous, and forms papilla. Outer layer is very strong and resilient. Zoospores release from sporangia as mononuclear cells without cell wall. Zoospores have two lateral flagella, which enable active movement. The two flagella differ in their length. Flagellum oriented in the direction of movement is shorter having mastigonemes, flagellum oriented to the back is

smooth (Erwin & Ribeiro, 1996). Zoospores are released from the mature zoosporangium when papilla absorbs great quantity of water and dissolves (Blackwell, 1943). Some works mention germination sporangia directly by hypha. This phenomenon occurs in old culture and under conditions unsuitable for forming zoospores. Dimensions of sporangia are often used for identification of *Phytophthora* species. However, a standardised method for measurement of sporangia are unavailable – different isolates produce sporangia under different conditions and quantity of atypical small sporangia in old cultures is increasing. Moreover, size of sporangia is dependent also on light and growth media (Erwin et al., 1983), their morphological variability is probably genetic (Hall, 1993). Despite of this, the shape in combination with dimensions of sporangia can provide rather reliable results at least on interspecies level (Fodor et al. 2015).

Chlamydospores are thick-walled vegetative spores formed intercalary or terminally from hypha, separated by septum. They are roughly globose, 33 μm (Blackwell, 1943; Waterhouse, 1963b; Waterhouse & Waterston, 1966), or 39.7 μm (Darmono & Parke, 1990) in diameter, can be multinucleate and contain high amount of storage substances. Walls are two-layered, each layer 1–1.5 μm thick. Chlamydospores are produced under light and dark conditions (Erwin Ribeiro, 1996), or when the culture was exposed to fluctuations of temperature or some other type of stress (Blackwell, 1943). Only some isolates of *P. cactorum* produce chlamydospores (Darmono & Parke, 1990), those isolates produce them at temperatures around 4°C. Chlamydospores are imporant for the survival of the isolate under unsuitable conditions. Darmono & Parke, 1990 showed that the mycelium could germinate even when chlamydospores were exposed to -23°C for 24 hours.

P. cactorum is a homothallic species, so its isolates produce antheridia and oogonia, with fertile oospores without the presence of isolate of another mating type (selfing). Gametangia are formed in the tissue of infested plants or spontaneously in cultures under laboratory conditions (Erwin & Ribeiro, 1996).

P. cactorum is diploid during most of its life-cycle, haploid phase is reduced to gametangia formation. Gametangia formation lasts several hours. They are formed when two hyphae make contact, their tips' shape change to a globose structure with several nuclei – young anteridium and oogonium. Both gametangia connects tightly through their walls, separate by septum from maternal hypha, and meiosis occurs in both of them. Only one haploid nucleus remains in oogonium, other nuclei migrate to the periphery of the

oogonium and are absorbed in periplasm. The transition of male nucleus from the anteridium into the oogonium is managed through the fertilization tube. The fusion of both haploid nuclei and the formation of the diploid nucleus continues during the ca. 30 days-long maturing of the oospore (Blackwell, 1943; Erwin & Ribeiro, 1996). One of the symptoms of maturity of the oospores is the fusion of both nuclei (Jiang et al., 1989). The whole sexual process depends on correct synchronisation of meiosis in both gametangia. In case of asynchronic meioses, the whole process collapses and the oospore is not developed (Hüberli et al., 1997). *P. cactorum* forms colourless oogonia with smooth surface, 25–32 μm (Waterhouse, 1963a) or 25–40 μm (Blackwell, 1943) in diameter. The oogonium wall is formed mainly by cellulose. Oogonia are globose, occasionally tapering to the base. Every oogonium has only one oospore. Oospores are resilient organs that could survive several years in the soil. During maturing, storage substances are deposited there, visible under microscope as typical off-centre located oily corpuscles occupying approximately one third of the oospore (Blackwell, 1943). *P. cactorum* forms plerotic oospores, i. e. oospores fully filling oogonium. Their diameter is usually 20–26 μm, with wall 2 μm thick (Waterhouse, 1963a). The oospore wall is two-layered; outer layer is formed mainly by pectins, inner layer by cellulose and proteins. On syntetic medium, oospores are produced when there is a nitrogen supply and a sugar decrease (Erwin & Ribeiro, 1996). Oospores germinate after the dormancy period even after several years. Under natural conditions, oospores germinate after resting winter period when soil temperature exceeds 7.5°C. Prior to germination, inner layer of oospore walls dissolves, oily corpuscle divides into several smaller and number of nuclei increases. Oospores germinate by hypha or by sporangium (Erwin & Ribeiro, 1996).

The antheridia of *P. cactorum* are single-celled, mostly paragynous (attached to the oogonium stalk) or amphigynous (antheridia surrounding oogonial stalk), more or less globose to clubshaped. Antheridia of mature oogonia measure 8.5–21×12–21 μm (Erwin et al., 1983) or 12–15 μm (Blackwell, 1943).

The measurement of cardinal temperatures during the cultivation on growth media is traditionally used as one of the features for characterization and identification of *Phytophthora* spp. The maximum, minimum, and the optimum temperatures for growth rate are measured (Erwin et al., 1983). Growth temperature range differs for cultivation on media and for plant tissue infestation.

Cardinal temperatures for *P. cactorum* are as follows: Minimum 2°C, optimum (20–)25(–28)°C, and maximum 30°C (Waterhouse, 1963a). The temperature values can vary among distinct isolates of the species by ±1- 2°C, optimum could be more variable (Erwin et al., 1983). Cardinal temperatures are often used as a less important identification character (Zentmyer & Jefferson, 1974).

Phytophthora nicotianae

Phytophthora nicotianae Breda de Haan was described in 1896 (Breda de Haan, 1896). In 1913, the same species was repeatedly described as *Phytophthora parasitica* Dastur (Dastur, 1913). According to the International Code of Botanical Nomenclature, the name *P. nicotianae* has priority over *P. parasitica.* However, the name *P. parasitica* is still often used in USA. Waterhouse (1963a) differentiates *P. nicotianae* var. *nicotianae*, and *P. nicotianae* var. *parasitica* based on morphological characters. Several studies had been conducted to better define *P. nicotianae*, but a holotype from the original description is unavailable (holotype determination was not necessary for valid publishing of the name in 1896). According to an extensive study (Hall, 1993), *P. nicotianae* subspecies seems not to be distinguishable, based on morphological characteristics due to substantial changes in morphology during the ageing process of the culture.

Some isolates of *P. nicotianae* var. *nicotianae* produce homothallic oogonia but the majority of the isolates are heterothallic. Isolates of *P. nicotianae* var. *parasitica* produce homothallic oogonia in the culture (Waterhouse, 1963a). However, according to Hall (1993) the homothalic oogonia may be larger than the heterothallic one. The author considered such fact as a possible reason for the original differetiation between both subspecies and stated oogonium/oospore diamater of *P. nicotianae* as 23–28/21–25 μm (Hall, 1993). Antheridia of *P. nicotianae* are always amphigynous, according to Waterhouse (1963), the size of var. *nicotianae* is 16×10 μm and var. *parasitica* 10×12 μm. Sporangia are produced in water environment, but many isolates also produce them on aerial mycelium cultivated on agar medium. Sporangia are formed mostly terminally, intercalar growth is less common. According to Waterhouse (1963), var. *nicotianae* usually forms globose sporangia in basal section and rostrally extended apically, 45×6 μm in size, with hemispherical apical thickening. The var. *parasitica* usually forms ovoid to globose sporangia of 38×30 μm, but without apical extension. Length to width ratio in var. *parasitica* is 1.4:1. Sporangia could be deciduous. According to Hall (1993), L:W

ratio in *P. nicotianae* sporangia is 1.1–1.3:1, sporangia are ovoid, oval to globose, measuring 25–60×21–40 μm.

Chlamydospores are produced only by a minority of isolates, they are 25×40 μm in diameter (Hall, 1993). According to Waterhouse (1963), their size and also chlamydospores production differ. Var. *parasitica* produces chlamydospores after ca. two weeks, and they have a diameter up to 60 μm, whilst var. *nicotianae* produces chlamydospores very early with a 20–40 μm diameter.

The growth of the isolates of *P. nicotianae* is determined by the temperature: *P. nicotianae* var. *nicotianae* grows from 12°C with optimum raging 25–30°C up to 36.5°C, *P. nicotianae* var. *parasitica* grows from 10°C with optimum ranging from 30–32°C up to 37°C (Waterhouse, 1963). According to Hall (1993), their temperatures' range is 5–35°C, but some isolates could even survive temperatures over 40°C.

P. nicotianae has not been recorded in Czech Republic yet.

Phytophthora hedraiandra

Phytophthora hedraiandra De Cock & Man in 't Veld was described in 2004. Its holotype was isolated from leaf of *Viburnum* sp. (De Cock & Lévesque, 2004). This species differs in nuclear (ITS) and mitochondrial (cox I) genes from *P. cactorum* and *P. pseudotsugae*, which are morphologically very similar species. *P. hedraiandra* has been recorded several times on woody plants in southern Europe (Munda et al., 2006; Moralejo et al., 2007), in Czech Republic it was confirmed by Hejna et al. in 2014.

P. hedraiandra is a homothallic species spontaneously producing gametangia in culture. Oogonia are formed terminally on short lateral hyphae and more rarely on the main hypha. The stalk of the oogonium is short and curved. Oogonia are smooth, colourless, older oogonia could be yellowish in colour, with diameter (26–)28–36(–38) μm. The oospore is aplerotic, (23–)24–32(–36) μm in diameter, with 2 μm thick wall. The club-shaped antheridium is usually paragynous, less commonly amphigynous, terminal or intercalar, 9–14(–20) μm in length (De Cock & Lévesque, 2004). The antheridium sessility to hypha is considered to be specific for *P. hedraiandra* and a distinguishing character from *P. cactorum*. However, the descriptions of *P. cactorum* in literature (Lebert & Cohn, 1870; Blackwell, 1943; Waterhouse, 1963b; Stamps et al., 1990) do not mention the length of the stalk of the antheridium.

Sporangia of *P. hedraiandra* are widely ovoid to globose, formed on sporangiophores either terminally or in loose sympodia, (21−)30−53(−64) µm long, (16−)23−34(−39) µm wide. The papilla is 5 µm thick and 5–6 µm wide is situated in the apical part of the sporangium. L:W ratio of sporangia is 1.22–1.41:1. Sporangia are deciduous, with pedicel up to 2 µm (De Cock & Lévesque, 2004).

The ex-holotype strain of *Phytophthora hedraiandra* (CBS 111725) is characterized by growth temperatures from 3°C with optimum 22–30°C (De Cock & Lévesque, 2004).

Phytophthora pseudotsugae

Phytophthora pseudotsugae Hamm & Hansen was described in 1983 (Hamm & Hansen, 1983) as a pathogen causing root rot of Douglas fir (*Pseudotsuga* spp.) in North America. Colony on agar medium lacks distinctive structure, the mycelium is sessile. It is homothallic, oogonia are globose, terminal, smooth, 29–45 µm in diameter. Antheridia are roughly globose, hyaline, 6–15×10–24 µm, paragynous. Oospores are smooth-walled, pigmented, aplerotic, 27–33 µm in diameter, usually aborted.

Sporangia are formed in temperatures between 10 to 20°C, they are globose to ovoid, with distinct papillae 49–39 µm. L:W ratio is 1.18:1 to 1.27:1. Sporangia are non-caducous and on agar medium are very rear. *P. pseudotsugae* does not form chlamydospores (Hamm & Hansen, 1983; Martin et al., 2012).

Species grows under 2–30°C, optimum at 20–25°C.

P. pseudotsugae is distinguished from *P. cactorum* mainly by long, infrequently branching sporangiophores and non-caducous sporangia. *P. pseudotsugae* has not been recorded in Czech Republic yet.

Phytophthora idaei

Phytophthora idaei D. M. Kenn was first isolated from red raspberry (*Rubus idaeus*) in the UK, and described as a new species in 1995 (Kennedy & Duncan, 1995). It seems to be specific for *R. idaeus*.

P. idaei colonies are uniform, with reduced aerial mycelium on agar media, but without any distinctive characters. Sporangia are globose to ovoid, 49×36 µm, formed at temperatures around 20°C. Sporangiophores are non-branched, in loose sympodium. Sporangia are non-caducous. It is homothallic, its oogonia are globose, smooth-walled,

26–38 µm in diameter. Antheridia are paragynous and rarely amphigynous. Oospores are aplerotic, 22–34 µm in diameter.

P. idaei differs from *P. cactorum* by non-caducous sporangia and lower optimal growth temperature under 20°C, while *P. cactorum* has 25°C. *P. idaei* does not form chlamydospores (Kennedy & Duncan, 1995).

This species has not been recorded in Czech Republic yet.

Phytophthora ×pelgrandis

Phytophthora ×pelgrandis Gerlach, Nirenberg & Gräfenhan (Nirenberg et al., 2009) is one of the hybrid species described in *Phytophthora* clade 1. *P. ×pelgrandis* was isolated from *Pelargonium grandiflorum* and recognised as hybrid of the parental species *P. cactorum* and *P. nicotianae* by Man in 't Veld et al., 1998, formally was described in 2009 (Nirenberg et al., 2009).

Colonies of *P. ×pelgrandis* form aerial, fine wooly mycelium of hyaline hyphae. Isolates are homothallic, oogonia are globose, 32–28 µm in diameter. Antheridia are both amphigynous and paragynous, 7 µm long. Oospores are aplerotic, 27–32 µm in diameter, with wall 2 µm thick. Sporangia are non-caducous, ovoid or nearly globose, with distinctive papilla, 48.8×41.4 µm in size. Chlamydospores are not described for this species. Cardinal temperatures are from minimum 7.5°C, optimum 25–30°C to maximum 32.5–35°C (Nirenberg et al., 2009).

Phytophthora ×serendipita

In 1995, some strains similar to *P. cactorum* but showing heterozygosity in malate dehydrogenase isozymes were detected (Man in 't Veld et al., 2007). This species was formally described as *Phytophthor*a *×serendipita* Man in 't Veld & K. Rosendahl (Man in 't Veld et al., 2012), the hybrid between *P. cactorum* and *P. hedraiandra*. The colony of the species on V8 agar is stellate with flattened aerial mycelium. Sporangia are papillate, ovoid to globose measuring 40.5×30.6 µm, single or sympodially arranged, non-caducous or caducous, with 2–5 µm long pedicel. It is homothallic, its oogonia are globose, 26–33 µm in diameter. Oospores are both plerotic and aplerotic, often aborted, 21–29 µm in diameter. Antheridia are paragynous. Chlamydospores were not recorded. Its optimal growth temperature is 27°C with 31°C maximum.

2.5 Ecology of *Phytophthora cactorum*

Growth temperature range, cardinal temperatures

Temperature is one of the most important factors that affect growth and sporulation of *P. cactorum*. Soil temperature in certain seasons could both inhibit oospore production and also plant tissue colonization. The optimal temperature for oospore production could differ from the optimal growth temperature, but it is rarely lower (Doster & Bostock, 1988). Temperature range for sporangia production is significantly narrower than the range for mycelium growth, 12.5–27.5°C (Grove et al., 1985). Germination of oospores and chlamydospores is also strongly influenced by temperature (Duncan, 1985).

