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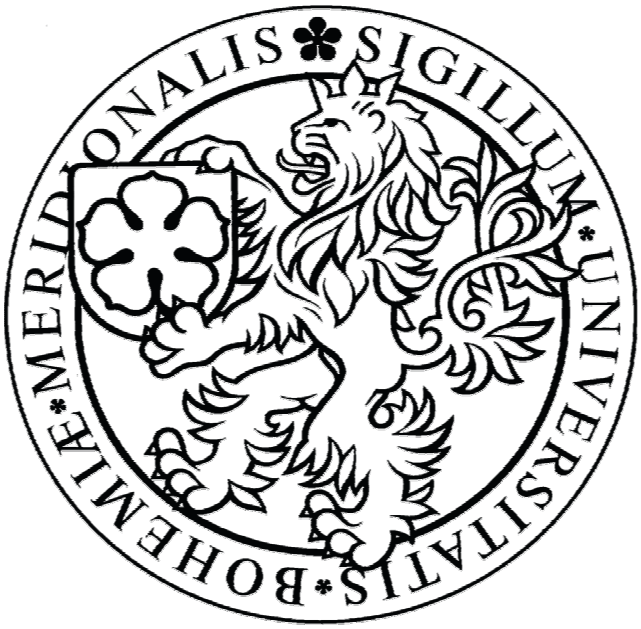

Master Thesis

The prevalance survey of oak powdery mildew *Erysiphe alphitoides* in Europe using molecular markers

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Annotation:

Powdery mildew *Erysiphe alphitoides* is one of the most important pathogenic fungus infecting pedunculate oak *Quercus robur* in Europe. As the identification of this species with morphologic markers is unreliable, the molecular markers development is necessary. In this work, we developed primers for PCR amplification of *cytochrome b* gene fragment. The results show high level of intraspecific variability of this mitochondrial gene and revealed the indistinct taxonomy relations within *E. alphitoides* s. lat group.

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V Českých Budějovicích, dne 30. 4. 2010

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Contents

1. Introduction

The powdery mildew fungus *Erysiphe alphitoides* (syn. *Microsphaera alphitoides*) is one of the most important representatives of fungal parasites on pedunculate oak (*Quercus robur*) in Europe. The first occurrence of *E. alphitoides* has been registered in 1907 in Portugal and during two years, this parasite invaded through the whole European continent, except high-mountain areas (Černý, 1970). The place of origin of this fungus is not sufficiently clear. Some studies define North America as the place of origin, although it may have been introduced to North America together with *Q. robur*, used as an ornamental tree or hedge (Takamatsu, 2007). This species of powdery mildew could be also domestic in Portugal, from where it has spread rapidly after a mutation causing higher level of pathogenicity (Příhoda, 1959). The anamorph (asexual stage) called *Oidium magniferum* of the fungus could be introduced to Portugal with the import of mango fruit. During the invasion, *E. alphitoides* replaced the original European powdery mildew *Phyllactinia roboris* (Gachet) Blumer. that has not been observed in Europe for several decades (Jančařík, 2000).

E. alphitoides adapts to the temperate climate of European continent extremely quickly. Epidemic outbreaks occur during hot and dry years but the fungus is also able to survive the extreme temperatures deep below zero during overwintering in leaf buds. The sexual reproduction is not strictly fixed on hot climate and fruiting bodies are developed in the whole area of its occurrence.

Today it is possible to find *E.alphitoides* thriving on the majority of oaks in Europe, Asia and Japan and it is also commonly found in area of North and South America, South Africa, Australia and New Zealand. It affects especially trees with lower fitness such as seedlings or shaded individuals, although recently we can find this fungus also on full grown trees.

1.1. The powdery mildew *Erysiphe alphitoides*

The powdery mildews are classified into phyllum: *Ascomycota*, subphyllum: *Pezizomycotina*, class: *Leotiomycetes*, order: *Erysiphales* (Hibbert, 2007).

Fig. 1. Mycelial film of *E. alphitoides* on the oak tree *Q. robur* leaf surface. (photo by Michálek 2008)

The symptoms of *E. alphitoides* infestation on the oaks are easily distinguishable (Figure 1.). White patches of mycelium appear on the leaf surface soon after leaf budding in the early spring. As mycelia patches grow, they connect together and cover the majority of the leaf surface in most cases. The thin-walled hyalinne mycelium germinates from the ascospore. Te mycelium is usually 3-7 μm thick, 6-10 μm in diameter with lobed appresoria and penetrates the layer of leaf epidermal cells with haustoria originating from these appresoria (Figure 2.). This anamorph (asexual) stage known as *Oidium quercinum* generate terminal obovoid-ellipsoid conidia, 13-25 μm × 20-45 μm, with the truncate base. These conidia induce the spread of infection during the spring and provides the secondary infection of the leaves. The teleomorph (sexual) fruiting body – cleistothecium, is generated by the fungus on the mycelial surface mainly in autumn (september, october). The cleistothecia are spheric, 75-140 μm large, with colour ranging from light yellow to dark brown in dependence on the level of maturation. During the maturation of cleistothecia, appendages grow in the equatorial plane. These appendages are straight, thick-walled, without septa, colourless, or only pigmented at the very base, 0.75-2 times as long as is the cleitothecium diameter (70-225

μm), and dichotomicaly branched. The purpose of these appendages has not been clearly explained. The cleitothecia of *E. alphitoides* contain higher amount of ellipsoid asci (4-15), 30-55 × 40-80 μm large, with each ascus containing 6-8 ovoid ascospores, 7-15 × 13-26 μm.

