SZENT ISTVÁN UNIVERSITY

INTRA- AND INTERSPECIFIC INTERACTIONS IN THE GENUS *PHYTOPHTHORA*

PhD thesis

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1. INTRODUCTION

1. 1. The genus *Phytophthora*

Having special cytological and biochemical features, species in the genus *Phytophthora* differ from the true fungi despite the similarities in colony structures. According to similarities in DNA sequences, *Phytophthora* species are related more closely to yellow and brown algae than to true fungi. Phytophthoras and their relatives, as fungal-like organisms along with Chrysophyta and Phaeophyta, were therefore classified within the Kingdom Chromista.

Phytophthoras propagate sexually by means of oospores, which are formed either within one thallus (homothallic species e.g. *Ph. fragariae*) or as a result of the interaction between two thalli of different mating type (heterothallic species e.g. *Ph. cambivora* or *Ph. infestans*).

Species of the genus *Phytophthora* as plant pathogens live in plant tissue but are also able to survive in soil by means of oospores or the asexually formed chlamydospores. However, diseases spreading quickly in one growing season are due to their asexually produced sporangia or zoospores formed in the sporangia.

1. 2. Intraspecific diversity of *Phytophthora infestans*

Phytophthora infestans, the causal agent of late blight of potato and tomato, may be one of the most devastating plant pathogens in history. During the 1840s it caused serious infection on potato in Ireland, destroying almost the whole crop in the country.

Having simple colony morphology it is difficult to use phenotypic markers to analyse populational structure in the genus *Phytophthora*. Resistance to fungicides and virulence are the most relevant features for practical purposes such as breeding and chemical control.

Increasing frequency of isolates which are resistant to the systemic phenylamides like metalaxyl, makes the chemical control less effective. Testing the response of the pathogen to this fungicide is therefore practiced worldwide.

Eleven dominant resistance genes have been identified in wild *Solanum* species (especially in *S. demissum*) which have been introduced

into potato during intensive breeding work. There are also eleven virulence phenotypes in *Ph. infestans* which are able to break through the protection of the resistance genes. Virulence phenotype of any isolate of *Ph. infestans* is the list of R genes which are ineffective against the tested isolate.

Mating type, the easiest phenotypic feature to determine, is tested by incubating the unknown isolate along with standards of known mating type in the same Petri dish. Production of oospores with one of the two tester isolates determines the mating type of the unknown isolate.

Genetic markers, for example isozymes, are more appropriate for describing the diversity of the *Ph. infestans* populations. Glucose-6-phosphate isomerase (Gpi, E. C. 5. 3. 1. 9.) and glycil leucine peptidase (Pep, E. C. 3. 4. 11/13.) are the most appropriate ones for analysis of populations of *Ph. infestans;* allele frequencies of these genes can be determined directly.

In comparison with isozymes, which always require clean, contamination-free samples of mycelium, DNA analysis using the polymerase chain reaction (PCR) can be carried out from even one-hundred-year-old herbarium samples. PCR therefore may give information on the structure of old populations. The moderately repetitive DNA probe RG57 hybridises to at least 25 different loci in the genome of *Ph. infestans.* Southern analysis after treatment with *Eco*RI restriction endonuclease gives a multilocus genetic fingerprint of isolates. The first worldwide genotypic database of *Ph. infestans* is also based on RG57 data. More recently microsatellites (SSR: simple sequence repeats) and mitochondrial haplotype data improve the molecular database for describing the population structure of *Ph. infestans*.

Central Mexico, where the genetic diversity of this pathogen has the highest level in the world due to the long presence of both mating types at the same frequency, may be the centre of origin of *Ph. infestans*. The first report on the occurrence of *Ph. infestans* outside Mexico dates from 1843 in the United States of America. Two years later the pathogen occurred in Europe and reached Hungary in the same decade. The pathogen colonised its new habitat quickly, because potato cultivars used were susceptible to late blight.

Isozyme data supports the hypothesis that populations of *Ph. infestans* consisted of only a few lines with limited variance of the same clonal lineage (US-1) during the last 150 years. Isolates from these old populations had *Gpi* 86/100 and *Pep* 92/100 genotype with limited virulence and high susceptibility to metalaxyl.