The pathogenicity of *P. cactorum* depends on temperature; at the optimum temperature (25°C) dieback of host plant it is enhanced when compared to 18°C (Thomidis, 2003). Necrotic lesions extend at temperature 20–25°C the fastest, and the growth stops under 15°C or above 30°C (Thomidis, 2003). Thus, temperature is considered one of the most important environmental factor influencing the pathogenic activity of *P. cactorum* (Thomidis, 2003; Matheron & Matejka, 1992).

Habitat and environmental conditions of *Phytophthora cactorum*

Phytophthora cactorum is widely distributed throughtout the entire temperate zone (Jung et al., 2005; Jung, 2009; Jung et al., 2016), mainly occuring at human-affected habitats with altitudes up to 600 metres above sea level (Jung & Blaschke, 1996; Jung, 2009). *P. cactorum* occurs mostly at humid localities enabling effective spreading by zoospores. Zoospore production is triggered by prolonged wet periods with temperatures around 2-8°C (Jung, 2009). Infestation and development of the disease are further supported by rapid change of climate extremes. Prolonged dry season causes weakening of plants and damage of roots and increases root susceptibility to infestation (Jung, 2009). Plant roots exposed to temperatures around 40°C are considerably more susceptible to the infestation (Macdonald, 1991). If prolonged wet season is followed by drought, the infectious pressure increases strongly and the pathogen spreads rapidly (Jung et al., 2005; Jung, 2009). Other factors increasing susceptibility of roots to *P. cactorum* infection are an increased oxygen concentration in soil or higher soil salinity (Macdonald, 1991).

Dispersal of *Phytophthora cactorum*

There are two basic strategies of dispersal in *Phytophthora*: (1) soil-borne species with non-caducous and non-papillate sporangia attacking mainly roots, and (2) air-borne species spreading by wind and infecting above-ground organs of plants. *P. cactorum* is mainly a soil-borne species, but also forms deciduous sporangia spread via air (Grove et al., 1985; Reynolds et al., 1989). Therefore, natural dispersal of *P. cactorum* is through sporangia and zoospores. Host plant infection usually occurs via roots or by contact of soft tissues (fruits, leaves) with infected soil. Zoospores are able to move in water or in soil saturated with water. After release from sporangia, zoospores are able to move over distance of tens of centimetres in water for several hours. When larger area is flooded with water, the transfer of sporangia and zoospores is possible over much greater distances, which increases the infection spread considerably (Jung, 2009). Zoospores are perceptive towards osmotic characteristics of water solution, and are able to actively look for suitable substrate through chemotaxis – they are attracted by root exudates (Raftoyannis & Dick, 2006), or electrotaxis –electric field generated by roots (van West et al., 2002). Some works (Raftoyannis & Dick, 2006) confirm that zoospores are able to find available roots of preferred host. Zoospores identify particular areas of roots (mainly the area of elongation and root hairs) and infect them (Raftoyannis & Dick, 2006). After contacting with a suitable substrate, zoospores loose the flagella, form the cell-wall, and encyst. During the encystation, adhesive substances are released and zoospores adhere to the surface of the plant tissue. Under suitable conditions, cyst could germinate and produce hypha (Blackwell, 1943), or zoospore detachs itself again (Erwin & Ribeiro, 1996). Its ability to directly germinate by hyphae is usually associated with more evolutionary advanced species of *Phytophthora* sp., which mainly spread by wind (Cooke et al., 2000). The spread of *P. cactorum* sporangia by wind for short distances was documented during an intense rainfall (Reynolds et al., 1989). Lower branches or fruits could be infected during intense rain via raindrops containing sporangia or zoospores, which is common at strawberry farms (Reynolds et al., 1989). The ability to spread by sporangia and zoospores, either by water and air considerably enhances the dispersion potential of *P. cactorum* (Stenlid et al., 2011).

Today, *P. cactorum* is quickly spreading due to global plant trade. The original area of distribution of *P. cactorum* is probably small. Isoenzyme and molecular analyses gave evidence of considerable genetic uniformity of *P. cactorum* (Förster & Coffey, 1991;

Oudemans & Coffey, 1991). It has spread from a presumably small area and has got a worldwide distribution mostly due to a human-mediated dispersal (Brasier et al., 1999; Brasier, 2008; Moralejo et al., 2009; Stenlid et al., 2011). The current increased speed of dispersal of *Phytophthora* pathogens is caused not only by transport of plants and plant products, but also by climate change, which affects the balance of complex relations among host, pathogen and environment, and increases host susceptibility to these pathogens (Jung et al., 2005). Rapid dispersal is also influenced by wide spectrum of *P. cactorum* hosts, including both, herbaceous and woody plants (Erwin & Ribeiro, 1996; Stenlid et al., 2001). *P. cactorum* is considered as non-native in majority of the current distribution area. The invasive pathogen exploits higher sensitivity of local hosts caused by lack of common evolutionary history in new areas of distribution, thus probability of further dispersal of the pathogen is increasing (Brasier, 2008).

P. cactorum is higly likely distributed throughout the whole area of the Czech Republic. The species was isolated in former Czechoslovakia in the 1960s, mainly from ornamental plants *Dahlia*, and *Antirrhinum* (Cejp, 1961a; Cejp, 1961b), and also on woody plants (Jančařík, 1961). Nevertheless, the distribution of pathogen has not been studied in detail. *P. cactorum* is reported mainly on ornamental trees and in gardens. Common hosts are *Rhododendron* spp., the pathogen was also recorded on numerous of forest trees, for example *Fagus sylvatica, Fraxinus excelsior*, *Alnus glutinosa*, *Quercus robur*, *Tilia cordata*, *Acer platanoides*, *A. pseudoplatanus*, *Aesculus hippocastanum* (Erwin & Ribeiro, 1996; Jung et al. 2013; Hayden et al. 2013). *P. cactorum* is also recorded on apple trees (*Malus domestica*), and strawberries (*Fragaria ×ananassa*) in gardens and agricultural plantations (Erwin & Ribeiro, 1996).

2.6 Phytopathology

Host specificity, intraspecific variability of pathogenicity in *Phytophthora cactorum*

Phytophora cactorum has wide host spectrum, comprising ca. 200 plant species from 150 genera. Almost all the important broadleaf trees in European forestry are potential hosts (*Fagus sylvatica*, *Quercus* spp., *Fraxinus* spp., *Acer* spp., *Castanea sativa*, *Aesculus hyppocastanum*, *Tilia* spp., *Juglans* spp., *Salix* spp., *Ulmus* spp., *Platanus occidentalis* etc.), also many coniferous trees can be infected by the pathogen (*Abies* spp., *Picea abies*, *Pinus* spp., *Larix decidua*, *Pseudotsuga menziesii*). It also causes losses in fruit production (*Malus* spp., *Pyrus communis*, *Prunus domestica*, *Fragaria ×ananassa*), it has

been documented on numerous crops (*Pisum sativum*, *Humulus lupulus*, *Brassica oleracea*, *Beta vulgaris*, *Capsicum annuum*, *Daucus carota*), and also infests many ornamental woody and herbaceous plants. Strains of *P. cactorum* significantly differ in aggressiveness and pathogenicity depending on host species. The pathogenicity level of *P. cactorum* strains is usually connected to a single host (Weres, 1995; Goss et al., 2011; Hantula et al., 2000; Bhat et al., 2006; Eikemo et al., 2004; Weiland et al., 2010). During pathogenicity tests isolates from strawberry could infect birch seedlings only when they were injured, and isolates from birch were not capable to infect strawberry at all. (Hantula et al., 1997). Differences among isolates from various hosts have physiological origin. The enzymatic activities of strains from strawberry are differ from those ones in other hosts (Oudemans & Coffey, 1991). The strawberry isolates form also a genetically homogenous group separated from isolates obtained from other hosts (Martin & Tooley, 2003; Eikemo et al., 2004). These isolates slightly differed also at morphological level. Therefore, specific pathotypes of *P. cactorum* specialized on particular host, or causing damage on different plant organs could be distinguished.

Population analyses have been conducted to elucidate the genetic variability of *P. cactorum*. It seems to be rather genetically uniform (Förster & Coffey, 1991; Oudemans & Coffey, 1991), but certain variability in affinity to different hosts is obvious. Bhat et al. (2006) tested its genetic diversity within species using Amplified Fragment Length Polymorphism (AFLP) method. The study confirms high genetic homogenity of the species. The low variability among the analysed isolates did not show a clear association of particular isolates to specific hosts or locality. Host-species association explained 31 % of discovered genetic variability, while geographic origin explained 24 %. Hantula et al. (2000), Bhat et al. (2006), and Eikemo et al. (2004) confirmed that at least two different pathotypes of *P. cactorum* could be distinguished on strawberry (*Fragaria ×ananassa*) in European, Californian and almost global population. A) Pathotype infesting strawberry crown (crown rot type) – this pathotype has low genetic variability. B) Pathotype causing strawberry fruit rot (leather rot type) – this pathotype has higher genetic variability and also attacks other hosts than strawberries. According to Eikemo et al. (2004), crown rot type was most likely originated in North America. Hantula et al. (1997, 2000) also mentioned a *P. cactorum* population specific to *Betula*. In population studies based on Random Amplified Microsatellites method (RAMS), these strains considerably differed from both, crown rot and leather rot types, which however were not distinguished well

by this method. Eikemo et al. (2004) also accepted this third pathotype, but in their AFLP study this pathogen was represented by only one strain. Bonants et al. (2000) explained the variability of different pathotypes by hybridization of *P. cactorum* with other *Phytophthora* spp.

The susceptibility of the plants to be infected is also an important factor within process of pathogenicity. In more susceptible plants, the bigger variation in damages caused by strains of *P. cactorum* differing in their agressiveness is observed, whereas in less susceptible hosts this variation is smaller. Therefore, pathogenicity of *P. cactorum* strains is more important for more susceptibile than more resistant hosts (Darmono et al., 1991). Host susceptibility to *P. cactorum* infection is also dependent on the season, when the infection starts, and on the physiological condition of the plant tissue (Boughaleb et al., 2006; Holub et al., 2010). The susceptibility of the plant tissue to the *P. cactorum* infection is influenced by the water content of the plant tissues – tissues with higher water content were proved to be more susceptible (Tippett et al., 1987). Susceptibility of plant issue is also dependent on temperature (Thomidis, 2003). In summary, damage severity caused by *P. cactorum* depends on complex conditions of interaction between pathogen and host under environmental conditions.

Plant infection by *Phytophthora cactorum*

The infection process and disease development of *P. cactorum* on woody plants is similar to other *Phytopthora* spp. The infection on a woody host starts when zoospores or whole sporangia attack fine roots or leaves. Encysted zoospores on the surface of plant tissues germinate by hyphae penetrating epidermal cells and entering intercellular space. On susceptible plants hyphae form mycelium and penetrate with haustoria in the mesophyll cells (Kamoun et al., 1999). *P. cactorum* is able to penetrate also into undamaged bark tissue, often through lesions caused by insect, herbivores, or abiotic factors. The most convenient season for infection is the begining of dormancy or during the wet, cold sesion in spring (Erwin & Ribeiro, 1996; Thomidis, 2003). *P. cactorum* enters the host tissue mostly through fine roots and root hairs and spreads to root collar and tree trunk. Infected fine roots and vascular tissue necrotize, the pathogen spreads intracellularly, and later also intercellularly. Infected roots turn black-brown, wet lesions are visible on older roots (Jung et al., 2005). During the first phase of infection, tissues of feloderm are affected, later also the cambium and adjacent layers of phloem and xylem. Later, the pathogen

spreads via the medullary rays. *Phytophthora* invades the xylem tissues up to ca. 25 mm, spread through the tissue and causes air embolism (Oßwald et al., 2014). Infected bark tissues of above-ground organs are characterized by remarcable dark necrotic lesions, so-called bleeding canker. They often occur on roots and stem near soil surface, but can occur also on higher part of tree stem. When outer bark of a bleeding canker is removed, tongue shaped, red-brown lesions appear, penetrating the inner bark and cambium (Jung et al., 2005). Cavities filled with liquid could appear under the surface of some necroses, they are 2–15 mm deep, 1–150 cm^2 across, often penetrating also xylem tissues. The liquid in cavities has pink colour, but darkens to dark red-brown due to the oxidation processes on air (Brown & Brasier, 2007). These symptoms are common for majority of trees. In some fruit trees bark could change colour to brownish rusty tints or bark tissue can peel off (Erwin & Ribeiro, 1996). Water and minerals supply to the tree crown decreases as a result of roots and vascular tissues damage. Stress caused by this water and nutrient defficiency forms extensive changes in the decline of the tree crown (Jung et al., 2005). On beech, terminal shoots growth is slowed over long periods, therefore these shoots are shorter and clustered around skeletal branches. At the same time lateral branches reduction occurs and leaves remain only in clusters around trunk and main branches. Leaves gain chlorotic colour, are smaller and fewer (Jung, 2009). As a side effects, the late shooting and dieback of shoots during vegetative season could be obvious (Erwin & Ribeiro, 1996). Decreased transport of assimilates from tree crown to roots occurs if large part of root collar is affected, and therefore flower and fruit production can increase. Tree crown becomes gradually thinner and finally declines (Jung & Blaschke, 1996). Root damage and xylem infection by pathogen also decrease transport of cytokinins from roots to above-ground parts of the plant, and their homeostatic equilibrium is disturbed (Davison, 2001). Collapse of xylem hydraulic conductivity caused by pathogen, accompanied by closing of leaf stomata in consequence of increased abscisic acid production in affected roots is considered as immediate cause of plant death (Davison, 2011). Lesions caused by *P. cactorum* are subsequently invaded by secondary pathogens which also increase susceptibility to damage by abiotic factors (Jung, 2009).

The infection of tree seedlings (mainly *Fagus sylvatica*) has different development. Seedlings are infected during germination already, the typical symptoms are large brownish stains on cotyledons Under high soil humidity and high temperature, infected

plants turn brown and die within few (Weres, 1995). Up to 80 % of germinated *F. sylvatica* seedlings infected with *P. cactorum* could die out (Weres, 1995).