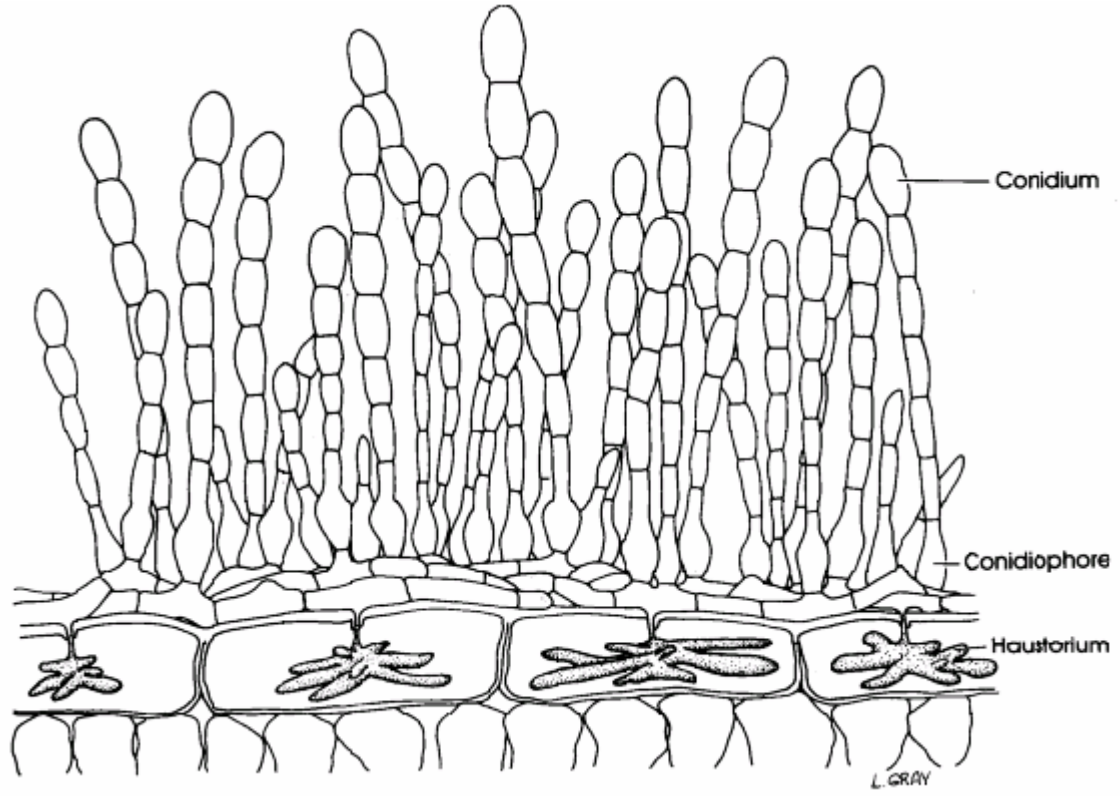
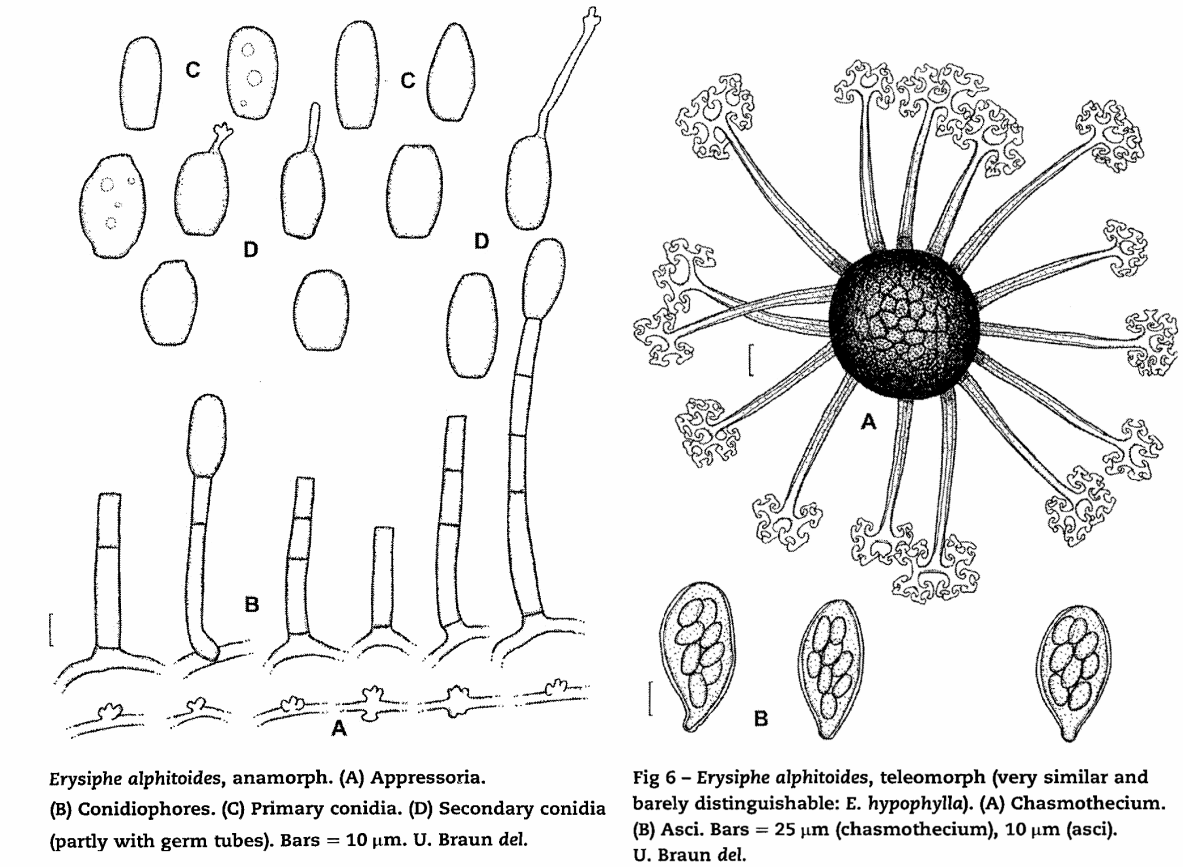


Fig. 2. *Erysiphe graminis* drawing; the fungus penetrates the epidermal cells with haustoria.
(Lenore Gray, http://www.ipm.uiuc.edu/diseases/series400/rpd406/406-2.gif)

The consequences of infection on the oak tree could be fatal especially for seedlings. The massive infection leads to necrosis, deformation of leaves and defoliation. Infected branches, annual growths or nurslings are deformed, dwarfed or otherwise undeveloped. The powdery mildew utilizes the nutrients from the leaves via the haustoria and causes the limitation of photosynthesis indirectly by shading by a thick film of mycelium growing on the leaf surface. This is the cause, namely for the small trees or branches, of host etiolation and forcing it, to make long growths, therefore making the plant more exhausted. Mature trees are heavily infected especially when attacked by leaf-eating insects. Combination of fungal and insectual infestation usually leads to decrease of fecundity (Gibbs, 1999).

In European conditions *E. alphitoides* infects mostly *Quercus robur* and occasionally *Quercus petrea*. The latter one is more resistant and so are its hybrids (Mirchev, 1985; Butin, 1995). The occurence of *E. alphitoides* is not limited on these two species. It is casually found

on other plants: *Quercus rubra*, *Fagus sylvatica*, *Aesculus hippocastaneum*, *Vaccinium* sp (Příhoda, 1959; Heuser, 2002). These species use to be infected rarely, especially when exposed to mutual contact with infected oaks in the areas with massive infestation. *E. alphitoides* was recognized also on some tropical species like *Anacardium occidentale*, *Bixa* sp., *Citrus* sp., *Mangifera* sp., *Acacia* sp., but it remains a question, if it was *E. alphitoides* s.str (Takamatsu, 2007).



Erysiphe alphitoides, anamorph. (A) Appressoria. (B) Conidiophores. (C) Primary conidia. (D) Secondary conidia (partly with germ tubes). Bars = 10 μm. U. Braun *del.*

Fig 6 – *Erysiphe alphitoides*, teleomorph (very similar and barely distinguishable: *E. hypophylla*). (A) Chasmothecium. (B) Asci. Bars = 25 μm (chasmothecium), 10 μm (asci). U. Braun *del.*

Fig. 3. (Takamatsu, 2007)

1.2. Identification of *E. alphitoides*

Morphological traits

Classic identification of fungi from the powdery mildew genera is based on morphological traits and also on the identification of the host species. Although these approaches of fungal classification are continually developing and specifying (SEM for example), they are unable to cover the detail and diversity. Molecular methods offer much more detailed study of taxonomy of *E. alphitoides*.