In spite of the fact that oospores were observed in old herbarium samples, there are no data about the presence of isolates with A2 mating type outside Mexico. The first isolate of A2 mating type outside Mexico was described in Switzerland in 1984. Following this report, profound changes in the populations of the pathogen were detected worldwide. New populations with increased aggressiveness and more complex virulence phenotype and containing both mating type displaced the old, clonally propagating populations. These changes were accompanied by an increase of the ratio of isolates resistant to metalaxyl threatening potato and tomato production.

The first study on a regional population in Hungary was published in 1965, revealing four races with simple virulence, but recent studies identified more complex races having isolates with up to eight virulence factors. The discovery of A2 mating type in 1996 suggested that the worldwide changes in the structure of *Ph. infestans* populations were also taking place in Hungary.

1. 3. Alder *Phytophthora,* a naturally formed species-hybrid

Production of species-hybrids in the nature is rare; moreover it is difficult to prove the origin of the progeny using only morphological features. An analysis of the progeny originating from an artificial crossbreeding of potential parents can explain the origin but this experiment is difficult to carry out with plant pathogens. Artificial creation of species hybrids may overcome the problem and hybridisation of *Phytophthora* species were carried out *in vitro* with induced zoospore fusion or sexual interactions.

Some species were suspected to have a hybrid origin such as the elm disease pathogen *Ophiostoma* species or *Melampsora* species causing poplar rust. There is evidence of formation of species hybrids under natural conditions in the genus *Phytophthora* as well. An unknown *Phytophthora* species was isolated in a hydroponic culture of *Primula* and *Spathiphyllum*. Isozyme and RAPD analysis together with Southern hybridisation of the PCR products proved the hybrid origin of this species from genetic interaction of *Ph. nicotianae* and *Ph. cactorum*. This new hybrid was able to infect *Cyclamen* on which neither parental species had been known before. According to chromosomal cytology, *Ph. meadii* may also be a species hybrid rather than an independently evolved species.

The first publication of alder *Phytophthora* in 1993-94 reported seriously damaged riparian alder *(Alnus glutinosa)* trees in southern

Britain. Necrotic lesions with tarry exudations were observed on the root collar of the trees. The canopy of these trees was frequently less dense than that of the healthy plants. The pathogen isolated from the necrotic lesions was a *Phytophthora* but it did not matched any other known species. This was the first report of *Phytophthora* disease on alder. The pathogen had some of the common features (*e.g.* warty oogonial wall) of *Ph. cambivora* which causes diseases on many different tree species but not alder. However, this new pathogen was homothallic and not heterothallic like *Ph. cambivora*. Following the first report in Great Britain, the pathogen was observed in several European countries including Hungary in 1999. A nationwide survey in Hungary revealed that 80% of some alder stands exhibited the typical symptoms of alder *Phytophthora* although isolation of the pathogen was not always successful.

According to the results of ITS sequence analysis and AFLP data, British scientists concluded that this pathogen was a species hybrid formed naturally between *Ph. cambivora* and a species similar to *Ph fragariae*. The species hybrid had a wide range of different variants. The chromosome number of *Ph. cambivora* and *Ph. fragariae* was n=5–6, but the hybrids had many more chomosomes; some hybrid strains were near tetraploid while others had lesser numbers of chromosomes. Due to the aneuploidy meiosis would rarely be completed successfully and the majority of the oospores were unable to germinate.

The pathogen has just recently been described as a new species, *Phytophthora alni* Brasier & S. A. Kirk, with subspecies according to the different variants. The standard type of the hybrid which was the most aggressive and genetically most homogenous of the several different variants, was named *Ph. alni* ssp. *alni*; and it had warty oogonia. The Swedish variant, which forms smooth walled oogonia, was named as *Ph. alni* ssp. *uniformis*. The less aggressive variants (German and Dutch variant together with a rare UK one) have warty oogonia and is various in colony morphology. On the basis of identical ITS sequence these variants were classified as *Ph. alni* ssp. *multiformis*.

1. 4. Objectives

Species in the genus *Phytophthora* cause serious economical losses. Moreover the genus exhibits great genetic diversity. The source of their diversity besides mutation originates from interspecific and intraspecific interactions. Our aim, therefore, was to assess pheno- and genotypic

diversity of populations of two species which exist in Hungary causing significant economical losses.

In analysing the Hungarian population of *Ph. infestans*, our aim was to reveal the genetic structure and the probability of sexual interactions in comparison with the results from other countries.

The new pathogen of alder trees seriously threatens the alder stands in Hungary. Our second objective was to isolate this new pathogen, evaluate the structure of its populations in Hungary using morphological and molecular markers and identify genetic markers to distinguish the different variants of alder *Phytophthora*.