2.7 Chemical protection against *Phytophthora cactorum*, acquiring of resistance and its inheritance

Fungicides, fungistatic chemicals and antibiotics are widely used for plant protection against infections by *Phytophthora* spp. (Ellis et al. 1982; Ellis et al. 1998; Fernandez-Cornejo et al. 2014). Nevertheless, phytopathogenic organisms have the ability to become resistant to such chemicals. The development of resistance in *Phytophthora* spp. has been repeatedly reported (Vaartaja 1960; Shaw and Elliott 1968; Ann and Ko 1988; Utkhede and Smith, 1993; Chabane et al. 1996b; Jeffers et al. 2004; Hu et al. 2008). The resistance of an isolate is defined by a more rapid mycelial growth of resistant individuals than that of sensitive ones or by enhanced sporulation in the presence of otherwise effective chemicals (Porter et al. 2007). The development and permanency of resistance to metalaxyl in *P. cactorum* were studied by Utkhede and Gupta (1988), and the resistance to mancozeb, metalaxyl and fosetyl-Al was studied by Utkhede and Smith (1993). The heritability of resistance was revealed by other works (Chang and Ko 1990; Bhat et al. 1993; Chabane et al. 1996b). While little is known about the heritability of resistance in *Phytophthora* spp., particularly in homothallic species, some studies have described the single nuclear genes that control the resistance of *P. sojae* to fungicides, such as metalaxyl and fluorophenylalanine (Utkhede and Smith 1993), and of *P. parasitica* (the synonym of *P. nicotianae*) to chloroneb and metalaxyl (Chang and Ko 1990). In contrast, the resistance to some antibiotics is likely controlled by mitochondrial genes (Ann and Ko 1988; Chang and Ko 1990; Chabane et al. 1996a; Whittaker et al. 1996). Once gained, resistance to a chemical persists as a property of an isolate. Thus, due to the prevalence of asexual reproduction in *Phytophthora* spp., resistance can reveal the history of exposure of the isolates to specific chemicals (Kai et al. 2013). If *P. cactorum* forms interspecific hybrids between two individuals that differ in their response to chemicals, the variability of resistance among *P. cactorum* and those species can be assessed.

3. Aims of the work

- To performe a complex analysis based on AFLP genotyping, sequencing of three genes, and morphological characters to elucidate the evolutionary relations of *P. cactorum* and other *Phytophthora* species hybridizing with *P. cactorum* mainly in European context.

- The main aim is to uncover the relations and gene flow among *P. cactorum*, *P. hedraiandra*, and *P.* × *serendipita*.

- To evaluate the applicability of ITS sequencing, morphological and physiological characters as an identification tool for *P. cactorum*, *P. hedraiandra*, and *P.* × *serendipita*.

- To examine the differences in resistance of *P. cactorum, P. hedraiandra, P.* ×*serendipita*, *P.* × *pelgrandis* and *P. nicotianae* to different chemical compounds applied for oomycete control.

4. Material and methods

4.1 Population and phylogenetic study of *Phytophthora cactorum* species complex in Europe (Paper 1)

Isolates

In total, 133 isolates of *P. cactorum*, *P. hedraiandra*, *P. nicotianae*, *P.* ×*serendipita*, and *P.* ×*pelgrandis* were obtained from 15 European countries (Austria, Belgium, Bulgaria, Czech Republic, Finland, Germany, Greece, Hungary, Iran, Italy, the Netherlands, New Zealand, Poland, Slovakia, Spain, Switzerland), and four countries outside Europe (Taiwan, and USA (Arizona and California), which were obtained from various culture collections (the list of isolates is presented in Tab. 1S in the supplementary material of paper 1).

DNA extraction

Small segments of a colony grown for 5–14 days on V8 agar were transferred into flasks containing

50 ml of V8 liquid medium (100 ml V8 Juice, 400ml distilled water and 1.5 mg $CaCO_3$, autoclaved at 121°C for 15 min). The flasks were placed onto an orbital incubator and kept at room temperature with slow shaking (50 rpm) for 5–7 days. Subsequently, the mycelium was washed in sterile deionized water and then powderized in liquid nitrogen using a mortar and a pestle. DNA extraction was performed using a QiaGen Plant mini kit (Qiagen) according to the manufacturer's protocol.

Amplified Fragment Length Polymorphisms (AFLP)

Genotyping of the AFLPs was carried out according to Vos et al. (1995). Total DNA (200 ng) was incubated at 37 °C for 3 hours in a thermocycler (Mastercycler® ep, Eppendorf) in 20 µl reactions with 2.5 U *Mse*I (NEB), 5 U *EcoR*I (NEB), 80 Cohesive End Units of T4 DNA ligase (NEB), 50 pmol *Mse*I- Adapter, 5 pmol *EcoR*I-Adapter, 1 mM ATP (Life Technologies), in 1× NEB Buffer 2 (NEB). The reactions were diluted 1:10 with $TE_{0.1}$ (20 mM Tris, pH 8.0; 0.1 mM EDTA, pH 8.0). Preamplification reactions contained 4 µl from the dilution of the restricted/ligated reaction, 0.2 mM dNTPs (Life Technologies), 2.5 pmol *EcoR*I+A primer, 2.5 pmol *Mse*I+C primer, 0.5 Taq DNA-

polymerase (Qiagen) in 1× QIAGEN® PCR Buffer (Qiagen). The reaction regime in the thermocycler was the following: 94 °C for 2 min; 20 cycles: 94 °C for 20 s, 56 °C for 30 s, 72 °C for 30 s; then 60 °C for 30 min. The preamplifications were diluted 1:20 with $TE_{0.1}$. Selective amplifications contained 4 µl from dilutions of the preamplification reaction, 0.2 mM dNTPs (Life Technologies), 2 pmol 5' fluorescence-labeled *EcoR*I primer (*EcoR*I+AT-PET, *EcoR*I+AG-FAM, or *EcoR*I+AC-NED), 5 pmol *Mse*I primer (*Mse*I+CA, or *Mse*I+CC), 0.5 Taq DNA-polymerase (Qiagen) in 1×QIAGEN® PCR Buffer (Qiagen). The primer combinations were *EcoR*I AG/*Mse*I-CC, *EcoR*I AT/*Mse*I-CA, and *EcoR*I AC/*Mse*I-CA. The cycling conditions were as follows: 94°C for 2 min; 10 cycles: 94 °C for 20 s, 66 °C – 1 °C per cycle for 30 s, 72 °C for 30 s; then 20 cycles: 94 °C for 20 s, 56 °C for 30 s, 72 °C for 30 s; final elongation was at 60 °C for 30 min. The AFLP fragments were separated using an ABI PRISM 3730XL automated sequencer (Applied Biosystems) at the facilities of Macrogen Ltd. As a size standard, the GS 500 (-250) LIZ was used.

AFLP data were analyzed using software GenMapper 4.1 (Applied Biosystems). Fragments of 50 – 500 bp length were scored, and the presence of all peaks was verified personally. Only unquestionable peaks narrower than 1 bp and at least 0.5 bp apart from neighboring peaks were considered for further analyses. Datasets were evaluated independently by two persons and then exported as a binary matrix. Reproducibility of AFLP data were calculated from 20 repeated independent samples for which all three steps of AFLP were carried out.

Evaluation of data

Genetic structure was inferred using Structure software, version 2.3.4 (Pritchard et al. 2000). The analysis utilized a recessive allele model; the length of the burn-in period was 10^5, and the number of MCMC repetitions after burning was 10^6; the tested K numbers, representing the number of populations, were 1-8; the number of iterations was 10. The visualization of the Structure results was carried out by the software Distruct 1.1 (Rosenberg 2004). Analysis of the similarity between single runs for given K (Nordborg et al. 2005) and analysis of ΔK and LnP(D) were performed using the R (R Development Core Team 2011) module Structure-sum (Ehrich et al. 2007). Nei's gene diversity (D) (Nei & Li 1979), the rarity index (DW) (Schönswetter & Tribsch 2005) and clonal analysis were calculated by the R module – AFLPdat (Ehrich 2006). The band (peak)

counting, AMOVA with Euclidean distance (Excoffier et al. 1992) and maximum likelihood hybrid index (Buerkle 2005) of single groups were calculated using FAMD (Schlüter & Harris 2006) after defining individual groups of isolates according to the K=3 and K=5 groupings of Structure results. The hybrid index of individual groups was calculated as the average hybrid index of all isolates within the group. A phylogenetic network was constructed by SplitsTree 4 (Huson & Bryant 2006) using the distance transformation of Neighbor-Net networks based on the neighbor-joining algorithm (Bryant & Moulton 2004). The bootstrap values were acquired after 10^4 iterations.

DNA sequencing

The sequence analysis of three genes, the ITS region of ribosomal RNA gene, Phenolic acid decarboxylase (Pheca I), and Cytochrome oxidase (Cox I) supplemented the AFLP data: for ITS primers ITS1/ITS4 (White et al. 1990), for Pheca I primers Pheca F/Pheca R (Hurtado-Gonzales et al. 2009), and for Cox I primers COXF4N/COXR4N (Kroon et al. 2004) were used.

The PCR mix contained 5 mM dNTP's (Fermentas), 0.2µM of both primer of the respective primer pair, 1U of Taq polymerase (Thermo Scientific DyNAzyme II DNA polymerase), and 20 ng of DNA in 1×DyNAzyme PCR buffer (containing 1.5 mM $MgCl_2$). The reaction parameters were identical for all three DNA regions except of the annealing temperatures. The cycling conditions were as follows: 95°C for 2 min; 35 cycles of 95 ° for 30 s, annealing for 30 s (55 °C for ITS, 56 °C for Pheca I, and 50 °C for Cox I), 72° for 60 s; then 72°C for 10 min. The PCR product was sequenced by MacroGen Ltd.

Phylogenetic analysis

The phylogenetic analyses were based on a combination of DNA sequences (ITS, Pheca I, Cox I) and AFLP data that were aligned manually in Bioedit (http://www.mbio.ncsu.edu/Bioedit/bioedit.html). The dataset is contained 1953 DNA characters (1868 constant, 63 variable, 22 parsimony-informative) and 364 AFLP binary characters. The partition Bayesian analysis (four partitions – the tree gene regions and the AFLP data) was run in MrBayes 3.2.3 (Ronquist et al. 2012) with following models adapted: JC (Cox I), K80 (Pheca I), TrNeF (ITS), and F81 (AFLP). The models for DNA data were selected according to AIC with jModeltest2 (Darriba et al. 2012). Four chains

of 5 million generations were run. The burn-in value (500,000 generations) was estimated using Tracer version.1.6 (http://tree.bio.ed.ac.uk/software/tracer/). Sampling frequency was set to every 100th generation. The data from the Bayesian analyses were deposited at Treebase (ID 18545).

Morphology

The morphological analyses were conducted on cultures cultivated on V8 agar (Miller 1955) at 22°C, 105 of total 133 isolates included into study were explored, 20 measurements of each character per isolate was performed. Oogonia were taken from the surface of colonies approximately 14 days old. The oogonial diameter, oospore diameter and the oospore wall thickness values were measured. In addition, for 30 isolates, including members of all groups derived from the Structure analysis except of *P. nicotianae* and *P.* x *pelgrandis* isolates, the characters of antheridia were recorded (amphigynous/paragynous, sessile/stalked; 40 antheridia per each isolate). Oogonia of *P. nicotianae* isolates were produced after pairing them with isolates of the opposite mating type using the polycarbonate membrane technique of Ko (1978). In the *P. nicotianae* mating tests, the agar in the V8 medium was replaced by agarose (Boutet et al. 2009). Sporangial production was induced by immersing an agar square (ca. 2×2 cm) cut from the margins of a fresh V8 culture in filtered pond water in a beaker (Jung 2009). Sporangia were produced after 2-7 days of incubation at natural light and room temperature. The microscopic mounts were stained and fixed by lactophenol with cotton blue. All measurements were conducted using an Olympus CX41 microscope at 1000 ×magnification with Quick Photo 2.3 software (Promicra Ltd.).

The oospore wall and aplerotic index were calculated according to Dick (1990). The statistical evaluation based on morphology was performed with the Statistica 7.0 software (StatSoft Inc.). Isolates were grouped according to the results obtained in Structure. Correlation analysis and Principal Component Analysis (PCA) were performed to evaluate correlations between characters. Only uncorrelated characters were used in a discriminant analysis, which was performed under default settings, with the redundant characters eliminated. The Wilk's λ of the whole model and of single roots was calculated, and Mahalanobis distances between the Structure-derived groups were determined. A subsequent canonical analysis determined the significance of single canonical roots and

their correlation to measured characters. The differences among the Structure groups in antheridial sessility were evaluated using Kruskall-Wallis test.

Cardinal temperatures

The growth of isolates at different temperatures was examined using 14 temperature steps conditions between 2 and 32°C. A 5 mm agar disks in diameter was cut from the margin of actively growing region, i.e. 5-7 days old colony on V8 agar and transferred to a fresh V8 agar plate in a 9 cm Petri dish. The inoculated plates were incubated at different temperatures for 5 days under darkness. The period was shortened to three days at temperatures around the growth optimum so that the colony margin did not reach the margin of the plate. The period was extended up to 12 days at temperatures close to the minimum and maximum temperature for growth so that the total growth remained still obvious. The colony area was measured in two orthogonal directions, and average daily growth at the relevant temperature was then calculated. Data were analyzed using a discriminant analysis in Statistica 7.0 software (StatSoft Inc.). Individual isolates were grouped according to the results of the Structure analysis. The Wilk's λ of the whole model and of single temperature values was calculated, and the Mahalanobis distances between isolate groups were determined. In canonical analysis, the correlation between the canonical roots and temperatures was ascertained.

4.2 Growth response of *Phytophthora cactorum* complex, *P. nicotianae* and *P.* × *pelgrandis* to antibiotics and fungicides (Paper 2)

Isolates

In total, 26 isolates of *Phytophthora cactorum* (including *P. cactorum sensu stricto*, *P. hedraiandra, P.* × *serendipita*), 8 isolates of *P.* × *pelgrandis* and 2 isolates of *P. nicotianae* were used (Tab. 1 in paper 2.). The isolates originated from the following 10 countries: Bulgaria, Czech Republic, France, Greece, Hungary, the Netherlands, New Zealand, Switzerland, Spain, and the USA (Arizona and California); the isolates originated from 18 different host species, water streams and soil samples. All isolates were divided into groups using the AFLP genotyping reported in our previous study (Pánek et al. 2016). Groups C1 (6 isolates), C2 (8 isolates), H (6 isolates) and F (4 isolates) were lineages within the *P. cactorum* complex, while other groups represented *P. nicotianae* (N, 2 isolates) and *P.* × *pelgrandis* (P, 8 isolates). Isolates were chosen as

typical representatives of relevant AFLP groups according to the genetic homogeneity of the groups identified in previous AFLP studies and the availability of isolates genotyped in this study. Therefore, group H in the present study comprised all isolates assigned to this group via AFLP. In both the current study and our previous AFLP study, the N group serves as outgroup; therefore, only two isolates were included in the present study. In the other groups (C1, C2, and F), the numbers of isolates included in the present study were determined by their genetic homogeneity, which was especially high in the F group.