The main parameters of teleomorphs are the number of asci in cleistothecium and the shape of its appendices (Figure 4.). This determination is not fully explicit; moreover the cleistothecia occur in the temperate climate only in autumn, which makes the identification more difficult during the vegetation period. Conidia from the anamorph stage are the other trait for determination of powdery mildews (Figure 5.), but their size and shape vary due to environmental influences (Takamatsu, 2007). Particularly sterile mycelia are absolutely indistinguishable.

The fungal taxonomy is rather complicated due to intraspecific morphological and physiological variation and the limited number of morphological markers. Such markers depend on gene expression, and are influenced by environmental conditions. Therefore morphological and physiological variation does not reflect genetic variation at the DNA level which is important for characterizing individuals. The variation of parasites also depends on the fitness of the host organism. For example cleistothecia in Europe are morphological rather uniform, unlike the cleistothecia in Asia and Japan, where they are more variable (Takamatsu, 2007).

Several species of powdery mildew are known to infect oaks. These include *E. abbreviata* (syn. *Microsphaera abbreviata*), *E. alphiotides* (syn. *M. alphiotides*), *E. calocladophora* (syn. *M. calocladophora*), *E. extensa* (syn. *M. extensa*) a *E. hypophylla* (syn. *M. hypophylla*). Phylogenetic analyses of the ITS (internal transcribed spacer) and rDNA sequences revealed their relationship and proved that they are different species, despite of their morphological similarity (Takamatsu, 2007).

In comparison to *E. alphitoides*, we find some morphological differences in species *E. hypophylla* and *E. quercicola*, (which are described as European species and they used to be sorted into the *E. alphitoides* s. lat. group), and these differences should be discussed. *E. hypophylla* was described on *Quercus robur* in the middle of 20th century in the European part of Russia and from this region it rapidly spreads westward (Braun, 1987). *E. hypophylla* is suspected to grow on the lower side of leaves and it is distinguishable only by the shape of conidia, while the cleistothecia from these two species are almost identical to *E. alphitoides*. In addition the shape of conidia could vary depending on theleaf side covering (lower or upper leaf surface). Therefore it is not proven whether *E. hypophylla* is a distinct species, or it is a form of *E. alphitoides* growing on the lower leaf surface. Sequencing the ITS region by Takamatsu et al. supports the hypothesis of separate species but the results may be misleading, because the samples were collected only in Japan and the part of the sequences was obtained from database. At the present time *E. hypophylla* does not occur in Europe.

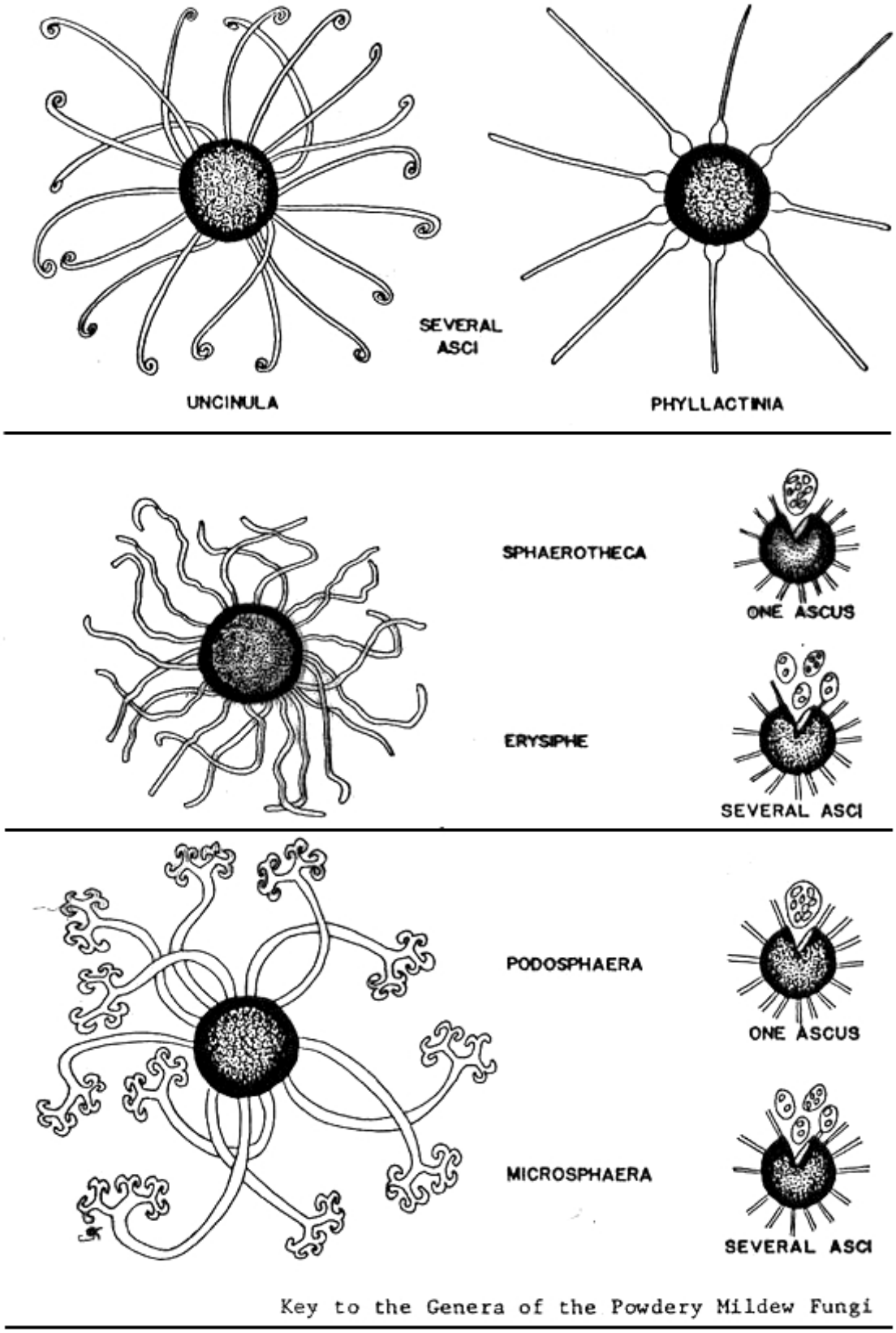


Fig. 4. Samples of genera-specific morpgological traits on cleistothecia.
(http://www.plantpath.wisc.edu/pp300-UW/images/image004.jpg)

The differences between *E. quercicola* and *E. alphitoides* are supported by morphological traits as well as by the sequences of the ITS region. *E. quercicola* infects predominantly tropical and subtropical plants (*Quercus phillyraeoides*), in the temperate zones of Europe it has not been yet detected (Takamatsu, 2007).