This dissertation concentrates on the changes in the populations of *Ph. infestans* in Hungary and on analysis of this newly formed species hybrid alder *Phytophthora*.

2. MATERIALS AND METHODS

2. 1. Isolates used

Isolatations of *Ph. infestans* were made from randomly chosen leaves of potato and tomato plants having disease symptoms. Four isolates originating from potato were also included as a reference by kind permission of W. E. Fry (Cornell University, Ithaca, NY, USA) and G. A. Forbes (International Potato Centre, Quito, Ecuador).

Isolations of the pathogen were made from alder trees in an alder forest in Hanság and another forest at Hévíz. Reference isolates of *Ph. cambivora* and *Ph. fragariae* var. *rubi* along with isolates of alder *Phytophthora* included in this study were kindly provided by C. M. Brasier (Forest Research Agency, Farnham, Surrey, U.K.).

2. 2. Culture media

Cultures were maintained on three different media made from pea or carrot broth. The third medium was prepared using commercially available V8 juice. For isolation, however, the same media amended with antibiotics such as pimaricin, ampicillin, rifampicin, hymexazol or pentachloronitrobenzene were used. A small cube of plant tissue or mycelium growing from plant material was surface sterilised then placed on the selective media.

2. 3. Phenotypic characteristics of *Phytophthora infestans*

The mating type of isolates of *Ph. infestans* was determined by means of incubating them in the same Petri dish along with standard isolates of known mating type. For testing the sensitivity to metalaxyl *in vitro* isolates were cultured on pea broth agar amended with 5 and 100 ppm metalaxyl. All metalaxyl sensitivity tests were replicated at least two times.

2. 4. Identification and physiological features of alder *Phytophthora*

Identification of an isolate of a *Phytophthora* species requires an analysis of its reproductive organs. Cultures were incubated in non sterile soil extract so as to induce formation of sporangia. Development, structure and size of sporangia and the shape and size of oogonia and antheridia along with their mode of joining were assessed using the light microscope. Additionally, ultrastructural studies on the surface of the oogonia were carried out with a scanning electron microscope.

Colony morphology was assessed on different media (pea broth, carrot- and V8-agar) in 9-cm-wide Petri dishes at the optimal temperature for colony growth. The optimal temperature was determined by measuring daily colony growth rate at different temperatures.

2. 5. Isozyme and DNA analysis

Mycelium was lyophilised and ground for molecular analysis. Native enzymes for isozyme analysis were then extracted into buffer from mycelial powder, whereas DNA was extracted with the phenol–chloroform method. Isozymes were separated by cellulose acetate gel electrophoresis and stained with an agar overlay method.

The RG57 genotype of *Ph. infestans* isolates were determined by Southern hybridisation of the 1.2-kb-long RG57 probe to the total genomic DNA treated with *Eco*RI restriction endonuclease. The probe was randomly labelled with DIG-11-dUTP (DIG).

The ITS region in alder *Phytophthora* was amplified by PCR using the universal primer pair ITS1–ITS4. Following the digestion with restriction endonucleases, restriction fragments were separated by agarose gel electrophoresis. RAPD PCR was used for the analysis of the whole genome of the alder *Phytophthora*. One of the RAPD-PCR products was DIG-labelled and then Southern hybridised to the PCR products from the same reaction to assess the relationship between *Ph. cambivora* and alder *Phytophthora*.

2. 6. Data analysis

The whole population of *Ph. infestans* was divided into subdivisions on the basis of host and mating type.

For assessing genotypic diversity within and between the subgroups of *Ph. infestans* in Hungary, allele frequency at the two isozyme loci were used in Nei's gene diversity analysis. Nei's unbiased genetic distance was also computed between these subgroups of the isolates. Shannon diversity indices of each subgroups were computed from multilocus genotypes after combining mating type and isozyme data with RG57 fingerprints.

3. RESULTS AND DISCUSSION

3. 1. Pheno- and genotypic features of isolates of *Phytophthora infestans*

During the growing season in 2001 and 2002, a total of 118 isolates were collected from Hungary. The majority of the isolates was collected in 2002 and represented two main potato producing regions. Twenty isolates originated from Northern Hungary (Ludányhalászi) whereas 39 isolates represented Northern Transdanubia (Borzavár, Porva, Olaszfalu, Tündérmajor).