Resistance tests

The tested substances were dimethomorph, mancozeb, and metalaxyl (fungicides), chloramphenicol and streptomycin (antibiotics). All fungicides and antibiotics were obtained from Sigma Aldrich Ltd. The V8 Juice agar medium (HiMedia according to Miller 1955) was autoclaved for 15 min at 121°C. After cooling to ca 45°C, medium was enriched by single chemicals, then poured into Petri dishes (9 cm in diam.). Previous optimisation of chemical concentration was tested on five isolates representing each genetic group. Preliminary results did not correspond with literature data (Ann et al. 1988; Bhat et al. 1993; Hu et al. 2008; Chabane et al. 1996a; Chang et al. 1990; Utkhede and Gupta 1988; Utkhede et al. 1993; Wagner et al. 2007), therefore on the base of preliminary tests the concentrations were adjusted to: metalaxyl – 6.25 mg/L, mancozeb – 30 mg/L, dimethomorph – 0.66 mg/L, chloramphenicol – 300 mg/L, streptomycin – 300 mg/L. The optimum concentration was defined by slow growth of all isolates − no isolate overgrew the dish after 7 days of cultivation under 23°C. The inoculation of dishes, cultivation and measuring was done according to Parra and Ristaino (2001). The plate was inoculated with the circular plug (5 mm in diam.) taken from the margin of actively growing colony on V8 Juice agar at the center of the dish. The dishes were cultivated in thermostat at 23° C (temperature close to growth optimum) in dark. After seven days of incubation the diameter of colony was measured in two orthogonal directions. The diameter of inoculation plug (5 mm) was subtracted from the measured value, acquired value was divided by two and by the number of days of incubation. The resulted value was the daily growth rate of an isolate. All combinations of isolates and tested substances were tested on three plates and the average value was counted. As a control, the isolates were also cultivated on dishes with V8 Juice medium without addition of a tested substances. The inhibition rate of isolates was calculated as the ratio of the growth rate on V8 Juice medium

enriched by a chemical to the growth rate of the same isolate growing on clean V8 Juice medium. To express the inhibition rate as percentage, those values were multiplied by 100, and then subtracted from 100 %.

Statistical analysis

The data normality of the inhibition rates was tested by the Kolmogorov-Smirnov and Lilliefors test, later ANOVA was performed. In tests, the hypothesis of coincidence of mean values was rejected based on the p-value ($p<0.05$). If the coincidence of mean values was not rejected by ANOVA, the Tukey's HSD post hoc test was performed to differentiate the groups into homogenous subsets. The inhibition rates of tested *Phytophthora* groups were graphically expressed in box and whisker plot with displayed mean value, its 95% confidence interval, minimum and maximum values.

To test the combined effect of all five chemicals, a discriminant analysis was performed using the standard settings of the analysis. To reveal the correlations of the effects of different substances, a correlation analysis was performed. The mean values of the inhibition rates were considered correlated when the value of the Spearman's rank correlation coefficient was higher than 0.3 and the p-value was lower than 0.05. For correlated substances, only one was used in the discriminant analysis. The Wilks' λ of the whole model and single canonical roots were calculated, and Mahalanobis distances between the isolate groups were determined. A subsequent canonical analysis determined the significance of single canonical roots and their meaning to measured characters in particular substances. To graphically depict the relationships between the groups, a cluster analysis based on the data of all combinations of isolate/chemical substance was performed. Simple joining method with Chebyshev distances was applied, distances of join among groups are expressed as percentage of the distance between the most distant groups. All analyses were processed using the Statistica 12.0 software (StatSoft, Oklahoma, USA).

5. Results

5.1 Population and phylogenetic study of the *Phytophthora cactorum* species complex in Europe (Paper 1)

Structure analysis and isolate grouping

The AFLP analysis revealed 364 well defined and reproducible AFLP loci; 360 of them (98.9 %) where polymorphic. The primer combination *EcoR*I AG/*Mse*I-CC provided 125 polymorphic and one monomorphic AFLP loci, the combination *EcoR*I AT/*Mse*I-CA yielded 135 polymorphic and two monomorphic AFLP loci, and the combination *EcoR*I AC/*Mse*I-CA provided 100 polymorphic and one monomorphic AFLP loci. The average number of fragments per individual was 109.24 (SD=24.95). The reproducibility of the AFLP data was 96.9 %.

According to the Structure analysis, the most probable model was K=3 followed by K=5 (Table 1). The values ΔK and LnP(D) in K=5 were lower than in K=3, but *P. hedraiandra* (H group) emerged only in the K=4 and K=5 models. Therefore, we tested also the K=5 model in other analyses to obtain information about this species.

Tab. 1: Probabilities of individual models in Structure analyse, which are presented by K values.

K	LnP(D) mean similarity coeff.	standard dev. of LnP(D)	ΔK	between runs mean similarity coeff.	standard dev.	Groups of isolates (details in Table 2)
1	-20454.80	3.33	/	/	/	/
2	-15908.10	671.18	0.97	0.54	0.61	C, N
3	-12009.80	11.76	274.95	0.99	0.00	C1, C2, N
4	-11345.10	2274.51	0.35	0.97	0.05	C1, C2, N, H
5	-9889.47	210.47	38.07	0.78	0.28	C1, C2, N, H, F
6	-16446.40	20481.63	0.63	0.70	0.21	/
7	-10014.70	859.08	10.67	0.70	0.23	/
8	-12751.50	6299.11	/	0.74	0.18	/

The bar plots were constructed from K=2 to K=5 (Fig. 1), displaying gradually segregating groups in the following order (Table 2): K=2 – one group includes isolates of *P. nicotianae* and *P.* × *pelgrandis* (N group), and all other isolates (*P. cactorum* complex) fell within the second group.

Tab. 1: Grouping of *Phytophthora* isolates according to the most probable Structure models K=3 and K=5.

Grouping	Group	Species according to ITS profile
K=3	C1	*P. cactorum, P. × serendipita, P. hedraiandra*
	C2	*P. cactorum, P. × serendipita, P. hedraiandra*
	N	*P. nicotianae* (NN), *P. × pelgrandis* (NP)
K=5	C1	*P. cactorum, P. × serendipita, P. hedraiandra*
	C2	*P. cactorum, P. × serendipita*
	F	Finnish *P. × serendipita*
	H	*P. hedraiandra, P. × serendipita*
	N	*P. nicotianae* (NN), *P. x pelgrandis* (NP)

K=3 – the isolates from the *P. cactorum* complex were further divided in two groups, C1 and C2,

both of which included *P. cactorum* and *P. × serendipita*, and *P. hedraiandra* (species indication according to ITS Pheca I and Cox I sequences). K=4 – the additional group H included some isolates of *P. hedraiandra* and Finnish isolates of *P. × serendipita*. K=5 – the F group, which contained only the Finnish isolates of *P. × serendipita*, separated from the H group. In all bar plots, *P. × pelgrandis* represented a combination of *P. nicotianae* and members of the C1 group, while the C2 group did not participate in the hybridization between *P. cactorum* and *P. nicotianae*. In further analyses, the N group was split into two subgroups: NN – *P. nicotianae* and NP – *P. × pelgrandis*. This identification of *P. × pelgrandis* was supported by the hybrid pattern of both the Structure and DNA sequence data.

Analysis of AFLP data

Peak analysis of AFLP markers: Results of the peak analysis are displayed in Table 3. The important results of this analysis were the determined numbers of private markers (present only in the respective groups), fixed markers (present in all individuals of the group) and fixed private markers (present in all individuals of the group, but not in another groups). Private markers were more frequent in the N group than in other groups in both the K=3 and K=5 groupings, and fixed private markers occurred only in the N group. A substantially low number of polymorphic markers was recorded in F and H groups.

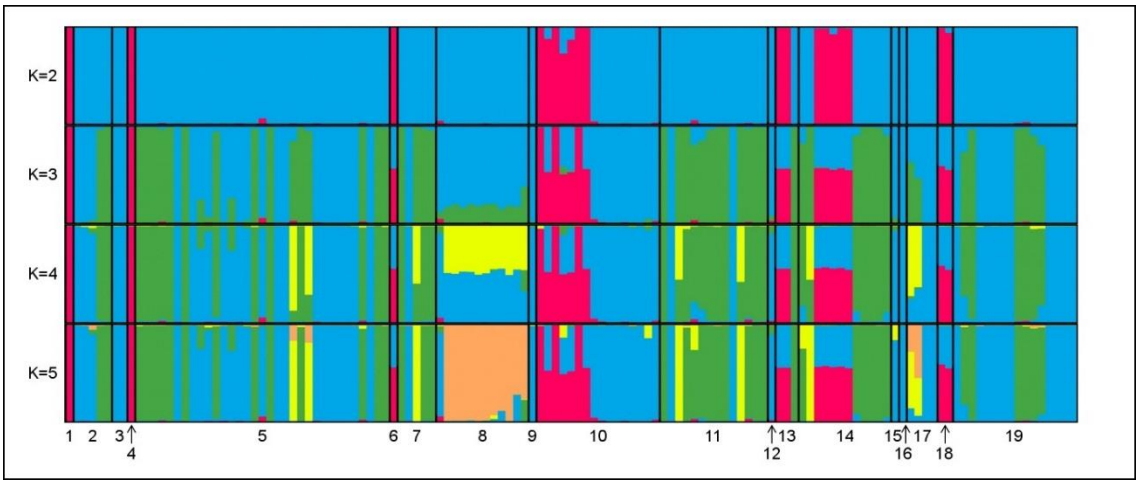

Fig. 1. Bar plots for Structure population models of Phytophthora cactorum complex for K=2 to K=5. Sample origin: 1-USA-Arizona, 2-Belgium, 3-Bulgaria, 4-USA-California, 5-Czech Republic, 6-Germny, 7-Spain, 8-Finland, 9-Greece, 10-Hungary, 11-Switzerland, 12-Iran, 13-Italy, 14-The Nederland, 15-New Zealand, 16-Poland, 17-Slovakia, 18-Tchai-wan, 19-Austria. Colours express the membership of isolates into groups: red – N, blue – C1, green – C2, yellow – H, orange – F.

Clonal analysis: Pairs of isolates with mutual difference lower than fifteen markers (the error rate based on the reproducibility of AFLP) were considered as clones. Clones were detected in all K=5 groups except in the NN subgroup. No clonal pairs were recorded across the groups. The number of clones in the groups is summarized in Table 4.

Hybrid analysis: This index estimates the proportion of alleles that were inherited from one of two parental species to quantify their genetic contribution to offspring, of which confidence interval is defined using maximum-likelihood. Because none of the K=3

Tab. 2 Peak analysis of the AFLP results.

	Markers in K=3 grouping								Markers in K=5 grouping							
	polymorphic		fixed		private		fixed private		polymorphic		fixed		private		fixed private	
	(No)	(%)	(No)	(%)	(No)	(%)	(No)	(%)	(No)	(%)	(No)	(%)	(No)	(%)	(No)	(%)
C1	204	56.7	50	13.9	10	2.8	0	0.0	149	41.4	66	18.3	6	1.7	0	0.0
C2	228	63.3	41	11.4	24	6.7	0	0.0	175	48.6	73	20.3	10	2.8	0	0.0
H	-	-	-	-	-	-	-	-	100	27.8	54	15.0	5	1.4	0	0.0
F	-	-	-	-	-	-	-	-	100	27.8	68	18.9	4	1.1	0	0.0
N	221	61.4	51	14.2	61	16.9	21	5.8	221	61.4	51	14.2	61	16.9	21	5.8
All data	360	-	3	0.8	-	-	-	-	360	-	3	0.8	-	-	-	-

groups can be a hybrid offspring of the two remaining groups, only an analysis of the K=5 groups was performed (Table 5). For the origin of the C2 group the most probable scenarios are the hybridization C1 × H or F × H. *Phytophthora* × *pelgrandis* (NP) is most likely a hybrid of the NN and C1 groups.

AFLP network: The neighbor network (Fig. 2) was constructed on the basis of effective genotype data, where each identified clone was present only once. Bootstrapping was performed using 10,000 bootstrap replicates. The analysis confirmed the homogeneity of the groups of isolates.

The main group comprised the C1, C2, H and F groups, and their approximate bootstrap values were

0.58, 0.59, 0.93, and 0.75, respectively. The more distant second cluster included the N group, which was divided into two groups − the NN (*P. nicotianae*) and NP (*P.* × *pelgrandis*) groups (for both, bootstrap values=1).

Tab. 3: Clonal lineages detected in Structure K=5 groups. In NN subgroup no clonal lineage was recorded.

Group	Number of isolates in this clonal lineage (% of isolates of the group)	Clonal lineage type (names selected arbitrarily)
C1	44 (87%)	P259
	2 (4%)	275/09
	5 (9%)[1]	Five rare genotypes found in only one isolate
C2	29 (71%)	109/07
	2 (5%)	78/07
	10 (24%)[1]	Ten rare genotypes found in only one isolate
H	4 (50%)	CBS 111 725
	4 (50%)[1]	Four rare genotypes found in only one isolate
F	7 (64%)	Ph20
	4 (36%)[1]	Four rare genotypes found in only one isolate
NP	5 (36%)	CBS 114 343
	2 (14%)	JA163
	2 (14%)	PD 93/1331
	5 (36%)[1]	Five rare genotypes found in only one isolate

[1]Number of unique genotypes present in only one isolate

AMOVA: Analysis of molecular variance (AMOVA) revealed relatively low gene flow among the groups. AMOVA performed on the base of the Structure K=3 grouping (Tab. 6) resulted in values of Φ_{ST} was 0.50 – 0.67. The variability rate was divided into 58.21

% among groups and 41.79 % within groups. The overall Φ_{ST} was 0.5821 ($p < 0.001$). When using the Structure K=5 grouping, the Φ_{ST} values were similar or increased (Tab. 7). Φ_{ST} between C1 and H was 0.75, which was the highest value in all analyses. The overall Φ_{ST} was 0.7036, ($p < 0.001$). The variability among and within groups was estimated as 70.36 %, and 29.63 %, respectively.