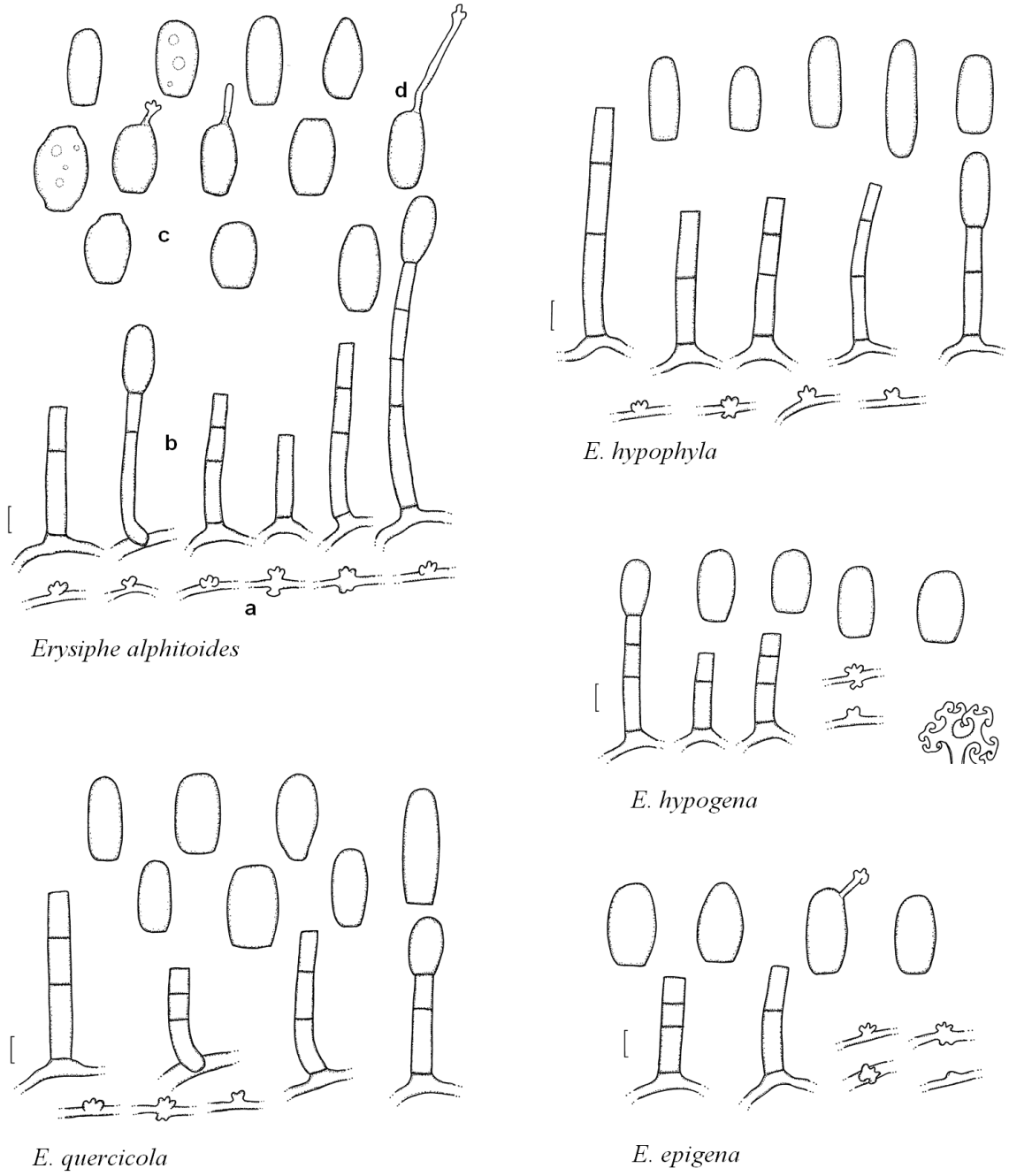


Fig. 5. The morphological differences between conidia of *Erysiphe* genera (Takamatsu, 2007).

Molecular markers

The ability to identify an organism is essential in diagnostics and fungal epidemiology, which is consequently an important source for plant pathology, environmental studies and biological control. Employing DNA-based characteristics in taxonomic studies alleviates problems with morphological and physiological variation, because genomic genomic DNA is stable under changing environmental conditions. Mutations in DNA do occur, but the frequency is low and the percentage of the genome affected over short periods (e.g. 100 years) is small (Foster, 2004).

From the examples above it is evident that the usage of conventional methods does not offer credible method for identification to distinguishing *E. alphitoides*. Conventional methods are based on identification of fungal symptoms, isolation and cultivation or morphology. All powdery mildews are obligate parasites, so they cannot be cultivated without their host (Nicot, 2002), which make DNA-based methods more appropriate for this study. Therefore the developing of reliable molecular markers (e.g. allozymes, RFLP, RAPD, SNP detection) for the separate species identification, resp. markers detecting intraspecific variability, is requested.

The most reliable marker to distinguish fungal species is the ITS segment and ribosomal RNA genes sequences (Hibbet, 2007). This is a universal marker, which can be applied throughout the fungal kingdom. Ribosomal DNA is important for phylogenetic studies because ribosomes and their sequences are in all organisms and the sequence similarity is a good indicator of their relationship.

The development of markers for fungal systematics must surmount many problems. Conventional methods, used for example in animals, could be more complicated or by principle impractical in fungi. Variability of certain segments of DNA could vary within each genus, so the development of reliable molecular markers applicable through fungal kingdom is an uneasy challenge. First, pathogenes, which are the most extensively studied species within fungi, have demographic and reproductive traits promoting a low genetic diversity. We must consider bottlenecks or host shifts, which can drastically reduce intraspecific genetic diversity. Furthermore, some specific life history traits of fungal pathogens, such as frequent asexual reproduction and recurrent bottlenecks in epidemic cycles, associated with low winter survival and selective sweeps following new virulence attributes, are also likely to result in low genetic diversity (Goodwin, 1994; Dutech, 2007). For example, microsatellites appeared to be less abundant in fungal genomes than in other organisms (Tóth, 2000). Microsatellites could be very short, usually formed with only one type of base, and in many species (like *E.*

alphitoides) the amplification of microsatellites by PCR is impossible (Dutech, 2007). Cytochome oxidase gene *cox1*, widely used in zoological barcoding, similarly shows very low degree of variability in fungi and therefore it is useless as a marker for most taxonomic studies (Seifert, 2008).

Other potential candidates for molecular markers include the gene for β-tubulin and mating-type genes. Mating-type genes (MAT1-2 sequence) are suitable for reliable determination of closely related species. The interspecific variation of these genes is relatively high with comparison to intraspecific variation (Du, 2005). The question is if these genes could be applied on fungi without observed sexual stage or on fungi, which lost the ability reproduce sexually. The phylogenetic analysis of fungus *Fusarium graminearum* based on this gene showed that mating-type genes are under purifying selection even in asexually reproduced species and it is possible to use them for these types of fungi (O´Donell, 2004).

Variability of the β-tubulin gene within one species is very low, for example in *Erysiphe necator* there is one-base substitution between two differently aggressive lineages of this fungus (Montarry, 2008).

Mitochondrial DNA and mitochondrial genes offers an excellent system to study fungal evolutionary genetics because of small size (20-80kb), maternal heredity and functional similarity. Fungal mtDNA is actually one genetic locus, in addition it is haploid, which eliminates recombination of this genome (Foster, 2004). In contrast to animals, mtDNA in fungi shows higher level of diversity in lenght and organisation. For example in the species Neurospora crassa we can find 25% diversity in the length of mitochondrial genome (Taylor, 1986). Analysis of mtDNA is used principally for detection phylogenetical differences between closely related species (Foster, 2004).