More than two thirds of the isolates tolerated (had intermediate or resistant response to) metalaxyl. The majority of these isolates originated from potato. Due to the high proportion of isolates resistant to metalaxyl, chemical control of the pathogen should be based on combination of chemicals with different mode of action.

Distribution of mating types were close to 50-50%. Presence of both mating types in one field provides the potential for sexual recombination and the production of oospores which allows the pathogen long survival in the soil even if the host is not present. Also genetic recombination increases the diversity in the population.

All the isolates exhibited 100/100 genotype at the *Gpi* locus. At the *Pep* locus, however, four genotypes 100/100 (34%) 96/96 (32%) 96/100 (27%) and 83/96 (7%) were resolved. The last genotype is rare throughout Europe and is associated with the A1 mating type.

Analysis of RG57 fingerprints uncovered great genetic diversity with 75 different genotypes; even the most frequent genotype occurred only 9 times. A high diversity has been detected in populations from other European countries but within these, the same few clonal lineages have been found to predominate at many sites and in many years. The high genetic diversity itself is not exceptional to Hungary, but there is no dominant genotypes which is always accompanied with the great genetic diversity in other countries. All the RG57 genotypes but twelve had not been reported from other countries before.

On the basis of combined traits of mating type, isozyme genotypes and RG57 fingerprint, the population had even higher diversity. The most frequent multilocus genotypes occurred only six times in the whole

population; the genotypic diversity of *Ph. infestans* seems almost as high as in Central Mexico, where *Ph. infestans* is thought to have originated.

Nei's genetic distance was small between subgroups of *Ph. infestans* on the basis of the host or mating type. Gene diversity analysis also did not exhibit significant differences, therefore these subgroups could not be distinguised by using these markers. The lack of any significant difference between any subdivisions of the population described by the Shannon indices indicates that the population is not substructured. These results imply an important role of the sexual recombination in the population of *Ph. infestans* in Hungary.

The role of the oospore in the renewal and variability of populations of *Ph. infestans* is not yet well understood. Oospore formation depends on many factors but both solanaceous weeds or remaining tissue of the host can be a significant source of inoculum. If oospores are a significant source for the next generation of the pathogen, the new generation may be different from the previous one due to genetic recombination.

The great genetic diversity in the populations of *Ph. infestans* in Hungary are due to many factors, but sexual recombination has a significant role in it. If asexual propagation had great influence in a life cycle of the pathogen, the genetic diversity within one growing season and at one site should be limited. If there were strong migration between the Hungarian and the neighbouring populations of the pathogen, the isozyme allele *Pep* 96 should appear at the same frequencies in other countries as in Hungary. If the propagation in the population is mainly asexual the same genotypes should be present in both areas. Neither of these options were observed in Hungary, therefore our results suggest, that the populations of *Ph. infestans* in Hungary are separated from their neighbourhood. The great genetic diversity observed indicates that sexual recombination has a significant role to play in the life cycle of *Ph. infestans* in Hungary.

3. 2. Morphological and molecular characterisation of alder *Phytophthora (Ph. alni)*

The pathogen, isolated from roots or the rhizosphere of alder trees showing symptoms of alder decline, conformed to an alder *Phytophthora* according to preliminary morphological results. All isolates had non-caducous, non-papillate sporangia. (These features are also typical of *Ph. cambivora* as well as *Ph. fragariae*.) The optimal temperature for mycelial growth was 25 °C.

The Hungarian isolates belonged to two different types. Three isolates from Hanság were similar to the Swedish type of this pathogen (*Ph. alni* ssp. *uniformis*) and formed smooth walled oogonia with a diameter of 24–64 μm under optimal conditions. The majority of the amphigynous antheridia were two celled. Artificial inoculation of two-year-old alder seedlings proved their pathogenicity. On the other hand, isolates from Hévíz had ornamented, warty-walled oogonia, varying in diameter from 23 to 54 μm. Antheridia were more often bicellular compared to the the isolates from Hanság. These features of isolates from Hévíz were identical to those of the standard type reference isolate (*Ph. alni* ssp. *alni*) and the heterothallic *Ph. cambivora*.

This evidence for two types of alder *Phytophthora* in Hungary was supported by molecular data. Isozymes with good diagnostic value (glucose-6-phosphate isomerase, malate dehydrogenase and leucil-tyrosine peptidase) enabled distinction between the two morphologically based groups, the standard isolates and the isolates representing the Swedish variant. There was no evidence of recombination of parental alleles in any of the hybrid types.