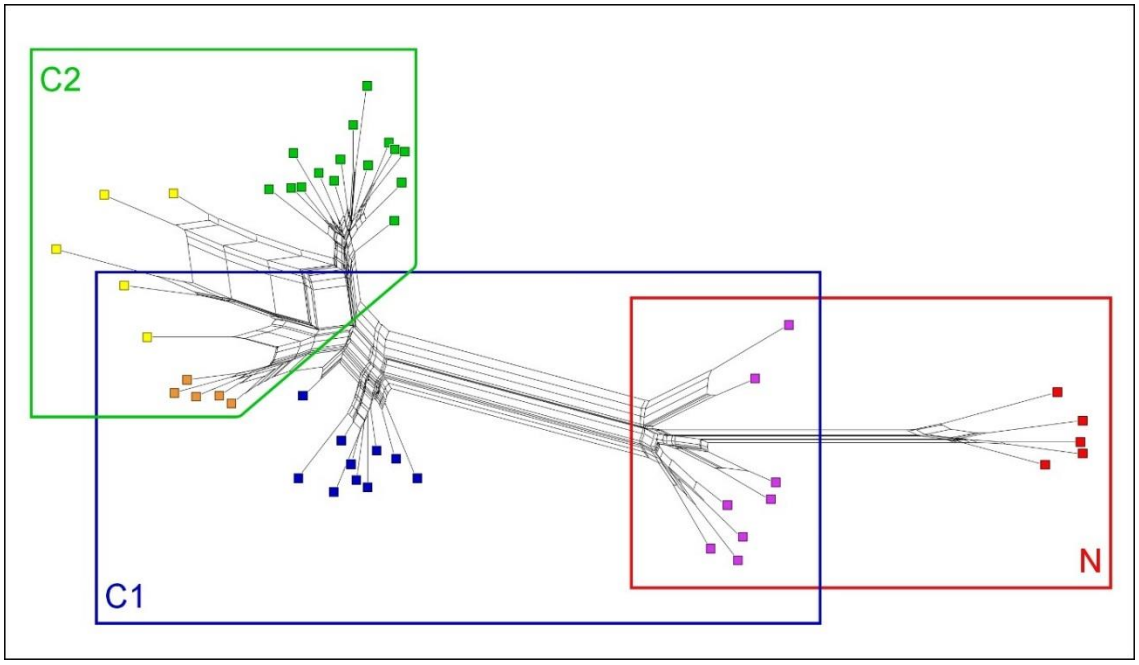

Fig 1. The neighbour network of effective genotypes of K = 3 and K = 5 groups. The tip labels indicate Structure K = 5 groups: C1 – blue, C2 – green, H – yellow, F – orange, and the group N divided into subgroups NP – violet, NN – red. The squares demarcates the groups of K = 3 Structure grouping. Isolates present in intersections of squares represents individuals, which were assigned by Structure to two groups with substantial probability. Intersection of blue and red square: P. × pelgrandis isolates. Intersection of blue and green square: small yellow squares – isolate P13 (belongs in K = 5 grouping to H-prob. 0.6, F-prob. 0.3, and C1-prob. 0.1), isolate P74 (belongs to F-prob. 0.6, and H-prob. 0.4); small orange squares – isolates of F group.

Nei's gene diversity: Nei's gene diversity (D_{Nei}) analysis (Tab. 8) demonstrated relatively low values within single groups (0.05-0.18). In the K=5 grouping, the H group had substantially higher D_{Nei} index than the C1, C2 and F groups, of which the D_{Nei} indexes were rather similar.

Rarity index (DW): The highest values of DW were recorded in the N group. In the K=3 grouping, the DW index of C2 was higher than that of C1. In K=5, the values of DW index of the F and H groups were substantially higher than C2 and C1 (Tab. 9).

Tab. 4: Maximal likelihood hybrid index counted for Structure K=5 groups as an offspring of different parental combinations.

Parent 1	Parent 2	Hybrid	Maximum likelihood hybrid index	Lower bound.	Upper bound.	Ln Likelihood
C1	C2	H	0,27	0,16	0,56	-114,36
C1	C2	F	0,68	0,52	0,73	-104,65
C1	H	F	0,66	0,47	0,71	-101,22
C2	H	F	0,51	0,35	0,56	-150,61
C1	H	C2	0,47	0,38	0,54	-78,86
F	H	C2	0,62	0,53	0,68	-79,25
C1	F	H	0,42	0,27	0,49	-158,70
C2	H	C1	0,66	0,59	0,75	-128,33
H	NN	NP	0,63	0,55	0,70	-153,28
C1	NN	NP	0,53	0,46	0,59	-117,26
C2	NN	NP	0,43	0,36	0,50	-144,73

Tab. 5: AMOVA pairwaise Φ_{ST} values of the Structure K=3 grouping.

	C1	C2	N
C1	0.00		
C2	0.50	0.00	
N	0.66	0.67	0.00

Tab. 6: AMOVA pairwaise Φ_{ST} values of the Structure K=5 grouping.

	C1	C2	H	F	N
C1	0.00				
C2	0.63	0.00			
H	0.75	0.57	0.00		
F	0.60	0.63	0.63	0.00	
N	0.70	0.71	0.63	0.63	0.00

Tab. 7: Nei's gene diversity index (D_{Nei}) values of K=3 and K=5 isolate groups, the values of (D_{Nei}) were calculated also for NN and NP subgroups.

	K=3			K=5		
	observed diversity	95% confidence interval		observed diversity	95% confidence interval	
		lower	upper		lower	upper
C1	0.078	0.065	0.091	0.050	0.041	0.061
C2	0.094	0.082	0.107	0.065	0.054	0.077
H	-	-	-	0.109	0.090	0.129
F	-	-	-	0.068	0.055	0.082
N	0.182	0.162	0.201	0.182	0.163	0.202
NN	-	-	-	0.130	0.108	0.152
NP	-	-	-	0.108	0.092	0.125

Tab. 8: Rarity indexes (DW) of both Structure K=3 and K=5 groups.

sample	n	DW index (K=3)	DW index (K=5)
C1	51	1.70	1.38
C2	41	2.56	2.44
H	7	-	3.26
F	12	-	3.07
N	19	6.85	6.85

Sequence analyses

ITS region: Five variable nucleotide positions in the ITS sequences were present, and the ITS genotypes were identified according to Man in 't Veld et al. (2007, 2012). The distribution of genotypes in the Structure K=5 groups is summarized in Table 10. The *P. cactorum* genotype was recorded only in the C1 group, while the *P. hedraiandra* genotype (identical to the ex-type isolate CBS 111725) was observed in the C1, C2 and H groups. *P.* × *serendipita* genotypes appeared in C2.

Pheca I: Three variable positions were found in the Pheca I sequences (Tab. 11). The genotypes were assigned to Structure K=5 groups. As no Pheca I sequence of *P. hedraiandra* nor *P.* × *serendipita* has been published at GenBank, the sequence of *P. hedraiandra* from the ex-type isolate was obtained in this study. The genotypes attributed to *P. cactorum* in the metadata of Genbank sequences KC109824 and FJ459731 (Faedda et al. 2013, Hurtado-Gonzales et al. 2009) occur in C1

and C2 groups, although the published sequences do not include position no. 52. We revealed three unpublished genotypes with various numbers of polymorphic positions, indicating hybridization between *P. cactorum* and *P. hedraiandra*; such intermediate genotypes occur only in the F group.

Tab. 10: Variable positions in the ITS rDNA region, and occurence of sequence types in Structure K=5 grouping.

ITS genotype	Genotype occurrence in Structure K=5 groups	74	100	101	611	686
P. cactorum[1]	C1	G	G	T	G	T
-	C1	G	G	T	R	T
-	C1	G	G	T	A	T
-	F	G	G	T	A	G
-	C2, H, F	G	G	T	G	G
P. × serendipita[1]	C2	R	R	Y	G	K
.-	C2	R	R	Y	G	T
P. hedraiandra[1]	C1, C2, H	A	A	C	G	G

[1]Assignment of sequence to Man in´t Velds (2007) species. (R=A+G, Y=C+T, K=T+G).

Tab 11: Variable positions in the Pheca I region of DNA, and occurence of genotypes in Structure K=5 groups.

Species	Genotype occurrence in Structure K=5 groups	52	146	305
***P. hedraiandra*[1]-**	**H**	T	G	A
.	**H**	T	G	G
.	**H**	G	G	A
.	**F**	K	G	A
.	**F**	K	R	R
.	**F**	K	R	G
.	**C1, C2**	T	A	G
.	**C1**	G	A	G
***P.cactorum*[2]**	**-**	-	A	G

[1] The sequence type recorded in the type isolate of *P. hedraiandra* – CBS111725

[2] The GenBank sequences KC109824 and FJ459731

Cox I: Ten variable positions were recorded in Cox I region (Tab. 12), which were capable of distinguishing *P. cactorum* from *P. hedraiandra* (according to GenBank sequences published by Robideau et al. 2011). A new genotype was detected, which

seemed to be a recombinant between *P. cactorum* and *P. hedraiandra* genotypes. This genotype occurred only in isolate no. P13 from the H group, which was isolated from *Quercus palustris* (a non-native species) in Slovakia.

All newly obtained sequences were submitted to the EMBL-EBI database (the accession numbers are given in supplementary materials, Table 1S).

Tab 12: Variable positions in the Cox I region of DNA, and occurence of individual sequence types in Structure K=5 grouping.

Species	Occurrence in Structure K=5 group	105	166	268	391	409	439	566	751	793	820
P. hedraiandra	C2, H	A	A	T	C	G	G	T	C	A	C
	H	C	A	A	A	T	T	T	A	A	T
P. cactorum	C1, C2, H, F	C	G	A	A	T	T	A	A	T	T

Phylogeny

The phylogenetic analysis of the combined dataset resulted in a phylogram (Fig. 3) composed of two main nested clades, roughly resembling the delimitation of Structure grouping K=3. The proximal clade with high support (1.00 PP) comprises most of the C2 and H isolates (K=5). The H group is well supported but is nested within the C2 group. The distal clade is composed of C1 and F isolates and one H isolate (P13). Isolate P13 (grouped into the H group by Structure with 60 % probability) has an isolated position and a unique Cox I genotype. The isolates 275/09 and 292/09 match the C1 group in the Structure K=5 grouping with the probabilities of 0.75 and 0.73 wheres clustering with the C2 group had probabilities of 0.24 and 0.27, respectively.

Summary of AFLP+DNA sequence data

P. hedraiandra belongs to the H group according to the Structure results and DNA sequence data. However, the species was not separated as a distinct group by the most probable Structure grouping K=3. According to the peak analysis, almost all groups did not contain fixed private markers, and the number of private markers was low. The results of AMOVA revealed higher, but not total genetic differentiation, which does not exclude appreciable gene flow between the H, C2 and F groups and probable gene flow between C1 (which most resembles *P. cactorum* sensu stricto) and *P. hedraiandra* (H). The level of gene flow between *P. cactorum* (C1) and *P. hedraiandra* (H) is comparable

to gene flow between *P. cactorum* (C1) and the N group (*P. nicotianae* and *P.* × *pelgrandis*). In addition, although the majority of isolates of the C1 group are characterized by an ITS genotype of *P. cactorum,* and H group isolates usually bear the ITS genotype of *P. hedraiandra*, some individuals with *P. hedraiandra* ITS genotype also grouped in C1. The situation is similar for the maternally inherited Cox I, where the *P. hedraiandra* genotype was recorded in four groups: H, C1, C2 and F. These results indicate that *P. hedraiandra* is probably taxonomic cluster within *P. cactorum* rather than a separate species. The pattern of diversity was different in the Pheca I region, where such ambiguity was not discovered.However, the variability of Pheca I sequences, particularly among members of the H group, indicate recombination between *P. cactorum* and *P. hedraiandra*. Heterozygous Pheca I genotypes were typical for isolates of the F group. The isolates identified as *P. hedraiandra* according to ITS sequences occured in three different Structure K=5 groups (C1, C2, H), while isolates with ITS genotypes of *P.* × *serendipita* were found in four groups (C1, C2, F, H).

In the C2 group isolates with ITS genotypes of *P.* × *serendipita* or *P. hedraiandra*, were more common than isolates with ITS genotypes of *P. cactorum.* Pheca I of C2 isolates was always of the *P. cactorum* type, whereas the mitochondrial Cox I region could be from *P. cactorum* or *P. hedraiandra* type. These results strongly support the hybrid origin of the C2 group, which is also confirmed by the ML hybrid index (ML= 0.47, Ln prob. -78.86) and low Φ_{ST} between C2 and H (0.57). Most likely, C2 is a hybrid of C1 × H. Although the possibility of C2 being an offspring of F and H cannot be excluded due to ML = 0.62 (Ln prob. -79.25), the DNA sequence data support the first hypothesis. The relatively high Φ_{ST} between the C1 and H groups (0.75) indicate a low probability of hybridization, but respective gene flow is not ruled out. The close relationships among the C1, C2 and F groups are demonstrated by the Φ_{ST} values (Table 7). The ML hybrid index indicates that the F group originated from a hybridization between either C1 and C2 (ML 0.68, Ln prob. -104.65) or C1 and H (ML 0.66, Ln prob. -101.22). The ITS sequences of the F group were either of the *P. cactorum* type or transitional between those of *P. cactorum* and *P. hedraiandra* (Table 10). Pheca I sequences included many heterozygous positions occurring exclusively in this group. The Cox I sequences were of the *P. cactorum* type. This sequence arrangement supports the possibility of the F group being a hybrid between C1 and H, while the rather high rarity index (DW=3.07) indicates its long-term isolation.

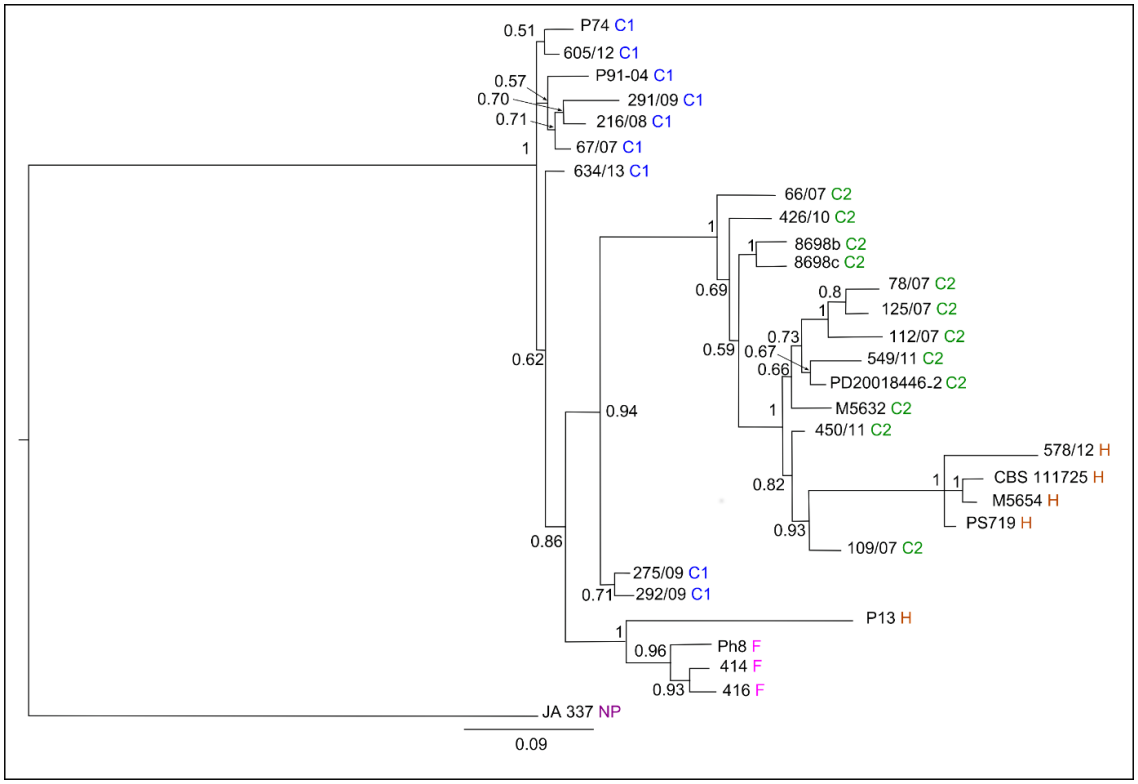

Fig. 2. Phylogram for *Phytophthora cactorum* complex calculated using Bayesian analysis on the base of combination squence and AFLP data. Values stated at branches express values of of Bayesian posterior probabilities. Scale bar express expected number of substitutions per one position. End branches are marked by number of isolate and its assignment to groups of K=5 Structure grouping. As an outgroup the isolate of *P.* ×*pelgrandis* was used.