In this work, we tried to compare two molecular markers, one residing on genomic and the other on mitochondrial DNA to assume a precise discerning of the E. alphitoides powdery mildew.

2. The aims of work

1. Verify the species classification of 30 samples from Europe by sequencing the ITS segment and compare it with database
2. Design primers to properly distinguish variable mitochondrial DNA segment
3. Determine the variability and usability of the segment as molecular marker for identifying *E. alphitoides* species

3. Material a methods

3.1. Experimental organisms

Erysiphe alphitoides

The European population of *Erysiphe alphitoides* fungus was analysed for variability and consequent phylogenetic relations. 30 samples were collected from infected trees during summer and autumn from different localities (for list see attachment). Leaves with mycelia were separately collected to sterile Petri dishes containing silicagel. Taxonomical identification and morphological analysis was carried out by Ing. Miloslava Kavková, Ph.D.

3.2. Isolation of genomic DNA

For isolation of genomic DNA we used a modified protocol of Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), Cleistothecia and mycelium with conidia from autumn and summer samples were used, respectively. Cleistothecia were removed from the leaf surface with an injection needle (100-150 clestothecia per one sample). Mycelium and conidia were scratched off from the leaf surface with a scalpel. This fungal matter was disintegrated with a little amount of sterile fiberglass in 200 μl of buffer AP1 (Quiagen DNeasy Plant Mini Kit) in a 1,5-ml test tube. After crushing the tissue, additional 200 μl of buffer AP1 and 4 μl of RNase A (stock solution 100mg/ml) were added. The manufacturer's instructions were followed thereafter.

3.3. Template preparation for sequencing

3.3.1. Amplification of the ITS segment and part sequence of *cytochrome b* gene

The amplification of the ITS segment and the fragment of *cytochrome b* gene was carried out by PCR method. For the amplification of the ITS segment the o-asc-for × o-asc-rev primers (stock solution 0,1 mM) were used, designed to be for *Erysiphe* genus specific as described by Heuser (Heuser, 2001). The sequence of primers is in the table 1. PCR reaction for ITS segment was performed in MJ Mini cycler (Bio-Rad, Berkeley, CA, USA). An initial denaturing step – 94 °C for 2 min, was followed by 35 cycles of 1 min at 94 °C, 45 sec at

48 °C for annealing and 1 min at 72 °C for extension. The final extension was at 72 °C for 10 min. A negative control, lacking template DNA, was included for each set of reactions.

Species-specific primers for *cytochome b* gene have not been described. For the PCR reactions primers infered from the *Podosphaera fusca* gene sequence (GeneBank EF137826) were used. The PCR profile for the *cytochrome b* fragment was: 2 min 94 °C , 35× [1 min 94°C, 45sec 52 °C, 1min 72 °C], 10 min 72 °C. A negative control, lacking template DNA, was included for each set of reactions.

The PCR mixture was set up according to the ExTaq manual (TaKaRa Biochemicals, Kyoto, Japan), in a volume of 25 μl:

19.4 μl ddH_2O

2.5 μl 10× Taq PCR pufr

1 μl dNTP (2.5 mM)

2×0.5 μl forward and reverse primers (both 5μM)

0.2 μl Taq polymerase (5 U/μl, Top-Bio s.r.o., Prague, Czech Republic),

1 μl genomic DNA (30-50 ng/μl).

One μl of PCR product was subjected to electrophoresis in 1% agarose gel in 1xTAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and 2-Log DNA ladder (New England Biolabs, Inc.) was used as a molecular size marker. The fragments were dyed with SYBR green fluorescent dye and detected on a UV transilluminator (TFX-35.M Vilber Lourmat).

Primer	**Sequence**
o-asc-for	5´ ATTACMGAGYGYGAGGCTCAGTCG 3´
o-asc-rev	5´ CTACCTGAWTCGAGGTCAACCTGTG 3´
cytb_pfF	5´ CAGGCTAATACAGCTTCAG 3´
Cytb_pfR	5´ CACTCAGGTACTATAGCAGG 3´

Tab. 1. The list of used primers with their sequences

3.3.2. The PCR product purification

PCR reactions were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Amplified DNA fragments were eluted in ddH_2O and stored at -20 °C.

The concentrations of DNA in resulting samples were quntified with a ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA)

3.3.3. Ligation of PCR fragments

The *cytochrome b* fragments were subsequently cloned into pGEM-T Easy Vector System II (Promega, Fitchburg, WI, USA). The ligation mixture contained 0.5 μl of pGEM-T Easy plasmid (50 ng/μl), 1.5 μl of PCR product, 0.5 μl of ligase enzyme (3 U/μl) and 2.5 μl ligation buffer. Reactions were incubated overnight at 4 °C.

3.3.4. Bacterial transformation and plasmid isolation

For transformation XL-1 Blue bacterial cells (Agilent Technologies, Santa Clara, CA, USA) were used according to manufacturer's instructions. 50 μl of thawed cells were added to the ligation reaction and incubated for 30 min on ice, followed by a heat-shock (30 sec in 42°C) and snap cooling and keeping on ice for 2 min. 200 μl of preheated SOC medium were added and the mixture was incubated for 1.5 hrs at 37 °C shaking at 220 rpm. After incubation, the competent cells were spread on LB agar (Langley, 1972) with ampicillin and with 80 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside, Serva), and 20 mM IPTG (isopropyl-1-thio-β-D-galacto-pyranoside). After incubation 14-16 hrs at 37 °C white colonies were analysed by PCR reaction for presence of required insert. Colonies were transferred into 50 μl of ddH_2O, 3 μl of this suspension were used for the PCR reaction. For positives, the remain suspensions were transferred into 3 ml of liquid LB medium with 12 μl of ampicillin (100 μg/ml) and incubated overnight in 37 °C (220 rpm).

App. 6 positive colonies from each *E. alphitoides* sample were used for plasmid isolation, using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Purified plasmids were eluted into 50 μl ddH_2O and stored at –20 °C. Plasmid concentations were detected by ND-1000 (Thermo Fisher Scientific, Waltham, USA)

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3.4. Sequencing reaction preparation and sequencing

The sequencing reaction was carried out by the Genomics Laboratory of the BC AS CR, v.v.i. in České Budějovice using an ABI PRISM 3130x1 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).The volume of sequencing reaction mixture was 15 μl: 0.5 μl primer (see above), 50 ng of purified PCR product or 200 ng of plasmid DNA for ITS segment and *cytochrome b* fragment, respectively, with ddH_2O added to the final volume of 7.5 μl combined with 7.5 μl of 2×PCR premix. The fragments were sequenced from both sides, for ITS segment using o-asc-for or o-asc-rev primer, for *cytochrome b* fragment using M13F or M13R primer.