Banding patterns of amplification products of RAPD-PCR were in agreement with the results of the isozyme electrophoresis and distinguished the two groups of alder *Phytophthora;* that is, the Swedish variant and the standard type. Nevertheless primer OPG-02 amplified a *ca*. 1.5 kb sequence in *Ph. cambivora* that was also present in all tested alder *Phytophthora* isolates but not in *Ph. fragariae*. This *Ph. cambivora* band was homologous with the co-migrating bands of the same size in the isolates of alder *Phytophthora* proving that these hybrids may contain sequences from *Ph. cambivora* in their genome. Some faint PCR products with the same size were present in *Ph. fragariae* and the hybrids as well but the amount of DNA were too low to test their sequence homology.

PCR with the universal primer pair ITS1 and ITS4 amplified the expected 0.9 kb long products. Following the digestion with restriction endonucleases, the banding pattern of the digested DNA was identical within each hybrid group but different between the groups. It is worth noting that the summarised length of digested DNA fragments was longer than the original PCR product indicating the presence of both parental sequences in the hybrids, as was observed in other *Phytophthora* species hybrids. Digesting the whole ITS region with *Msp*I restriction endonuclease there were no significant qualitative difference between the two hybrid groups but the banding pattern exhibited quantitative differences and the Swedish variants were rather similar to *Ph. cambivora*.

Isolates of the standard hybrid type, however, were similar to *Ph. fragariae*.

The evidence that there are no detectable genetic differences within the two groups is consistent with the evidence that sexual propagation of this new pathogen is not succesful. The majority of the oospores were unable to germinate under the conditions tested therefore the main way of propagation of alder *Phytophthora* is asexual. If we were able to clarify how species can interact in the nature we would be able to understand how the hybrids were originally generated. An increasing number of succesful hybrid species could result in immense changes in certain ecosystems. Species hybrids may be able to survive if they change their host range or settle down in new, unoccuppied niches. Species hybrids have a good chance of spreading and the human activity along with environmental changes may accelerate these otherwise slow ecological processeses.

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Finally the genus *Phytophthora* as one of the most devastating group of plant pathogens causing serious economical losses but it is still able to raise new challenges to the human race in the near future. Increasing pathogenicity in some of its populations owing to intra- and interspecific interactions demands new control strategies. It is therefore vital to know the state and the changes of the genetic structure in the populations of any pathogen. New molecular biological methods may give a powerful tool to the scientists, though it is almost impossible to control perfectly such a genetically diverse pathogen. According to the increasing number of isolates resistant to metalaxyl it is risky to use chemical control with only one compound. But a mixture of chemicals with different mechanisms promises better results and decreases the risk of new resistance to chemicals. On the other hand it is impossible to use chemical control over pathogens in a fragile ecosystem as in the case of alder *Phytophthora*. The only way to control pathogens in these ecosystems is breeding for resistance which can also be applicable in traditional agroecosystems. First and foremost the seed must be free of pathogens. Screening the seed stocks needs efficient and quick methods to detect possible contamination. Our aim is therefore to design new molecular methods to detect alder *Phytophthora* in symptomless plant materials.

4. NEW SCIENTIFIC RESULTS

This work is part of those researches going on recently in the Plant Protection Intitute of the Hungarian Academy of Sciences which concentrate on changes in the populations as a result of intra- and interspecific interactions caused by the late blight pathogen *(Ph. infestans)* and the species hybrid causing a new alder disease *(Ph. alni).* New scientific results are as follows:

- We provided further evidence that the old populations of *Ph. infestans* were completely displaced in Hungary. The new populations of *Ph. infestans*, however, are different from the populations observed recently in Europe, because the diversity is much greater than anywhere else in Europe.
- Two types of a new pathogen causing alder disease were identified in two habitats in Hungary. These have recently been described as *Ph. alni* ssp. *uniformis* (Swedish variant) and *Ph. alni* ssp. *alni* (the standard type).
- Isozyme and DNA markers were identified for characterisation of the two subspecies of *Ph. alni*. The two subspecies can be distinguished with isozyme and RAPD markers as well but isolates within one type (subspecies) were genetically homogenous.
- The presence of DNA from *Ph. cambivora* were detected in the isolates of the species hybrid *Ph. alni* by means of DNA–DNA hybridisation.

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