Our results confirmed the repeated occurrence of combinations of different genes that are characteristic of different species in a single isolate. For example, the isolate M5632 (C2 in both K=3 and K=5 grouping) had ITS and Cox I sequences of *P. hedraiandra*, while the Pheca I sequence resembles *P. cactorum*. Most of the isolates from the C2 and F groups include polymorphism in variable positions of the ITS or Pheca I genotypes. The ITS intragenomic polymorphism in C2 (according to the K=5 grouping) matches to a hybrid species *P.* × *serendipita* (Man In 'T Veld et al. 2007), but C2 isolates lack polymorphism in Pheca I. In contrast, Pheca I polymorphism is restricted to the isolates from the F group having ITS and Cox I genotypes mostly of typical *P. cactorum*.

Morphology

Morphology of oogonia: Isolates of the C1, C2, F, H and NP groups had homothallic breeding system, while isolates of the NN group were heterothallic. In the NN group, all antheridia were amphigynous, while in the other groups, the presence of both paragynous and amphigynous antheridia was recorded. The oospores were nearly plerotic in all groups, with the lowest aplerotic index on the plerotic/aplerotic margin (0.60) being recorded in the NN and H groups. The values of the other groups (C1, C2, F, NP) were 0.63-0.67 (Table 13). The highest value of the oospore wall index was recorded in the H group (0.50), while the lowest value was in the NN group (0.42). Values of the other groups (C1, C2, F, NP) were 0.45-0-49. Both oogonium and oospore diameters were highest in the NP group, and the lowest values were recorded in the F group (Table 13).

Morphology of sporangia: Sporangia of all isolates were papillate, ellipsoid to ovoid. The on average largest sporangia were recorded in the NN group. Sporangia of the C1 and C2 groups were rather similar; they were approximately 20 % shorter and narrower than in the NN group. Dimensions of F group sporangia were even smaller, and the lowest values were recorded in the H group. The sporangia of the NP group were intermediate between the NN, C1 and C2 groups. The sporangial papilla dimensions were highest in the NP group, and the values of NN group were a slightly lower. The values of C1 and C2 groups were intermediate, and the lowest values were recorded in the F and H groups (Tab. 13).

Discriminant analysis among groups: According to the principal component analysis and correlation analysis, two morphological characters and two calculated indexes were chosen for discriminant analysis: oogonial diameter, sporangial width, aplerotic index and oospore wall index. Discriminant analysis revealed that the chosen characters and indexes are partly capable to distinguish the K=5 groups included in this study, although the total differential capability of the model was rather low : Wilk's λ_{total}=0.419, $p<0.0001$ (The value of Wilk's λ_{total} could be on the scale 0 – the best discrimination, to 1 – none discrimination and express the total discriminatory ability of model). The values of λ_p of single characters were the following: oogonial diameter λ_p=0.573 $p<0.0001$, sporangial width λ_p=0.850, $p<0.0001$, aplerotic index λ_p=0.944, $p<0.0001$ and wall index λ_p=0.918, $p<0.0001$(the values of λ_p of the single variables express the discriminatory ability of model when the particular variable is eliminated from model).

The differences among the K=5 groups inferred on the basis of Mahalanobis distances are rather low (Table 14); only NN and NP groups are well discriminable from the others. A separate analysis including only the C1, C2, F and H groups lead to substantially lower discrimination of the groups, Wilks λ_{total}=0.745, p<0.0001.

Tab. 93: Mean values (µm) of morphological characters in K=5 groups defined by Structure analysis.

	C1		C2		F		H		NN		NP	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Oogonial diameter	29.31	2.71	27.70	2.77	27.14	3.21	30.78	2.75	25.89	2.83	35.72	3.64
Oospore diameter	25.46	2.58	24.23	2.49	23.42	2.73	25.83	2.47	21.73	2.17	30.59	3.57
Oospore wall thickness	2.28	0.43	2.26	0.35	2.31	0.51	2.70	0.58	1.82	0.41	3.11	0.69
Sporangial length	39.43	6.76	39.40	8.21	36.41	5.89	32.69	4.57	54.04	6.31	43.03	8.11
Sporangial width	30.17	5.41	28.88	5.98	28.67	4.03	26.62	3.59	36.67	6.74	34.48	6.37
Papilla length	6.91	1.01	6.93	1.09	6.46	1.04	6.31	0.87	7.40	0.88	7.76	1.49
Papilla width	4.99	1.00	5.24	1.24	4.68	0.89	4.38	0.75	6.30	1.17	5.99	1.49
Aplerotic index	0.66	0.09	0.67	0.07	0.65	0.08	0.60	0.08	0.60	0.06	0.63	0.07
Oospore wall index	0.45	0.07	0.46	0.05	0.48	0.08	0.50	0.07	0.42	0.07	0.49	0.08

Tab. 104. Mahalanobis distances based on Wilks λ_{total} among the K=5 groups defined by Structure analysis. The N group was divided into NN and NP subgroups.

	C2	C1	F	H	NN	NP
C2	0.000					
C1	0.361	0.000				
F	0.947	0.375	0.000			
H	2.357	3.200	2.327	0.000		
NN	6.182	5.698	7.143	12.831	0.000	
NP	6.346	8.854	10.156	5.501	16.298	0.000

Sesility of antheridia: According to De Cock & Lévesque (2004), the sessility of antheridia is an important discriminative character between *P. cactorum* and *P. hedraiandra*. The analysis included isolates of C1, C2, H and F groups. In all four groups, both sessile antheridia and antheridia with stalk were detected. Two types of sessile antheridia were further distinguished – intercalary antheridia and terminal antheridia on

very short stalks which were similar to a lateral hyphal branch. The stalked antheridia were always terminal. Intercalary antheridia were separated from hypha by two septa, while terminal antheridia were separated by only one septum. A Kruskall – Wallis test based on grouping according to Structure results K=3 (C1, C2) revealed no significant differences (p=0.833) between groups. The same test using the K=5 grouping (C1, C2, H, F) revealed differences between the groups C2 (*P.* × *serendipita*) and H (*P. hedraiandra*), p=0.0228. *P. hedraiandra* has significantly lower proportion of sessile antheridia than *P.* × serendipita. There were no significant differences between other K=5 groups.

Tab 115: Cardinal temperatures and average growth (mm/day) of K=5 groups. The values in brackets were recorded in a minority of samples (less than 33%).

	Maximum temperature	Optimum temperature	Minimum temperature	Growth at temperatures used in discrimate analysis			Average growth at optimum temperature
				32°C	15°C	3°C	
C1	32°C	23°C	3°C	1.02	4.88	0.13	7.19
C2	30 (32)°C	23°C	3 (5)°C	0.71	5.15	0.12	7.35
F	30 (32)°C	25°C	(3) 5 (5<n<10)°C[1]	0.10	2.75	0.09	4.88
H	30 (32)°C	25°C	(3) 5°C	0.22	4.13	0.19	6.37
NN	33°C	25°C	(5<n<10)°C[1]	5.21	2.98	0.00	5.71
NP	(33) 35°C	30°C	(5<n<10)°C[1]	8.16	4.94	0.00	9.83

[1] If no temperature between 5 and 10°C was measured, the value was estimated for isolates which did not grow at 5°C, but the growth at 10°C was already substantial. Those values were excluded from discriminant analysis.

Cardinal temperatures

The temperature-growth relations of all isolates tested are summarized in Table 15. C1 and C2 of K=5 groups showed almost identical growth at the different temperatures. The H group had higher optimum temperatures than C1 and C2. The growth of a majority of isolates from the F group started at higher minimum temperature and was substantially slower than in C1 and C2. The growth of NN and NP groups started and terminated at considerably higher temperatures with 33°C the maximum; the maximum temperature of the NP group was even higher - 35°C. The correlation analysis revealed that only three of

the fifteen temperatures tested were uncorrelated (32 °C, 15 °C and 3 °C). The daily growth rate values of individual isolates at these temperatures were used in discriminant analysis. The analysis based on K=3

Tab. 16: Mahalanobis distances between groups based on cardinal temperatures. The subdivision into the groups is based on Structure analysis K=3, the N group is divided in NN and NP subgroups.

	C1	C2	NN	NP
C1	0.000			
C2	0.984	0.000		
NN	25.846	34.247	0.000	
NP	42.412	48.650	7.653	0.000

Tab. 127: Mahalanobis distances between groups based on cardinal temperatures. The subdivision into the groups is based on Structure analysis K=5, the N group is divided in NN and NP subgroups.

	C1	C2	H	F	NN	NP
C1	0.000					
C2	0.722	0.000				
H	2.183	4.732	0.000			
F	17.114	23.843	8.450	0.000		
NN	42.146	52.811	36.467	20.852	0.000	
NP	43.237	50.420	48.074	49.370	14.606	0.000

grouping revealed that the growth at 32 °C (λ_p=0.701, p<0.0001) and 15 °C (λ_p=0.199, p<0.0001) were useful for discriminating between K=3 groups. The temperature 3°C was excluded during analysis as redundant by the model. Two main canonical roots were calculated, corresponding to the two temperatures. The first root corresponded to 32 °C with a correlation coefficient of -1.096, and the second root corresponded to 15 °C with coefficient of -0.973. The total efficiency of the model in discriminating individual groups was relatively high: Wilk's λ_{total}=0.127, p<0.0001. The differences among groups are summarized in Table 16. The model differentiating efficiency was higher for K=5 grouping than for K=3 grouping (Wilk's λ_{total}=0.044, p<0.0001); λ_p of the growth rate at 32 ° was 0.248, whereas for 15°C it was 0.179. In this model, the highest Mahalanobis

distances were detected between the C1 and C2 group and the N and P groups. The distances between C1, C2 and H groups were low, and the distance between C1 and C2 groups was almost identical (Table 17).

5.2 Growth response of *Phytophthora cactorum* complex, *P. nicotianae* and *P.* × *pelgrandis* to antibiotics and fungicides (Paper 2)

Effect of substances

ANOVA test showed significant differences among isolate groups against all chemicals tested, except streptomycin.

Metalaxyl: The average growth inhibition caused by metalaxyl ranged from 73.5 % in *P. nicotianae* to 97.5 % in *P.* × *pelgrandis*. The values of inhibition in the *P. cactorum* groups were 81.3-93.2 % (Tab. 18). The differences among those three groups and some groups within *P. cactorum* were significant (Tab. 18, Fig. 4).

Mancozeb: The lowest growth inhibition was recorded in the F and P groups – 40.4 % and 46.1 %, respectively, while the highest value – 62.7 % was in the C1 and N groups (Tab. 18). The results of an ANOVA and subsequent Tukey's HSD test divided all isolate groups into two subsets (Tab. 18). The first one included the C1, C2, H, and N groups, while the second one included the N, P and F groups ($p<0.001$). The N group did not differ significantly from any other group; therefore, it was placed in both subsets (Tab. 18, Fig. 4).

Dimethomorph: The highest value of growth inhibition was recorded in the H group – 83.5 %, while the lowest value was in *P. nicotianae* – 46.8 %. ANOVA and subsequent Tukey's HSD test confirmed significant differences between most of the *P. cactorum* groups, *P. nicotianae*, and *P.* × *pelgrandis*. Differences between groups F − P and F − C2 were insignificant (Tab. 18, Fig. 4).

Chloramphenicol: The range of inhibition rates was 33.1 – 88.1 % (Tab. 18, Fig. 4). The ANOVA and subsequent Tukey's HSD test results confirmed significant differences between the groups as follows: all the *P. cactorum* groups except H were different from either *P. nicotianae* or *P.* × *pelgrandis,* and those two groups were also significantly different from each other. There was no significant difference revealed between groups C1, C2, and F.

Streptomycin: The highest growth inhibition was recorded in the C1 group – 78.9 %, while the lowest value was in the N and H groups – 63.6 % and 64.8 %, respectively.

All differences among the groups were evaluated as insignificant by ANOVA. The growth inhibition rates and results of the tests are summarized in Tab. 18.

Tab. 18. Results of ANOVA and subsequent multiple comparisons in Tukey's HSD tests. The table includes average values of growth inhibition caused by chemicals of *Phytophthora cactorum* groups (C1, C2, F and H). *P.nicotianae* (N) and *P.* ×*pelgrandis* (P) and their placement in homogenous subsets (1-4) determined by Tukey's HSD test. Some groups belong to two distinct subsets.

Dimethomorph			Mancozeb			Streptomycin		
Group	Inhibition rate (%)	Homogenous subset	Group	Inhibition rate (%)	Homogenous subset	Group	Inhibition rate (%)	Homogenous subset
N	46.8	4	F	40.4	1	N	63.6	1
P	64.5	2	P	46.1	1	H	64.8	1
F	68.2	2, 3	H	46.6	1	F	74.9	1
C2	74.5	1, 3	N	55.0	1, 2	C2	76.4	1
C1	82.9	1	C2	61.8	2	P	77.2	1
H	83.5	1	C1	62.7	2	C1	78.9	1

Metalaxyl			Chloramfenicol		
Group	Inhibition rate (%)	Homogenous subset	Group	Inhibition rate (%)	Homogenous subset
N	73.5	2	C2	33.1	1
F	81.3	2, 3	F	41.2	1, 2
C2	90.1	1, 3	C1	44.2	2
H	92.0	1	P	55.8	4
C1	93.2	1	H	79.3	3
P	97.5	1	N	88.1	3

Growth responses of the isolate groups

This study confirmed the variability of resistance in closely related *Phytophthora* species. *P. nicotianae* seems to be more resistant to metalaxyl, mancozeb and dimethomorph, while chloramphenicol was less tolerated. The *P. cactorum* groups C1 and C2 were similar in their response to all tested chemicals. Both groups were rather sensitive to metalaxyl, streptomycin, mancozeb and dimethomorph but more tolerant to chloramphenicol. The H group had similar responses to metalaxyl and dimethomorph and was more resistant to chloramphenicol than other groups. The F group was more tolerant to metalaxyl and mancozeb then other groups of *P. cactorum* complex, while the responses to dimethomorph, streptomycin and chloramphenicol were similar as in C1 and C2 groups. *P.* × *pelgrandis* had an average resistance to almost all chemicals, except for metalaxyl, for which its reaction was the most susceptible of all tested groups.