3.5. Data processing

Sequencing data were further edited prior to phylogenetic analyses. Vector contamination in case of plasmid sequencing was identified using VecScreen query at NCBI (www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) and removed using BioEdit software (Hall, 1999). Edited sequences were submitted to query at NCBI database using BLAST search and identified. Initial alignment of datasets was carried out in BioEdit software (Hall, 1999). Sequences were aligned via ClustalW Multiple Alignment tool (BioEdit) including sequences of nearest taxa downloaded from GenBank. Before aligning, the coding sequences (*cytochrome b* gene) were temporarily translated into amino acid sequences using toggle translation tool (BioEdit). This operation provided datasets which were exported as NEXUS format for further analysis. As outgroups, we used sequences from ascomycetous fungi: *Blumeria graminis f. sp. tritici* (AF343441), *Podosphaera fusca* (EF088827), *Glomerella graminicola* (AY285743), *Rhynchosporium secalis* (DQ463419), *Aspergillus niger* (DQ178141).

3.6. Phylogenetic analyses

Datasets were analysed by maximum parsimony (MP) and maximum likelihood (ML) method in PAUP* 4.10b (Swofford, 2001). MP analyses were done with heuristic search option using the TBR (tree bisection and reconnection) algorithm. All sites were treated as

unordered and unweighted. The strength of the internal branches of the resulting trees was tested with bootstrap analysis (1000 replications for MP and 100 replications for ML). The sequence divergence was computed using dnasp5 software (Librado, 2009). Computed trees were edited by TreeView 1.6.6 (Page, 1996).

4. Results

4.1. Gene amplification and sequencing

The ITS segment

For the amplification of ITS segment, we used a primer pair o-asc-for × o-asc-rev as described by Heuser (2001), designed to be specific for the *Erysiphe* group and amplifying strictly *E. alphitoides*, resp. *E. quercicola* species. The PCR product was 503 bp long.

Cytochrome b

For the amplification of the part of *cytochrome b* gene we used degenerated primers cytb_pfF × cytb_pfR. These primers amplify sequences from a spectrum of ascomycetous fungal species, since sequence sequence similarity between *cytochrome b* orthologues is about 80% among ascomycetes (unpublished), thus cloning and individual sequencing of each PCR product was necessary. The PCR product was app 550 bp long.

4.2. Phylogenetic analyses

The ITS segment

The sequences obtained from amplified ITS segments were uniform except for one sample from Poland – Gdansk. Comparison of these sequences with the GeneBank database showed that the majority of samples were amplicons from *E. alphitoides* and the only different sequence was from *E. quercicola*. The value of divergence between *E. alphitoides* and *E. quercicola* ITS segment sequences is 0.0203 (10 mutations).

Cytochrome b

Cloning and sequencing the PCR fragments of the *cytochrome b* gene revealed variability even within one leaf sample. All obtained sequences were phylogenetically analysed to distinguish *E. alphitoides cytochrome b* sequence group, since any *cytochrome b* sequence is available for comparison in GeneBank database for this fungus. The majority of analyzed sequences formed a monophyletic group related to *Podospahera fusca*, which is the

closest related species from all outgroups used. Several sequences attached to other fungal taxons were eliminated from subsequent phylogenetic analyses. The phylogenetic tree obtained from the analysis of *cytochrome b* sequences of the *Erysiphe* group is shown in figure 6., 7. and 8.

This phylogenetic analysis revealed high level of intraspecific variability of *cytochrome b* fragment in contrast to uniformity of the ITS segment. The sample from Poland – Gdansk (*E. quecicola* according to the ITS sequence) is allied with other *E. alphitoides* samples and did not create a separated group.

Not all samples are included in the phylogenetic tree of *cytochrome b* fragment, because in many cases the PCR reaction from leaf isolates amplified other ascomycetous fungi and even cloning this PCR fragments into a plasmid did not capture the required sequences. MP tree was created to verify the taxonomic relationship with distant taxa. From figure 6. it is evident, that these sequences created isolated group of fungi related to outgroup sequences.

Fig. 6 A phylogenetic tree constructed by *cytochrome b* fragment analysis, used MP method with different ascomycetous outgroups. Pecentage BS support (1000 replications; >50%) are shown on the branches.

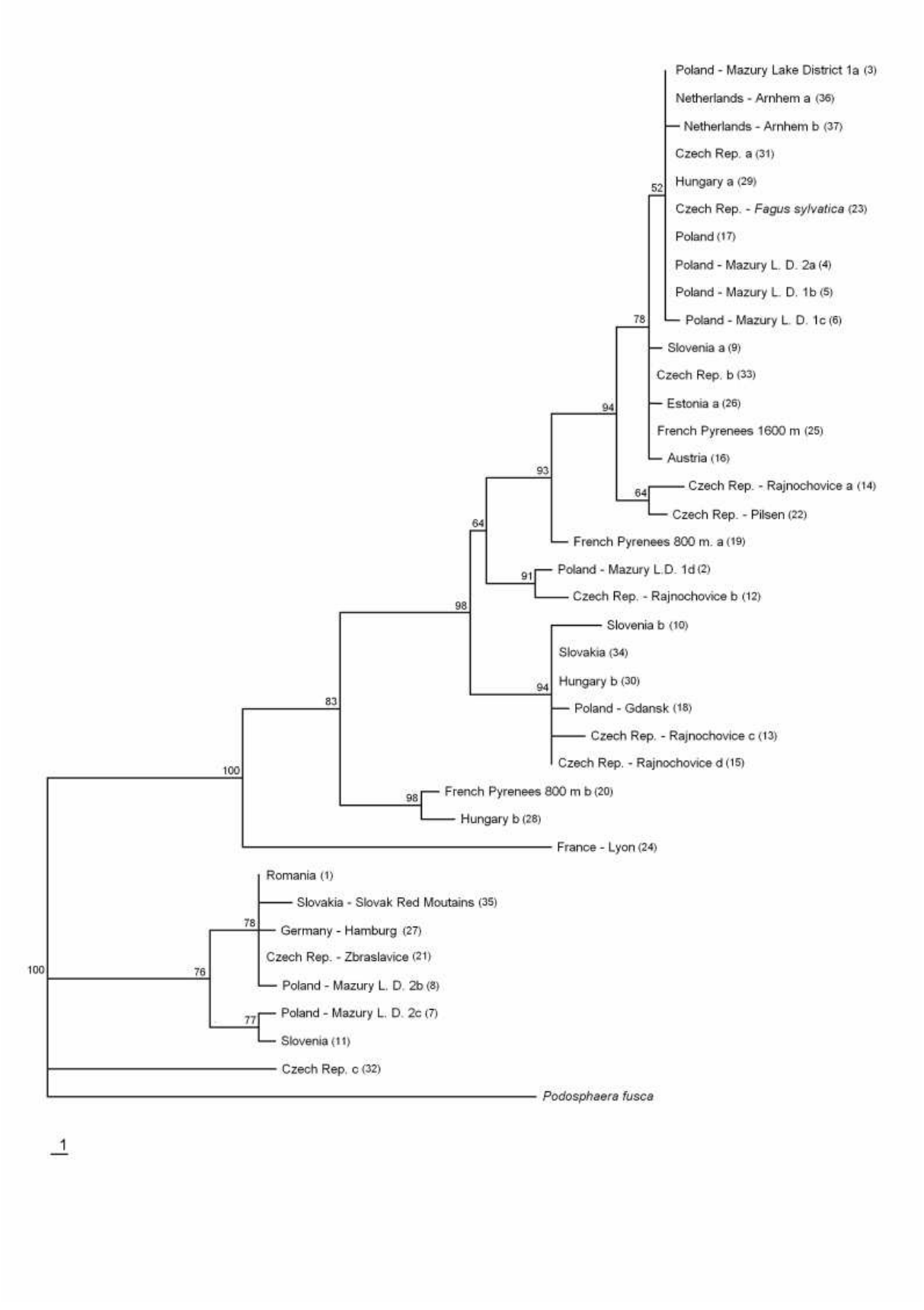

Fig. 7 A phylogenetic tree constructed by *cytochrome b* fragment analysis, used MP method. Pecentage BS support (1000 replications; >50%) are shown on the branches. In phylogram only *E. alphitoides* sequences are included.