Discriminant analysis among growth inhibition rates of the isolate groups

A discriminant analysis was performed to evaluate the joint effect of all tested substances on the growth of the examined isolates. Since this analysis demands uncorrelated values between evaluated groups, a correlation analysis was performed, resulting in mostly low correlations between the growth inhibition values of different chemicals. Values of metalaxyl were correlated to those of streptomycin, and dimethomorph (Spearman's rank correlation coefficient = 0.33, and 0.29, $p<0.05$). Another correlations were rather weak: streptomycin/chloramphenicol (-0.20, $p<0.05$), metalaxyl/mancozeb (0.19, $p<0.05$), chloramphenicol/mancozeb (-0.21, $p<0.05$). Based on the correlation analysis, the values of streptomycin were excluded from the discriminant analysis. The main results of the discriminant analysis is the determination of Wilks' λ values, which describes the ability of the analytical model to differentiate between *Phytophthora* groups based on the resistance to each chemical. This quantity could fall between 0 (the best discrimination between the groups) and 1 (no ability to discriminate between the groups).

Tab. 19. Mahalanobis distances between *Phytophthora cactorum* groups (C1, C2, F, and H). *P.nicotianae* (N) and *P. ×pelgrandis* (P). The distances are calculated in discriminant analysis based on inhibition rates caused by chemical substances.

	C1	C2	F	H	N	P
C1	**0.00**	**0.88**	**4.84**	**10.36**	**39.10**	**9.03**
C2	**0.88**	**0.00**	**3.80**	**14.52**	**41.92**	**9.30**
F	**4.84**	**3.80**	**0.00**	**8.97**	**27.57**	**4.04**
H	**10.36**	**14.52**	**8.97**	**0.00**	**15.01**	**5.69**
N	**39.10**	**41.92**	**27.57**	**15.01**	**0.00**	**19.78**
P	**9.03**	**9.30**	**4.04**	**5.69**	**19.78**	**0.00**

Moreover, the values of Wilks' λ corresponding to single canonical roots were determined, which reflect the contribution of the root to the differentiation ability of the model. The relationships between the canonical roots and the effects of the tested substances on isolate growth and their contribution to differentiating ability of model were ascertained. The Wilks' λ of our complete model was 0.089 ($p<0.001$), indicating a strong ability to discriminate between the groups. The relevance of a single chemical to the model was expressed by particular Wilks' λ values as follows: metalaxyl 0.12, mancozeb 0.13, chloramphenicol 0.31, and dimethomorph 0.18. A second important

result of the discriminant analysis was the determination of the Mahalanobis distances between the *P. cactorum* groups based on the growth inhibition. According to the Mahalanobis distances (Tab. 19 and 20), the most distant group is *P. nicotianae*. The distances among the remaining groups are smaller, with the smallest distance between C1 and C2. If the N and P groups were excluded from the analysis, the distances between members of *P. cactorum* groups – C1, C2, F and H groups (Tab. 20) were similar to those of the previous results (Tab. 19), but the differentiating ability of the model decreased (total Wilks' $\lambda = 0.217$, $p<0.001$). Distances among isolate groups based on growth inhibition rate of *Phytophthora* isolates caused by tested chemicals are graphically displayed by cluster analysis (Fig. 4).

Table 20. Mahalanobis distances between *Phytophthora cactorum* groups (C1, C2, F, and H). The distances are calculated in discriminant analysis based on inhibition rates caused by chemical substances.

	C1	C2	F	H
C1	**0.00**	**1.03**	**4.84**	**9.64**
C2	**1.03**	**0.00**	**3.72**	**13.08**
F	**4.84**	**3.72**	**0.00**	**7.23**
H	**9.64**	**13.08**	**7.23**	**0.00**

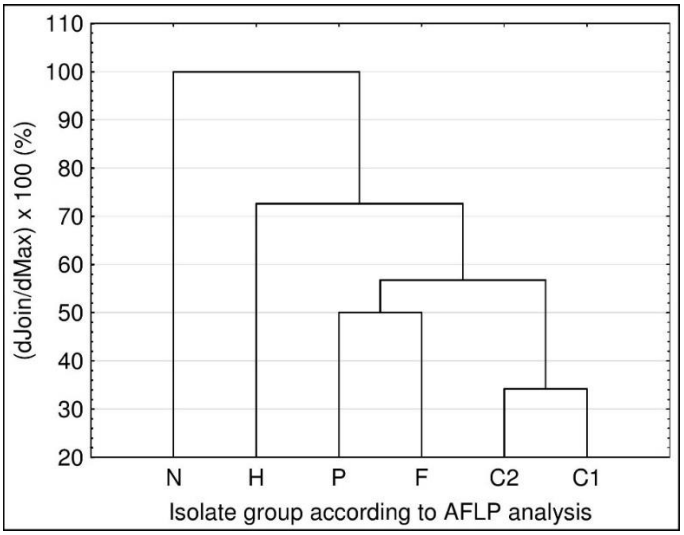

Figure 4. The cluster analysis of similarities among *Phytophthora cactorum* groups (C1, C2, H, F), *P.* ×*pelgrandis* (P) and *P. nicotianae* (N) based on average values of growth inhibiton. The inhibition rates caused by tested chemicals (metalaxyl, mancozeb, dimethomorph, streptomycin and chloramphenicol) are included in analyses. Simple joining method with Chebyshev distances was applied, distances of join among groups are expressed as percentage of the distance between the most distant groups.

6. Discussion

The results unraveled evolutionary relationships between *P. cactorum, P. hedraiandra, P. nicotianae* and two hybrid species *P.* × *serendipita* and *P.* × *pelgrandis. Phytophthora nicotianae* and *P.* × *pelgrandis* are clearly distinguishable from the other taxa by morphological, molecular and growth characters, but the relations among the other groups are more complicated. The results conclude that the data from individual DNA sequences (like ITS), which are frequently used in DNA barcoding, are insufficient for the identification of the species of the *P. cactorum* complex.

The conventional morphological identification of *Phytophthora* species is based on a combination of both sporangial and oogonial dimensions (Waterhouse 1963). All morphometric data from this study correspond to the published characters of *P. cactorum* (Blackwell 1943, Waterhouse 1963), although there were some differences between groups in particular characters. The summarized morphological differences among groups were insignificant and are in agreement with those published by Hantula et al. (2000) for *P. cactorum*. Morphological delimitation of *P. cactorum* from *P. hedraiandra* is questionable. De Cock & Lévesque (2004) mentioned the type of antheridia as an important morphological character for discriminating between *P. hedraiandra* and *P. cactorum*. According to their work, *P. hedraiandra* should have predominantly sessile antheridia. However, according to the results of the present study the difference in antheridia sessility between *P. cactorum* and *P. hedraiandra* is insignificant. Only the difference between *P. hedraiandra* (H group) and C2 group (mainly *P.* × *serendipita*) was significant, but the proportion of sessile antheridia was higher in *P.* × *serendipita* than in *P. hedraiandra*. Moreover, due to high variability of *antheridial features* (paragynous/amphigynous; sessile/stalked) within a single isolate, the morphology of antheridia does not seem to be a useful character for species discrimination.

This study did not show any geographical structure of groups from the *P. cactorum* complex except for the F group, which has been recorded mainly in Finland. The isolates of other groups are dispersed across Europe, mostly clonally. For example, the clonal type of isolate ICMP11853 from the C1 group has a distribution across all of Europe (from Spain and the Netherlands to Bulgaria and Greece), Iran and New Zealand. Such results are in agreement with the clonal nature of *P. cactorum* described by Hantula et al. (1997).

Our results indicate that *P. hedraiandra* (H group) is the group with the highest genetic diversity (D_{Nei}=0.11) as well as the highest rarity index (DW=3.26), indicating long term isolation of the population (Schönswetter & Tribsch 2005). Because genetic diversity is usually higher in indigenous populations, these data suggests a European origin of this group, although Man In'T Veld et al. (2007) treated *P. hedraiandra* as a recently introduced species in the Netherlands. Furthermore, the ITS genotype of *P. hedraiandra* was noted by Cooke et al. (1996) as an atypical isolate of *P. cactorum* from Wales, thus arguing that this genotype was present in Europe for more than ten years before the formal description of the species. On the contrary, *P. cactorum* in the narrow sense (C1 group) has the lowest genetic diversity as well as the lowest rarity index (DW=1.38), which may indicate a non-European origin of the C1 group. The origin of *Phytophthora* species, and even the whole clade (Brasier et al. 1999, Man In 'T Veld et al. 2007, Goss et al. 2011, Mammella et al. 2013) is largely unclear. Nevertheless, the North American origin of *Phytophthora* clade 1 is expected (Jung et al. 2015). Our results indicate a possible European origin of *P. hedraiandra*, although the data may be influenced by gene flow among the groups within the *P. cactorum* complex, and it cannot be ruled out that *P. hedraiandra* might have been introduced on multiple occasions from an exotic origin and therefore has a relatively high genetic diversity.

P. cactorum was described in central Europe (Wroclaw in Poland), however, on non-indigenous species of cacti – *Cereus giganteus* and *Melocactus nigrotomentosus* (Lebert & Cohn 1870). The spceies recently has worldwide distribution, so both European and non-European origin is possible. The results of presented study supports hypothesis, that at least *P. cactorum sensu stricto* (the group C1) is of non – European origin. Such hypthesis is supported by low genetic variability of *P. cactorum*, and by their phylogenetic proximity to another non-native species, of which non-European origin is possible infer on the base of indirect evidence mentioned by Jung et al. (2016). The gene flow between *P. cactorum* and well distinguishable *P. nicotianae* was repeatedly confirmed. From our results arised, that only *P. cactorum senu stricto* (C1 group) participates on hybridization between *P. cactorum* and *P. nicotianae*. Other species related to *P. cactorum* from *Phytophthora* clade 1 are *P. pseudotsugae* (Hamm & Hansen 1983) from the Western USA and *P. idaei* from Scotland (Kennedy & Duncan 1995, Greenh et al. 1999). Both species are closely related to *P. cactorum* (Cooke et al. 1996, Martin et al. 2012); therefore, hybridization events between them and species from the *P. cactorum* complex

are possible. However, the ITS sequences of the two species obtained from the GenBank (HQ643330, HQ261655, HQ261654, FJ172263, HQ643246 and HQ261579) are not identical to any sequence from our dataset, and our data do not support such hybridization. Hantula et al. (2000), Bhat et al. (2006), and Eikemo et al. (2004) reported that in Europe, California, and on a global scale, respectively, a similar host specificity of two to three different pathotypes of *P. cactorum* according to the preference of attacked organs of strawberry and host ranges exist: A) the crown rot pathotype with lower genetic variability and B) the leather rot pathotype, which attacs strawberry fruits and also another host species than *Fragaria*. The differences between lineages are confirmed by molecular analysis. According to Eikemo et al. (2004), the crown rot pathotype could be indigenous to North America, and on the base of our results is possible assume connection between this pathotype and our C1 group. The Czech isolates no. 434/11 and 634/13 induced crown rot in *Fragaria* and belonged to the C1 group. The association of the crown rot pathotype with *P. cactorum sensu stricto* is also supported by similarly low genetic diversity found in the C1 group in this study and in crown rot type isolates found by Eikemo et al. (2004). The leather rot pathotype could be associated with the C2 or H groups (*P. hedraiandra)*, which have higher genetic diversity like the leather rot isolates (Hantula et al. 2000, Eikemo et al. 2004). These studies also mention a separate *P. cactorum* lineage specific to *Betula*. Some of these isolates (Ph4, Ph8 and Ph20) were included in this study and grouped separately as the F group, which differs from the others by substantially lower growth, high variability of minimum and maximum temperature, and commonly high oogonia abortion rates (100 % in isolates nos. 415, 417, 420 and 451) and intraspecific polymorphism of at least three positions of Pheca I sequences. Since the F group also included isolates from other hosts than *Betula* (*Amelanchier* – isolate no. P74, *Rhododendron* – isolate no. 440, *Sorbus* – isolate no. 451), this group is not restricted to *Betula* as a host.

The interspecific gene flow between *Phytophthora* species is still insufficiently understood. This process between either two heterothallic or one heterothallic and one homothallic species was described by Goodwin & Fry (1994) in a laboratory experiment. Another type of gene transfer that has been artificially achieved under laboratory conditions is zoospore fusion (Ersek et al. 1995, Ersek & English 1998, English et al. 1999). The theoretical mechanism of hybridization is somatic fusion, as mentioned by English et al. (1999). Other confirmed hybridization events between *Phytophthora*

species are well documented (Hurtado-Gonzales et al. 2009, Hurtado-Gonzales & Lamour 2009, Brasier et al. 2004, Bertier et al. 2013, Nagel et al. 2013, Burgess 2015), but the mechanism of gene flow between two homothallic species remains unclear. Homothalic species are primarily inbreeding, but occasional outcrossing obviously does occur. The high proportion of clonal genotypes, low gene flow among groups, as defined by the Structure analysis and AMOVA, as well as the rather low D_{Nei} values confirm the homothallic life cycle of all the groups except of *P. nicotianae*. Although AMOVA results revealed low gene flow between the groups, the low number of fixed markers indicate the impact of such gene flow upon the genetic diversity of the population. The results of this study confirm both intraspecific and interspecific (*P.* × *pelgrandis*) gene flow in natural populations.

The evolutionary relations defined on the base of AFLP are reflected also by combined results of test of resistence to chemicals, although resistance levels to single chemicals were not so clear. The combined data of several substances provided significant results, represented by a very low Wilks' λ, as ascertained by the discriminant analysis. Determined Mahalanobis distances based on the growth inhibition caused by chemicals of the *P. cactorum*, *P. nicotianae* and *P.* ×*pelgrandis* isolates describes mutual relationships between the tested groups which are in row congruence to results of molecular part of study. The groups of *P. cactorum* C1, C2, F were rather similar in their growth inhibiton caused by tested chemical substances, *P. nicotianae* was distant and differed significantly from them, while H group and *P.* × *pelgrandis* were intermediate between both of those two species.

The development of fungicide resistance in pathogenic *Phytophthora* spp. presents a substantial problem for effective disease control, especially as the formation of resistance can be a relatively quick process (Ellis et al. 1998). The probability of resistance formation to at least some chemicals depends on the frequency of contact between the pathogen and the chemical (Morales-Rodríguez et al. 2013; Tamietti and Valentino 2001), or frequency of multiple contacts with concentrations of the chemical that are too low to be effective due to inaccurate application (Davis and Dennis, 1981; Utkhede and Gupta 1988; Hu et al., 2008).

The variability of responses to chloramphenicol, which inhibits protein synthesis (Xalpanteri et al. 2003), most closely resembles the total variability revealed by the discriminant analysis. The response to this chemical significantly differed between *P.*

cactorum and *P. nicotianae.* In contrast, the response to streptomycin, which causes an erroneous reading of the genetic code on ribosomes (Pelechovich et al. 2013), did not show any significant variability amongst the different groups. The variability of *Phytophthora* responses to some of these substances has also been previously reported. Chloramphenicol-resistant strains were detected in *P. infestans* (Rooke and Shattock 1983) and *P. nicotianae* (Chang and Ko 1990; Ann and Ko 1992). Resistance to streptomycin has been confirmed in some strains of *P. cactorum* (Shaw and Elliott 1968) and *P. nicotianae* (Chang and Ko 1990, Ann and Ko 1992), although we did not detect any isolates with a decreased sensitivity to streptomycin. These two chemicals have different mechanisms of action, and while streptomycin can be either fungistatic or fungicidal (the reaction depends on its concentration and the duration of exposure), chloramphenicol is considered fungistatic (Rooke and Shattock 1983).