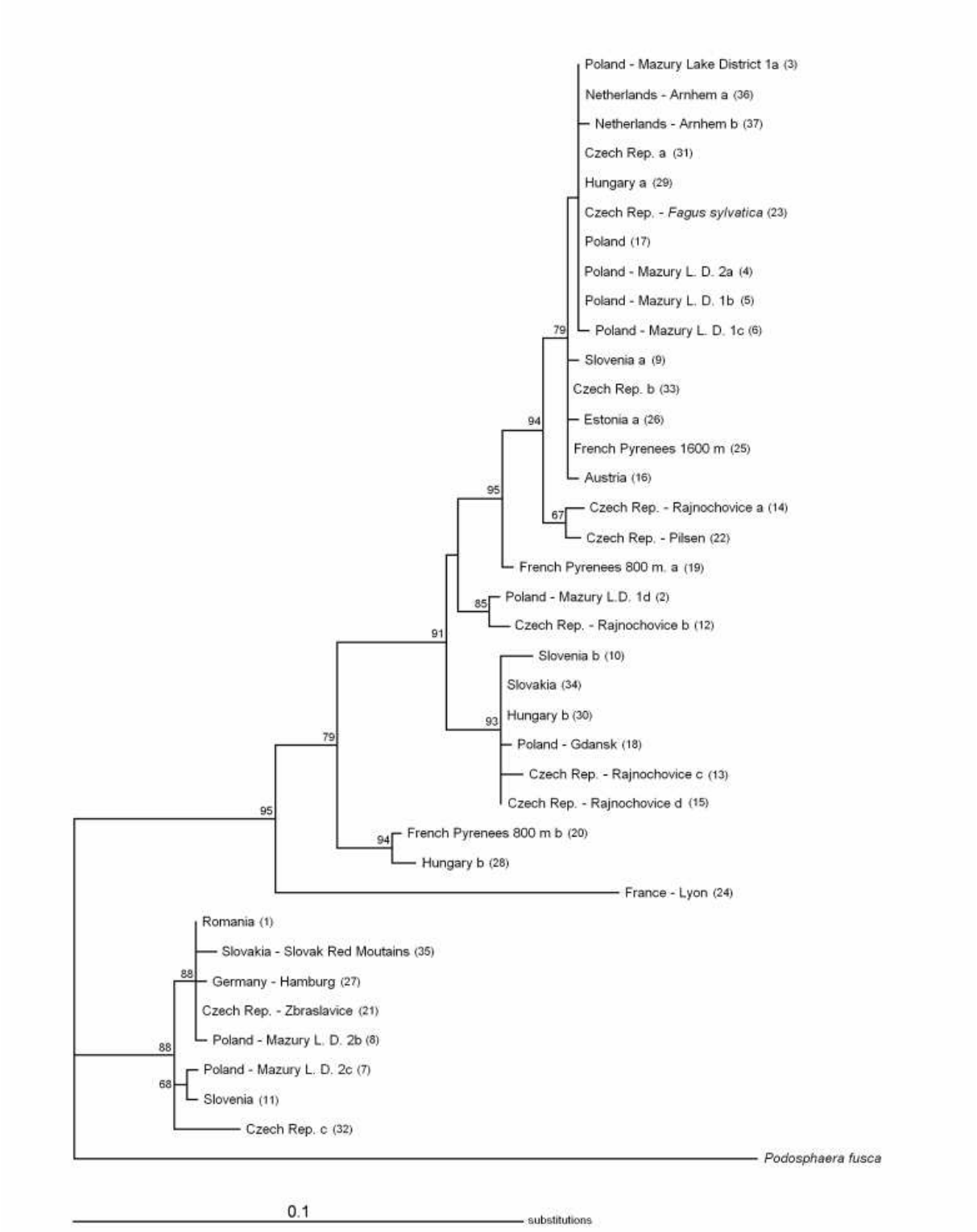

Fig. 8 A phylogenetic tree constructed by *cytochrome b* fragment analysis, used ML method. Pecentage BS support (100 replications; >50%) are shown on the branches. In phylogram only *E. alphitoides* sequences are included.

5. Discussion

The taxonomy of genus *Erysiphe* is a challenging task, not only because morphologic characteristics may be variable in dependence on environmental conditions but also few molecular data are available. Modern taxonomy of these fungi is based on the ITS sequence comparison (Hibbet, 2007; Takamatsu, 2007).

Sequences of ITS segment obtained from all collected samples of *E. alphitoides* showed absolute uniformity and are promising to be a perfect molecular marker for identifying species within genus *Erysiphe* as they are described in current taxonomy (Braun, 2002). Invariably only *E. alphitoides* species-related sequences were found, except one sample of *E. quercicola* from the Polish port of Gdansk. *E. quercicola* has not been observed in European continent yet and until recently it was considered to be a subtropical species (Takamatsu, 2007). It remains a question whether the species was introduced into Poland due to the import of tropical plants or fruits (Gdansk is a major seaport) and adapted to local conditions, or whether it is a rare domestic species (Mougou-Hamdane, 2009) and its presence has not been described. The morphological similarity between *E. quercicola* end *E. alphitoides* makes their distinguishing more difficult and in fact they are unquestionably discernible only by molecular methods.

From these results we can conclude that the population of *E. alphitoides* is rather unifom in Europe, which corresponds to the premise that it is a recently introduced species which underwent a bottleneck event. Due to its aggressiveness, in Europe, we can mostly find *E.alphitoides* displacing the original powdery mildew *Phyllactinia roboris* and notably suppressing the prevalence of other *Q. robur* powdery mildews.

The sequencing of ITS segment did not reveal the *E. hypophylla* species, which should be common in Europe (Takamatsu, 2007).

Results of this work revealed considerable variability of mitochondrial *cytochrome b* gene within the genus *Erysiphe*. The mitochondrial genome is haploid and its recombination is limited, so the variability should indeed be higher. Interrestingly, by phylogenetic analyses of *cytochtome b* fragments, the species *E. quercicola* is classified into the *E. alphitoides* group. Within this group *E.quercicola* created a well-supported branch with three other samples of *E. alphitoides*. This result may demonstrate that *E. quercicola* is not a separate species and the different ITS sequence only reflects intraspecific variability within the *Erysiphe alphitoides* s. lat. species. Another possible conclusion is that ITS region sequencing

cannot identify the DNA variability among species. Nevertheless, the nature of mitochondrial genome must be taken into consideration. Fungi as parasites tend to have reduced genomes and genetic variability because of their specific life style and mostly rapid asexual reproduction (Dutech, 2007). These presumptions, however, cannot be fully applied to the mitochondrial genome.