The variability we observed in response to the dimethomorph treatment was rather high. The mode of action of this compound is disruption of cell wall biosynthesis (Bagirova et al. 2001). Resistance to dimethomorph has been described in various *Phytophthora* species, e.g., *P. nicotianae* (as *P. parasitica,* Chabane et al. 1996b), *P. capsici* (Tamietti and Valentino 2001; Keinath 2007), and *P. infestans* (Rekanović et al. 2012; Stein and Kirk 2004). In contrast, dimethomorph has been considered more effective against *P. cactorum* than metalaxyl because *P. cactorum* has not been reported to be resistant to this substance generally (Hill and Hausbeck 2008). Similar results were also confirmed by Matheron and Porchas (2000) in *P. nicotianae* (as *P. parasitica*). Our results are in agreement with these studies as *P. cactorum*, except for the more resistant F group, exhibited low resistance to dimethomorph, which is significantly different from the more resistant *P. nicotianae.* F group together with *P.* × *pelgrandis* performed intermediate reaction. As the F group slightly differs from the typical physiological and molecular characteristics of *P. cactorum* (Pánek et al. 2016), the difference in the response to dimethomorph is not surprising.

Mancozeb is a fungicide considered to have a low risk of resistance formation due to its control of at least six different biochemical processes – the chemical disrupts lipid synthesis and the membrane integrity of the cell wall, mitochondrial respiration processes, nucleic acid synthesis, mitosis and cell division and cell wall biosynthesis. Therefore, the development of resistance would require the simultaneous appearance of mutations in multiple genes (Ludwig and Thorn 1960; Gullino et al. 2010). However, due

to its frequent application against *Phytophthora* (Fernandez-Cornejo et al. 2014), evidence of its low effectiveness, even in hundredfold higher concentrations compared to manufacturer's recommendations, was published (Wagner et al. 2007). We observed obvious differences in the sensitivity to this chemical among the isolate groups. The *P.* × *pelgrandis* and F groups appeared most resistant. Due to the hybrid origin of both groups, the development of tolerance to this chemical may be related to these hybridisation events. Metalaxyl (and the related active isomer mefenoxam) has been one of the most frequently used fungicides against *Phytophthora* since 1977 (Hu et al. 2008). This chemical disrupts nucleic acids synthesis, with the main target site lying in RNA polymerase I (Wollgiehn et al. 1984). The resistance of some *P. cactorum* strains to metalaxyl has been previously documented since 1988 (Utkhede and Gupta 1988). It is estimated that the proportion of resistant isolates could reach up to 80 % in areas where metalaxyl is frequently used (Utkhede and Gupta 1988; Jeffers et al. 2004; Reeleder et al. 2007; Hill and Hausbeck 2008). Resistance to metalaxyl is also documented for *P. nicotianae* (Timmer et al. 1998), *P. infestans* (Rekanović et al. 2012), *P. erythroseptica* (Goodwin and McGrath 1995), *P. capsici* (Jackson et al. 2012) and *P. ramorum* (Wagner et al. 2007). In *P. infestans,* a correlation between the sensitivity to metalaxyl and clonal lineages was demonstrated (Goodwin et al. 1996). In *P. ramorum,* only European isolates were sensitive to this substance (Wagner et al. 2007). In our study, all isolates showed sensitivity to metalaxyl, and *P. nicotianae* was more tolerant to metalaxyl than *P. cactorum*. However, *P.* × *pelgrandis* was more sensitive than both of its parental species.

7. Conclusions

The Structure group C1 is identified as *Phytophthora cactorum sensu stricto* because the C1 group usually includes the ITS, Pheca I and Cox I genotypes typical for this species. *Phytophthora hedraiandra* can be distinguished using ITS sequence analysis, but the H group is not separated by the most probable Structure grouping K = 3 and does not contain any fixed private markers. On the other hand, the AFLP data indicate a native, European origin of the H group. The sequence analysis revealed numerous hybridization events that decrease the species cohesion of *P. hedraiandra.* Because discrimination of *P. hedraiandra* from *P. cactorum* is unreliable according to both AFLP genotyping and DNA sequence data as well as morphological and growth characters, we do not

recommend to keep *P. hedraiandra* as a distinct species. In a consequence, also distinction of *Phytophthora. × serendipita* as the hybrid species of *P. cactorum* and *P. hedraiandra* lose meaning.

The *P. cactorum* complex seems to be an assemblage of closely related genetic lineages, which occasionally hybridize. The C2 and F groups are probably the progeny of hybridization events between H and C1 groups. C2 isolates usually have the ITS genotype of *P. × serendipita*.

The reactions of isolates of *Phytophthora cactorum* complex, *P. nicotianae* and *P. × pelgrandis* to metalaxyl, mancozeb, dimethomorph, streptomycin and chloramphenicol were tested to obtain information about the variability of resistance in these pathogens. Distinct genetic groups showed significant differences in resistance to all tested substances except streptomycin. In response to streptomycin, the growth inhibition rates of distinct groups did not differ significantly. The most remarkable differences were detected in the reactions to chloramphenicol and metalaxyl. Discriminant analysis evaluating the effect of all substances confirmed the differences among the groups, which are in agreement with the differences revealed by earlier DNA analyses. Results confirm importance of correct identification of pathogen for correct selection of effective substance in chemical control.

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9. Supplementary materials

Paper 1.

Pánek, M. Fér, T., Mráček, J., Tomšovský, M., 2016. Evolutionary relationships within the *Phytophthora cactorum* species complex in Europe. *Fungal Biology*, 120(6-7), pp.836–851.

Supplementary materials - Tab. 1S: Detail isolate description (host species, origin), GenBank numbers of sequences acquired during works on *Phytophthora cactorum* population analysis, analyses performed in isolates. Isolates were kindly provided by: 1- Aňa Péres Sierra, Universidad Politecnica de Valencia, 2 - Jarkko Hantula and Arja Lilja Finnish Forest Research Institute, 3 - József Bakónyi, Plant Protection Institute of the Hungarian Academy of Sciences, 4 - Karel Černý, RILOG, 5 - Kris van Poucke and Kurt Heungens, Institute for Agricultural and Fisheries Research, 6 - Simone Prospero and Corine Schoebel, Swiss Federal Institute for Forest, Snow and Landscape Research, 7 - Thomas Jung, Phytophthora Research and Consultancy, Nußdorf, Germany, 8 - Willem A. Man in 't Veld and Karin Rosendahl, Plant Protection Service, Wageningen, Gelderland, Netherlands.

Isolate descritpion				Performed analyses						
Isolate ID	Host species	Origin	Provided by	ITS	Cox	Pheca	AFLP	Cardinal temperatures	Morphology	Antheridia
407	Rhododendron	FL	2				x	x	x	
414	Water	FL	2	LN907642	LN907701	LN907671	x	x	x	
415	Betula	FL	2				x	x	x	
416	Betula	FL	2	LN907643	LN907702	LN907672	x	x	x	
417	Betula	FL	2				x	x	x	x
420	Betula	FL	2				x	x	x	x
421	Water	FL	2				x	x	x	
440	Rhododendron	FL	2				x	x	x	x
451	Sorbus	FL	2				x	x	x	x
Ph 20	Betula	FL	2				x	x	x	
Ph4	Betula	FL	2				x	x	x	x
Ph8	Betula	FL	2	LN907645	LN907704	LN907674	x	x	x	x
1383	Arbutus	E	1				x	x	x	x
PS-668	Photinia	E	1				x	x	x	
PS-719	Aucuba	E	1	LN907669	LN907728	LN907699	x	x	x	
PS-840	Cistus	E	1				x	x	x	
PS-902	Viburnum	E	1				x	x	x	
D/05/1076	Prunus	B	5				x	x	x	
M/05/0001	Soil	B	5				x	x	x	
M/05/0011	Malus	BG	5				x	x	x	
M/06/0001	Fragaria	BG	5				x	x	x	x
M/07/0003	Viola	B	5				x	x	x	
P/05/021	Rhododendron	B	5				x	x	x	
P/05/025	Rhododendron	B	5				x	x	x	
8676/8677a	Aesculus	A	7				x			
8676/8677b	Aesculus	A	7				x			
8676/8677c	Aesculus	A	7				x			
8692cac	Aesculus	A	7				x			
8698a (2)	Aesculus	A	7				x			
8698b	Aesculus	A	7	LN907649	LN907708	LN907678	x			
8698c	Aesculus	A	7	LN907650	LN907709	LN907679	x			x
8698d	Aesculus	A	7				x			
8702	Aesculus	A	7				x			
8706a	Aesculus	A	7				x			
8706b	Aesculus	A	7				x			
8706c	Aesculus	A	7				x			
8796/8952	Aesculus	A	7				x			
8854/8857a	Aesculus	A	7				x			
8854/8857b	Aesculus	A	7				x			
8892/8895d	Aesculus	A	7				x			

P13	Quercus	SK	7	LN907658	LN907717	LN907687	x			x
P52/2	Gymnocladus	SK	7				x			
P64	Taxodium	SK	7				x			
P74	Amelanchier	SK	7	LN907644	LN907703	LN907673	x			x
PI	Unknown	PL	7				x			
34-3-9	Pistachio	CA/USA	3				x	x	x	
C-2-CL	Citrus	AZ/USA	3				x	x	x	
CBS 100425	Rhododendron	NL	3				x	x	x	
CBS 114343/P537	Lavandulla	NL	3				x			
CBS 123385/P538	Pelargonium	GE	3				x			
CBS111725	Viburnum	NL	3				x	x	x	x
H-1/00	Lylly	H	3				x	x	x	
ICMP11853	Malus	NZ	3				x	x	x	
IMI354975	Prunus	IR	3				x	x	x	
JA 163	Chamaecyparis	H	3				x	x	x	
JA 175	Abies	H	3				x	x		
JA 337	Chamaecyparis	H	3	LN907670		LN907700	x	x	x	
JA 512	Lavandulla	H	3				x		x	
JA 98	Rhododendron	H	3				x			
JA 99	Buxus	H	3				x	x	x	
P 175	potting soil	H	3				x	x	x	
P 302	Apple soil	H	3				x	x	x	
P 42/04	Fragaria	FR	3				x			
P 632	Malus	H	3				x	x	x	x
P 74/04	Malus	GR	3				x	x	x	
P 81/04	Apple soil	H	3				x	x	x	
P 82/04	Apple soil	H	3				x	x	x	
P163	Naseberry	H	3				x	x	x	
P259	orchard soil	H	3				x	x	x	
P610	Lavandulla	IT	3				x			
P611	Lavandulla	IT	3				x			
P91/04	Soil	GE	3	LN907654	LN907713	LN907683	x			
T1LRd(4)	Unknown	IT	3				x	x	x	
PD 07/034866598	Loquat	TWN	8				x		x	
PD 07/03486667	Loquat	TWN	8				x	x	x	
PD 93/1339	Spathiphyllum	NL	8				x		x	
PD 94/1166	Spathiphyllum	NL	8				x		x	
PD 97/10235	Cyclamen	NL	8				x		x	
PD 97/8771	Lavandulla	NL	8				x		x	
PD20017401	Penstemon	NL	8				x	x	x	
PD20018446-2	Rhododendron	NL	8	LN907668	LN907727	LN907698	x	x	x	x
PD20025453-1	Rhododendron	NL	8				x	x	x	
PD2002685	Rhododendron	NL	8				x	x	x	
PD95/5111	Idesia	NL	8				x	x	x	
101/07	Aesculus	CZ	4					x	x	
109/07	Rhododendron	CZ	4	LN907663	LN907722	LN907692	x	x	x	x
111/07	Rhododendron	CZ	4					x	x	
112/07	Rhododendron	CZ	4	LN907664	LN907723	LN907693	x	x	x	
113/07	Rhododendron	CZ	4				x	x	x	
116/07	Rhododendron	CZ	4				x	x	x	x
125/07	Rhododendron	CZ	4	LN907665	LN907724	LN907694	x	x	x	
216/08	Rhododendron	CZ	4	LN907657	LN907716	LN907686	x	x	x	x
251/08	Rhododendron	CZ	4				x	x	x	
272/09	Aesculus	CZ	4				x	x	x	

275/09	Rhododendron	CZ	4	LN907651	LN907710	LN907680	x	x	x	
277/09	Populus	CZ	4				x	x	x	
282/09	Tilia	CZ	4				x	x	x	
291/09	Tilia	CZ	4	LN907655	LN907714	LN907684	x	x	x	x
292/09	Acer	CZ	4	LN907652	LN907711	LN907681	x	x	x	
293/09	Acer	CZ	4				x	x	x	
300/09	Rhododendron	CZ	4				x	x	x	
426/10	Tilia	CZ	4	LN907648	LN907707	LN907677	x	x	x	
434/11	ragaria colar rot	CZ	4				x	x	x	
450/11	Rhododendron sp	CZ	4	LN907661	LN907720	LN907690	x		x	
458/11	Viburnum	CZ	4				x	x	x	
503/11	Malus	CZ	4				x	x	x	
505/11	Quercus	CZ	4				x	x	x	
549/11	Rhododendron	CZ	4	LN907660	LN907719	LN907689	x	x	x	
578/12	Fagus silvatica	CZ	4	LN907662	LN907721	LN907691	x	x	x	x
602/12	Malus	CZ	4				x		x	x
604/12	Malus	CZ	4				x		x	
605/12	Malus	CZ	4	LN907653	LN907712	LN907682	x			
634/13	Fragaria colar rot	CZ	4	LN907646	LN907705	LN907675	x	x	x	x
634/13b	Malus	CZ	4				x			
634/13c	Malus	CZ	4				x			
66/07	Populus	CZ	4	LN907647	LN907706	LN907676	x	x	x	
67/07	Fagus silvatica	CZ	4	LN907656	LN907715	LN907685	x	x	x	x
78/07	Fagus silvatica	CZ	4	LN907659	LN907718	LN907688	x	x	x	x
82/07	Rhododendron	CZ	4				x	x	x	
M5618	Nursery soil	CH	6				x	x	x	
M5620	Nursery soil	CH	6				x	x	x	
M5624	Nursery soil	CH	6				x	x	x	x
M5625	Nursery soil	CH	6				x	x	x	
M5630	Nursery soil	CH	6				x	x	x	
M5631	Nursery soil	CH	6				x	x	x	
M5632	Nursery soil	CH	6	LN907666	LN907725	LN907696	x	x	x	
M5637	Nursery soil	CH	6				x	x	x	x
M5641	Nursery soil	CH	6				x	x	x	
M5652	Nursery soil	CH	6				x	x	x	x
M5654	Nursery soil	CH	6	LN907667	LN907726	LN907697	x	x	x	
M5658	Nursery soil	CH	6				x	x	x	
M6280	Nursery soil	CH	6				x	x	x	
PI100	Nursery soil	CH	6				x	x	x	x

Paper 2.

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