From the phylogenetic tree, it is evident, that the sequence variability does not reflect the geographic distribution. The cloning of amplified *cytochrome b* fragment allowed us to recognize more than one variants of this sequence in several samples although each sample was obtained from one leaf. This supports the hypothesis, that the leaves are being infected with more species or varieties of powdery mildew fungi at the same time (Mougou-Hamdane, 2009), which also contributes to morphological data misinterpretation. The ocurence of higher variability between mitochondrial genomes could be evocated by interspecific hybridisation, but about this subject no relevant information is available. From the phylogenetic trees, it is also notable that we can find up to three different variants of *cytochrome b* sequence we can find in one leaf sample. This result could support the theory of interspecific hybridisation and clarify the phylogenetic submission of *E. quercicola* sample into the *E. alphitoides* group.

Further investigation in at this field should be accomplished. It is possible that mitochondrial genes' variability of *E. alphitoides* could reflect the level of hybridisation between *Q. robur* and *Q. petrea*, as we know that *Q. petrea* and its hybrids are more resistant to *E. alphitoides* pathogen *than Q. robur* s. str. (Gibbs, 1997).

These findings reveal that at least one variable nuclear marker herewith the ITS segment is necessary to ivestigate. Additionally, to verify the *E. quercicola* taxonomic status it is of great importnce to test another *E. quercicola* or *E. hypophylla* isolates from different locations.

6. Conclusions

We can find many species of powdery mildew fungi in Europe but only few of them infect oaks. Among these species *E. alphitoides* is the most significant pathogen through the continent and other oak powdery mildew species are less common. Investigating the *cytochrome b* gene sequence, we observed unsuspected intraspecific variability of this gene within *Erysiphe alphitoides* isolates. Our results revealed the unsuitability of *cytochrome b* gene sequence as relevant marker for identifying *E. alphitoides* species. At the same time, a new question is raised; what is the mechanism that keep the intraspecific variability of this mitochondrial gene so high? The answer to this question represents a challenge for further investigation and may lead to subsequent taxonomic revision of this interesting genera.

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Attachment

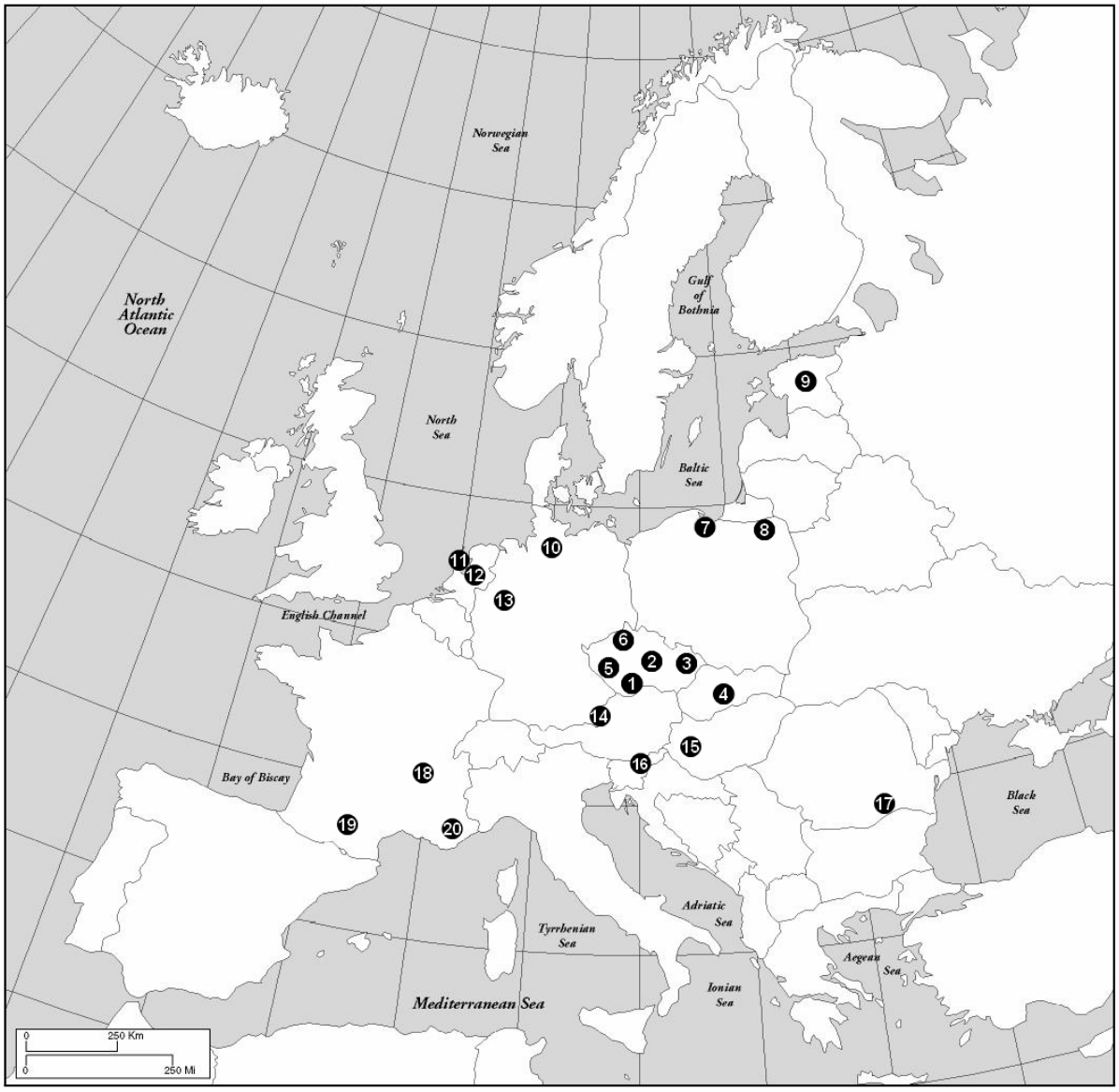

Fig. 9. – Distribution of samples: 1– four samples were taken in Southern Bohemia including sample of powdery mildew growing on *Fagus silvatica*. 2, 3, 5 and 6 – Czech Republic (Zbraslavice, Rajnochovice, Pilsen, Jablonec nad Nisou). 4 – Slovakia (Slovak Red Moutains, Martin). 7,8 – Poland (Gdańsk and several samples around Mazury Lake District). 9 – Estonia. 10, 13 Germany (Hamburg, Dortmund). 11, 12 – Netherlands (Alkmaar, Arnhem). 14 – Austria (Salzburg). 15 – Hungary (Lake Balaton). 16 – Slovenia (Maribor). 17 – Romania (Bucuresti). 18, 19, 20 – France (Lyon, French Pyrenee – three samples, Provence)
(picture taken from the internet: http://alabamamaps.ua.edu/contemporarymaps/world/europe/europe3.jpg and modified by Paint.NET software)